Frontiers in Bioscience Scholar

Front. Biosci. (Schol Ed) **2025**; 17(4): 46225
https://doi.org/10.31083/FBS46225

Review

Genetic Regulation of DNA Double-Strand Breaks and Repair Pathways

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Academic Editor: Alexey V Polonikov
Submitted: 1 September 2025 Revised: 14 October 2025 Accepted: 31 October 2025 Published: 17 December 2025

Abstract

DNA double-strand breaks (DSB) represent one of the most severe forms of genomic damage. Thus, cells have evolved a complex network of DSB repair pathways, including homologous recombination, classical and alternative end joining, and single-strand annealing, which are tightly regulated by genetic and epigenetic factors. The selection and efficiency of these pathways influence genome integrity, oncogenesis, and therapeutic response. This comprehensive review synthesizes recent findings on the genetic regulation of DSB repair, with emphasis on pathway-specific regulators, chromatin context, and post-translational modifications. Moreover, this review integrates primary research from mammalian systems, including CRISPR-based studies, proteomics, and imaging, with a focus on publications from 2020 to 2025. We discuss the role of key players, such as MRE11–RAD50–NBS1 (MRN), ataxia telangiectasia mutated (ATM), mediator tumor suppressor p53-binding protein 1 (53BP1), breast cancer type 1 susceptibility protein (BRCA1), anti-silencing function 1 (ASF1), ring finger protein (RNF)8/168, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and RAD51 recombinase (RAD51), in orchestrating the associated pathway choice. Epigenetic modifications, RNA-mediated mechanisms, and chromatin remodeling dynamically influence the efficiency and fidelity of repair. Particular attention is provided to emerging regulators, including thyroid hormone receptor interactor 13 (TRIP13), ubiquitin-like with plant homeodomain (PHD) and RING finger domains 1 (UHRF1), Shieldin, and polymerase theta. This review highlights novel insights into transcription-associated DSB repair, the interplay of replication stress with repair pathway engagement, and context-dependent synthetic lethality. We also examine implications for cancer biology, including therapy resistance and biomarker development. Ultimately, understanding the genetic regulation of DSB repair pathways can provide critical insights into genome stability maintenance and reveal new therapeutic opportunities in cancer. Future work should focus on pathway crosstalk, phase-specific regulation, and integrating repair modulation into personalized medicine.

Keywords: double-strand breaks; DNA repair; homologous recombination; nonhomologous end-joining; chromatin remodeling; ubiquitination; RNA-DNA hybrid; neoplasms

1. Introduction

Among all DNA lesions, double-strand breaks (DSB) pose the highest threat to genome integrity. Eukaryotic cells rely mainly on two major routes for their resolution: the high-fidelity homologous recombination (HR) and the versatile, ligation-driven classical non-homologous end joining (C-NHEJ) (Fig. 1) [1]. HR is a high-fidelity repair mechanism active during the S and G2 phases of the cell cycle, when a sister chromatid is available as a repair template. In contrast, C-NHEJ functions throughout the cell cycle and directly ligates DNA-ends without the need for homology. More recently, an additional error-prone pathway — alternative end joining (Alt-EJ), also referred to as microhomology-mediated end joining (MMEJ) has been characterized (Fig. 1). This pathway utilizes short microhomologous sequences (typically 5–25 base pairs) flanking the break site to mediate repair [2]. The decision between these pathways is tightly regulated and influenced by multiple factors, including chromatin state at the break locus [3], the extent of 5′ end resection, and the phase of the cell cycle [1]. In addition, numerous proteins act as key regulators either promoting or inhibiting specific repair routes. The review focuses on the molecular mechanisms underlying pathway choice in DSB repair, emphasizing the roles of DNA-end processing factors, chromatin modifiers, and regulatory proteins that determine the engagement of HR, C-NHEJ, or MMEJ/Alt-EJ.

This review was prepared as a narrative synthesis of peer-reviewed research articles published between 2020 and 2025, identified through comprehensive searches of the PubMed and Scopus databases using defined keywords combinations. Approximately 75% of all cited references originate from this five-year period, ensuring that the review reflects the most recent and authoritative advances in the field. Priority was given to primary research studies in mammalian systems providing mechanistic insights confirmed by biochemical, imaging, or CRISPR-based approaches. Earlier seminal works were included selectively to provide historical or conceptual context.

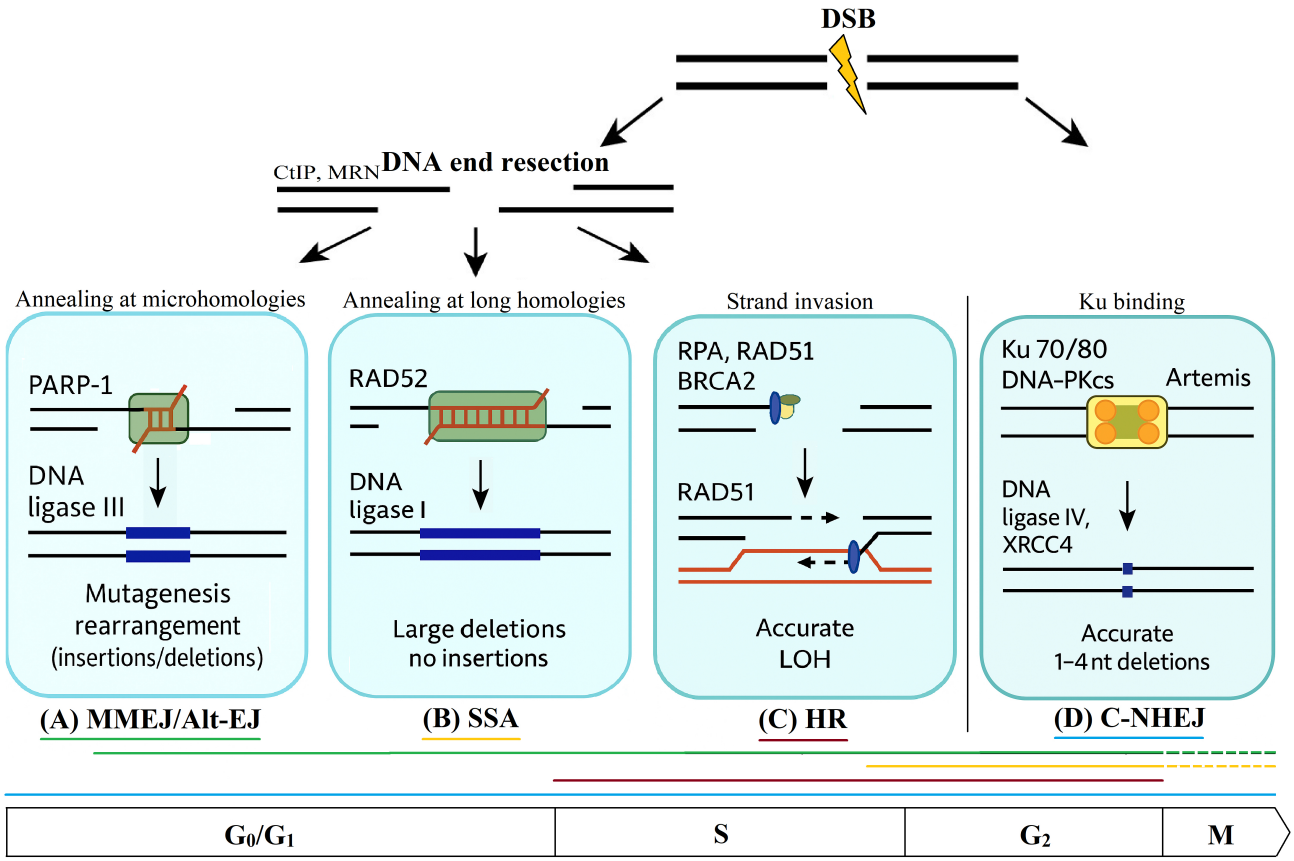

Fig. 1. DNA double-strand breaks (DSB) repair pathways and their activity throughout the cell cycle. (A–D) The choice of DNA DSB repair pathway primarily depends on whether DNA end resection takes place. When resection is inhibited, repair proceeds mainly through C-NHEJ. In contrast, once DNA ends are resected, three resection-dependent pathways (HR, MMEJ/Alt-EJ, and SSA) can compete for DSB repair. Thus, two levels of competition exist during the repair process: first, between C-NHEJ and the resection-dependent mechanisms during the initiation of resection; and second, among HR, MMEJ/Alt-EJ, and SSA once resection has occurred. Each pathway produces distinct genetic outcomes (e.g., loss of heterozygosity, deletions, or insertions), and the fidelity of each repair mechanism is indicated. Black solid lines represent DNA strands; black dashed lines indicate newly synthesized DNA or resected single-stranded regions; arrows denote the direction of DNA synthesis or strand invasion during repair. C-NHEJ functions with relatively constant efficiency throughout the cell cycle, whereas HR, MMEJ/Alt-EJ, and SSA predominate during the S and G2 phases, when resection activity is elevated. The color-coded horizontal bars below the schemes indicate the relative activity of each pathway across the cell cycle phases: solid lines denote active engagement, and dashed lines represent reduced or minimal activity. Abbreviations: C-NHEJ, classical non-homologous end joining; DSB, double-strand break; HR, homologous recombination; LOH, loss of heterozygosity; MMEJ/Alt-EJ, microhomology-mediated end joining/alternative end joining; nt, nucleotides; SSA, single-strand annealing.

2. DSB Repair Pathway Selection and Regulation — Factors Involved in DNA Damage Response

While the molecular components of DSB repair have been extensively characterized, the mechanistic hierarchy governing pathway choice remains under active investigation. Emerging evidence suggests that multiple, sometimes conflicting, regulatory layers act simultaneously, underscoring the need for integrated models of DSB repair regulation (Table 1, Ref. [4–91]) [92].

Upon DSB detection, the sensor complex MRE11-RAD50-NBS1 (MRN) recruits and activates the kinase ATM (ataxia-telangiectasia mutated), which initiates a phosphorylation cascade central to the DNA damage response (DDR) [93]. Activated ATM undergoes autophosphorylation and dimer dissociation, enabling phosphorylation of downstream effectors such as breast cancer type 1 susceptibility protein (BRCA1), checkpoint kinase 2 (CHK2), tumor suppressor protein p53 (p53), and the histone variant H2AX, producing the phosphorylated H2AX (γH2AX) mark that scaffolds assembly of repair proteins [94,95].

Mediator of DNA damage checkpoint protein 1 (MDC1) is subsequently recruited to γH2AX sites to reinforce signal amplification and promote accumulation of repair factors. The ubiquitin ligases ring finger protein

Table 1. Key molecular regulators of DNA double-strand break (DSB) repair pathways and their mechanistic roles.

Pathway	Key regulators	Primary function	Cell-cycle phase preference	Mechanistic features	Representative references
C-NHEJ	Ku70/80, DNA-PKcs, XRCC4, LIG4, Artemis	Direct ligation of minimally processed DSB ends	G1–G2	Fast, error-prone repair leading to small (1–4 nt) deletions	[4–31]
HR	BRCA1, BRCA2, RAD51, MRN complex, CtIP	Homology-directed repair using sister chromatid as template	S–G2	High-fidelity repair, may result in LOH	[32–49]
MMEJ/Alt-EJ	PARP-1, POLθ, XRCC1, LIG3	Microhomology-mediated end joining of resected ends	S–G2	Error-prone, frequently causes insertions/deletions and rearrangements	[50–59]
SSA	RAD52, LIG1	Annealing of homologous repeat sequences	S–G2	Produces large deletions, no insertions	[60–64]
Chromatin modulators	RNF8, RNF168, 53BP1, UHRF1, ASF1	Control end accessibility, ubiquitination, and resection	Cell-cycle dependent	Epigenetic and post-translational regulation of repair choice	[65–78]
Emerging regulators	AATF, TRIP13, DYNLL1, PIN1	Checkpoint control and resection regulation	Context dependent	Unresolved or newly characterized mechanisms	[79–91]

This table summarizes the major pathways involved in DNA DSB repair, the principal regulatory proteins, their preferred phases of the cell cycle, and characteristic molecular outcomes. C-NHEJ functions as a rapid repair mechanism with low fidelity, whereas HR ensures accurate DNA restoration. The error-prone MMEJ/Alt-EJ and SSA pathways compete with HR under resection-permissive conditions. References correspond to sections within this review.

Abbreviations: AATF, apoptosis-antagonizing transcription factor; ASF1, antisilencing function 1; ATM, ataxia telangiectasia mutated; BRCA1, breast cancer 1; BRCA2, breast cancer 2; C-NHEJ, classical non-homologous end joining; CtIP, CtBP-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; DYNLL1, dynein light chain LC8-type 1; HR, homologous recombination; LIG1, DNA ligase I; LIG3, DNA ligase III; LIG4, DNA ligase IV; LOH, loss of heterozygosity; MMEJ/Alt-EJ, microhomology-mediated end joining/alternative end joining; MRN, MRE11-RAD50-NBS1 complex; nt, nucleotides; PARP-1, poly(ADP-ribose) polymerase 1; PIN1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; POLθ, DNA polymerase theta; RAD51, RAD51 recombinase; RAD52, DNA repair protein RAD52; RIF1, replication timing regulatory factor 1; RNF8, ring finger protein 8; RNF168, ring finger protein 168; SHLD1-3, Shieldin complex subunits 1–3; SSA, single-strand annealing; UHRF1, ubiquitin-like with PHD and RING finger domains 1; XRCC1, X-ray repair cross-complementing protein 1; XRCC4, X-ray repair cross-complementing protein 4.

(RNF)8 and RNF168 extend this signal by catalyzing K63-linked polyubiquitin chains on histone H1 and H2A [96], providing binding platforms for the mediator p53-binding protein 1 (53BP1), which limits excessive resection of DNA ends and favors C-NHEJ [97–99]. 53BP1 executes this protective function in cooperation with the Shieldin complex and Rap1-interacting factor 1 (RIF1), thereby preventing nucleolytic degradation and facilitating repair through end joining. Conversely, recruitment of BRCA1 to DSB together with BRCA1-associated RING domain 1 (BARD1) and CtBP-interacting protein (CtIP) promotes resection and drives the shift toward HR [100,101]. Notably, the Bloom syndrome helicase (BLM) has been shown to function in both HR and C-NHEJ pathways, participating in the recruitment of repair factors during S and G1 phases, respectively, and thus influencing pathway choice based on the cell cycle context [102].

2.1 Classical Non-Homologous End-Joining and Proteins Promoting C-NHEJ Repair

In higher eukaryotic organisms, the C-NHEJ pathway serves as the predominant mechanism for repairing DSB, responsible for correcting approximately 80% of such lesions. The repair process initiates with the Ku70/Ku80 heterodimer, which rapidly associates with the DNA termini, safeguarding them from resection and serving as a scaffold for subsequent recruitment of repair factors (Fig. 1) [4]. DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs) associates with Ku to form the DNA-PK holoenzyme that bridges broken DNA ends and coordinates end-processing enzymes. Upon activation, DNA-PKcs undergoes autophosphorylation and phosphorylates several downstream effectors, including Artemis, polynucleotide kinase phosphatase (PNKP), X-ray repair cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV (LIG4), which collectively execute end processing and ligation. The cooperation among these enzymes ensures restoration of DNA continuity while maintaining genome stability [5]. The nucleolytic activities of Artemis, exonuclease 1 (EXO1), tyrosyl-DNA phosphodiesterase 1 (Tdp1), and PNKP prepare DNA-ends, while polymerases from the Pol X family (such as polymerase μ and λ) participate in gap-filling synthesis [5]. The final ligation is executed by the XRCC4/XLF/DNA ligase IV complex [6].

Recent cryo-electron microscopy (cryo-EM) structural studies have elucidated the direct interaction interfaces between Ku70/Ku80 and DNA polymerases λ and μ (Pol λ and Pol μ), detailing the role of their BRCA1 C-terminal (BRCT) domains in mediating efficient gap-filling and C-NHEJ fidelity [7]. It has been recently revealed that glioblastoma stem-like cells (GSCs) bolster their DNA repair proficiency by activating the C-NHEJ pathway through the upregulation of apoptosis antagonizing transcription factor (AATF), thereby contributing to treatment resistance in glioblastoma (GB). AATF directly binds to XRCC4, a critical component of the C-NHEJ machinery, and protects it from ubiquitin-dependent proteasomal degradation. Upon induction of DNA damage, AATF is phosphorylated at serine 189 by ATM kinase, which facilitates its release from XRCC4 and enables the prompt recruitment of XRCC4 to DNA break sites, ensuring efficient NHEJ-mediated repair. Notably, silencing AATF or disrupting its phosphorylation impairs C-NHEJ efficiency in GSCs and sensitizes GB xenograft models to chemoradiotherapy [8].

A pivotal factor influencing DSB repair pathway choice is the phosphorylation state of DNA-PKcs [9]. As a central orchestrator of the C-NHEJ process, DNA-PKcs phosphorylates itself and other C-NHEJ-related proteins (Ku, XRCC4, XLF, Artemis), promoting repair fidelity and completion [10]. During G1 phase, DNA-PKcs inhibits ATM signaling through phosphorylation of ATM, thereby favoring C-NHEJ [11]. In contrast, during G2 phase, autophosphorylation of DNA-PKcs leads to its release from DNA-ends, permitting EXO1-mediated resection and subsequent HR activation [12]. During the S phase, the association of BRCA1 with DNA-PKcs counteracts its kinase self-activation, thereby diminishing end-joining efficiency while promoting DNA-end resection and facilitating the transition toward HR [13].

Also, DNA-PKcs exerts indirect control over pathway choice via modulation of *RBX1* expression, which targets EXO1 for degradation during G1 phase, suppressing HR [14]. Furthermore, recent reviews underscore the multifaceted role of DNA-PKcs beyond DNA repair, including its regulation of RNA metabolism, transcription machinery interactions, alternative splicing, and noncoding RNA functions in cancer biology [15].

The Ku70/Ku80 heterodimer also plays a crucial role in shielding DNA-ends from nucleolytic degradation, preserving the structural integrity of DSB and enforcing C-NHEJ fidelity [16]. Additionally, the C-terminal SAP domain of Ku70 has been shown to be essential for optimal cellular responses to DNA damage, promoting Ku70 retention at DSB and enhancing DNA ligase IV stability at these sites [17]. Another central regulator is 53BP1, which limits end resection, thereby antagonizing BRCA1-mediated HR and reinforcing C-NHEJ preference [18]. 53BP1 promotes end joining via two primary routes: the replication timing regulatory factor 1 (RIF1)-REV7 (also known as mitotic arrest-deficient protein 2, MAD2L2)-REV7/Shieldin complex and the PTIP-Artemis pathway [19]. Nevertheless, 53BP1 has additional roles in HR under replication stress, including both BLM helicase-dependent and -independent activities [20], and it suppresses HR in BRCA1-deficient contexts by blocking end resection [21]. Importantly, post-translational acetylation of 53BP1 impedes its chromatin recruitment, thus favoring HR [22]. Phosphorylation of 53BP1 by ATM at its N-terminal domain allows for the recruitment of RIF1 to damaged DNA, a process that inhibits

end resection during G1 phase. In contrast, during S/G2 phases, CDK-mediated phosphorylation of CtIP activates the BRCA1-CtIP complex, suppressing RIF1 recruitment and thereby enabling HR progression [23].

Acting downstream of RIF1, the Shieldin complex (comprising SHLD1, SHLD2, SHLD3, and REV7) protecs DSB termini from extensive resection [11,24, 99]. The SHLD2 subunit possesses an RPA70-like oligonucleotide/oligosaccharide-binding fold that anchors to single-stranded DNA, thereby preventing resection nucleases such as EXO1 and DNA2 from accessing DNA ends [24]. Dynein light chain LC8-type 1 (DYNLL1), a 53BP1-interacting protein, further reinforces this blockade by associating with MRE11 - its depletion enhances Shieldin accumulation at break sites [25]. REV7, a vital Shieldin component, suppresses 5′–3′ end resection and bolsters C-NHEJ by dimerizing and coordinating interactions with SHLD1 and SHLD3. These combined events recrute the CST complex (CTC1-STN1-TEN1) together with DNA polymerase α, which mediates limited fill-in synthesis to prepare DNA ends for ligation via the C-NHEJ pathway [26]. Notably, even in Shieldin-deficient BRCA1-null cells, an alternative fill-in pathway mediated by CST/Polα/primase complex can partially substitute the protective function [27]. When REV7 is inactivated, SHLD3 fails to localize to DSBs, allowing BRCA1 to displace 53BP1 and initiate extensive resection, thereby enabling HR [28]. Both RIF1 and PTIP are independently recruited via distinct phosphorylation motifs on 53BP1 and are indispensable for C-NHEJ commitment [29,30]. PTIP specifically facilitates the recruitment of Artemis, which, via its endonuclease activity, processes overhangs at DSB to render them ligatable, completing the repair through the C-NHEJ pathway [31].

2.2 Homologous Recombination and Proteins Promoting HR Repair

HR is a high-fidelity DNA repair mechanism that restores genomic integrity by exchanging homologous or identical DNA sequences between a damaged strand and an intact homologous template. The process is initiated by the MRN complex (comprising MRE11, RAD50, and NBS1) which detects the break and promotes 5′ to 3′ resection at the DSB-ends, generating 3′ single-stranded DNA (ssDNA) overhangs (Fig. 1). These overhangs are initially coated by replication protein A (RPA), which stabilizes ssDNA and prevents secondary structure formation. RPA is subsequently displaced by the BRCA2-mediated loading of RAD51, leading to the formation of a presynaptic nucleoprotein filament [93]. This filament performs a homology search and invades a homologous duplex DNA, forming a displacement loop (D-loop) that serves as a primer for DNA synthesis.

Following strand invasion and extension, the repair process can proceed through one of three distinct subpathways [94]: (a) DSB repair (DSBR), which involves second-end capture and the formation of a double Holliday junction, later resolved by structure-specific endonucleases such as MUS81-EME1, SLX1-SLX4, or flap endonuclease GEN homolog 1 (GEN1), leading to gene conversion events with or without crossover; (b) Synthesis-dependent strand annealing (SDSA), where the extended invading strand dissociates and anneals to the resected second end without forming a Holliday junction, generally resulting in non-crossover outcomes; (c) Break-induced replication (BIR), a less common but highly mutagenic pathway wherein the D-loop is converted into a replication fork, facilitating extensive DNA synthesis to the end of the chromosome and often causing loss of heterozygosity.

RAD54 plays dual roles in HR and chromatin remodeling [32]. It stabilizes the RAD51-ssDNA presynaptic filament, enhancing its strand invasion activity during HR [32]. Beyond the synaptic phase, RAD54 facilitates RAD51 turnover by dissociating it from heteroduplex DNA, thus exerting a postsynaptic regulatory function *in vivo* [1]. Additionally, RAD54 accelerates joint molecule formation and promotes ATP-dependent branch migration during later stages of HR [1,32]. Notably, BLM helicase has been shown to augment RAD54-driven chromatin remodeling, contributing to chemotherapy resistance [33]. RAD51, a core HR recombinase, suppresses non-conservative pathways such as SSA and Alt-EJ, while favoring gene conversion (GC). This preference is largely dictated by its loading efficiency onto ssDNA [34].

BRCA1 functions as a central coordinator of HR, engaging at several distinct checkpoints of the repair cascade. Upon the formation of a DSB, BRCA1 associates with CtIP and the MRN complex to initiate limited DNA-end resection, generating stretches of ssDNA that enable RAD51 loading while counteracting 53BP1-mediated end protection. This interplay redirects the balance from end joining toward recombination-based repair [35]. Furthermore, the BRCA1-BARD1 heterodimer enhances the homologous pairing activity of RAD51, thereby supporting recombination fidelity [35,36].

The human RecQ helicases BLM and WRN also critically influence pathway selection. BLM participates in both DNA end resection and Holliday junction resolution during HR [37,38], and can stimulate EXO1-mediated resection to promote HR initiation [39]. Additionally, BLM coordinates repair factor recruitment in a cell cycle-dependent manner: in G1, it facilitates XRCC4 loading to support C-NHEJ, while in S phase, it aids RAD51 recruitment to promote HR [102].

WRN functions similarly, participating in end resection via interactions with MRE11, NBS1, EXO1, and DNA2 [40]. Its activity is modulated by posttranslational modifications, including CDK2-dependent phosphorylation at Ser426, which enhances its binding to RPA and stabilizes 3′ ssDNA intermediates [41]. In early HR stages, WRN also promotes RAD51 loading through CHK1 signal-

ing [42]. Recent work shows that the WRN-RPA complex is crucial for replication fork recovery following replication stress, thereby limiting DSB formation and indirectly supporting HR fidelity [43]. Another important regulator of repair pathway choice is TRIP13 (also known as HPV16E1BP), an ATP-ase that inactivates REV7 by inducing a conformational switch. This process disrupts the REV7-Shieldin complex and promotes HR [44].

UHRF1 (Ubiquitin-like with PHD and RING finger domains 1) is an epigenetic modulator that contributes to transcriptional repression via HDAC1 induction [45] and also plays a direct role in DSB repair. Loss of UHRF1 leads to defective DSB resolution, heightened sensitivity to genotoxic stress, chromosomal instability, and increased sister chromatid exchange (SCE) [46]. Phosphorylated UHRF1 promotes HR through its E3 ligase activity at break sites [46,47], and its methylated form binds PCNA to enhance its polyubiquitination, further stimulating HR [46]. Furthermore, DNMT1 inhibition triggers UHRF1-dependent H3K18ub and concomitant H3K9me3 increases, indicating a chromatin-mediated layer of HR regulation via UHRF1 as shown in 2025 study [48]. Importantly, UHRF1-deficient cells exhibit hypersensitivity to PARP inhibitors, suggesting a pivotal role in DSB repair downstream of BRCA1. Recent pancancer analysis confirms high HR deficiency rates associated with mutations in *RAD54L*, *RAD51C/D*, and *PALB2*, underscoring their functional significance in HR and therapeutic vulnerability [49].

2.3 Microhomology-Mediated End Joining/Alternative End Joining and Its Regulation

The recently identified MMEJ/Alt-EJ, constitutes an error-prone mechanism for DSB repair that can function independently of C-NHEJ [103]. MMEJ/Alt-EJ operates even in cells with intact C-NHEJ components, provided that microhomologous regions (typically 5 nucleotides or longer) are available near the DNA break sites (Fig. 1) [50]. These short homologies are recognized and utilized by the MRN complex in conjunction with PARP1 or potentially other yet-unidentified factors. Following initial recognition, the MRN complex resects DNA ends until complementary microhomologies are exposed on either side of the break. This facilitates strand annealing, while endonucleases such as xeroderma pigmentosum complementation group F–excision repair cross-complementation group 1 complex (XPF)/ERCC1 or flap endonuclease 1 (FEN1) remove non-homologous 3′ flaps [51]. After reannealing of microhomologous sequences, Polθ performs limited DNA synthesis to bridge the remaining gaps, followed by end sealing carried out by the XRCC1-DNA ligase III complex, which restores DNA continuity [52]. As MMEJ/Alt-EJ typically deletes sequences between the microhomology regions, it is highly mutagenic and therefore must be tightly regulated during DSB repair pathway selection [53].

CtIP- and MRE11-driven DNA-end resection initiates the MMEJ/Alt-EJ pathway by revealing microhomologous regions. Notably, depletion of BLM or EXO1 disrupts this process and shifts repair pathway utilization [54, 103]. BRCA1, a central player in HR, acts to suppress MMEJ/Alt-EJ by promoting extended end resection and RAD51-mediated strand invasion [54]. Similarly, replication protein A (RPA) antagonizes MMEJ/Alt-EJ by binding to ssDNA and preventing spontaneous annealing of short homologous sequences close to the break [55].

Further, loss of the tumor suppressor *RB1* has been associated with elevated MMEJ/Alt-EJ activity, pointing to its role as another regulatory factor influencing DSB repair pathway choice [56]. Conversely, DNA polymerase theta (Polθ), which possesses both polymerase and helicase activity, is a core effector of MMEJ/Alt-EJ and promotes end joining at sites with short microhomology [57,58]. In HR-deficient tumors, elevated *POLθ* expression has been linked to increased reliance on MMEJ/Alt-EJ, suggesting that Polθ might serve as a potential therapeutic target in cancers with defective HR machinery, reinforced by recent preclinical data showing that *POLθ* inhibition effectively sensitizes HR-deficient cells to DNA damage [59].

2.4 Single-Strand Annealing

Another repair mechanism for DSB, in addition to HR and end-joining pathways, is the error-prone single-strand annealing (SSA) pathway (Fig. 1). Unlike HR, SSA does not require a homologous donor template but instead utilizes direct tandem repeat sequences that flank the break site [60]. Following the generation of DSB, the MRN complex initiates DNA end resection via the exonuclease activity of MRE11, similarly to the HR pathway. However, SSA typically involves more extensive resection than MMEJ/Alt-EJ, requiring long ssDNA regions generated by CtIP and EXO1 to expose homologous repeats on both sides of the break. This mechanism proceeds independently of RAD51 but requires RAD52 to mediate strand annealing between the complementary repeat sequences. Once annealing occurs, ERCC1/XPF endonuclease removes non-homologous 3′-ssDNA overhangs that result from the resection process [60]. Subsequently, DNA polymerases fill in the remaining gaps, and the DNA ends are ligated by either DNA ligase I or ligase III to restore the strand continuity. However, SSA inevitably results in the deletion of one repeat and the intervening sequence between the repeats, rendering the pathway inherently mutagenic.

Previous studies have proposed that SSA pathway might contribute to precise donor integration by facilitating the annealing of homology arms in the donor construct to complementary genomic sequences [61–63]. However, to date, no direct experimental evidence has substantiated this hypothesis. Findings from Tei *et al.* (2025) [64] demonstrate that inhibition of the SSA pathway did not diminish the frequency of precise homology-directed repair (HDR),

but rather led to a reduction in asymmetric HDR events. These results imply that SSA may mediate accurate integration at one end of the donor construct, while the opposite end undergoes imprecise resolution via alternative repair mechanisms, ultimately resulting in asymmetrical integration. Therefore, targeted suppression of SSA could reduce erroneous integration events and represents a promising strategy to enhance the efficiency of precise knock-in.

2.5 Outstanding Questions and Future Directions in Canonical DSB Repair

Despite major advances in defining the molecular architecture of canonical DSB repair pathways, several key regulatory principles remain unresolved. One major gap concerns how cells dynamically reconcile competing repair cues from 53BP1-RIF1-Shieldin complexes and BRCA1-CtIP-MRN signaling, particularly during S and G2 phases when both HR and C-NHEJ machineries are accessible. It is not fully understood how post-translational modifications (PTMs) of shared factors (e.g., phosphorylation, acetylation, or SUMOylation of 53BP1, RIF1, or BRCA1) bias repair toward one pathway in different chromatin contexts. Further biochemical and single-molecule studies are needed to clarify whether these modifications act as transient molecular "toggles" or as stable determinants of pathway choice.

Likewise, several recently identified proteins such as AATF and TRIP13 play dual or context-dependent roles, but their *in vivo* relevance remains only partially defined. AATF has been shown to stabilize XRCC4 during C-NHEJ and confer therapeutic resistance in glioblastoma models, yet whether its phosphorylation by ATM also influences HR efficiency or replication fork recovery is still unclear. TRIP13-mediated conformational regulation of REV7 has emerged as a central switch between Shieldin-dependent C-NHEJ and HR, but the temporal hierarchy and cell-cycle specificity of this process are incompletely mapped. Further *in vivo* models and time-resolved interactome analyses will be required to delineate these transitions.

Another conceptual uncertainty involves histone modifications. While certain chromatin marks such as H2AK15ub, H4K20me1/2, and H3K79me2 are firmly established as C-NHEJ-promoting features, others (including H3K9me2/3 or H3K36me2) show more correlative associations with pathway bias and remain under debate. Similarly, the histone chaperone ASF1 has been implicated in promoting both HR and C-NHEJ. The determinants of this bifunctionality are not yet known. It is possible that ASF1's binding partners (RIF1 versus MMS22L-TONSL) or chromatin accessibility around DSB dictate its functional polarity, a hypothesis requiring targeted biochemical validation.

Collectively, these open questions emphasize that DSB repair pathway selection is not governed by linear hierarchies but by highly dynamic, context-dependent signaling networks. Integrating spatial chromatin organization, local transcriptional activity, and real-time post-translational regulation into systems-level models represents a major challenge for the next decade of DSB repair research.

3. Transcription-Coupled DNA Repair and Genomic Instability

Transcription plays a multifaceted role in genome maintenance, exerting both protective and detrimental effects on genomic stability. On the one hand, transcription enhances genome integrity by facilitating the recruitment of lesion-specific DNA repair proteins to transcribed regions, thereby ensuring efficient surveillance and repair of transcriptionally active loci. On the other hand, transcription can contribute to genomic instability under certain conditions, such as when it becomes dysregulated, when transcription machinery encounters replication forks leading to conflicts, or when aberrant formation of (RNA-DNA loops) R-loops occurs. These scenarios can impair genome maintenance and promote mutagenic outcomes.

3.1 Transcription-Coupled Nucleotide Excision Repair

Genotoxic insults such as UV irradiation induce bulky DNA lesions, which are resolved through two major nucleotide excision repair pathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER). These pathways differ in the initial lesion recognition steps. GG-NER relies on the detection of DNA damage by two distinct protein complexes: UV-DDB (comprising DDB1 and DDB2) [104] and the XPC-RAD23B-CETN2 complex [105–107]. In contrast, TC-NER identifies lesions via the stalling of RNA polymerase II (Pol II) at sites of transcription-blocking damage. Despite these different lesion recognition mechanisms, both pathways converge on a shared downstream set of repair proteins, including transcription factor IIH (TFIIH), xeroderma pigmentosum group A protein (XPA), xeroderma pigmentosum group G endonuclease (XPG), RPA, ERCC1-XPF, DNA polymerases, and ligases. A key player in TC-NER is cockayne syndrome protein B (CSB), which is recruited to stalled Pol II, and in turn facilitates the assembly of CSA and UV-stimulated scaffold protein A (UVSSA), initiating the repair cascade by targeting TFIIH to the lesion, halting transcription, and mediating the removal of the stalled Pol II complex [108,109].

3.2 Transcription-Coupled Homologous Recombination Repair

Although TC-NER has been recognized since the 1980s, only recently has transcription-coupled repair of DNA DSB been described. Chaurasia *et al*. (2012) [110] reported that DSB in actively transcribed genes are resolved more efficiently than those in transcriptionally silent regions [111]. DSB repair generally occurs via two major pathways: C-NHEJ and HR. C-NHEJ is a rapid but error-

prone mechanism that simply ligates broken DNA-ends, whereas HR is a more accurate process requiring a homologous sequence - typically the sister chromatid as a template for repair [112]. The availability of sister chromatids during specific cell cycle phases heavily influences the choice between these two pathways. Furthermore, several studies suggest that HR is preferentially used at sites of active transcription, possibly due to chromatin openness or associated structural features [113,114]. Factors such as RAD52 and RAD51AP1 have been implicated in facilitating HR specifically at transcribed loci [115].

3.3 RNA Transcription DNA Repair

The preference for HR repair at actively transcribed regions may stem from various factors including open chromatin, specific epigenetic marks, the presence of transcriptional machinery, or the RNA transcripts themselves [116–118]. Using an inducible DSB model in which transcription can be activated by doxycycline, it was demonstrated that active transcription enhances HR efficiency [119]. Notably, even a tethered RNA transcript homologous to the repair template was sufficient to mimic this effect, indicating a direct functional role of RNA in transcription-coupled HR [119]. This RNA-mediated stimulation of HR depends on sequence homology, suggesting that RNA-DNA hybrid structures form during repair. Several studies in both yeast and mammalian cells have confirmed the presence of RNA-DNA hybrids at DSB sites. The RNA component of these hybrids may originate from various sources including pre-existing transcripts or RNA newly synthesized by Pol II or Pol III [120,121]. However, genome-wide analyses indicate that pre-existing RNA is the predominant contributor to the RNA-DNA hybrids observed at break sites [122,123]. In special conditions, such as in yeast lacking both RNase H and reverse transcriptase, RNA can even serve as a direct template for DSB repair [124]. Similarly, in human G0/G1-phase cells where sister chromatids are absent, ROS-induced DSB can be repaired in a manner dependent on RNA-DNA hybrids, implying that RNA templates may also play a role [125]. It remains to be determined whether such repair in G0/G1 represents a form of non-canonical, RNA-templated HR.

3.4 Transcription as a Source of Genomic Instability

Transcription and its associated RNA products can represent significant endogenous sources of genome instability, particularly when transcriptional activity interferes with replication processes or facilitates the formation of aberrant R-loop structures. While pathological R-loops are recognized threats to genome stability, physiological R-loops fulfill critical regulatory roles, including in transcriptional initiation and termination, immunoglobulin class switch recombination, and the maintenance of telomeric and centromeric integrity [126–128]. Concurrent activity of transcription and replication on the same DNA template can lead to physical encounters between the transcription machinery and the replication fork, resulting in either head-on or co-directional collisions. These events may compromise genome integrity by generating DNA breaks. Much of the foundational understanding of transcription-replication collisions (TRCs) originates from prokaryotic systems, which possess a single replication origin. In *E. coli*, most highly expressed operons are oriented co-directionally with replication to minimize head-on conflicts, a likely outcome of evolutionary pressure. In contrast, head-on TRCs, which are more mutagenic, may contribute to rapid genomic evolution [129]. A recent study emphasizes the complex role of nascent RNA at transcription-replication conflict (TRC) sites, where the emerging transcript can critically influence conflict outcomes [130].

Reviews now increasingly consider chromatin context and DNA damage signaling in TRC regulation as potential therapeutic targets [131]. In mammalian systems, where thousands of replication origins are activated during each cell cycle [132], and where transcription is tightly regulated in a spatially and temporally complex manner, TRCs are more challenging to characterize. Utilizing an episomal model containing an R-loop-forming sequence and an inducible promoter system, Hamperl *et al.* (2017) [133] demonstrated that both head-on and co-directional TRCs provoke DNA damage. Notably, these collision orientations elicit distinct types of DNA lesions that are resolved through different DNA repair pathways [134]. Moreover, interference at TRCs has been shown to drive R-loop-dependent DNA damage responses in mammalian cells, corroborating the mechanistically detrimental role of R-loops in genomic instability [135]. Noteworthy, Hartono *et al.* (2018) [136] provided a critical methodological refinement, revealing that the widely used S9.6 antibody exhibits high affinity not only for DNA-RNA hybrids but also for double-stranded RNA. This discovery demonstrated that previous genome-wide R-loop maps may contain false-positive signals, emphasizing the need for orthogonal validation methods and improved specificity in R-loop detection.

Transcriptional dysregulation is a frequent source of pathological R-loop formation. Oncogene activation, such as by ewing sarcoma RNA-binding protein fused with Friend leukemia integration 1 transcription factor (*EWS-FLI1*) or harvey rat sarcoma viral oncogene homolog with glycine-to-valine substitution at codon 12 (*HRASG12V*), has been shown to enhance R-loop accumulation at target genes regulated by these factors [137,138]. This phenomenon is thought to arise from an overabundance of RNA transcripts available for hybridization with the template DNA. Interestingly, reduced transcriptional output due to RNA polymerase II (Pol II) pausing can also increase R-loop formation, likely due to prolonged retention of nascent transcripts within the transcription bubble [139,140]. R-loop formation is tightly coupled to co-

transcriptional RNA processing, including splicing, editing, nuclear export, and RNA-binding protein interactions. Impairment in these processes can induce transcriptional stress and facilitate aberrant R-loop accumulation, thereby linking RNA metabolism to transcription-associated genomic instability [141,142]. Interestingly, in *Leishmania major*, replication timing and chromosome stability correlate with formation of R-loops and RNase H1 distribution, highlighting how R-loop resolution factors interface with chromatin accessibility and replication programming [143].

To maintain genome integrity, pathological R-loops must be efficiently resolved. The RNA moiety of an R-loop can be specifically degraded by RNase H enzymes. Alternatively, RNA-DNA helicases such as SETX, aquarius intron-binding spliceosomal factor (AQR), DEAD-box helicase 5 (DDX5), and DEAH-box helicase 9 (DHX9) can unwind the hybrid structure. Additionally, nucleases including CtIP, FEN1, XPF, and XPG are implicated in excising R-loops formed during transcription [144,145], whereas enzymes like MUS81 and MRE11 resolve DNA structures arising from R-loop-replication fork collisions [146,147]. Furthermore, DNA repair proteins such as BRCA1, BRCA2, and the FANCD2-FANCI complex have been shown to suppress R-loop formation in diverse biological contexts [148,149]. Errors introduced during transcription, defined as sequence mismatches between nascent RNA transcripts and the DNA template, may stem from the inherent infidelity of RNA polymerases or be triggered by DNA damage and unfavorable chromatin environments. Although typically transient, these errors can exert meaningful effects on genome stability, especially under specific conditions or in repetitive genomic contexts [150,151].

3.5 Outstanding Questions and Future Directions in Transcription-Coupled DNA Repair

Although the relationship between transcription and genome maintenance has been intensively studied, the mechanistic and regulatory landscape of transcription-coupled DNA repair (TCR) remains only partially understood. One unresolved issue concerns how cells prioritize between RNA polymerase removal, lesion bypass, and DSB repair at sites of transcriptional blockage. The temporal coordination between TC-NER, HR, and C-NHEJ in active chromatin is still poorly defined, particularly in non-dividing or terminally differentiated cells where repair template availability is limited.

Another major question relates to the functional role of RNA transcripts and R-loops in the repair of transcription-associated DSB. While accumulating evidence supports a positive contribution of RNA templates to HR through DR-loop formation, the molecular determinants that distinguish physiological R-loops from pathological structures remain elusive. It is also unclear which RNA species (pre-existing transcripts, antisense RNAs, or small noncoding RNAs) serve as *bona fide* repair templates in mammalian cells. Identifying the factors that mediate RNA-directed repair without triggering deleterious R-loop-dependent genome instability will be an important direction for future work. In addition, TRCs represent an emerging source of DSBs, but the molecular sensors and signaling cascades that determine whether these lesions are resolved by HR, C-NHEJ, or replication restart mechanisms are still being elucidated. Understanding how transcription factors, chromatin remodelers, and topoisomerases cooperate to prevent or resolve TRCs in human cells remains a key open question. Furthermore, how the phase separation of transcription and repair factors at damage sites affects repair pathway selection and chromatin restoration is an area only recently being explored.

Finally, many questions remain regarding the integration of TCR with epigenetic and metabolic signaling. The influence of histone modifications, RNA modifications (such as m6A), and chromatin topology on TCR efficiency and fidelity is still largely correlative. Future studies combining live-cell imaging, nascent RNA sequencing, and high-resolution proteomics are needed to map these interdependent layers and to define how transcriptional programs shape DNA damage responses at a systems level.

These challenges underscore that transcription-associated DNA repair represents a dynamic and multilayered regulatory network. Deciphering how transcriptional activity, chromatin context, and RNA metabolism jointly determine repair outcome will be essential for understanding genome stability maintenance and its perturbation in cancer and neurodegenerative disease.

4. Posttranslational Regulation of Repair Pathway Choice and Chromatin Modifications

Posttranslational modifications (PTM) orchestrated by chromatin-bound E3 ubiquitin ligases RNF8 and RNF168 critically influence the selection of DSB repair pathways and, ultimately, cell fate [65]. The RNF8-RNF168 ubiquitin ligase cascade constitutes a pivotal signaling module that shapes the outcome of DSB repair. Although the RNF168-ubiquitin-53BP1 branch predominantly favors end joining, and the RNF8-K63-linked ubiquitin-BRCA1/RAD51 route facilitates recombination, both ligases can participate in either pathway depending on the chromatin and cell-cycle context. RNF8 acts as the initial ubiquitin E3 ligase at DNA damage sites, assembling K63-linked chains on histone substrates that recruit both 53BP1 and BRCA1 adaptors. Subsequently, RNF168 amplifies this ubiquitin signal and extends histone modification spreading around the lesion, with its expression levels and chromatin occupancy influencing the balance between C-NHEJ and HR engagement. Evidence from RIDDLE syndrome cells harboring *RNF168* mutations and from *Rnf168*-knockout mice demonstrates that insufficient RNF168 impairs 53BP1 recruitment but not BRCA1 or RAD51, suggesting that higher RNF168 lev-

els favor C-NHEJ, while reduced levels shift preference toward HR [66,67]. Notably, RNF8 and RNF168 do not act in a mutually exclusive manner. Rather than functioning in isolation, RNF8 and RNF168 establish an interconnected ubiquitin-dependent signaling framework that allows the repair machinery to dynamically choose between pathways. This network supports the rapid but error-prone C-NHEJ response as an initial attempt, with the potential to shift toward HR during the S and G2 phases, when sister chromatids provide an available template. Their coordinated ubiquitin activity also influences the behavior of the BLM helicase: RNF8/RNF168-dependent ubiquitylation facilitates BLM recruitment to stalled replication forks, an event linked to the transient attenuation of recombination activity [65]. The principle remains strongly supported by recent investigations. For instance, Chauhan *et al*. (2025) [68] uncovered a SUMOPIN1-dependent mechanism limiting RNF168 chromatin retention and preventing excessive ubiquitin signaling and 53BP1 over-recruitment. Furthermore, Yang *et al*. (2024) [69] described how RNF168 directs ubiquitin-dependent recruitment of DDR factors including 53BP1, reflecting its central role in tuning repair pathway choice.

ASF1, along with its paralogs ASF1A and ASF1B, functions as a histone chaperone facilitating the transfer of H3-H4 dimers to CAF1 for nucleosome assembly. In response to DNA damage, DNA-PKcs-mediated phosphorylation of ASF1A at Ser192 enhances its interaction with histones and promotes HR by supporting MMS22L-TONSL-driven RAD51 loading onto ssDNA [70]. Conversely, phosphorylation of ASF1A at Ser166 by CHK1 (activated via ATM signaling) facilitates C-NHEJ by promoting ASF1A-MDC1 interaction, which enhances MDC1 phosphorylation by ATM, leads to histone ubiquitination, and facilitates 53BP1 recruitment through the H2AX-MDC1-ATM-RNF8 pathway [71]. ASF1, together with RIF1, also promotes C-NHEJ by inducing chromatin compaction around DSBs, thus influencing pathway selection [72]. ASF1's role in repair pathway preference depends on chromatin accessibility and protein recruitment. In heterochromatin, ASF1 associates with 53BP1 and RIF1 to promote C-NHEJ. When resected DNA is present and C-NHEJ is compromised, ASF1 may instead facilitate RAD51 loading and activate HR. Beyond its chaperone activity, ASF1 may exert additional regulatory functions, and its paralogs likely exhibit context-dependent roles in DNA repair.

Specific histone marks and variant incorporation strongly influence which DSB repair pathway becomes engaged. The replacement of canonical histones variants: H3.3 (by CHD2) or H2A.Z (by p400) facilitates the recruitment of proteins associated with end joining [73]. Additional post-translational changes: H4Y51 phosphorylation by TIE2, conversion of H2BK120ub to acetylated H2BK120 via the SAGA complex, and H3K36 dimethylation catalyzed by SETMAR have also been linked to promoting C-NHEJ. Modifications including H2AK15ub (mediated by RNF168), H4K20me1/2, H3K416me1, and H3K79me2 contribute to the recruitment of anti-resection proteins such as 53BP1, thus stabilizing DNA ends and favoring C-NHEJ [74,75]. Furthermore, SETD1A-BOD1L-dependent methylation of H3K4 supports RIF1 recruitment and biases repair toward C-NHEJ [73]. In contrast, HR is favored under chromatin states enriched in macroH2AX and depleted of marks such as H2A.Z, H2BK120ub, H3K4me3, H3K79me2, or H4K12ac near DSB. H3K9 dimethylation promotes the accumulation of BRCA1 and BRAD1, thereby stimulating HR activity. During the G2 phase, acetylation of H2AK15 and H4K16 interferes with 53BP1 binding and facilitates DNA-end resection through BRCA1- and BRAD1-dependent mechanisms [73].

Despite major progress in understanding how PTM and chromatin remodeling influence DSB repair, several mechanistic aspects remain unresolved. It is still unclear how sequential ubiquitination and deubiquitination events mediated by RNF8, RNF168, BRCC36, and USP48 are coordinated to balance 53BP1 versus BRCA1 recruitment. The temporal coupling between histone modification and chromatin accessibility during repair progression also requires clarification, particularly regarding how histone chaperones such as ASF1 and remodelers like INO80 or SMARCA5 interpret and reset specific histone marks. In addition, growing evidence suggests that metabolic intermediates directly modulate chromatin-based repair regulation. Nuclear acetyl-CoA availability shapes histone acetylation landscapes and impacts damage signaling [76,77] while NAD^+ functions as a critical substrate for sirtuins and PARPs involved in chromatin regulation and DSB repair [78]. How cells locally regulate the availability of these metabolites at DNA lesions, and whether metabolic fluctuations bias repair pathway selection toward HR or C-NHEJ, remains an open and important question for future research. Addressing these interconnected issues will be critical for developing a unified model of how PTM networks, chromatin dynamics, and cellular metabolism collectively determine genome stability.

5. Dysregulation of DNA Damage Response and its Implications in Tumorigenesis

Disruptions in DDR mechanisms are key contributors to oncogenesis and malignant transformation. Cells with defective DNA repair systems accumulate genomic instability, which in turn accelerates cancer progression. In addition to chromatin modifications, the stability and activity of DNA repair proteins can be modulated through epigenetic mechanisms, contributing to homologous recombination deficiency (HRD). Epigenetic silencing of *BRCA1* through promoter hypermethylation occurs in a variety of malignancies. Most notably breast and ovarian cancer, but also in uterine, gastric, colorectal, pancreatic, and non-small cell lung tumors [79].

The FANC-BRCA repair axis is frequently compromised in ovarian cancers due to CpG island methylation of HR-related genes; for example, BRCA1 promoter hypermethylation has been directly associated with poor prognosis in high-grade serous ovarian carcinoma [80]. Somatic or germline mutations in several HR components have been implicated in diverse malignancies. Alterations in *CtIP* and *RAD54* have been reported in non-Hodgkin's lymphoma and colorectal cancer, whereas *RAD51B* mutations are recurrent in benign mesenchymal tumors such as lipomas and uterine leiomyomas. Changes affecting *RAD51C* are frequently detected in breast and ovarian cancers, while *PALB2* inactivation is observed in gastro-intestinal tumors. Moreover, defects in the RecQ helicases *BLM* and *WRN* have been broadly linked to genomic instability across multiple cancer types.

Paradoxically, upregulated expression of *RAD51* has been observed in several tumor types; this trend is reinforced by a recent report indicating that *RAD51* upregulation correlates with metastasis and therapy resistance across solid tumors [81]. This overexpression is frequently driven by promoter hyperactivation [82,83] and mutant *KRAS* signaling [84], resulting in elevated DSB repair capacity. However, excessive RAD51 levels are associated with poor clinical outcomes and chemoresistance [81,85], prompting the development of RAD51-targeted inhibitors as a therapeutic strategy [86]. Moreover, comprehensive functional screening such as the "HR eye" assay not only allows ultra-rapid detection of HR activity but has already uncovered previously unrecognized mutations in HR genes like *RAD51D* [87].

Enhanced activity of the C-NHEJ pathway also promotes malignant transformation. Aberrations in *XRCC4*, *LIG4*, and *XLF* have been correlated with worse prognosis and therapy resistance [88], which has justified the development of DNA ligase IV inhibitors [88,89]. A recent study by Hildebrand *et al*. (2025) [90] further exemplifies this approach by testing SCR130, a ligand IV inhibitor, in HNSCC cell lines, where it demonstrated moderate radiosensitizing effects. On the other hand, reduced expression of *53BP1*, *XRCC5*, and *DNA-PKcs* has been shown to favor MMEJ/Alt-EJ, potentially increasing genomic instability through frequent chromosomal rearrangements [2]. This is supported by a study from 2025 showing that the senataxin helicase and DNA-PKcs act redundantly to promote C-NHEJ, underscoring the critical role of DNA-PKcs in maintaining DNA repair fidelity and suggesting that loss of DNA-PKcs function could shift repair toward more error-prone MMEJ/Alt-EJ pathways [91]. Loss of HR capacity forces tumor cells to exploit MMEJ/Alt-EJ as a compensatory repair route. This synthetic vulnerability forms the mechanistic basis for the clinical efficacy of PARP inhibitors in HR-deficient malignancies [2].

Despite growing understanding of how defective DNA repair drives tumorigenesis, several key questions remain unresolved. It is not fully clear how the balance between HR and C-NHEJ is dynamically rewired during malignant transformation or therapy resistance, nor which signaling networks determine whether enhanced repair confers survival advantage or triggers lethal genomic instability. The causal hierarchy among epigenetic events such as *BRCA1* promoter hypermethylation, histone mark alterations, and chromatin compaction also remains to be established. Furthermore, the molecular mechanisms linking oncogenic signaling pathways (e.g., *KRAS*, *MYC*) to the upregulation of *RAD51* and other repair factors are incompletely understood. How tumors fine-tune partial HR deficiency to achieve "repair plasticity" that supports survival under genotoxic stress is an emerging concept that warrants further study. Addressing these questions will be critical for identifying vulnerabilities that can be exploited therapeutically through synthetic lethality and pathway-specific inhibitors.

6. Pathway Choice and Its Impact on Genomic Stability

The selection among DSB repair pathways - C-NHEJ, MMEJ, or HR is a dynamic process that critically influences genome stability. The selection of a repair pathway is influenced by multiple parameters such as the cell-cycle phase, the nature and complexity of the DNA break, the chromatin landscape surrounding the lesion, and the local concentration of repair factors. Central to this regulation is DNA end resection and its tight coordination with cell cycle cues. C-NHEJ predominates in G1-phase, offering rapid, albeit error-prone repair in the absence of a sister chromatid. The S and G2 phases of the cell cycle favor HR, as sister chromatids act as faithful templates for DNA synthesis-based repair. Under conditions where C-NHEJ or HR are defective or saturated, the cell can engage the MMEJ/Alt-EJ mechanism, which relies on short microhomologies and frequently introduces small insertions or deletions.

The pathway selected for repair directly impacts genomic integrity. For instance, during S/G2, ionizing radiation-induced DSB are resolved through both C-NHEJ and HR [10], whereas in G1, C-NHEJ may process resected ends in a way that promotes chromosomal translocations [152,153]. C-NHEJ, while efficient, may introduce small insertions or deletions, and excessive reliance on it can cause gross chromosomal abnormalities. Although HR tends to restore DNA faithfully, alternative processing routes like SSA or MMEJ/Alt-EJ following end resection can lead to deletions and genomic rearrangements [98,114,154]. MMEJ/Alt-EJ, being intrinsically inaccurate, is a known driver of such mutagenic outcomes.

Hence, while the plasticity in repair pathway choice supports cellular adaptability, its dysregulation (frequent in cancer) exacerbates genomic instability, fostering malignancy and therapeutic resistance. Understanding the molecular control of DNA repair provides the foundation for pre-

cision treatments that leverage cancer-specific repair weaknesses.

7. Conclusions

This review has summarized the complex regulatory networks that govern the cellular preference between error-prone DNA DSB repair pathways such as C-NHEJ and MMEJ/Alt-EJ and the high-fidelity HR pathway. We have outlined how key molecular players (ranging from chromatin modifiers and posttranslational regulators to transcription-coupled and metabolic factors) coordinate the dynamic "switch" between these routes. In each thematic section, we have highlighted outstanding questions that remain unresolved, underscoring the multifaceted nature of DSB repair regulation and its context dependence.

Given the central role of these pathways in maintaining genomic integrity, many of their core regulators have emerged as therapeutic targets, and several inhibitors are currently being evaluated for the treatment of repair-deficient cancers [155–157]. Advances in functional genomics, particularly CRISPR/Cas9-based screening technologies, continue to uncover novel repair components and to identify synthetic lethal interactions among HR, C-NHEJ, and accessory genes [158–161]. Nevertheless, a major clinical obstacle remains the development of resistance to existing inhibitors — most notably PARP, ATR, ATM, WEE1, and PRMT inhibitors — often through the restoration of HR activity [162,163]. Consequently, strategies aimed at achieving complete HR suppression, or at dual inhibition of HR and C-NHEJ, may offer improved therapeutic efficacy in specific tumor contexts [164–166]. The integration of mechanistic insight, functional screening, and targeted therapy design will be essential for translating our growing understanding of DSB repair into precision oncology. By systematically addressing open questions across each level of DSB regulation, future research can bridge molecular mechanisms with clinical applications, ultimately enabling rational combination therapies that exploit the vulnerabilities of repair-deficient cancers.

Author Contributions

Conceptualization, methodology, resources, writing-original draft preparation, writing-review and editing, visualization, project administration, funding acquisition — LZ. The author has read and approved the final version of the manuscript and is accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

I sincerely thank the reviewers for their constructive and insightful comments, which greatly contributed to improving the clarity and quality of this manuscript.

Funding

This research was funded by Vedecká grantová agentúra (VEGA) - Grant Agency of the Slovak Republic, grant number 2/0140/23 and the Recovery and Resilience Plan of the Slovak Republic under the project [CU-ESG-2024-05-PD] - administered by the RESEARCH AGENCY under the Grant Agreement No. 09I03-03-V05-00019.

Conflict of Interest

The author declares no conflict of interest.

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