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CRISPR in the Tumor Microenvironment: From Diagnostic Profiling to Precision Therapy

Lirenhui Zhou^{1*}, Meiya Mu^{1*}, Meihua Yang^{1*}, Xiaojing Liu^{1*}, Guangrong Zou^{2#}, Chaoxing Liu^{3#}

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Correspondence: Chaoxing Liu - Email: liuchx69@mail.sysu.edu.cn

Guangrong Zou - Email: zougr3@mail.sysu.edu.cn

The author's information is available at the end of the article.

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Abstract

The tumor microenvironment (TME) is a dynamic and heterogeneous ecosystem that shapes tumor initiation, progression, immune evasion, and therapeutic resistance. Its cellular and molecular composition evolves throughout disease progression. The CRISPR–Cas gene-editing system has emerged as a transformative platform for decoding TME biology and enabling innovative diagnostic and therapeutic strategies. In this review, we outline the fundamental principles of CRISPR–Cas technologies and summarize their applications in functional genetic screening, interrogation of cell–cell interactions, and dissection of dynamic signaling networks within the TME. We highlight advances in CRISPR-based diagnostic platforms that allow highly sensitive and specific detection of cancer-associated signals across in vitro systems, patient-derived organoids, and ex vivo tumor samples. Furthermore, we discuss emerging CRISPR-enabled therapeutic approaches targeting the TME, including genetic modulation of tumor cells, reprogramming of immune and stromal compartments, and disruption of tumor vasculature and metabolic niches to enhance antitumor efficacy. Particular emphasis is placed on delivery strategies that achieve cell-type specificity and spatial precision. Finally, we examine key challenges that limit clinical translation, such as off-target editing, immunogenicity, and the inherent plasticity and heterogeneity of the TME, and discuss future directions to improve safety, robustness, and therapeutic durability. Collectively, this review provides a comprehensive framework for leveraging CRISPR–Cas technologies to advance TME-focused cancer diagnostics and therapeutics.

Keywords: Biomarkers, CRISPR-Cas, Tumor microenvironment, Drug resistance, Gene editing, Targeting therapies, Precision oncology, Oncogenic signaling pathways, Cancer immunotherapy

1. Introduction

CRISPRs are widely investigated as versatile biotechnological tools. The CRISPR locus contains CRISPR-associated (Cas) genes and an array of repeats separated by variable spacers. These spacers, taken from foreign genetic elements (1), store memory of exogenous nucleic acids and enable immunity by specifically recognizing and cutting matching pathogens (2) (Figure 1a). Guide RNAs are programmed with distinct spacer sequences that match the target DNA "protospacer" near the PAM, underscoring the key roles of spacers in gene editing and diagnostics (3). The protospacer-adjacent motif (PAM), a short, conserved DNA sequence adjacent to the CRISPR cleavage site, allows Cas proteins to distinguish self from foreign DNA. This interplay among CRISPR components, particularly spacers and PAMs, underpins the broad applications of CRISPR in gene editing and diagnostics. Cancers are complex biological systems that integrate tumor cells with various non-cancerous cells within a modified extracellular environment. The tumor microenvironment (TME), comprising cancer cells, immune cells, stromal cells, vasculature, and extracellular matrix, is essential for cancer initiation and progression and is associated with immune suppression, immune evasion, sustained proliferation, metastasis, and angiogenesis. Due to the pathophysiological complexity of the TME, current diagnostic and therapeutic approaches remain limited, highlighting the need for new technologies. CRISPR-Cas technology shows promise for effective, precise cancer diagnosis and treatment. In this review, we discuss the applications of CRISPR-Cas technology in analyzing and targeting the TME, highlighting its potential in molecular diagnostics and therapeutic strategies.

2. Mechanisms and Applications of CRISPR-Cas

2.1 Mechanisms and Functionality

The CRISPR-Cas system is categorized as class 1 and class 2. Class 1 systems are composed of multi-subunit protein complexes, while class 2 systems consist of a single-effector multidomain protein. These are commonly used as genome-editing technologies because of their simpler architecture. Class 2 is classified into types II, V, and VI, with the corresponding effector proteins Cas9, Cas12, and Cas13 (5). V-A and V-B refer to Cas12a and Cas12b (6), previously named Cpf1 and C2c1, respectively. While the CRISPR-Cas system shares a fundamental mechanism for targeting and cleaving DNA, it differs in aspects such as guiding RNA, PAM sequences, cleavage ends, and capacity for trans-cleavage. Cas9 is a DNA endonuclease guided by single-guide RNA (sgRNA), which consists of trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) (7). The upstream region of the CRISPR locus transcribes tracrRNA, which induces the maturation of another pre-CRISPR RNA (pre-crRNA) to generate active crRNA (8,9). The tracrRNA:crRNA complex, commonly referred to as sgRNA, can combine with CRISPR-associated nuclease 9 (Cas9) to form the CRISPR-Cas9 system (10). In the absence of sgRNA, Cas9 remains inactive and cannot cleave the target DNA sequence. PAM sequences, typically 2-5 bp located downstream of the CRISPR-targeted cleavage site, are essential for the CRISPR system first to recognize PAM and then unwind the DNA duplex to facilitate base pairing between the target DNA strand and the crRNA guide sequence (11). *Streptococcus pyogenes* Cas9 (SpCas9) was the first Cas9 nuclease used in genome editing (12), whose sgRNA could be easily programmed to guide the cleavage of almost any sequence preceding a 5'-NGG-3' PAM sequence in mammalian cells, enabling various edits to the target locus (13). CRISPR-Cas9 variants exhibit PAM specificity, with

StCas9 (*S. thermophilus*) requiring 5'-NNAGAAW-3' and SaCas9 (*S. aureus*) recognizing 5'-NNGRRT-3'.

After binding to the PAM and forming a DNA-sgRNA hybrid complex, the Cas9 proteins can create a double-strand break (DSB) at a site three base pairs upstream of the PAM with its RuvC and the HNH nuclease domain (14), predominantly generating blunt-ended DSBs (Figure 1b). DSBs are one of the most genetically toxic DNA damages. If not repaired, they may lead to chromosomal rearrangement, genomic instability, and cell death. Thus, distinct pathways are employed in eukaryotic cells, resulting in differences in genome-editing accuracy and efficiency (15). HDR and NHEJ are two main strategies that cells use to deal with DSBs. The former is precise but limited by the requirement for large amounts of exogenous DNA templates, whereas the latter is efficient but error-prone. DSBs in CRISPR-Cas9 are commonly repaired by the NHEJ pathway, resulting in either complete repair or the formation of small insertions and deletions (16). Consequently, gene editing can be achieved by generating DSBs at specific genomic sites, enabling processes such as gene knock-in, knock-out, gene repair, and transcriptional regulation (17).

The fundamental mechanism of CRISPR-Cas9 and CRISPR-Cas12 is substantially the same. For Cas12a, only crRNA is sufficient for mediating DNA targeting without tracrRNA (18), while the CRISPR-Cas12b system has both crRNA and tracrRNA (19). Cas12 family proteins have predominantly T-rich PAM sequences but differ in the number of Ts18. The PAM of CRISPR-Cas12a is TTTV, whereas that of Cas12b is TTN (20). Both Cas12a and Cas12b proteins contain a RuvC-like endonuclease domain and a nuclease lobe (NUC) domain for DNA cleavage, but they lack the HNH domains found in Cas9 (21). After the crRNA recognizes the PAM sequences that are located 18-23 nt upstream of the spacer and produces a DNA-sgRNA hybrid complex, Cas12 generates DSBs with staggered ends (Figure 1b), which can accelerate non-homologous end joining (NHEJ)-based gene insertion into the mammalian genome. Different from Cas12, Cas9 produces blunt-ended DNA breaks (21,22). After completing the cis-cleavage, Cas12 is activated and no longer relies on PAM; instead, it nonspecifically binds to any single-stranded DNA (ssDNA) to cleave, a process called trans-cleavage (23). Trans-cleavage can generate shorter DNA fragments, thereby facilitating signal amplification of the target DNA and revealing its nucleic acid detection ability (24). The unique characteristics of CRISPR-Cas12 can be effectively integrated with nucleic acid amplification (NAA) techniques to achieve point-of-care testing (POCT). CRISPR Cas9 and Cas12 are both DNA-targeting systems, while Cas13 is an RNA-targeting system. To efficiently target small single-stranded RNAs (ssRNAs), Cas13 requires a protospacer flanking site (PFS) rich in A and U at the 3' end instead of PAM (25). Cas13 proteins typically contain two nucleotide-binding (HEPN) domains, one from higher eukaryotes and the other from prokaryotes, to cleave ssRNA (26). Compared to RNAi-mediated knockdown, Cas13-based RNA knockdown demonstrates a substantial reduction in off-target effects (27), making it particularly suitable for precise RNA manipulation and diagnostic applications.

2.2 Applications in Molecular Diagnosis and Precise Therapy

As a revolutionary gene-editing tool, CRISPR-Cas9 has demonstrated significant potential in molecular diagnostics and precise therapy in recent years (Figure 1c). By designing specific single-guide RNAs (sgRNAs), the system can precisely recognize and cleave target DNA, making it an ideal platform for clinical diagnostics and treatment. In antiviral therapy, CRISPR technology has been developed to edit CCR5 and HIV-1 to eliminate HIV-1 infection (28). In oncology, the CRISPR-Cas9 system is driving revolutionary advances in precision cancer medicine. CRISPR-Cas9 can be combined with next-generation sequencing (NGS) to analyze genomic variations and realize personalized cancer medicine (17). CRISPR-based platforms have been successfully applied to detect clinically significant

biomarkers, such as pancreatic cancer-specific tsRNAs (29) and breast cancer markers (PIK3CA E545K ctDNA), enabling early cancer screening (30). Previous studies have demonstrated that employing CRISPR technology to knock out PD-1 in CAR-T cells can effectively augment T cell immune responses and enhance their capacity to eliminate cancer cells (31). With the successful application of CRISPR-Cas9, more CRISPR-Cas variants have been discovered, and they have demonstrated fantastic superiority as well.

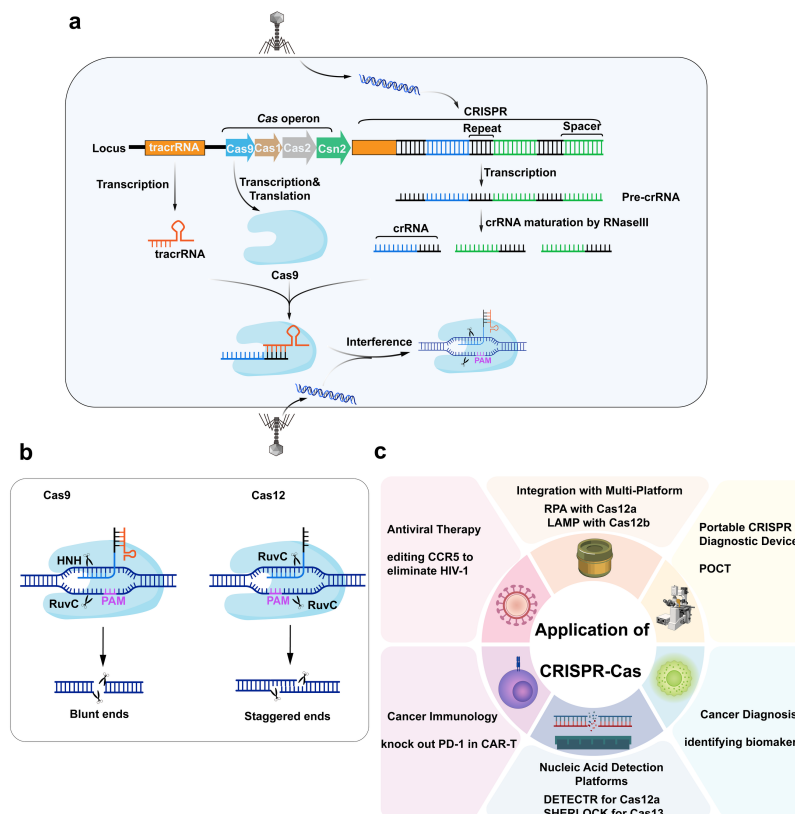


Figure 1. Molecular mechanisms and applications of the CRISPR–Cas system. (a) The CRISPR–Cas9 adaptive immune system in prokaryotes. Its locus typically comprises an array of repetitive sequences interspersed with spacer sequences derived from invasive genetic elements, along with a suite of CRISPR-associated (*Cas*) genes. Preceding the *cas* operon is the *tracrRNA* gene. Upon phage infection, a new spacer acquired from the invading DNA is integrated into the CRISPR array. The transcribed *pre-crRNA* is processed into mature *crRNAs* by RNase III-mediated cleavage. These mature *crRNAs* assemble with *tracrRNA* and *Cas* proteins to form a ribonucleoprotein (RNP) complex. During interference, the RNP identifies invading DNA via an *sgRNA* (*tracrRNA:crRNA*), and the *Cas* protein cleaves the foreign DNA, thereby eliminating the foreign gene. (b) The CRISPR–Cas9 system can cleave at 3 bp upstream of the dsDNA PAM, guided by the *sgRNA*, yielding blunt ends via the HNH and RuvC domains of Cas9. The CRISPR–Cas12a system relies solely on *crRNA* to recognize the PAM, then cleaves the non-complementary strand of the target DNA downstream of the PAM, producing staggered ends exclusively through the nuclease RuvC. (c) The application of the CRISPR–Cas system in different scenarios. Figure 1 was created by the authors using graphical components from Servier Medical Art (<https://smart.servier.com/>), licensed under the Creative Commons Attribution 4.0 (CC BY 4.0) license.

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Because CRISPR–Cas12 exhibits trans-cleavage, it has been applied across multiple fields. For example, in nucleic acid detection, DETECTR is a key mechanism that leverages the trans-cleavage capability of the Cas12a (23). In addition to DETECTR, other nucleic acid detection technologies based on CRISPR–Cas13 include SHERLOCK (32) and SUREST (33). Further details of these technologies will be elaborated in subsequent sections. CRISPR–Cas systems can be combined with microfluidic platforms for densely multiplexed assays, enabling rapid assessment of complex diseases and guiding precise treatment (34). Thus, CRISPR-based molecular diagnosis retains significant potential to advance clinical applications, particularly in oncology therapeutics. After Cas9 and Cas12a, Cas12b is the third promising CRISPR system for genome engineering (35). Different types of Cas12b have distinct advantages. Among the Cas12b family, AaCas12b from the Alicyclobacillus acidiphilus maintains optimal nuclease activity over a wide temperature range (31 °C–59 °C) and shows high

specificity and minimal off-target effects, which can be a versatile tool for mammalian genome engineering (36). Cas12b can also be applied to DNA detection, a Cas12b-mediated DNA detection (CDetection) platform showing sensitivity and accuracy (37). Because specific subtypes of Cas12b exhibit high-temperature resistance, they are suitable for use in LAMP-based pathogen detection and screening (38). In contrast, the CRISPR-Cas12a system is temperature compatible with RPA (39). Compared to CRISPR-Cas9 and Cas12a, Cas12b holds potential applications in extreme-environment bioengineering and precision medicine due to its unique thermal stability and editing properties. However, its full potential remains to be further explored.

3. The Application of CRISPR-Cas in Analyzing the Complexity of TME

3.1 Functional Gene Screening

CRISPR-Cas9-based functional genetic screening (Figure 2a) has been extensively employed across diverse research domains, including investigations of tumorigenesis mechanisms, drug discovery, immunotherapy, and gene-function characterization. Compared to alternative methodologies such as RNA interference (RNAi) and cDNA overexpression, CRISPR-Cas9-based genome-wide screening offers distinct advantages of high throughput, enhanced efficiency, and reduced cost. Among CRISPR-based strategies, gene knockout (KO) screening represents the most established paradigm. For instance, KO screening in CD8⁺ T cells revealed that deletion of the transcription factor FLI1 induces 5- to 30-fold cellular expansion and enhances tumoricidal capacity (40). Separately, ADCY7KO was shown to increase CD8⁺ T-cell infiltration via transcriptional upregulation of the cytokine CCL5 (41). In polymorphonuclear myeloid-derived suppressor cells, the surface protein CD300ld is enriched in the TME, and its genetic deletion significantly suppresses neoplastic progression (42). Similarly, Genetic ablation of the E3 ubiquitin ligase COP1 in malignant cells reduces tumor-associated macrophage infiltration, thereby impeding tumorigenesis (43).

Beyond CRISPR KO, CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) constitute widely utilized functional genomic screening methodologies. CRISPR employs catalytically deactivated Cas9 (dCas9), which lacks endonuclease activity and therefore cannot introduce double-strand breaks in DNA. Instead, dCas9 is fused to transcriptional activators such as VP64, p65, and NF- κ B to induce targeted gene transcription. Using this approach, studies have identified PRODH2 as a critical target whose activation significantly enhances the anti-tumor efficacy of CD8⁺ T cells (44). Joung et al. (45) discovered that transcriptional induction of BCL-2 and B3GNT2 drives tumor cell resistance to cytotoxic T lymphocytes (CTLs). Similarly, CRISPRi utilizes dCas9 fused to transcriptional repressor domains, such as KRAB or MeCP2, to suppress gene transcription. For instance, Li et al. (46) identified several genes, including ARPC4, PI4KA, ATP6V1A, UBA1, and NDUFV1, whose repression significantly enhances the tumor-killing efficacy of CAR T cells. In comparison to CRISPR KO, CRISPRa/I offer distinct advantages that include reduced off-target effects and reversible perturbation. However, these techniques may have limitations, including limited transcriptional modulation efficiency and potential epigenetic constraints at specific loci. Furthermore, researchers (47) have enhanced screening accuracy under high Multiplicity of Infection (MOI) conditions by incorporating unique internal barcodes (iBARs) within sgRNA constructs. This methodology significantly reduces experimental workload and improves screening fidelity compared to conventional approaches.

Base editor (BE) enables the precise introduction or correction of specific point mutations, allowing for the faithful recapitulation of the functional impact of single-nucleotide variants and single-nucleotide

polymorphisms prevalent in cancer. Utilizing the cytosine base editor (CBE), researchers have demonstrated the feasibility of base editing for high-throughput screening of point mutations (48,49). This approach has been successfully applied to conduct functional screens for specific mutations in DNA damage response genes, such as BRCA1/2. However, the editing efficiency of base-editing tools varies significantly across genomic loci. Furthermore, an sgRNA can generate multiple types of mutations at its target site.

Consequently, the functional consequence associated with a screened sgRNA may not accurately reflect the intended variant effect. To address this limitation, an efficiency-correction model (50) can be implemented to transform the cellular effect induced by an sgRNA into a variant pathogenicity score, thereby enhancing the accuracy of functional variant assessment. iBARed Cytosine Base Editing-mediated Gene KnockOut (BARBEKO) is a novel high-throughput screening methodology that integrates CBE with sgRNAs incorporating iBARs (47,51). This method significantly reduces the number of cells required for library construction and is unaffected by copy number effects or editor-induced cytotoxicity. In colorectal cancer cell lines, employing cytosine base editors (CBEs) and adenine base editors (ABEs) to target core genes in the IFN- γ signaling pathway enables assessment of how distinct mutations affect the IFN- γ response and T cell-mediated cytotoxicity (52). To achieve base editing in primary human T cells, Schmidt et al. (53) employed optimized BE and screened multiple allelic pairs that regulate T cell activation and cytokine production.

Integrating CRISPR screening with single-cell analytical modalities, single-cell CRISPR screening (scCRISPR) provides a transformative tool for identifying cancer-associated genes within the TME. Building upon this platform, several sequencing methodologies have been developed, including Perturb-Seq (54,55), CROP-Seq (56), and Direct Capture Perturb-Seq (57). Using Perturb-Seq, Fagerberg et al. (58) first elucidated the pivotal role of the transcription factor KLF2 in governing the differentiation and exhausted states in CD8⁺ T cells. Specifically, KLF2 maintains effector T cell lineage stability by promoting TBET activity while suppressing TOX expression. In another study employing CROP-Seq, researchers identified a TNFR1-dependent macrophage tumor necrosis factor (TNF) signaling module that functions as a universal driver of clonal expansion in epithelial tissues (59). This discovery provides a mechanistic foundation for developing therapeutic strategies targeting the TNF signaling pathway.

Beyond Cas9-based screening platforms, Cas12a is also a viable tool for functional genetic screening. Utilizing the optimized AsCas12a variant (enAsCas12a), researchers identified 11 pairs of established synthetic lethal gene interactions in OVCAR8 and A375 cancer cell lines (60). This demonstrates the utility and potential of Cas12a for complex functional genomic screens. Furthermore, Cas13 enables programmable RNA targeting and knockdown in mammalian cells (61, 62). Unlike Cas9, Cas13 directly cleaves non-coding RNAs without altering genomic DNA sequences. Cas13-based screening of highly expressed circular RNAs (circRNAs) in cervical and colon carcinoma cell lines identified circRNAs essential for cell-type-specific proliferation (63). A parallel Cas13 screen across five human cell lines, including the THP-1 monocytic cell line, identified 778 essential long non-coding RNAs (lncRNAs) that play indispensable roles in human cancer pathogenesis and development (64). These findings highlight the promise of Cas13 as a powerful platform for interrogating non-coding RNA function in the TME. Following the widespread use of Cas9, Cas13 is poised to become a key tool in elucidating the roles of non-coding RNAs in tumorigenesis and cancer progression.

3.2 Dissecting Intercellular Interaction

Cell–cell interactions are central to tissue function and microenvironmental regulation, and CRISPR-Cas technologies provide powerful tools to interrogate their molecular regulators. Within the tumor microenvironment (TME), cytotoxic T lymphocytes (CTLs) exhibit marked heterogeneity, including progenitor-exhausted T cells (Tpex) and terminally exhausted T cells (Tex). Tex cells constitute the dominant CTL population in tumors, mediate tumor cell killing, but progressively lose proliferative capacity. To overcome this limitation, Zhou et al. (65) applied scCRISPR to map in vivo fate-regulatory networks of tumor-infiltrating T cells (Figure 2b). This analysis identified the transcription factors IKAROS, ETS1, and RBPJ as key regulators that control Tpex exit from quiescence and promote proliferation of terminal Tex cells, thereby revealing strategies for functional T cell reactivation.

MHC class I (MHC-I) antigen presentation (AP) is a critical determinant of CD8⁺ T cell specificity and activation and represents a significant target in cancer immunotherapy. CRISPR-based screens have uncovered multiple positive and negative regulators of the MHC-I AP pathway. For instance, interferon (IFN)-mediated upregulation of classical MHC-I molecules and the non-classical MHC-I molecule Qa-1b suppresses CD8⁺ T cells and NK cells, respectively, thereby facilitating tumor immune evasion (66). Polycomb Repressive Complex 2 acts as a negative regulator by transcriptionally silencing genes involved in MHC-I antigen processing (67). In addition, SUSD6, TMEM127, and MHC-I form a ternary complex that recruits the E3 ubiquitin ligase WWP2, promoting MHC-I ubiquitination and lysosomal degradation (68). Genetic disruption of SUSD6, TMEM127, or WWP2 enhances MHC-I AP and suppresses tumor growth in a CD8⁺ T cell-dependent manner.

Additionally, BIRC2 interacts with the NF-κB-inducing kinase. It promotes ubiquitin-dependent degradation, thereby inactivating the non-canonical NF-κB signaling pathway and downregulating MHC-I expression (69). Collectively, these findings underscore the complexity of the regulatory networks governing immune surveillance and identify potential targets to enhance antigen presentation and T-cell-mediated tumor clearance. Notably, a recently developed methodology termed Perturb-FISH (70) integrates CRISPR perturbations with spatial transcriptomics to capture how genetic perturbations influence intercellular interaction. The methodology has been employed to investigate the impact of NF-κB signaling pathway gene knockouts on tumor cell-immune cell interactions, thereby validating its utility in dissecting microenvironmental crosstalk. As a versatile molecular toolkit, CRISPR enables high-precision elucidation of intercellular interactions within the TME. This capability provides strategic frameworks for enhancing tumor cell immunogenicity and potentiating T-cell-mediated antitumor immunity, thereby advancing cancer immunotherapy.

3.3 Investigating TME Dynamics

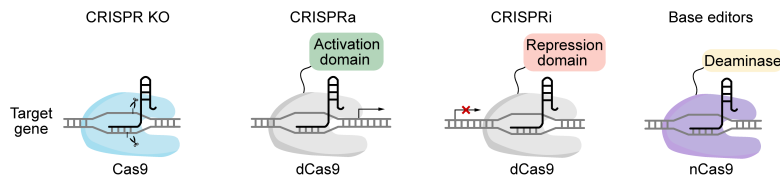
CRISPR-Cas9 enables novel in vivo lineage tracing strategies by utilizing insertion/deletion (indel) mutations generated through targeted DNA double-strand breaks as heritable genetic barcodes. This approach reconstructs lineage relationships between cellular clones and quantifies dynamic clonal evolution. For example, Rogers et al. (71) combined CRISPR-Cas9 technology with DNA barcoding to enable precise tracking of every cell in mouse lung adenocarcinoma, providing a scalable, high-throughput platform for delineating cancer gene interactions. Sarah Bowling et al. (72) first reported a CRISPR-Cas9-based mouse model for lineage tracing, termed CARLIN, which is used in the CRISPR screening process (Figure 2c). However, its utility is constrained by limited barcode diversity and indiscriminate labeling of all cells. Addressing these limitations, the DARLIN (73) methodology innovatively substitutes Cas9 with Cas9-TdT fusion and implements a triple-target array, substantially enhancing barcoding resolution. The CREST system (74) employs analogous principles to resolve lineage relationships among diverse midbrain cell types in mice at specific developmental timepoints.

Its key advantage lies in elucidating molecular mechanisms governing fate decisions at single-clone resolution.

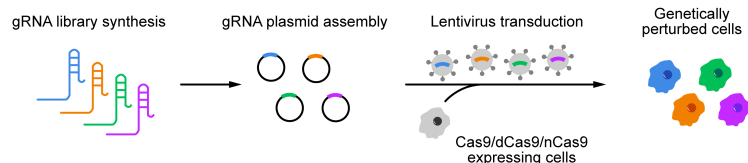
In addition, Jonathan S. Weissman's team (75) developed an alternative high-resolution CRISPR-Cas9 tracing system. This system incorporates a random integration barcode (IntBC) to encode clonal lineage information. It uses sgRNA-directed Cas9 targeting of three distinct loci to induce continuous indel formation, thereby capturing subclonal lineage trajectories over time. Leveraging this system, the team constructed a single-cell lineage tracer that mapped metastatic dissemination routes and identified metastasis-specialized subclonal clusters in lung adenocarcinoma, generating comprehensive cancer lineage trees that delineate spatial trajectories of disseminated tumor cells (76). Collectively, these methodologies provide powerful tools for investigating cellular lineage hierarchies and fate determination, offering strategic frameworks to dissect spatiotemporal dynamics and heterogeneity within the TME.

a

Step 1: Different screening system



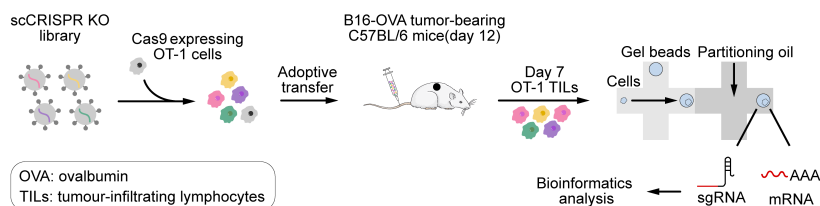
Step 2: Generation of genetically perturbed cells



Step 3: Phenotypic screening



b



c

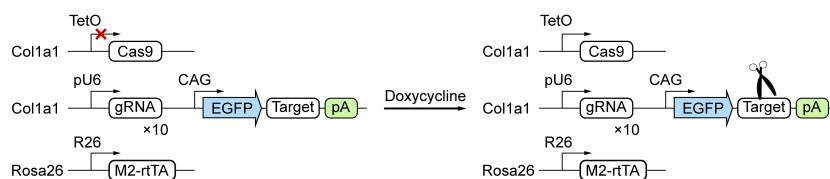


Figure 2. CRISPR-based functional screening strategies and single-cell applications. (a) *CRISPR screening process.* First, an appropriate screening system must be selected: (I) *CRISPR KO:* Cas9 is guided by gRNA to locate the target DNA sequence and cleave the DNA double strand. (II) *CRISPRa:* dCas9 fused to transcriptional activators promotes the transcription of target genes. (III) *CRISPRi:* dCas9 fused to transcriptional repressors suppresses the transcription of target genes. (IV) *Base editor:* Composed of dCas9 or nickase Cas9 (nCas9) combined with cytidine deaminase or adenine deaminase, this system enables the introduction of single-nucleotide mutations without inducing double-strand breaks. After selecting the screening system, the designed gRNA library is introduced into cells. After applying selection conditions (e.g., drugs), gRNA is extracted from surviving cells, amplified, and analyzed using next-generation sequencing to determine its target gene. (b) Researchers transduced Cas9-expressing activated OT-I CD8⁺ T cells (specific for ovalbumin (OVA)) with the scCRISPR library, then transferred cells to B16-OVA melanoma tumor-bearing mice. Seven days later, single-cell sgRNA and transcriptome libraries from tumor-infiltrating lymphocytes (TILs) in the mice were assessed via droplet-based sequencing. Subsequent bioinformatics analysis helped map the T cell gene regulatory network. (c) Schematic of the CARLIN system. The *Col1a1* genetic locus contains gRNA arrays, target sites, and a doxycycline-inducible Cas9 cassette. Ten gRNA sequences are independently controlled by individual U6 promoters. The *Rosa26* locus harbors an enhanced reverse tetracycline-controlled transactivator (M2-rtTA). Fig. 2a was adapted from Refs 97 and 98, licensed under CC-BY 4.0. Fig. 2b and 2c were created by the authors using graphical components from Servier Medical Art (<https://smart.servier.com/>), licensed under the Creative Commons Attribution 4.0 (CC BY 4.0) license.

4. Application of CRISPR Technology in Tumor Microenvironment-Related Diagnostic Analysis

4.1 Applications of CRISPR Technology in Nucleic Acid Detection

Liquid biopsy is a noninvasive diagnostic method that enables early screening and diagnosis of tumors by detecting biomarkers, such as ctDNA, miRNA, DNA methylation, and exosomes, in samples such as blood and saliva (77). However, because ctDNA levels are low in blood (only 1% of cell-free DNA), amplifying variable signals is crucial. Currently, clinical quantitative detection is mainly based on polymerase chain reaction (PCR) techniques. Still, the expensive instruments and cumbersome operational procedures limit the use of PCR in basic home assays and point-of-care testing (POCT) (78). Recently, the CRISPR-Cas system has become widely applicable for molecular detection (79). Several CRISPR-Cas-based detection platforms, including DETECTR, HOLMES, and SHERLOCK, have been developed as point-of-care diagnostic tools enabling higher specificity and sensitivity in pathogen detection (80). ctDNA is a free DNA fragment released into the blood by tumor cells that carries information on gene mutations, copy number variations, aberrant methylation, and other genomic alterations. However, the low abundance of wild-type DNA in blood and the high background of wild-type DNA impede precise and specific measurement of ctDNA (78). Cas9 enables ctDNA detection through its DNA-targeting and precise cleavage capabilities. The primary detection mechanisms include pre-amplification, engaged amplification, and post-amplification. Using Cas9 before amplification removes background nucleic acids and enriches for rare targets. DeRisi et al. developed the DASH (81) technique, which uses Cas9 to enrich for targeted ctDNA and reduce interference from background wild-type DNA. The precise cleavage properties of Cas9 can also be used to distinguish amplification products after amplification, thereby improving detection specificity. During amplification, Cas9 relies on a specific protospacer-adjacent motif (PAM) for cleavage; however, not all target sequences meet the PAM design requirements. To address this limitation, we can engineer Cas9 derivatives by altering PAM specificities. For example, *Streptococcus pyogenes* Cas9 (SpCas9) (82) can be modified to recognize alternative PAM sequences by leveraging structural information, directed evolution via bacterial screening, and combinatorial design. Later, researchers confirmed that Type II CRISPR-Cas9 systems, produced by crRNA and tracrRNA, exhibit trans-cleavage activity (83) on both ssDNA and ssRNA substrates, depending on the RuvC domain (84). Cas9 exhibits sequence preference for trans-cleavage substrates, preferring to cleave T- or C-rich ssDNA substrates. Based on the trans-cleavage activity of Cas9 and nucleic acid amplification technology, a

DNA/RNA-activated Cas9 detection platform has been developed, enabling detection of trace nucleic acids via signal amplification.

CRISPR-Cas12a proteins are used in molecular diagnostics. Unlike Cas9, Cas12a is guided by a T-rich CRISPR RNA and recognizes specific PAM sequences to bind and cleave double-stranded DNA. Upon site-specific cleavage, Cas12a undergoes a conformational activation and non-specific trans-cleavage activity against single-stranded DNA. This unique cleavage property has been exploited for nucleic acid detection. By integrating the trans-DNA cleavage activity of Cas12a with isothermal amplification methods, researchers developed the DETECTR platform (23). DETECTR is based on target-dependent activation of Cas12a, which induces indiscriminate cleavage of engineered ssDNA reporter substrates labeled with paired fluorophore-quencher moieties, resulting in a detectable fluorescence signal. This signal amplification strategy enables highly sensitive detection of target DNA sequences at the attomole level and provides a rapid, scalable approach for molecular diagnostics (Figure 3a).

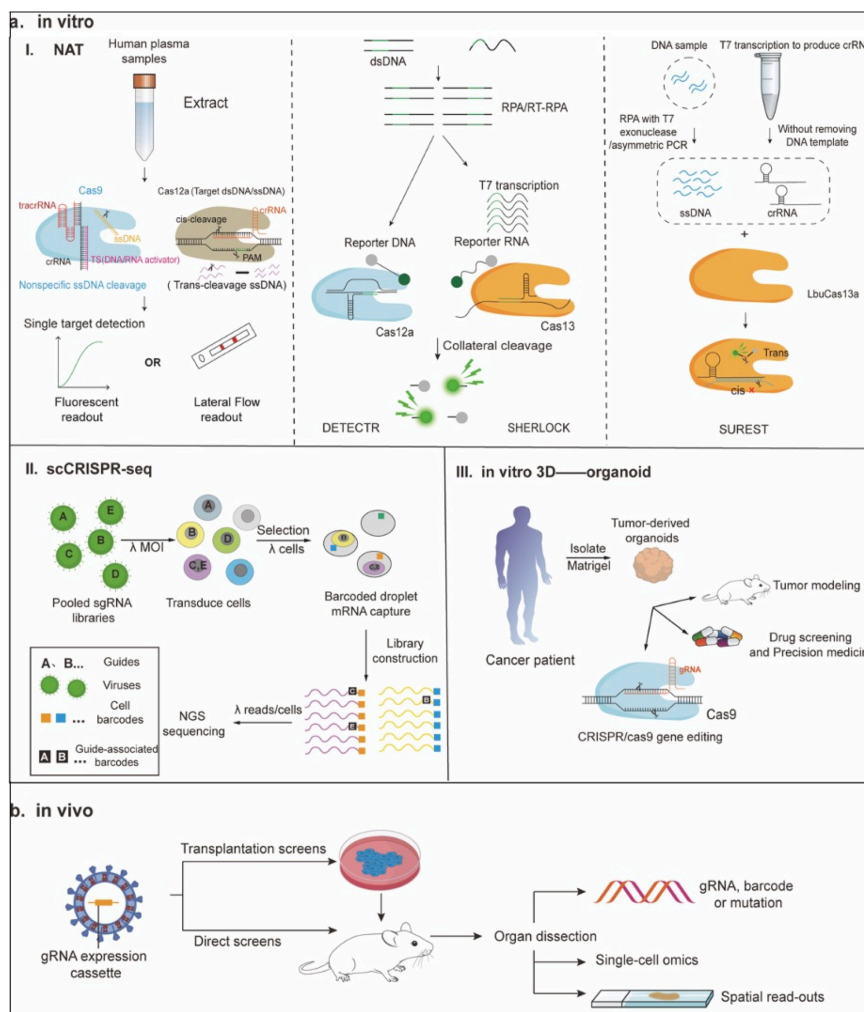


Figure 3. CRISPR-enabled platforms for molecular diagnostics and functional genomics. (I) *Nucleic acid detection: The trans-cleavage activity of Cas9 is activated upon binding to DNA or RNA targets. Cas12a allows for cis-cleavage and trans-cleavage. Subsequently, fluorescence detection or lateral flow readout is performed. DNA or RNA is amplified through RPA or RT-RPA, respectively. The amplified RPA products are converted into RNA through T7 transcription. When crRNA binds to its complementary target sequence, it activates the Cas enzyme, triggering collateral cleavage that quenches the fluorescent reporter. Thus, Cas13a (for the SHERLOCK system) or Cas12a (for the DETECTR system) indicates the presence of RNA or DNA target sequences, respectively. For Cas12 detection, the T7 transcription step is omitted, allowing direct detection of the amplified target. The fundamental principle of the SUREST platform is that the LbuCas13a-crRNA-ssDNA ternary complex exhibits significant trans-cleavage activity when targeted to DNA.* (II) *Perturb-Seq: By constructing the pooled sgRNA library and infecting cells with a pool of viral vectors encoding sgRNA, each cell's mRNA (including GBC) is labeled with a unique cell barcode (CBC) and unique molecular identifier (UMI) for scRNA-seq.* (III) *Organoid technology can be used to model various human cancers, test drug efficacy and toxicity in precision medicine, and develop novel targeted therapies. In addition, synergistic use of CRISPR-Cas9 gene editing can elucidate the pathophysiology of various cancers and investigate the effects of specific genetic changes on tissue function and disease progression.* (b) *In vivo CRISPR screening is divided into transplant screening and other types of in vivo screening. In transplantation screening, cells are transduced in vitro and then transplanted*

the amplified target. The fundamental principle of the SUREST platform is that the LbuCas13a-crRNA-ssDNA ternary complex exhibits significant trans-cleavage activity when targeted to DNA. (II) Perturb-Seq: By constructing the pooled sgRNA library and infecting cells with a pool of viral vectors encoding sgRNA, each cell's mRNA (including GBC) is labeled with a unique cell barcode (CBC) and unique molecular identifier (UMI) for scRNA-seq. (III) Organoid technology can be used to model various human cancers, test drug efficacy and toxicity in precision medicine, and develop novel targeted therapies. In addition, synergistic use of CRISPR-Cas9 gene editing can elucidate the pathophysiology of various cancers and investigate the effects of specific genetic changes on tissue function and disease progression. (b) In vivo CRISPR screening is divided into transplant screening and other types of in vivo screening. In transplantation screening, cells are transduced in vitro and then transplanted

into adult animals. In direct screening, the gRNA library is delivered directly to the target tissue or cell type in the mouse. Subsequently, screening readouts are performed, with phenotypes measured as changes in gRNA or barcode abundance or frequency, or using single-cellomics or imaging techniques to record multidimensional phenotypes for each perturbation. Fig. 3a was adapted from Ref 89, licensed under CC-BY-NC-ND 4.0 (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), Fig. 3b was created using graphical components from Servier Medical Art (<https://smart.servier.com/>), licensed under Creative Commons Attribution 4.0 (CC BY 4.0).

SHERLOCK (32) is a nucleic acid detection technology based on CRISPR-Cas13 and RPA, which amplifies DNA or RNA using RPA or RT-RPA, respectively. For CRISPR enzymes targeting RNA (including Cas13a), amplified RPA products are converted into RNA by T7 transcription. When crRNA binds to its complementary target sequence, it activates the Cas enzyme, triggering collateral cleavage that quenches the fluorescent reporter. Additionally, Wu et al. developed a new nucleic acid detection platform, the Superior Universal Rapid Enhanced Specificity Test (SUREST), using LbuCas13a (33). Studies have shown that the LbuCas13a-crRNA-ssDNA ternary complex exhibits significant trans-cleavage activity against DNA. Compared with conventional CRISPR-Cas systems, SUREST can target DNA specifically and directly without being restricted by the PAM sequence, opening new avenues for its application in molecular diagnostics. Without amplification, the detection limit of the CRISPR-Cas12a RNA-guided complex is only at the pico-molar level. Therefore, a platform called CRISPR-ENHANCE (85) (Enhanced Analysis of Nucleic acids with CrRNA Extensions) was developed. The trans-cleavage ability of target-activated LbCas12a is enhanced when the 3' end of crGFP is extended by ssDNA and ssRNA. At the same time, the specificity of target recognition was significantly improved. The platform, based on engineered crRNAs and optimized conditions, enables the detection of multiple clinically relevant nucleic acid targets with higher sensitivity, achieving femtomolar detection limits without any target pre-amplification steps.

miRNAs are endogenous, short-stranded non-coding RNAs (86) (18-23 nucleotides), whose abnormal expression is closely related to the occurrence and development of tumors. However, miRNAs are short, low in abundance, and exhibit high sequence similarity within miRNA families; therefore, the sensitivity and specificity of their detection are of particular importance. RT-qPCR is the gold standard technique for detecting miRNA (87), but due to its high cost, researchers have turned to the CRISPR-Cas enzyme system. Researchers have developed a series of detection methods based on the CRISPR-Cas12a system, combining CRISPR-Cas12a with technologies such as fluorescence in situ hybridization (FISH), exponential amplification reaction (EXPAR), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), and microfluidic systems. They have the advantages of high sensitivity and low detection limits. Despite its advantages in simplicity and specificity, the CRISPR-Cas system still relies on independent pre-amplification, which limits its clinical applications.

As a result, Jain et al. developed the Split Activator for Highly Accessible RNA Analysis (SAHARA) (88). In the presence of a split activator, multiple Cas12a homologs will activate trans-cleavage. The method detects picomolar concentrations of RNA without sample amplification, strand replacement, or reverse-transcription by simply supplying a short DNA sequence complementary to the seed region. However, picomolar levels (250-700 pM) of RNA are not sufficient for clinical assays. The researchers then improved the "Cas12a-crRNA split-activator" complex into the split Cas12a system (SCas12a (89)), in which the Cas12a enzyme binds to a split crRNA containing both scaffold RNA and spacer RNA components. By utilizing miRNA targets as spacer RNA and another ssDNA as the activator, SCas12a-based fluorescence and rapid lateral flow assays were developed. It can distinguish between mature miRNA and pre-miRNA with identical sequences (89). This method enables highly sensitive and selective miRNA detection without the need for reverse transcription or pre-amplification, with a limit of detection (LoD) of 100 femtomoles (fM). Similarly, another study developed an asymmetric

CRISPR assay that does not require pre-amplification or reverse transcription, utilizing the asymmetric trans-cleavage behavior of competitive crRNA. The competitive reaction between a full-sized crRNA and a split crRNA in CRISPR-Cas12a induces cascade signal amplification and significantly improves target detection.

Research found that Cas12a could directly recognize RNA targets when the RNA was located at the 3' end of the crRNA and supported by a DNA at the 5' end of the crRNA, implying that CRISPR-Cas12a could recognize fragmented RNA/DNA targets, enabling direct detection of RNA (90). The assay sensitivity is 856 aM. In contrast to SCas12a, asymmetric CRISPR not only involves a complex reaction system but also relies on the full-length crRNA, effectively inhibiting Cas12a system activation in the presence of split crRNA and corresponding substrates. This effectively prevents false-positive results, but achieving this suppression requires relatively stringent conditions.

Not only can we reduce false positive results by avoiding pre-amplification, but we can also reduce them through a one-step, one-pot, isothermal CRISPR-Cas12a assay called "Endonucleolytically Exponentiated Rolling Circle Amplification with CRISPR-Cas12a" (EXTRA-CRISPR (86)). This method integrates multiple reactions, including target-mediated ligation, RCA, Cas12a binding, and nucleolytic cleavage, into a single reaction network. It uses cis-cleavage activity to convert conventional linear RCA into one that exponentially amplifies the target sequence, while employing the trans-cleavage reaction for amplicon detection and signal amplification. Its analytical performance is comparable to RT-qPCR, including high sensitivity with a single-digit femtomolar detection limit, single-nucleotide specificity, and rapid, flexible turnaround (analysis times ranging from 20 minutes to 3 hours, depending on the target and sample). This dramatically simplifies the assay process and facilitates POC diagnosis.

Additionally, a Cas12a-assisted in vitro diagnostic tool can be used to discriminate single-CpG-site methylation in DNA. This method uses methylation-sensitive restriction endonucleases (MSREs) (91) to digest non-methylated sequences, thereby fragmenting the target sequences and affecting R-loop formation between the crRNA and the target DNA. By analyzing the effects of fragment size, cleavage position, and fragment quantity on the trans-cleavage activity of ssDNA, the methylation sites can be inferred. This approach enables the study of individual CpG methylation sites for disease detection. Limitations of CRISPR-Cas diagnostic techniques include cleavage of off-target DNA (92) and the difficulty of developing multiplexed detection formats for single-tube assays. The cleavage of off-target DNA can lead to false-positive diagnoses, and specificity can be improved by engineering the guide RNA (93) or by modifying it to incorporate DNA (94) into the Cas9 gRNA (95). It isn't easy to develop multiplexed detection formats in single-tube detection. Because the CRISPR system relies on an undifferentiated trans-cleavage mechanism. The researchers divided the process into two steps, each with a separate NAA and CRISPR assay. However, the liquid transfer process is cumbersome and susceptible to aerosol contamination, which may lead to false-positive results.

The research developed "The one-pot CRISPR detection strategy (96)", which can be achieved by optimizing reaction components, performing physical or chemical separation, and selectively targeting amplification products. To achieve optimal detection efficiency, precise control of Cas12 cleavage activity is essential. For example, the cleavage activity of the CRISPR-Cas12a system can be regulated by altering the PAM site. When mediated by non-optimal PAM sites, cleavage efficiency is reduced, but INA is hardly affected. The altered PAM-site strategy was shown to be infeasible in CRISPR-Cas12b, possibly due to loss of cleavage activity resulting from mutation of the TTN site. Therefore, a one-pot assay strategy combining CRISPR-Cas12b mutants and LAMP amplification was developed to

weaken the binding affinity of Cas12b proteins for target DNA by modifying their PAM recognition domains.

Additionally, the RG4 structure (97) can be added to the 5' end of crRNA to control the activity of Cas12a. By introducing a PC linker between the RG4 and DR regions (G4-PC-DR), a light-controlled system can be established to activate any Cas12a target by replacing the spacer region. In addition, we can modify and demodify the 3' end of the direct repeat (DR) region (98) to control Cas12a activity.

In response to the limitation that the CRISPR system relies on indiscriminate trans-cleavage mechanisms and the difficulty of differentiating between highly similar nucleic acid sequences, the researchers developed a method termed the Cas12a cis-cleavage-mediated lateral flow assay (cc-LFA) (99). This method uses a dual-key recognition mechanism based on CRISPR-Cas12a cis-cleavage and invasive hybridization of the released sticky-end DNA product. It integrates multiplexed nucleic acid amplification, a dual-key Cas12a detection mechanism, and a lateral-flow detection platform. Compared with the three mainstream CRISPR diagnostic technologies based on trans-cleavage, such as SHERLOCK, DETECTOR, and the Cas9-based CASLFA platform, cc-LFA exhibits higher specificity for the same DNA target. This technique enables single-base-resolution detection without interference from high concentrations of wild-type DNA.

4.2 Advances and Applications of Single-Cell CRISPR Screening Technology in Analyzing Tumor Heterogeneity

Cellular heterogeneity is manifested as phenotypic differences among cells, but ensemble-averaging methods mask these differences and the unique characteristics of individual cells (100). Single-cell sequencing analysis enables in-depth examination of cell populations by focusing on intercellular differences in expression profiles, preventing heterogeneity within individual cells from being masked by the homogenization of large numbers of cells. CRISPR technology, combined with highly multiplexed assays such as single-cell sequencing and spatial omics, facilitates analysis of tumor cell heterogeneity. They are the primary approaches for rapidly identifying cancer driver genes or tumor immune regulatory factors.

Perturb-seq (55) and CRISP-seq (101) are the first scCRISPR-seq platforms developed to analyze the transcriptional changes after gene perturbation at the single-cell level. Both Perturb-seq and CRISP-seq rely on indirectly labeled sgRNA, but there is a certain probability of error in pairing GBC or UGI with sgRNA (Figure 3a). Later, CROP-seq was developed (102). It reads the gRNA directly without requiring an additional barcode. However, the loading capacity of the vector is limited, and CROP-seq is not compatible with the delivery of multiple sgRNAs. Direct Capture Perturb-seq technology (103) addresses the limitations of CROP-seq by detecting multiple different sgRNA sequences in a single cell. We designed platforms for direct capture with both 5' and 3' scRNA-seq. The approach enables sequencing of expressed sgRNAs alongside the single-cell transcriptome, thereby directly pairing sgRNAs with phenotypes. At the same time, hybridization-based target enrichment technology can be used to sequence thousands of specifically selected transcripts. While maintaining a high intronic phenotype, the required sequencing is also reduced. Targeted Perturb-seq (TAP-seq) (104) can also enrich for target genes, thereby significantly decreasing sequencing depth and enhancing the detection of low-expression genes. By defining a predefined target gene set, the multiple-testing problem in traditional Perturb-seq (105) was addressed. Subsequently, some studies were conducted to reduce costs. Researchers developed the "compressed Perturb-seq105" technology which, based is nbased of compressed sensing theory. Two methods are included: cell pooling (overloading the microfluidic chip

to generate multicellular droplets) and guided pooling (high MOI infection to make cells contain multiple guides).

DNA barcoding technology can be analyzed only at the mixed cellular level and provides limited phenotypic analysis. In response, the researchers developed Pro-Codes (106). Pair each Pro-Code with different CRISPR gRNAs and conduct high-dimensional proteomic phenotypic analysis at the single-cell resolution for hundreds of genes. However, existing pooled CRISPR screening methods cannot provide information on extracellular events. Removing genes from the tissue environment results in the loss of much information. Therefore, the Perturb-map (107) was developed. It combines multiple imaging and spatial transcriptomics (108) while preserving spatial structure (109) and simultaneously analyzes the phenotypes of dozens of genes in tissues or tumors at the cellular resolution. The technique revealed that knocking out *Tgfr2* promotes tumor growth and transforms the TME into a fibromuscular state that excludes T cells.

In contrast, knocking out *Socs1* promotes tumor growth and also leads to T-cell accumulation in the tumor. Perturb-map can be combined with an ovarian cancer mouse model (110) that highly simulates the dissemination and heterogeneity characteristics observed in human ovarian cancer. It can reveal how factors influence tumor cell-immune cell interactions and ultimately affect immune therapy responses. However, Perturb-map sacrifices the advantages of the pool-cloning workflow by using protein barcodes, and the spatial resolution of Visium limits its applicability to non-clonal growing cells. Therefore, the researchers turned to imaging spatial transcriptomics (iST) and developed Perturb-FISH (111). This method combines multiplexed error robust fluorescence in situ hybridization (MERFISH) with in situ amplification of gRNA to achieve simultaneous spatial detection of gRNA and mRNA. The feasibility of the technique can be verified by screening for ASD risk genes in astrocytes and by recording calcium activity responses to ATP stimulation. OPS research uses information-rich phenotypic and functional readouts, such as live-cell imaging or cell staining. It uses a library covering the entire human genome to explore the functional consequences of gene disruption. However, traditional OPS is limited to low-throughput phenotyping because it is difficult to efficiently and accurately detect disturbed barcodes. PerturbView (112) was developed as a novel optical pool screening (OPS) technology. This technology uses in vitro transcription (IVT) to amplify barcodes before in situ sequencing, enabling screening with highly multiplexed phenotypic readouts across various systems, including primary cells and tissues. PerturbView is compatible with multi-phenotype analysis and spatial omics reads, enabling more comprehensive interpretation of the effects of genetic perturbations and tracking of cell status in both healthy and diseased conditions. PerturbView can detect barcodes in situ within tissues, promoting the development of in vivo operations in animal models.

In addition to transcriptomics, the integration of CRISPR functional screening with proteomics and epigenomics provides new perspectives for cancer research. ECCITE-seq (113) can simultaneously detect the transcriptome, proteins, clonal types, and CRISPR perturbations. ECCITE-seq has higher sensitivity in detecting expression phenotypes. Another parallel method, "Perturb-CITE-seq (114)", combines scRNA-seq analysis and CITE-seq to study single-cell surface proteins under perturbed conditions. It can be applied to patient-derived tumor immune models to analyze immune regulatory factors. Most single-cell multi-omics techniques used to analyze chromatin accessibility (115,116) are derived from the transposase-accessible chromatin sequencing (ATAC-seq) method (117). It utilizes the hyperactive transposase (Tn5) to measure the activity of regulatory DNA elements.

Subsequently, the Perturb-ATAC was developed. It is based on ATAC-seq and simultaneously detects CRISPR-guided RNA and open chromatin sites. It realizes a direct association between genotype and epigenetic phenotype. However, it is limited by high cost and low throughput. Spear-ATAC (118) and

CRISPR-sciATAC (119) were developed. Spear-ATAC reads sgRNA intervals directly from genomic DNA, unlike previous techniques that read from RNA transcripts. CRISPR-sciATAC employs a two-step combinatorial indexing process to label DNA molecules and assign them unique cellular barcodes, without requiring specialized equipment. Both techniques break through the limitations of high cost and low throughput. A single-cell CRISPR screen enables simultaneous analysis of genetic disruption and high-dimensional phenotypes in single cells. However, its application is limited to a few hundred genetic perturbation studies. Subsequently, CRISPRi (120) was developed to analyze the effects of thousands of loss-of-function genetic perturbations in different cell types. Moreover, this technology has produced the first comprehensive map that integrates the genotype and phenotype of human cells. Using CRISPR KO screening in pancreatic ductal adenocarcinoma (PDAC) cells (121), it was found that gene deletion either positively or negatively alters PDAC cell survival when MEK signaling is inhibited. CRISPR screening can be applied to identify drug targets and predict drug responses in cancer cells. The DREBIC method can capture the relative essentiality of drug targets (CRISPR screening activity score) and their relative abundance. It enables precise medicine by simulating overall drug responses and identifying drug-specific vulnerabilities associated with carcinogenic mutations.

CRISPR screening using organoids is the next level in *in vitro* systems, providing a model for revealing the genetic mechanisms of gene-drug interactions. Patient-derived organoids (PDOs) have been widely used in the study of various types of cancer, including colorectal cancer (CRC), lung cancer, pancreatic cancer, breast cancer, and liver cancer (122) (Figure 3a). For example, genome-wide CRISPR screening in 3D spheroids and xenograft tumors based on human non-small cell lung cancer cell lines showed that depletion of carboxypeptidase D (CPD) prevented tumor growth in spheroids and *in vivo* but was ineffective in 2D cultures. Growth phenotypes in 3D more accurately reflect observations in tumors. CRISPR screening can also be applied to primary human 3D gastric organoids (123) to identify genes that affect cisplatin sensitivity. In addition, CRISPR-mediated gene-engineered mouse models (CRISPR-GEMMs) (124) have been developed. For example, by combining the RCAS-TVA (125) and CRISPR-Cas9 systems, researchers developed an *in vivo* somatic-cell genome-editing mouse model. It can perform precise genetic modification on specific cell types to accurately model human tumors. The successful application of CRISPR-GEMMs depends on the effective delivery of interference reagents to target cells, which varies across different organs and cell types. Therefore, to date, CRISPR-GEMMs have been limited to cancers of the liver, lung, and brain. However, traditional 3D organoid cultivation techniques cannot precisely control the various factors in the TME over both time and space. It is essential to identify interacting regulators in TME. MEN1 (126) was identified as the most significant target, leading to differential shedding effects *in vitro* and *in vivo*. It has tumor microenvironment-dependent carcinogenic and anticancer functions. Therefore, *in vivo* screening via allogeneic transplantation of homologous mouse cancer cells, or via *ex vivo* or *in situ* transplantation of patient-derived cells (PDXs), is more closely aligned with a functional TME. These methods have led to the identification of genes associated with cancer cell immune escape or immune checkpoint blockade.

Therefore, CRISPR-based *in vivo* screening can be employed to identify immune regulatory genes that may serve as cancer prognostic markers, diagnostic markers, or potential drug targets. However, errors during cancer cell transplantation may confound screening results. Therefore, researchers developed an *in situ* CRISPR-Cas9 lung cancer screening method. By combining this approach with the adoptive transfer of cytotoxic T cells targeting tumor antigens in the model, the function of genes embedded in their native tissue structures was evaluated. It identified the known immune-escape factors Stat1 and Serpinb9, and the cancer testicular antigen Adam2 (127) as an immune-regulatory factor.

Furthermore, through an *in vivo* pooled CRISPR-Cas9 mutagenesis screening, it was discovered that Regnase-1 (128) is the primary negative regulator of antitumor responses. PTPN2 and SOCS1 can, in

the context of Regnase-1 deficiency, enhance antitumor immunity. Regnase-1-deficient CD8⁺ T cells are reprogrammed into long-lasting effector cells within the TME, resulting in significantly improved therapeutic effects in mouse melanoma and leukemia through enhanced BATF function and mitochondrial metabolism. In addition, additional screening was conducted in the E0771-OVA triple-negative breast cancer (129) and GL261 glioblastoma models (130) implanted in situ. These screens identified negative regulators of T cell responses, including DHX37, PDIA3, and MGAT5.

High-throughput in vivo genetic perturbation screening facilitates a deeper understanding of cell interactions. In vivo CRISPR screening (131), including transplantation-based screening and direct in vivo screening. Screening readouts are categorized as count-based and information-rich. In count-based screening, the phenotype is measured as the change in the abundance or frequency of a specific perturbation or perturbation identifier (e.g., gRNA or barcode). Information-rich screening uses single-cell omics or imaging to document multidimensional phenotypes for each perturbation (Figure 3b). In vivo screening yields less reliable gene identification due to bottleneck effects, increased overall noise, and delivery problems. Noise primarily arises from intercellular heterogeneity (132) and library-bottleneck effects. CRISPR screening relies on the hypothesis that cellular phenotypes are directly caused by experimentally induced perturbations. Intercellular heterogeneity threatens this hypothesis. For transplantation screening, bottlenecks include the model's upper limit on injection volume and the possibility that transplanted tumor cells may die. To address these issues, we could increase library representation, but this would significantly reduce the size of viable screening libraries, thereby averaging out behavioral inconsistencies across many cells carrying the same sgRNA. We can also create monoclonal cell lines. CRISPR nucleases are delivered in batches to the cell lines, which are then isolated as single cells through FACS sorting or serial dilution, followed by the growth of clonal lines. In direct in vivo screening, the process is often hampered by poor delivery efficiency. Faced with high noise in CRISPR-Cas9 in vivo screening, researchers developed CRISPR-STAR (133). By randomly activating sgRNAs and employing an internal control mechanism, the interference of cell growth heterogeneity and genetic drift with the experimental results was effectively mitigated, thereby significantly reducing experimental noise. It also conducted a genome-wide screen in Braf inhibitor-resistant mouse melanoma cells to identify specific genetic dependencies in vivo. It highlights the relevance of functional genetics in identifying potential novel drug targets.

5. CRISPR-Cas Delivery Systems: A Critical Determinant of Therapeutic Success

Efficient delivery of the CRISPR-Cas gene-editing system to cancer or immune cells is pivotal to its successful application in cancer therapy. Currently, three primary modalities are employed to achieve CRISPR-Cas9-mediated gene editing in target cells, including delivery of plasmids encoding the Cas9 protein and sgRNA, delivery of Cas9 mRNA together with sgRNA, and delivery of the Cas9 protein and sgRNA, either as separate components or as a pre-assembled ribonucleoprotein (RNP) complex (134).

Delivery strategies are broadly categorized into viral vectors, non-viral vectors, and physical methods (Figure 4). Viral vectors are relatively mature delivery platforms, including adeno-associated virus (AAV), lentivirus, and adenoviral vectors. While viral delivery offers high transfection efficiency, potential off-target effects and safety concerns limit its broader applicability. In contrast, non-viral vectors offer notable advantages, including low immunogenicity, high biocompatibility, enhanced safety, and lower production costs. This category encompasses liposomes, lipid-like nanoparticles, gold nanoparticles, virus-like particles, and cell-penetrating peptides. Among these, nanoparticle-based carriers demonstrate considerable potential for clinical translation (135) (Figure 4).

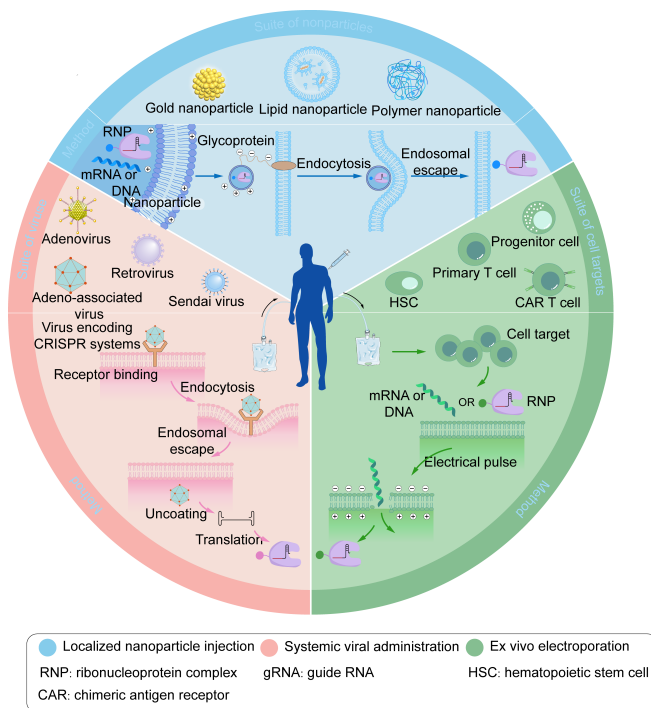


Figure 4. Three representative CRISPR delivery strategies include localized nanoparticle injection, systemic viral administration, and ex vivo electroporation. Nanoparticles loaded with mRNA, DNA, RNP, or viruses encoding CRISPR systems undergo receptor recognition, endocytosis, and endosomal escape, ultimately releasing the mRNA, DNA, or RNP into the cytoplasm. Electroporation utilizes high-voltage electrical pulses to deliver substances through transient tiny pores in cell membranes. Blue: Localized nanoparticle injection. RNP: ribonucleoprotein complex. CAR: chimeric antigen receptor. Pink: Systemic viral administration. gRNA: guide RNA. Green: Ex vivo electroporation. HSC: hematopoietic stem cell. Figure 4 was created by the authors using graphical components from Servier Medical Art (<https://smart.servier.com/>), licensed under the Creative Commons Attribution 4.0 (CC BY 4.0) license.

Physical methods facilitate entry by transiently disrupting the cellular membrane, including microinjection, electroporation, and sonoporation. However, the clinical application of physical methods is hindered by the challenge of determining optimal parameters that preserve the native properties of human tissues. The characteristics, optimization strategies, and suitable application scenarios for each of these delivery approaches have been extensively reviewed (8, 136-138). Collectively, these three delivery modalities have been successfully applied in vitro, ex vivo, and in vivo to enable CRISPR-Cas-mediated gene editing in cancer cells or immune cells (135, 139). AAV represents a classic vector platform for gene therapy (140). However, the commonly used nuclease *Streptococcus pyogenes* Cas9 (SpCas9) has a coding sequence of approximately 4.2 kb. The addition of essential gene regulatory elements brings the total size close to the AAV packaging limit (~4.7 kb), thereby constraining practical utility (141). The development of miniaturized CRISPR-Cas systems constitutes an alternative solution. The early-reported *Staphylococcus aureus* Cas9 (SaCas9), with a gene size of 3.2 kb, can be co-packaged with sgRNA into a single AAV vector and has been successfully employed for in vivo gene editing (142). Numerous other compact Cas9 orthologs have since been identified and characterized, including NmCas9 (*Neisseria meningitidis*) (143,144), CjCas9 (*Campylobacter jejuni*) (145), SauriCas9 (*Staphylococcus auricularis*) (146), BlatCas9 (*Blattella germanica*) (146), Nme2Cas9 (*Neisseria meningitidis*) (147), and IscB (putative ancestor of Cas9) (148). Commonly utilized Cas12a variants, such as *Acidaminococcus sp* Cas12a (AsCas12a) and *Lachnospiraceae bacterium* Cas12a (LbCas12a), exhibit sizes comparable to SpCas9 and are similarly constrained by the AAV payload capacity. Consequently, more minor Cas12 variants have been actively explored, including AaCas12b (*Alicyclobacillus acidiphilus*), BhCas12b (*Bacillus hisashii*), DpbCas12e (*Desulfofundulus piezophilus*), Cas12j (Uncultivated phage), and Cas12f (also known as Cas14-derived) (149-153). Among these, Cas12f nucleases represent the most compact CRISPR nucleases identified to date, typically half the size of conventional Cas9 and Cas12a, but their editing efficiency is generally lower. Cas12f engineered through rational mutagenesis exhibits markedly enhanced editing activity and reduced off-target effects and has been successfully applied to ameliorate

choroidal neovascularization in mouse models of macular degeneration (154). The incorporation of an α -helical domain at the N-terminus of the Un1Cas12f1 variant (CasMINI) (155), or the fusion of T5 exonuclease to either its N or C terminus (156), enables hyper editing efficiency in mammalian cells. Furthermore, compact members of the Cas13 family, such as Cas13bt (157), Cas13X (158), and Cas13Y (158), are sufficiently small for single-AAV packaging and effectively mediate RNA editing.

The efficacy of gene editing at tumor sites is affected by non-specific delivery and multiple intra- and extracellular barriers. TME-based stimuli-responsive CRISPR-Cas delivery systems provide a promising strategy for targeted editing, including redox-, pH-, enzyme-, ATP-, and microRNA-responsive platforms (159,160). For instance, a pH- and light-dual-responsive CRISPR nanotherapeutic (161), comprising a thioether-cross-linked polyplex core and an acid-cleavable polymer shell, maintains structural stability in systemic circulation while preferentially accumulating in acidic TMEs. Subsequent laser irradiation triggers spatiotemporally controlled activation of the CRISPR system, enabling precision therapy at tumor sites.

Incorporation of targeted ligands can further enhance delivery efficiency. The short peptide Angiopep-2 binds explicitly to low-density lipoprotein receptor-related protein 1 (LRP-1), which is abundantly expressed on both blood-brain barrier (BBB) endothelial cells and glioblastoma cells (162). Therefore, Angiopep-2-functionalized glutathione-responsive nanoparticles can penetrate the BBB and selectively target glioblastoma (163,164). Similarly, nanoparticle surface coatings composed of hyaluronic acid and trimethylamine N-oxide (TMAO) at optimized ratios exploit TMAO's anti-adhesive properties to facilitate penetration of intestinal mucus, thereby accumulating in colorectal tumor tissues via specific transcytosis across intestinal epithelia (165). Notably, erythrocyte membrane coatings (166) or erythrocyte-tumor cell hybrid biomimetic membranes (167) substantially outperform conventional chemical modifications by leveraging inherent immune-evasive properties, thereby enhancing targeting specificity, prolonging systemic circulation, and reducing immune clearance.

6. The Application of CRISPR Technology in Precision Therapy Targeting the TME

CRISPR-Cas gene-editing technology programmability and target specificity offers a powerful tool for cancer treatment (168). The tumor microenvironment (TME), a critical factor in tumor progression and treatment resistance, is heterogeneous. This heterogeneity makes it difficult for traditional therapies to achieve precise targeting, limiting efficacy and causing significant toxicity (169). However, the CRISPR-Cas system enables precise intervention within the TME; the following section details its specific applications.

6.1 Application of CRISPR Technology for Direct Gene Editing in Tumor Cells

Oncogenes and tumor suppressor genes play crucial roles in cancer pathogenesis, influencing tumor treatment resistance and progression (170). Abnormalities in oncogenes are typically associated with genetic mutation or abnormal amplification, which lead to overexpression of their encoded proteins, thereby causing uncontrolled cell growth and proliferation (171). CRISPR-Cas9 technology can specifically target and knock out oncogenes such as MYC, KRAS, HER-2, MIEN1, MASTL, and EGFR, providing precise intervention strategies for tumor treatment (172,173). For instance, MYC is overexpressed in most human tumors, driving cancer cell growth and proliferation by affecting physiological processes such as gene expression, cell differentiation, and angiogenesis, and by interfering with apoptosis and DNA repair. Therefore, MYC-targeted therapy has become an essential strategy for treating malignant tumors (174). The KRAS oncogene is also a crucial target for cancer therapy (Figure 5a). Research has shown that using CRISPR-Cas9 to target mutant KRAS genes can

effectively inhibit the survival and proliferation capacities of cancer cells both *in vitro* and *in vivo*. It opens up new avenues for the treatment of malignant tumors such as colorectal cancer, lung cancer, and pancreatic cancer (175). Additionally, another study has shown that knockdown of the HPV E6 and E7 genes using CRISPR-Cas9 can reduce cell viability, increase p53 and p21 expression, and significantly inhibit proliferation of HPV-driven cancer cells (176).

Reversing drug resistance is also an essential strategy in cancer cell therapy. The fundamental reason lies in the emergence of mutations in drug resistance genes in tumor cells. At the same time, knockout chemotherapy resistance genes using CRISPR-Cas9 is expected to play a significant role in overcoming drug resistance. For example, the Nuclear Factor Erythroid 2-Related Factor (NRF2) is involved in the evolution of drug resistance in lung cancer. Studies have shown that after knocking out the NRF2 gene in chemotherapy-resistant lung cancer cells using CRISPR-Cas9, the efficacy of anticancer drugs such as cisplatin, carboplatin, and vinorelbine is restored, inhibiting tumor growth (177,178). Similar findings have also been reported in breast cancer, where tumor resistance arises from HER2 gene mutation (179). Therefore, therapeutic regimens combining standard treatment options (e.g., chemotherapy) with gene-editing technologies may address disease recurrence or treatment failure due to drug resistance (170).

CRISPR-Cas9 can be used to restore the function of various tumor suppressor genes (e.g., TP53, PTEN, BRCA). Upon reaching the target DNA site, Cas9 cleaves and forms specific double-strand breaks, which activate cellular repair mechanisms and subsequently repair the genome via NHEJ (Non-Homologous End Joining) or HRR (Homologous Recombination Repair) pathways¹⁵. Phosphatase and Tensin Homolog (PTEN) is an essential, multifunctional tumor suppressor gene that suppresses various cellular processes by antagonizing the phosphoinositide 3-kinase (PI3K)/AKT pathway. The loss of its activity is associated with the development of many malignant tumors and the emergence of drug resistance (180). Research has confirmed that the CRISPR-dCas9 system can specifically activate PTEN expression in BRAF-mutant melanoma or triple-negative breast cancer (TNBC) human cell lines, thereby remarkably inhibiting relevant downstream carcinogenic signaling pathways and ultimately suppressing cell proliferation and migration (Figure 5a) (181). Despite the demonstrated potential of this therapy, comprehensive and careful evaluation of its safety and reliability is still required to establish CRISPR-based repair of tumor suppressor genes as a viable treatment strategy. Technical challenges such as the low efficiency and incidence of HDR repair, or the lower accuracy of NHEJ repair compared to HDR, remain to be addressed (182).

CRISPR-Cas12a is a common RNA-guided endonuclease. Compared with Cas9, Cas12a possesses several distinct advantages, including greater accuracy in targeted editing and the ability to perform multiplex targeting (183,184). The human epidermal growth factor receptor (EGFR) is frequently overexpressed in tumor cells. Its dysregulation can activate carcinogenic signaling pathways, affect the cell cycle, apoptosis, and metastasis, thereby aggravating malignant phenotypic alterations (185). The therapy that delivers a single oncolytic adenovirus (Ad) intratumorally, co-expressing Cas12a and CRISPR RNA (crRNA) targeting the EGFR gene (Ad/Cas12a/crEGFR), has been proven to be specific and precise in editing the targeted EGFR gene (Figure 5a). It efficiently downregulates EGFR expression and ultimately exerts a potent anti-tumor effect by inducing cancer cell killing and inhibiting cancer cell proliferation (172,186).

Furthermore, leveraging its ability for multiplex gene targeting, Cas12a has been employed to simultaneously target and knock out three oncogenic mutant genes in colorectal cancer patients, such as TP53, the Adenomatous Polyposis Coli gene (APC), and the Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene (PIK3CA), thereby reducing the proliferation of cancer cells

(187). Targeting and identifying synthetic lethal interactions in tumors through CRISPR-Cas12a also represents a promising therapeutic strategy. Synthetic lethality refers to a phenomenon in which the simultaneous inactivation of two genes results in cell death, whereas inactivating either alone is tolerated (188). For instance, studies have revealed that certain breast or ovarian cancer cells carrying BRCA1 or BRCA2 mutations are sensitive to PARP inhibitors. The simultaneous loss of both genes can lead to cell death by suppressing DNA repair (189-191). Integrating this principle with CRISPR technology may enable the selective elimination of tumor cells without damaging normal cells, by targeting genes other than the specific gene that is absent in tumor cells (192,193).

CRISPR-Cas13a is a novel crRNA-guided, RNA-targeting Cas enzyme capable of specifically knockdown single-stranded RNA levels without altering the host genome, with significantly reduced off-target effects (62,194). In a study, researchers specifically used CRISPR-Cas13a to knock down KRAS-G12D mRNA in pancreatic cancer cells. The results showed that the expression of this mRNA was efficiently reduced, significantly restraining tumor cell growth and proliferation both in vitro and in vivo, and increasing cell apoptosis (195). Nevertheless, its practical application strategies remain to be further explored.

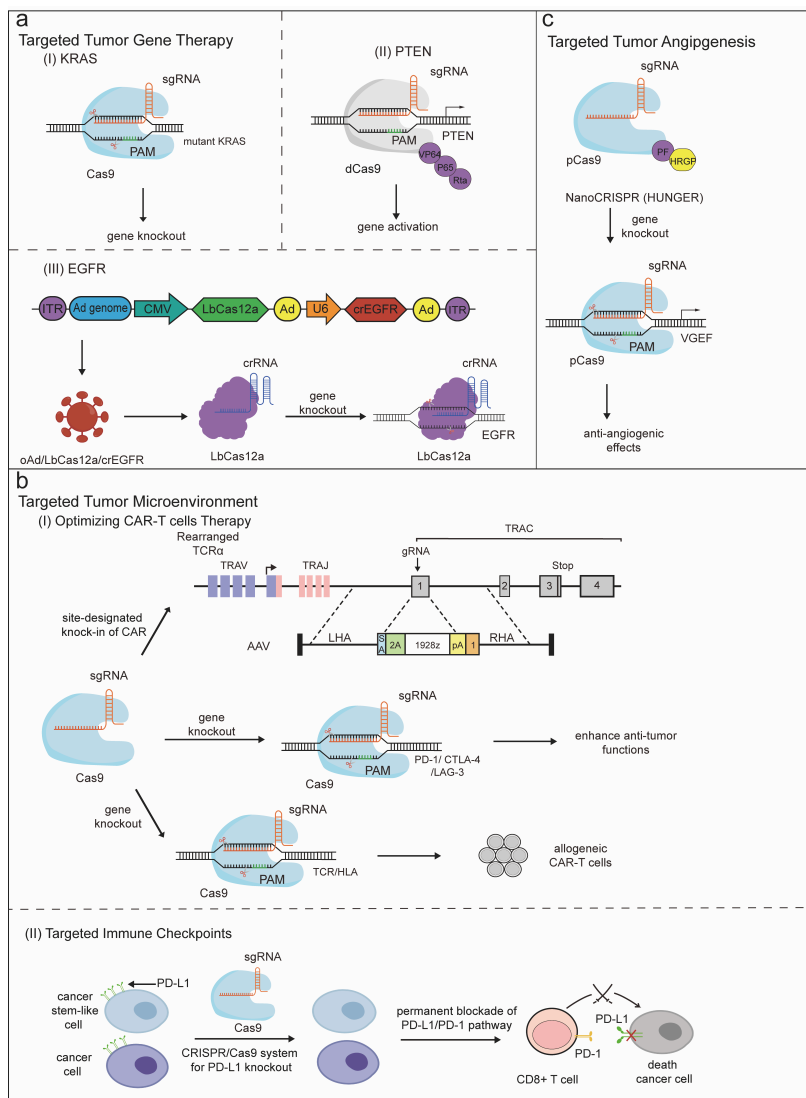


Figure 5. CRISPR-based precision therapy for tumors. (a) The therapeutic strategies for targeting tumor genes. (I) Using CRISPR-Cas9 to knock out targeted mutant KRAS genes. (II) The sgRNA-dCas9-VPR complex mediates PTEN activation. (III) EGFR gene knockout mediated by vectors co-expressing Cas12a and crRNA targeting the EGFR gene. (b) The therapeutic strategies for targeting the tumor microenvironment. (I) Enhancing tumor therapeutic efficacy through different CRISPR-mediated optimized CAR-T cell therapy strategies, including Cas9-targeted CAR gene integration into the TRAC locus, knockout of inhibitory genes such as PD-1, CTLA-4, and LAG-3, as well as editing allogeneic T cells. (II) Permanent blockade of the PD-L1/PD-1 pathway via the CRISPR-Cas9 system. (c) Therapeutic strategies targeting tumor angiogenesis are being explored. Inhibition of tumor growth and promotion of anti-angiogenic effects can be significantly achieved by precisely disrupting VEGF autocrine and paracrine pathways using HUNGER. Figure 5 was created by the authors using graphical components from Servier Medical Art (<https://smart.servier.com/>), licensed under the Creative Commons Attribution 4.0 (CC BY 4.0) license.

6.2 CRISPR-Cas for Remodeling the Immune Microenvironment

The efficacy of cancer immunotherapy largely depends on the tumor microenvironment, particularly the tumor immune microenvironment (TIME), which includes tumor cells, immune cells, cytokines, and other components (196). The roles of these components are divided into anti-tumor and pro-tumor. The former primarily includes cytotoxic T lymphocytes (CTLs), natural killer cells (NK cells), macrophages, dendritic cells (DCs), IFN- γ , and TNF- α , among others. In contrast, the latter primarily comprises regulatory T cells (Tregs), tumor-associated macrophages (TAMs) or myeloid-derived suppressor cells (MDSCs), TGF- β , IL-10, and PD-1 (197). Tumors can evade immune surveillance by constructing an immunosuppressive microenvironment. Immunotherapies targeting this mechanism aim to activate or restore the immune system's inherent tumor-suppressive function, thereby remodeling the immune microenvironment and improving the efficacy of anti-tumor treatment. CRISPR-Cas technology is a vital tool in immunotherapy, exerting therapeutic effects by enhancing anti-tumor immune responses or weakening pro-tumor responses (198). Strategies of CRISPR-Cas in therapies aimed at enhancing immune responses include modifying immune cells such as T cells and NK cells leveraging targeted gene-editing capabilities to improve their therapeutic efficacy, deleting genes that negatively regulate the immune system such as immune checkpoints to enhance immune activity, and precisely inserting beneficial genes such as cytokines or other immune mediators to strengthen immune responses to specific antigens (199).

With its capabilities for targeted delivery, gene editing, precision, and high efficiency, CRISPR-Cas9 can enhance the convenience, cost-effectiveness, and safety of CAR-T cell therapy. It also serves as a key support for the development of next-generation CAR-T cells, which hold broad application prospects (200). CAR-T cell therapy is based on genetic modification, enabling autologous or allogeneic cells to efficiently express a chimeric antigen receptor (CAR), thereby allowing immune cells to target tumor-associated antigens (TAAs) specifically. This precise targeting endows CAR-T cells with powerful anti-tumor capabilities (201,202). Although CAR-T cell therapy has achieved significant success in treating hematological tumors, its efficacy and applicability in solid tumors remain unsatisfactory, constrained by factors such as limited tumor infiltration, T-cell exhaustion, and toxicities (203,204). To overcome these limitations, CRISPR technology has been employed to engineer CAR-T cells with improved efficacy and reduced toxicity (Figure 5b). In the traditional manufacturing of CAR-T cells, transduction is typically performed by retroviral vectors carrying chimeric receptor sequences, which randomly integrate into the T-cell genome. This may lead to issues like carcinogenic transformation, unstable transgene expression, and transcriptional silencing. CRISPR-Cas9, however, enables delivery of the DNA cassette encoding CAR to specific genomic loci, enabling site-specific knock-in of CAR at target sites. For example, integrating CD19 CAR into the TRAC locus of T cells can result in more uniform and stable CAR expression, thereby increasing therapeutic efficacy (205). Additionally, using CRISPR-Cas9 to knock out inhibitory genes such as PD-1, CTLA-4, and LAG-3 on T cells has been certified to reverse T-cell exhaustion and enhance anti-tumor functions (206).

Furthermore, leveraging CRISPR-Cas9 to edit allogeneic T cells through the knockout of TCR and HLA genes can reduce alloreactivity and prevent graft-versus-host disease (GVHD). Such a strategy will reduce costs and expand access to CAR-T cell therapy for patients with insufficient healthy lymphocytes and for critically ill cancer patients (207,208). Cas9-mediated multiplex gene editing can be applied to establish universal CAR-T cells. Studies have shown that triple knockout CAR-T cells (with TRAC, B2M, and PD-1 knocked out) not only fail to induce GVHD but also display enhanced anti-tumor activity (209,210). Severe toxic reactions are a significant factor limiting the application of CAR-T. Among them, cytokine release syndrome (CRS) is a common acute toxic condition triggered

by excessive cytokine secretion from activated CAR-T cells. It may lead to fever, hypotension, vascular leakage, and multiple organ dysfunction (211). One strategy to overcome such toxicity is to control the activity of CAR-T cells by integrating suicide genes. For example, genetically modified CAR-T cells that express iCasp9 can be utilized to induce apoptosis when severe toxicity occurs, thereby controlling cytotoxicity during treatment (204,212). Another strategy is using CRISPR-Cas9 to knock out genes encoding cytokines that drive CRS, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6. Studies have shown that GM-CSF-deficient CAR-T cells can exhibit improved antitumor activity, longer survival, and fewer adverse events (213,214).

Optimizing the anti-tumor efficacy of CAR-NK cells via CRISPR-Cas9 is also the subject of ongoing research and development. Natural killer cells (NK) are important innate immune cells that can exert immune effects without antigenic stimulation and exhibit potent antitumor effects. Therefore, the development of CAR-NK cells and their application in immunotherapy have also attracted much attention (215). Compared with CAR-T cells, CAR-NK cells possess several unique advantages. Firstly, CAR-NK cells exhibit superior safety, with a lower risk of GVHD, CRS, and neurotoxicity. Secondly, since CAR-NK cells can kill tumor cells via either CAR-dependent pathways or their inherent cytotoxic activity, they may effectively eliminate specific heterogeneous tumor cells that do not express the CAR-targeted antigen. Thirdly, CAR-NK cells have a broader range of donor sources, facilitating large-scale production (216). However, CAR-NK cells still face a range of challenges, including limited efficacy within the suppressive TME, short-term persistence, and greater difficulty with genetic modification compared with other immune cells, thereby requiring further optimization (217). As an emerging method for delivering the CAR gene into NK cells, CRISPR-Cas9 technology allows for more precise localization of the CD19-CAR cassette. It may lead to higher expression levels, yet the efficiency of gene delivery remains suboptimal (215,218). CD38 is a potential immunotherapeutic target, with generally elevated expression in malignant plasma cells, and is also expressed on mature myeloid cells and their precursors. A study proposed an approach that merits further exploration. Transducing affinity-optimized anti-CD38 CAR variants into CAR-NK cells can enhance cytotoxicity against CD38-positive cells. Meanwhile, to reduce toxicity toward normal cell populations, researchers used CRISPR-Cas9 to target and knock out CD38 in NK cells, thereby preventing fratricide among NK cells and prolonging their therapeutic duration (219).

Knockout of specific genes, such as TIM3, TIGIT, and CISH, through CRISPR-Cas9, may also be an effective strategy to optimize CAR-NK cells (220). T cell immunoglobulin mucin family member 3 (TIM3) is a receptor expressed on NK cells, which is believed to play a vital role in inhibiting NK cell function. Studies have shown that using Cas9 to knock out TIM3 in NK cells enhances NK-cell growth-inhibitory activity against glioblastoma (GBM) cells, a promising therapeutic approach (221). TIGIT, a checkpoint receptor expressed on T cells and NK cells, is believed to be involved in inducing T-cell exhaustion in the TME. Studies have also indicated that blocking TIGIT can prevent NK cell exhaustion and amplify anti-tumor immune effects (222,223). Therefore, generating CAR-NK cells that overexpress the anti-EGFR-CAR gene and knock out TIGIT via CRISPR-Cas9 genetic engineering may be a feasible strategy to enhance anti-tumor activity (224). In addition, CRISPR-Cas9 can also be applied to develop “off-the-shelf” CAR-NK cells. For instance, researchers have demonstrated that targeting the B2M gene in NK cells with Cas9 to downregulate HLA-I surface expression can reduce T-cell-mediated destruction of CAR-NK cells. Furthermore, upregulating HLA-E and CD47 expression can inhibit fratricide among NK cells and reduce the destruction of CAR-NK cells (225,226).

Targeted modification of macrophages via CRISPR-Cas9 may also be a potential strategy for immunotherapy. Macrophages are innate immune cells that participate in immune responses through antigen presentation and phagocytosis. Under the influence of the TME, tumor-associated macrophages

(TAMs) can differentiate into two subtypes: M1 and M2. Particularly, M1 macrophages exert anti-tumor effects, while M2 macrophages promote tissue repair and tumor growth. Notably, TAMs predominantly exhibit the M2 phenotype in the TME, which is often associated with worse prognosis (227). Compared with CAR-T cells, CAR-M cells exhibit advantages, including a higher rate of tumor infiltration, an enhanced capacity to promote antigen presentation, and reduced toxic effects. Therefore, leveraging CRISPR-Cas9 gene-editing technology to develop and optimize CAR-M cell therapy holds significant potential in immunotherapy (228). Notably, the CRISPR-Cas9-mediated strategy that precisely knocks in DNA templates has been successfully applied to optimize CAR-T cells, demonstrating its potential in CAR-M cells (229). For example, a study employed CRISPR-Cas9 gene editing to integrate the anti-GD2 CAR into human pluripotent stem cells (hPSCs), which were then differentiated into anti-GD2 CAR-M cells. As a result, these CAR-M cells exhibited potent antitumor cytotoxic activity against GD2-expressing neuroblastoma cells (230,231). Moreover, other therapeutic directions worth exploring include genetically engineering CAR-M cells to be HLA-deficient to produce more universally applicable hypoimmunogenic macrophages, and modifying CAR-M cells to express anti-CD47 CARs or SIRP α -deficient variants to enhance their anti-tumor capabilities (230,232).

The CRISPR-Cas12a gene-editing technology can also be applied in the development and optimization of CAR-T cell therapies. For instance, it can introduce CAR genes into specific genomic loci, generating CAR-T cells with enhanced specificity and efficacy. Additionally, CRISPR-Cas12a can knock out the PD-1 gene with higher efficiency than Cas9, thereby reducing T-cell exhaustion and preserving their anti-cancer activity (212,233). A novel editing platform, CRISPR-Cas12b, has also been experimentally applied for multiple editing of CAR-T cells, mediating efficient and specific disruption of three genes (B2M, CIITA, and TRAC). Results indicate that these CAR-T cells demonstrated potent antitumor efficacy and eliminated GVHD; however, this strategy still requires further clinical investigation (234).

The CRISPR-Cas system can diminish immunosuppression by the knockout of immune checkpoint ligands or targeting inhibitory cytokines, thereby enhancing anti-tumor efficacy. CRISPR-Cas9-mediated targeted knockout of immune checkpoints, such as programmed death ligand 1 (PD-L1), is a key strategy in immunotherapy that can effectively block tumor immune escape (235). PD-L1 is often abnormally overexpressed in tumor cells, and the programmed death ligand-1/programmed death-1 (PD-L1/PD-1) signaling pathway represents a crucial mechanism underlying tumor immunosuppression. It can inhibit T-cell activation, enhance tumor cell immune tolerance, and ultimately facilitate tumor immune escape (236). Therefore, precise knockout of PD-L1 in tumor cells using CRISPR-Cas9 technology enables a permanent blockade of the PD-L1/PD-1 signaling pathway, thereby remodeling the suppressive tumor microenvironment (TME) and activating immune cells to enhance anti-tumor immunity (Figure 5b). Meanwhile, it may also increase tumor cell sensitivity to chemotherapy, thereby improving chemotherapeutic efficacy and broadening its applications (237). X. Luan et al. reported a nanoplatform (HMnMPH) designed for dual activation of the cGAS-STING pathway. Upon combination with CRISPR-Cas9 plasmids targeting PD-L1, this nanoplatform can promote the release of type I interferons (IFNs) and proinflammatory cytokines, including IFN- β and IL-6, and activate cytotoxic T lymphocytes (CTLs). It can thereby impair the tumor's immunosuppressive effect and, consequently, induce a stronger anti-tumor immune response (238).

Targeting inhibitory cytokines or their receptors is also a viable approach for the CRISPR-Cas9 system to impair immunosuppression. For instance, transforming growth factor-beta (TGF- β) is a crucial cytokine that helps maintain immune homeostasis. Its dysfunction would disrupt immune-cell function, establish an immune-tolerant microenvironment, and drive tumor progression (239). Lee et al. knocked out TGF- β receptor 2 (TGFBR2) in patient-derived endogenous tumor-infiltrating lymphocytes (TILs) using CRISPR-Cas9. The resulting TGF- β -resistant TILs were insulated from the immunosuppressive

effects of TGF- β , exhibiting enhanced proliferative capacity and anti-tumor activity (240). Furthermore, targeted knockout of endogenous TGFBR2 in CAR-T cells using CRISPR-Cas9 can reduce Treg conversion, prevent exhaustion, improve CAR-T cell function within the suppressive immune microenvironment, and thereby enhance therapeutic efficacy (241). Moreover, growth differentiation factor 15 (GDF15), which is often overexpressed in malignant tumor cells, has been shown to alter the TME and regulate Treg cell activity, thereby facilitating tumor immune escape (242). Zhang et al. designed a TME-responsive nanocapsule system that is capable of specifically delivering CRISPR-Cas9 to hepatocellular carcinoma cells and targeting GDF15 for knockout. Results showed that this approach enhanced immune cell infiltration, including CTLs, NK cells, and M1 macrophages, while reducing infiltration by M2 macrophages, improving the suppressive TME and tumor immune elimination (243).

Furthermore, CRISPR-Cas9 has proven effective in the knockout of novel cancer drivers to improve cancer outcomes. One example is interleukin-30 (IL-30), a recently identified regulator of cancer stem cell (CSC) behavior with notable autocrine and paracrine effects. IL-30 may promote tumor initiation, progression, and metastasis by upregulating oncogenes, growth factors, chemokine receptors, and inflammatory mediators, while facilitating tumor immune escape (244). Studies show that IL-30 exhibits immunosuppressive activity in the microenvironments of breast, prostate, and colon cancers. CRISPR-Cas9-mediated deletion of IL-30 suppresses tumor growth and metastasis and improves host survival and overall survival duration (244–246).

6.3 CRISPR-Cas Targeting the Tumor Vasculature and Metabolic Microenvironment

The abnormal remodeling of the tumor vasculature and disorder of the metabolic microenvironment serve as the basis for tumors to acquire nutrients, evade immunity, and sustain proliferation, directly driving tumor progression and therapeutic resistance. Leveraging its precise editing capacity, CRISPR technology enables novel strategies to target vascular regulatory genes and metabolic reprogramming nodes, thereby improving vascular function and reversing metabolic abnormalities. CRISPR-Cas9 is a vital tool for blocking tumor angiogenesis, exerting anti-angiogenic effects by disrupting genes encoding pro-angiogenic molecules in tumor cells. Vascular endothelial growth factor (VEGF) is overexpressed in most malignant tumors. It can promote the formation of new blood vessels through paracrine and autocrine pathways, while also facilitating tumor proliferation and migration. Consequently, anti-VEGF therapy has become an attractive strategy targeting solid tumors. Complete disruption of the VEGF gene via CRISPR-Cas9 can effectively downregulate VEGF expression in tumor cells and impair the VEGF/VEGFR signaling pathway, thereby inhibiting tumor growth (Figure 5c) (247,248). While this strategy can restrict tumor angiogenesis, it may be insufficient for a comprehensive anticancer effect on its own. Thus, it can be combined with other therapies (e.g., radiotherapy, chemotherapy, and immunotherapy) to enhance overall therapeutic efficacy.

Using CRISPR-Cas9 technology to modulate the tumor metabolic microenvironment has emerged as a promising therapeutic strategy. To sustain their rapid proliferation, tumor cells would typically reprogram the metabolic microenvironment, relying extensively on metabolic pathways involving glucose, lipids, and amino acids. These processes contribute to a series of alterations, including hypoxia, TME acidification, and increased oxidative stress (249). These changes significantly affect tumor growth, proliferation, immune evasion, and therapeutic responses. For example, lactate accumulation resulting from elevated glycolysis would impair the cytotoxicity of CTLs or NK cells and regulate Treg differentiation. Targeting glucose transporter 1 (GLUT-1) and hypoxia-inducible factor-1 α (HIF-1 α) genes with Cas9 can inhibit glucose uptake and lactate production in tumor cells, thereby suppressing tumor progression (250).

Furthermore, Huang et al. developed a synergistic nanoplatform that employs CRISPR-Cas9 to reduce SLC43A2 expression. This can restrict methionine uptake by tumor cells and restore normal methionine metabolism in CD8⁺ T cells, thereby recovering their immune activity. Meanwhile, the platform synergistically promotes activation of the cGAS/STING signaling pathway, thereby enhancing T-cell infiltration in the TME and improving the efficacy of tumor therapy (251).

Discussion

CRISPR-Cas systems have shown immense potential in oncology research, yet their clinical translation remains challenging. One primary concern is the unintended off-target cleavage by Cas nucleases, which can lead to unpredictable genomic alterations. Following DNA cleavage, large-scale deletions encompassing thousands to millions of base pairs may occur near the target site, with additional risks of chromosomal breaks, translocations, or losses (252,253). The subsequent repair of double-strand breaks also introduces uncertainty. For example, NHEJ can generate small insertions or deletions, potentially inactivating tumor suppressor genes or disrupting normal cellular functions. Immunogenicity represents another significant barrier. Pre-existing antibodies against Cas9 and Cas13d have been detected in human plasma (254), and Cas-specific T cell responses have been documented (255). These responses not only reduce genome-editing efficiency but may also provoke inflammatory reactions and increase diagnostic and therapeutic risks. With respect to delivery strategies, despite innovations in vector engineering and CRISPR-Cas optimization, achieving high concurrent editing efficiency, specificity, minimal immunogenicity, and safety, remains technically elusive (5). Within the TME, hypoxia, acidosis, and cytokine gradients further diminish editing efficiency, while dynamic TME heterogeneity impairs targeting precision. Future directions to address these obstacles include the development of novel Cas's variants, rational sgRNA design, and the optimization of delivery systems. The integration of CRISPR technology with cutting-edge methodologies, including single-cell sequencing, spatial transcriptomics, and artificial intelligence, will expand its diagnostic and therapeutic applications in oncology. Moreover, combinatorial therapeutic strategies that integrate CRISPR-based genome editing with immunotherapy, targeted therapy, and other modalities may provide effective means to overcome current limitations. CRISPR-Cas has emerged as a powerful tool for investigating the TME. With its robust capabilities in genetic screening and editing, CRISPR-Cas has facilitated in-depth dissection of the complexity of the TME and has driven groundbreaking advances in refining diagnostic strategies and developing precisely targeted therapies. Although challenges remain, continuous innovation in CRISPR-based technologies, coupled with their integration with other cutting-edge approaches, holds great promise for advancing precision oncology in both diagnosis and treatment.

References

1. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. 2014;157(6):1262-1278. <https://doi.org/10.1016/j.cell.2014.05.010>
2. Vink JNA, Baijens JHL, Brouns SJJ. PAM-repeat associations and spacer selection preferences in single and co-occurring CRISPR-Cas systems. *Genome Biol*. 2021;22(1):281. <https://doi.org/10.1186/s13059-021-02495-9>
3. Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol*. 2020;38(7):824-844. <https://doi.org/10.1038/s41587-020-0561-9>
4. Deepak KGK, Vempati R, Nagaraju GP, Nagini S, Dasari VR, Rao DN, et al. Tumor microenvironment: challenges and opportunities in targeting metastasis of triple negative breast cancer. *Pharmacol Res*. 2020;153:104683. <https://doi.org/10.1016/j.phrs.2020.104683>

5. Tao J, Bauer DE, Chiarle R. Assessing and advancing the safety of CRISPR-Cas tools: from DNA to RNA editing. *Nat Commun.* 2023;14(1):212. <https://doi.org/10.1038/s41467-023-35886-6>
6. Yan WX, Hunnewell P, Alfonse LE, Carte J, Keston-Smith E, Sothiselvam S, et al. Functionally diverse type V CRISPR-Cas systems. *Science.* 2019;363(6422):88-91. <https://doi.org/10.1126/science.ava7271>
7. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* 2012;337(6096):816-821. <https://doi.org/10.1126/science.1225829>
8. Wang SW, Gao C, Zheng YM, Yi L, Lu JC, Huang XY, et al. Current applications and future perspective of CRISPR/Cas9 gene editing in cancer. *Mol Cancer.* 2022;21(1):57. <https://doi.org/10.1186/s12943-022-01518-8>
9. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature.* 2011;471(7340):602-607. <https://doi.org/10.1038/nature09886>
10. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science.* 2014;346(6213):1258096. <https://doi.org/10.1126/science.1258096>
11. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature.* 2014;507(7490):62-67. <https://doi.org/10.1038/nature13011>
12. Bhatia S, Pooja, Yadav SK. CRISPR-Cas for genome editing: classification, mechanism, designing and applications. *Int J Biol Macromol.* 2023;238:124054. <https://doi.org/10.1016/j.ijbiomac.2023.124054>
13. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell.* 2014;156(5):935-949. <https://doi.org/10.1016/j.cell.2014.02.001>
14. Sapranaukas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. The streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Res.* 2011;39(21):9275-9282. <https://doi.org/10.1093/nar/gkr606>
15. Xue C, Greene EC. DNA repair pathway choices in CRISPR-Cas9-mediated genome editing. *Trends Genet.* 2021;37(7):639-656. <https://doi.org/10.1016/j.tig.2021.02.008>
16. Bothmer A, Phadke T, Barrera LA, Margulies CM, Lee CS, Buquicchio F, et al. Characterization of the interplay between DNA repair and CRISPR/Cas9-induced DNA lesions at an endogenous locus. *Nat Commun.* 2017;8:13905. <https://doi.org/10.1038/ncomms13905>
17. Selvakumar SC, Preethi KA, Ross K, Tusubira D, Khan MWA, Mani P, et al. CRISPR/Cas9 and next generation sequencing in the personalized treatment of cancer. *Mol Cancer.* 2022;21(1):83. <https://doi.org/10.1186/s12943-022-01565-1>
18. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell.* 2015;163(3):759-771. <https://doi.org/10.1016/j.cell.2015.09.038>
19. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, et al. Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. *Mol Cell.* 2015;60(3):385-397. <https://doi.org/10.1016/j.molcel.2015.10.008>

20. Badon IW, Oh Y, Kim HJ, Lee SH. Recent application of CRISPR-Cas12 and OMEGA system for genome editing. *Mol Ther.* 2024;32(1):32-43. <https://doi.org/10.1016/j.ymthe.2023.11.013>
21. Yang H, Gao P, Rajashankar KR, Patel DJ. PAM-dependent target DNA recognition and cleavage by C2c1 CRISPR-Cas endonuclease. *Cell.* 2016;167(7):1814-1828.e12. <https://doi.org/10.1016/j.cell.2016.11.053>
22. Manghwar H, Lindsey K, Zhang X, Jin S. CRISPR/Cas system: recent advances and future prospects for genome editing. *Trends Plant Sci.* 2019;24(12):1102-1125. <https://doi.org/10.1016/j.tplants.2019.09.006>
23. Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science.* 2018;360(6387):436-439. <https://doi.org/10.1126/science.aar6245>
24. Li F, Li J, Yang W, Yang S, Chen C, Du L, et al. Framework-Hotspot Enhanced Trans Cleavage of CRISPR-Cas12a for Clinical Samples Detection. *Angew Chem Int Ed Engl.* 2023;62(32):e202305536. <https://doi.org/10.1002/anie.202305536> <https://doi.org/10.1002/anie.202305536>
25. Wu S, Tian P, Tan T. CRISPR-Cas13 technology portfolio and alliance with other genetic tools. *Biotechnol Adv.* 2022;61:108047. <https://doi.org/10.1016/j.biotechadv.2022.108047>
26. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, et al. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nat Rev Microbiol.* 2020;18(2):67-83. <https://doi.org/10.1038/s41579-019-0299-x>
27. Bot JF, van der Oost J, Geijsen N. The double life of CRISPR-Cas13. *Curr Opin Biotechnol.* 2022;78:102789. <https://doi.org/10.1016/j.copbio.2022.102789>
28. Dash PK, Chen C, Kaminski R, Su H, Mancuso P, Sillman B, et al. CRISPR editing of CCR5 and HIV-1 facilitates viral elimination in antiretroviral drug-suppressed virus-infected humanized mice. *Proc Natl Acad Sci U S A.* 2023;120(19):e2217887120. <https://doi.org/10.1073/pnas.2217887120>
29. Wu J, Xu H, Hu F, Jiang Y, Fan B, Khan A, et al. CRISPR-Cas and catalytic hairpin assembly technology for target-initiated amplification detection of pancreatic cancer specific tsRNAs. *Front Bioeng Biotechnol.* 2023;11:1169424. <https://doi.org/10.3389/fbioe.2023.1169424>
30. Wei Y, Fu Y, Li C, Chen S, Xie L, Chen M. Ultrasensitive detection of circulating tumor DNA using a CRISPR/Cas9 nickase-driven 3D DNA walker based on a COF-AuNPs sensing platform. *Mikrochim Acta.* 2024;191(11):671. <https://doi.org/10.1007/s00604-024-06749-8>
31. Wang Z, Li N, Feng K, Chen M, Zhang Y, Liu Y, et al. Phase I study of CAR-T cells with PD-1 and TCR disruption in mesothelin-positive solid tumors. *Cell Mol Immunol.* 2021;18(9):2188-2198. <https://doi.org/10.1038/s41423-021-00749-x>
32. Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science.* 2017;356(6336):438-442. <https://doi.org/10.1126/science.aam9321>
33. Wu X, Luo S, Guo C, Zhao Y, Zhong J, Hu R, et al. LbuCas13a directly targets DNA and elicits strong trans-cleavage activity. *Nat Biomed Eng.* 2025. <https://doi.org/10.1038/s41551-025-01424-6>
34. Hao L, Zhao RT, Welch NL, Tan EKW, Zhong Q, Harzallah NS, et al. CRISPR-Cas-amplified urinary biomarkers for multiplexed and portable cancer diagnostics. *Nat Nanotechnol.* 2023;18(7):798-807. <https://doi.org/10.1038/s41565-023-01372-9>

35. Ming M, Ren Q, Pan C, He Y, Zhang Y, Liu S, et al. CRISPR-Cas12b enables efficient plant genome engineering. *Nat Plants*. 2020;6(3):202-208. <https://doi.org/10.1038/s41477-020-0614-6>
36. Teng F, Cui T, Feng G, Guo L, Xu K, Gao Q, et al. Repurposing CRISPR-Cas12b for mammalian genome engineering. *Cell Discov*. 2018;4:63. <https://doi.org/10.1038/s41421-018-0069-3>
37. Teng F, Guo L, Cui T, Wang XG, Xu K, Gao Q, et al. CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity. *Genome Biol*. 2019;20(1):132. <https://doi.org/10.1186/s13059-019-1742-z>
38. Sam IK, Chen YY, Ma J, Li SY, Ying RY, Li LX, et al. TB-QUICK: CRISPR-Cas12b-assisted rapid and sensitive detection of *Mycobacterium tuberculosis*. *J Infect*. 2021;83(1):54-60. <https://doi.org/10.1016/j.jinf.2021.04.032>
39. Hu M, Liu R, Qiu Z, Cao F, Tian T, Lu Y, et al. Light-start CRISPR-Cas12a reaction with caged crRNA enables rapid and sensitive nucleic acid detection. *Angew Chem Int Ed Engl*. 2023;62(23):e202300663. <https://doi.org/10.1002/anie.202300663>
40. Chen Z, Arai E, Khan O, Zhang Z, Ngiow SF, He Y, et al. In vivo CD8⁺ T cell CRISPR screening reveals control by Flil in infection and cancer. *Cell*. 2021;184(5):1262-1280.e22. <https://doi.org/10.1016/j.cell.2021.02.019>
41. Chen J, Jiang Y, Hou M, Liu C, Liu E, Zong Y, et al. Nuclear translocation of plasma membrane protein ADCY7 potentiates T cell-mediated antitumour immunity in HCC. *Gut*. 2024;74(1):128-140. <https://doi.org/10.1136/gutjnl-2024-332902>
42. Wang C, Zheng X, Zhang J, Jiang X, Wang J, Li Y, et al. CD300ld on neutrophils is required for tumour-driven immune suppression. *Nature*. 2023;621(7980):830-839. <https://doi.org/10.1038/s41586-023-06511-9>
43. Wang X, Tokheim C, Gu SS, Wang B, Tang Q, Li Y, et al. In vivo CRISPR screens identify the E3 ligase Cop1 as a modulator of macrophage infiltration and cancer immunotherapy target. *Cell*. 2021;184(21):5357-5374.e22. <https://doi.org/10.1016/j.cell.2021.09.006>
44. Ye L, Park JJ, Peng L, Yang Q, Chow RD, Dong MB, et al. A genome-scale gain-of-function CRISPR screen in CD8 T cells identifies proline metabolism as a means to enhance CAR-T therapy. *Cell Metab*. 2022;34(4):595-614.e14. <https://doi.org/10.1016/j.cmet.2022.02.009>
45. Joung J, Kirchgatterer PC, Singh A, Cho JH, Nety SP, Larson RC, et al. CRISPR activation screen identifies BCL-2 proteins and B3GNT2 as drivers of cancer resistance to T cell-mediated cytotoxicity. *Nat Commun*. 2022;13(1):1606. <https://doi.org/10.1038/s41467-022-29205-8>
46. Li X, Sun S, Zhang W, Liang Z, Fang Y, Sun T, et al. Identification of genetic modifiers enhancing B7-H3-targeting CAR T cell therapy against glioblastoma through large-scale CRISPRi screening. *J Exp Clin Cancer Res*. 2024;43(1):95. <https://doi.org/10.1186/s13046-024-03027-6>
47. Zhu S, Cao Z, Liu Z, He Y, Wang Y, Yuan P, et al. Guide RNAs with embedded barcodes boost CRISPR-pooled screens. *Genome Biol*. 2019;20(1):20. <https://doi.org/10.1186/s13059-019-1628-0>
48. Hanna RE, Hegde M, Fagre CR, DeWeirdt PC, Sangree AK, Szegletes Z, et al. Massively parallel assessment of human variants with base editor screens. *Cell*. 2021;184(4):1064-1080.e20. <https://doi.org/10.1016/j.cell.2021.01.012>
49. Cuella-Martin R, Hayward SB, Fan X, Chen X, Huang JW, Tagliabatella A, et al. Functional interrogation of DNA damage response variants with base editing screens. *Cell*. 2021;184(4):1081-1097.e19. <https://doi.org/10.1016/j.cell.2021.01.041>

50. Huang C, Li G, Wu J, Liang J, Wang X. Identification of pathogenic variants in cancer genes using base editing screens with editing efficiency correction. *Genome Biol.* 2021;22(1):80. <https://doi.org/10.1186/s13059-021-02305-2>
51. Xu P, Liu Z, Liu Y, Ma H, Xu Y, Bao Y, et al. Genome-wide interrogation of gene functions through base editor screens empowered by barcoded sgRNAs. *Nat Biotechnol.* 2021;39(11):1403-1413. <https://doi.org/10.1038/s41587-021-00944-1>
52. Coelho MA, Cooper S, Strauss ME, Karakoc E, Bhosle S, Gonçalves E, et al. Base editing screens map mutations affecting interferon- γ signaling in cancer. *Cancer Cell.* 2023;41(2):288-303.e6. <https://doi.org/10.1016/j.ccell.2022.12.009>
53. Schmidt R, Ward CC, Dajani R, Armour-Garb Z, Ota M, Allain V, et al. Base-editing mutagenesis maps alleles to tune human T cell functions. *Nature.* 2024;625(7996):805-812. <https://doi.org/10.1038/s41586-023-06835-6>
54. Adamson B, Norman TM, Jost M, Cho MY, Nunez JK, Chen Y, et al. A multiplexed single-cell CRISPR screening platform enables systematic dissection of the unfolded protein response. *Cell.* 2016;167(7):1867-1882.e21. <https://doi.org/10.1016/j.cell.2016.11.048>
55. Dixit A, Parnas O, Li B, Chen J, Fulco CP, Jerby-Aron L, et al. Perturb-Seq: dissecting molecular circuits with scalable single-cell RNA profiling of pooled genetic screens. *Cell.* 2016;167(7):1853-1866.e17. <https://doi.org/10.1016/j.cell.2016.11.038>
56. Datlinger P, Rendeiro AF, Schmidl C, Krausgruber T, Traxler P, Klughammer J, et al. Pooled CRISPR screening with single-cell transcriptome readout. *Nat Methods.* 2017;14(3):297-301. <https://doi.org/10.1038/nmeth.4177>
57. Replogle JM, Norman TM, Xu A, Hussmann JA, Chen J, Cogan JZ, et al. Combinatorial single-cell CRISPR screens by direct guide RNA capture and targeted sequencing. *Nat Biotechnol.* 2020;38(8):954-961. <https://doi.org/10.1038/s41587-020-0470-y>
58. Fagerberg E, Attanasio J, Dien C, Singh J, Kessler EA, Abdullah L, et al. KLF2 maintains lineage fidelity and suppresses CD8 T cell exhaustion during acute LCMV infection. *Science.* 2025;387(6735):eadn2337. <https://doi.org/10.1126/science.adn2337>
59. Renz PF, Ghoshdastider U, Baghai Sain S, Valdivia-Francia F, Khandekar A, Ormiston M, et al. In vivo single-cell CRISPR uncovers distinct TNF programmes in tumour evolution. *Nature.* 2024;632(8024):419-428. <https://doi.org/10.1038/s41586-024-07663-y>
60. DeWeirdt PC, Sanson KR, Sangree AK, Hegde M, Hanna RE, Feeley MN, et al. Optimization of AsCas12a for combinatorial genetic screens in human cells. *Nat Biotechnol.* 2021;39(1):94-104. <https://doi.org/10.1038/s41587-020-0600-6>
61. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD. Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell.* 2018;173(3):665-676.e14. <https://doi.org/10.1016/j.cell.2018.02.033>
62. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, et al. RNA targeting with CRISPR-Cas13. *Nature.* 2017;550(7675):280-284. <https://doi.org/10.1038/nature24049>
63. Li S, Li X, Xue W, Zhang L, Yang LZ, Cao SM, et al. Screening for functional circular RNAs using the CRISPR-Cas13 system. *Nat Methods.* 2021;18(1):51-59. <https://doi.org/10.1038/s41592-020-1011-4>

64. Si J, Su X, Jin Z, Duan S. Uncovering essential lncRNAs through transcriptome-scale CRISPR-Cas13 screening. *Adv Biotechnol (Singap)*. 2025;3(3):27. <https://doi.org/10.1007/s44307-025-00082-8>
65. Zhou P, Shi H, Huang H, Sun X, Yuan S, Chapman NM, et al. Single-cell CRISPR screens in vivo map T cell fate regulomes in cancer. *Nature*;624(7990):154-163. <https://doi.org/10.1038/s41586-023-06733-x>
66. Dubrot J, Du PP, Lane-Reticker SK, Kessler EA, Muscato AJ, Mehta A, et al. In vivo CRISPR screens reveal the landscape of immune evasion pathways across cancer. *Nat Immunol*. 2022;23(10):1495-1506. <https://doi.org/10.1038/s41590-022-01315-x>
67. Burr ML, Sparbier CE, Chan KL, Chan YC, Kersbergen A, Lam EYN, et al. An evolutionarily conserved function of Polycomb silences the MHC class I antigen presentation pathway and enables immune evasion in cancer. *Cancer Cell*. 2019;36(4):385-401.e8. <https://doi.org/10.1016/j.ccell.2019.08.008>
68. Chen X, Lu Q, Zhou H, Liu J, Nadorp B, Lasry A, et al. A membrane-associated MHC-I inhibitor y axis for cancer immune evasion. *Cell*. 2023;186(18):3903-3920.e21. <https://doi.org/10.1016/j.cell.2023.07.016>
69. Fu L, Li S, Mei J, Li Z, Yang X, Zheng C, et al. BIRC2 blockade facilitates immunotherapy of hepatocellular carcinoma. *Mol Cancer*. 2025;24(1):113. <https://doi.org/10.1186/s12943-02502319-5>
70. Binan L, Jiang A, Danquah SA, Valakh V, Simonton B, Bezney J, et al. Simultaneous CRISPR screening and spatial transcriptomics reveal intracellular, intercellular, and functional transcriptional circuits. *Cell*. 2025;188(8):2141-2158.e18. <https://doi.org/10.1016/j.cell.2025.02.012>
71. Rogers ZN, McFarland CD, Winters IP, Seoane JA, Brady JJ, Yoon S, et al. Mapping the in vivo fitness landscape of lung adenocarcinoma tumor suppression in mice. *Nat Genet*. 2018;50(4):483-486. <https://doi.org/10.1038/s41588-018-0083-2>
72. Bowling S, Sritharan D, Osorio FG, Nguyen M, Cheung P, Rodriguez-Fraticelli A, et al. An engineered CRISPR-Cas9 mouse line for simultaneous readout of lineage histories and gene expression profiles in single cells. *Cell*. 2020;181(6):1410-1422.e27. <https://doi.org/10.1016/j.cell.2020.04.048>
73. Li L, Bowling S, McGeary SE, Yu Q, Lemke B, Alcedo K, et al. A mouse model with high clonal barcode diversity for joint lineage, transcriptomic, and epigenomic profiling in single cells. *Cell*. 2023;186(23):5183-5199.e22. <https://doi.org/10.1016/j.cell.2023.09.019>
74. Xie L, Liu H, You Z, Wang L, Li Y, Zhang X, et al. Comprehensive spatiotemporal mapping of single-cell lineages in developing mouse brain by CRISPR-based barcoding. *Nat Methods*. 2023;20(8):1244-1255. <https://doi.org/10.1038/s41592-023-01947-3>
75. Chan MM, Smith ZD, Grosswendt S, Kretzmer H, Norman TM, Adamson B, et al. Molecular recording of mammalian embryogenesis. *Nature*. 2019;570(7759):77-82. <https://doi.org/10.1038/s41586-019-1184-5>
76. Quinn JJ, Jones MG, Okimoto RA, Nanjo S, Chan MM, Yosef N, et al. Single-cell lineages reveal the rates, routes, and drivers of metastasis in cancer xenografts. *Science*. 2021;371(6532):eabc1944. <https://doi.org/10.1126/science.abc1944>
77. Wang S, Shen X, Chen G, Zhang W, Tan B. Application and development of CRISPR-Cas12a methods for the molecular diagnosis of cancer: a review. *Anal Chim Acta*. 2025;1341:343603. <https://doi.org/10.1016/j.aca.2024.343603>

78. Chen M, Wu D, Tu S, Yang C, Chen D, Xu Y. CRISPR/Cas9 cleavage triggered ESDR for circulating tumor DNA detection based on a 3D graphene/AuPtPd nanoflower biosensor. *Biosens Bioelectron*. 2021;173:112821. <https://doi.org/10.1016/j.bios.2020.112821>
79. Sahel DK, Giriprasad G, Jatyan R, Guha S, Korde A, Mittal A, et al. Next-generation CRISPR/Cas-based ultrasensitive diagnostic tools: current progress and prospects. *RSC Adv*. 2024;14(44):32411-32435. <https://doi.org/10.1039/d4ra04838e>
80. Dong JL, Xu Z, Zhang L, Chen M, Yu J, Zhou H, et al. Comparative evaluation of PCR-based, LAMP and RPA-CRISPR/Cas12a assays for the rapid detection of *Diaporthe aspalathi*. *Int J Mol Sci*. 2024;25(11):15773. <https://doi.org/10.3390/ijms25115773>
81. Gu W, Crawford ED, O'Donovan BD, Wilson MR, Chow ED, Retallack H, et al. Depletion of abundant sequences by hybridization (DASH): using Cas9 to remove unwanted high-abundance species in sequencing libraries and molecular counting applications. *Genome Biol*. 2016;17:41. <https://doi.org/10.1186/s13059-016-0904-5>
82. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015;523(7561):481-485. <https://doi.org/10.1038/nature14592>
83. Li SY, Cheng QX, Liu JK, Nie XQ, Zhao GP, Wang J. CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA. *Cell Res*. 2018;28(4):491-493. <https://doi.org/10.1038/s41422-018-0022-x>
84. Chen J, Chen Y, Huang L, Lin X, Chen H, Xiang W, et al. Trans-nuclease activity of Cas9 activated by DNA or RNA target binding. *Nat Biotechnol*. 2025;43(4):558-568. <https://doi.org/10.1038/s41587-024-02255-7>
85. Nguyen LT, Smith BM, Jain PK. Enhancement of trans-cleavage activity of Cas12a with engineered crRNA enables amplified nucleic acid detection. *Nat Commun*. 2020;11(1):4906. <https://doi.org/10.1038/s41467-020-18615-1>
86. Yan H, Wen Y, Tian Z, Hart N, Han S, Hughes SJ, et al. A one-pot isothermal Cas12-based assay for the sensitive detection of microRNAs. *Nat Biomed Eng*. 2023;7(12):1583-1601. <https://doi.org/10.1038/s41551-023-01033-1>
87. Yan H, Wen Y, Tian Z, Hart N, Han S, Hughes SJ, Zeng Y. A one-pot isothermal Cas12-based assay for the sensitive detection of microRNAs. *Nat Biomed Eng*. 2023;7(12):1583-1601. <https://doi.org/10.1038/s41551-023-01033-1>
88. Rananaware SR, Vesco EK, Shoemaker GM, Anekar SS, Sandoval LSW, Meister KS, et al. Programmable RNA detection with CRISPR-Cas12a. *Nat Commun*. 2023;14(1):5409. <https://doi.org/10.1038/s41467-023-41006-1>
89. Chen Y, Wang X, Zhang J, Jiang Q, Qiao B, He B, et al. Split crRNA with CRISPR-Cas12a enabling highly sensitive and multiplexed detection of RNA and DNA. *Nat Commun*. 2024;15(1):8342. <https://doi.org/10.1038/s41467-024-52691-x>
90. Moon J, Liu C. Asymmetric CRISPR enabling cascade signal amplification for nucleic acid detection by competitive crRNA. *Nat Commun*. 2023;14(1):7504. <https://doi.org/10.1038/s41467-023-43389-7>

91. van Dongen JE, Berendsen JTW, Eijkel JCT, Segerink LI. A CRISPR/Cas12a-assisted in vitro diagnostic tool for identification and quantification of single CpG methylation sites. *Biosens Bioelectron.* 2021;194:113624. <https://doi.org/10.1016/j.bios.2021.113624>
92. Kryslar AR, Cromwell CR, Tu T, Jovel J, Hubbard BP. Guide RNAs containing universal bases enable Cas9/Cas12a recognition of polymorphic sequences. *Nat Commun.* 2022;13(1):1617. <https://doi.org/10.1038/s41467-022-29202-x>
93. Kocak DD, Josephs EA, Bhandarkar V, Adkar SS, Kwon JB, Gersbach CA. Increasing the specificity of CRISPR systems with engineered RNA secondary structures. *Nat Biotechnol.* 2019;37(6):657-666. <https://doi.org/10.1038/s41587-019-0095-1>
94. Yin H, Song CQ, Suresh S, Kwan SY, Wu Q, Walsh S, et al. Partial DNA-guided Cas9 enables genome editing with reduced off-target activity. *Nat Chem Biol.* 2018;14(3):311-316. <https://doi.org/10.1038/nchembio.2559>
95. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature.* 2016;529(7587):490-495. <https://doi.org/10.1038/nature16526>
96. Chen L, Hu M, Zhou X. Trends in developing one-pot CRISPR diagnostics strategies. *Trends Biotechnol.* 2025;43(1):98-110. <https://doi.org/10.1016/j.tibtech.2024.07.007>
97. Huang W, Wang J, Wang C, Liu Y, Li W, Chen Q, et al. Expanding Cas12a activity control with an RNA G-quadruplex at the 5' end of CRISPR RNA. *Adv Sci (Weinh).* 2025;12(7):e2411305. <https://doi.org/10.1002/advs.202411305>
98. Zhang W, Zhong Y, Wang J, Zou G, Chen Q, Liu C. Direct repeat region 3' end modifications regulate Cas12a activity and expand its applications. *Nucleic Acids Res.* 2025;53(3):gkaf040 <https://doi.org/10.1093/nar/gkaf040>
99. Lin M, Qiu Z, Hao M, Qi W, Zhang T, Shen Y, et al. Cas12a cis-cleavage mediated lateral flow assay enables multiplex and ultra-specific nucleic acid detection. *Nat Commun.* 2025;16(1):5597. <https://doi.org/10.1038/s41467-025-60917-9>
100. Labib M, Kelley SO. Single-cell analysis targeting the proteome. *Nat Rev Chem.* 2020;4(3):143-158. <https://doi.org/10.1038/s41570-020-0162-7>
101. Jaitin DA, Weiner A, Yofe I, Lara-Astiaso D, Keren-Shaul H, David E, et al. Dissecting immune circuits by linking CRISPR-pooled screens with single-cell RNA-seq. *Cell.* 2016;167(7):1883-1896.e15. <https://doi.org/10.1016/j.cell.2016.11.039>
102. Li Y, Huang Z, Xu L, Fan Y, Ping J, Li G, et al. UDA-seq: universal droplet microfluidics-based combinatorial indexing for massive-scale multimodal single-cell sequencing. *Nat Methods.* 2025;22(6):1199-1212. <https://doi.org/10.1038/s41592-024-02586-y>
103. Metzner E, Southard KM, Norman TM. Multiome perturb-seq unlocks scalable discovery of integrated perturbation effects on the transcriptome and epigenome. *Cell Syst.* 2025;16(1):101161. <https://doi.org/10.1016/j.cels.2024.12.002>
104. Schraivogel D, Gschwind AR, Milbank JH, Leonce DR, Jakob P, Mathur L, et al. Targeted Perturb-seq enables genome-scale genetic screens in single cells. *Nat Methods.* 2020;17(6):629-635. <https://doi.org/10.1038/s41592-020-0837-5>

105. Yao D, Binan L, Bezney J, Simonton B, Freedman J, Frangieh CJ, et al. Scalable genetic screening for regulatory circuits using compressed Perturb-seq. *Nat Biotechnol.* 2024;42(8):1282-1295. <https://doi.org/10.1038/s41587-023-01964-9>
106. Wroblewska A, Dhainaut M, Ben-Zvi B, Rose SA, Park ES, Amir ED, et al. Protein barcodes enable high-dimensional single-cell CRISPR screens. *Cell.* 2018;175(4):1141-1155.e16. <https://doi.org/10.1016/j.cell.2018.09.022>
107. Dhainaut M, Rose SA, Akturk G, Wroblewska A, Nielsen SR, Park ES, et al. Spatial CRISPR genomics identifies regulators of the tumor microenvironment. *Cell.* 2022;185(7):1223-1239.e20. <https://doi.org/10.1016/j.cell.2022.02.015>
108. Tang L. Spatial CRISPR screens in tumors. *Nat Methods.* 2022;19(5):517. <https://doi.org/10.1038/s41592-022-01501-7>
109. Park YM, Lin D-C. Moving closer towards a comprehensive view of tumor biology and microarchitecture using spatial transcriptomics. *Nat Commun.* 2023;14(1):7017. <https://doi.org/10.1038/s41467-023-42960-6>
110. Rausch M, Bartels K, Leibold J. Immunotherapy in ovarian cancer: spatial functional genomics to unravel resistance mechanisms. *Signal Transduct Target Ther.* 2025;10(1):17. <https://doi.org/10.1038/s41392-024-02110-w>
111. Binan L. Investigating spatial gene circuits and gene-phenotype mechanisms with Perturb-FISH. *Nat Rev Genet.* 2025;26(8):507-508. <https://doi.org/10.1038/s41576-025-00857-8>
112. Kudo T, Meireles AM, Moncada R, Chen Y, Wu P, Gould J, et al. Multiplexed, image-based pooled screens in primary cells and tissues with PerturbView. *Nat Biotechnol.* 2025;43(7):1091-1100. <https://doi.org/10.1038/s41587-024-02391-0>
113. Hu Y, An Q, Sheu K, Trejo B, Fan S, Guo Y. Single cell multi-omics technology: methodology and application. *Front Cell Dev Biol.* 2018;6:28. <https://doi.org/10.3389/fcell.2018.00028>
114. Frangieh CJ, Melms JC, Thakore PI, Geiger-Schuller K, Ho P, Luoma AM, et al. Multimodal pooled perturb-CITE-seq screens in patient models define mechanisms of cancer immune evasion. *Nat Genet.* 2021;53(3):332-341. <https://doi.org/10.1038/s41588-021-00779-1>
115. Vandereyken K, Sifrim A, Thienpont B, Voet T. Methods and applications for single-cell and spatial multi-omics. *Nat Rev Genet.* 2023;24(8):494-515. <https://doi.org/10.1038/s41576-023-00580-2>
116. Minnoye L, Marinov GK, Krausgruber T, Pan L, Marand AP, Secchia S, et al. Chromatin accessibility profiling methods. *Nat Rev Methods Primers.* 2021;1:10. <https://doi.org/10.1038/s43586-020-0008-9>
117. Grandi FC, Modi H, Kampman L, Corces MR. Chromatin accessibility profiling by ATAC-seq. *Nat Protoc.* 2022;17(6):1518-1552. <https://doi.org/10.1038/s41596-022-00692-9>
118. Pierce SE, Granja JM, Greenleaf WJ. High-throughput single-cell chromatin accessibility CRISPR screens enable unbiased identification of regulatory networks in cancer. *Nat Commun.* 2021;12(1):2969. <https://doi.org/10.1038/s41467-021-23213-w>
119. Liscovitch-Brauer N, Montalbano A, Deng J, Méndez-Mancilla A, Wessels H-H, Moss NG, et al. Profiling the genetic determinants of chromatin accessibility with scalable single-cell CRISPR screens. *Nat Biotechnol.* 2021;39(10):1270-1277. <https://doi.org/10.1038/s41587-021-00902-x>

120. Replogle JM, Saunders RA, Pogson AN, Hussmann JA, Lenail A, Guna A, et al. Mapping information-rich genotype-phenotype landscapes with genome-scale Perturb-seq. *Cell*. 2022;185(14):2559-2575.e28. <https://doi.org/10.1016/j.cell.2022.05.013>
121. Szlachta K, Kuscu C, Tufan T, Adair SJ, Shang S, Michaels AD, et al. CRISPR knockout screening identifies combinatorial drug targets in pancreatic cancer and models cellular drug response. *Nat Commun*. 2018;9(1):4275. <https://doi.org/10.1038/s41467-018-06676-2>
122. Qu J, Kalyani FS, Liu L, Cheng T, Chen L. Tumor organoids: synergistic applications, current challenges, and future prospects in cancer therapy. *Cancer Commun (Lond)*. 2021;41(12):1331-1353. <https://doi.org/10.1002/cac2.12224>
123. Lo YH, Horn HT, Huang MF, Yu WC, Young CM, Liu Q, et al. Large-scale CRISPR screening in primary human 3D gastric organoids enables comprehensive dissection of gene-drug interactions. *Nat Commun*. 2025;16(1):7566. <https://doi.org/10.1038/s41467-025-62818-3>
124. Shi H, Doench JG, Chi H. CRISPR screens for functional interrogation of immunity. *Nat Rev Immunol*. 2023;23(6):363-380. <https://doi.org/10.1038/s41577-022-00802-4>
125. Oldrini B, Curiel García Á, Marques C, Matia V, Uluçkan O, Graña-Castro O, et al. Somatic genome editing with the RCAS-TVA-CRISPR-Cas9 system for precision tumor modeling. *Nat Commun*. 2018;9(1):1466. <https://doi.org/10.1038/s41467-018-03731-w>
126. Su P, Liu Y, Chen T, Xue Y, Zeng Y, Zhu G, et al. In vivo CRISPR screens identify a dual function of MEN1 in regulating tumor-microenvironment interactions. *Nat Genet*. 2024;56(9):1890-1902. <https://doi.org/10.1038/s41588-024-01874-9>
127. Dervovic D, Malik AA, Chen ELY, Narimatsu M, Adler N, Afuni-Zadeh S, et al. In vivo CRISPR screens reveal Serpinb9 and Adam2 as regulators of immune therapy response in lung cancer. *Nat Commun*. 2023;14(1):3150. <https://doi.org/10.1038/s41467-023-38841-7>
128. Wei J, Long L, Zheng W, Dhungana Y, Lim SA, Guy C, et al. Targeting REGNASE-1 programs long-lived effector T cells for cancer therapy. *Nature*. 2019;576(7787):471-476. <https://doi.org/10.1038/s41586-019-1821-z>
129. Dong MB, Wang G, Chow RD, Ye L, Zhu L, Dai X, et al. Systematic immunotherapy target discovery using genome-scale in vivo CRISPR screens in CD8 T cells. *Cell*. 2019;178(5):1189-1204.e23. <https://doi.org/10.1016/j.cell.2019.07.044>
130. Ye L, Park JJ, Dong MB, Yang Q, Chow RD, Peng L, et al. In vivo CRISPR screening in CD8 T cells with AAV-Sleeping Beauty hybrid vectors identifies membrane targets for improving immunotherapy for glioblastoma. *Nat Biotechnol*. 2019;37(11):1302-1313. <https://doi.org/10.1038/s41587-019-0246-4>
131. Mimitou EP, Cheng A, Montalbano A, Hao S, Stoeckius M, Legut M, et al. Multiplexed detection of proteins, transcriptomes, clonotypes and CRISPR perturbations in single cells. *Nat Methods*. 2019;16(5):409-412. <https://doi.org/10.1038/s41592-019-0392-0>
132. Tang L. Tackling in vivo screening complexity. *Nat Methods*. 2025;22(2):224. <https://doi.org/10.1038/s41592-025-02605-6>
133. Uijttewaal ECH, Lee J, Sell AC, Botay N, Vainorius G, Novatchkova M, et al. CRISPR-StAR enables high-resolution genetic screening in complex in vivo models. *Nat Biotechnol*. 2025;43(11):1848-1860. <https://doi.org/10.1038/s41587-024-02512-9>

134. Lu M, Shao W, Xing H, Huang Y. Extracellular vesicle-based nucleic acid delivery. *Interdiscip Med.* 2023;1:100007. <https://doi.org/10.1002/inmd.20220007>
135. Xu X, Liu C, Wang Y, Koivisto O, Zhou J, Shu Y, et al. Nanotechnology-based delivery of CRISPR/Cas9 for cancer treatment. *Adv Drug Deliv Rev.* 2021;176:113891. <https://doi.org/10.1016/j.addr.2021.113891>
136. Glass Z, Lee M, Li Y, Xu Q. Engineering the delivery system for CRISPR-based genome editing. *Trends Biotechnol.* 2018;36(2):173-185. <https://doi.org/10.1016/j.tibtech.2017.11.006>
137. Porto EM, Komor AC, Slaymaker IM, Yeo GW. Base editing: advances and therapeutic opportunities. *Nat Rev Drug Discov.* 2020;19(12):839-859. <https://doi.org/10.1038/s41573-020-0084-6>
138. Madigan V, Zhang F, Dahlman JE. Drug delivery systems for CRISPR-based genome editors. *Nat Rev Drug Discov.* 2023;22(11):875-894. <https://doi.org/10.1038/s41573-023-00762-x>
139. Song X, Liu C, Wang N, Huang H, He S, Gong C, et al. Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy. *Adv Drug Deliv Rev.* 2021;168:158-180. <https://doi.org/10.1016/j.addr.2020.04.010>
140. Wang X, Wei M, Miao R, Hao X, Li M, Wang W, et al. Adeno-associated virus vectors for gene therapy—focusing on melanoma. *Interdiscip Med.* 2024;2(4):100031. <https://doi.org/10.1002/inmd.20240031>
141. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov.* 2019;18(5):358-378. <https://doi.org/10.1038/s41573-019-0012-9>
142. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, et al. In vivo genome editing using Staphylococcus aureus Cas9. *Nature.* 2015;520(7546):186-191. <https://doi.org/10.1038/nature14299>
143. Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods.* 2013;10(11):1116-1121. <https://doi.org/10.1038/nmeth.2681>
144. Hou Z, Zhang Y, Propson NE, Howden SE, Chu L-F, Sontheimer EJ, et al. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc Natl Acad Sci U S A.* 2013;110(39):15644-15649. <https://doi.org/10.1073/pnas.1313587110>
145. Kim E, Koo T, Park SW, Kim D, Kim K, Cho H-Y, et al. In vivo genome editing with a small Cas9 orthologue derived from *Campylobacter jejuni*. *Nat Commun.* 2017;8:14500. <https://doi.org/10.1038/ncomms14500>
146. Hu Z, Zhang C, Wang S, Gao S, Wei J, Li M, et al. Discovery and engineering of small SlugCas9 with broad targeting range and high specificity and activity. *Nucleic Acids Res.* 2021;49(7):4008-4019. <https://doi.org/10.1093/nar/gkab148>
147. Edraki A, Mir A, Ibraheim R, Gainetdinov I, Yoon Y, Song C-Q, et al. A compact, high-accuracy Cas9 with a dinucleotide PAM for in vivo genome editing. *Mol Cell.* 2019;73(4):714-726.e4. <https://doi.org/10.1016/j.molcel.2018.12.003>
148. Schuler G, Hu C, Ke A. Structural basis for RNA-guided DNA cleavage by IscB- ω RNA and mechanistic comparison with Cas9. *Science.* 2022;376(6600):1476-1481. <https://doi.org/10.1126/science.abq7220>
149. Teng F, Cui T, Gao Q, Guo L, Zhou Q, Li W. Artificial sgRNAs engineered for genome editing with new Cas12b orthologs. *Cell Discov.* 2019;5:23. <https://doi.org/10.1038/s41421-019-0091-0>

150. Strecker J, Jones S, Koopal B, Schmid-Burgk J, Zetsche B, Gao L, et al. Engineering of CRISPR-Cas12b for human genome editing. *Nat Commun.* 2019;10(1):212. <https://doi.org/10.1038/s41467-018-08224-4>
151. Liu JJ, Orlova N, Oakes BL, Ma E, Spinner HB, Baney KLM, et al. Casx enzymes comprise a distinct family of RNA-guided genome editors. *Nature.* 2019;566(7743):218-223. <https://doi.org/10.1038/s41586-019-0908-x>
152. Pausch P, Al-Shayeb B, Bisom-Rapp E, Tsuchida CA, Li Z, Cress BF, et al. CRISPR-CasΦ from huge phages is a hypercompact genome editor. *Science.* 2020;369(6501):333-337. <https://doi.org/10.1126/science.abb1400>
153. Kim DY, Lee JM, Moon SB, Chin HJ, Park S, Lim Y, et al. Efficient CRISPR editing with a hypercompact Cas12f1 and engineered guide RNAs delivered by adeno-associated virus. *Nat Biotechnol.* 2022;40(1):94-102. <https://doi.org/10.1038/s41587-021-01009-z>
154. Chen L, Zhou X, Huang C, Zhang Y, Xin C, Hong J, et al. Engineered Un1Cas12f1 with boosted gene-editing activity and expanded genomic coverage. *Proc Natl Acad Sci U S A.* 2025;122(32):e2501292122. <https://doi.org/10.1073/pnas.2501292122>
155. Ma S, Liao K, Chen K, Cheng T, Yang X, Chen P, et al. hpCasMINI: An engineered hypercompact CRISPR-Cas12f system with boosted gene editing activity. *Nat Commun.* 2025;16(1):5001. <https://doi.org/10.1038/s41467-025-60124-6>
156. Liao K, Chen K, Ma S, Yang X, Chen P, Li S, et al. exoCasMINI: A T5 exonuclease fused CRISPR-Cas12f system with enhanced gene editing efficiency. *iScience.* 2025;28(8):113171. <https://doi.org/10.1016/j.isci.2025.113171>
157. Kannan S, Altae-Tran H, Jin X, Madigan VJ, Oshiro R, Makarova KS, et al. Compact RNA editors with small Cas13 proteins. *Nat Biotechnol.* 2022;40(2):194-197. <https://doi.org/10.1038/s41587-021-01030-2>
158. Xu C, Zhou Y, Xiao Q, He B, Geng G, Wang Z, et al. Programmable RNA editing with compact CRISPR-Cas13 systems from uncultivated microbes. *Nat Methods.* 2021;18(5):499-506. <https://doi.org/10.1038/s41592-021-01124-4>
159. Yu C, Li L, Hu P, Yang Y, Wei W, Deng X, et al. Recent advances in stimulus-responsive nanocarriers for gene therapy. *Adv Sci (Weinh).* 2021;8(14):2100540. <https://doi.org/10.1002/advs.202100540>
160. Tang N, Ning Q, Wang Z, Tao Y, Zhao X, Tang S. Tumor microenvironment based stimuli-responsive CRISPR/Cas delivery systems: A viable platform for interventional approaches. *Colloids Surf B Biointerfaces.* 2022;210:112257. <https://doi.org/10.1016/j.colsurfb.2021.112257>
161. Xing Y, Yang J, Wang Y, Wang C, Pan Z, Liu FL, et al. Remodeling tumor immunogenicity with dual-activatable binary CRISPR nanomedicine for cancer immunotherapy. *ACS Nano.* 2023;17(6):5713-5726. <https://doi.org/10.1021/acsnano.2c12107>
162. Costagliola di Polidoro A, Cafarchio A, Vecchione D, Donato P, De Nola F, Torino E. Revealing Angiopep-2/LRP1 molecular interaction for optimal delivery to glioblastoma (GBM). *Molecules.* 2022;27(19):6696. <https://doi.org/10.3390/molecules27196696>
163. Zhao Y, Qin J, Yu D, Liu Y, Song D, Tian K. Polymer-locking fusogenic liposomes for glioblastoma-targeted siRNA delivery and CRISPR-Cas gene editing. *Nat Nanotechnol.* 2024;19(12):1869-1879. <https://doi.org/10.1038/s41565-024-01769-0>

164. Zou C, Liu X, Wang W, He L, Yin A, Cao Z, et al. Targeting GDF15 to enhance immunotherapy efficacy in glioblastoma through tumor microenvironment-responsive CRISPR-Cas9 nanoparticles. *J Nanobiotechnology*. 2025;23(1):126. <https://doi.org/10.1186/s12951-025-03182-8>
165. Zhao K, Yan Y, Jin XK, Pan T, Zhang SM, Yang CH, et al. An orally administered gene editing nanoparticle boosts chemo-immunotherapy in colorectal cancer. *Nat Nanotechnol*. 2025;20(7):935-946. <https://doi.org/10.1038/s41565-025-01904-5>
166. Zhao W, Zhang L, Guo J, Xu Q, Zhang M, Liu H, et al. Intelligent nano-cage for precision delivery of CRISPR-Cas9 and ACC inhibitors to enhance antitumor cascade therapy through lipid metabolism disruption. *Adv Funct Mater*. 2025;35(13):2418090. <https://doi.org/10.1002/adfm.202418090>
167. Liu H, Yang Y, Zhang N, Hou Y, Zhang Z, Yu X, et al. Overcoming photothermal resistance of gastric cancer by bionic 2D iron-based nanoplatfoms with precise CRISPR/Cas9 delivery. *ACS Nano*. 2025;19(19):18188-18202. <https://doi.org/10.1021/acsnano.4c16846>
168. Awwad SW, Serrano-Benitez A, Thomas JC, Gupta V, Jackson SP. Revolutionizing DNA repair research and cancer therapy with CRISPR-Cas screens. *Nat Rev Mol Cell Biol*. 2023;24(7):477-494. <https://doi.org/10.1038/s41580-022-00571-x>
169. Zhang A, Miao K, Sun H, Deng CX. Tumor heterogeneity reshapes the tumor microenvironment to influence drug resistance. *Int J Biol Sci*. 2022;18(7):3019-3033. <https://doi.org/10.7150/ijbs.72534>
170. Azangou-Khyavy M, Ghasemi M, Khanali J, Boroomand-Saboore M, Jamalkhah M, Soleimani M, et al. CRISPR/Cas: From tumor gene editing to T cell-based immunotherapy of cancer. *Front Immunol*. 2020;11:2062. <https://doi.org/10.3389/fimmu.2020.02062>
171. Chehelgerdi M, Chehelgerdi M, Khorramian-Ghahfarokhi M, Shafieizadeh M, Mahmoudi E, Eskandari F, et al. Comprehensive review of CRISPR-based gene editing: mechanisms, challenges, and applications in cancer therapy. *Mol Cancer*. 2024;23(1):9. <https://doi.org/10.1186/s12943-023-01925-5>
172. Di Carlo E, Sorrentino C. State of the art CRISPR-based strategies for cancer diagnostics and treatment. *Biomark Res*. 2024;12(1):156. <https://doi.org/10.1186/s40364-024-00701-x>
173. Martinez-Lage M, Torres-Ruiz R, Puig-Serra P, Moreno-Gaona P, Martin MC, Moya FJ, et al. In vivo CRISPR/Cas9 targeting of fusion oncogenes for selective elimination of cancer cells. *Nat Commun*. 2020;11(1):5060. <https://doi.org/10.1038/s41467-020-18875-x>
174. Duan Y, Liu Z, Wang Q, Zhang J, Liu J, Zhang Z, et al. Targeting MYC: Multidimensional regulation and therapeutic strategies in oncology. *Genes Dis*. 2024;12(4):101435. <https://doi.org/10.1016/j.gendis.2024.101435>
175. Kim W, Lee S, Kim HS, Song M, Cha YH, Kim YH, et al. Targeting mutant KRAS with CRISPR-Cas9 controls tumor growth. *Genome Res*. 2018;28:374-382. <https://doi.org/10.1101/gr.223891.117>
176. Wei Y, Zhao Z, Ma X. Description of CRISPR-Cas9 development and its prospects in human papillomavirus-driven cancer treatment. *Front Immunol*. 2022;13:1037124. <https://doi.org/10.3389/fimmu.2022.1037124>
177. Jin X, Lou X, Qi H, Zheng C, Li B, Siwu X, et al. NRF2 signaling plays an essential role in cancer progression through the NRF2-GPX2-NOTCH3 axis in head and neck squamous cell carcinoma. *Oncogenesis*. 2024;13:35. <https://doi.org/10.1038/s41389-024-00536-z>
178. Hussen BM, Najmadden ZB, Abdullah SR, Rasul MF, Mustafa SA, Ghafouri-Fard et al. CRISPR/Cas9 gene editing: a novel strategy for fighting drug resistance in respiratory disorders. *Cell Commun Signal*. 2024;22:329. <https://doi.org/10.1186/s12964-024-01713-8>

179. Chen Y, Zhang Y. Application of the CRISPR/Cas9 system to drug resistance in breast cancer. *Adv Sci (Weinh)*. 2018;5:1700964. <https://doi.org/10.1002/adv.201700964>
180. Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell*. 2000;100(4):387-390. [https://doi.org/10.1016/s0092-8674\(00\)80674-1](https://doi.org/10.1016/s0092-8674(00)80674-1)
181. Vitiello M, Evangelista M, Zhang Y, Salmena L, Pandolfi PP, Poliseno L. PTENP1 is a ceRNA for PTEN: it's CRISPR clear. *J Hematol Oncol*. 2020;13:73. <https://doi.org/10.1186/s13045-020-00894-2>
182. Tang W, Liu J, Ding B. Nucleic acid nanostructure for delivery of CRISPR/Cas9-based gene editing system. *Interdiscip Med*. 2023;1:e20220014. <https://doi.org/10.1002/inmd.20220014>
183. Guo LY, Bian J, Davis AE, Liu P, Kempton HR, Zhang X, et al. Multiplexed genome regulation in vivo with hyper-efficient Cas12a. *Nat Cell Biol*. 2022;24:590-600. <https://doi.org/10.1038/s41556-022-00870-7>
184. Li P, Zhang L, Li Z, Xu C, Du X, Wu S. Cas12a mediates efficient and precise endogenous gene tagging via MITI: microhomology-dependent targeted integrations. *Cell Mol Life Sci*. 2020;77:3875-3884. <https://doi.org/10.1007/s00018-019-03396-8>
185. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007;316(5827):1039-1043. <https://doi.org/10.1126/science.1141478>
186. Yoon AR, Jung BK, Choi E, Chung E, Hong J, Kim JS, et al. CRISPR-Cas12a with an oAd induces precise and cancer-specific genomic reprogramming of EGFR and efficient tumor regression. *Mol Ther*. 2020;28(10):2286-2296. <https://doi.org/10.1016/j.ymthe.2020.07.003>
187. Choi E, Hwang H-Y, Kwon E, Kim D, Koo T. Expanded targeting scope of LbCas12a variants allows editing of multiple oncogenic mutations. *Mol Ther Nucleic Acids*. 2022;30:131-142. <https://doi.org/10.1016/j.omtn.2022.09.005>
188. Huang A, Garraway LA, Ashworth A, Weber B. Synthetic lethality as an engine for cancer drug target discovery. *Nat Rev Drug Discov*. 2020;19:23-38. <https://doi.org/10.1038/s41573-019-0046-z>
189. Gralowska P, Gajek A, Marczak A, Rogalska A. Participation of the ATR/CHK1 pathway in replicative stress targeted therapy of high-grade ovarian cancer. *J Hematol Oncol*. 2020;13:39. <https://doi.org/10.1186/s13045-020-00874-6>
190. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*. 2005;434:913-917. <https://doi.org/10.1038/nature03443>
191. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921. <https://doi.org/10.1038/nature03445>
192. Parrish PCR, Thomas JD, Gabel AM, Kamlapurkar S, Bradley RK, Berger AH. Discovery of synthetic lethal and tumor suppressor paralog pairs in the human genome. *Cell Rep*. 2021;36(9):109597. <https://doi.org/10.1016/j.celrep.2021.109597>
193. Ryan CJ, Mehta I, Kebabei N, Adams DJ. Targeting synthetic lethal paralogs in cancer. *Trends Cancer*. 2023;9(5):397-409. <https://doi.org/10.1016/j.trecan.2023.02.002>

194. Mohanraju P, Makarova KS, Zetsche B, Zhang F, Koonin EV, van der Oost J. Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science*. 2016;353(6299):aad5147. <https://doi.org/10.1126/science.aad5147>
195. Bender G, Yamaci RF, Taneri B. CRISPR and KRAS: a match yet to be made. *J Biomed Sci*. 2021;28:77. <https://doi.org/10.1186/s12929-021-00772-0>
196. Ma J, Huang L, Hu D, Zeng S, Han Y, Shen H. The role of the tumor microbe microenvironment in the tumor immune microenvironment: bystander, activator, or inhibitor? *J Exp Clin Cancer Res*. 2021;40:327. <https://doi.org/10.1186/s13046-021-02128-w>
197. Lv B, Wang Y, Ma D, Cheng W, Liu J, Yong T, et al. Immunotherapy: Reshape the Tumor Immune Microenvironment. *Front Immunol*. 2022;13:844142. <https://doi.org/10.3389/fimmu.2022.844142>
198. Bai R, Cui J. Development of immunotherapy strategies targeting tumor microenvironment is fiercely ongoing. *Front Immunol*. 2022;13:890166. <https://doi.org/10.3389/fimmu.2022.890166>
199. Liu Z, Shi M, Ren Y, Xu H, Weng S, Ning W, et al. Recent advances and applications of CRISPR-Cas9 in cancer immunotherapy. *Mol Cancer*. 2023;22(1):35. <https://doi.org/10.1186/s12943-023-01738-6>
200. Lu Y, Xue J, Deng T, Zhou X, Yu K, Deng L, et al. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. *Nat Med*. 2020;26(5):732-740. <https://doi.org/10.1038/s41591-020-0840-5>
201. June CH, Sadelain M. Chimeric antigen receptor therapy. *N Engl J Med*. 2018;379(1):64-73. <https://doi.org/10.1056/NEJMra1706169>
202. Jogalekar M, Rajendran RL, Khan F, Dmello C, Gangadaran P, Ahn B, Ahn B-C. CAR T-cell-based gene therapy for cancers: new perspectives, challenges, and clinical developments. *Front Immunol*. 2022;13:925985. <https://doi.org/10.3389/fimmu.2022.925985>
203. Tong C, Zhang Y, Liu Y, Ji X, Zhang W, Guo Y, et al. Optimized tandem CD19/CD20 CAR-engineered T cells in refractory/relapsed B-cell lymphoma. *Blood*. 2020;136(14):1632-1644. <https://doi.org/10.1182/blood.2020005278>
204. Azeez SS, Yashooa RK, Smail SW, Salihi A, Ali AS, Mamand S, et al. Advancing CAR-based cell therapies for solid tumours: challenges, therapeutic strategies, and perspectives. *Mol Cancer*. 2025;24:191. <https://doi.org/10.1186/s12943-025-02386-8>
205. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJC, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*. 2017;543:113-117. <https://doi.org/10.1038/nature21405>
206. Yang C, Liu Y, Huang Z, Liu S, Zhang X, Liu Q, et al. Recent advances and challenges of cellular immunotherapies in lung cancer treatment. *Exp Hematol Oncol*. 2025;14(1):94. <https://doi.org/10.1186/s40164-025-00679-8>
207. Benjamin R, Graham C, Yallop D, Jozwik A, Mirzi-Danicar OC, Lucchini G, et al. Genome-edited, donor-derived allogeneic anti-CD19 chimeric antigen receptor T cells in paediatric and adult B-cell acute lymphoblastic leukaemia: results of two phase 1 studies. *Lancet*. 2020;396(10266):1885-1894. [https://doi.org/10.1016/s0140-6736\(20\)32334-5](https://doi.org/10.1016/s0140-6736(20)32334-5)
208. Guo YL, Tong C, Su L, Zhang W, Jia H, Liu Y, et al. CRISPR/Cas9 genome-edited universal CAR T cells in patients with relapsed/refractory lymphoma. *Blood Adv*. 2022;6(8):2695-2699. <https://doi.org/10.1182/bloodadvances.2021006232>

209. Dimitri A, Herbst F, Fraietta JA. Engineering the next-generation of CAR T-cells with CRISPR-Cas9 gene editing. *Mol Cancer*. 2022;21:78. <https://doi.org/10.1186/s12943-022-01559-z>
210. Stadtmauer EA, Fraietta JA, Davis MM, Cohen AD, Weber KL, Lancaster E, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science*. 2020;367(6481):eaba7365. <https://doi.org/10.1126/science.aba7365>
211. Morris EC, Neelapu SS, Giavridis T, Sadelain M. Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy. *Nat Rev Immunol*. 2022;22:85-96. <https://doi.org/10.1038/s41577-021-00547-6>
212. Naeem M, Hazafa A, Bano N, Ali R, Farooq M, Razak SIA, et al. Explorations of CRISPR/Cas9 for improving the long-term efficacy of universal CAR-T cells in tumor immunotherapy. *Life Sci*. 2023;316:121409. <https://doi.org/10.1016/j.lfs.2023.121409>
213. Sterner RM, Sakemura R, Cox MJ, Yang N, Khadka RH, Forsman CL, et al. GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function in xenografts. *Blood*. 2019;133(7):697-709. <https://doi.org/10.1182/blood-2018-10-881722>
214. Razeghian E, Nasution MKM, Rahman HS, Gardanova ZR, Abdelbasset WK, Aravindhan S, et al. A deep insight into CRISPR/Cas9 application in CAR-T cell-based tumor immunotherapies. *Stem Cell Res Ther*. 2021;12:428. <https://doi.org/10.1186/s13287-021-02510-7>
215. Gong Y, Klein Wolterink RGJ, Wang J, Bos GMJ, Germeraad WTV. Chimeric antigen receptor natural killer (CAR-NK) cell design and engineering for cancer therapy. *J Hematol Oncol*. 2021;14:73. <https://doi.org/10.1186/s13045-021-01083-5>
216. Xie G, Dong H, Liang Y, Ham JD, Rizwan R, Chen J. CAR-NK cells: a promising cellular immunotherapy for cancer. *eBioMedicine*. 2020;59:102975. <https://doi.org/10.1016/j.ebiom.2020.102975>
217. Wang Y, Jin S, Zhuang Q, Liu N, Chen R, Adam SA, et al. Chimeric antigen receptor natural killer cells: a promising antitumor immunotherapy. *MedComm (2020)*. 2023;4(6):e422. <https://doi.org/10.1002/mco2.422>
218. Kong JC, Sa'ad MA, Vijayan HM, Ravichandran M, Balakrishnan V, Tham SK, et al. Chimeric antigen receptor-natural killer cell therapy: current advancements and strategies to overcome challenges. *Front Immunol*. 2024;15:1384039. <https://doi.org/10.3389/fimmu.2024.1384039>
219. Gurney M, Stikvoort A, Nolan E, Kirkham-McCarthy L, Khoruzhenko S, Shivakumar R, et al. CD38 knockout natural killer cells expressing an affinity-optimized CD38 chimeric antigen receptor successfully target acute myeloid leukemia with reduced effector cell fratricide. *Haematologica*. 2022;107(2):437-445. <https://doi.org/10.3324/haematol.2020.271908>
220. Daher M, Basar R, Gokdemir E, Baran N, Uprety N, Nunez Cortes AK, et al. Targeting a cytokine checkpoint enhances the fitness of armored cord blood CAR-NK cells. *Blood*. 2021;137(5):624-636. doi:<https://doi.org/10.1182/blood.2020007748>
221. Morimoto T, Nakazawa T, Matsuda R, Nishimura F, Nakamura M, Yamada S, et al. CRISPR-Cas9-mediated TIM3 knockout in human natural killer cells enhances growth inhibitory effects on human glioma cells. *Int J Mol Sci*. 2021;22(7):3489. <https://doi.org/10.3390/ijms22073489>
222. Zhang Q, Bi J, Zheng X, Chen Y, Wang H, Wu W, et al. Blockade of the checkpoint receptor TIM3 prevents NK cell exhaustion and elicits potent anti-tumor immunity. *Nat Immunol*. 2018;19:723-732. <https://doi.org/10.1038/s41590-018-0132-0>

223. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8⁺ T cell effector function. *Cancer Cell*. 2014;26(6):923-937. <https://doi.org/10.1016/j.ccell.2014.10.018>
224. Jo D-H, Kaczmarek S, Shin O, Wang L, Cowan J, McComb S, et al. Simultaneous engineering of natural killer cells for CAR transgenesis and CRISPR-Cas9 knockout using retroviral particles. *Mol Ther Methods Clin Dev*. 2023;29:173-184. <https://doi.org/10.1016/j.omtm.2023.03.006>
225. Kong R, Liu B, Wang H, Lu T, Zhou X. CAR-NK cell therapy: latest updates from the 2024 ASH annual meeting. *J Hematol Oncol*. 2025;18:22. <https://doi.org/10.1186/s13045-025-01677-3>
226. Hoerster K, Uhrberg M, Wiek C, Horn PA, Hanenberg H, Heinrichs S. HLA class I knockout converts allogeneic primary NK cells into suitable effectors for “off-the-shelf” immunotherapy. *Front Immunol*. 2021;11:586168. <https://doi.org/10.3389/fimmu.2020.586168>
227. Mukhopadhyay M. Macrophages enter CAR immunotherapy. *Nat Methods*. 2020;17(6):561. <https://doi.org/10.1038/s41592-020-0862-4>
228. Chen Y, Yu Z, Tan X, Jiang H, Xu Z, Fang Y, et al. CAR-macrophage: A new immunotherapy candidate against solid tumors. *Biomed Pharmacother*. 2021;139:111605. <https://doi.org/10.1016/j.biopha.2021.111605>
229. Lu J, Ma Y, Li Q, Xu Y, Xue Y, Xu S. CAR Macrophages: a promising novel immunotherapy for solid tumors and beyond. *Biomark Res*. 2024;12(1):86. <https://doi.org/10.1186/s40364-024-00637-2>
230. Zhang J, Webster S, Duffin B, Bernstein MN, Steill J, Swanson S, et al. Generation of anti-GD2 CAR macrophages from human pluripotent stem cells for cancer immunotherapies. *Stem Cell Reports*. 2023;18(2):585-596. <https://doi.org/10.1016/j.stemcr.2022.12.012>
231. Chettri D, Satapathy BP, Yadav R, Uttam V, Jain A, Prakash H. CAR-macrophages: tailoring cancer immunotherapy. *Front Immunol*. 2025;15:1532833. <https://doi.org/10.3389/fimmu.2024.1532833>
232. Sloas C, Gill S, Klichinsky M. Engineered CAR-macrophages as adoptive immunotherapies for solid tumors. *Front Immunol*. 2021;12:783305. <https://doi.org/10.3389/fimmu.2021.783305>
233. Siegler EL, Simone BW, Sakemura R, Tapper EE, Horvei P, Cox MJ, et al. Efficient gene editing of CART cells with CRISPR-Cas12a for enhanced antitumor efficacy. *Blood*. 2020;136(Suppl 1): 6–7. <https://doi.org/10.1182/blood-2020-141115>
234. Chaivorapol C, Beauchesne P, De Jesus R, Dowdle WE, Chen X, Bayley A, et al. Efficient and specific multi-locus editing of allogeneic CAR T cells for hypoimmunity during large scale manufacture using Cas12b. *Blood*. 2022;140(Suppl 1):1606–1607. <https://doi.org/10.1182/blood-2022-167627>
235. Zhao L, Luo Y, Huang Q, Cao Z, Yang X. Photo-enhanced CRISPR/Cas9 system enables robust PD-L1 gene disruption in cancer cells and cancer stem-like cells for efficient cancer immunotherapy. *Small*. 2020;16:e2004879. <https://doi.org/10.1002/sml.202004879>
236. Jiang X, Wang J, Deng X, Xiong F, Ge J, Xiang B, et al. Role of the tumor microenvironment in PD-L1/PD-1-mediated tumor immune escape. *Mol Cancer*. 2019;18:10. <https://doi.org/10.1186/s12943-018-0928-4>
237. Park H, Kang YK, Shim G. CRISPR/Cas9-mediated customizing strategies for adoptive T-cell therapy. *Pharmaceutics*. 2024;16(3):346. <https://doi.org/10.3390/pharmaceutics16030346>

238. Lu Q, Chen R, Du S, Chen C, Pan Y, Luan X, et al. Activation of the cGAS-STING pathway combined with CRISPR-Cas9 gene editing triggering long-term immunotherapy. *Biomaterials*. 2022;291:121871. <https://doi.org/10.1016/j.biomaterials.2022.121871>
239. Chen B, Mu C, Zhang Z, He X, Liu X. The love-hate relationship between TGF- β signaling and the immune system during development and tumorigenesis. *Front Immunol*. 2022;13:891268. <https://doi.org/10.3389/fimmu.2022.891268>
240. Fix SM, Forget M-A, Sakellariou-Thompson D, Wang Y, Griffiths TM, Lee M, et al. CRISPR-mediated TGFBR2 knockout renders human ovarian cancer tumor-infiltrating lymphocytes resistant to TGF- β signaling. *J Immunother Cancer*. 2022;10(7):e003750. <https://doi.org/10.1136/jitc-2021-003750>
241. Tang N, Cheng C, Zhang X, Qiao M, Li N, Mu W, et al. TGF- β inhibition via CRISPR promotes the long-term efficacy of CAR T cells against solid tumors. *JCI Insight*. 2020;5(4):e133977. <https://doi.org/10.1172/jci.insight.133977>
242. Haake M, Haack B, Schäfer T, Harter PN, Mattavelli G, Eiring P, et al. Tumor-derived GDF-15 blocks LFA-1 dependent T cell recruitment and suppresses responses to anti-PD-1 treatment. *Nat Commun*. 2023;14(1):4253. <https://doi.org/10.1038/s41467-023-39817-3>
243. He L, Li Z, Su D, Du H, Zhang K, Zhang W, et al. Tumor microenvironment-responsive nanocapsule delivery CRISPR/Cas9 to reprogram the immunosuppressive microenvironment in hepatoma carcinoma. *Adv Sci (Weinh)*. 2024;11(26):e2403858. <https://doi.org/10.1002/advs.202403858>
244. Sorrentino C, D'Antonio L, Ciummo SL, Fieni C, Landuzzi L, Ruzzi F, et al. CRISPR/Cas9-mediated deletion of Interleukin-30 suppresses IGF1 and CXCL5 and boosts SOCS3 reducing prostate cancer growth and mortality. *J Hematol Oncol*. 2022;15(1):145. <https://doi.org/10.1186/s13045-022-01357-6>
245. Sorrentino C, Ciummo SL, D'Antonio L, Fieni C, Lanuti P, Turdo A. Interleukin-30 feeds breast cancer stem cells via CXCL10 and IL23 autocrine loops and shapes immune contexture and host outcome. *J Immunother Cancer*. 2021;9(10):e002966. <https://doi.org/10.1136/jitc-2021-002966>
246. D'Antonio L, Fieni C, Ciummo SL, Vespa S, Lotti L, Sorrentino C, et al. Inactivation of interleukin-30 in colon cancer stem cells via CRISPR/Cas9 genome editing inhibits their oncogenicity and improves host survival. *J Immunother Cancer*. 2023;11(3):e006056. <https://doi.org/10.1136/jitc-2022-006056>
247. Ma S, Li Y, Liu F, Wang X, Qin Z, Wang L, et al. Hierarchical-unlocking virus-esque NanoCRISPR precisely disrupts autocrine and paracrine pathway of VEGF for tumor inhibition and antiangiogenesis. *J Control Release*. 2024;366:505-518. <https://doi.org/10.1016/j.jconrel.2024.01.001>
248. Hariprabu KNG, Sathya M, Vimalraj S. CRISPR/Cas9 in cancer therapy: a review with a special focus on tumor angiogenesis. *Int J Biol Macromol*. 2021;192:913-930. <https://doi.org/10.1016/j.ijbio.2021.10.029>
249. Dey P, Kimmelman AC, DePinho RA. Metabolic codependencies in the tumor microenvironment. *Cancer Discov*. 2021;11(5):1067-1081. <https://doi.org/10.1158/2159-8290.CD-20-1211>
250. Ma K, Mao Q, Fei B, Ni T, Zhang Z, Ni H. Metabolic reprogramming and immune microenvironment characteristics in laryngeal carcinoma: advances in immunotherapy. *Front Immunol*. 2025;16:1589243. <https://doi.org/10.3389/fimmu.2025.1589243>

251. Huang Y, Qin G, Cui T, Zhao C, Ren J, Qu X. A bimetallic nanoplatform for STING activation and CRISPR/Cas mediated depletion of the methionine transporter in cancer cells restores anti-tumor immune responses. *Nat Commun.* 2023;14(1):4647. <https://doi.org/10.1038/s41467-023-40345-3>
252. Leibowitz ML, Papathanasiou S, Doerfler PA, Blaine LJ, Sun L, Yao Y, et al. Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. *Nat Genet.* 2021;53(6):895-905. <https://doi.org/10.1038/s41588-021-00838-7>
253. Aussel C, Cathomen T, Fuster-Garcia C. The hidden risks of CRISPR/Cas: structural variations and genome integrity. *Nat Commun.* 2025;16(1):7208. <https://doi.org/10.1038/s41467-025-62606-z>
254. Tang XZE, Tan SX, Hoon S, Yeo GW. Pre-existing adaptive immunity to the RNA-editing enzyme Cas13d in humans. *Nat Med.* 2022;28(7):1372-1376. <https://doi.org/10.1038/s41591-022-01848-6>
255. Charlesworth CT, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat Med.* 2019;25(2):249-254. <https://doi.org/10.1038/s41591-018-0326-x>

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Authors' affiliations

- 1- School of Medicine, Sun Yat-sen University, Shenzhen, Guangdong, 518107, P. R. China.
- 2- Department of Clinical Laboratory, The Seventh Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, 518107, P. R. China.

3- Guangdong Provincial Key Laboratory of Digestive Cancer Research, Digestive Diseases Center, Scientific Research Center, The Seventh Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, 518107, P. R. China.

*These authors contributed equally to this work.

#Correspondence: Chaoxing Liu - Email: liuchx69@mail.sysu.edu.cn

Guangrong Zou - Email: zougr3@mail.sysu.edu.cn

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ORCID ID

Chaoxing Liu, <https://orcid.org/0000-0003-3077-9660>

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