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Emerging role of CRISPR-Cas9 in precision medicine for retinoblastoma

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ABSTRACT

Loss or mutation of the RB1 tumor suppressor, a crucial regulator of the G1-to-S cell cycle transition, gene expression, cellular differentiation, chromosomal integrity, and apoptosis, is the main cause of retinoblastoma. RB1 inactivation causes genomic instability, poor DNA repair, metabolic reprogramming, and increased metastatic potential. While CDK-mediated phosphorylation of RB1 promotes unchecked proliferation and alters the response to CDK4/6 inhibition, other genetic disruptions such as RBL1/RBL2 loss and MYCN amplification further increase malignancy aggressiveness. Genome-editing technologies such as CRISPR-Cas9 provide an accurate and adaptable toolkit for retinoblastoma mechanistic research and therapeutic exploration. Targeted RB1 correction, gene disruption, base and prime editing, and transcriptional regulation are made possible by developments in the biology of Cas9, its optimized variations, and its delivery methods, which include viral vectors and nonviral nanoparticles. In order to support functional genomics, high-throughput drug screening, and customized treatment discovery, patient-derived iPSC retinal organoids and non-mammalian model systems replicate tumor initiation, secondary genetic processes, and epigenetic remodeling. Base and prime editors provide accurate mutation correction with few off-target effects. At the same time, synthetic lethality screens identify actionable vulnerabilities in RB1-deficient cells, such as disruptions in mitotic regulators and DNA repair machinery. Additionally, CRISPR-mediated epigenome and transcriptome modification enables reversible regulation of gene expression, which may be achieved using RNA-targeting Cas13 or CRISPRi/CRISPRa. Although retinal delivery, safety, scalability, and ethical application remain significant barriers to clinical translation, these integrated techniques collectively provide a strong foundation for genotype-guided precision therapy in retinoblastoma.

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Introduction

In newborns and young children, retinoblastoma, a frequent malignant ocular tumor, poses a major risk to life and eyesight. Nearly 75% of instances are unilateral; however, a small percentage of individuals present with bilateral involvement. The disorder often appears between the ages of two and three and is frequently associated with inherited genetic changes (1, 2). Retinoblastoma occurs in approximately 1 in 16,000 to 24,000 live births, according to epidemiological data from the United States and the United Kingdom. In high-income countries, mortality is relatively low, but in some regions of Asia and Africa, where death rates exceed 40%, it rises significantly (3). Most retinoblastoma cases are caused by basic genetic changes (4). By controlling the transcription factor E2F1, the RB1 gene, located on chromosome 13q14, acts as a crucial tumor suppressor by preventing the unchecked growth of immature retinal cells (5, 6). Retinoma, a benign lesion that might progress to malignant retinoblastoma with the potential to spread, is usually the first clinical consequence

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(7). The PubMed literature has examined several treatment modalities for retinoblastoma. Intravenous chemotherapy (IVC) is frequently used as the primary treatment, and standard care typically includes targeted therapies, systemic drugs, and surgical procedures, with selection depending on tumor size and stage (8, 9), as well as intra-arterial chemotherapy (IAC)(10). Important therapeutic options include intravitreal chemotherapy (IVitC) and intra-cameral chemotherapy (ICaC). Adjuvant treatments, including cryotherapy and transpupillary thermotherapy (TTT), are used to remove smaller tumor foci (11). External beam radiation treatment (EBRT) and plaque radiotherapy are two examples of radiation-based therapeutic techniques that are used during particular phases of illness care (12). Enucleation, or surgical removal of the eye, is considered the most appropriate and definitive treatment strategy when ocular preservation is no longer possible (13).

The invention of CRISPR–Cas9 genome editing technology has enabled new, highly accurate possibilities for genetic intervention in retinoblastoma (14, 15). These experimental models demonstrate CRISPR's potential as a preclinical platform for assessing and refining targeted therapy approaches, as well as elucidating the molecular underpinnings of tumor initiation and development (16). CRISPR-based methods have the potential to address the drawbacks of traditional medicines, which are sometimes non-specific and frequently linked to serious ocular side effects, by precisely editing or modifying RB1 and other crucial components of the Rb signaling system (17). This capability has been shown in both Drosophila and mammalian model systems (18).

These techniques improve our understanding of gene regulation across a variety of biological contexts by enabling the discovery of paralog-specific roles of retinoblastoma family members in both *in vitro* and *in vivo* models. In light of this, this review critically assesses new CRISPR–Cas9 applications in retinoblastoma, emphasizing their importance for precision medicine and reviewing current and potential future approaches for gene-targeted treatments.

Methodology

This narrative review aims to provide a comprehensive and critical overview of recent developments in CRISPR-based therapeutic strategies for RB. Unlike a systematic review, this review aimed to synthesize and critically evaluate mechanistic evidence, preclinical studies, and recent translational findings from the current literature, rather than conduct a quantitative meta-analysis. A thorough search was performed using PubMed, Web of Science, and specifically Google Scholar to identify pertinent publications, including both established evidence and newly published research. The search primarily focused on articles published between 2018 and 2026, while some essential studies published before this period were included for their substantial historical or scientific context. The search method included combinations of the terms retinoblastoma, RB1, CRISPR/Cas9, gene editing, base editing, prime editing, CRISPRi, CRISPRa, and ocular gene therapy. Eligible papers were peer-reviewed original scientific articles in English that investigated CRISPR-based techniques for retinoblastoma or ocular gene editing with clear translational significance. Supplementary content was also provided through carefully selected, high-quality review articles. Excluded from consideration were conference abstracts, editorials, duplicate publications, non-English articles, studies not relevant to ocular diseases, and reports without enough methodological detail. The selection focused on both scientific quality and relevance to the review's objectives, rather than publication availability alone. The evaluation of the included studies included methodological quality, experimental design, originality, repeatability, translational significance, and consistency with the broader corpus of published data. To provide a more objective and equitable evaluation of the available data, the review discussed any conflicting or divergent findings. The use of a structured search methodology and predefined inclusion criteria aimed to enhance transparency, mitigate the risk of selection bias, and augment the reliability of the evidence synthesis provided, despite the review not being designed as a systematic review. This approach provides a crucial, up-to-date overview of CRISPR-based therapeutic strategies for retinoblastoma.

Molecular basis of retinoblastoma

Beyond E2F-dependent regulation, pRB interacts with a variety of nuclear partners and chromatin-modifying complexes to govern gene activation and repression. It also affects cellular differentiation, chromosomal architecture, and apoptotic signaling pathways (Figure 1)(19, 20). A mutation or loss of RB1 inhibits mitochondrial function, causes chromosomal instability, changes metabolic processes, and interferes with proper cell cycle exit (21, 22). The context-dependent nature of these effects' magnitude and specificity emphasizes the broad and integrative function of pRB in preserving genomic integrity and averting neoplastic transformation (23). Because of its extensive interaction network, pRB is a critical target for treatments targeting RB1-deficient malignancies and acts as a central regulatory hub in tumor suppression (24). Tumor suppressor activity is eliminated, and unchecked progression into the S phase is permitted when both RB1 alleles completely stop functioning (25, 26).RB1-deficient cells often have higher quantities of reactive oxygen species (ROS) and spontaneous DNA damage (25). Since these cells also show a decreased ability to repair homologous recombination, this links unchecked growth to genomic stress (25). Non-homologous end joining (NHEJ), by contrast, is largely preserved, indicating a specific flaw in how the DNA repair pathway is used (Figure 1). This imbalance promotes aneuploidy and causes mitotic abnormalities such as anaphase bridges and ultrafine DNA bridges (25, 27). Xenograft experiments further indicate that RB1-deficient cells possess greater dissemination capability, with a notable tendency toward pulmonary spread, suggesting that cell cycle dysregulation is accompanied by enhanced metastatic behavior. The majority of the information that is now available comes from xenograft models and experimental cell lines, even though these findings consistently indicate the crucial role that RB1 depletion plays in genomic instability.

The genesis and timing of the two RB1 mutations determine the different pathogenic mechanisms by which sporadic and hereditary RB develop (28, 29). In hereditary conditions, the first mutation occurs in the germline (29). However, because each retinal precursor cell already has one dormant RB1 gene, the second mutation happens after

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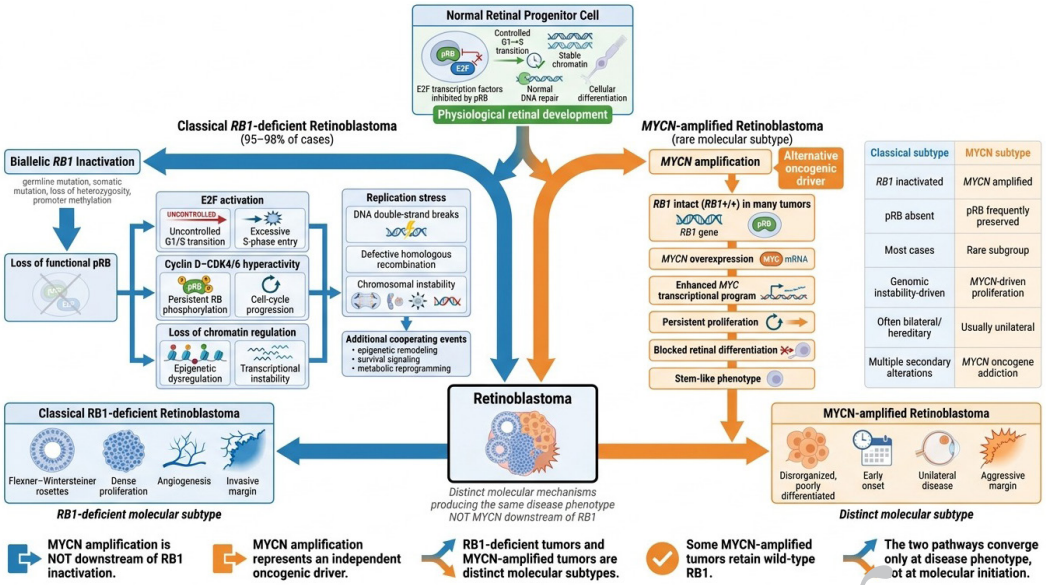

Figure 1. Molecular pathogenesis of retinoblastoma: Distinct RB1-deficient and MYCN-amplified molecular subtypes
In normal retinal progenitor cells, functional retinoblastoma protein (pRB) restrains E2F transcription factor activity and G1-to-S phase progression, thereby maintaining normal retinal development. The classical retinoblastoma subtype arises from biallelic RB1 inactivation, leading to pRB loss, E2F deregulation, cyclin–dependent kinase (CDK) activation, replication stress, defective homologous recombination, chromosomal instability, oxidative stress, and progressive genomic alterations, with additional epigenetic and pro-survival signaling changes promoting tumor progression. In contrast, MYCN amplification represents an alternative oncogenic subtype that drives proliferation and impairs retinal differentiation, often independently of RB1 loss. Despite distinct initiating mechanisms, both pathways ultimately converge on retinoblastoma development, highlighting the disease's two principal molecular subtypes. (The figure was created using Adobe Illustrator)
CDK, cyclin-dependent kinase; E2F, E2F transcription factor family; G1, gap 1 phase; MYCN, MYCN proto-oncogene, basic helix–loop–helix transcription factor; pRB, retinoblastoma protein; RB1, RB transcriptional corepressor 1; S, synthesis phase

birth and results in a tendency for bilateral and multifocal tumors (28, 30). However, spontaneous RB, which usually results in unilateral illness and drastically reduces the risk of multiple tumors, occurs when both mutations occur somatically within a single retinal cell lineage (30, 31). Because certain germline RB1 mutations are located in just a fraction of cells, mosaicism further complicates inherited cases by altering clinical presentation and transmission patterns (32).

Although the main oncogenic event in RB is loss of RB1 function, other genetic alterations may affect illness severity and onset. Changes in other members of the RB family, particularly RBL1 (p107) and RBL2 (p130), have been associated with defective cell cycle regulation in RB cells. These changes may either promote tumor growth or partially compensate for RB1 loss (33-35). Amplification of the MYCN oncogene can independently start tumor growth in some malignancies that retain wild-type RB1, and it is frequently linked to earlier onset, unilateral presentation, and more aggressive clinical behavior. Nevertheless, in experimental settings, MYCN overexpression alone seldom results in malignancies, suggesting that malignant transformation typically requires disruption of RB pathway components or other cellular alterations (36, 37). Overall, these subsequent genetic changes, such as MYCN amplification or RBL1/RBL2 dysregulation, significantly influence tumor aggressiveness and cell proliferation in RB, beyond the initial loss of RB1 function (Figure 1)(33). Collectively, these findings suggest that although MYCN amplification can function as an alternative oncogenic driver in a subset of retinoblastomas, its tumorigenic capacity appears highly context-dependent and often requires cooperation with additional molecular alterations. Differences among experimental models may explain the variability reported across studies.

RB1 gene structure and regulation

One of the earliest findings emphasizing the crucial role of tumor suppressor genes in cancer initiation was the identification of genetic changes at the RB1 locus (38). According to preliminary research, the primary genetic event causing infantile retinoblastoma is a homozygous deletion of RB1, which mostly eliminates RB1's ability to regulate the cell cycle (39, 40). Subsequent research linked changes in the RB1 locus to the emergence of lung and ovarian cancers, as well as several other cancers, including sarcomas (41).

Transcriptional regulation of RB1 by growth factors and environmental signals

The most frequent method of RB1 inactivation is phosphorylation by cyclin-dependent kinases (CDKs), notwithstanding the significance of genetic deletion. E2F is activated when CDK-mediated phosphorylation disrupts RB1's interaction with E2F transcription factors. Therefore, oncogenic overexpression of CDK4/6 suppresses RB1 function and promotes transcription of genes necessary for mitosis and cell cycle progression, whether through activating mutations in CDK4/6, amplification of positive regulators like CCND1, or loss of CDK4/6 inhibitors like p16 (38, 42-45).

Cell cycle deregulation in RB

Numerous studies have examined RB1's genetic and epigenetic changes, as well as its biological functions, since it was identified as the first tumor suppressor protein. Most malignancies interfere with this checkpoint either directly through changes to RB1 (e.g., deletion, mutation, or epigenetic silencing) or, more frequently, by functional inactivation via CDK-mediated hyperphosphorylation (46). Clinically licensed CDK4/6 inhibitors, including

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palbociclib, ribociclib, and abemaciclib, effectively decrease tumor cell proliferation in accordance with this mechanism, particularly in estrogen receptor-positive breast cancer (47, 48). Functional RB1 protein is crucial for these medicines' ability to suppress proliferation (49, 50). RB1 expression and clinical response to CDK4/6 inhibitors are highly associated, and acquired resistance is frequently caused by loss of functional RB1 due to deletion or point mutations (51, 52). Alterations in other pathways can also cause resistance; two clinically important examples include amplification of Cyclin E or increased Cyclin D levels, both of which cause RB1 inactivation (53-57). This dependence offers a therapeutic justification for developing drugs that directly increase RB1 activity by stabilizing its contacts with binding partners, thereby strengthening and prolonging cell cycle arrest, since effective cell cycle advancement ultimately depends on RB1 inactivation (38).

Numerous mechanistic findings derive from preclinical investigations using cell lines, genetically engineered animal models, or xenografts, notwithstanding considerable progress in elucidating the molecular functions of RB1. Variations in experimental methods, genetic backgrounds, and disease models have led to inconsistencies in the precise role of RB1 deletion and associated oncogenic mechanisms in retinoblastoma initiation. Subsequent investigations using therapeutically relevant human retinal organoid models and standardized experimental frameworks will be essential to improve repeatability and refine the molecular basis of CRISPR-mediated therapeutic strategies.

Although RB1 inactivation is universally recognized as the initiating genetic event in most retinoblastomas, accumulating evidence indicates that RB1 loss alone is not invariably sufficient for complete malignant transformation (58). Rather than representing a true biological contradiction, the apparently conflicting findings reported in the literature likely reflect differences in experimental models, developmental stage, retinal cell of origin, genetic background, duration of observation, and the presence of cooperating oncogenic events. For example, Zheng *et al.* (59) demonstrated that CRISPR-mediated RB1 deletion in human retinal organoids induced aberrant S-phase entry and extensive apoptosis but was insufficient to generate overt tumors (59). In contrast, Deniz Kanber *et al.* reported that prolonged differentiation of RB1-deficient retinal organoids resulted in tumor-like transformation, while *in vivo* studies further showed that simultaneous disruption of additional RB pathway components such as RBL1 or cooperation with MYCN amplification markedly accelerates tumorigenesis (60). Collectively, these findings support a unified model in which RB1 inactivation represents the essential initiating event of retinoblastoma, whereas complete malignant transformation depends on additional genetic, epigenetic, developmental, and microenvironmental factors that influence disease progression (Table 1).

CRISPR-Cas9 technology in precision medicine

Using a 20-nucleotide guide RNA sequence, Cas9 functions as an RNA-guided endonuclease, causing DNA breaks at particular chromosomal locations (91, 92). Comprising a repeat–anti-repeat duplex, a guide–target heteroduplex region, and many stem–loop elements that support complex stability, the single-guide RNA (sgRNA) creates a characteristic T-shaped secondary structure (92). When Cas9 recognizes the PAM motif using its PAM-interacting (PI) domain, an R-loop is formed at the target site, resulting in target recognition (Figure 2)(93, 94).

Mechanistic insights into CRISPR/Cas9 genome editing

Shreya Bhattacharya *et al.* (95). Furthermore, molecular dynamics investigations, supported by principal component

Table 1. Key genetic and molecular alterations in retinoblastoma

Gene / Pathway	Type of Alteration	Functional Consequence	Clinical Relevance	Primary Evidence	Therapeutic Implications	Ref
RB1	Point mutations, deletions, promoter methylation, loss of heterozygosity (LOH), or biallelic inactivation (germline or somatic). Rare MYCN-amplified germline tumors retain intact RB1	Loss of functional pRb disrupts E2F regulation, promoting an inappropriate G1–S transition and uncontrolled proliferation. RB1 loss is sufficient for tumor initiation but usually requires additional genetic or epigenetic alterations for full malignant progression	Germline RB1 alterations are associated with bilateral, multifocal disease, earlier onset, and increased risk of secondary malignancies, whereas most sporadic cases are unilateral	Human: clinical and genomic studies. Animal: RB1/TP53-deficient mouse models; retinoma models	Supports genetic counseling and surveillance. Radiotherapy is generally avoided in hereditary disease because of secondary malignancy risk. Therapeutic strategies restoring p53 signaling remain investigational	(61-66)
MYCN	Amplification or overexpression. Acts as an alternative initiating oncogenic event in a rare molecular subtype	Promotes proliferation, suppresses retinal differentiation, activates MYC/E2F transcriptional programs, and enhances mTOR signaling	Associated with an aggressive, poorly differentiated subgroup of retinoblastoma; many MYCN-amplified tumors retain wild-type RB1	Human: MYCN-amplified tumors. Experimental: retinal organoids, xenografts, engineered cell lines	Preclinical studies suggest sensitivity to PLK1 inhibitors (Volasertib), MYC transcriptional inhibitors (THZ1, Flavopiridol), and PI3K/mTOR inhibition; clinical benefit remains unproven	(67-70)
RBL1 (p107)	Rare alterations, predominantly amplifications	Functions as a compensatory RB-family tumor suppressor regulating cell-cycle progression	Clinical significance remains uncertain and appears context-dependent	Animal: Rb1/Rbl1-deficient mouse models. Limited human genomic evidence	May contribute to tumor progression when combined with RB1 loss; no established targeted therapies	(71-75)
RBL2 (p130)	Rare alterations, including occasional amplifications	Regulates cell-cycle exit and maintenance of differentiated retinal cells	Likely contributes modestly to tumor progression in cooperation with RB1 deficiency	Animal: Rb1/Rbl2-deficient mouse models. Limited human evidence	Therapeutic significance remains largely unexplored	(71, 76)
CDK4/6–Cyclin D Axis	Hyperactivation or overexpression	Enhances G1–S progression and cellular proliferation	Increased activity has been associated with aggressive behavior in multiple cancers; its specific role in retinoblastoma remains under investigation	Human: limited RB datasets. Preclinical: cell lines and animal models	CDK4/6 inhibitors (palbociclib, abemaciclib) have demonstrated preclinical activity in retinoblastoma models; their clinical efficacy in retinoblastoma has not yet been established	(77-80)
E2F Transcription Factors	Increased activity, particularly E2F1	Activates genes involved in proliferation, DNA replication, DNA repair, and cell-cycle progression	Contributes to tumor growth and malignant progression following RB1 loss	Human: tumor specimens. Experimental: Y79, WERI-Rb-1 cells, xenograft models	Experimental inhibition of E2F1 suppresses tumor proliferation; therapeutic approaches remain investigational	(81-86)
SKP2	Functional activation; increased dependence in RB1-deficient cells	Promotes degradation of p27 through SCF–SKP2-mediated ubiquitination, facilitating survival of RB1-deficient cells	Genetic ablation suppresses tumorigenesis in RB1-deficient experimental models	Animal: RB1/Skp2 mouse models. Experimental: human RB cell lines	Pharmacologic inhibition of SKP2 or disruption of p27-T187 phosphorylation has shown promising preclinical activity. Notably, MLN4924 does not directly inhibit SKP2; instead, it inhibits the NEDD8-activating enzyme (NAE), thereby indirectly suppressing SCF–SKP2 ubiquitin ligase activity	(87-90)

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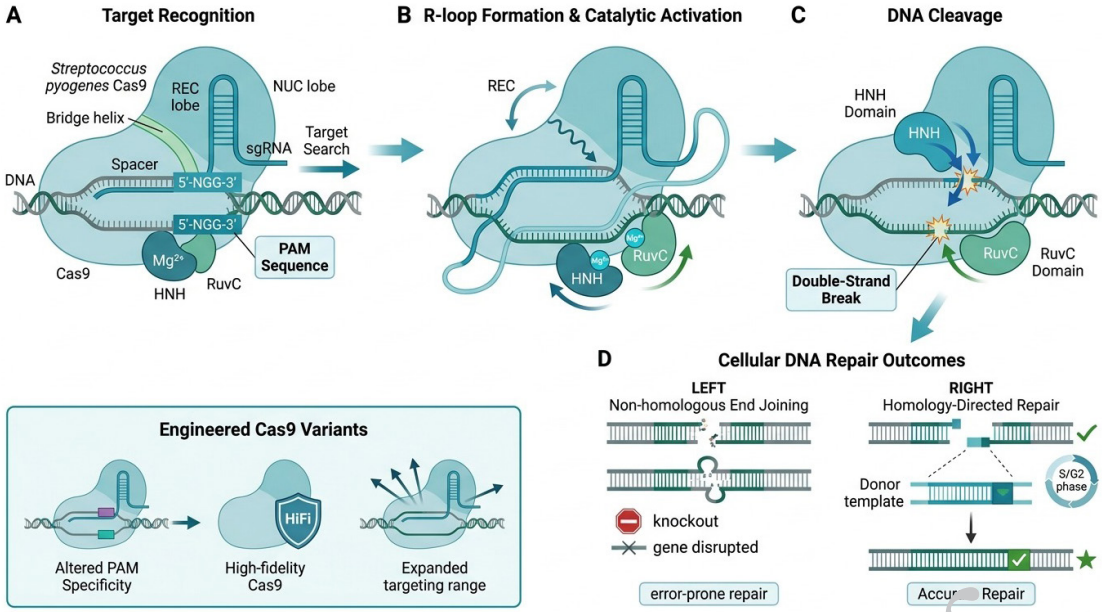

Figure 2. Structural activation and genome-editing mechanism of CRISPR–Cas9
(A) CRISPR-associated protein 9 (Cas9) first recognizes the protospacer-adjacent motif (PAM; 5′-NGG-3′, where N represents any nucleotide and G represents guanine) and binds the target deoxyribonucleic acid (DNA). (B) PAM recognition induces DNA unwinding, R-loop formation, and conformational rearrangements that position the histidine–asparagine–histidine (HNH) and RuvC-like nuclease domains for catalysis. (C) The HNH domain cleaves the target DNA strand, whereas the RuvC domain cleaves the non-target strand in a magnesium ion (Mg^{2+})-dependent reaction, generating a site-specific double-strand break. (D) DNA repair proceeds through either non-homologous end joining (NHEJ), resulting in insertions or deletions, or homology-directed repair (HDR), enabling precise sequence correction in the presence of a donor template. An inset highlights engineered Cas9 variants with altered PAM recognition and improved targeting specificity that have expanded genome-engineering applications. (The figure was created using Adobe Illustrator)
Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; DNA, deoxyribonucleic acid; HDR, homology-directed repair; HNH, histidine–asparagine–histidine nuclease domain; Mg^{2+}, magnesium ion; NHEJ, non-homologous end joining; PAM, protospacer-adjacent motif; RuvC, RuvC-like nuclease domain; 5′-NGG-3′, a PAM sequence in which N represents any nucleotide and each G represents guanine

analysis, have demonstrated that PAM binding functions as an allosteric regulator, promoting a critical open-to-closed conformational transition required for nucleotide accommodation while enhancing the dynamic interaction and coordinated conformational changes between the HNH and RuvC catalytic domains, which are essential for efficient double-stranded DNA cleavage (Figure 2)(96). Cas9 binds to PAM-containing DNA in different conformations than it does to PAM-lacking substrates, according to structural modeling. Principal component analysis shows that PAM binding promotes a critical open-to-closed transition necessary for nucleotide accommodation and enhances the dynamic interaction between the HNH and RuvC domains. Certain residues, such as Q770 and E584, represent important nodes in the allosteric signaling network that is conveyed across the L1 and L2 linker regions by establishing electrostatic interactions with K775 and R905 (96). Changes to these charged residues can alter Cas9's enzymatic specificity; experimental evidence indicates that modifications such as K775A improve targeting precision while reducing off-target effects (95-97). Engineered Cas9 variants like xCas9, SpG, and SpRY increase PAM compatibility without appreciably sacrificing cleavage precision, whereas specific mutations like D1135E enhance discrimination between PAM sequences, particularly against noncanonical motifs (Figure 2)(98-100).

Nevertheless, greater PAM compatibility does not always translate into safer treatment, even as tailored Cas9 variants enhance targeting versatility. Further validation in therapeutically relevant animals is necessary to confirm the long-term specificity of certain variations, which may introduce new possible off-target locations.

Additionally, Mg^{2+} ions in the RuvC active site strengthen DNA binding, favor staggered cleavage patterns, and facilitate the shift of the HNH domain from a precatalytic to a catalytic state, as shown by classical molecular dynamics simulations. A rotation of around 180 degrees occurs when the nontarget strand is loaded, allowing the completely bound precatalytic complex to form (101, 102). Although these mechanistic insights provide a basis for enhancing RB1-targeted genome editing techniques, their therapeutic use in retinoblastoma still depends on resolving issues with long-term safety, editing specificity, and delivery efficiency.

Overall, this thorough understanding of Cas9's molecular role provides a strong foundation for precisely targeting RB1 and related pathways in retinoblastoma and informs broader genome-editing techniques (102). Importantly, despite substantial advances in understanding Cas9 structure and engineering, most mechanistic insights have been obtained from biochemical analyses, engineered cell systems, or general cancer models rather than retinoblastoma-specific experimental settings. Therefore, the direct applicability of these optimized Cas9 platforms for RB1 targeting remains to be fully established, as validation in disease-relevant models, including retinoblastoma cell lines, retinal organoids, and *in vivo* tumor models, is still comparatively limited. Differences in genetic background, target-sequence accessibility, and experimental design may further influence editing outcomes and complicate the translation of mechanistic findings into therapeutic applications.

Mechanisms of Cas9-Mediated gene editing in retinoblastoma

While the RuvC domain cuts the non-complementary

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DNA strand, the HNH domain cleaves the complementary DNA strand (46, 94). Numerous genome-editing techniques are made possible by CRISPR-Cas9, such as gene knockout to create loss-of-function models, homology-directed repair (HDR) for gene correction, and sophisticated precision techniques including base and prime editing for single-nucleotide modifications (103-105). CRISPR provides greater specificity, simpler guide RNA construction, and the ability to target many genomic loci at once compared with previous genome-editing techniques like TALENs and ZFNs (106, 107). Despite these advantages, CRISPR-based systems are not universally superior, as concerns regarding off-target activity, immune recognition of Cas proteins, and DNA damage responses remain important limitations compared with earlier genome-editing platforms.

Achieving safe and efficient administration in ocular applications is still a significant problem. Adeno-associated virus (AAV) vectors, including single- and dual-AAV systems, can effectively target photoreceptors and retinal pigment epithelium cells (108). Larger genetic payloads can be carried by lentiviral vectors, but transport limitations and concerns about genome integration limit their *in vivo* use (109). Lipid nanoparticles and virus-like particles are examples of nonviral techniques that offer temporary expression, lower immunogenicity, and the potential for tailored delivery to post-mitotic retinal cells (110). Although AAV vectors have demonstrated success in retinal gene therapy, constraints including limited cargo capacity, pre-existing immunity, and challenges in achieving homogeneous delivery throughout tumor tissues remain unresolved. Similarly, nonviral approaches require further optimization to achieve sufficient editing efficiency while maintaining safety.

Safety considerations and challenges of CRISPR-based ocular gene therapy

Despite the transformative impact of CRISPR-based genome editing on ocular gene therapy, many safety concerns that extend beyond conventional off-target mutations continue to hinder its widespread use in clinical environments. The operational mechanism of CRISPR nucleases, including Cas9 and Cas12, which induce double-strand DNA breaks (DSBs) to initiate genomic modifications, is a significant reason for concern (111-114). Using intrinsic DNA repair mechanisms, these DNA breaks enable efficient editing; however, they can generate diverse repair outcomes that may threaten genomic stability (114, 115). DSB repair may lead to significant chromosomal alterations, including substantial deletions, inversions, translocations, the integration of foreign DNA segments, and, in exceptional instances, profound genomic anomalies such as chromothripsis, aneuploidy, and total chromosome loss, alongside the minor insertions and deletions commonly generated by NHEJ (116). The enduring presence and potential clonal proliferation of cells harboring these mutations post-transplantation or after *in vivo* modification raise concerns about long-term carcinogenic hazards, despite the infrequency of such events. Consequently, comprehensive examination of extensive structural genomic alterations must be included in the safety evaluation of CRISPR-derived therapies, including the detection of off-target mutations (117).

The inherent traits of endogenous DNA repair mechanisms provide an additional limitation. The predominant repair mechanism in mammalian cells is NHEJ, which often surpasses HDR, particularly in quiescent tissues (118, 119). Consequently, endeavors to achieve accurate gene modification can provide undesirable repair outcomes. NHEJ-mediated repair may lead to substantial deletions or unforeseen sequence rearrangements that disrupt adjacent coding sequences or regulatory genomic regions, as well as small insertions and deletions (120). Given that the majority of retinal neurons are terminally differentiated and exhibit no HDR activity, this limitation is especially significant in ocular tissues. Consequently, template-directed repair is inherently inefficient in these cells, whereas editing precision diminishes owing to a much heightened reliance on error-prone NHEJ.

Endogenous mechanisms for responding to DNA damage are activated when CRISPR nucleases create double-strand breaks (DSBs)(121). In properly modified cells, persistent DNA damage signaling may trigger the tumor suppressor protein p53, leading to cell cycle inhibition or apoptosis. In principle, this creates a selection pressure that favors cells with compromised p53 function (122). These results underscore the importance of meticulously evaluating genome-editing methods that intentionally create DSBs, particularly in therapeutic contexts aimed at implementing enduring genomic alterations, even though the biological implications of this occurrence in ocular tissues remain inadequately understood.

A further considerable barrier to the practical use of ocular genome editing is immunogenicity. The eye is an appealing target for genome-editing therapies because its relatively immune-privileged setting facilitates precise gene delivery and minimizes inflammatory responses (123). Nevertheless, this immunologic privilege is not entirely actualized. Prolonged intracellular presence of exogenous editing elements may enhance immunogenicity, and Cas proteins from bacteria and viral delivery systems continue to stimulate both innate and adaptive immune responses (124, 125). Consequently, minimizing the duration of genome-editing equipment use has become an essential strategy for improving therapeutic safety. Transient delivery platforms, including ribonucleoprotein (RNP) complexes and messenger RNA (mRNA)-based systems, are often considered safer than plasmid or viral DNA methods because they facilitate rapid editing and subsequent efficient intracellular destruction. This diminishes extended nuclease expression and the likelihood of vector DNA incorporation into the host genome (126, 127).

Ensuring enduring genomic stability remains essential for clinical application, despite the rapid advances in genome editing. The ability to identify undesirable editing events has markedly improved owing to substantial progress in genome-wide off-target detection technologies (128); nonetheless, no existing platform can comprehensively detect the full spectrum of off-target effects, particularly complex structural genomic changes or rare mutations (129, 130). Moreover, the chromatin structure, cellular environment, and delivery conditions seen in therapeutic ocular tissues may not be accurately mirrored by analytical methods validated in experimental frameworks (129). Consequently, before the advancement of genome-editing therapies for eye disorders into clinical use, a comprehensive safety assessment must include several supplementary detection

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methods alongside extensive sequencing techniques and extended longitudinal observation.

Numerous safety limitations might be mitigated by the advancement of next-generation genome-editing technologies. Base editors and prime editors achieve very precise sequence modifications without inducing canonical double-strand breaks or requiring donor DNA templates, unlike conventional CRISPR nucleases (131, 132). Substantial chromosomal alterations, extensive deletions, viral DNA incorporation, and other genomic irregularities associated with DNA double-strand break repair are markedly reduced when DSB production is eradicated (133). Additionally, prime editing includes multiple free base-pairing validation steps, improving editing precision and reducing off-target modifications (134).

Ultimately, the sustained efficacy of CRISPR-mediated ocular gene therapy will depend on preventing unintended genomic alterations that might compromise therapeutic safety, alongside enhancing editing precision. Based on existing data, the optimal strategy for achieving clinically acceptable safety profiles integrates sophisticated editing technologies such as base editors and prime editors with comprehensive genome-wide safety assessments, high-fidelity Cas enzymes, meticulously optimized guide RNAs, and transient RNP-based delivery mechanisms. Integrating these complementary techniques provides the strongest foundation for the safe use of precision genome editing in future ophthalmic therapies (135).

Applications of CRISpr-cas9 in retinoblastoma research & therapy

Disease modeling

Naert *et al.* (16) disrupted the RB1 and Retinoblastoma-like 1 (RB11) genes in Xenopus tropicalis using CRISPR/Cas9. While double mosaic mutants lacking both RB1 and RBL1 rapidly developed retinoblastoma, mosaic single mutants for either RB1 or RBL1 did not develop tumors (Figure 3)(136). This work produced the first hereditary retinoblastoma model in a non-mammalian system and the first CRISPR/Cas9-based cancer model in X. tropicalis, creating a platform for preclinical research that may aid in identifying new therapeutic targets (136). Although the Xenopus tropicalis model offers important insights into RB1-associated cancer, direct extrapolation to human retinoblastoma may be limited because of the animal's non-mammalian genetic background and differences in retinal development. RB1-null retinal organoid models are discussed in Section 3.

These results show that CRISPR/Cas9 may be used as a research platform and as a method for employing induced pluripotent stem cells (iPSCs) to correct genetic defects *in vitro*. These cells can then be implanted into afflicted retinas (137). Bassuk *et al.*(138) used skin fibroblasts from individuals with X-linked hereditary retinitis pigmentosa to create induced pluripotent stem cells (iPSCs). These patient-derived iPSCs had harmful mutations; therefore, CRISPR/Cas9 was used *in vitro* to fix them. This produced genetically repaired cells. According to the sequencing data, around 13% of the mutations were effectively fixed. Theoretically, autologous transplantation techniques may use these modified iPSCs, which would lessen the possibility of immunological rejection. Additionally, Burnight *et al.* (139) examined several genes, including MAK, CEP290, and BBS, and showed that patient-derived iPSCs could be effectively edited regardless of whether the mutations were in deep intronic sequences, exonic regions, or represented dominant alleles. These results demonstrate CRISPR/Cas9's potential to alter patient-derived iPSCs for autologous cell therapy and validate its feasibility (136, 140).

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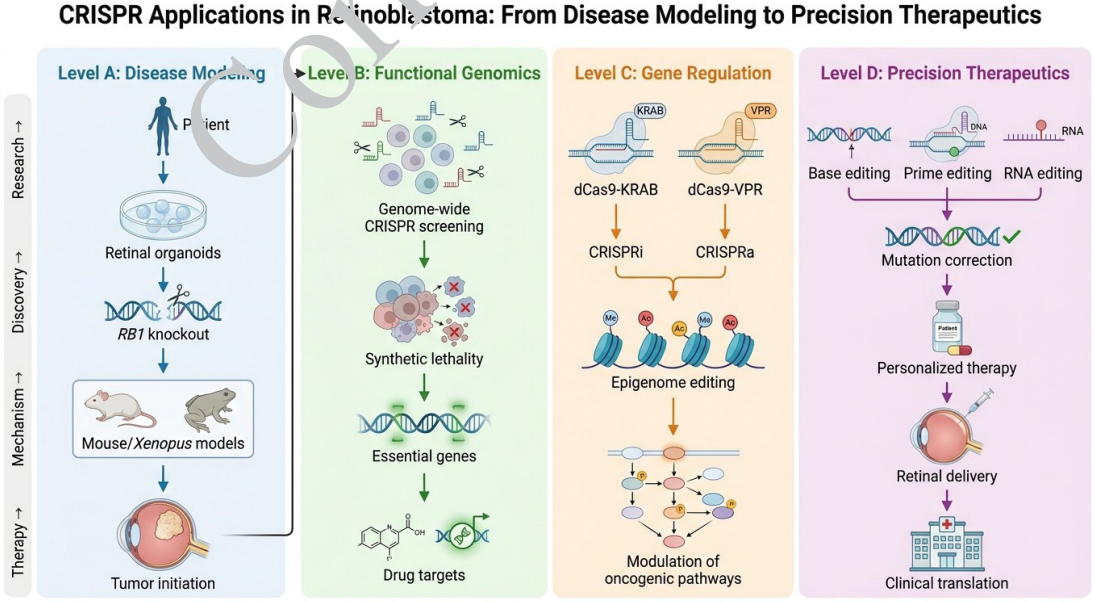

Figure 3. CRISPR applications in retinoblastoma: From disease modeling to precision therapeutics
(A) Clustered regularly interspaced short palindromic repeats (CRISPR)-mediated RB1 editing in retinal organoids and in vivo models enables investigation of retinoblastoma initiation and progression. (B) Genome-wide CRISPR loss-of-function screening identifies synthetic lethal interactions and context-specific genetic dependencies associated with RB1 deficiency. (C) Nuclease-deficient CRISPR-associated protein 9 (Cas9) platforms enable transcriptional activation, repression, and epigenetic modulation of genes involved in retinoblastoma biology. (D) Advanced precision-editing technologies, including base editing, prime editing, and ribonucleic acid (RNA)-targeting systems, facilitate mutation correction and personalized therapeutic development. Together, these complementary CRISPR modalities form a continuous pipeline from mechanistic investigation to precision medicine (The figure was created using Adobe Illustrator)
Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; RB1, RB transcriptional corepressor 1; RNA, ribonucleic acid

Generation of in vitro retinoblastoma models

Expanding on the widely accepted theory that retinoblastoma arises from progenitors of immature retinal neurons (63, 141, 142), Liu *et al.* differentiated retinal organoids from mutant human embryonic stem cell (hESC) lines to investigate Rb carcinogenesis. They used a previously described stepwise induction protocol to create human retinal organoids (143-145). These organoids replicated the main molecular indicators and morphogenetic traits of typical human retinal development (146, 147). There were no discernible changes between RB1-mutant organoids (hRBOs) and control retinal organoids (hROs) by differentiation day 45 (148). However, by day 75, organoids generated from both RB1−/− and RB1Mut/Mut hESCs showed clear tumor-like primary foci. Compared with normal structures, these aberrant regions showed increased overall size, ambiguous boundaries, and variable internal density (148). These foci quickly grew out of the original masses once they were formed. Deep optical coherence tomography, a sophisticated live-imaging technique, revealed the mutant organoids' complex internal architecture and uneven surface patterns. Increased nucleocytoplasmic ratios, multiple mitochondria with different morphologies, densely packed cellular aggregates, rosette structures like Flexner–Wintersteiner rosettes typical of retinoblastoma and fleurettes typical of retinoma, and condensed chromosomal material were all visible under transmission electron microscopy as hallmarks of primary retinoblastoma (Figure 3)(148). Overall, these results show that RB1-mutant human retinal organoids can develop into tumors and are useful as a disease-relevant model derived from genetically altered hESC lines (148). Retinal organoids do not fully replicate the complexity of the tumor microenvironment, immune interactions, and *in vivo* developmental context, but they do mimic certain histopathological and molecular features of retinoblastoma (149). Consequently, findings from organoid systems are highly informative; however, they require validation using animal models and patient samples (150).

Recapitulating patient-specific genotypes

Two separate clones with biallelic RB1 knockouts (RB1−/−) were generated by targeting the first exon of the RB1 gene to further examine the consequences of RB1 mutagenesis (148). Immunocytochemical staining independently confirmed the Western blot analysis's finding that all altered lines had significantly lower pRB levels (148). RB1, p107, and p130 are among the members of the RB protein family that are known to be important regulators of self-renewal in human embryonic stem cells (hESCs)(151). By using flow cytometry to analyze pluripotency markers and cell cycle patterns, the impact of RB1 silencing on hESC identity was evaluated (148). There were no discernible alterations in the overall cell cycle distribution or the percentage of OCT3/4-positive cells in RB1-mutant clones. These results were supported by RNA sequencing, which revealed that RB1 deletion had no effect on gene expression programs linked to pluripotency, self-renewal, and cell cycle control. Cytogenetic studies showed that the modified hESC lines preserved a normal 46, XX karyotype. Despite these encouraging outcomes, developing patient-specific CRISPR-engineered models requires a comprehensive assessment of editing accuracy, unintended alterations, and long-term genomic stability, particularly for prospective therapeutic or regenerative applications (152).

Modeling secondary genetic events

Liu *et al.* (148) found significant overlap in gene expression patterns by comparing the transcriptomes of natural retinoblastoma tumors, human RB organoids (hRBOs), and the Y79 retinoblastoma cell line. The expression profiles seen in primary retinoblastoma samples were closely matched by the 1,014 differentially expressed genes—574 up-regulated and 440 down-regulated—that were seen in hRBOs after more than 75 days of differentiation (153). The number of genes with greater than twofold expression alterations grew gradually as differentiation progressed, according to genome-wide comparisons between control retinal organoids (hROs) and RB organoids. Additionally, KEGG pathway enrichment analysis revealed disruption of important oncogenic signaling networks implicated in retinoblastoma, including those linked to PI3K–Akt signaling and cell cycle control (148). To gain deeper insight into transcriptional regulation during Rb tumorigenesis, Liu *et al.* (148) investigated this process. Since methylation plays a key role in tumor-associated gene regulation, genome-wide DNA methylation patterns were analyzed to look into epigenetic contributions to transcriptional dysregulation in retinoblastoma. 8,968 differentially methylated regions (DMRs) were found in this investigation, including 4,371 hypomethylated and 4,597 hypermethylated loci. Compared with control organoids, hRBOs showed notable methylation alterations in several genes intimately associated with retinoblastoma biology, including SYK, RXRG, MKI67, CCNE2, VIM, and CDH11. Notably, one of the genes most substantially increased during tumor-like transformation in hRBOs was SYK, a crucial part of the PI3K–Akt pathway. While VIM, which is not a key Rb driver, exhibited increased methylation in line with its expression pattern, CpG island shore areas inside the SYK promoter showed decreased methylation in both hRBOs and primary retinoblastoma samples in comparison to controls (154).

Moreover, Liu *et al.* (148) found that DMRs were broadly dispersed and more frequently found in noncoding areas than in coding sequences, according to genome-wide mapping. Spatial analysis showed that roughly 77% of DMRs were located more than 4 kb from CpG islands (CGIs), and more than half of the DMRs linked to CGIs were localized to CGI shore areas. These methylation alterations were linked by functional pathway enrichment to axon guidance, general cancer pathways, and PI3K–Akt signaling, among other biological processes (148). However, interpretation of epigenomic and transcriptomic alterations in organoid-based models requires caution, as some observed changes may reflect differences in maturation state rather than tumor-specific events (155).

Functional genomics and drug discovery

Aubry *et al.* described prioritizing potential genes for *in vivo* shRNA screening using the DyNeMo framework (156, 157). Transcriptome analysis of 12 prenatal retinal samples and 21 human RB1-null tumors revealed 27 disrupted regulatory hubs (156). BRCA1, RAD51, and XRCC6 were among the DNA repair-related factors for which these potential hubs were substantially concentrated (156). These

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27 network hubs, along with 34 other tumor suppressors and oncogenes, including known positive controls implicated in retinoblastoma evolution, were then used for functional *in vivo* shRNA screening. 647 shRNAs that targeted 147 genes in total were evaluated (156). Of these constructs, 100 shRNAs in Y79 tumors and 72 shRNAs in WERI-RB1 cancers showed significant depletion, suggesting functional significance. Nine of the 18 genes that were consistently found in both tumor models were supported by at least two significantly decreased shRNAs. A second iterative *in vivo* screen was conducted to improve candidate selection and guarantee repeatability, verifying 15 of the 18 first positives (156). In both RB1-null and MYCN-amplified retinoblastoma cell lines, seven genes showed significant concordance and shared oncogenic networks, consistently scoring across three or four examined models. This collection contained ID2, which is known to have oncogenic activity in both RB1-deficient and RB1-wildtype/MYCN-amplified settings, as well as SKP2 and MYCN, supporting earlier findings (87, 156, 158-160). Numerous high-ranking candidates, such as RAD51 and BRCA1, were connected to DNA repair pathways (115). These results show that DyNeMo-based network analysis may detect physiologically significant targets and uncover common oncogenic dependencies among retinoblastoma molecular subtypes, with DNA repair proteins playing key roles. Functional investigations have demonstrated that retinoblastoma cell survival is specifically reduced by RAD51 inhibition (156). It has been demonstrated that therapeutic strategies that combine drugs that damage DNA with inhibitors of DNA repair pathways increase the effectiveness of treatment (161, 162). In this case, RAD51 inhibition or depletion results in selective cytotoxicity, whereas disruption of homologous recombination increases retinoblastoma cell susceptibility to clinically utilized chemotherapeutics. Thus, novel synergistic treatment options for juvenile retinoblastoma have been identified using the combination of dynamic network modeling and functional genomic screening. The RAD51/BRCA1 complex, several interacting partners, and documented RB drivers that validated the screen were among the highest-confidence targets. Furthermore, when used with frontline retinoblastoma medications such as topotecan (TPT) or etoposide (ETO), the RAD51 inhibitor B02 showed synergistic results (156).

Agrawal *et al.* 2024 (165). To find out how RB1 deletion impacts retinal progenitor cell proliferation and differentiation *in vitro*, they created three CRISPR/Cas9-edited iPSC lines with targeted RB1 disruption from a healthy parental iPSC background. Comprehensive transcriptomic, proteomic, and genomic analyses confirmed significant RB1 suppression while demonstrating that the edited RB1−/− homozygous lines preserved normal chromosomal organization and genetic identity, together with the expression of differentiation-associated genes, their corresponding proteins, and key pluripotency markers. These animals provide a strong platform for studying cellular processes and signaling pathways that promote tumor cell survival in retinoblastoma because of their near-isogenic background (Figure 3)(163).

Drug response profiles were evaluated in MYCN-overexpressing retinal organoid-derived cells (MYCNO/E) and compared with the well-known retinoblastoma cell line Y79, which is RB1-deficient and MYCN-amplified, in order to uncover therapeutic vulnerabilities associated with MYCN-driven retinoblastoma (164). WST-1 assays were used to assess cell viability across a panel of MYC-targeted agents grouped by mechanism of action. These agents included compounds that affect MYC protein stability (Alisertib, Volasertib, Prexasertib), inhibitors of translational signaling (Dactolisib, Rapamycin), and inhibitors of MYC-associated transcription (THZ1, Flavopiridol, Roscovitine, JQ1, I-BET)(164). Compared to Y79 cells, MYCNO/E cells showed noticeably greater sensitivity to certain MYC-targeted drugs (164). The CDK7-selective drug THZ1 was the most potent transcriptional inhibitor in MYCNO/E cells (IC50=21 nM), while Y79 cells exhibited significantly lower sensitivity (IC50=2384 nM). Flavopiridol, a broad-spectrum CDK inhibitor, showed a similar pattern. It was quite active in MYCNO/E cells (IC50=50.46 nM), but it was unable to reach a detectable IC50 in Y79 cells, indicating relative resistance (164). The mTORC1-selective inhibitor Rapamycin showed minimal activity in both MYCNO/E and Y79 models, whereas the combination PI3K/AKT/mTOR inhibitor Dactolisib caused considerable growth suppression in MYCNO/E cells (IC50=370.8 nM) within the family of translational pathway inhibitors (164). Among the substances that target MYC stability, the PLK1 inhibitor Volasertib significantly reduced sensitivity in Y79 cells (IC50=220 nM) and viability in MYCNO/E cells (IC50=273.3 nM)(164). Overall, MYCN-overexpressing retinoblastoma cells have a unique therapeutic sensitivity profile, with particularly strong reactivity to PLK1 inhibition with Volasertib and transcriptional CDK inhibitors like THZ1 and Flavopiridol (164). An increasing dependence on MYC-driven transcriptional and proliferative processes, which is sometimes referred to as oncogene addiction, is consistent with this selective sensitivity. In contrast, Y79 cells, which are mainly influenced by RB1 loss and more extensive genomic changes, appear to be less reliant on MYC signaling and may activate other survival pathways, resulting in decreased drug sensitivity. These variations highlight the need to adjust treatment plans for MYCN-driven retinoblastoma based on the main oncogenic driver (164). However, the limitations of preclinical drug-screening models should be considered when interpreting these results. Due to variations in tumor heterogeneity, pharmacokinetics, and microenvironmental interactions, drug sensitivity shown in established cell lines or organoid-derived cells may not accurately predict clinical responses (165, 166). Before these vulnerabilities can be converted into treatment procedures, independent validation utilizing additional patient-derived models will be necessary (167).

Further investigations confirmed the significant susceptibility of MYCNO/E cells to MYC-directed treatments, especially transcriptional inhibitors like THZ1 and flavopiridol (168, 169). THZ1's strong action is consistent with CDK7's critical role in maintaining MYCN-driven transcriptional programs, which are necessary for the continuous growth and survival of tumor cells (170). There was also notable sensitivity to the PLK1 inhibitor Volasertib (164, 170). This further emphasizes that MYCN-driven retinoblastoma relies on mitotic signaling pathways and cell cycle control (171). Y79 cells likewise have MYCN amplification; however, they showed a clear difference in drug reactivity compared to MYCN0/E cells (164). The decreased response of Y79 cells supports the conclusion that

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RB1 deletion is the main oncogenic driver in this model, as it permits the activation of compensatory mechanisms that reduce dependence on MYCN signaling (85, 172). MYCNO/E cells, on the other hand, have a strong MYCN-centered dependence, making them extremely vulnerable to specific inhibition of MYC-associated pathways (173). Taken together, our findings emphasize MYCN as a top therapeutic target and the importance of precision-guided, genetically stratified therapy approaches for this aggressive subtype of retinoblastoma (164). These therapeutic vulnerabilities remain in the preclinical stage, and because of variations in tumor heterogeneity, drug delivery, pharmacokinetics, and microenvironmental interactions, drug sensitivity seen in engineered organoid-derived models may not reliably predict clinical efficacy (174). Before being translated into patient treatment plans, further validation in clinically relevant models is needed.

CRISPR-mediated gene editing offers a promising way to directly correct RB1 mutations in patient-derived animals after functional genomics has identified key therapeutic targets.

CRISPR synthetic lethality in RB1-deficient RB

Synthetic lethality and CRISPR applications in RB1-deficient cancers

It is intrinsically difficult to target inactivated or deleted tumor suppressor genes therapeutically because pharmacologic treatments cannot directly reverse their loss (175). A different strategy is based on gene–drug synthetic lethality, which uses therapeutically exploited secondary vulnerabilities caused by the loss of a particular gene. This approach can be used for targeted cancer treatment by identifying context-dependent vulnerabilities that arise after tumor suppressor inactivation (175, 176). Oser MG and associates performed synthetic lethal screenings to find targetable dependencies in RB1-deficient small cell lung tumors (SCLCs) using CRISPR/Cas9-based screening platforms (177). However, synthetic lethal dependencies identified in RB1-deficient cancers are highly context-dependent. Findings obtained from small cell lung cancer or other RB1-deficient malignancies cannot be directly extrapolated to retinoblastoma, as tissue-specific transcriptional programs, developmental stage, and cooperating genetic alterations may influence therapeutic vulnerabilities (46, 178). Numerous interdependent elements within this network were included in the vulnerabilities that were found to be enriched for genes related to chromosomal segregation and mitotic control. Upstream mitotic regulators such as AURKB, PLK1, and INCENP, as well as condensin complex members like SMC2, NCAPG, and SMC4, were important contenders (175). Independent research has linked RB1 pathway status to SKP2 activity, demonstrating that the RB1 tumor suppressor functionally regulates SKP2 (175, 179). The inhibitor MLN4924 has been used to study the therapeutic potential of SKP2 inhibition in retinoblastoma models since SKP2 depletion causes a synthetic lethal impact in RB1-null cells (180).In both *in vitro* and *in vivo* models, MLN4924 therapy specifically suppressed retinoblastoma tumor development, causing G1-phase arrest with apoptosis and G2/M arrest with endoreplication phenotypes (175). Although these findings support SKP2 inhibition as a potential therapeutic strategy, the available evidence remains primarily preclinical. Reproducibility across independent retinoblastoma models and assessment of systemic toxicity will be essential before considering clinical translation (181).

Changes in RB1 loss of function are also known to give estrogen receptor-positive (ER+) metastatic breast cancer resistance to CDK4/6 inhibitors (182, 183). In ER^+/RB1-deficient breast cancer cells, a genome-wide CRISPR screen revealed a context-specific vulnerability to protein arginine methyltransferase 5 (PRMT5) inhibition (184). Mechanistic research shows that PRMT5 inhibition causes RNA polymerase II Ser2 hyperphosphorylation and increases intron retention in many genes necessary for DNA synthesis, ultimately resulting in proliferative arrest in RB1-deficient cells. This impairs G1-to-S phase progression regardless of RB1 status (175). This impact was further enhanced by combination therapy: in ER^+/RB1-deficient models, including patient-derived xenografts (PDXs) *in vivo*, co-treatment with the selective estrogen receptor degrader fulvestrant and the PRMT5 inhibitor pemrametostat produced synergistic tumor growth suppression (175).

Identifying dependencies using RBNSig and CRISPR screens

With 61 out of 100 lines falling into the RBNSig-high categorization group (odds ratio=11.12; $P=3.66\times10^{-16}$, Fisher's exact test), cell line models with RB1 faults were significantly overrepresented in this group. This enrichment supports applying the patient-derived RBNSig-core transcriptional signature to *in vitro* cell line systems (185). Publicly accessible genome-wide CRISPR–Cas9 dependence datasets from the same cell lines, acquired through the DepMap resource, were methodically examined using RBNSig-core stratification in order to find genetic vulnerabilities associated with RB pathway malfunction (186, 187). However, because cancer cell lines cannot fully retain the molecular heterogeneity and microenvironmental features of real tumors, computational studies based on DepMap datasets should be interpreted with caution. Consequently, experimental validation in patient-derived models is necessary for potential synthetic lethal interactions found using in silico stratification (188).

RB1-defective against RBNSig-low models and RBness-classified versus RBNSig-low models were the two parallel comparison frameworks used. This dual-comparison method made it possible to identify the genes whose loss results in synthetic lethality under both general RB pathway impairment and RB1 deficiency (185). 90 potential synthetic lethal genes were linked to RB1-defective status and 87 to RBness categorization, according to an analysis of CRISPR gene-effect dependence patterns ($P<0.05$, one-sided Welch's t-test). Between the two vulnerability groups, 34 genes overlapped, indicating a highly significant intersection ($P=2.08\times10^{-57}$, Fisher's exact test). These genes may mimic therapeutic response patterns seen with RB1-targeted therapies in RBness-defined malignancies, given this common dependent set (185).

Validation and identification of key synthetic lethal genes

A genome-wide CRISPR–Cas9 loss-of-function dataset obtained from an isogenic breast epithelial model consisting of MCF10A TP53−/− cells with or without RB1 deletion was used for independent *in vitro* validation (187). Further validating this paired model system for researching RB1-linked genetic vulnerabilities, targeted RNA interference

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targeting important RB1-associated dependence genes SKP2, E2F3, and TFDP1 in the RB1-deleted background produced results comparable with reliance patterns seen in DepMap analysis (185). 764 genes were identified as possible synthetic lethal partners of RB1 deletion by comparing the CRISPR screening findings between MCF10A TP53−/− RB1−/− cells and their RB1-proficient counterparts (185). For synthetic lethal targets that the RBNSig-core framework has previously predicted using DepMap data, this candidate list was substantially enhanced. Notably, 13 of the 34 genes SKP2, CDK2, GTF2A1, TIMM44, TAF1, and TAF7 that were shown to be common synthetic lethal candidates across the RBness-classified and RB1-defective cell line groups were independently confirmed in the RB1-deleted isogenic screen (185). These validation results suggest that RB1-targeted therapeutic approaches may apply to tumors displaying RBness features, even in the absence of RB1 genomic alterations, and provide evidence that the RBness transcriptional phenotype captures important dependency patterns typical of RB1-deficient cancers (185).

Therapeutic gene editing

Building upon the genome-editing strategies described in Section 4, Target-AID cytosine base editing has been successfully applied to generate RB1 disease models by introducing clinically relevant nonsense mutations without relying on conventional nuclease-mediated disruption (189, 190). A cytosine base-editing method was selected to recreate patient-relevant mutations rather than using conventional nuclease-based editing to disrupt RB1 (59, 191). Low editing efficiency and poor cell viability were the major reasons why attempts to employ Cas9 and single-stranded oligodeoxynucleotide (ssODN) repair templates to construct an RB1 mutant cell line with the R552X variation failed (189). Although double-strand break-based CRISPR/Cas9 editing has made it possible to treat certain point-mutation illnesses through damage-and-repair processes, its broader clinical use has been constrained by inconsistent repair results and variable editing efficiency (192). The first known use of gene-edited cells in people occurred in October 2016, when autologous T lymphocytes genetically modified using CRISPR/Cas9 were reinfused into patients (193). The first clinical use of CRISPR-edited cells was reported in 2016, when ex vivo PD-1-edited autologous T cells were administered to patients; however, this study was unrelated to retinoblastoma (194, 195). In future research, we want to produce a corresponding mutant cell line by generating RB1-deficient retinal organoids using our cytosine base editor (CBE) technique. It is anticipated that retinal organoids produced from iPSCs that faithfully replicate patient-specific RB1 mutations will provide important new information on how these mutations contribute to tumor growth and evaluate the effectiveness of gene repair techniques (196). Nonetheless, instead of being an established therapeutic approach, the use of base and prime editing for retinoblastoma remains a potential option. Before evaluating these technologies in clinical trials, critical challenges such as efficient transport to tumor cells, immune responses, unintended alterations, and long-term genetic stability must be addressed (197, 198)(Table 2).

Epigenome & transcriptome editing in RB

Epigenetic dysregulation in RB: Methylation, chromatin remodeling

Eight genes linked to prominent morphological traits were investigated: Pex2 (peroxisomal function), Acf (histone remodeling), mcm6 (DNA replication), InR, wg, and dpp (signaling pathway components), and E2F2 and Mpp6 (cell cycle control)(203). It is necessary to take into account the local chromatin environment, which is abundant in transcription factor binding sites and histone modifications, when interpreting data at this chromosomally embedded locus. This environment likely alters interactions between Rb proteins and the transcriptional machinery at the common regulatory region (204). According to data from S2 cells, Rb protein activity may be affected by variations in chromatin arrangement between endogenous loci and reporter constructs on temporary plasmids, with cell-type-specific effects likely playing a role as well. Although suppression at Mpp6 reporter sites 4 and 5 was anticipated in wing discs, other regulatory elements in region 6 could further alter gene expression (204). However, the constraints of non-

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Table 2. CRISPR-based applications in retinoblastoma research and therapy

Experimental System	Gene(s) Investigated	CRISPR-Based Approach	Primary Application	Key Outcomes	Clinical/Translational Significance	Ref
Xenopus tropicalis (mosaic *in vivo* model)	RB1, RBL1	CRISPR/Cas9-mediated gene disruption	Retinoblastoma disease modeling	Concurrent inactivation of RB1 and RBL1 rapidly initiated retinoblastoma development, whereas loss of either gene alone failed to trigger tumorigenesis, highlighting their cooperative tumor-suppressive function	Serves as a rapid and efficient *in vivo* model for investigating early tumor initiation and evaluating potential therapeutic targets	(16)
Human embryonic stem cell (ESC)-derived retinal organoids	RB1	CRISPR/Cas9 knockout	Modeling early disease mechanisms	Deletion of RB1 induced inappropriate S-phase re-entry and increased apoptotic activity; however, RB1 loss alone was insufficient to drive complete malignant transformation	Provides valuable mechanistic insight into the earliest cellular alterations underlying retinoblastoma initiation	(59)
Human ESC-derived retinal organoids	RB1 (biallelic deletion)	CRISPR/Cas9-mediated biallelic knockout	Tumor modeling	Complete biallelic disruption of RB1 resulted in retinal lesions exhibiting morphological and molecular characteristics closely resembling primary human retinoblastoma	Represents a human-specific organoid platform suitable for mechanistic investigations and preclinical therapeutic evaluation	(199)
CRISPR-engineered induced pluripotent stem cells (iPSCs)	RB1	Precision genome editing	Functional genomic analysis	RB1-deficient iPSCs maintained pluripotency despite displaying significant alterations in cellular proliferation dynamics	Provides a near-isogenic experimental system for dissecting RB1-regulated signaling pathways and cellular functions	(200)
Y79 and WERI-RB1 xenograft models	DNA repair genes (e.g., RAD51, BRCA1)	CRISPR/Cas9-based and shRNA functional screening	Functional dependency screening	Loss-of-function genomic screens uncovered critical DNA repair vulnerabilities associated with RB1 deficiency, identifying pathways essential for tumor cell survival	Offers a rationale for developing combination therapeutic strategies targeting DNA repair mechanisms in retinoblastoma	(156)
DepMap pan-cancer cell line panel (n=563)	RB1 and RBness-associated synthetic lethal genes (SKP2, CDK2, TAF1, PABPC1, OPA1, etc.)	Genome-wide CRISPR-Cas9 loss-of-function screening	Synthetic lethality discovery	Large-scale functional screening revealed common synthetic lethal dependencies shared by RB1-mutant and RBness tumors, with key interactions subsequently validated in isogenic cellular models	Facilitates prioritization of novel therapeutic targets and broadens precision oncology strategies beyond tumors harboring canonical RB1 mutations	(201)

mammalian experimental systems should be considered when interpreting these results. While Drosophila models provide useful mechanistic insights into Rb-associated chromatin control, direct extrapolation to human RB1 regulation in retinoblastoma may be limited because of differences in enhancer design, genome organization, and developmental programs (205).

CRISPRi/CRISPRa and dCas9 effectors for reversible gene control

FLAG-epitope-tagged dCas9-Rbf1 and dCas9-Rbf2 fusion proteins were created in order to assess the impact of Rb paralogs on endogenous Drosophila genes. FLAG-tagged dCas9 alone served as a negative control (204). A proof-of-principle approach was used to rapidly evaluate the *in vivo* consequences of dCas9-Rb chimeras because they had never been investigated in any setting before. To assess the effects of dCas9-Rb targeting of gene promoters, tissue-specific production of these chimeras in the developing Drosophila wing was achieved using the GAL4/UAS system (204). When attached to the InR promoter, dCas9 alone occasionally impacted wing development, most likely as a result of steric obstruction at the promoter. The activation effects were also compared using a dCas9-VPR chimera, which is known to function as a strong transcriptional activator in Drosophila (204, 206). To increase target gene expression without changing the DNA sequence, CRISPR activation (CRISPRa) uses dCas9 linked to transcriptional activator domains such as VP64, p65, or Rta, the Epstein-Barr virus transcriptional activator (207, 208). CRISPR interference (CRISPRi), in contrast, blocks transcription and reduces gene expression without changing the underlying DNA sequence by fusing dCas9 to transcriptional repressor domains like KRAB (207, 209). The dCas9-KRAB complex, when directed to promoters or enhancers, draws chromatin-remodeling factors and co-repressor proteins, resulting in a restrictive chromatin state that successfully inhibits the target gene's transcription (210). For example, in cancer research, a CRISPR-Cas9-based demethylation system that combines dCas9 with the TET1 catalytic domain has been used to specifically remove DNA methylation marks at target gene promoters, reactivating silenced genes (207). This technique has made it possible to reactivate epigenetically silenced tumor suppressor genes, such as BRCA1, which are frequently down-regulated in malignancies and accelerate disease progression when deactivated (211). The ability to systematically evaluate the effects of targeting Rbf1 and Rbf2 on transcriptional programs and developmental consequences across many endogenous genes is a major advantage of the CRISPRi technique (204). However, the outcome of CRISPRi-mediated transcriptional repression is strongly influenced by genomic context, chromatin accessibility, and target locus characteristics (212). Furthermore, the longevity and reversibility of epigenetic alterations need to be carefully considered, especially for therapeutic applications where long-term unintentional suppression might have negative effects (Table 3)(213).

Direct comparison of these platforms indicates that their suitability for RB1-associated retinoblastoma depends primarily on the type of pathogenic alteration and the intended therapeutic outcome (219). HDR can theoretically restore the native RB1 sequence with high fidelity but is constrained by low activity in post-mitotic retinal cells and by DSB-associated genotoxicity. Base editing may provide a more efficient and safer option for compatible RB1 transition mutations, whereas prime editing offers the broadest sequence-correction capacity and may address substitutions and small insertions or deletions that are inaccessible to current base editors (134). In contrast, CRISPRi and CRISPRa do not permanently correct RB1 mutations. Their principal value lies in reversible pathway modulation: CRISPRi may suppress cooperating oncogenic or synthetic-lethal targets, while CRISPRa could potentially increase expression from a residual functional RB1 allele or activate compensatory tumor-suppressive pathways. Nevertheless, no standardized study has directly compared these platforms in matched RB1-mutant retinoblastoma models (220, 221). Therefore, reported editing frequencies should be interpreted cautiously, and future studies should evaluate the platforms under identical delivery, cellular, and sequencing conditions.

Emerging tools: RNA-targeting CRISPR (Cas13), temporal control

Because they target and cleave single-stranded RNA (ssRNA) rather than DNA, type VI CRISPR systems like Cas13a are unique in that they allow for programmed transcriptome modification without changing the genome (207, 222). The CRISPR toolset has grown with the discovery of RNA-targeting Cas13 enzymes, which enable precise RNA molecule modification without altering the underlying DNA. Cas13a's ability to selectively bind and cleave certain transcripts provides a prospective treatment approach for RNA-mediated illnesses such as viral infections and neurodegenerative diseases, allowing temporary gene knockdown for functional research (207, 223-225). Cas13's RNA-targeting capability has enabled sensitive viral diagnostics. The significance of Cas13-based RNA targeting to retinoblastoma is still mostly experimental, despite its growing uses. The majority of the data now available originates from models of infectious diseases, and its possible use in RB would need the identification of transcripts that cause the illness, effective delivery to retinal tumor cells, and confirmation of specificity in models that are clinically relevant (226, 227).

In addition to cleaving its target, Cas13 also causes collateral cleavage of neighboring reporter RNAs by identifying particular viral RNA sequences, including those from SARS-CoV-2. This produces a detectable signal that enables quick, highly specific virus identification (207, 223, 228). These results show that Cas13 is a flexible antiviral agent that can temporarily inhibit viral replication without modifying the host genome by directly cleaving viral RNA. Because of its RNA specificity, it can precisely target pathogens such as HIV and SARS-CoV-2, offering a promising platform for prospective therapeutic uses and rapid-response antiviral therapies (207, 225). Crucially, Cas13's capacity to target RNA enables it to eradicate both latent viral transcripts and actively replicating HIV within CD4 T cells, underscoring its potential as a potent tool for tackling viral reservoirs that would otherwise be resistant to traditional antiretroviral treatments (229).

Overall, emerging CRISPR-based regulatory and RNA-targeting technologies offer promising opportunities to study RB biology beyond conventional genome editing (Table 4)(230).

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Table 3. Comparative characteristics and therapeutic potential of CRISPR-based platforms for RB1-associated retinoblastoma

CRISPR platform	Editing mechanism and outcome	Representative editing efficiency/frequency	Precision and principal safety considerations	Potential application to RB1 mutations	Major translational limitations	Reference
Cas9-mediated HDR	This strategy relies on Cas9-induced double-strand DNA breaks (DSBs), after which HDR uses an exogenous donor template to accurately replace or correct the target genomic sequence. When HDR is successful, the endogenous allele can be restored with high sequence fidelity	Editing efficiency is generally low to moderate and varies by genomic locus, cell type, proliferative status, and donor-template delivery efficiency. In patient-derived retinal induced pluripotent stem cells (iPSCs), approximately 13% correction of RPGR mutant alleles has been reported	HDR enables highly accurate sequence correction; however, because NHEJ frequently competes with HDR, unwanted repair products such as indels, extensive deletions, and heterogeneous genomic alterations may occur. Furthermore, DSB formation may activate p53 signaling and contribute to structural genomic instability	This approach is well suited for the precise correction of pathogenic RB1 point mutations or other small sequence defects when efficient donor-template delivery can be achieved. It can also replace larger dysfunctional genomic regions	Clinical application is restricted by the inherently low HDR activity of terminally differentiated retinal cells, the technical difficulty of donor-template delivery, and the need to carefully evaluate DSB-associated genotoxicity	(214)
Base editing	Base editors combine a catalytically impaired or nickase Cas protein with a nucleotide deaminase, allowing direct base conversion without introducing canonical DSBs or requiring donor DNA. Cytosine base editors primarily mediate C→T substitutions, whereas adenine base editors catalyze A→G conversions	Editing efficiencies are typically moderate to high, although they remain highly dependent on genomic context and delivery strategy. In an RPE65 retinal disease model, adenine base editing achieved approximately 29% correction, accompanied by restoration of visual function, while optimized retinal studies have reported substantially higher efficiencies	By avoiding DSB formation, base editing markedly reduces the incidence of large deletions, chromosomal rearrangements, and other nuclease-associated genomic abnormalities. Nevertheless, bystander nucleotide conversions, Cas-mediated off-target editing, and deaminase-dependent DNA or RNA off-target effects remain important safety considerations	Base editing is particularly attractive for correcting RB1 single-nucleotide pathogenic variants compatible with CBE or ABE editing windows, including selected nonsense and missense mutations, thereby restoring endogenous gene function without requiring HDR	This technology cannot correct every type of mutation, particularly many insertions, deletions, or nucleotide substitutions outside compatible editing windows. PAM constraints and unintended bystander edits may further limit therapeutic applicability, while the biological consequences of RB1 restoration in established tumors remain uncertain	(215)
Prime editing	Prime editing employs a Cas9 nickase fused to a reverse transcriptase together with a prime editing guide RNA (pegRNA) to directly write the desired genetic sequence into the genome without generating canonical DSBs or requiring donor DNA. This platform can introduce all twelve possible base substitutions, as well as selected small insertions and deletions	Editing efficiency is generally moderate and highly variable across different loci and cell types. *In vivo* correction of an RPE65 mutation using a dual-AAV split-prime editor achieved approximately 16% editing efficiency without detectable off-target modifications at examined loci, whereas higher efficiencies have been reported under optimized experimental conditions	Prime editing provides broader editing capability with fewer indels and bystander mutations than conventional Cas9 or base editing. However, PE3 strategies involving dual nicking may increase indel formation, and unintended pegRNA-scaffold incorporation, along with infrequent off-target events, should still be carefully evaluated	Owing to its broad editi... versatility, prime editing represe... one of the most promi... platforms for correc... ing diver... RB1 mutations, including ... transversions and selec... small insertions or deletio... s that are beyond the capa... ities of current base editors	Clinical translation is challenged by the large molecular size of the editing machinery, which complicates ocular delivery and often necessitates dual-AAV or alternative delivery systems. Moreover, editing efficiency remains highly dependent on guide design, genomic locus, and cell type, and therapeutic correction of RB1 in retinoblastoma has not yet been demonstrated *in vivo*	(216)
CRISPR interference (CRISPRi)	CRISPRi utilizes catalytically inactive Cas9 (dCas9), typically fused to transcriptional repressors such as KRAB, to inhibit transcription without altering the genomic DNA sequence. Because no permanent genetic modification occurs, the effect is potentially reversible and can be modulated	Conventional editing efficiency is not applicable because genomic DNA remains unchanged. Instead, efficacy is measured by the degree of transcriptional repression, which depends on guide RNA positioning, chromatin accessibility, and the selected repressor domain	Since CRISPRi does ...t generate DSBs, ... avoids permanent ge... me alterations, including large deletio... and chromosomal rearrangements. Pote...tial con... may include unintended repressio... of non-target genes, prolonged expression o... bacterial Cas proteins, and immune responses associated wit... delivery	CRISPRi is not designed to repair defective RB1 alleles. Rather, its therapeutic potential lies in transient suppression of oncogenic pathways, synthetic-lethal targets, or downstream signaling cascades activated following RB1 loss. It also serves as a valuable platform for functional genomics and target validation	Long-term therapeutic benefit requires sustained expression of the repression machinery, while gene silencing may remain incomplete or reversible. Successful application also depends on identifying suitable disease-promoting targets and efficiently delivering the relatively large CRISPRi complex	(217)
CRISPR activation (CRISPRa)	CRISPRa employs dCas9 fused to transcriptional activation domains to enhance expression of endogenous genes without modifying their DNA sequence. The magnitude of activation can be adjusted through guide RNA selection and engineering of transcriptional activators	Conventional editing frequency is not applicable. Instead, efficacy is e...luated by the extent of endogenous ...e activation, which varies accordi... promoter acces...ibi... basal transcription... activity, ...ide positioning, and the ...ctivation domain ...ploye...	...se no DNA cleavage occurs, CRISPRa avoids DSB-associated genomic ...xicity. Nevertheless, excessive or ectopic gene activation, unintended transcriptional effects, prolonged Cas expression, and delivery-related immunogenicity remain important safety considerations	CRISPRa may provide a strategy for enhancing expression from a residual functional RB1 allele, thereby compensating for partial RB1 deficiency or stimulating additional tumor-suppressive pathways. However, it is unlikely to restore function when both RB1 alleles are deleted or produce nonfunctional proteins	Therapeutic application in retinoblastoma remains largely theoretical. Clinical success would require precise regulation of RB1 expression, preservation of a functional endogenous allele, and efficient delivery of relatively large dCas9-activator constructs into tumor cells	(218)

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The existing evidence reveals a distinct albeit partial transition from mechanistic modeling to therapeutic use. CRISPR-modified cell lines and induced pluripotent stem cells are manageable resources for investigating RB1 function and personalized repair, whereas retinal organoids more accurately reflect human retinal maturation, cellular diversity, and early tumorigenesis. Further insights into pharmacological reactions, *in vivo* safety, tumor progression, and administration may be acquired from research performed on animals and xenografts. However, most advanced therapeutic information on base editing, prime editing, and *in vivo* CRISPR delivery now comes from hereditary retinal degeneration rather than retinoblastoma. The translational implications of EDIT-101 are discussed in Section 8.5. Consequently, most CRISPR applications in RB remain in the preclinical, organoid, and mechanistic phases. Additional verification in mammalian eye models, patient-derived neoplasm models, and official safety assessments will be necessary before clinical use.

Challenges and limitations

The outer segments and the external limiting membrane of the densely packed photoreceptor layer physically limit the particles that may enter the retina through the subretinal channel (237). The majority of Cas9 molecules gathered on the surface of the neural retina following subretinal delivery of Cas9 ribonucleoproteins (RNPs), suggesting a restricted capacity to pass through the extracellular matrix and the densely packed outer segments of the outer nuclear layer (Figure 4)(237).To evaluate the limited activity of Cas9 RNPs within the neuronal retina, we examined distribution using 3D whole-eye imaging three days after injection. Analysis of the photoreceptor layer revealed that the main physical obstacles preventing RNP penetration are the stacked outer segment discs and the thick extracellular matrix of the external limiting membrane (237). Cas9 protein was nearly entirely broken down seven days after injection. The most common technique for gene editing in the neural retina is the use of adeno-associated virus (AAV)

Table 4. Maturity of evidence for CRISPR-based approaches relevant to retinoblastoma and ocular gene editing

Evidence category	Representative study	CRISPR strategy	Experimental model and disease context	Principal outcome	Translational maturity and relevance to RB	Reference
Cell-based study	Bassuk *et al*., 2016	Cas9-mediated HDR	Patient-derived induced pluripotent stem cells (iPSCs) carrying a pathogenic RPGR mutation associated with X-linked retinitis pigmentosa	CRISPR-mediated HDR successfully generated genetically corrected patient-derived iPSC clones, with approximately 13% repair efficiency observed among edited cells, thereby demonstrating accurate restoration of the mutant allele	Initial proof-of-concept. This study established the feasibility of personalized ex vivo genome correction in patient-derived ocular cells; however, it did not evaluate therapeutic efficacy in retinal tissue, animal models, or RB1-associated retinoblastoma	(214)
Cell-based study	Agrawal *et al*., 2024	CRISPR/Cas9-mediated knockout of RB1	Human control iPSCs genetically engineered to generate three independent RB1-null cell lines.	Stable RB1-deficient iPSC lines were established while preserving pluripotency and normal chromosomal architecture, providing a reproducible platform for investigating the biological consequences of RB1 deficiency during retinal differentiation	Mechanistic disease model. Although directly relevant to retinoblastoma biology, the study focused on disease modeling and did not evaluate tumor development, therapeutic genome correction, delivery strategies, or *in vivo* efficacy	(231)
Organoid study	Zheng *et al*., 2020	CRISPR/Cas9-mediated RB1 knockout	Human embryonic stem cell-derived retinal organoids with subsequent transplantation into immunodeficient mice	Loss of RB1 induced abnormal cell-cycle re-entry and increased apoptosis; however, neither retinal organoids nor transplanted tissues developed overt retinoblastoma, indicating that RB1 loss alone may not be sufficient for malignant transformation	Mechanistic evidence. The findings provide important insights into early RB pathogenesis while suggesting that additional oncogenic events are likely required for complete tumor development. The study did not investigate therapeutic genome editing	(59)
Organoid study	Liu *et al*., 2020	CRISPR/Cas9-mediated biallelic disruption of RB1	Human embryonic stem cell-derived retinal organoids carrying engineered RB1 mutations	RB1-deficient organoids developed tumor-like lesions exhibiting histopathological, transcriptomic, epigenetic, and molecular characteristics closely resembling human retinoblastoma	Advanced human disease model. This work established one of the most physiologically relevant *in vitro* models of retinoblastoma for studying tumor initiation and evaluating candidate therapies, although systemic pharmacology, immune interactions, and clinical validation remain absent	(148)
Organoid study	Kanber *et al*., 2022	CRISPR/Cas9-mediated inactivation of RB1	Human embryonic stem cell-derived retinal organoids lacking functional RB1	RB1 deficiency resulted in sustained proliferation of cone photoreceptor precursors together with impaired retinal maturation, supporting the hypothesis that cone lineage cells represent a principal origin of retinoblastoma	Human-relevant mechanistic evidence. The study strengthens the biological relevance of retinal organoids for investigating RB pathogenesis but remains limited to disease modeling rather than therapeutic intervention	(232)
Organoid study	Li *et al*., 2022	Patient-specific iPSC modeling incorporating acquisition of the second RB1 mutation	Retinal organoids generated from an individual carrying a heterozygous germline RB1 mutation	Acquisition of the second RB1 alteration triggered tumorigenic changes within retinal organoids, providing direct experimental validation of Knudson's two-hit hypothesis in a patient-specific human model	Personalized disease-model evidence. This platform closely reproduces hereditary retinoblastoma and may facilitate individualized therapeutic testing; however, genome-editing therapy and *in vivo* efficacy were not investigated	(233)
Animal disease model	Naert *et al*., 2016	Mosaic CRISPR/Cas9-mediated disruption of RB1 and RBL1	*Xenopus tropicalis* model	Individual disruption of either RB1 or RBL1 failed to induce tumors, whereas simultaneous inactivation of both genes resulted in rapid and highly penetrant retinoblastoma formation	*In vivo* mechanistic evidence. The study highlights the importance of cooperating RB-pathway alterations in tumorigenesis and provides a rapid platform for biological investigation, although species-specific differences limit direct clinical translation	(16)
Preclinical therapeutic study—RB-specific	Aubry *et al*., 2020	Pharmacological targeting of an RB1-associated synthetic lethal pathway	RB1-mutant/MYCN-amplified retinoblastoma cells together with human xenograft models treated with intravitreal MLN4924	MLN4924 significantly inhibited tumor growth *in vitro* and suppressed progression of multiple human retinoblastoma xenografts following intravitreal administration while demonstrating a measurable therapeutic window	Advanced preclinical therapeutic evidence. Unlike mechanistic studies, this investigation demonstrated *in vivo* efficacy, although treatment relied on pharmacological intervention rather than direct CRISPR-mediated correction of RB1	(234)
Preclinical therapeutic study—ocular proof-of-concept	Suh *et al*., 2021	Adenine base editing	Adult rd12 mouse model of RPE65-associated inherited retinal degeneration	*In vivo* adenine base editing corrected the pathogenic RPE65 mutation, restored retinal protein expression, and significantly improved retinal function and visual performance while generating substantially fewer indels than DSB-dependent genome editing approaches	Advanced ocular preclinical evidence. This work demonstrates the feasibility of DSB-free therapeutic editing within the retina, although it addresses inherited retinal degeneration rather than retinoblastoma or RB1 deficiency	(235)
Preclinical therapeutic study—ocular proof-of-concept	She *et al*., 2023	Dual-AAV split prime editing	rd12 mouse model carrying an RPE65 mutation	Prime editing achieved precise correction of the disease-causing mutation with editing efficiencies reaching approximately 16%, restored RPE65 expression, improved retinal and visual function, and showed no detectable off-target editing at examined genomic sites	Advanced ocular preclinical evidence. The study highlights the therapeutic potential of precise *in vivo* retinal prime editing, although challenges associated with large-vector delivery and the absence of RB1-targeted tumor studies currently limit its direct application to retinoblastoma	(216)
Clinical trial—ocular genome editing	Pierce *et al*., 2024 (BRILLIANCE/EDIT-101)	*In vivo* AAV-mediated CRISPR/Cas9 excision	Phase I/II clinical trial involving patients with CEP290-associated inherited retinal degeneration	Subretinal administration of EDIT-101 demonstrated an acceptable preliminary safety profile together with evidence of biological activity, and several participants experienced improvements in selected measures of visual function	Clinical proof-of-concept. This represents the most clinically advanced demonstration of ocular CRISPR therapy to date. However, it does not involve retinoblastoma, nor does it establish the safety or therapeutic efficacy of RB1 genome editing in ocular tumors. At present, no clinical CRISPR trial specifically targeting retinoblastoma has been reported	(236)

vectors that carry Cas9 and sgRNAs (238). By avoiding the size restrictions connected to AAV-mediated delivery, direct administration of RNPs offers a potential advantage. However, RNP-based retinal gene editing remains only about half as effective as AAV vectors (237). These findings highlight a major translational challenge for CRISPR-based therapy in retinoblastoma, as efficient delivery requires not only access to tumor cells but also sufficient editing activity within a heterogeneous and developmentally complex retinal environment (239). Therefore, delivery strategies that are effective in experimental models may not achieve comparable performance in pediatric patients (239).

Using non-viral vectors will likely be necessary to increase the effectiveness of Cas9 RNP delivery in the retina while lowering dose-related toxicity (Figure 4) (238).In this case, peptide-based carriers and cationic lipids such as Lipofectamine 2000 failed to improve editing efficiency and caused retinal toxicity, underscoring the need to create and refine other vector systems or conjugates for efficient transport into retinal cells (237). There was no decrease in

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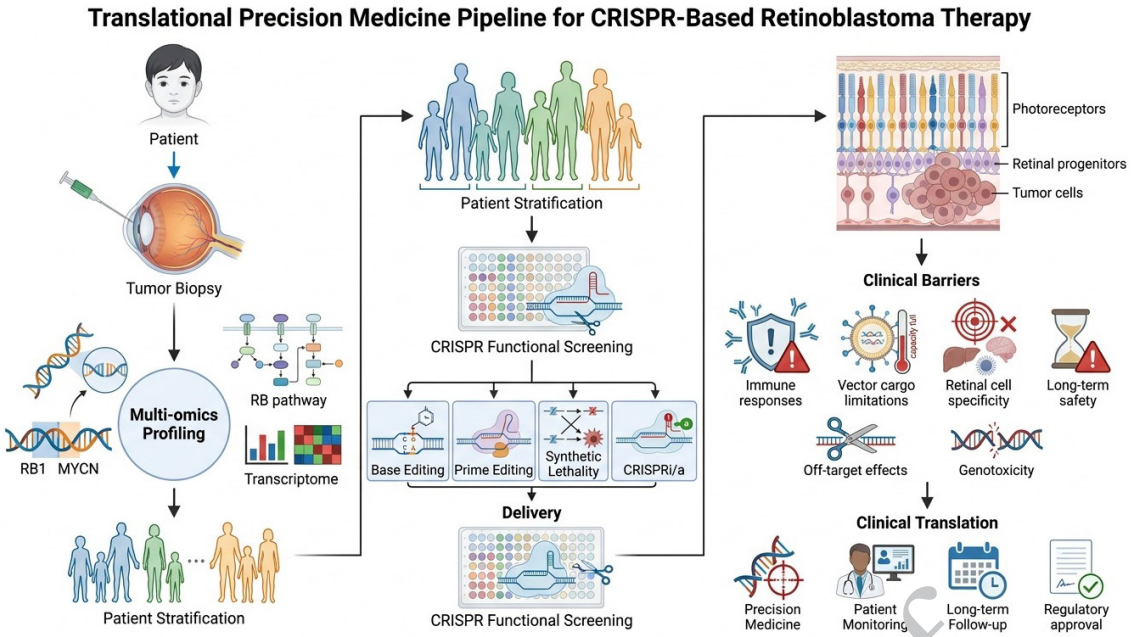

Figure 4. Translational pipeline for precision CRISPR-based therapy in retinoblastoma
Molecular profiling, including assessment of RB transcriptional corepressor 1 (RB1) status, MYCN proto-oncogene (MYCN) amplification, retinoblastoma protein (pRB) pathway activity, and genomic alterations, enables patient stratification and identification of subtype-specific therapeutic opportunities. Functional clustered regularly interspaced short palindromic repeats (CRISPR) screening defines genetic dependencies that guide the selection of precision genome-editing strategies, including base editing, prime editing, CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), and synthetic lethal targeting. Candidate therapies are subsequently optimized using retinal delivery platforms before clinical translation. The final stage highlights key translational challenges, including immune responses, vector cargo limitations, retinal cell specificity, off-target activity, and long-term safety, all of which must be addressed before routine clinical implementation

size variability or more consistent complexes after switching from lipid to peptide carriers. The results also highlighted how much more difficult it is to distribute mRNA encoding gene editing tools than reporter gene mRNA, with the former being noticeably more difficult (237). Since photoreceptors make initial contact with RNPs upon subretinal injection, gene editing was mostly seen in these cells inside the neural retina. These findings imply that the degree of target gene expression in each cell type influences editing effectiveness. To understand the causes of variation in editing results among retinal cell types *in vivo*, more research is needed (237).

Beyond RB1 loss, several genetic changes that interfere with different signaling pathways influence the course of retinoblastoma (240). Recent research has used a variety of omics techniques, such as microarray analysis, whole-genome sequencing, and RNA sequencing, in addition to RB1 and MYCN alterations, to discover important driver genes in both localized and metastatic retinoblastoma (240, 241). Research shows that retinoblastomas with MYCN amplification or without RB1 react well to targeted therapies, causing tumor cells to undergo cell cycle arrest, induce apoptosis, and inhibit SKP2 (240). The relationship between gene expression patterns and tumor phenotype has significant clinical relevance, informing prognostic, therapeutic, and diagnostic approaches (240). Identifying these biomarkers enables more individualized, accurate treatment plans, which can improve patient outcomes. The development of multi-omics tools, including proteomics and next-generation sequencing, to decipher the molecular landscape of retinoblastoma has therefore produced a robust collection of possible targets for precision therapeutics (Figure 4)(240). However, discovering molecular targets using multi-omics techniques does not always imply immediate therapeutic relevance. Before these options can be deemed appropriate for CRISPR-based intervention, functional validation, target-dependence assessment, and investigation of possible toxicity in retinal-specific models are necessary (242).

CRISPR-based therapy ethics emphasize the need for informed consent, equitable access to the technology, and responsible usage to prevent unforeseen consequences, such as the possible production of "designer" progeny (243). Potential immunological responses to viral delivery vectors and the creation of novel oncogenic mutations are additional safety concerns with CRISPR-mediated cancer treatment (Figure 4)(243, 244). These concerns are particularly relevant for retinoblastoma because most patients are young children with long life expectancy after treatment (245). Therefore, even rare genomic alterations or persistent immune complications may have significant long-term consequences, emphasizing the need for extended safety monitoring (246).

To overcome these obstacles, gene-editing tools must be continuously optimized alongside rigorous review and validation processes (247). To ensure that base editors and prime editors are safe for therapeutic use, extensive preclinical research is essential (243). The massive cell volumes required for clinical usage cannot be produced using current CRISPR-edited cell production techniques, highlighting the need for additional efficiency and safety improvements (243, 248, 249).

Next-generation genome editing: Base and prime editors

Phenylalanine levels can be safely and permanently normalized with a single therapy using gene-editing platforms, particularly base editors (BEs) and prime editors (PEs)(258). Base editors can effectively fix disease-causing mutations in the livers of PKU mouse models, according to a preclinical study (258, 259). Prime editing has also

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been swiftly used to treat PKU, leveraging its technological advantages, with encouraging outcomes that highlight its therapeutic promise (258). In mouse models, base and prime editing technologies have shown promise as effective therapeutic approaches for Duchenne muscular dystrophy (DMD) by accurately correcting mutations in the DMD gene, restoring dystrophin expression, and improving associated phenotypes (259). The results provide the groundwork for future clinical translation, even though the adeno-associated virus (AAV) dosages used in these investigations were higher than what would likely be required in human patients. The c.8713C>T point mutation in the DMD gene has been effectively fixed by an additional study employing prime editing, with patient-derived muscle cells showing an editing effectiveness of about 22% (260). CRISPR-based systems' high efficiency and programmability allow for quick adaptation to a variety of applications, such as *in vitro* cell engineering and *in vivo* gene repair prior to cell therapy. Extensive use of CRISPR-Cas9 in clinical studies has established a solid basis for developing base and prime editors (258, 261).

Combination therapies: CRISPR and targeted agents

When paired with the antisense oligonucleotide nusinersen, which controls SMN2 splicing, this approach demonstrated synergistic effects that improved treatment results (262, 263). The treatment of cancer has been significantly impacted by advancements in precision and personalized therapies, particularly in hematologic malignancies where targeted immunotherapies, such as chimeric antigen receptor (CAR) T cells, antibody-drug conjugates (ADCs), and bispecific T-cell engagers (BTEs), have shown notable clinical success (258, 264-266). Wellhausen and associates created a unique CAR T-cell platform that integrates adenine base editors (ABEs) to enable an "epitope editing" technique to overcome these restrictions. This alteration enables anti-CD45 CAR T cells to effectively target blood cancer cells that express unchanged CD45, such as patient-derived acute myeloid leukemia, B-cell lymphoma, and acute T-cell leukemia, while avoiding detection of edited hematopoietic cells (258). Epitope-edited stem cells may engraft, survive over time, and differentiate, promoting the development of functional blood lineages, in contrast to hematopoietic stem cells that have undergone total CD45 deletion (266). In a separate work, surface epitopes on transplanted hematopoietic stem and progenitor cells were altered via base editing, which prevented CAR T-cells from recognizing healthy cells while preserving the malignant cells' susceptibility to CAR T-mediated cytotoxicity (258, 267).

Patient-derived models for preclinical testing

Topotecan and TW-37 therapy significantly decreased the percentage of proliferative cone precursors in Rb organoids, highlighting the usefulness of these *in vitro* models for evaluating novel Rb therapies (268, 269). Researchers can monitor tumor progression, record complex genetic and epigenetic traits, track tumor cell sources, and assess potential treatment agents by using gene-editing methods on pluripotent stem cells and directing their differentiation into Rb organoids (268, 270). Single-cell RNA sequencing showed that retinal progenitor cells are the major population in RB-derived organoids. Additionally, transcriptome analysis revealed no variations in the cell type composition between patient-derived xenografts and RB organoids (268, 271).

Furthermore, Knudson's "two-hit" theory was empirically supported by the development of Rb tumors in retinal organoids due to second-hit mutations in iPSCs containing heterozygous patient-derived RB1 mutations.

Near-term versus long-term clinical translation of CRISPR-based strategies for retinoblastoma

Although multiple CRISPR-based technologies have potential applications in retinoblastoma, their translational maturity differs substantially. Prioritization for clinical development should consider not only editing precision but also the availability of disease-specific efficacy data, compatibility with heterogeneous RB1 mutations, ability to reach a sufficient proportion of tumor cells, durability of the therapeutic effect, ocular delivery feasibility, and the risk of irreversible genomic injury. Based on these criteria, the most realistic strategies for the next 5–10 years are unlikely to involve universal restoration of RB1 in all tumor cells. Instead, near-term translation will probably be led by CRISPR-enabled identification and validation of druggable vulnerabilities, followed by mutation-selective base or prime editing only after efficacy and safety have been demonstrated in clinically relevant retinoblastoma models. Table 5 summarizes the proposed translational prioritization of these approaches.

Taken together, the most realistic near-term clinical application of CRISPR in retinoblastoma is likely to be CRISPR-enabled target discovery and synthetic-lethal drug development rather than direct genome editing in patients, because this strategy is supported by retinoblastoma-specific cell-line and xenograft evidence and does not require modification of every tumor cell. Among direct editing approaches, mutation-selective base editing currently appears more clinically advanced than prime editing owing to stronger retinal efficacy and delivery data, although both still require demonstration of effective RB1 restoration and tumor control in retinoblastoma models. In contrast, Cas9/HDR-mediated correction, CRISPRi, CRISPRa, Cas13, and multiplex genome or epigenome editing remain less mature because of inefficient or complex delivery, genotoxicity, limited disease-specific evidence, and regulatory challenges. Although EDIT-101 supports the feasibility of delivering CRISPR components to the human retina, successful translation to retinoblastoma will require tumor-selective delivery, preservation of surrounding retinal tissue, and effective treatment of genetically heterogeneous tumors (Table 6).

Accordingly, a rational translational roadmap for the next decade should prioritize three sequential objectives: first, validating CRISPR-identified vulnerabilities in patient-derived retinoblastoma organoids and independent *in vivo* models; second, optimizing tumor-selective and transient ocular delivery systems; and third, evaluating base or prime editing only in molecularly selected RB1-mutant subgroups for which restoration of functional pRB is biologically and technically achievable. Progression toward clinical testing should require evidence of durable tumor control, preservation of surrounding retinal function, absence of clinically relevant structural genomic alterations, and a clear advantage over existing local and systemic treatments (Table 6).

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Table 5. Retina-specific challenges and emerging solutions for CRISPR-based gene editing in retinoblastoma

Challenge Category	Retina-Specific Limitation	Biological / Technical Basis	Optimization Strategy	Emerging Technologies	Clinical Impact	Ref
Off-target editing	Unintended editing of retinal neurons or adjacent ocular tissues may impair vision	High sequence similarity among genomic loci and variable chromatin accessibility in retinal cells	High-fidelity Cas9 variants, optimized sgRNA design, multi-platform off-target validation in retinal cells	SpCas9-HF1, eSpCas9, CRISPRme, ONE-seq, GUIDE-seq	Improves retinal safety and minimizes irreversible visual toxicity	(250)
Retinal delivery efficiency	Efficient delivery to intraocular tumor cells remains challenging because of retinal architecture and internal limiting membrane barriers	Limited penetration of viral and non-viral vectors; restricted vector tropism	Intravitreal/subretinal delivery optimization, engineered AAV capsids, lipid nanoparticles	AAV2.7m8, Anc80L65, SORT-LNPs, virus-like particles	Enhances editing efficiency while reducing systemic exposure	(251)
Vector cargo limitations	Large CRISPR editors (SpCas9, Prime editors) exceed AAV packaging capacity	AAV cargo limit (~4.7 kb).	Dual-AAV systems, compact Cas orthologs, split-intein systems, non-viral nanoparticles	SaCas9, CasMINI, Dual-AAV, LNP-mediated RNP delivery	Enables delivery of advanced editing systems to retinal tissue	(252)
Retinal cell specificity	Therapeutic editing must selectively target retinoblastoma cells while preserving healthy retinal neurons	Cellular diversity of the retina and overlapping receptor expression	Cell-specific promoters, tumor-selective regulatory elements, targeted nanoparticles	Cone- or retinal-specific promoters, ligand-directed nanoparticles	Improves therapeutic precision and preserves visual function	(251)
DNA damage response	Double-strand breaks may activate p53 signaling, reducing editing efficiency and increasing toxicity in retinal cells	DSB-induced activation of DNA damage pathways	Base editing, prime editing, transient modulation of DDR pathways	Prime editing, base editing, Cas9 nickase systems	Reduces genotoxicity and improves long-term retinal safety	(253, 254)
Immune responses	Ocular immune privilege does not completely prevent immune responses against Cas proteins or viral vectors	Pre-existing immunity to bacterial Cas proteins and adaptive immune responses to AAV vectors	Immune-orthogonal Cas proteins, transient expression, non-viral delivery, transient immunosuppression	Anti-CRISPR systems, LNP delivery, self-limiting CRISPR expression systems	Improves safety and enables repeat intraocular administration	(255, 256)
Long-term safety and durability	Persistent editing outcomes and delayed ocular adverse effects remain insufficiently characterized	Permanent genomic modification and prolonged vector persistence	Long-term ophthalmic follow-up, genomic surveillance, optimized editing windows	Single-cell sequencing, long-read sequencing, retinal imaging biomarkers	Essential for regulatory approval and clinical translation	(257)

Table 6. Prioritization of CRISPR-based strategies for clinical translation in retinoblastoma over the next 5-10 years

CRISPR-based strategy	Current supporting evidence	Principal translational advantage	Critical barriers to clinical testing in retinoblastoma	Plausible status within the next 5-10 years	Translational priority	Ref
CRISPR-enabled target discovery and synthetic-lethal therapy	CRISPR and functional-genomic screens have identified dependencies associated with RB1 loss, including mitotic regulators, SKP2-related signaling, and DNA-repair factors such as RAD51 and BRCA1. Pharmacological inhibition of the SKP2-associated neddylation pathway with MLN4924 has produced antitumor effects in retinoblastoma cell and xenograft models, including following intravitreal administration	Does not require correction of RB1 in every tumor cell. Identified vulnerabilities can be targeted with small molecules or locally administered agents whose manufacture, dosing, and pharmacology are generally more tractable than *in vivo* genome editing. It also allows genotype-guided treatment of RB1-deficient or MYCN-driven subgroups	CRISPR identifies the target but is not necessarily the administered therapeutic product. Candidate dependencies may be model-specific, and efficacy must be reproduced in patient-derived organoids, independent xenografts, and appropriate mammalian ocular models. Ocular and systemic toxicity of the final drug must also be defined	Most clinically near-term application. CRISPR-prioritized drugs or drug combinations could plausibly enter early clinical evaluation within 5–10 years, provided that robust disease-specific validation and an acceptable therapeutic window are demonstrated	High	(272)
Mutation-selective RB1 base editing	Base editing has restored gene function and visual phenotypes in multiple inherited retinal disease models without canonical DSB formation. Optimized ocular base-editing systems have achieved substantial correction in mouse retina, and more recent studies have shown high editing levels in nonhuman primate retina and human retinal tissues. However, direct therapeutic correction of RB1 in retinoblastoma models has not yet been established	Avoids canonical DSBs and donor DNA, thereby reducing DSB-associated large deletions and chromosomal rearrangements. It may efficiently correct selected RB1 transition mutations, including some nonsense or missense variants, while preserving endogenous gene regulation	Only a subset of RB1 variants is compatible with current base-conversion rules. Bystander editing, PAM restrictions, and deaminase-associated off-target effects remain concerns. More importantly, heterogeneous delivery throughout the tumor and correction of a sufficient proportion of malignant cells have not been demonstrated. Restoring RB1 in established tumors may also be insufficient if secondary oncogenic alterations have become dominant	Plausible medium-term candidate, particularly for carefully selected recurrent or hereditary RB1 variants. Entry into clinical testing within 5–10 years is conceivable but conditional on successful tumor-control studies in RB1-mutant organoids and mammalian retinoblastoma models	Moderate–high	(215, 273)
Mutation-selective RB1 prime editing	Dual-AAV prime editing has corrected pathogenic retinal mutations *in vivo* and improved retinal function in mouse models. Compared with base editing, prime editing can install all classes of base substitutions and selected small insertions or deletions, making it theoretically applicable to a wider range of RB1 variants. Disease-specific therapeutic evidence in retinoblastoma is not yet available	Provides the broadest mutation-correction scope without requiring a canonical PAM or an external donor template. It may address RB1 transversions, small insertions, deletions, and variants unsuitable for current base editors, with fewer bystander edits	Prime editors are large and frequently require split or dual-vector delivery. Efficiency varies by locus, guide design, and cell state. Reconstitution of split editors, intratumoral distribution, and manufacturing complexity remain substantial obstacles Long-term safety in developing pediatric ocular tissues also requires evaluation	Promising but less mature than base editing. Selected applications may approach late preclinical development in the next 5–10 years, but clinical testing for retinoblastoma is likely to depend on major advances in compact editors and tumor-selective delivery	Moderate	(216, 254)
Localized delivery of transient CRISPR components using nonviral nanoparticles or virus-like particles	AAV-mediated CRISPR delivery has already reached clinical evaluation in inherited retinal disease, demonstrating that genome-editing components can be administered locally to the human retina. Nonviral nanoparticles and engineered virus-like particles are being developed to transiently deliver editor mRNA or ribonucleoproteins	Local ocular administration may limit systemic exposure. Transient systems reduce prolonged nuclease expression, vector integration concerns, and potentially immunogenic exposure. Nanoparticles may also offer greater cargo flexibility than AAV and could be engineered for tumor-selective delivery	Retinoblastoma is a heterogeneous three-dimensional tumor rather than a uniform retinal cell layer. Achieving homogeneous penetration, tumor-cell selectivity, and sufficient intracellular delivery without damaging the surrounding retina remains unresolved. Clinical ocular delivery experience from inherited retinal degeneration cannot be assumed to apply directly to an intraocular malignancy	A critical enabling technology rather than a stand-alone treatment. Local transient delivery could reach advanced preclinical testing within 5-10 years and may determine whether base or prime editing becomes clinically feasible for RB	Moderate–high as an enabling platform	(236)
Direct Cas9/HDR-mediated restoration of RB1	HDR has corrected pathogenic variants in patient-derived ocular iPSCs, providing proof of principle for precise sequence restoration. Nevertheless, HDR is intrinsically inefficient in quiescent or post-mitotic retinal cells, and there is no convincing *in vivo* evidence of therapeutic RB1 correction and tumor control	In principle, HDR can restore the exact endogenous RB1 sequence and may correct variants or sequence regions beyond the scope of current base editors	Requires a DSB and donor template; competes with error-prone NHEJ; and can generate indels, large deletions, rearrangements, and p53-mediated DNA-damage responses. Low HDR activity, donor delivery, and the need to edit a large proportion of heterogeneous tumor cells markedly limit feasibility	Unlikely to become a leading *in vivo* RB strategy within 5–10 years. It may remain useful for ex vivo generation or correction of research models, but direct clinical application to ocular tumors is a longer-term prospect	Low	(214, 274)
CRISPRi-mediated suppression of cooperating oncogenes	CRISPRi can reversibly suppress endogenous transcription without changing DNA sequence and is widely used for functional screening. In RB, potential applications include inhibition of MYCN-associated programs, mitotic dependencies, or downstream pathways activated after RB1 loss. Direct therapeutic efficacy in retinoblastoma has not been demonstrated	Avoids DSB-associated genotoxicity and permits tunable, potentially reversible suppression. It may be more appropriate than RB1 correction when tumor maintenance depends on an active oncogene or synthetic-lethal pathway	Durable benefit requires sustained and sufficiently homogeneous expression of large dCas9-repressor complexes. Incomplete repression, off-target transcriptional effects, delivery limitations, and the possibility of tumor escape remain important concerns	Primarily a research and target-validation platform in the next 5–10 years. Therapeutic testing is possible only after a strongly validated, tumor-specific transcriptional dependency and a practical delivery system are established	Low–moderate	(217)

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Continued Table 6.

CRISPRa-mediated activation of residual RB1 or compensatory tumor-suppressor pathways	CRISPRa can activate endogenous promoters and has rescued haploinsufficiency phenotypes in non-retinoblastoma experimental models. Its application to restoration of RB1 expression in retinoblastoma remains conceptual	Does not produce a DSB and preserves the endogenous genomic sequence. It could potentially increase expression from a remaining functional RB1 allele or activate compensatory tumor-suppressive programs	Most retinoblastomas contain biallelic RB1 inactivation; therefore, increasing transcription may not restore a functional protein when both alleles are deleted, truncated, or otherwise nonfunctional. Large dCas9 activators, sustained delivery, and excessive or ectopic activation create additional challenges	Long-term and mutation-context-dependent. It is unlikely to become a broadly applicable clinical strategy for RB in the next 5-10 years	Low	(218)
Cas13-mediated RNA targeting	Cas13 platforms can transiently degrade or edit selected RNA molecules and have shown emerging potential in experimental retinal and other disease models. Disease-specific efficacy against RB1-associated retinoblastoma has not been established	Does not permanently modify genomic DNA. It may permit reversible targeting of oncogenic transcripts, fusion transcripts, or mutation-specific RNA and could be discontinued if toxicity develops	Therapeutic effects are temporary and may require repeated administration. Efficient delivery, transcript selectivity, collateral RNA activity, durability, and tumor escape must be addressed. No validated Cas13 target or therapeutic delivery system currently exists for retinoblastoma	Exploratory or long-term strategy. Clinical evaluation for RB within 5–10 years appears less likely than CRISPR-informed pharmacological treatment or DNA base editing	Low	(275, 276)
Multiplex genome or epigenome editing	Multiplex CRISPR systems can simultaneously modify several genomic or regulatory targets in experimental models, potentially addressing RB1 loss together with cooperating events such as MYCN or RB-family pathway alterations. Evidence in retinoblastoma remains predominantly conceptual or mechanistic	Could theoretically address tumor heterogeneity, cooperating oncogenic alterations and acquired resistance more effectively than single-target editing	Multiplexing increases cargo size, delivery complexity, off-target burden, genomic rearrangement risk, manufacturing difficulty, and regulatory uncertainty. Predicting the combined biological effects of permanently modifying several pathways is especially difficult in pediatric patients	Currently speculative and unlikely to enter RB clinical testing within 5–10 years, except possibly as an ex vivo research or screening method	Very low	(277)

Conclusion

CRISPR-based technologies have fundamentally altered retinoblastoma research by providing a versatile framework for disease modeling, functional genomics, therapeutic target discovery, and precision genome editing. CRISPR platforms are more than gene-editing tools; they have greatly increased our understanding of RB1 biology, revealed context-dependent oncogenic pathways, and enabled the systematic identification of molecular vulnerabilities that may guide customized therapy. All things considered, the material now available suggests that CRISPR's greatest contributions to retinoblastoma are the development of physiologically relevant experimental models and the identification of actionable therapeutic dependencies, rather than direct clinical gene repair. Despite these advancements, many important knowledge gaps remain. The molecular mechanisms leading from RB1 loss to completely malignant transformation, including the involvement of developmental stage, retinal cell of origin, epigenetic remodeling, and cooperative genetic alterations, are still poorly understood. Furthermore, the biological significance of several potential synthetic lethal interactions discovered by genome-wide CRISPR screens has not yet been demonstrated in clinically relevant patient-derived systems. Similarly, the predictive value of retinal organoids and engineered cell models for human treatment responses is yet uncertain since these platforms do not accurately mimic the tumor microenvironment, immunological interactions, vascularization, and intratumoral heterogeneity seen in patients. Efficiency in editing is no longer the sole significant barrier to translation. A number of major challenges must be addressed before therapeutic genome editing is considered clinically feasible. These challenges include safe and consistent delivery to intraocular tumor cells, minimization of extensive and off-target genomic alterations, long-term genomic stability, immunological compatibility, and standardized evaluation of editing outcomes. Crucially, editing technological breakthroughs by themselves are unlikely to overcome these limitations without parallel improvements in ocular delivery methods, safety assessment frameworks, and clinically significant experimental models. The most practicable short-term therapeutic impact on retinoblastoma is expected to come from employing CRISPR as a discovery platform rather than as a curative genome-editing therapy, based on the existing evidence. CRISPR-guided functional genomic screening, synthetic lethality mapping, and patient-derived retinal organoids are anticipated to expedite the identification of druggable molecular vulnerabilities that might be incorporated into precision oncology approaches. However, direct base or prime editing correction of RB1 mutations should now be seen as a longer-term objective requiring substantial preclinical validation. Future research should thus prioritize integrating patient-derived organoids, single-cell multi-omics, longitudinal lineage monitoring, high-fidelity editing techniques, and therapeutically relevant nonviral delivery platforms into standardized experimental workflows. Prospective evaluation of possible therapeutic targets across genetically diverse retinoblastoma animals will be equally important to distinguish universal causes from context-dependent alterations. Such an integrated strategy will be essential to bridge the current gap between molecular discoveries and clinically relevant precision medicine. CRISPR in retinoblastoma will unlikely depend on a single genome-editing method. Instead, substantial clinical translation will need precise genetic editing in combination with functional genomics, disease modeling, rational target selection, and safe delivery techniques. Establishing this thorough translational framework is the most practical way to develop long-lasting, genotype-guided medicines that might improve the prognosis of retinoblastoma patients.

Corrected Proof

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Authors' Contributions

NSS S conceived the study, designed the review framework, supervised the manuscript preparation, and served as the corresponding author. All authors contributed to the literature search, data interpretation, manuscript drafting, critical revision of the intellectual content, and approved the final version of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the authorship, preparation, or publication of this article.

Declaration

The authors acknowledge using ChatGPT (OpenAI) to assist with writing and editing portions of the manuscript, including language refinement and grammatical corrections. The authors critically reviewed, verified, and revised all AI-assisted content. The authors take full responsibility for the manuscript's scientific accuracy, integrity, originality, and final content. The AI tool was not listed as an author and was not used to make autonomous scientific or clinical decisions.

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Corrected Proof