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Investigating the DNA Damage Response Elicited by Transposable Element Integration

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ABSTRACT

All organisms encounter DNA damage daily through UV exposure, carcinogens, and more. Therefore, there must be a conserved system in place to repair such damage. The DNA Damage Response (DDR) is the conserved system that protects and repairs DNA lesions and breaks. It is known that some mobile genetic elements, such as transposable elements (TEs), can elicit the DDR to aid the transposition efficiency while maintaining a low mutagenesis rate. However, other TE and CRISPR/Cas9 studies propose that DDR activation can lead to off target and mutagenic effects. With the search for a better genetic editor, the CRISPR/Cas12k system has become a hot target due to its precise prokaryotic genome editing through transposition. By considering the mechanisms at play in endogenous TEs, retrotransposons, and CRISPR/Cas9, we can achieve a clearer understanding of the eukaryotic cell's response to genetic modification through the CRISPR/Cas12k system.

Introduction

DNA damage is the primary cause of many human pathologies including cancer, neurodegenerative diseases, and aging (Jackson & Bartek, 2009). DNA damage occurs when there is a break in the DNA due to numerous reasons such as UV exposure, carcinogen exposure, increase of intracellular reactive oxygen species (ROS), and more (Jackson & Bartek, 2009). These breaks include single stranded breaks (SSBs) which are the most common and easily repairable. However, more intense DNA damage can occur to lead to double stranded breaks (DSBs) which elicit a DNA Damage Response (DDR). There are two primary DDR pathways: Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR) (Fig. 1). NHEJ uses the Ku70/80 complex to recognize DSBs which are then phosphorylated by DNA-PK to recruit end-processing enzymes, polymerases, and finally DNA ligases (Jackson & Bartek, 2009). Microhomology mediated end joining (MMEJ) is a KU independent NHEJ pathway, but it results in greater sequence deletion and therefore greater chances of mutation upon repair (Jackson & Bartek, 2009). HDR utilizes the homologous single stranded DNA (ssDNA) or the homologous chromosome as a template for repair (Jackson & Bartek, 2009). The MRN complex, composed of MRE11, RAD50, and NBN proteins, recruits the ATM/ATR kinases to recruit further repair proteins such as RPA and RAD51 through phosphorylation events (Jackson & Bartek, 2009). RPA protects the ends of the severed DNA to reduce the chance of degradation while DNA-repair-proteins transcribe the damaged DNA, hence making a stable and less defective repair. RAD51 aides in the search of the homologous DNA which will act as the template. Once the homologous DNA sequence is identified, strand invasion and a Holliday junction is formed for the repairment of the DSB (Jackson & Bartek, 2009). While NHEJ and MMEJ are extremely error prone, they're typically used more often for DNA repair due to its ability to operate in any phase of the cell cycle. Conversely, HDR is relatively error-free but it can only occur in S and G_2 due to its use of sister chromatids to create accurate repairs (Jackson & Bartek, 2009).

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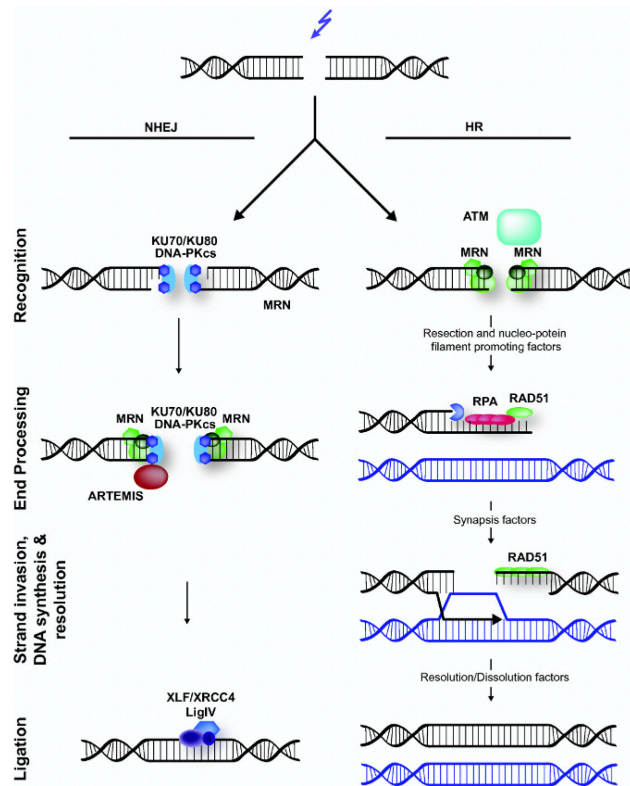

Figure 1. Overview of NHEJ and HDR DNA Repair Pathways (Lans et al., 2012)
Non-Homologous End Joining (NHEJ) is the error-prone DNA repair pathway. It involves Ku70/80 binding the the blunt ends of the DSB, which then recruits DNA-PK. DNA-PK auto phosphorylates and recruits the MRN repair complex including MRE11, RAD50, and NBN. Artemis is also recruited to undergo exonuclease action prior to ligation of the two ends of DNA. Because exonuclease activity can delete or insert DNA bases upon repair (indels), this process is error prone.
Homology Directed Repair (HDR) is the relatively error-free DNA repair pathway. MRN complex sense the DSB, which then recruits ATM kinase for further recruitment of resection factors, RPA, and RAD51. RPA coats and protects the resected ssDNA, while RAD51 is involved in the homology searching process. Once the homologous template is identified, strand invasion occurs and is resolved through a holiday junction and subsequent polymerases and ligases.

While the DDR is triggered when DNA is damaged due to exogenous factors as explained above, the DDR can also be triggered by the excision and insertion of transposable elements. Transposable elements, or transposons, are mobile genetic elements that can integrate in typically any location in the host genome. However, these mobile genetic elements are only DNA sequences and cannot move themselves. Transposases are the protein(s) that move the transposons/mobile genetic elements from one location to the next. There are endogenous transposable elements, such as the *Sleeping Beauty* element and RAG1/2 V(D)J recombination elements which are embedded in the host DNA to increase immune genetic diversity. However, there are also exogenous elements such as retrotransposons, including viruses such as HIV. Their ssRNA genomes are reverse transcribed into dsDNA which is integrated into the human host genome and repaired via the NHEJ pathway (Jackson & Bartek, 2009). Integration into the host genome allows the retrovirus to be replicated with the cell replication machinery, making it difficult to treat due to its stability in its expression (Jackson & Bartek, 2009).

In the 21st century, scientists have found a way to elicit the DDR which results in gene perturbation through the discovery of CRISPR/Cas9. The CRISPR/Cas9 system uses crRNA to be inputted into the host genome to edit the gene (Cubbon et al., 2018). The crRNA aims to evade the cell's cytosolic DNA detection protein called cGAS which can reduce the efficacy of the DNA. The CRISPR/Cas9 induces DSBs at a target area, however there are many off target effects due to the lack of efficacy (Cubbon et al., 2018). With the help of NHEJ mechanisms, the genome is modified (from the inaccuracy of the DDR pathway) and creates the intended effect of a “gene knockout” (Cubbon et al., 2018). Due to CRISPR/Cas9 off target effects and lack of substantial gene editing, a transposable element alternative is being researched. Similar to retrotransposons, CRISPR/Cas12k carries a transposable element that can be integrated into the host DNA for gene targeting effects or perturbing open reading frames (Moon et al., 2019). With the limited research and understanding of Cas12k, there is no certainty on how it will affect the eukaryotic DDR. In this review, the goal is to evaluate the current data on integration events and DNA damage to synthesize hypotheses for the DDR elicited by CRISPR/Cas12k function.

Class I TEs: Retrotransposons and DDR

Class I TEs, or retrotransposons, are a category of mobile genetic elements that function through reverse transcription. Class I TEs are termed “copy-and-paste” transposons. This is due to their mechanism of transposition. In short, they function by first “copying” the genetic material of the TE through transcription into an RNA intermediate (Bourque et al., 2018). Then the RNA intermediate is reverse transcribed into cDNA (complementary DNA) to be integrated, or “pasted” into the genome (Bourque et al., 2018). The location of integration is typically arbitrary, which makes these integration events mutagenic. The Class I TE can be integrated into the open reading frame (ORF) of a gene, disrupting the expression of said ORF (Bourque et al., 2018).

There are three types of Class I TEs:

1) Retrotransposons: elements that contain Long Terminal Repeats (LTRs) and encode reverse transcriptase, retroviruses are an example of this.

2) Retroposons: elements that contain Long Interspersed Nuclear Elements (LINEs), lack LTRs, and encode reverse transcriptase. They utilize RNA PolII to copy from the DNA, then reverse transcriptase to convert back to cDNA for integration.

3) Short Interspersed Nuclear Elements (SINEs): elements that are transcribed by RNA PolIII and do not encode reverse transcriptase.

For this review we will be focusing on Type I Class I TEs, retrotransposons. Retrotransposons, such as Human Immunodeficiency Virus type 1 (HIV-1), initially infect a host a host cell and reverse transcribe their ssRNA genome to cDNA to then be integrated into the host genome through integrase activity. Once the retroviral cDNA is integrated into the host genome it is called a provirus. Proviruses are specialized eukaryotic retrotransposons that can produce RNA intermediates that can leave the cell. HIV-1 and other retroviruses do this through creating viral particles after the viral DNA is transcribed and translated for viral replication (Yoshinaga et al., 2019). This cycle of creating a vector for further infection, then integration of the genetic material in a new host cell is very similar to the transposition cycle for prokaryotic organisms, suggesting there is a distant relationship between the two. Furthermore, it is known that mechanisms of viral assembly are highly conserved amongst retroviruses, suggesting that this mechanism is highly advantageous for further TE propagation (Dick et al., 2020).

It’s long been a curiosity to the DNA damage field, what DDR hallmarks are displayed upon HIV-1 integration? Several studies have identified a few key genomic stability and chromatin remodeling players that are stimulated by HIV-1 integration events. RAD18 suppressed HIV-1 infection (during reverse transcription) while EXO1, TP53BP1, and WRN promoted integration events (Yoshinaga et al., 2019). RAD18, an E3 ubiquitin ligase involved in trans-lesion synthesis post-replication repair, suppresses HIV-1 proliferation. Studies have shown that RAD18 only suppresses HIV-1 proliferation through ssDNA binding, the E3 ligase function and dsDNA binding function has no significant HIV-1 suppression phenotypes. This suggests that RAD18 is involved in suppressing viral proliferation at the integration step (Yoshinaga et al., 2019). Furthermore, it was also found that ATM-dependent DDR is stimulated upon integrase cleaving the genomic DNA for integration of HIV cDNA (Yoshinaga et al., 2019). With this evidence that canonical DDR mechanisms are activated upon integration, it brings up a plethora of questions regarding mutagenesis of chromosomal genes at and adjacent to the integration site (Fig. 2a).

Class II TEs: DNA Transposons and DDR

Class II transposons, or DNA transposons, undergo a "cut-and-paste" transposition that does not involve an RNA intermediate prior to integration (Bourque et al., 2018). Instead, sequences of DNA already within the host chromosomal genome are cut out of their current location then pasted into a new location within the host genome by a series of enzymes called transposases (Bourque et al., 2018).

For decades the most widely studied Class II TE was the *Sleeping Beauty* (SB) element. It was thought that all Class II TEs exclusively elicit NHEJ repair. This was due to evidence demonstrating that DNA-dependent protein kinase (DNA-PK) regulates DNA repair signaling from damage done by SB element excision and reinsertion (Yant & Kay, 2003). SB mobilization is done through cut-and-paste methods with a DNA intermediate (Yant & Kay, 2003). Through Ku-dependent NHEJ, SB excision was efficiently and accurately processed (Yant & Kay, 2003). Without Ku present, there is greater degradative loss of DNA termini and frequent recombination (Yant & Kay, 2003). Ku protects the end segments of the DNA from degradation, making it crucial for maintaining genomic integrity in DNA transposition (Yant & Kay, 2003).

This idea was even corroborated by the endogenous RAG1/2 transposases. RAG1/2 is an ancestral transposase from the *Transib* transposon. It was demonstrated to have maintained its' transposition activity in the 1990s (Yakovenko et al., 2021). Now in eukaryotic cells, RAG1/2 is involved in immune receptor diversification through the variable generation of antigen protein T cell receptors (TCRs). TCR generation utilizes the transposases RAG1 and RAG2 to cleave DNA sequences to undergo V(D)J recombination, the select removal of some V(D)J segments and recombination of the remaining segments, in order to create greater diversity in the immune system (Henssen & Kentsis, 2018; Sadofsky, 2001). To elaborate, TCR heterodimers have polypeptides that have a variable side that is created through V(D)J recombination and a constant region that is later joined to the variable end through mRNA splicing (Henssen & Kentsis, 2018; Sadofsky, 2001). This process starts by RAG1/2 binding to the Recombination Signal Sequences (RSS), which is analogous to the Terminal Inverted Repeats (TIRs) in other cut-and paste TEs, which allows for cut-site recognition for excision from the genome.

The recombination aspect of this process was once thought to be only through the NHEJ pathway. A field-wide hypothesis for NHEJ repair preference was the ligation of the DNA after cleavage is intentionally imprecise to create gene diversity through small deletions or random insertions since the mutation increases the yield of different receptor molecules (Henssen & Kentsis, 2018; Sadofsky, 2001). However, recent studies have shown that RAG1/2 excision repair can undergo the HDR pathway in DNA-PK or Ku70 depleted cells (Weinstock & Jasin, 2006). This demonstrates that cut-and-paste TEs can elicit HDR repair, which was previously only speculated (Fig. 2b).

CRISPR/Cas9 and DDR

In 2020, the Nobel Prize in Chemistry was awarded to Jennifer Doudna and Emmanuelle Charpentier for the pioneering of the CRISPR/Cas9 gene editing technology (Ledford & Callaway, 2020). With the relevance in treating genetic diseases, it is important that research is diligently done to confirm that genetic editing by CRISPR/Cas9 will not elicit an overwhelming DNA damage response. The CRISPR/Cas9 system can be programmed to target specific genetic code with guide RNA (gRNA). The gRNA guides the CRISPR/Cas9 system to the specified region of the genome, where the Cas9 endonuclease creates a DSB and relies on DDR to fix the repair and create mutations (indels) in the process which will result in genetic modification of the gene at hand (Cubbon et al., 2018). The DDR elicited by the Cas9 system is NHEJ, which is known for its common insertion/deletion mutations (indels) which can lead to the resulting protein being truncated or inactive (Cubbon et al., 2018). This system relies on the R-loop target site which facilitates the excision after the DNA is found through RNA-DNA pairing (the RNA of the Cas9 protein pairing to the DNA complementary strand) (Cubbon et al., 2018). Cas1 and Cas2 aid in inserting foreign DNA fragments into the CRISPR locus of a host chromosome which creates guide RNA (gRNA) that binds to the host genome (Moon et al., 2019). The hope is to encourage HR repair from the Cas9 disruption in order to create a new code that can be

inserted into the genome and has been tested through Cas9 protein fusion (Cubbon et al., 2018). However, Cas9 remains ineffective in deciding the code that takes the place of the target DNA.

In recent years, studies have given cells the CRISPR/Cas9 cassette + template repair DNA that is complementary to the gRNA. They found that HDR became the primary repair pathway, with NHEJ still being present as the minor pathway (van Kampen & van Rooij, 2019). Regardless of the precision of repair (lacking unwanted mutations at the target locus), this method still struggles with off-target editing just as CRISPR/Cas9 without the repair template does. This creates a demand for a gene editing system that can target efficiently and repairs via HDR (Fig. 2c).

CRISPR/Cas12k, Tn7, and DDR

With the desire for a site-specific gene editor that repairs via HDR, it was found that the CRISPR Associated Transposase (CAST), CRISPR/Cas12k derived from cyanobacteria *Scytonema hofmanni* may be a solution. Cas12k is a Type V-K CRISPR effector, that targets dsDNA and integrates the programmed dsDNA into the targeted site via a cut-and-paste transposase mechanism with 80% efficacy without positive selection as of 2019 (Strecker et al., 2019). The mechanism by which CRISPR/Cas12k acts is assumed to be like Tn7, due to all its effector proteins being Tn7 family proteins: TnsA, TnsB, TnsC, TnsD, and TniQ.

The current proposed mechanism by which it is thought CRISPR/Cas12k acts based on the Tn7 mechanism of action is as follows. Instead of the typical gRNA to direct the Cas machinery as seen in many other Cas effector proteins, Cas12k utilizes Tn7-like TnsC and TniQ (similar to TnsD in Tn7) to work together as the target site selector machinery. TnsC/TniQ define the fixed point of insertion of the transposon by recognizing the specific attachment site in the host genome to create a controlled gene modification (Park et al., 2021). After TnsC/TniQ localization, CRISPR/Cas12k utilizes a cassette of transposases (TnsA/TnsB) similar to Tn7 to execute DNA integration at a precise distance and orientation (Park et al., 2021). Furthermore, TnsC/TniQ function is multifaceted. Alongside target site selection, TniQ recruits TnsA and TnsB transposases to bind to the ends of the substrate DNA to integrate into the host genome target-site (Park et al., 2021). Lastly, TnsC induces a distortion (unwinding) in the DNA in order for the transposon to insert; however, the implications are unknown at this time (Park et al., 2021).

Currently it is speculated that CRISPR/Cas12k site-specific integration does not induce DSBs (Moon et al., 2019). However, due to the infancy of Cas12k research, this has yet to be experimentally verified. The idea that the Cas12k mechanism lacks DSB inducing integration is based on the current knowledge of other Class II TEs, specifically the PiggyBac transposase which uses DNA ligase for insertion (Zhao et al., 2016). However, it is controversial if we can fully compare the Cas12k mechanism to simply other Class II TEs. CRISPR/Cas12k expression, let alone editing has yet to be achieved in eukaryotic cells, which speaks to a major difference between Cas12k and typical Class II TEs. In order to consider all of the potential hypotheses in relation to DNA damage induction, a broader point of view should be taken.

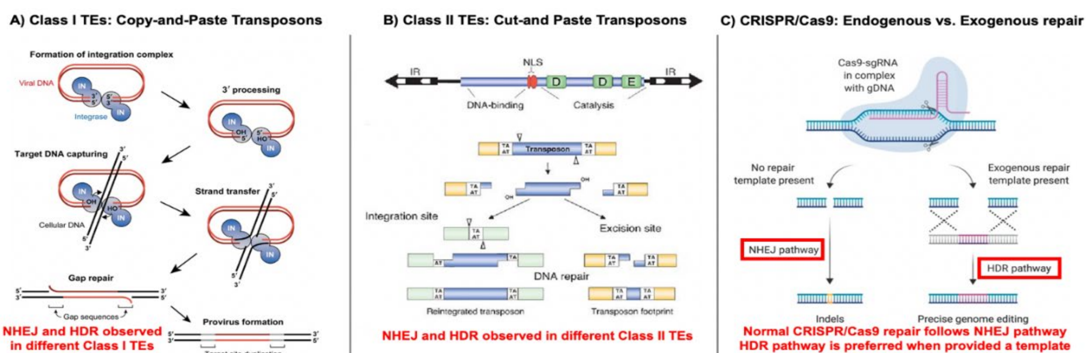

Figure 2. Overview of Class I TEs, Class II TEs, and CRISPR/Cas9 Repair Pathways

A) Class I TEs: Copy-and-Paste Transposons are those that contain an RNA intermediate prior to cDNA integration into the chromosome. It has been identified in HIV-1 that gap repair can occur by NHEJ or HDR, but evidence of which is preferred is still not clear but hypothesized to be HDR. (Suzuki et al., 2012)

B) Class II TEs: Cut-and Paste Transposons are those that do not have an RNA intermediate yet excise the transposon from one locus of the genome to another. NHEJ has been observed in all Class II TEs, but recent evidence has found that RAG1/2 can undergo HDR repair when depleted with NHEJ factors such as Ku70 and DNA-PK. (Miskey et al., 2005)

C) CRISPR/Cas9: When CRISPR/Cas9 editing occurs endogenously, meaning without any further scientific intervention, the primary repair pathway is NHEJ, creating indels through this error-prone pathway. When CRISPR/Cas9 editing occurs with an exogenous repair template provided, the primary repair pathway is HDR, resulting in precise genome editing. (van Kampen et van Rooij, 2019)

Discussion

DNA is the biological blueprint of cellular function; it encodes proteins and other regulatory regions we don't fully understand that allow all cellular functions to be tightly regulated. As such, maintaining the integrity of the genetic code is the most important aspect of cellular regulation. However, maintaining genomic integrity is challenging with the daily DNA damage due to UV exposure, carcinogens, etc. Therefore, a robust DDR is required for the maintenance of the genome. While NHEJ is the fastest DDR pathway, it also is the most error prone (Jackson & Bartek, 2009). As such, HDR is preferred to uphold genomic integrity. In addition to exogenous DNA damaging agents, the DDR can also be triggered by transposable elements. Class I TEs copy their genetic code through transcription into an RNA intermediate, which is then reverse transcribed into cDNA which is integrated into the host genome (Bourque et al., 2018). Studies have found that upon retrotransposon integration, a type of Class I TEs, ATM is phosphorylated through an unknown mechanism, which elicits an HDR response (Yoshinaga et al., 2019). Conversely, Class II transposons do not use an RNA intermediate. Instead, they use transposases to cut their DNA from one locus in the chromosome and integrate it into another region of the chromosome (Bourque et al., 2018). Most endogenous TEs are Class II, such as the notable *Sleeping Beauty* and RAG1/2 TEs. Class II integration typically elicits an NHEJ response as it is the goal of these endogenous TEs to increase genetic diversity through mutations. However, recent evidence has shown that RAG1/2 insertion repair undergoes the HDR pathway when DNA-PK or Ku70 are depleted in the cell (Weinstock & Jasin, 2006). This evidence that HDR can be elicited upon cut-and-paste transposition, opens many possibilities to the fact that perhaps these Class II TEs can be "programmed" to prefer HDR repair, which is ideal due to its stability and lower chances of mutations.

This same line of reasoning is the goal for genetic modification technology, it is ideal to elicit HDR over NHEJ. In CRISPR/Cas9, a specific region is targeted via sgRNA to create a DSB. It then relies on DDR to fix the repair. However, since the preferred DNA repair response for CRISPR/Cas9 is NHEJ, the repair creates indels which create minimal genetic modification and potential unwanted effects to the protein product due to its unpredictability (Cubbon et al., 2018). However, scientists have found a way to elicit HDR upon CRISPR/Cas9 editing by providing the cells with a repair template in addition to the CRISPR/Cas9 cassette (van Kampen & van Rooij, 2019). Regardless of the increase in repair fidelity, the problem of off-target editing still remains, which creates the demand for another more reliable gene editing system. CRISPR/Cas12k is the newest candidate for high precision and fidelity gene editing. The CRISPR/Cas12k system acts similarly to the E. coli indigenous TE Tn7, due to its use of Tn7-like proteins TnsA, TnsB, TnsC, and TniQ (Park et al., 2021). However, like Tn7, research on Cas12k is only known in prokaryotic organisms thus far. The dsDNA transposon is for CRISPR/Cas12k can be programmed and is then enacted through a Class II TE mechanism with an 80% efficacy rate under no positive selection (Strecker et al., 2019). However, it is unknown what DDR CRISPR/Cas12k will elicit in eukaryotes. Currently, there are propositions stating CRISPR/Cas12k does not induce DSBs due to examples of other Class II TEs such as the PiggyBac TE (Zhao et al. 2016). However, it is unknown how similar Cas12k is to TEs that function in eukaryotic cells since all research has been in E. coli thus far. As such, the hope for the future of genetic editing is that the host cell will react CRISPR/Cas12k in a similar manner as retrotransposons to elicit the ideal HDR.

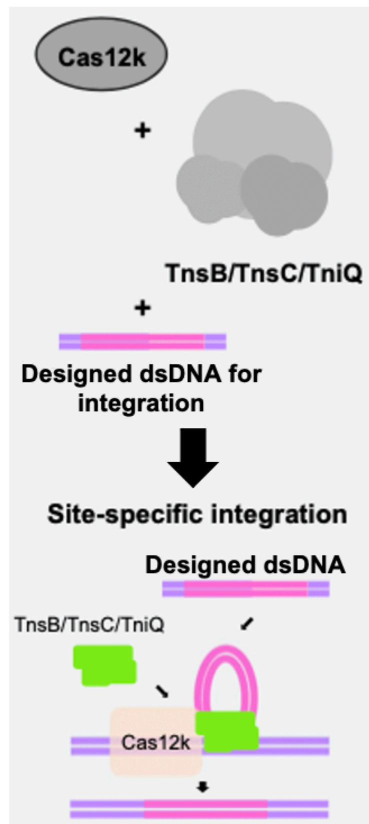

Figure 3. Hypothesized Repair Mechanism of CRISPR/Cas12k Genome Editing
Moon et al. (2019) illustrated how CRISPR/Cas12k + TnsB/TnsC/TniQ + transposable DNA act together to undergo a site-specific integration event. However, there is yet to be any literature that proposes what DNA repair pathway is predominant upon integration. There are three possible mechanisms:

1) NHEJ or HDR repair: Like in class I TEs, NHEJ or HDR is the predominant repair pathway depending on the TE.
2) NHEJ major and HDR minor repair: Like in Class II TEs, most undergo NHEJ, but can undergo HDR in certain circumstances.
3) HDR major and NHEJ minor repair: Like in CRISPR/Cas9 genome editing when provided a repair template.

Because CRISPR/Cas12k is considered a cut-and-paste transposase (Class II) which can undergo HDR in certain circumstances, and because Cas12k editing must provide a dsDNA transposon, similar in concept to the template provided in CRISPR/Cas9 editing, ***we hypothesize that the preferred repair pathway in CRISPR/Cas12k editing is HDR.***

Based on the evidence of known TEs and CRISPR/Cas9 presented in this paper, we propose the following hypothesis (Fig. 3) We propose that CRISPR/Cas12k will elicit the HDR repair pathway. This is due to CRISPR/Cas12k being a foreign entity in the eukaryotic cells, like retroviruses. Additionally, it was presented that CRISPR/Cas9 prefers HDR when provided a repair template. Due to dsDNA that is homologous to the insertion site being required for CRISPR/Cas12k activity, it is likely the gap will be repaired via HDR due to this presence of homology. While it is a large possibility that CRISPR/Cas12k could elicit NHEJ, or possibly no DDR like PiggyBac, we believe that the evidence combined with retroviruses and Cas9 modification is sufficient to propose this HDR hypothesis.

With the recent surge in research on CRISPR/Cas12k, the first aspect that needs to be achieved is the successful transfection and transposition of CRISPR/Cas12k programmed dsDNA into the eukaryotic genome. Once transposition into eukaryotic cells is achieved, off-target transposition events should be analyzed. Lastly, an extensive study on DDR elicitation should be carried out to determine what DNA repair pathway, if any, is elicited upon CRISPR/Cas12k transposition. While we are many years out from seeing the benefits from CRISPR/Cas12k genetic editing, it is currently a promising editing machinery to study for the next decade to come.

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