# Genome maintenance in retinoblastoma: Implications for therapeutic vulnerabilities (Review)

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**Abstract.** Retinoblastoma (RB) is a pediatric ocular malignancy that is initiated mostly by biallelic inactivation of the RB transcriptional corepressor 1 (*RB1*) tumor suppressor gene in the developing retina. Unlike the prevailing prediction based on multiple studies involving *RB1* gene disruption in experimental models, human RB tumors have been demonstrated to possess a relatively stable genome, characterized by a low mutation rate and a few recurrent chromosomal alterations related to somatic copy number changes. This suggests that RB may harbor heightened genome maintenance mechanisms to counteract or compensate for the risk of massive genome instability, which can potentially be driven by the early *RB1* loss as a tumor-initiating event. Although the genome maintenance mechanisms might have been evolved to promote RB cell survival by preventing lethal genomic defects, emerging evidence suggests that the dependency of RB cells on these mechanisms also exposes their unique vulnerability to chemotherapy, particularly when the genome maintenance machineries are tumor cell-specific. This review summarizes the genome maintenance mechanisms identified in RB, including findings on the roles of chromatin regulators in DNA damage response/repair and protein factors involved in maintaining chromosome stability and promoting survival in RB. In addition, advantages and challenges for exploiting these therapeutic vulnerabilities in RB are discussed.

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## 1. Introduction

Retinoblastoma (RB) is an intraocular malignancy occurring in young children. For the vast majority of cases, RB develops as a result of biallelic inactivation of the RB transcriptional corepressor 1 (*RB1*) gene in the developing retina, followed by genetic and epigenetic alterations during tumor progression (1-3). Since *RB1* mutations are required for RB initiation and are also frequently found in other human cancers, especially during cancer progression, extensive research efforts have been made to elucidate the functions of RB protein (pRB) in tumor suppression for the past decades. This has unraveled the multifaceted roles of pRB in a wide variety of cellular events, ranging from canonical cell cycle regulation at local gene promoters to organization of higher-order chromatin structures and chromosomes (4-7). Furthermore, a deeper understanding of pRB functions in the context of cancers has enabled envisioning of novel therapeutic strategies for cancers with *RB1* loss, which frequently develop therapy resistance by varied mechanisms (8,9).

Genomic instability is a characteristic of most human malignancies and the impact of genome maintenance mechanisms on neoplastic transformation and subsequent development of cancer has been well established (10). Notably, most of the chromatin-associated functions exerted by pRB at diverse genomic locations bear important relevance to the maintenance of genomic stability (5,11). Functional inactivation or gene disruption experimental models have revealed at least three major mechanisms by which pRB participates in genome maintenance: First, pRB is recruited to DNA double-strand breaks (DSBs) and directly promotes DNA repair (12,13). A study reported that pRB interacts with repair factors X-ray repair cross complementing (XRCC)5 and XRCC6 at DSBs to facilitate canonical nonhomologous end-joining (C-NHEJ) repair, while another study demonstrated that pRB recruits BRM/SWI2-related gene 1 (BRG1) chromatin remodeler to alter the chromatin structure and stimulate DNA end resection for homologous recombination (HR) repair (12,13). Notably,

E2 factor (E2F) transcription factor 1 (E2F1) has been demonstrated to be required for the recruitment of pRB and BRG1 to DNA breaks for HR repair, which suggests a transcription-independent function of E2F1 in DNA repair (13,14). Second, pRB ensures the fidelity of DNA replication and chromosome segregation (15-20). Inactivation of RB family proteins by human papilloma virus oncoprotein E7 causes the stalling of replication forks prevalently at repetitive regions of the genome and results in DSBs (15). Furthermore, pRB has been reported to be essential for recruitment of condensin II and cohesin, which are involved in structural maintenance of chromatin during DNA replication and mitosis, and pRB loss-driven defects in the recruitment of such factors results in aberrant replication followed by chromosome segregation errors, which can directly contribute to aneuploidy and facilitate tumor development (16-20). Third, pRB serves critical roles in silencing repetitive sequences across the genome and maintaining heterochromatin by recruiting repressive histone modifiers such as enhancer of zeste 2 polycomb repressive complex 2 subunit, suppressor of variegation 4-20 and histone deacetylase (HDAC) complexes for stable maintenance of the genomic regions via deposition of distinct histone modification marks (21-24). This illustrates that the roles of pRB in genome maintenance are further extended to the maintenance of epigenetic stability in genomes. As aforementioned for HR repair, some of these genome maintenance functions exerted by pRB have been demonstrated to be dependent on the presence of E2F1 at the sites in a sequence-independent manner (19,21). Thus, these findings further support the notion that E2F1 may actively participate in genome-wide functions of pRB in chromatin regulation, independently of its canonical roles in cell cycle control and transcription.

As pRB protects against genomic instability, and functional pRB is deficient following initiation of RB due to biallelic inactivation of *RB1*, the present review begins with an overview of RB genomic analysis results to understand the relationship between the intrinsic *RB1* loss and the status of genome stability observed in primary RB tumors. Subsequently, previous findings on genome maintenance mechanisms in RB are presented, revealing the possibility of novel therapeutic opportunities. Finally, advantages and challenges for exploiting the newly identified therapeutic vulnerabilities in RB are discussed.

## 2. Genomic attributes of RB

In contrast to the widespread roles of pRB in genome maintenance as demonstrated by *RB1* gene mutation and pRB depletion in the aforementioned experimental models, whole-genome sequencing (WGS) of human RB tumors has revealed that RB genomes are relatively stable compared with those of other cancer types (25). Although only four RB specimens were used for the WGS analysis, the study also demonstrated that, despite multiple passaging over a prolonged time, orthotopic xenografts of the same human RB displayed only a modest increase in passenger mutations without gross defects in chromosome stability, suggesting that human RB genomes are maintained stably *in vivo* and massive genome instability may not be a strong driver for RB progression. Subsequent genomic analyses in larger RB cohorts have employed WGS, exome sequencing and targeted next-generation sequencing (NGS) (26-31). These studies consistently identified driver mutations in MYCN proto-oncogene, bHLH transcription factor (*MYCN*) and BCL6 corepressor (*BCOR*), and verified recurrent copy number alterations on chromosomes 1q, 2p, 6p and 16q that had also been identified by previous cytogenetic analyses (3,32). Notably, two molecular subtypes of human RB tumors identified by a recent multi-omics approach were also associated with these genomic characteristics, represented by subtype 1 harboring few genetic alterations other than *RB1* mutations and subtype 2 presenting *MYCN* amplification or recurrent 1q gain and/or 16q loss (33). In addition to these known genomic changes, the targeted NGS approach on cancer-related gene panels led to the identification of several genetic alterations beyond *RB1* inactivation. Although these additional gene mutations were found to occur at low frequency except for *BCOR* (14-23%), the presence of the non-*RB1* alterations was demonstrated to be associated with aggressive histopathologic features and poor prognosis when combined with the corresponding clinicopathological data (29,30). Given the limited cohort size in most studies, further investigations are required to verify the clinical significance of the non-*RB1* mutations as biomarkers for prognosis. Of particular relevance, if the RB subtypes identified by the recent multi-omics approach and their close association with the known genomic attributes can be validated in larger cohorts, this may impact the therapeutic decision-making process through subtype-based patient stratification, since subtype 2 tumors have been demonstrated to possess stemness features and a higher predilection for metastasis (33).

Since targeted NGS studies interrogate specific genes and select genomic regions, assessment of overall genome stability in the RB specimens may be limited (29,30). A recent WGS study on 21 RB samples has revealed that the overall mutation burden is consistently low in these tumors, as evidenced by the average count of 275 substitutions at a frequency of 0.085 per Mb, 70 small insertions/deletions with a frequency of 0.021 per Mb and 17 structural rearrangements at a frequency of 0.005 per Mb (27). This low somatic mutational burden in RB was also demonstrated by exome sequencing of 71 RB samples, suggesting that RB is among the least mutated cancer types (28). Notably, this genomic feature appears to be common in a number of pediatric neoplasms as a pan-cancer genomic analysis of 24 types of childhood cancer has demonstrated that overall somatic mutation frequencies of pediatric cancers are markedly lower than those of adult cancers (34). The low mutational burden in pediatric malignancies may be related to the age at diagnosis or tumor resection as somatic mutations tend to accumulate with age by DNA replication errors and environmental factors throughout life (35). Indeed, albeit being low in terms of overall mutation frequency, the total number of substitution mutations in RB exhibited a positive association with the age of enucleation, indicative of the absence of specific mutational mechanisms other than cell division-related mutational processes (27). In agreement with this interpretation, another recent study reported that variability in RB genomic alterations is associated with patient age at diagnosis but not with the possession of germline *RB1* mutations (36). Not only for single nucleotide variants but also for somatic copy number alterations (SCNAs), RB genomes

have been revealed to harbor relatively few SCNAs compared with other cancer types (28). Considering that most genomic analyses have been carried out with advanced tumors from enucleated eyes, the observation of low mutation burden and fewer SCNAs in these specimens suggests that RB genomes are relatively stable and there may be genome maintenance mechanisms operating in RB to counteract or compensate for the risk of *RB1* deficiency-driven genomic instability.

The next section presents an overview of mechanisms and factors contributing to genome maintenance in RB, which include the gene signature associated with the DNA repair/DNA damage pathway in primary RB tumors, emerging roles of chromatin regulators in DNA damage response/repair, and protein factors which have been proposed to be important for maintaining chromosome stability and promoting survival in RB.

## 3. Genome maintenance mechanisms in RB

*Upregulation of genes involved in DNA damage response and repair in RB*. Unlike the majority of human cancers where *RB1* mutations occur during cancer progression for acquisition of more malignant phenotypes and therapy resistance (4,5), RB is initiated by biallelic inactivation of the *RB1* gene (1-3). The timing of *RB1* loss during tumor development has been proposed as an important consideration for understanding the varied roles of pRB in non-canonical pathways of chromatin and genome regulation, which highlights the pRB loss later in disease progression as a driving force for genomic instability and therapy resistance (5). Given the causal roles of pRB loss in genome instability (11) and relatively stable genomes observed in primary RB tumors (25,27), it is hypothesized that RB may harbor mechanisms to protect and maintain genome stability from the beginning of tumorigenesis, in addition to the mechanisms to tolerate any potentially deleterious genomic alterations driven by *RB1* deficiency. Since the *TP53* gene is intact and the p53 signaling pathway is functional in RB tumors (37,38), these genome maintenance mechanisms would be crucial for tumor survival particularly in early stages of tumorigenesis. High MDM2 expression in cone precursors, the cellular origin of human RB, would be instrumental for evasion of p53-mediated tumor surveillance in early stages of tumor development (39,40). MDM4 expression during tumor progression may also serve a critical role in suppression of p53-mediated apoptosis (41); however, active genome maintenance mechanisms may still be required to restrain *RB1* loss-related genomic alterations. In support of the notion, a number of gene expression profiling studies have demonstrated that genes involved in DNA damage response and various DNA repair pathways constitute a highly conserved gene signature in primary RB tumors, in addition to the well-known proliferation-related signatures (42-45) (Table I). The enhanced expression of DNA repair genes in RB may account for the low somatic mutation burden observed in primary tumors (25,27), indicating that these genes are functional to counteract the risk of *RB1* deficiency-driven genomic instability. Consistent with the aforementioned interpretation, a recent *in vivo* RNA interference (RNAi) screen study in two orthotopic RB xenograft models ($RB1^{null}$ and *MYCN*-amplified $RB1^{wt}$; $MYCN^{amp}$) has identified *BRCA1* and RAD51 recombinase (*RAD51*) as indispensable genes for RB cell survival among 647 short hairpin RNAs targeting 147 genes selected by a perturbed molecular hub analysis in RB tumors compared with human fetal retina (46). The tumor-promoting functions of these genes were associated with DNA repair but not with other known functions, suggesting that HR repair and associated genes in human RB serve an essential role in RB cell survival by error-free DNA damage recovery (46). Notably, gene expression studies in multiple models of *RB1* deletion other than RB tumors have also revealed that enriched gene sets are involved not only in DNA replication and cell cycle progression but also in DNA damage response/repair and mitotic segregation (8,47-49). The conserved gene signatures in various *RB1*-deficient cells are in part attributed to the fact that a number of these genes are E2F targets whose expression is driven by pRB loss and consequent release of E2Fs (50-52). Therefore, these findings support the notion that *RB1* inactivation is intrinsically linked to the transcriptional activation program via deregulated E2Fs to prevent any lethal genomic defects that would compromise RB cell survival, while promoting robust proliferation. Since high DNA repair activity can affect tumor progression and response to chemotherapy (53,54), upregulation of genes in diverse DNA repair pathways by which various DNA lesions are recognized and repaired efficiently represents an important mechanism for maintenance of overall genome stability in RB, thereby sustaining tumor growth despite the constant threat of DNA damage from both endogenous and exogenous sources.

*Chromatin regulators in DNA damage response and repair in RB*. The DNA damage response serves a pivotal role in ensuring genome integrity throughout the cell cycle by sensing DNA damages, activating cell cycle checkpoints, and engaging multiple DNA repair pathways or apoptosis (55). Defects in DNA damage response and repair could be detrimental to cancer cells, particularly in cancer cells with a functional p53 signaling pathway. As shown in Table I, RB tumors exhibit high expression levels of genes implicated in DNA damage response and diverse DNA repair pathways, which may facilitate the repair of DNA lesions and subsequent cell cycle progression. Efficient DNA damage detection and repair also requires close cooperation with chromatin-associating proteins to alter the local chromatin environment near the DNA lesions and promote recruitment of DNA repair factors (56). Notably, RB tumors harbor a number of aberrantly expressed chromatin regulators, which are not expressed in the normal retina, and some of these chromatin regulators are direct E2F1 targets (57).

Ubiquitin-like with PHD and RING finger domains 1 (UHRF1) is an epigenetic regulator that is frequently upregulated in cancer and promotes tumor development by altering gene expression through changes in DNA methylation and histone modifications via recruitment of various chromatin modifiers (58,59). Furthermore, several studies have reported that UHRF1 is implicated in diverse aspects of DNA damage response and repair by sensing DNA damages such as interstrand crosslinks, interacting with relevant repair factors and regulating the cell cycle-dependent choice of DSB repair pathways (60-63). All of these findings may mechanistically explain the observation that *Uhrf1*-null embryonic stem (ES)

Table I. Gene signature of DNA damage response and repair in primary RB.

| First author/s, year | Functional category | Gene symbol | Name | (Refs.) |
|---|---|---|---|---|
| Chakraborty, 2007; Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | DNA damage checkpoint | *CHEK1* | Checkpoint kinase 1 | (42-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | Chromatin regulators in DNA damage response and repair | *UHRF1* | Ubiquitin-like with PHD and RING finger domains 1 | (43-45) |
| Ganguly, 2010 | | *DOT1L* | DOT1-like histone lysine methyltransferase | (43) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *HMGA1* | High mobility group AT-hook 1 | (43-45) |
| Kapatai, 2013; Rajasekaran, 2019 | | *HMGA2* | High mobility group AT-hook 2 | (44,45) |
| Ganguly, 2010; Rajasekaran, 2019 | | *SMARCA6* | Helicase, lymphoid specific | (43,45) |
| Ganguly, 2010; Rajasekaran, 2019 | | *SMARCAD1* | SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily A, containing DEAD/H box 1 | (43,45) |
| Ganguly, 2010; Rajasekaran, 2019 | HR repair | *BRCA1* | BRCA1 DNA repair associated | (43,45) |
| Kapatai, 2013; Rajasekaran, 2019 | | *BRCA2* | BRCA2 DNA repair associated | (44,45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *RAD51* | RAD51 recombinase | (43-45) |
| Kapatai, 2013; Rajasekaran, 2019 | | *XRCC2* | X-ray repair cross complementing 2 | (44,45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *RAD54L* | DNA repair and recombination protein RAD54-like | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *RAD18* | RAD18 E3 ubiquitin protein ligase | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *BARD1* | BRCA1-associated RING domain 1 | (43-45) |
| Ganguly, 2010 | | *BLM* | BLM RecQ-like helicase | (43) |
| Ganguly, 2010 | C-NHEJ repair | *XRCC5* | X-ray repair cross complementing 5 | (43) |
| Ganguly, 2010; Rajasekaran, 2019 | MMEJ repair | *XRCC1* | X-ray repair cross complementing 1 | (43,45) |
| Ganguly, 2010 | | *PARP1* | Poly(ADP-ribose) polymerase 1 | (43) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *POLQ* | DNA polymerase θ | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | MMR | *MSH5* | MutS homolog 5 | (43-45) |
| Chakraborty, 2007; Ganguly, 2010; Rajasekaran, 2019 | | *MSH6* | MutS homolog 6 | (42,43,45) |
| Ganguly, 2010; Rajasekaran, 2019 | | *MSH2* | MutS homolog 2 | (43,45) |

Table I. Continued.

| First author/s, year | Functional category | Gene symbol | Name | (Refs.) |
|---|---|---|---|---|
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | BER | *UNG* | Uracil DNA glycosylase | (43-45) |
| Ganguly, 2010; Rajasekaran, 2019 | | *LIG1* | DNA ligase 1 | (43,45) |
| Ganguly, 2010 | | *PARP1* | Poly(ADP-ribose) polymerase 1 | (43) |
| Ganguly, 2010; Rajasekaran, 2019 | | *PARP2* | Poly(ADP-ribose) polymerase 2 | (43,45) |
| Ganguly, 2010; Rajasekaran, 2019 | | *XRCC1* | X-ray repair cross complementing 1 | (43,45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | FA pathway | *FANCA* | FA complementation group A | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *FANCD2* | FA complementation group D2 | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *FANCI* | FA complementation group I | (43-45) |
| Ganguly, 2010; Rajasekaran, 2019 | | *FANCE* | FA complementation group E | (43,45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *FANCL* | FA complementation group L | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *FANCG* | FA complementation group G | (43-45) |
| Ganguly, 2010; Kapatai, 2013; Rajasekaran, 2019 | | *EME1* | Essential meiotic structure-specific endonuclease 1 | (43-45) |

Upregulation of the listed genes is detected in primary human RB tumors relative to normal retina by gene expression profiling in the indicated references. BER, base excision repair; C-NHEJ, canonical nonhomologous end-joining; FA, Fanconi anemia; HR, homologous recombination; MMEJ, microhomology-mediated end-joining; MMR, mismatch repair; RB, retinoblastoma.

cells are more sensitive to genotoxic insults induced by irradiation and DNA-damaging agents than *Uhrf1*$^{+/+}$ and *Uhrf1*$^{+/-}$ ES cells (64). Our previous study demonstrated that UHRF1 knockdown sensitizes RB cells to chemotherapeutic drugs by impeding DNA repair via downregulation of XRCC4 involved in C-NHEJ repair and consequential impairment of DNA ligase IV loading onto damaged chromatin (65). In addition, another recent study revealed that UHRF1 depletion in RB cells increases the sensitivity to HDAC inhibitors by enhancing oxidative stress-mediated apoptosis via downregulation of the redox-responsive genes encoding glutathione S-transferase α4 and thioredoxin 2 (66). In agreement with the results in a cell study, UHRF1 depletion in RB cells increased the therapeutic efficacy of the HDAC inhibitor MS-275 in murine orthotopic xenografts (66). The detailed underlying mechanisms of how UHRF1 modulates these distinct sets of effector genes in DNA repair and redox homeostasis remain to be elucidated; however, both studies point to the role of UHRF1 in genome maintenance in RB cells by functionally linking NHEJ to redox homeostasis since oxidative stress even at low levels has been demonstrated to induce DSBs and NHEJ repair-deficient cells are hypersensitive to oxidative stress (67,68). Therefore, the findings support the hypothesis that enhanced DNA repair capacity and ROS homeostasis driven by UHRF1 may protect RB cells against endogenous DNA damage or chemotherapeutics-induced cell death (Fig. 1A). Furthermore, in contrast to normal retina lacking UHRF1 expression, its constitutive expression in RB as a direct E2F1 target gene makes UHRF1 an attractive therapeutic target for RB treatment (57).

Similar to the case of UHRF1, disruptor of telomeric silencing 1-like (DOT1L) is highly and exclusively expressed in RB although it is thus far unknown whether DOT1L is also an E2F1 target (69). DOT1L is the only known histone methyltransferase catalyzing H3K79 methylation, which

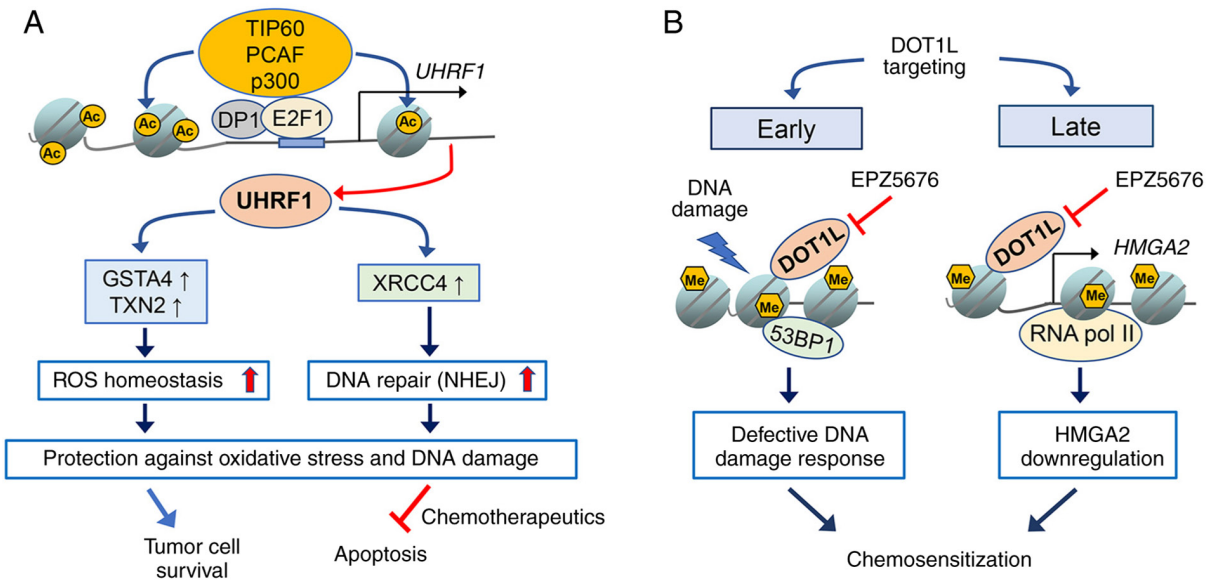


Figure 1. Models depicting the roles of select chromatin regulators in DNA damage response and modulation of chemosensitivity in RB. (A) Tumor-promoting functions of UHRF1 in RB. UHRF1 expression is aberrantly induced in RB cells by deregulated E2F1 in collaboration with activating chromatin modifiers, such as histone acetyltransferases (TIP60, PCAF and p300). Subsequently, UHRF1 upregulates downstream effectors implicated in ROS homeostasis and DNA repair, which assists RB cells in coping with oxidative stress and endogenous DNA damage arising from robust proliferation. In addition, the augmentation of cellular stress-managing capacity driven by UHRF1 expression also contributes to resistance against chemotherapeutics, endowing RB cells with a selective advantage to evade apoptosis and thereby promoting their survival and outgrowth. (B) Dual role of DOT1L targeting in chemosensitization of RB cells. DOT1L inhibition by EPZ5676 immediately interferes with the early DNA damage response mediated by DOT1L itself following treatment with genotoxic drugs. Furthermore, prolonged inhibition of DOT1L leads to epigenetic downregulation of HMGA2, which is a direct DOT1L target gene and is also involved in DNA damage response by a distinct mechanism. Through this late effect of DOT1L inhibition on HMGA2 downregulation, RB cells which might have evaded apoptosis during the early defective DNA damage response may get doubly targeted and eliminated upon combined chemotherapy. 53BP1, tumor protein p53 binding protein 1; DOT1L, disruptor of telomeric silencing 1-like; DP1, DRTF1-polypeptide 1; E2F1, E2F transcription factor 1; GSTA4, glutathione S-transferase α4; HMGA2, high mobility group AT-hook 2; NHEJ, nonhomologous end-joining; PCAF, p300/CBP-associated factor; RB, retinoblastoma; RNA pol II, RNA polymerase II; ROS, reactive oxygen species; TIP60, Tat interacting protein, 60 kDa; TXN2, thioredoxin 2; UHRF1, ubiquitin-like with PHD and RING finger domains 1; XRCC4, X-ray repair cross complementing 4.

is considered mostly as an activating mark for gene transcription (70,71). Notably, DOT1L and H3K79 methylation have been demonstrated to be indispensable for ionizing radiation-induced tumor protein p53 binding protein 1 foci formation during $G_1/G_2$ phase, and pharmacological inhibition of DOT1L in combination with DNA-damaging agents further decreased the proliferation of colorectal cancer cells and mixed-lineage leukemia (MLL)-rearranged leukemia cells (72-74). Consistent with the findings in other cancer cells, DOT1L targeting by EPZ5676 (pinometostat) sensitized RB cells to chemotherapeutic drugs by impairing the DNA damage response and thereby enhancing apoptosis, while it was largely inefficacious as a single-agent therapy in both RB cells and an orthotopic xenograft model (69). In addition to verifying the role of DOT1L in DNA damage response and chemosensitization in RB cells, the study also revealed that high mobility group AT-hook 2 (HMGA2) is a novel DOT1L target gene and its expression is epigenetically upregulated by DOT1L. Notably, HMGA2 has been reported to promote RB cell proliferation and participate in the regulation of DNA damage response in cancer cells (75-78). HMGA2 depletion reduces checkpoint kinase 1 phosphorylation during the etoposide-induced DNA damage response and potentiates the drug sensitivity in RB cells (69). The aforementioned study suggested that DOT1L targeting has a dual role in chemosensitization of RB cells by immediately hindering the early DNA damage response mediated by DOT1L itself upon genotoxic insults, and also by downregulating HMGA2 expression as a late effect of DOT1L inhibition (Fig. 1B).

In addition to the aforementioned epigenetic regulators, several chromatin remodelers may also exhibit chemosensitization properties in RB cells upon their co-inhibition in combination with conventional genotoxic drugs. Chromatin remodelers, including BRG1, helicase, lymphoid specific (HELLS) and SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1, are known to be recruited to DSBs and facilitate HR repair (13,79,80). In particular, HELLS (also known as SMARCA6) is an E2F1 target gene and has been demonstrated to be crucial for RB tumor initiation and progression in genetically engineered mouse models (81). Given the importance of HELLS for RB development and high dependence of RB cells on HR repair for their survival, it would be of great interest to investigate the functions of HELLS in the context of the DNA damage response and repair in RB cells as well as its chemosensitization properties in preclinical animal models.

*Mechanisms contributing to chromosome stability and survival in RB*. In addition to alterations at the level of DNA bases and small stretches of DNA, aneuploidy generated by gains and losses of whole chromosomes is a major indicator of genomic instability, which is a common feature of a number of cancer cells such as ovarian, breast and prostate cancer,

and is often related to *RB1* loss (82). However, RB tumors appear to maintain overall chromosome stability with a few recurrent chromosome arm-level alterations but limited whole-chromosome aneuploidy (25). This raises the question of how RB cells can achieve chromosomal stability despite the *RB1* loss from the initiation of tumors. One study attempted to investigate this question by examining genes expressed prominently in cones, under the hypothesis that RB cells may use the intrinsic molecular network of the cell-of-origin to restrain *RB1* deficiency-associated chromosome instability for their survival and proliferation (83). The authors revealed that thyroid hormone receptor β1 and 2 (TRβ1 and TRβ2), which are highly expressed in both cones and RB cells, inhibit the expression of PTTG1 regulator of sister chromatid separation, securin (PTTG1). Since PTTG1 prevents separase from promoting sister chromatid separation (84), PTTG1 accumulation in RB cells upon TRβ1 and TRβ2 knockdown led to an increase in polyploidy, demonstrating the role of the TRβ1/β2-PTTG1 signaling pathway in maintaining chromosome stability in RB cells (83). Although the aforementioned study reported that both TRβ1 and TRβ2 knockdown resulted in E2F1 accumulation in RB cells and E2F1 depletion led to a decrease in PTTG1 expression, it remains unclear whether E2F1 acts on the same pathway mediated by TRβ1 and TRβ2. Furthermore, *PTTG1* has been found to be one of mitotic genes that are highly expressed in primary RB tumors compared with normal retinal tissues (43,44), which requires a comparative analysis of PTTG1 expression in purified retinal cone cells relative to whole retinal tissues and RB tumors in order to firmly establish the role of TRβ1 and TRβ2 in suppression of polyploidy by PTTG1 downregulation.

Defects in mitotic checkpoint signaling are one of the prime causal factors for chromosome missegregation and consequential generation of aneuploidy (85). Notably, inhibition of mitotic kinases, such as aurora kinase A and B (AURKA and AURKB), has been found to be synthetic lethal with *RB1* deficiency in cancer cells in pharmacological or CRISPR/CRISPR associated protein 9 (Cas9)-based screens (86,87). Since AURKA and AURKB contribute to correct mitotic spindle assembly and chromosome segregation (88), these kinases serve critical roles in ensuring mitotic fidelity and their inhibition is lethal for *RB1*-deficient cancer cells, which upregulate a number of mitotic genes as a result of E2F deregulation (50,51,82). As is the case with other *RB1*-deficient cancer cells, primary RB tumors display high expression levels of several mitotic genes, including *AURKB*, polo-like kinase 1 (*PLK1*), mitotic arrest deficient 2 like 1 and BUB1 mitotic checkpoint serine/threonine kinase (43,44,89,90). Two recent studies have demonstrated that pharmacological inhibition of AURKB and PLK1 in RB cells resulted in cell cycle arrest and increased apoptosis, whereas the effects of the inhibitors on a nontumoral retinal pigment epithelial cell line (ARPE-19) were negligible under identical conditions, which was indicative of a higher sensitivity of RB cells to these inhibitors (89,91). Although both studies have not examined whether inhibition of these upregulated mitotic kinases causes chromosomal aberrations that may eventually lead to cell death, the results support the possibility that cancer cells with hyperactive mitotic checkpoint signaling due to *RB1* loss might depend on AURKB and PLK1 for efficient mitotic exit and survival, establishing a synthetic lethal relationship with *RB1* deficiency upon their inhibition. Notably, PLK1 targeting by ON 01910.Na (rigosertib) has been found to be efficacious for local therapy in orthotopic xenografts of RB (91). Since PLK1 is known to have other genome maintenance functions beyond mitosis, in particular during DNA replication and the DNA damage response (92), further studies are required to achieve an improved mechanistic understanding of PLK1 targeting in RB.

## 4. Exploiting genome maintenance mechanisms as therapeutic vulnerabilities in RB

In RB, targeted therapies are currently lacking as a standard treatment option in clinics. For past decades, research efforts have been directed toward the identification of potential driver genes or pathways that promote RB development and can also be targeted therapeutically (93). This has led to numerous discoveries in RB cells and the proposal of potential therapeutic targets involved in diverse cellular processes (93); however, at present, none of the proposed targets has advanced into clinical trials and some of these targets, including microRNAs, are not amenable to specific targeting by small-molecule inhibitors (94). Although conventional genotoxic drugs, which are widely used for chemotherapy in RB, are efficacious in saving eyes and lives upon early diagnosis and timely treatment, high doses of such non-specific genotoxic drugs would be detrimental to young children and may result in multiple adverse effects during treatment or later in their life, as exemplified by ocular toxicities such as maculopathy and uveal effusion, ocular motility restriction due to fibrosis of orbital tissues, and rare incidence of secondary leukemia associated with cumulative doses and high-intensity treatment schedules (95-97). In this regard, strategies to selectively sensitize RB tumors to conventional chemotherapeutics may serve as a practical and viable approach to achieve the same therapeutic outcome with lower doses of the drugs, while minimizing any undesired toxicity in normal cells. An approach that can be taken for such endeavors would be to exploit the known genome maintenance mechanisms in RB and leverage them to sensitize RB cells to chemotherapy in a selective manner. As aforementioned, the identification of *BRCA1* and *RAD51* as the most critical genes for RB cell survival from a recent functional RNAi screen in $RB1^{null}$ and $RB1^{wt}$; $MYCN^{amp}$ orthotopic xenografts (46) provides strong evidence that genome maintenance mechanisms serve a pivotal role in RB cell survival, and these attributes can be exploited therapeutically to develop more effective chemosensitization strategies. Furthermore, the tumor-promoting functions of the identified genes were associated with DNA repair but not with other known functions, such as centrosome duplication and heterochromatin integrity, and RAD51 targeting by a small-molecule inhibitor engaged the classical p53-mediated apoptotic pathway and synergized with topoisomerase inhibitors, which suggests that targeting of these factors may not involve other unknown cellular pathways, which can potentially complicate the assessment of therapeutic effects (46). Notably, the DNA-repair hub has been found to be overlapping for survival of both *RB1*-inactivated tumors and *MYCN*-amplified tumors harboring intact *RB1* gene (46), which implies a wide-range applicability of the

chemosensitization strategies toward different RB subtypes. In line with this notion, poly (ADP-ribose) polymerase (PARP) inhibition was revealed to be efficacious for proliferation inhibition of *RB1*-mutated osteosarcoma cells, and the hypersensitivity to PARP inhibitors was associated with rapid activation of DNA replication checkpoint signaling, while no apparent defects in HR repair were observed in the *RB1*-mutated cells (98). These findings collectively suggest that the therapeutic vulnerabilities identified to be associated with *RB1* loss hinge on DNA damage response and repair, albeit with variability in the detailed mechanisms of action.

When common genome maintenance mechanisms, such as DNA repair pathways, are directly targeted for therapies, a key consideration for effective therapy is how to minimize their non-selective toxicity to normal cells, while eliciting a favorable response to therapy. A series of dosing and drug combination schemes has to be tested to achieve optimal therapeutic regimens. Alternatively, co-targeting of molecules involved in DNA damage response and repair, which are expressed exclusively in RB tumors, may enable more effective therapy by selectively sensitizing RB cells to chemotherapy. As aforementioned, chromatin regulators, including UHRF1, DOT1L and HMGA2, are exclusively expressed in RB without any detectable expression in normal retina, and their targeting by gene knockdown or pharmacological inhibition sensitizes RB cells to chemotherapeutics by employing diverse mechanisms involved in DNA damage response and repair (65,66,69). Currently, only DOT1L has several types of small-molecule inhibitors available for clinical trials; however, their poor pharmacokinetic properties limit the therapeutic efficacy and necessitate combinations with other drugs (99-101). Since pinometostat, a DOT1L inhibitor, has been reported to be generally safe in patients with MLL-rearranged leukemia even after prolonged continuous intravenous infusion (101), local combination therapies with a DOT1L inhibitor by intra-arterial or intravitreal chemotherapy for patients with RB may reduce the effective dose of standard chemotherapeutic drugs, thereby preventing systemic toxicity and mitigating any adverse effects in the eyes (102-104). Given the proven effectiveness of local therapies for the management of RB as both primary care and secondary treatment (102-104), this approach for DOT1L inhibitors appears to hold great promise as a novel therapy.

Development of small-molecule inhibitors for other chromatin regulators may also benefit a wide range of patients with cancer as these epigenetic regulators are upregulated in a number of cancer types of different cellular origins and their genome maintenance functions are conserved across various cancer cells including breast, lung, and colorectal cancer cells (57,105,106). In particular, UHRF1 is a known E2F1 target (107), which allows its constitutive expression in *RB1*-deficient tumors, including RB, and thereby obviates the patient selection process for UHRF1 targeting. UHRF1 has also been identified as one of the top 21 synthetic lethal genes in a recent CRISPR/Cas9 screen in *RB1*$^{-/-}$ small cell lung cancer cells (87). Therefore, development of UHRF1 inhibitors may impact the cancer therapy beyond RB if specificity and toxicity profiles of the inhibitors are in acceptable ranges for clinical trials. Since most epigenetic regulators are considered to be druggable (108), a complete understanding of their functions and comprehensive validation of clinical relevance would be a prerequisite to prioritize the targets that are amenable to selective inhibition by small-molecule inhibitors.

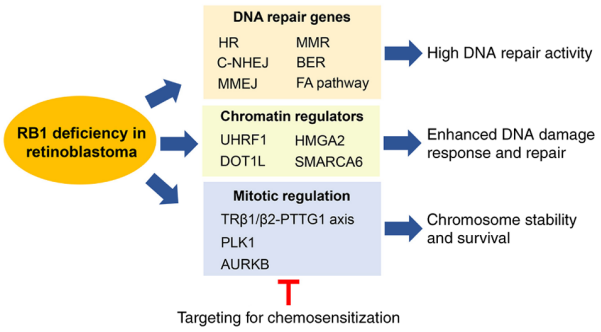


Figure 2. Heightened genome maintenance in RB. Genes involved in various DNA repair pathways, chromatin regulation during DNA damage response and repair, and mitotic regulation are highly upregulated in RB, and serve varied roles in restraining *RB1* deficiency-associated genomic alterations to promote RB survival and growth. Co-targeting of these factors is postulated to increase the sensitivity to conventional chemotherapeutics in RB. AURKB, aurora kinase B; BER, base excision repair; C-NHEJ, canonical nonhomologous end-joining; DOT1L, disruptor of telomeric silencing 1-like; FA, Fanconi anemia; HMGA2, high mobility group AT-hook 2; HR, homologous recombination; MMEJ, microhomology-mediated end-joining; MMR, mismatch repair; PLK1, polo-like kinase 1; PTTG1, PTTG1 regulator of sister chromatid separation, securin; RB, retinoblastoma; RB1, RB transcriptional corepressor 1; SMARCA6, SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 6; TRβ, thyroid hormone receptor β; UHRF1, ubiquitin-like with PHD and RING finger domains 1.

Another potentially important group of therapeutic targets in RB is mitotic kinases, some of which have a synthetic lethal relationship with *RB1* deficiency in other cancer cells (86,87). Although rigosertib, a PLK1 inhibitor, does not target RB cells selectively and is also known to have a short half-life and rapid clearance, it has shown remarkably less eye toxicity upon local therapy in orthotopic xenografts than melphalan, which is most commonly used for intravitreal therapy in clinics (91,103,109). Therefore, despite the mixed results in clinical trials with advanced solid tumors (110-112), comprehensive preclinical studies with rigosertib as a single agent or in combination with other drugs by local administration may result in promising outcomes, which could encourage clinical trials for RB.

## 5. Concluding remarks

Given the well-known functions of pRB in the maintenance of genome stability, lack of functional pRB by biallelic inactivation of the *RB1* gene in the vast majority of human RB cases suggests that human RB genomes would display a high degree of genomic instability. However, several whole-genome analyses (25-27) have revealed that RB genomes are relatively stable, characterized by low mutation burden and certain recurrent chromosomal alterations associated with somatic copy number changes. These findings have brought a novel perspective on the genome maintenance mechanisms in RB that may operate actively to attenuate the *RB1* deficiency-associated risk of genomic instability and thereby avoid any catastrophic genomic defects that would jeopardize survival and growth of RB. As summarized in Fig. 2, RB tumors possess multiple mechanisms to invigorate their DNA damage response and

repair processes in response to genotoxic insults (including the examples shown in Fig. 1) (42-45,65,66,69), and to prevent chromosome instability, such as aneuploidy (83). Although these genome maintenance mechanisms might have been evolved and adapted to promote RB cell survival and proliferation, the dependency of RB cells on these mechanisms may expose their unique vulnerability to chemotherapy, particularly when the genome maintenance mechanisms are tumor cell-specific. In order to achieve tumor cell-specific chemosensitization by selective targeting of the genome maintenance machineries, a thorough understanding of their functions and comprehensive evaluation of therapeutic efficacy in preclinical models, as well as development of an efficient methodology for monitoring toxicity in normal tissues, are required. This combination-based therapeutic approach exploiting genome maintenance mechanisms in RB as susceptibility factors may improve the efficacy of current chemotherapy, and may at least partially compensate for the lack of targeted therapies in RB by enabling more efficient control of treatment-related toxicity.

As massive induction of DNA damages and genomic instability to a lethal level is the basis for chemotherapy. Loss of pRB in cancer cells has been associated with inherent sensitivity to DNA-damaging agents due to the roles of pRB in promoting DNA repair and genome stability (8). While *RB1*-deficient cancer types respond to genotoxic drug-based therapies, findings in RB (25,27) suggest that the sensitivity is not strictly based on the compromised genome stability driven by pRB loss that would affect the sensitivity threshold to genotoxic drugs. The difference observed in RB may be related to the timing and prevalence of *RB1* loss. RB is initiated by biallelic inactivation of the *RB1* gene, and thus, the frequency of *RB1* mutations is exceptionally high, whereas the majority of human cancer types acquire *RB1* mutations during cancer progression and the mutation frequency is relatively low (4). In the case of RB with a functional p53 signaling pathway (38), heightened genome maintenance mechanisms may be indispensable for tumor initiation and progression by preventing the occurrence of any lethal genomic defects, while tolerable genomic alterations may still be allowed to occur for an improved chance of survival and outgrowth. This suggests that *RB1* deficiency in cancer does not necessarily indicate a high degree of genome instability in the cancer, and the context of *RB1* loss during the course of tumor development and the presence of other gene mutations should be considered to better understand the etiology of the disease. The information obtained for RB may provide novel insights into the understanding of the biology for other cancer types with early pRB loss and may guide toward improved therapeutic strategies for such cancer types.

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**Competing interests**

The authors declare that they have no competing interests.

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