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Review

Double-Stranded Break Repair in Mammalian Cells and Precise Genome Editing

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Abstract: In mammalian cells, double-strand breaks (DSBs) are repaired predominantly by error-prone non-homologous end joining (NHEJ), but less prevalently by error-free template-dependent homologous recombination (HR). DSB repair pathway selection is the bedrock for genome editing. NHEJ results in random mutations when repairing DSB, while HR induces high-fidelity sequence-specific variations, but with an undesirable low efficiency. In this review, we first discuss the latest insights into the action mode of NHEJ and HR in a panoramic view. We then propose the future direction of genome editing by virtue of these advancements. We suggest that by switching NHEJ to HR, full fidelity genome editing and robust gene knock-in could be enabled. We also envision that RNA molecules could be repurposed by RNA-templated DSB repair to mediate precise genetic editing.

Keywords: DSB repair; NHEJ; HR; RNA template; genome editing

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1. DSB Induction and Pathway Choice

In mammalians, genome editing through CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) nuclease systems can induce double-strand breaks (DSBs), initiating one of the conserved repertoire repair pathways, depending on the type of damage, cellular context, and phase of the cell cycle [1,2]. CRISPR/Cas9 genome editing actually takes advantage of DSB repair pathways to introduce variations into the user-defined genomic loci. Eukaryotes possess highly coordinated repair processes, and DSBs can be repaired through non-homologous end joining (NHEJ) or homologous recombination (HR) pathways. Broken DSB ends activate phophotidylinositol-3 kinase-like kinases (PIKKs) (Figure 1a), which phosphorylates histone H2AX (known as γH2AX) leading to cell cycle arrest, a pause in the local transcription of RNA polymerase-I (RNAP-I), RNA polymerase-II (RNAP-II) activation, and up-regulation of genes encoding DNA damage repair factors [3–6]. γH2AX facilitates in the activation of NHEJ through 53BP1 and KU (Ku70/Ku80) heterodimers [7–9], or it can interact with NBS1, through the localization of the MRN (MRE11-RAD50-NBS1) and BRCA1-CtIP (C-terminal binding protein interacting protein) complex at the DSB site to initiate HR repair pathway, as shown in Figure 1b [1].

In this review, we outline the molecular processes of NHEJ and HR and envision the future developments of genome editing by taking advantage of these insightful comprehensions. We also highlight the damage-induced RNAs and RNA-template DSB repair for increasing the genome editing fidelity and efficiency.

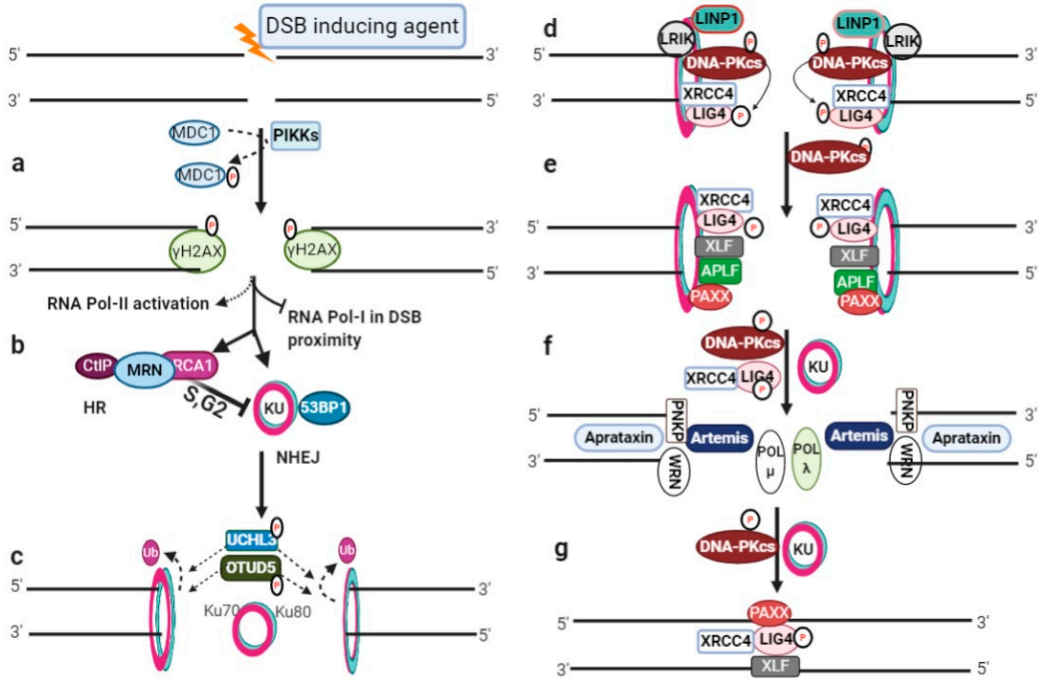

Figure 1. DSB induction and NHEJ repair pathway: (**a**) DSB initiates phosphorylation by activated phophotidylinositol-3 kinase-like kinases (PIKKs) and phosphorylation of H2AX (γH2AX) for cascade signaling; (**b**) RNA polymerase I-based local transcription inhibition in the DSB proximity, RNA polymerase II activation for damage-induced RNA generation, and the recruitment of NHEJ or HR repair machinery; (**c**) KU heterodimer binding at broken ends for NHEJ repair initiation and deubiquitylation of the Ku80 subunit; (**d**) DNA-PKcs autophosphorylates and then phosphorylates LIG4. LINP1 and LRIK are non-coding RNAs that stabilize the KU–DNA complex; (**e**) recruitment of XLF, APLF, and PAXX; (**f**) end processing by Artemis nuclease and other end processing molecules, and gap filling by polymerases; (**g**) LIG4 ligate-processed ends assisted with XRCC4, PAXX and XLF.

2. Non-Homologous End Joining (NHEJ)

NHEJ is the predominant, template-independent repair pathway and can potentially re-ligate any type of damaged DNA ends throughout the cell cycle [2,3]. NHEJ can be subdivided into three main sub-sequential steps, namely, (i) DSB end recognition and recruitment of NHEJ machinery, (ii) DNA ends processing, and (iii) DNA ends ligation. The schematic presentation of NHEJ repair pathway is illustrated in Figure 1c–g.

2.1. DSB Ends Recognition and Recruitment of NHEJ Machinery

DSB ends are encircled by a stable ring-shaped heterodimer KU protein (150 kDa) composed of Ku70 (70 kDa) and Ku80 (86 kDa) molecules (Figure 1c). Ku70 and Ku80 have a conserved secondary structure; the N-terminal (von Willebrand) and central core bind with DNA while the C-terminal is mainly involved in protein–protein interactions [4–6]. KU binding to DSB ends is essential and is an initial key step in NHEJ-repair pathway [7–9]. KU binds with the sugar backbone and possesses a high binding affinity with linear dsDNA compared with supercoiled, circular, or ssDNA [4,7,10,11]. KU retention at the broken ends is enhanced by Ku80 deubiquitylation mediated by UCHL3 and OTUD5 [12,13]. This KU–DNA complex acts as scaffold for the recruitment of other NHEJ molecules (Figure 1d). KU recruits DNA-PKcs or XRCC4–LIG4, depending on the nature and/or complexity of the DNA damage [2]. DNA-PKcs autophosphorylates and its retention is facilitated by the Ku80 C-terminal domain [9,14–16]. Long noncoding RNAs (LRIK and LINP1) also increase the binding ability of KU with DSB ends [17,18].

The XRCC4–LIG4 complex could also be localized at DSB in a DNA-PKcs-independent manner [19]. The N-terminal domain of XRCC4 interacts with the Ku70 subunit and the

C-terminal mediates LIG4 binding with KU [20]. Hence, XRCC4 serves as a tether between KU and LIG4 [8]. LIG4 possesses two BRCT domains known as BRCT1 and BRCT2. The inter-BRCT region and BRCT2 domain bind with the C-terminal region of XRCC4 [21]. The formation of the KU–XRCC4–LIG4 complex is an important rate-limiting step in the DNA-ends joining process. DNA-PKcs phosphorylates LIG4 to stabilize the KU–XRCC4–LIG4 complex [22,23].

Other important molecules (Figure 1e) such as XLF and APLF bind with the vWA domain of the Ku80 subunit [24]. In turn, APLF increases XRCC4–LIG4 and XLF retention [25]. XLF interacts with DNA-PKcs [26]. PAXX binds with the Ku70 unit of the KU heterodimer and is stabilized with DNA extension [27].

2.2. DNA Ends Processing

Depending on the nature of the DNA broken ends, different molecules may be required (Figure 1f) for removing the functional groups involved in blocking ends, resecting ends, making ends ligatable, and gap filling. A number of factors are involved in DNA ends processing (Table 1).

The primary and abundant nuclease that is required is Artemis, which interacts with the FAT domain of DNA-PKcs and the N-terminus region of LIG4 [28–31]. Mentase and Artemis can trim 3′overhangs, but are more efficient later [32]. Artemis and TDP1 have the ability to catalyze the removal of the 3′phosphoglycolate (3′PG) group from DNA ends [33,34]. Similar to Artemis, the KU–XRCC4–LIG4–APLF complex can also reset 3′overhangs [35]. DNA broken ends may have non-ligatable groups such as 3′phosphate or 5′hydroxyl. These blocking groups can be removed in order to convert into ligatable. PNKP has a kinase domain to incorporate phosphate groups at 5′-OH, preferentially at a double-stranded substrate, while the phosphatase catalytic domain can dephosphorylate single-stranded and double-strand termini 3′-phosphate groups [36]. Aprataxin can specifically release adenylate groups covalently attached with the 5′-phosphate termini of single-strand nicks and gaps to rejoin ends efficiently [37]. The lyase activity of KU has been demonstrated by the Ku80 subunit, which can cleave the apurinic/apyimidinic (AP) site from the partial DNA duplex with 5′- and 3′-protruding ends [38,39]. Gap filling in NHEJ is mainly performed by DNA polymerase μ (Polμ) and λ (Pol λ), which possess functional redundancy [40]. Polμ and Polλ interact with KU and XRCC4–LIG4 via BRCT domains [41,42]. Polλ performs a fill-in activity of terminally compatible overhangs in a template-dependent manner [43]. Polμ has both template-dependent and -independent activities [44,45]. It is also competent to add dNTP as well as rNTP generating regions of the terminal microhomology for subsequent pairing and ligation processes. Thus, Polμ strongly promotes the joining of two DNA strands with incompatible 3′-overhangs [46,47]. TdT also has the ability to incorporate nucleotides in a template-independent manner, but is mainly transcribed in lymphocyte [48].

2.3. DNA Ends Ligation

LIG4 is the key molecule and belongs to the superfamily of nucleotidyl transferases that can introduce phosphodiester bonds to join broken ends (Figure 1g). PAXX and XLF are also important components of the ligation process. Despite a small sequence homology, both PAXX and XLF share structural similarity with XRCC4 [49]. PAXX has been demonstrated to promote the ligation of blunt ends in the presence of the KU–XRCC4–LIG4 complex [50]. XLF can stimulate the ligation of incompatible 3′-overhangs interacting with KU and XRCC4 [46,49]. However, in the presence of DNA-PKcs, XLF has the ability to ligate non-cohesive overhangs [51]. Both PAXX and XLF can also promote KU stability and possess redundancy in the ligation of DNA ends [52]. Recently, it has been revealed that XRCC4 is essential for DNA ligation in association with LIG4, PAXX, and XLF [53].

Table 1. NHEJ-associated key factors with gene location based on human genome assembly (GRCh38.p14) and their functions.

No.	Factor	Gene	Location (*H. sapiens*)	Function in NHEJ
1	DNA-dependent protein kinase catalytic subunit	*PRKDC/XRCC7*	8q11.21	Associated with phosphorylation and essential complex formation [54]
2	Polo-like kinase 1	*PLK1/STPK13*	16p12.2	Phosphorylates DNA-PKcs [55]
3	Polynucleotide kinase/phosphatase	*PNKP/PNK*	19q13.33	Removal of 3′-phosphates [56]
4	Artemis	*DCLRE1C*	10p13	Endonuclease 5′ to 3′ and prevents ends resection [57]
5	DNA ligase IV	*LIG4*	13q33.3	Ligates DNA ends [58]
6	Aprataxin- and PNK-Like Factor	*APLF/PALF*	2p13.3	Scaffold for recruitment of XRCC4–LIG4 and XLF [25] and competent to reset 3′-overhangs [35].
7	Ku70	*XRCC6*	22q13.2	Core component of KU, acts as scaffold for recruitment of NHEJ machinery [59]
8	Ku80	*XRCC5*	2q35	Part of KU and acts as scaffold
9	lncRNA NHEJ pathway 1	*LINP1*	10p14	Modulator scaffold for KU and DNA-PKcs [18,60]
10	Paralog of XRCC4 and XLF	*PAXX/XLS*	9q34.3	Interacts with KU and Polλ to stimulate fill-in gap as well as ends ligation [27,61]
11	Rap1-Interacting Factor 1	*RIF1*	2q23.3	Forms a 53BP1–RIF1 complex to protect from ends resection [62]
12	XRCC4-like factor	*XLF/NHEJ1*	2q35	Interacts with KU and XRCC4 enabling synaptic complex formation required for ends ligation [63,64]
13	X-ray cross complementing protein 4	*XRCC4*	5q14.2	Core component of NHEJ complex [65]
14	Zinc-finger protein 281	*ZNF281/ZBP−99*	1q32.1	Promotes XRCC4 recruitment and interacts with DNA-PKcs and Ku70 [66]
15	Ubiquitin carboxyl terminal hydrolase L3	*UCHL3*	13q22.2	Deubiquitylates Ku80 and enhances its retention [12]
16	OTU Deubiquitinase 5	*OTUD5*	Xp11.23	Deubiquitylates Ku80 and regulates NHEJ [13]
17	TP53 binding protein 1	*53BP1/TP53BP1*	15q15.3	Inhibitor of BRCA1 and key player defining the DSB repair pathway choice [67,68]

3. Homologous Recombination (HR)

In eukaryotes, accurate DSB repair is performed by HR, which requires sister-chromatid/repair-template generally restricted to the S/G2 phase of the cell cycle. HR can be categorized into subsections (i) DNA ends resection, (ii) RAD51-nucleofilament formation, (iii) homology searching, (iv) hDNA formation and D-loop extension, and (vi) D-loop dissolution.

3.1. DNA Ends Resection

In HR, DSB ends must undergo 5′- and 3′-nucleolytic degradation to generate 3′-ssDNA tails, referred to as ends resection. Activated γH2AX histone interacts with NBS1 and facilitates in the localization of MRN (MRE11–RAD50–NBS1) complex at the DSB site [1]. NBS1 also binds with ATM to render a signaling role of MRN and the recruitment of other molecules [69]. ATM phosphorylates and activates BRCA1 to bind with MRN and facilitates in 53BP1 removal [70], and thus inhibits the NHEJ repair pathway (Figure 2a). RAD50 regulates the MRN affinity with DNA in an ATP-dependent manner [71]. MRE11 binds with DNA ends to perform 3′- and 5′-exonuclease on dsDNA and 5′- and 3′-endonuclease on the ssDNA activity, generating 3′-ssDNA overhangs [72,73]. MRE11 also interacts with CDK2 to facilitate CtIP phosphorylation and stability [74]. This phosphor-mimic CtIP promotes the endonuclease activity of MRN [75]. Short resected 3′-ssDNA tails are covered by RPA in a sequential manner to prevent KU binding, thereby inhibiting the NHEJ repair pathway [76,77].

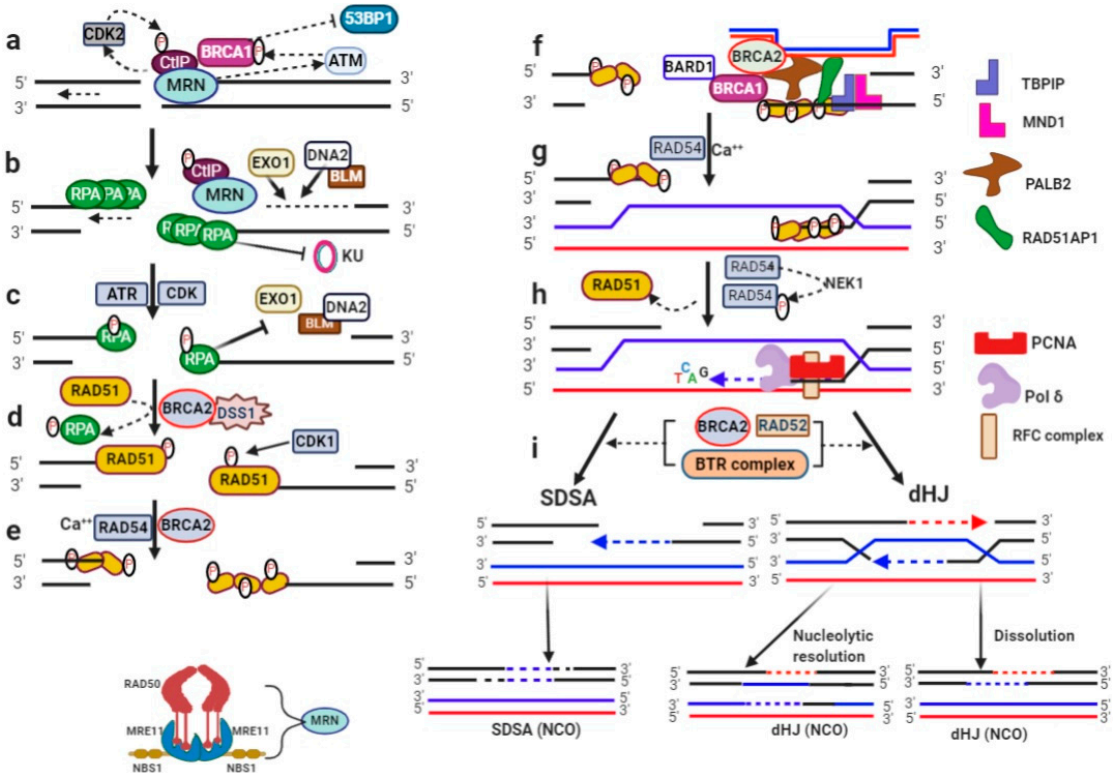

Figure 2. DSB repair via HR: (**a**) MRN complex binding with DSB ends, phosphorylation of CtIP and BRCA1 to activate MRN complex and inhibiting 53BP1; (**b**) long-range resection initiation via the EXO1 or BLM-DNA2 pathways, RPA covering of 3′-ssDNA tails, and KU inhibition; (**c**) RPA phosphorylation (pRPA) by ATR and CDK to negatively regulate resection; (**d**) replacement of pRPA with RAD51 mediated by BRCA2–DSS1 complex; (**e**) RAD51-nucleofilament formation; (**f**) RAD51AP1 and the TBPIP–MND1 complex stabilize RAD51-NF, PALB2 forms a complex with BRCA1, BRCA2, and DNA, and the BRAD1–BRCA1 complex in homology searching; (**g**) synaptic complex and D-loop formation; (**h**) DNA synthesis and D-loop extension; (**i**) D-loop dissociation and annealing via SDSA, in which newly synthesized DNA anneals with ssDNA tails or dHJ formation and dissolution into non-crossover products.

RPA, MRN, and CtIP stimulate extensive resection preceded by two non-overlapping pathways (Figure 2b). One is executed by EXO1 and the other via DNA2 nuclease in concert with BLM helicase. BLM forms a BTR complex with TOPOIIIα-RMI1/2. BLM enforces 5′- and 3′-resection by DNA2, and also enhances the EXO1 affinity for DNA ends [78]. CtIP influences long-range ends resection beyond MRN regulation, it can activate the BLM helicase and motor activity of DNA2 [79,80]. CtIP also interacts with EXO1 for localization at DSB [81], whereas MRN and RPA stimulate EXO1-dependent resection [78]. CtIP can also inhibit over-resection, thus acting as a DNA resection regulator [82].

RPA is necessary for 3′-ssDNA strand protection from degradation, DNA unwinding by BLM, and the accelerated 5′-3′ nucleolytic function of DNA2 [78,83]. However, phosphorylated RPA (pRPA) inhibits BLM and ssDNA accumulation (Figure 2c), therefore pRPA negatively regulates DNA ends resection [84,85].

3.2. RAD51-Nucleofilament Formation

After ends resection, the ssDNA–RPA interaction is destabilized and pRPA is replaced with RAD51 [86]. RAD51 mediator proteins stimulate it to form a bridge with ssDNA to displace RPA [87,88]. In vertebrates, BRCA2 is suggested to be a more critical mediator in RPA–RAD51 exchange [89]. It has been revealed that BRCA2 forms a complex with DSS1 (Figure 2d) and replaces RPA with RAD51 [90]. RAD51 forms a nucleoprotein filament (NF), a necessary event for the subsequent homology search, strand invasion, and

replication fork protection. RAD51-NF assembly/disassembly represents the ability of rapid and high covering ssDNA via a decreased binding tendency with the DNA sequence. RAD51 ATPases show a complex behavior based on bound nucleotide co-factors during HR [87,91,92]. ATP increases RAD51 affinity with DNA, and RAD51-ATP binds with ssDNA and stretches the DNA B-form length to 150% [93]. RAD51 binds with non-hydrolyzable ATP analogs and forms extended form filaments (99 Å), while the RAD51-ADP filament is compressed and low pitch (76 Å) [92]. RAD51-NF formation is mainly driven by BRCA2, but RAD54 also stabilizes it, independent of its ATPase activity [94,95]. Calcium promotes RAD51-NF formation and stabilizes RAD51-ATP (Figure 2e) [96,97].

3.3. Homology Searching

RAD51-NF interacts with dsDNA to find a complementary sequence for homology search in the DSB vicinity. In vertebrates, RAD51AP1, TBPIP-MND1 (heterodimer of TBPIP and MND1), and PALB2 (partner and localizer of BRCA2) are important in RAD51-NF bridging with dsDNA (Figure 2f) [98–100]. RAD51AP1 is capable of binding with ssDNA and dsDNA, but possesses the highest affinity for branched DNA structures obligatory during HR [101]. TBPIP-MND1 stabilizes the RAD51-NF structure and enhances its ability to capture duplex DNA, which is an essential intermediate step during the synaptic complex [102]. BRCA1 stimulates PALB2 to form a ternary BRCA1–PALB2–BRCA2 complex [103]. PALB2 binds with DNA and stimulates RAD51 recombinase to form a D-loop structure. The BRCA1–BARD1 complex has also been reported in RAD51-mediated homologous pairing [104].

The RAD51–ssDNA–dsDNA complex uses a nucleotide-length specific recognition mechanism for homology searching. The microhomology of the eight-nucleotide tract is sufficient for robust kinetic selection. Successful pairing with the ninth base reduces the binding free energy and the subsequent strand exchange initiates precisely in three base steps, indicating the triplet-base arrangement of the presynaptic complex [105,106].

3.4. Heteroduplex DNA Formation and D-Loop Extension

During the homology search, RAD51-NF probes and interacts with donor dsDNA to form a synaptic complex. The invading strand 3′-ends intertwine with the donor complementary sequence to form heteroduplex DNA (hDNA). Original base pairing is disrupted during hDNA formation, and this intermediate is known as displacement or D-loop. In humans, RAD51 can accomplish D-loop formation on its own in the presence of Ca++, while RAD54 accelerates the process (Figure 2g) [97,107,108]. RAD54 possesses ATPase to prevent non-productive intermediates during D-loop formation [109], as well as RAD51-promoted translocase activity to create the hDNA junction [110]. In the late G2 phase, never-in-mitosis A-related kinase 1 (NEK1) phosphorylates RAD54 to turn over RAD51 from hDNA to orchestrate HR [108].

As shown in Figure 2h, hDNA formation is favored at the 3′-ends sequences compared with the internal homologous region [110]. DNA synthesis essentially requires 3′-ends of invading strands to be intertwined in hDNA, forming a primer–template junction [111]. The minimum required components for D-loop extension, as shown in Figure 2f,g, are RAD51, RAD54, DNA polymerase δ (Polδ), proliferating cell nuclear antigen (PCNA), and replication factor-c (RFC1-5) [112–114].

Polδ can replicate both invading and complementary strands [115]. Polδ is stimulated and accelerated for hDNA extension by PCNA and its loader RFC1-5 [112]. It was recently revealed that Cft18-RFC and Polε form a stable clamp loader/polymerase complex that favors leading strand DNA synthesis, and it is helpful in leveling the PCNA balance in the replication fork [116,117]. Lengths of hDNA sequence vary and may be hundreds of bases long [110]. Extension continues until a topological block is encountered. Topoisomerases can induce negative supercoiling (∼10.5 bp/turn) to relax donor DNA ahead of D-loop extension [118,119]. However, the exact mechanism of bubble migration during DNA synthesis in hDNA is to be determined. Interestingly, MCM8 and MCM9 have been

proposed as HR helicases involved downstream of RAD51 processing [120]. However, the detailed mechanism has not been established yet.

3.5. D-Loop Dissolution

D-loop disruption is ultimately required to anneal extended strands with the second resected ends [121,122]. However, it is also essential to remove partial homologous donors and to prevent ssDNA invasion to more than one donor [123]. Additionally, it also decreases the probability that both DSB ends will concurrently invade donor DNA, allowing DNA synthesis as well as maturation to the double Holliday junction (dHJ) and cross-over (CO) outcomes [124,125]. Several proteins are associated with D-loop disruption, but Sgs1, Mph1, and Srs2 are major helicases in *S. cerevisiae* that act in distinct ways, without significant overlapping [126–128]. In vertebrates, functional orthologs to Sgs1, Mph1, and Srs2 are BLM, FANCM, and FBH1, respectively [129–131].

After D-loop disruption, the annealing of nascent DNA homologous is the next step. RAD52 and BRCA2 are the key driving molecules to anneal ssDNA in vertebrates [132,133]. However, second-end annealing is unclear, either by annealing or invasion, regarding restoring the genomic integrity and CO avoidance [118]. It is evident that CO avoidance tendency favors synthesis-dependent strand annealing (SDSA), an alternate processing of extended D-loops by the dissociation and annealing of newly synthesized DNA with ssDNA tails from the other end (Figure 2i) [134]. Evidence shows that all three helicases promote SDSA, thus avoiding a loss of heterozygosity [127]. Although SDSA is a preferred pathway, sometimes dHJs are formed either by second-end annealing with displaced strands of D-loop or by both resected ends to invade donor DNA, and are prolonged through DNA synthesis [135]. These dHJs can be processed to generate CO or non-CO (NCO) products (Figure 2i). The BTR complex has the ability to induce the branch shift of two HJs and to resolve topological limitations resulting in NCO outcomes [136,137]. However, long hDNA sequences favor dHJs and COs [138,139]. These COs may lead to a risk of loss of heterozygosity [140].

4. Roles of RNA Transcripts in DSB Repair

4.1. dincRNAs in DSB Repair

DSB induction inhibits RNA polymerase I (RNAP-I) to down-regulate ongoing local transcription, while it activates RNAP-II promoter-independent activity to generate damage-induced ncRNAs (dincRNA), as shown in Figures 1 and 3 [141]. These dincRNAs can be categorized on the basis of size into long (dilncRNA) >200 nt and small ncRNAs with about 21 nt [142]. Long ncRNAs (lncRNAs) act as a precursor for small ncRNAs, analogous to miRNA biosynthesis [143]. These dincRNAs are exported to the cytoplasm via RNA binding proteins (RBPs), and protect the cell by degrading truncated mRNA [142,144,145]. The role of dincRNAs in the DSB repair process is controversial, as Michelini et al. identified that the MRN complex recruits RNAP-II in the DSB vicinity for DICER or AGO2-mediated HR repair [142]. However, DICER inactivation is reported to reduce 53BP1 foci formation, suggesting its role in the NHEJ repair pathway [146]. Similarly, pre-existing dincRNA transcripts have been reported prior to DSB induction by Bader and Bushell [147], indicating that additional studies are needed in order to understand the detailed molecular mechanisms.

4.2. RNA-Template Mediated DSB Repair

The RNAP-I transcription pause transcribing DSB region is essential to halt truncated mRNA synthesis. Moreover, it is also important, as nascent RNA is proposed to serve as a template [148]. As proposed by Lavigne et al., nascent transcripts or pre-mRNAs are available for transcript-templated repair shortly after the initiating lesion [149]. It is widely accepted that RNAs can pair with homologous DNA strands to form RNA–DNA hybrids [144,150]. RNA-binding proteins (RBPs) such as THRAP3, DICER, DHX9, Drosha, and Senataxin are important driving factors [146,151–154]. These possess different functions, for example Drosha promotes RNA–DNA hybrids, while Senataxin and RNase

H2 resolve [144,154–156]. A plethora of studies indicate that RNA–DNA hybrids have direct roles in DSB repair [145,157,158]. Mazina et al. revealed that RPA has a higher RNA binding affinity than DNA, and RAD52 is able to invert the strand exchange between homologous DNA and RNA [159]. It is evident that defective H-RNases could stimulate homologous RNA-mediated DNA repair more than the homologous DNA-template in the presence of RPA [160]. The emerging picture is that dincRNAs are important for truncated mRNA degradation as well as cascade signaling, and RNA molecules homologous to DSB ends could serve as a template for faithful repair through HR. An interesting finding about DNA polymerase θ (Polθ) has revealed its reverse transcription ability, similar to reverse transcriptases in retrovirus. Polθ is efficient in the incorporation of deoxyribonucleotides on RNA versus DNA, and promotes RNA-templated DNA repair in mammalian cells [161]. This suggests the significance of RNA as a template in DNA repair.

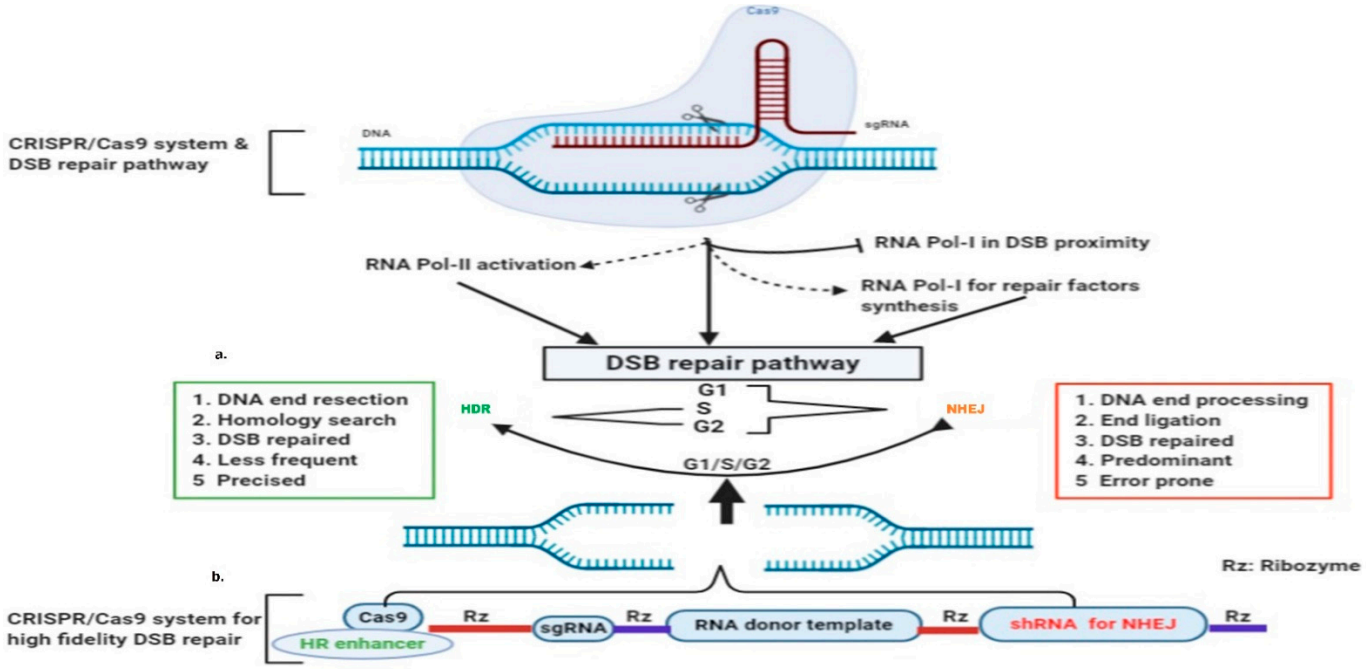

Figure 3. CRISPR-Cas9 induced DSB and RNA-templated high-fidelity repair system: (**a**) Cas9 induced broken ends pause RNA polymerase-I transcription in the DSB vicinity, and activate RNA Polymerase-II and RNA polymerase-I for the synthesis of DSB repair factors; (**b**) single construct for Cas9 expression ribozyme mediated transcription of sgRNA, donor template with desired sequence, and shRNA for NHEJ suppression.

4.3. RNA versus DNA as a Donor Repair Template

Both single-stranded oligonucleotides (ssODNs) and double-stranded DNA (dsDNA) are used in genome editing (GE). ssODNs provide higher insertion efficiencies over the dsDNA template [162]. However, ssODNs require efficient delivery in the nuclear region to avoid degradation from ssDNAases [163]. ssODNs can also generate null alleles and are inefficient when applied at scale [164]. The other main disadvantage of ssODNs is their size limitation. Although limited data are available, it has been demonstrated that DSBs can be repaired via the RNA donor template repair pathway [160,165,166].

5. DSB-Oriented Genome Editing and Future Prospects

The discovery of the CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated) system has revolutionized the genome editing (GE), owning to its high efficiency in introducing DSB into the user-defined locus. Accelerated advancements in CRISPR/Cas9 technology have validated its therapeutic applications [167]. Precise GE mainly depends on the DSB repair pathway, and Cas9-induced DSB repair tends to be

error prone [168]. Considering the aforementioned DSB repair pathways and the role of RNA molecules, we recommend that faithful and effective GE requires further assistance to ensure HR-mediated DSB repair. Plenty of evidence has revealed that NHEJ is error prone but predominant, so precise editing essentially requires the inhibition of the NHEJ repair pathway. This could be achieved through RNA interference degradation, drug mediated inhibition, or altering the protein sequence of NHEJ driving factors (listed in Table 1) such as 53BP1, DNA-PKcs, Ku70, Ku80, and LIG4 [169–173]. Similarly, plenty of evidence has shown that HR is inherently less efficient in DSB repair, which could be improved through HR enhancers (Table 2). RAD51, RAD52, MRE11, and CtIP have shown a significant increase in template dependent repair when tethered with Cas9 [174–176]. Here, we also suggest CtIP as a preferred choice due to its dual role as an HR enhancer and regulator for over-resection [82]. In addition to these, covalently-linked donor repair-oligonucleotides with benzylguanine to Cas9 could also substantially favor HR [177,178]. Single-stranded oligonucleotides (ssODNs) used in GE provide higher insertion efficiencies over the dsDNA template [162]. However, ssODNs require efficient delivery in the nuclear region to avoid degradation from ssDNAases [163]. It has been demonstrated that DSBs can be repaired via the RNA donor template repair pathway [160,165,166]. It is proposed (Figure 3) that nascent RNA or pre-mRNA transcribed from the DSB spanning region could act as a template [149]. In order to enhance co-localization of the RNA donor repair template, we suggest that it could be generated endogenously through ribozymes, and then annealing of this donor to the complement homologous DSB ends can be catalyzed by RAD52 even more efficiently than DNA–DNA [179–182].

Table 2. Key molecules associated with the HR repair pathway and their gene location based on human genome assembly (GRCh38.p14).

No.	Factor	Gene	Location (*H. sapiens*)	Function in HR
1	Breast cancer gene 1	*BRCA1*	17q21.31	Inhibits 53BP1 to prevent NHEJ
2	Breast cancer gene 2	*BRCA2*	13q13.1	Promotes RPA replacement with RAD51 [90]
3	Bloom's helicase	*BLM*	15q26.1	Unwinds DNA substrates including Holliday junction [130]
4	CtBP (C-terminal binding protein) interacting protein	*CtIP*	18q11.2	Multifunctional role in HR [79,80]
5	DNA replication helicase/nuclease 2	*DNA2*	10q21.3	Long-range end resection role concerted with BLM [78]
6	Deleted in split hand/split foot type 1	*DSS1*	7q21.3	Forms a complex with BRCA2 to replace RPA with RAD51 [90]
7	Exonuclease 1	*EXO1*	1q43	Executes long-range end resection [183]
8	Meiotic nuclear divisions protein 1	*MND1*	4q31.3	TBPIP-MND1 complex facilitates in homologous strand search [98,102]
9	Meiotic recombination 11	*MRE11*	11q21	Perform nuclease activity generating 3′-ssDNA overhangs [72,73]
10	Nijmegen breakage syndrome 1	*NBS1*	8q21.3	Binds with DSB and ATM to render a signaling role of MRN [69]
11	Partner and localizer of BRCA2	*PALB2*	16p12.2	Forms a BRCA1-PALB2-BRCA2 complex to stimulate RAD51 [103]
12	Proliferating cell nuclear antigen	*PCNA*	20p12.3	Stimulates Polδ-based hDNA extension [112].
13	RAD50	*RAD50*	5q31.1	Forms an ATP-dependent compact structure with dsDNA [71]
14	RAD51	*RAD51*	15q15.1	Forms a nucleoprotein filament (NF), necessary for subsequent homology search, strand invasion, and replication fork protection [87,91,92]
15	RAD51 associated protein 1	*RAD51AP1*	12p13.32	Binds with ssDNA and dsDNA to facilitate D-loop formation [101]
16	RAD54	*RAD54*	Xq21.1	Multifunctional and possess an ATPase activity to prevent non-productive intermediates in D-loop formation [109]
17	Replication protein A1	*RPA1*		Multifunctional, 70kDa main DNA binding subunit of RPA
18	TBP-1 interacting protein	*TBPIP*	17q21.2	TBPIP forms a complex with MND1 during homology search [102]

We conclude that the silencing of NHEJ driving factors as well as up-regulation of the HR repair pathway through modified Cas9 could be promising to uplift RNA-template-mediated genome editing efficiency and fidelity.

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Abbreviations

DSB	Double-strand break
NHEJ	Non-homologous end joining
HR	Homologous recombination
dsDNA	Double-strand DNA
ssDNA	Single-strand DNA
hDNA	Heteroduplex DNA
KU	Ku70/Ku80 heterodimer
LIG4	DNA ligase 4
MRN	MRE11-RAD50-NBS1
RPA	Replication protein A
dHJ	Double holliday junction
SDSA	Synthesis dependent strand annealing
NCO	Non-crossover
CtIP	CtBP (C-terminal binding protein) interacting protein
UCHL3	Ubiquitin carboxyl-terminal hydrolase isozyme L3
OTUD5	OTU Deubiquitinase 5
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
XRCC4	X-Ray Repair Cross Complementing 4
LIG4	DNA ligase-4
LRIK	lncRNA interacting with Ku
LINP1	LncRNA in non-homologous end joining (NHEJ) pathway 1
BRCT1	BRCA1 C-terminal
BRCT2	BRCA2 C-terminal
XLF	XRCC4-like factor
APLF	Aprataxin and PNKP-like factor
PAXX	Paralog of XRCC4 and XLF
PNKP	Polynucleotide Kinase 3′-Phosphatase
dNTP	Deoxynucleoside triphosphate
rNTP	Ribonucleoside triphosphate

TdT	Terminal deoxynucleotidyl transferase
RAD51	RAD51 Recombinase
MRE11	Meiotic recombination 11
NBS1	Nijmegen breakage syndrome 1 protein
H2AX	H2A histone family member X
CDK2	Cyclin-dependent kinase 2
EXO1	Exonuclease 1
BLM	Bloom Syndrome Helicase
BTR complex	Bloom helicase, topoisomerase IIIα (TOP-IIIα), and the RMI1/2
TOPOIIIα	Topoisomerase IIIα
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
RAD51-NF	RAD51-nucleoprotein filament
RAD51AP1	RAD51 associated protein 1
TBPIP	TBP-1 interacting protein
MND1	Meiotic nuclear divisions protein 1
PALB2	Partner and Localizer of BRCA2
NEK1	Never-in-mitosis A-related kinase 1
POL δ	DNA polymerase δ
PCNA	Proliferating cell nuclear antigen
RFC1-5	Replication factor-c 1-5
dilncRNA	Damage-induced long ncRNAs
DICER	Endoribonuclease encoded by DICER1 gene
AGO2	Argonaute RISC catalytic component 2
THRAP3	Thyroid hormone receptor associated protein 3
DHX9	DExH-Box Helicase 9
ssODNs	Single-stranded oligonucleotides
FANCM	Fanconi anemia, complementation group M
FBH1	F-box DNA helicase 1

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