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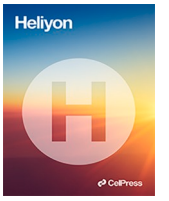

Review article

CRISPR screens in mechanism and target discovery for AML

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ABSTRACT

CRISPR-based screens have discovered novel functional genes involving in diverse tumor biology and elucidated the mechanisms of the cancer pathological states. Recently, with its randomness and unbiasedness, CRISPR screens have been used to discover effector genes with previously unknown roles for AML. Those novel targets are related to AML survival resembled cellular pathways mediating epigenetics, synthetic lethality, transcriptional regulation, mitochondrial and energy metabolism. Other genes that are crucial for pharmaceutical targeting and drug resistance have also been identified. With the rapid development of novel strategies, such as barcodes and multiplexed mosaic CRISPR perturbation, more potential therapeutic targets and mechanism in AML will be discovered. In this review, we present an overview of recent progresses in the development of CRISPR-based screens for the mechanism and target identification in AML and discuss the challenges and possible solutions in this rapidly growing field.

1. Introduction

Acute myeloid leukemia (AML) is one of the common hematological malignancies in adults. It is characterized by clonal malignant proliferation of myeloid progenitor cells and suppression of proper hematopoietic cell function. Currently, the 5-year relative survival of AML patients is 30.5 % in the United States. Another Swedish national registry data shows that the 5-year survival in patients under the age of 50 is estimated at 62 %, falling to 37 % for those aged 50 to 64, and only 9.4 % for those aged 65 and older [1]. Allogeneic bone marrow or hematopoietic stem cell transplantation (Allo-BMT or Allo-HSCT), which is often used as a sequential treatment after the "3 + 7 program" and standard treatment for AML, is one of the effective therapies for AML [2]. However, patients may have a high recurrence rate due to the lack of graft-versus-leukemia (GVL) effects in Auto-HSCT [3].

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Since 2017, several new targeted drugs, including IDH1/2 inhibitors, FLT3 inhibitors, BCL-2 inhibitors, CPX-351 (Dual drug liposome formulation of cytarabine and daunorubicin with a fixed drug ratio of 5:1) have gradually entered the clinic, triggering major changes in AML treatment. On the one hand, for some AML patients, who cannot tolerate high-intensity chemotherapies or are too old to receive standard regimen treatment, usually received low-intensity chemotherapy or demethylation drugs based on cytarabine in the past. Compared with others, these patients have lower remission rate, more limited survival time and poorer prognosis. The introduction of targeted drugs has greatly improved the remission rate and prognosis of these patients. On the other hand, for AML patients who can tolerate the standard "3 + 7 regimen", the introduction of targeted drugs has also brought new treatment options. Some targeted drug treatment programs have gradually shaken classical treatment programs [4,5]. Although some progress has been made in the treatment of AML, effective treatment remains dramatically challenging and many mysteries remain to be solved. Developing high-throughput and high-efficiency screening technologies for exploring novel AML targets will contribute to the development of new therapeutics for improving prognosis of the patients [6].

CRISPR Screen is a powerful platform to interrogate phenotypic loss-of-function genes at a high-throughput level based on the CRISPR-Cas9 system. The specific sgRNA libraries in combination with next-generation sequencing (NGS) have made genetic screening feasible, efficient, and prevalent. In recent years, advanced technologies such as barcodes, ATAC-seq, multiplexed mosaic CRISPR perturbation have enabled high-dimensional, single-cell CRISPR to identify new targets through high-throughput gene screening methods. Over the past few years, the collision between the CRISPR screen and AML has produced creative and innovative sparks for AML target discovery. In this review, we present an overview of recent progresses in the development of CRISPR-based screens for mechanism and target identification in AML and discuss challenges and possible solutions in this rapidly growing field.

2. Strategies for CRISPR screens of AML targets

In brief, the CRISPR screen process is mainly 3 steps. Firstly, establish a randomized, unbiased mutant cell pool. Introducing Cas9 into AML cells to obtain $Cas9^+$ AML cells; Subsequently transfecting $Cas9^+$AML cells with CRISPR libraries in low MOI to make each cell contain one sgRNA; Secondly, select the "winning" or "eliminating" cells with a presupposed readout from the cell pool; Thirdly, extract RNA from selected cells for deep sequencing and identify the enriched or depleted genes through comparison to the original sgRNA library. Usually, the selection of sgRNA libraries, cell lines and readout is based on specific purposes. Here we provide a review on the CRISPR screen strategies and summarize the main findings in AML (Supplemental table 1).

2.1. Discovering therapeutic targets for AML cell survival

Cell survival is a common readout for the CRISPR library to screen tumor therapeutic targets [7]. Zhou Y et al. conducted CRISPR screens in 15 AML cell lines and identified 94 genes that met the following three criteria: First, it is important for the survival of AML cells. Second, it is not important for the survival of normal cells. Third, it is structurally able to be a drug target. Among these 94 genes, 44 were previously well-defined drug targets, and the remaining 50 were potential drug targets that have not been reported. The 50 genes are related to uridine monophosphate synthesis and dihydrofolate metabolism [8].

Currently, most studies using CRISPR screen to identify AML targets have been performed *in vitro* using established cell lines [9–11]. Given that evaluating the physiologic relevance of these targets *in vivo* is critical, Lin et al. developed an integrated *in vivo* screening approach to prioritize and validate AML dependencies with high translational potential. They identified SLC5A3 and MARCH5, a metabolic vulnerability and a critical regulator of apoptosis in AML, both of which represent novel therapeutic opportunities [12].

2.1.1. Targeting the initiation of AML

Analyzing early molecular events in AML pathogenesis not only helps to understand the mechanism of AML development [13], but may also provide new targets for AML treatment. However, the early stages of leukemia transformation are typically inaccessible from both human patients and conventional mouse models. The researchers speculated that the Hoxb8-FL cell line, which can be conditionally blocked at the stem/progenitor cell stage and still maintain intact differentiation potential, can be used to establish an MLL-ENL induced AML mouse model [14]. Therefore, they transformed MLL-ENL into Hoxb8-FL cells (ME-transformed Hoxb8-FL cells) and cultured them in specific conditions. Then the survival cells were injected into lethally irradiated mice. In this model, ME-transformed Hoxb8-FL cells developed AML within 75 days, simulating MLL-ENL-driven AML starting from hematopoietic progenitor cells. Using these ME-transformed Hoxb8-FL cells, researchers performed a genome-wide CRISPR-Cas9 drop-out screen. Surviving cells under specific culture conditions were collected and deep sequenced. Enriched or depleted gRNAs were determined by comparison to the original library. 127 candidate genes critical in ME-Transformed cells were obtained. In order to further identify potential drug targets, the researchers used drug gene interaction database (dgidb, https://dgidb.org/) to predict the 127 genes and found that 47 of them could become potential drug targets. Subsequently, they validated these genes with commercially available inhibitors, demonstrating that *Atm*, *Cdc7* and *Ldha* were potential targets for AML treatment [14].

Myelodysplastic syndromes (MDS) are a group of heterogeneous myeloid clonal diseases originating from hematopoietic stem cells. It is characterized by abnormal differentiation and development of myeloid cells, manifested as ineffective hematopoiesis, refractory hemocytopenia, hematopoietic failure, and high risk of transformation to secondary acute myeloid leukemia (sAML). At present, the mechanism of MDS to sAML conversion is still unclear [15]. To identify the key regulators that accelerate MDS to sAML, Michael Schieber et al. performed a genome-wide CRISPR screen in human MDS-L cell line. The cells that survived for 4 weeks without IL-3 were selected and sequenced. 22 enriched candidate genes were determined by comparison with the original library. They verified

that FBXO11 was required for cytokine-independent survival of MDS-L cells *in vitro* [16]. Loss of FBXO11 led to significant changes in transcriptional regulation of some genes, thereby affecting the proliferation, differentiation, and apoptosis of leukocyte. They also found that FBXO11 expression was significantly down regulated in patients with sAML, suggesting that FBXO11 is a promising target

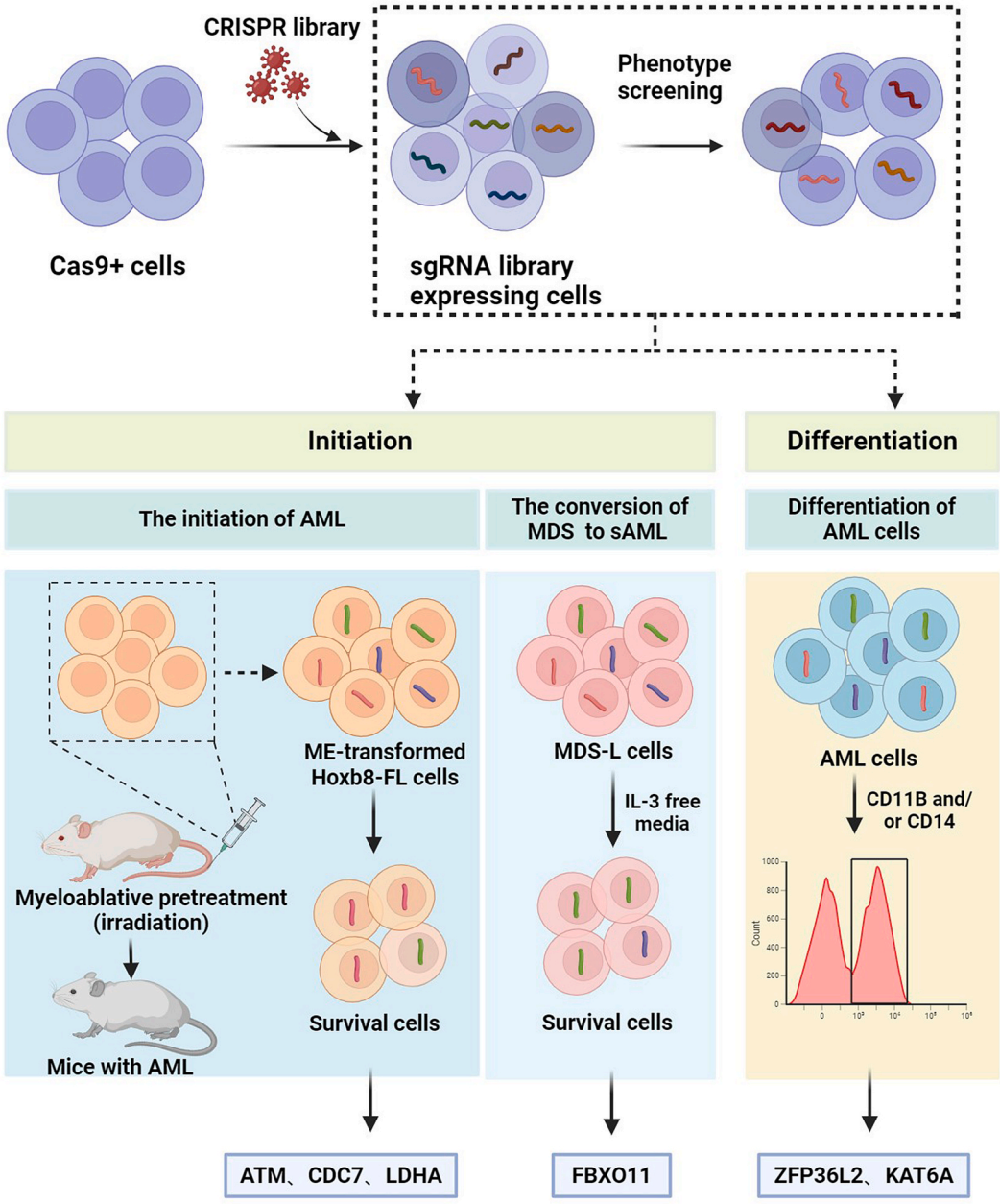

Fig. 1. Strategies for CRISPR screens of AML Initiation and differentiation. Briefly, AML cells are transduced with an sgRNA library in low MOI. Elutriating the cell pool with a presupposed readout model prompts the "winning" or "eliminating" cells to stand out, and then sgRNAs enriched or depleted in the sorted cells are analyzed to identify potential candidate genes. To discover the critical genes in the initiation of AML, the MLL-ENL-driven leukemia model is established based on Hoxb8-FL cells. Transplantation the ME-transformed cells into lethally irradiated mice will lead to the development of AML in mice. The ME-transformed Hoxb8-FL cells are transduced with a CRISPR library to establish a mutagenized cell pool. The candidate targets are identified through sequencing and analysis of the enriched sgRNAs in the survival cells [14]. MDS-L cells obtain leukemic cell properties required cytokine-dependent signals. Therefore, a CRISPR/Cas9 screen is performed based on the IL-3 free media to identify the critical genes in the conversion of MDS to sAML. FBXO11 is identified through sequencing and analysis of the enriched sgRNAs in the survival cells [16]. AML is characterized by a failure to terminally differentiate into functional mature myeloid cells. Cells in the mutagenized cell pool are sorted on the basis of high CD14 and/or CD11B expression and individual sgRNA read counts are evaluated by deep sequencing. ZFP36L2 and KAT6A are identified as the critical regulators for AML differentiation [17,18].

for treating MDS to sAML conversion [16] (Fig. 1).

2.1.2. Targeting cell differentiation

AML is characterized by failure to differentiate terminally into functional mature myeloid cells and targeting differentiation blockade represents one of the promising therapeutic strategies for AML [19]. To explore the key manipulators of AML differentiation, Eric Wang et al. used a genome-wide sgRNA library and selected AML terminal differentiation markers CD14 and CD11B as alternative readouts for CRISPR screening in THP-1 cells. 12 genes related to AML cell survival and differentiation were determined by intersecting with previously reported genes. The top-scoring candidate ZFP36L2, an RNA binding protein, was verified to be a potential target for differentiation therapy *in vitro* and *in vivo*. Mechanistically, ZFP36L2 interacted with the 3′-UTR of ZFP36L1, CEBPB, and ITGB2, which belong to ZFP36 paralogs and are key myeloid maturation genes, to promote mRNA degradation and suppress terminal differentiation of myeloid cells. Genetic inhibition of ZFP36L2 restored mRNA stability of these targeted transcripts and ultimately triggered myeloid differentiation in leukemia cells [17]. Another group designed a CRISPR screen with CD11B as readout to explore key regulators of AML differentiation, and identified KAT6A [18]. KAT6A may serve as a transcriptional control module, in which KAT6A-catalyzed promoter H3K9ac provides a substrate for binding the acetyl-lysine reader ENL and facilitates the later stages of transcriptional activation of MYC, MYB, and other prominent regulators of AML cell fates to induce transcriptional elongation [18] (Fig. 1).

2.1.3. Epigenetic regulators

Epigenetic abnormalities are a common feature of AML. Epigenetic changes, such as DNA methylation or histone modifications, may lead to the occurrence, progression, and resistance of AML. Multiple studies have found that 70 % of recurrent mutations in AML patients occur in factors regulating gene expression, such as epigenetic proteins, transcription factors, and splicing mechanism components [18,20–23]. Therefore, CRISPR screen using sgRNA libraries targeting epigenetic factors is promising to discover therapeutic targets for AML. Gu Z et al. established a CRISPR library focusing on epigenetic regulators [24]. Briefly, 498 evolutionarily conserved chromatin-interacting domains were identified from 266 human epigenetic regulators, and 3248 sgRNAs were designed for these domains; 1800 sgRNAs were designed for the 5 ’exons of 311 transcription regulators. By using this library, CRISPR-based negative-selection screen was performed in MOLM-13 cells. Among the determined 12 candidates, they focused on the top ranked MPP8. And they further used MPP8 deficient cells and mice to confirm its role in AML development *in vivo*. MPP8 is usually inactivated

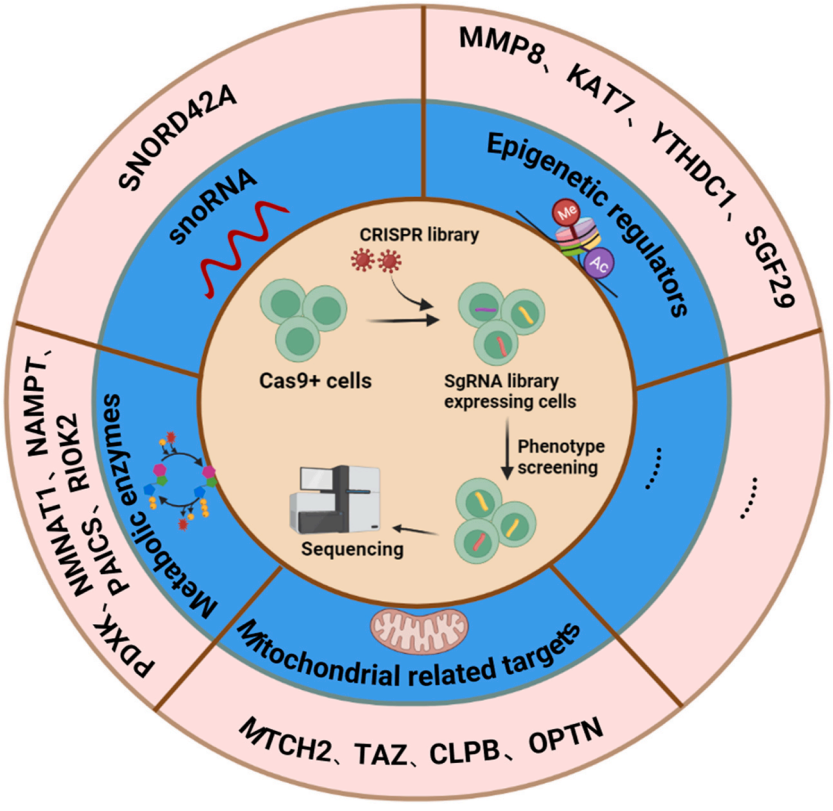

Fig. 2. Multiple therapeutic targets for AML cell survival Several physiological processes are crucial for AML cell survival, and based on that, multiple therapeutic targets were identified, including MMP8 [24], KAT7 [26], YTHDC1 [30], SGF29 [32], SNORD42A [33], PDXK [34], NMNAT1 [35], NAMPT [36], PAICS [37], RIOK2 [38], MTCH2 [39], TAZ [40] CLPB [41] and OPTN [42].

in AML. Deletion of MPP8 reactivates the L1 retrotransposon, inducing DNA damage response and cell cycle arrest of tumor cells [24]. Interestingly, unlike previous reports [25], this study revealed the tumor suppressor role of L1 retrotransposons for the first time.

Another group employed a genome-wide CRISPR library and found KAT7, an epigenetic regulator, might be a promising target for treating AML [26]. As a writer, KAT7 induces acetylation modification of histones H3K14 and H4K12 and promotes MLL fusion associated adapter proteins (BRD4 and AF4) to be recruited as promoters of target genes, including MEIS1, PBX3 and SENP6, which are important for AML survival and growth. Blocking KAT7 not only reduces proliferation but also increases apoptosis and differentiation of AML cells [26]. In another study published at the same period, researchers also revealed that KAT7 affected the maintenance of AML stemness through shRNA library screening and CRISPR domain screening [27,28].

Aberrant epigenetic modifications of RNA also play vital roles in the malignant progression of AML. More than 100 chemical modifications of RNA have been identified, and m6A is the most abundant RNA modification in eukaryotic mRNA. M6A is written into mRNA by the methyltransferase complex (writer), removed by demethylase (eraser), and recognized by various m6A readers, thereby regulating gene expression at post-transcriptional level [29]. Yuanming Cheng et al. analyzed published CRISPR screen data from 14 AML cell lines and found that the m6A reader YTHDC1 was critical for AML cell survival [30]. Binding to m6A modified MYC mRNA, YTHDC1 enhanced the stability of MYC mRNA through liquid phase separation. The researchers also found that YTHDC1 was significantly overexpressed in several subtypes of AML clinical samples [30]. In the same period, Sheng et al. found that YTHDC1 was highly expressed in AML with different karyotypes by mining the AML patient database [31]. These findings suggest that YTHDC1 may become a target for AML treatment.

MEIS, a key leukemia stem cell associated gene, plays an important role in leukemia pathogenesis in diverse subsets of AML. Researchers first used a library comprising all categories of epigenetic regulators to perform a CRISPR screen in UB3 cells. They inferred positive MEIS1 regulators based on the expression level of MEIS1. MAGeCKFlute analysis revealed several novel hits. Then they generated and cloned a small pooled CRISPR library targeting top hits and conducted CRISPR droplet sequencing. Combined with single-cell RNA-seq profile, new candidates SGF29 and AFF2 were identified. They focused on SGF29 due to its uncharacterized role in AML. It has been demonstrated that SGF29 regulates AML oncogene transcription and KAT2A chromatin localization. As the chromatin reader protein, SGF29 is critical in AML cell growth and leukemogenesis (Fig. 2).

2.1.4. Mitochondrial related targets

AML cells have unique mitochondrial and metabolic features with greater reliance on oxidative phosphorylation [43]. Inhibiting mitochondrial processes, such as mitochondrial protein translation, will impair oxidative phosphorylation and have selective cytotoxicity on AML cells [44]. Dilshad Khan et al. used the CRISPR library including 91,320 gRNAs targeting 17,237 nuclear-encoded genes cells to explore the necessary genes related to mitochondrial pathways for the survival of AML cells in OCI-AML2 cell line [39]. When analyzing the screening results, they focused on 1050 nuclear-encoded mitochondrial genes. In the top 20 hits, mitochondrial outer membrane protein mitochondrial carrier homolog 2 (MTCH2) stood out. Knocking down MTCH2 expression significantly reduced the survival and proliferation ability of AML cells, and promoted AML cell differentiation [39]. Decreased expression of MTCH2 did not affect mitochondrial function, but may increase the levels of pyruvate and pyruvate dehydrogenase, thereby promoting differentiation of AML cells [39].

Using an identical CRISPR screen strategy, a mitochondrial transacylase Tafazzin (TAZ) which is necessary for AML cell viability was identified [40]. Inhibiting TAZ does not impair normal hematopoiesis but inhibits stemness and promotes differentiation of AML cells [40]. Using the genome-wide CRISPR library, two groups independently demonstrated that mitochondrial chaperonin CLPB and several genes involved in mitochondrial translation intimately participate in AML cells resistance to Venetoclax, a specific B cell lymphoma 2 (BCL-2) inhibitor [41,45,46]. These studies indicate that targeting mitochondrial biological processes is one of the promising approaches for developing therapeutic strategies for AML.

In addition, mitochondrial DNA (mtDNA)-encoding genes may also play an important role in the initiation and development of AML. David Liu's lab developed mitochondrial DNA-editing base editors, DdCBEs, that catalyze C•G to T•A conversion in human mtDNA [47]. Some studies have confirmed the feasibility of editing mtDNA with DdCBEs [48,49], but its efficiency is low and there is severe sequence preference, and may cause serious off-target effects on nuclear DNA [50]. Recently, David Liu's lab has carried out multiple optimizations on DdCBEs, and developed an upgraded version of DdCBEs with higher editing efficiency and stronger sequence compatibility, which has more precise editing capabilities for mtDNA [51] (Fig. 2). The development of these new tools may provide the possibility to screen mtDNA-encoded AML targets.

Mitophagy is essential for the AML maintenance and progression. However, the role of redundancies between different mitophagy receptors in AML remains unexplored. The researchers performed a multiplexed CRISPR screen with a library containing pairwise combinations of mitophagy receptors in MV4–11 cells. After mitophagy induction, cells with the 5 % highest and lowest pH 4: pH 7 mt-mKEIMA ratio were sorted for sequencing. By mapping the mitophagy receptor network using the changed genes, they identified OPTN (optineurin). As a sole non-redundant mitophagy receptor, OPTN has been demonstrated to limit AML cell proliferation both *in vitro* and *in vivo*. Mechanistically, OPTN increases mitochondrial ROS and impairs mitochondrial respiration and function, leading to AML proliferation defect [42].

2.1.5. Metabolic enzymes

Recent studies have revealed that AML, especially leukemia stem cells (LSCs) that are largely responsible for relapse and therapy resistance, have unique metabolic requirements, such as low reactive oxygen species and dependence on oxidative phosphorylation (OXPHOS) and adenosine 5′-monophosphate–activated protein kinase (AMPK), a master regulator of metabolism [52]. These metabolic dependencies offer opportunities for therapeutic intervention and implicate the need to identify novel metabolic vulnerabilities

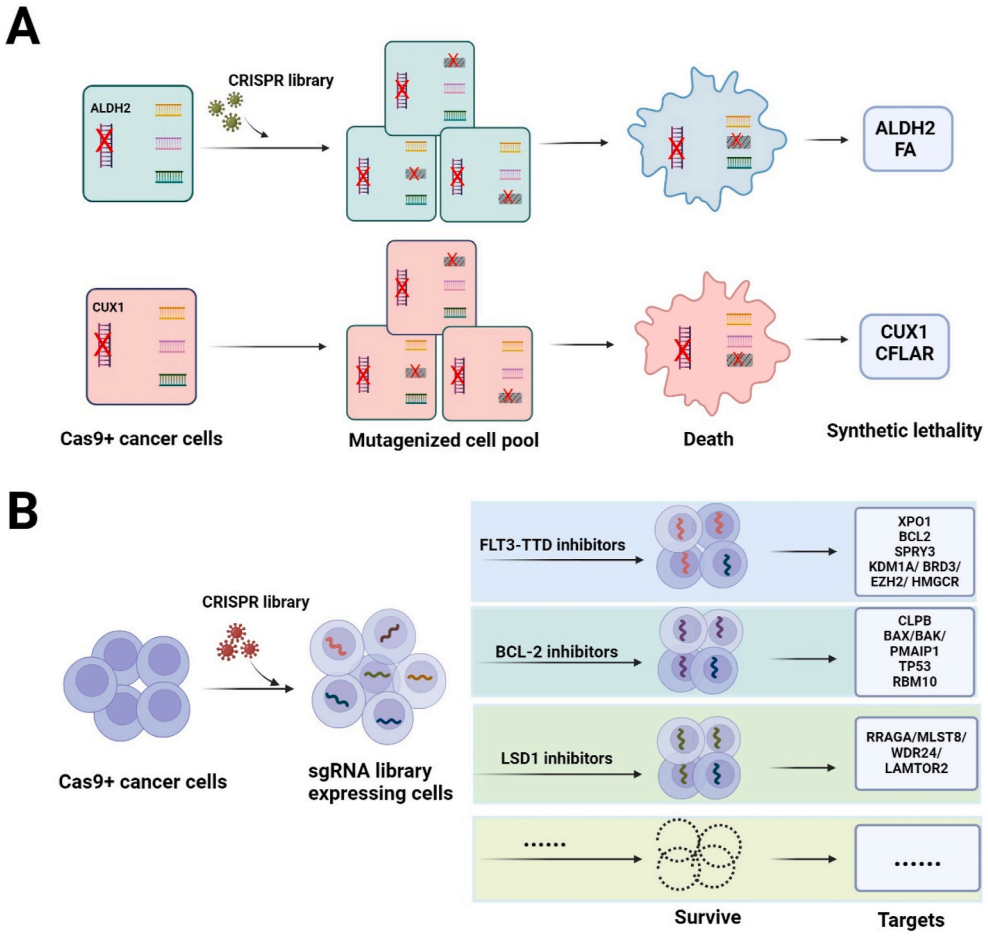

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Fig. 3. CRISPR screen in discovery targets for combination therapy **A.** Screening synthetic lethal genes. The CUX1 (or ALDH2) deficient AML cells are transduced with a CRISPR library to establish a mutagenized cell pool. The candidate targets are identified through sequencing and analysis of the enriched sgRNAs in the survival cells. The gene induced cell death in CUX1 (or ALDH2) deficient AML cells but not wide type AML cells, is a synthetic lethal gene [59,60]. **B.** Target discovery for overcoming drug resistance. The AML cells expressing Cas9 are transduced with a CRISPR library to establish a mutagenized pool. Then these transduced cells are stimulated with the specific target inhibitors. The candidate targets are identified through sequencing sgRNAs enriched in survival AML cells and evaluated by functional studies [37,64–66,77]. **C.** Screening targets based on an evolutionary trap. In the mutagenized AML cell pool, each cell lost a gene, such as A、B or C. The mutagenized AML cell pool is treated with drugs X or Y. The level of sgRNA is analyzed to identify genes exhibited different characteristics between two drug-treatment groups. In this figure, C is the evolutionary trap under drug X treatment [61].

for better therapeutic intervention in AML. Chen C C et al. analyzed the expression levels of 2752 metabolism-related genes in AML and showed that 236 genes were highly expressed in tumor cells [34]. The researchers constructed an sgRNA library targeting 236 genes and screened AML fragile genes using Nras (G12D)/MLL-AF9 leukemic cell. Through comparing screening results with previously reported data in more than 400 cell lines, it was found that sgRNA targeting PDXK was specifically missing in survival leukemia cells, but not in normal cells or other tumor cells. The results indicate that PDXK may become a target for AML treatment with high specificity and safety [34]. Further studies support this hypothesis that knocking out or inhibiting PDXK expression reduces the proliferation of a variety of AML cell lines *in vitro* and *in vivo*, but has no effect on mouse fibroblasts or sarcoma cells. PDXK metabolizes vitamin B6 into pyridoxal phosphate (PLP). Targeting PDXK or PLP with drugs could produce anti-AML proliferation effects similar to PDXK knockout, indicating that the vitamin B6 metabolic pathway may be a specific target for AML treatment [34].

Nicotinamide adenine dinucleotide (NAD^+), as a coenzyme or protease substrate (cosubstrate) that transfers protons, is widely involved in various metabolic reactions that are required for a variety of biological processes, including cell survival and genome integrity. Through combining genome-wide CRISPR screening results in AML cell lines with the cancer genetic dependence database (https://depmap.org/portal/), the team led by Professor Daisuke Nakada identified NMNAT1 and NAMPT metabolic enzymes involved in the NAD^+ remedial synthesis pathway are important regulators for maintaining the stemness of leukemia [35]. NMNAT1 enhances the deacetylation activity of the NAD^+ dependent enzyme Sirtuin 6/7, inhibits p53 acetylation, and enhances genomic stability. Blocking NMNAT1 not only inhibits the proliferation of AML cells, but also significantly reduces the number of leukemia stem cells and delays the progression of leukemia. Equally important, knocking out of NMNAT1 has little effect on the normal hematopoietic system of mice [35]. Another study revealed that antagonizing the activity of NAMPT specifically induced apoptosis of AML stem cells by destroying cell lipid homeostasis [36]. These studies illustrate that the key regulators of the NAD^+ remedial synthesis pathway might be effective and safe targets for the treatment of AML.

By employing the CRISPR screen, recent studies have identified other metabolic enzymes might become therapeutic targets for AML, including PAICS, an enzyme involved in de novo purine biosynthesis, and ATPase RIOK2 [37,38,53] (Fig. 2). Future studies based on CRISPR screen may uncover more metabolic vulnerabilities to AML and develop better therapeutics.

2.1.6. SnoRNAs

Noncoding RNA plays an important role in the development of AML [54]. Small nucleolar RNAs (snoRNAs) are important members of noncoding RNAs and participate in post-transcriptional modification of ribosomal RNA. Recently, Pauli C et al. designed a sgRNA library for snoRNAs and utilized leukemia cell lines Kasumi-1, MV4-11, HL60 and U937 to screen for snoRNAs that were critical to the survival of AML cells [33]. Among the screening results of several cell lines, SNORD42A stood out. Subsequently, AML clinical data also confirmed that compared with monocytes, granulocytes and $CD34^+$ hematopoietic progenitor cells, AML cells expressed SNORD42A at a higher level. Mechanism studies showed that SNORD42A, as a member of the C/D box snoRNA family, enhanced the translation of ribosomal proteins by directly inducing 2′-O methylation modification of uridine 116 in 18S ribosomal RNA, thereby promoting the survival and proliferation of AML cells [33]. With the development of gene editing technologies, CRISPR screen has been applied to the screening of functional noncoding RNAs, including long noncoding RNAs and circRNAs [55–57], which may promote the identification of more noncoding RNAs that may become potential targets for AML treatment in the near future (Fig. 2).

2.2. Screening synthetic lethal genes as therapeutic targets for AML

The so-called “synthetic lethality" means that mutations in A or B gene alone will not affect cell survival, but inhibition of A and B genes together can lead to cell death (Fig. 3A). For example, PARP is a synthetic lethal gene of BRCA1, and tumor cells lacking BRCA1 are apt to rely more on PARP for DNA damage repair. PARP inhibitors exhibit ideal therapeutic efficacy in tumors with BRCA deletion or inactivation mutations [58]. Searching for “synthetic lethal genes" in AML with specific gene subtypes is one of the important ways to achieve accurate treatment of AML.

2.2.1. CUX1

CUX1 gene deletion or mutation exists in tumor cells from some AML patients. CUX1 is a tumor suppressor gene in AML, and its loss of expression or loss-of-function mutation is closely related to the poor prognosis of patients [62]. Supper E et al. screened the synthetic lethal genes of CUX1 by employing a genome-wide CRISPR library in CUX1 deficient AML cells, and identified CASP8 and FADD like apoptosis regulator (CFLAR) [59]. CUX1 is a transcriptional inhibitor of CFLAR, and CUX1 deletion will increase the expression level of CFLAR, thereby allowing AML cells to escape apoptosis. Therefore, CFLAR and anti-apoptotic pathways may become therapeutic targets for CUX1-deficient AML (Fig. 3A). Given that specific CFLAR inhibitors are unavailable currently, a CFLAR pathway inhibitor

birinapant was used to detect “synthetic lethality" effect. Birinapant showed significant therapeutic effects on both CUX1 deficient cell lines and patient derived tumor cells [59].

2.2.2. ALDH2

During the development of AML, tumor cells rapidly evolve through increased gene mutations to adapt to environmental pressures. ALDH2 reduces the genotoxicity of endogenous acetaldehyde by metabolizing acetaldehyde to acetic acid. However, the clinical data showed that the molecular classification of some relapsed AML is ALDH2 deletion. Yang Z et al. applied domain-focused CRISPR screening [27] to discover the synthetic lethal gene of ALDH2 [60]. They designed sgRNA libraries for 564 genes with ubiquitin binding domain or ubiquitin connecting domain. Then, the screening was conducted in six AML cell lines and identified Fanconi anemia (FA) proteins, including UBE2T and FANCL. AML cells with ALDH2 deficiency relied on FA to complete acetaldehyde metabolism. Importantly, targeting FA greatly inhibited the survival of AML cells with ALDH2 deficiency but did not cause toxicity to normal ALDH2 expressing tissues [60] (Fig. 3A).

2.3. Application of CRISPR screen in target discovery for overcoming drug resistance

The past few years witness the remarkable advances in our understanding about AML that promote the development of several targeted drugs, including inhibitors of FLT3 and BCL2 [6] (Fig. 3B). Nevertheless, inherent or acquired drug resistance is a main contributor of treatment failure. Therefore, screening new candidates for overcoming drug resistance and then establishing corresponding strategies are key to improving the therapeutic efficacy and prolonging AML patient survival.

2.3.1. FLT3 inhibitors

In the gene mutation map of AML, FMS like receptor tyrosine kinase 3 (FLT3) gene mutation is the most common, which exists in about one third of newly diagnosed patients. FLT3 mutation mainly includes tandem repeat mutation (ITD) in transmembrane region and point mutation (TKD) involving activation loop. Although the molecular mechanism of these mutations affecting cell function is still unclear, a large number of clinical studies have shown that AML patients with FLT3 mutations have a poor prognosis, which has also led to the development and clinical application of a series of FLT3 inhibitors (FLT3i). So far, three FLT3 inhibitors have been approved for clinical use in Japan, Europe and the United States, but drug resistance limits further improvement in their clinical efficacy [63,64]. Brinton L T et al. applied the genome-wide CRISPR library to screen genes related to the resistance of FLT3-ITD targeted drugs (midostaurin and gilteritinib) in MOLM-13 cells, and found XPO1 and BCL2 are candidate targets for enhancing the efficacy of FLT3-ITD targeted drugs [65,66]. The combination of FLT3-ITD targeted drugs with XPO1 or BCL2 inhibitors can significantly improve the clinical efficacy of AML patients with FLT3-ITD mutations and significantly delay tumor recurrence. In addition, some researchers applied the CRISPR library to screen the resistance genes of the FLT3 inhibitor AC220, and the results showed that knocking out SPRY3 could lead to the resistance of AML cells to AC220 [67]. A significant decrease in SPRY3 expression was also observed in clinical samples of AC220 resistant patients [67]. Recently, Suruchi Pacharne et al. have suggested that AML carrying FLT3-ITD and highly expressing SETBP1 is a new subtype, and identified *Kdm1a*, *Brd3*, *Ezh2* and *Hmgcr* are potential therapeutic targets for this subtype through CRISPR library screening [68] (Fig. 3B).

2.3.2. BCL-2 inhibitors

Among those members of the BCL-2 family that play important roles in AML, B cell lymphoma 2 (BCL-2) is found to be upregulated in AML cells, especially in leukemic stem cells (LSCs) [69,70]. Enhanced BCL-2 expression is an adverse risk factor for AML and is also correlated with poor response to standard cytotoxic therapy [71]. Mechanistically, BCL-2 overexpression supports cell survival because it inhibits the mitochondrial-mediated apoptosis by binding to proapoptotic members of the same family [72]. Venetoclax, a selective BCL-2 inhibitor, is able to promote caspase-dependent cell death in AML [73]. It has received FDA approval for the treatment of newly-diagnosed elderly AML patients [74]. To elucidate the mechanisms of Venetoclax resistance, Chen X et al. applied a genome-wide CRISPR loss-of-function screen in MOLM-13 cells with or without Venetoclax and identified mitochondrial chaperonin CLPB. As one of the top candidates, CLPB inactivation sensitizes AML blasts to Venetoclax [41]. Mechanistically, CLPB interacts with the cristae-shaping protein OPA1 to maintains the mitochondrial cristae structure, whereas its loss promotes apoptosis by inducing cristae remodeling and mitochondrial stress responses [41]. In the same year, Sharon D et al. employed the CRISPR screen and found that inactivation of genes involved in mitochondrial translation or using the inhibitor of mitochondrial translation tedizolid restored sensitivity to venetoclax in resistant AML cells [46]. Using a similar screening strategy, Nechiporuk and colleagues validated that the absence of pro-apoptotic proteins BAX/BAK or PMAIP1 (gene encoding for NOXA) confers venetoclax resistance [75]. Likewise, TP53 deletion has similar effects, consistent with p53’s central role as a transcriptional regulator of proapoptotic proteins under cellular stress [41].

Recently, Wang E et al. performed a genome-wide CRISPR screen and found RNA splicing factors modulating venetoclax response in human AML cell line, MOLM-13 [76]. Next, they applied a CRISPR library targeting functional domains of 492 RNA-binding proteins and identified that the loss of several RNA splicing factors, such as *RBM10*, *SRSF11*, *SRSF8*, *HNRNPD*, *HNRNPAB*, and *HNRNPF*, sensitized AML cells to venetoclax. Mechanistically, RBM10 deletion combined with BCL2 inhibition promotes XIAP mis-splicing and downregulates the anti-apoptotic protein BCL2A1. SM09419, an inhibitor of splicing-dependent kinases, overcomes venetoclax resistance by impairing XIAP and splicing factors [76] (Fig. 3B).

2.3.3. Other novel targets for overcoming drug resistance

LSD1 is another candidate target for the treatment of AML. LSD1 inhibitors have shown favorable security and biological activity in clinical trials for the treatment of R/R AML patients [77]. Deb G et al. applied the whole genome CRISPR library to screen the drug-resistance related genes of LSD1 inhibitor OG-86 in the AML cell line THP-1 [78]. The results showed that multiple amino acid sensor genes in the mTORc signaling pathway were related to the sensitivity of AML cells to OG-86, including: RRAGA, MLST8, WDR24, and LAMTOR2. The combination of the mTORc inhibitor and LSD1 inhibitor could significantly promote the differentiation of AML cells and reduce the minimal residual disease in animal models [78]. Other candidate targets, such as genes correlated with resistance to sorafenib, MEK/ERK inhibitors, TAK-243 or CC-90009 have also been identified through CRISPR screens [79–81].

2.3.4. Targets based on an evolutionary trap

With supernormal evolutionary capacities, cancer cells can survive and multiply in response to various treatments and environmental changes. Usually, cancer cells randomly adapt to the environment of local tissue or host systemic pressures in untreated patients, or acclimatize to cytotoxic chemotherapies through multiple mechanisms and exhibit diverse genotypes. However, under pressure from more targeted or molecularly defined therapeutics, developing adaptive resistance of cancer cells mainly dependents on the specific signaling pathway and certain genetic or epigenetic changes, which provide vulnerabilities for cancer cells to target and are nominated as the evolutionary trap [82,61].

Recently, Lin et al. developed an approach based on CRISPR to systematically identify evolutionary traps of AML [61]. Firstly, the AML cell lines MOLM-13 and THP-1 were transduced with an sgRNA library which targeting 2240 genes from major cellular and oncogenic signaling pathways to establish a mutagenized cell pool. Secondly, it was treated with 9 kinds of chemotherapy drugs, respectively. Then, through deep sequencing, they compared the level of sgRNA between each group to identify whether the potential

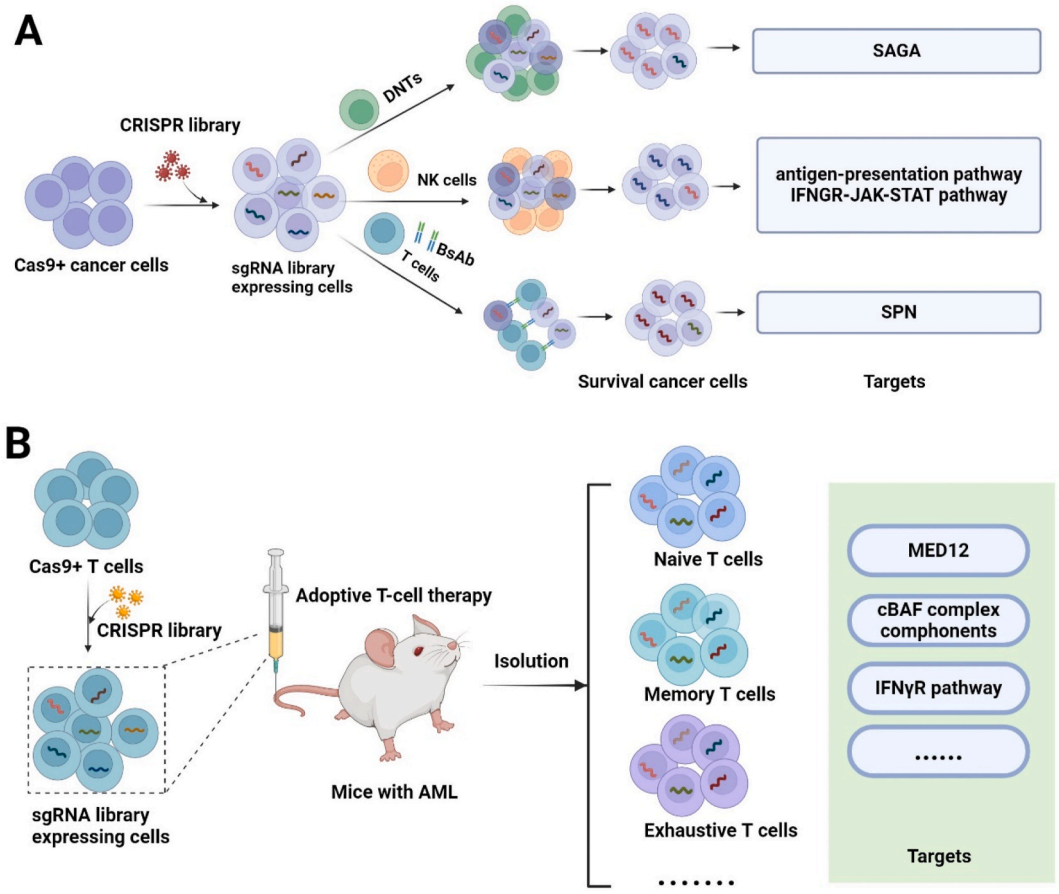

Fig. 4. Discovering targets to enhance the efficacy of immunotherapy for AML A. Discovering targets in cancer cells. The AML cells transduced with a CRISPR library are co-cultured with effector cells (DNTs, NK cells or T cells with bsAb). The sgRNAs enriched in survival AML cells are identified through sequencing [86,87]. The candidate targets are evaluated by functional studies. DNTs, Double negative T cells; bsAb, bispecific antibodies. **B.** Discovering targets in T cells. The tumor specific T cells isolated from Cas9 transgenic mice are transduced with a CRISPR library to establish a mutagenized pool. The mutagenized T cells are transferred into the mouse allograft model. The candidate targets are identified through sequencing and analysis of the enriched or depleted sgRNA in several types of T cells [88–91]. Whether these targets are suitable for enhancing T cell-based immunotherapies of AML remain to be evaluated.

candidate genes led to drug tolerance, sensitivity, or inertia. If a gene exhibited different characteristics in two drugs, then an “evolution trap" appeared (Fig. 3C). Many interesting pitfalls have been discovered. Based on this, they tested a group of drugs that may constitute an evolutionary trap, ABT-199 and JQ-1. Animal studies have confirmed that drug resistance induced by JQ-1 can enhance the sensitivity of tumor cells to ABT-199, and the signal axis mediated by MYC is involved in regulating this biological phenomenon [61].

2.4. Discovering targets to enhance the efficacy of immunotherapy for AML

Immunotherapies are one of the most promising manners for treating AML, including therapies based on monoclonal antibodies, bispecific antibodies, cell-based therapies or vaccines [83,84]. Studies have shown that varying responses to immunotherapy among different patients, and even some of them develop resistance to immune cell treatments. Therefore, it is crucial to identify key molecules that synergistically enhance the efficacy of immunotherapy. CRISPR screen is an effective tool for discovering mechanisms and identifying targets in immunotherapy [85] (Fig. 4A).

2.4.1. Double negative T (DNT) cells

Although some encouraging efficacy has achieved [92], CAR-T cell therapy has been severely limited in AML due to the high heterogeneity of AML. DNTs are mature T cells that comprise 3–5 % of peripheral T cells and express CD3 but not CD4, CD8, or invariant-NKT cell markers [93–95]. Compared to primary activated NK cells, DNTs from healthy donors show a greater killing effect on AML cell lines and longer persistence *in vivo*, which allow for the use of DNTs as a universal adoptive cellular therapy (ACT). However, about 30 % of AML patients have resistance to DNT cell-mediated cytotoxicity, and there are no clear markers or mechanisms for such resistance [96]. Researchers designed a focused sgRNA library against 317 epigenetic regulators and 657 genes that are targets of FDA-approved drugs to identify epigenetic modifications involved in sensitizing or developing resistance to DNTs. Using this sgRNA library, they found inactivation of the Spt-Ada-Gcn5-acetyltransferase (SAGA) deubiquitinating complex components increased susceptibility of AML cells to DNT-mediated cytotoxicity in AML3 and AML2 cells [86] (Fig. 4A). In contrast, CD64 inactivation confer AML resistance to DNT-mediated killing [86]. Overall, these findings can identify potential positive biomarkers for AML patient stratification, ultimately increasing the therapeutic efficacy of DNTs.

2.4.2. Other immunotherapies

Because of their robust effector function and intrinsic anti-cancer activity, NK cells are crucial for cancer immune surveillance. Xiaoxuan Zhuang et al. carried out a genome-wide CRISPR screen to identify genes that modulate CML resistance to NK-mediated killing [97]. CML cell line K562 was transduced with the GeCKO V2 CRISPR-knockout lentivirus library and then co-incubated with IL-2-activated NK cells. They showed that upregulation of genes involved in the antigen-presentation pathway and the IFNGR-JAK-STAT pathway resulted in resistance to NK cytotoxicity with the MAGeCK-VISPR pipeline (Fig. 4A). Mechanistically, the upregulation of MHC class I which were upregulated by NK-derived IFN-γ dependent on IFNGR2 results in resistance to NK cytotoxicity. In addition, RNA-seq data from TCGA revealed that a strong association of low IFNGR2 mRNA expression in cancer tissues with improved overall survival in KIRC and AML, further supporting the above findings [97].

Bispecific antibodies (bsAb) are emerging as promising immunotherapy for AML [98]. Corinne e. Decker et al. employed a genome-scale CRISPR activation screen in BLL cell lines. They identified that sialophorin (SPN) with predicted effects on cell-cell adhesion could regulate T cell-tumor cell clustering and bsAb-mediated AML cell killing [87] (Fig. 4A).

3. Conclusion and Perspective

In conclusion, CRISPR screens represent an effective strategy to characterize the functional role of genetic changes [99] and have already been implied in the discovery of new therapeutic vulnerabilities in AML. For CRISPR screen design, several points should be considered: Firstly, a single, stable, repeatable screening model is critical. Secondly, design a rational sgRNA library based on the experimental purpose. Finally, select a clear, appropriate and feasible readout. In this review, we summarized effector genes with previously unknown roles for AML survival through CRISPR screen. The novel targets related to AML survival resembled cellular pathways mediating epigenetics, synthetic lethality, transcriptional regulation, mitochondrial and energy metabolism. The CRISPR genetic screens also identified an array of candidate targets that were crucial for combating drug resistance in AML. Regarding cancer immunotherapy, CRISPR screens strategies have also achieved significant advances in improving the DNT-mediated cytotoxicity, NK cytotoxicity and bsAb-mediated cytotoxicity.

Although CRISPR screen is one of the effective ways for us to find targets, genes discovered through this way may not translate into viable therapeutic targets. For example, Zhou Y et al. determined 94 genes from the CRISPR library that are crucial for the survival of AML cells, but only 44 of them are currently approved therapeutic drugs. Whether a gene can become a therapeutic target depends not only on its key role in pathogenesis, but also on its drug interactions and safety. Online databases, such as Dgidb and CanSAR Black, are commonly used tools for predicting druggability. Most importantly, candidate genes should be validated *in vitro* and *in vivo* to confirm whether they can become therapeutic targets.

So far, the application of CRISPR screen in AML target discovery is still in its early stages. T cell-based immunotherapies are promising in the field of AML treatment [83,100]. Identifying targets to enhance the capacities of T cells to eliminate tumor cells is a feasible strategy for AML treatment. Recent advances enable genome-scale pooled CRISPR screening in mouse or human primary T cells [101–104]. Using *in vivo* or *in vitro* readout models combining with CRISPR screens, several groups recently identified potential

targets in T cells for the cancer immunotherapy, such as MED12, the components of cBAF complex and interferon-γ receptor (IFNγR) signalling pathway [88–91] (Fig. 4B). Whether these targets are suitable for the T cell-based immunotherapies of AML remains to be evaluated. Future studies to identify T cells targets in AML models are appreciated. Recently, the combination with other technologies, such as adenine base editors [105,106], immunomagnetic cell sorting [107], BARC-FISH, chromatin tracing [108] and dual CRISPR-Cas9 [109], enables CRISPR screen visible and accurate, allowing the identification of new targets undetectable previously. These novel CRISPR screen methods might be applied to screen candidate therapeutic targets for AML in the future. Moreover, developing ingenious readout models and novel CRISPR screen strategies will hold great promise for decoding AML mechanisms, identifying promising targets, and providing lifesaving treatments.

Data availability statement

All data used in this article are included in the main text, supplementary materials and references.

CRediT authorship contribution statement

Tian Lin: Writing – original draft, Investigation, Conceptualization. **Dan Liu:** Writing – original draft, Investigation, Conceptualization. **Zhangchun Guan:** Writing – original draft, Investigation, Conceptualization. **Xuan Zhao:** Writing – review & editing, Visualization, Investigation. **Sijin Li:** Writing – review & editing, Visualization, Investigation. **Xu Wang:** Writing – review & editing, Visualization, Investigation. **Rui Hou:** Writing – review & editing, Visualization, Investigation. **Junnian Zheng:** Writing – review & editing, Supervision, Funding acquisition. **Jiang Cao:** Writing – review & editing, Supervision, Funding acquisition. **Ming Shi:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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