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**Mammalian Genome special issue**

**Control of Gene Editing by Manipulation of DNA Repair Mechanisms**

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## Abstract

DNA double-strand breaks (DSBs) are produced intentionally by RNA guided nucleases to achieve genome editing through DSB repair. These breaks are repaired by one of two main repair pathways, classic non-homologous end joining (c-NHEJ) and homology-directed repair (HDR), the latter being restricted to the S/G2 phases of the cell cycle and notably less frequent. Precise genome editing applications rely on HDR, with the abundant c-NHEJ formed mutations presenting a barrier to achieving high rates of precise sequence modifications. Here, we give an overview of HDR- and c-NHEJ-mediated DSB repair in gene editing and summarize the current efforts to promote HDR over c-NHEJ.

## 1. Introduction

DNA double-strand breaks (DSBs) are among the most dangerous lesions for DNA, resulting in cancer or cell death if ineffectively unrepaired. DSBs occur spontaneously due to endogenous metabolic products such as reactive oxygen species or by exposure to exogenous agents such as irradiation or mutagenic reagents (Jackson and Bartek 2009) (Mladenov et al. 2016). Furthermore, programmed DSBs occur as normal developmental intermediates in meiosis of germ cells and for the specification of antigen receptors in lymphocytes (Baudat et al. 2013) (Alt et al. 2013). In order to maintain genome integrity and survival cells constantly express biochemical components for the recognition and repair of DSBs. These repair mechanisms can be utilized for gene editing by the induction of artificial, targeted DSBs in regions of interest using programmable, sequence-specific nucleases. The first generations of sequence-specific nucleases included zinc-finger nucleases (ZFN), meganucleases and TALEN (Urnov et al. 2010) (Boch 2011). In recent years the bacterial CRISPR/Cas9 nuclease system became the preferred gene editing method due to its efficiency and simplicity (Komor et al. 2016) (Hsu et al. 2014). (Hsu et al. 2014). Research involving DSBs formed from Cas9 is highlighted in this review as Cas9 has become the system of choice for most researchers. For the creation of DSBs by CRISPR/Cas9 two basic components are introduced into cells: the Cas9 nuclease and a short, single guide (sg)RNA which associates with Cas9. The first twenty nucleotides of guide RNAs direct Cas9 to the complementary DNA target sequence located upstream of an invariant 5´-NGG-3´ (for SpCas9) protospacer adjacent motif (PAM) sequence. Correct pairing of the sgRNA to the DNA leads to DSB formation 3 bp upstream of the PAM site (Jinek et al. 2012) (Ran et al. 2013). The advantage of the CRISPR/Cas9 system is that new genomic sites can be easily targeted by modification of the sgRNA sequence and no changes are required for the Cas9 protein. The initial demonstration of CRISPR/Cas9 activity in mammalian cells in 2013 (Cong et al. 2013) (Mali et al. 2013), has been expanded

to edit the genome of somatic and germline cells in a wide range of species (Barrangou and Doudna 2016).

For genetic editing efficient DSB formation is but the first step in a two-step process. The actual sequence editing is performed by the cell`s DSB repair machinery. Presently it is possible to dictate the site of gene editing by DSB formation but we have much less control on the outcome of DNA repair. Unfortunatly, often the native DSB repair outcomes do not meet specific experimental needs. In this article first we give an overview on the usage of homology-directed repair (HDR) and classic non-homologous end joining (c-NHEJ) mediated DSB repair for gene editing, and then summarize the efforts undertaken to promote HDR over c-NHEJ.

## 2. Harnessing DSB Repair Mechanisms for Gene Editing

Two main branches of DSB repair mechanisms exist in mammalian cells. These either process DSB ends by strand resection and initiate repair by homology-directed repair (HDR), or seek for the immediate protection of free ends through religation by non-homologous end joining (c-NHEJ) (Lieber 2010) (Fig. 1). While the c-NHEJ pathway operates throughout all phases of the cell cycle, HDR is restricted to the S and G2 phases when intact sister chromatids are available as ideal repair templates (Hustedt and Durocher 2016). During mitosis DSB repair is entirely shut down to guard the chromosomes against the fusion of telomeres (Orthwein et al. 2014). In the G1 phase and in resting cells, c-NHEJ repair is exclusive since HDR is silenced. Both pathways are active and competing for in the S/G2 phases. In summary, DSB induction in a population of cycling cells leads to a variety of edited alleles, with c-NHEJ as the dominant outcome.

Under natural conditions HDR is mediated by the homologous recombination (HR) pathway (Heyer et al. 2010) (San Filippo et al. 2008). HR uses the intact identical sequences of sister chromatids as template for the repair of DSB sites, and leads to the reconstitution of a wildtype allele. To achieve precise sequence modifications at targeted DSBs, the HR pathway can be hacked by providing an artificial DNA repair template which contains sequence regions homologous to the DSB ends. The sequence between the homologous ends, either an insertion or replacement, is then transferred into the targeted locus during HDR, enabling the generation of precisely modified \`knockin´ alleles, e.g. for codon replacements or the insertion of reporter genes (Fig. 1). Large sequence insertions require the use of double-stranded, plasmid-based gene targeting vectors with homology regions of > 500 bp. Shorter sequence modifications can be introduced by using synthetic single-stranded DNA oligodeoxynucleotides (ssODN) as repair templates. Repair using ssODNs is mediated by a poorly defined mechanism designated as single-strand template repair (SST-R).

Alternatively to HDR, short resected DSB ends can be religated via stretches of a few complementary nucleotides through the mechanism of microhomology-mediated end joining (MMEJ) (Sfeir and Symington 2015). MMEJ is active in the S and G2 phases at lower levels than HR. Its mode of function is based on the alignment of microhomologies between the sequences at the broken DNA ends. Under natural conditions this type of repair results into small sequence deletions resembling the products of c-NHEJ. However, this is a distinct pathway and can be harnessed for targeted sequence insertion by providing DNA fragments which include short homology regions to the DSB ends (Fig. 1) (Sakuma et al. 2016). Using this PITCh (Precise Integration into Target Chromosomes) approach for gene addition obviates the laborious construction of gene targeting vectors with long homology regions. Although PITCh is not yet widely used, it has been shown as an efficient tool for the integration of large donor DNAs into target sites in human cells, silk worm embryos, frogs, and mice (Nakade et al. 2014) (Hisano et al. 2015) (Aida et al. 2016).

In contrast to HDR, the c-NHEJ pathway mediates the religation of DSB ends without the involvement of a repair template. Although some fraction of NHEJ repair events likely results in precisely reconstituted wildtype sequences, a fraction of cleaved sequences gain a random insertion or deletion of one or more nucleotides (Indels). Therefore, DSB repair by the error-prone c-NHEJ pathway is frequently used to generate Indels within coding regions, which often will cause a frameshift knockout mutation (Fig. 1). The formation of Indels is enriched at Cas9-mediated DSBs as any error-free religation reforms the original target site, which can then be re-cleaved. This cleavage-ligation loop proceeds until a mutation is formed or the nuclease is diluted or degraded.

NHEJ repair has been shown to frequently ligate exogenously provided DNA segments in-between the ends of DSBs. In this approach, designated as Non-Homology (NH) Targeting (He et al. 2016), the orientation of the inserted fragments is stochastic and cannot not be controlled. This stochastic insertion has been recently overcome by a modification in the technique that allows a high degree of preference in the insertion orientation (Suzuki et al. 2016). This Homology-Independent Targeted Integration (HITI) method was shown to be remarkably efficient in primary cells *in vitro* and *in vivo,* suggesting that c-NHEJ can be a method of choice for achieving targeted integrations in non-dividing cells. As expected for c-NHEJ repair events, the ligation of the fragment into the genome frequently results in Indels at one or both junctions.

**- FIGURE 1 -**

## DSB Repair Signaling and Pathway Choice

For many gene editing experiments the frequency of alleles repaired by HDR is often low, increasing and complicating experiments. This is due to the inherent dominance of c-NHEJ repair and therefore efforts for influencing the cell`s choice of DSB repair often aim to suppress c-NHEJ, as well as to enhance HDR. This section gives an overview on the key molecules which execute DSB repair and regulate its pathway choice.

The initiation of DBS repair is identical for both c-NHEJ and HDR The ATM (ataxia telangiectasia mutated) protein kinase is a key initial regulator of the DNA damage response coordinates DSB repair (Fig. 2). ATM is activated by the MRN (Mre11-Rad50-Nibrin) complex and other factors at DNA breaks (Stracker and Petrini 2011) (Lavin et al. 2015). Upon monomerization and autophosphorylation, ATM phosphorylates Serine 139 of histone H2AX, forming γH2AX. The phosphorylated residue on γH2AX is recognized by MDC1, which in turn recruits more MRN complexes (Stucki et al. 2005). These further activate ATM and create a positive feedback loop driving the expansion of γH2AX chromatin domains into γH2AX foci (Nakamura et al. 2010). MDC1 becomes phosphorylated by ATM at its TQXF repeats and initiates downstream signaling by recruiting the E3 ubiquitin ligase RNF8 (Fig. 2) (Smeenk and Mailand 2016) (Schwertman et al. 2016). RNF8 and its E2 enzyme partner UBC13 polyubiquitinate the H1 linker histone (Thorslund et al. 2015). This further promotes the recruitment of the E3 ubiquitin ligase RNF168 that ubiquitinates histone H2A at Lysine 13 and 15 (Mattiroli et al. 2012). H2A-K15Ub together with dimethylated Lysine 20 of histone H4 (H4K20me2) are chromatin marks for the recruitment of the checkpoint protein 53BP1 (Fig. 2) (Fradet-Turcotte et al. 2013) (Zimmermann and de Lange 2014). The control of accumulation of 53BP1 determines if the DSB event is repaired by c-NHEJ, or through resection and subsequent HDR (Symington and Gautier 2011) (Panier and Durocher 2013) (Daley and Sung 2014) (Zimmermann and de Lange 2014).

The classical c-NHEJ pathway initiates with the localization of 53BP1 to a DSB and blocks 5' resectioning. 53BP1 blocks CtIP based resectioning (Bunting et al. 2010) and recruits Rif1, which further blocks resectioning and inhibits BRCA1 accumulation (Escribano-Díaz et al. 2013) (Zimmermann et al. 2013). Unresected ends allow Ku70/80 to bind, further inhibiting resection. Ku proteins form a scaffold and recruit DNA-PKcs, which then recruits end-processing factors (like Artemis) and the XRCC4/XLF/DNA Ligase-IV complex. The XRCC4/XLF factors stabilize and align the DNA fibers and DNA Ligase IV ligates the two strands (Waters et al. 2014). Repair of chemically or irradiation-induced DSBs is greatly complicated by the need to excise and repair damaged bases. However this will be left out of this review as DSBs from Cas9 nucleases form blunt ends with 5' phosphorylated DNA, the substrate for DNA Ligase IV. The ability to excise damaged bases and then ligate

noncomplementary strands has resulted in c-NHEJ being often thought of as a mutagenic process. However given a complimentary cut, such as created by Cas9 error-free events can be 75% or higher (Bétermier et al. 2014). Error-prone mutations that have previously been attributed to c-NHEJ are often a result of DSB resectioning and annealing through the similarly named but mechanistically distinct a-NHEJ.

The homologous recombination pathway requires the exclusion of 53BP1 and resection to occur. H2A is de-ubiquitinated upon mitotic entry so 53BP1 is excluded from the chromatin (Giunta et al. 2010). During the S/G2 phase, BRCA1 excludes Rif1 from the foci, and recruits CtIP and the MRN complex. This complex initiates a cleavage step which is then further 5'-resected by Exo1 (Symington and Gautier 2011) (Sartori et al. 2007) (Symington 2016). The resection extends 2-4 kb on each side of the DSB (Zakharyevich et al. 2010). The exposed single-stranded DNA (ssDNA) is quickly bound by RPA for protection. RPA is replaced by Rad51 through the action of BRCA2 and Rad52 to form a nucleofilament competent for homology search (Liu et al. 2011). The Rad51 filaments maintain the ssDNA in a B-form which has triplets open for Watson-Crick paring with complementary triplets in homologous duplex DNA. It should be noted that this review highlights only some of the key factors of the HR pathway and more complete reviews are available (Heyer et al. 2010) (San Filippo et al. 2008) (Symington and Gautier 2011) (Jasin and Rothstein 2013).

Alternative Non-Homologous End Joining pathways (a-)NHEJ encompass Microhomology-Mediated End Joining (MMEJ), Single Strand Annealing (SSA), and Theta-Mediated End Joining (Rodgers and McVey 2016). Once thought to only be a backup pathway, a-NHEJ can in some cases occur up to 10% of the frequency of c-NHEJ (Corneo et al. 2007). These repair events can result in deletions of various sizes, and only sometimes anneal and ligate through microhomologies. However, they always begin with the same resection steps as in homologous recombination, involving the MRE11 complex and CtIP. Resection can be <20 bp for microhomology or up to thousands of bps for SSA. The choice between a-NHEJ and HR comes from the inability of RPA to be replaced by Rad51 by Rad52/BRCA2. This limits the ssDNA to proceed through the HR pathway. Importantly, the extensive resection, when repaired by a-NHEJ, results in increased chromosomal translocation frequency, a major driver of human cancer (Deriano and Roth 2013).

**- FIGURE 2 -**

**Manipulation of DSB repair pathways**

The key molecules of the c-NHEJ pathway such as DNA Ligase-IV, Ku70/80, and DNA-PKcs have been targeted to transiently suppress c-NHEJ activity (Table 1). By transient suppression of c-NHEJ in cycling cells DSB repair can be biased in favor of HDR. SCR7 is a small molecule inhibitor that prevents the binding of Ligase IV to DNA and by this mean inhibits the ability of Ligase IV to join DSB ends (Srivastava et al. 2012). . Maruyama et al. used SCR7 to inhibit c-NHEJ repair for CRISPR/Cas9-induced DSBs in mammalian cell lines and mouse zygotes (Maruyama et al. 2015). The treatment of cells with SCR7 increased the rate of HDR events up to 19-fold. In a similar study DNA Ligase-IV was targeted in human and murine cell lines by SCR7, shRNA-mediated gene silencing, or by adenoviral (Ad) directed Ligase IV proteasomal degradation (Chu et al. 2015). A combined knockdown of Ku70/Ligase IV, SCR7 treatment, or Ad protein expression were all effective for c-NHEJ suppression and increased HR events in HEK293 cells up to 8-fold. However, SCR7 has shown unequal effectiveness when applied to other systems. It has been noted that its actual structure differs from the original description and furthermore its selectivity towards other DNA ligases has been questioned (Greco et al. 2016). The search for more potent and specific DNA Ligase-IV inhibitors will certainly continue since Ligase IV is a promising target in cancer therapy. A step into this direction was reported by Menchon *et al.* who identified a lead compound disrupting the interaction of the DNA Ligase-IV clamp domain to its binding partner XRCC4 (Menchon et al. 2016).

Various small molecule inhibitors against DNA-PK have been tested for suppression of c-NHEJ repair at CRISPR/Cas9-induced DSBs. Robert et al. confirmed previous results (Chu et al. 2015) on the suppression of Ligase IV and Ku70/80 to increase HDR. They additionally identified the DNA-PK inhibitors NU7441 and KU-0060648 effective for c-NHEJ suppression and resulted in increasing HDR for both double- and single-stranded DNA repair templates (Robert et al. 2015). Another approach antagonizes mammalian DNA-PK by depletion of its cofactor inositol hexakisphosphate (InsP6) using calmodulin inhibitors W7 and Chlorpromazine (Byrum et al. 2004). InsP6 depletion by these compounds reduces the binding of the Ku complex to DSBs affecting the recruitment of other c-NHEJ factors. Although not yet tested for its effect on CRISPR/Cas9-induced DSBs in mammalian cells, both inhibitors phenocopy the positive effect on HDR seen in Ku deletion strains of *Cryptococcus* fungi (Arras and Fraser 2016).

53BP1 is a key determinant of DSB repair pathway choice, promoting c-NHEJ by blocking DSB end resection. 53BP1 is therefore a prime target for interventions aiming to suppress c-c-NHEJ. Earlier studies of the role of 53BP1 in DSB repair used siRNA-mediated knockdown (Tang et al. 2013), the ectopic expression of dominant-negative (DN)53BP1 subdomains including the tandem Tudor domain (Xie et al. 2007) (Yoo et al. 2005), or 53BP1 inactivated

cell lines (Orthwein et al. 2015). Only recently have specific inhibitors against the 53BP1 protein become available. In a recent preprint, Canny et al. describe the development of `i53´ as a genetically coded high affinity peptide inhibitor of 53BP1 (Canny et al. 2016). i53 is a mutant ubiquitin with 7 amino acid substitutions that binds to the Tudor domain of 53BP1, preventing the recognition of the H2A-K15Ub ubiquitin mark for damaged chromatin. Expression of i53 suppresses the recruitment of 53BP1 at DSB sites in U2OS cells, leading to rates of HDR that are comparable to 53BP1 deficient cells. Combining both inhibitors i53 and NU7441, the rate of HDR was further increased (Canny et al. 2016).

Enrichment for HDR events

In addition to inhibiting the c-NHEJ pathway, considerable effort has focused on enhancing the efficiency of the HDR pathway. In the following part we will discuss different approaches that have been shown to promote HDR (Table 1).

Small molecules

RS-1, an enhancer of the single-strand DNA binding protein Rad51, was identified by library screening to support HR by stimulating the formation of active presynaptic Rad51 filaments (Jayathilaka et al. 2008). RS-1 was found to increase the insertion of reporter genes by 3-6 fold at CRISPR/Cas9-induced DSBs in HEK293 and U2OS cells. Coadministration of RS-1 and SCR7 had no additional effect on HDR (Pinder et al. 2015). Furthermore, RS-1 was recently shown in Cas9 microinjected rabbit embryos to improve HDR and knockin efficiencies by 25 fold *in vitro* and *in vivo* (Song et al. 2016). In the same study, the effect of SCR-7 was minimal, but the coinjection of mRNA for the overexpression of Rad51 had a comparable effect to the treatment with RS-1.

In a screen for regulators of DNA repair L755507 and Brefeldin A were identified as enhancers of HR in several mammalian cell lines at /Cas9-induced DSBs (Yu et al. 2015). The β3-adrenergic receptor agonist L755507 increased HDR efficiency up to 3-fold when using a plasmid-based repair template and 9 fold when using a ssODN repair template. The addition of 0.1 μM Brefeldin A, an inhibitor of the intracellular protein transport, showed a further enhancement in HR.

Synchronized Cas9 Delivery

The HR pathway is restricted to the S/G2 cell cycle phases to enable precise DSB repair by recombination of sister chromatids. Therefore, higher HR rates can be expected in a population of dividing cells enriched for the S/G2 phases by cell cycle synchronization. Lin et al. delivered pre-assembled Cas9/sgRNA ribonucleoprotein (RNP) complexes into synchronized primary

fibroblasts, HEK293, or human embryonic stem (ES) cells. These cells were blocked in S-phase by Aphidicolin, in M-phase by Nocodazole, or by both compounds sequentially (Lin et al. 2014). The timed delivery of RNPs into M-phase synchronized HEK cells led to a 4-fold increase in HR as compared to non-treated cells (38% vs 9%). In fibroblasts and ES cells Aphidicoline was more efficient. Yang et al. describes the synchronization of induced pluripotent stem (iPS) cells and neural progenitors using Nocodazole or ABT-751. They demonstrated a 3-6 fold increase for on-target gene editing in synchronized cells, and the edited iPS cells could be successfully differentiated into multiple lineages (Yang et al. 2016).

A complementary approach limits Cas9 activity to S/G2 phase of the cell cycle. In this study, Cas9 has been fused to a peptide from the Geminin protein which causes protein degradadation during the G1 phase of the cell cycle. The N-terminus of Geminin includes a destruction box motif that is recognized and tagged for degredation by the E3 ubiquitin ligase complex APC/Cdh1, active in the late M and G1 phase. Using this strategy, the rate of HR in HEK293 cells was found to be increased up to 87% as compared to unfused Cas9 (Gutschner et al. 2016). Using the same fusion in human iPS cells, Howden et al. did not observe increased HDR frequencies, but found a reduction of c-NHEJ-mediated Indels. This reduction in Indels facilitates the generation of heterozygous knockin cell lines that are free of mutations in the second, wildtype locus (Howden et al. 2016).

Reactivation of HDR in G1

The mechanistic basis for the suppression of HDR in the G1 phase of the cell cycle had long proven elusive. Subsequently it was not known whether HDR could be reactivated in this phase. Recently, the work of Orthwein et al. details the mechanism of HR containment in the S/G2 phase (Orthwein et al. 2015). They show how the cell cycle controsl the interaction of BRCA1 with PALB2–BRCA2 and constrains BRCA2 function to the S/G2 phases. In the G1 phase the ubiquitination of PALB2 leads to its degradation while end resection at DSBs by CtIP is prevented by the lack of phosphorylation controlled by cyclin-dependent kinases. Hence the ectopic expression of a degradation resistant PALB2 mutant and a phosphomimetic mutant of CtIP, together with the inhibition of 53BP1 restores HDR in G1 cells at least to some extent. Alternatively to the inactivation of the 53BP1 by gene knockout, the ectopic expression of the i53 inhibitory peptide is equally efficient (Canny et al. 2016).

Structure of HDR Donors

In common practice templates used as HDR donors are either plasmid-based dsDNA or synthetic ssODNs. Plasmid-based vectors are suitable for introducing large sequence changes and insertions, and ssODNs are suitable for short (<50 nt) modifications. Typically ssODNs

are used with symmetric homology regions of 50-80 nt flanking each side of the DSB site, representing the forward or reverse strand of the target site. Nevertheless, an optimized ssODN design has been recently described in a study based on the interaction of the Cas9 protein with DSB ends, leading to improved rates of HDR (Richardson et al. 2016). The authors observed that in *in vitro* assays after DSB induction, Cas9 remains associated with the DNA for up to 5.5 hours. When dissociating from its target site Cas9 first releases the 3' end of the cleaved DNA strand that is non-complementary to the sgRNA. This led to the hypothesis that an ssODN complementary to the non-target strand could result into increased HDR rates. It was found that asymmetric ssODNs against the non-target strand with homology regions of 36 nt distal to the PAM site and of 91 nt proximal to the PAM site indeed result in almost 60% increase of HDR at multiple loci in HEK293 and K562 cells. In a follow-up study Liang et al. (Liang et al. 2017) observed that asymmetric ssODN donors that had short 30 nt 3´-homology and long 60 nt 5´-homology regions resulted in highest HDR efficiencies, regardless of which genomic strand was used. Contrary to Richardson et al. it appears that both DNA strands are resected upon the release of Cas9 and can initiate repair by annealing to appropriate ssODNs.

Another structural feature that can be modified in synthetic ssODNs is the replacement of the regular phosphodiester bonds by phosporothioate bond, increasing exonuclease resistance. Renaud et al. found that ssODNs with two phosporothioate bonds at both ends lead to higher knockin rates in U2OS cells and rodent embryos. Furthermore, since the homology regions of phosporothioate modified ssODNs can be kept short it is possible to increase the length of inserts up to 100 nt (Renaud et al. 2016). The efficiency of ssODNs is related to the distance between the mutation and DSB. Further, modulation of this distance can be used to generate heterozygous and homozygous knockins (Paquet et al. 2016).

## Enrichment of Cells with HDR Alleles

The enrichment of cells that underwent HDR successfully is a potential alternative to increasing the absolute rate of HDR. The use of drug-selectable gene-targeting vectors for this purpose is long established and mandatory for gene targeting in mouse embryonic stem cells since the frequency of spontaneous HR, without assistance by site-specific nucleases, is very low (Hasty et al. 2000). The `pop in, pop out´ strategy described by Xi et al. (Xi et al. 2015) (Kühn and Chu 2015) uses the classic targeting vector design in a new way to improve the isolation of targeted clones obtained by CRISPR/Cas9 induced gene editing. Briefly, targeting vectors are used that include an expression cassette for a GFP reporter for the isolation of cells harboring a stable vector integration by fluorescence activated cell sorting (FACS). The use of a GFP cassette flanked with loxP recombination sites allows its removal in a subsequent transfection step with Cre recombinase, followed by the FACS enrichment of $GFP^-$ cells. This

approach, leaves a single loxP site in the target locus and so the authors also used a second targeting vector for the scarless removal of the selection cassette to introduce a single nucleotide replacement. Altogether, this method is a useful option for the isolation of cells harboring precisely modified alleles using CRISPR/Cas9-induced DSBs, but requires a second working step for removal of the marker gene.

A related, single step approach without marker removal has been described by Dever et al. for targeting of the β–globin (HBB) gene in human hematopoietic stem cells using AAV6 based HDR templates (Dever et al. 2016). The use of either GFP or a membrane bound truncated NGF receptor (tNGFR) allowed to enrich for cells undergoing HDR, reaching levels of up to 90%. Since in primary cells it is not possible to apply a second step for marker removal, HDR templates for correction of the HBB sickle cell mutation (G6V) introduced a functional HBB cDNA, followed by the tNGFR expression cassette.

**Measurement and Quantification of DSB Repair Events**

Following a DSB cells can and do follow numerous pathways to repair the lesion. This results in a multitude of genotypes within the cellular population for a single experiment. Whether working to optimize a specific outcome or simply understand an event's frequency for protocol design – i.e. picking an appropriate number of single cell derived colonies - one must be able to asses and quantify the population of repair outcomes following a cleavage event. This section provides a brief overview of some methods for measuring these events.

**Knockout/Indel Analysis**

A DNA break resulting in an Indel is a commonly sought for change to the genome. These Indels are considered knockouts when disrupting a gene and frequently introduced to produce a frameshift in a coding region. The variety of insertions and deletions can be considerable and outcomes difficult to predict *in silico* as repair comes through a combination of the c-NHEJ and a-NHEJ pathways. The T7 endonuclease assay (T7EI) is a simple way to gain a rough quantification of the overall Indel frequency. It is done through amplification of the Indel region, hybridization to a native sequence, and exposure to the T7EI mismatch cleaving endonuclease. Agarose gel-separated band intensities give an approximate quantification of alleles that contain an Indel (Wyvekens et al. 2015).

Often a more detailed characterization of the Indel is necessary. PCR amplicons of the cut-site of interest can be Sanger sequenced. The resulting mixture of sequencing traces can

be computationally deconvoluted by either TIDE (Brinkman et al. 2014) or CRISPR-ID (Dehairs et al. 2016). A functional readout of knockout effectiveness can be easily done if the target site encodes a surface expressing protein. In such cases FACS sorting provides a rapid and simple method for function based KO quantification and the enrichment of KO cells (Ren et al. 2016).

**Knockin/HR Analysis**

Optimization or quantification of HDR in a specific locus often begins by knockin of a fluorescent reporter into the locus of interest for rapid quantification by FACS (Dever et al. 2016) (Suzuki et al. 2016). This simple readout gives information about HDR events but no insight into the amount of Indels that concurrently formed. An easy readout to quantify both the HDR events and Indel events occurring in a population is to use the synthetic Traffic Light Reporter System. In this system an Indel can shift an out-of-frame RFP into the correct frame or repair a disrupted GFP by HDR (Certo et al. 2011) (Chu et al. 2015). In another approach cell lines with an integrated GFP reporter are utilized to asses HDR efficacy. GFP can be converted to BFP by a single nucleotide substitution by HR with ssODNs (Glaser et al. 2016) (Richardson et al. 2016). In this assay HDR events can be measured by the shift from GFP expression to BFP expression and NHEJ by the loss of fluorescence. Furthermore, Droplet Digital (dd)PCR is a method to distinguish and quantify HDR events vs Indel formation. This technique can be done rapidly for many samples or conditions, for any region of interest, and even if the HDR event only results in a single nucleotide substitution (Miyaoka et al. 2016).

Deep sequencing the region of interest provides the most detailed analysis of the repair events. The hardware and the software for amplicon profiling has become increasingly accessible for the specialist or non-specialist. Basic Indel profiling and simple HDR events, such as point mutations, can be easily analyzed by the Crispresso (Pinello et al. 2016) or Outknocker (Schmid-Burgk et al. 2014) online tool. For those more bioinformatically minded the R toolkit, CrispRvariant, has been developed (Lindsay et al. 2016) as well as Batch-GE (Boel et al. 2016).

Off-target mutations are often a point of concern, and perturbation of DNA repair pathways may alter the expected mutation frequency of off-targets. These off-target events can be monitored by PCR amplification of the predicted off-target locations (Haeussler et al. 2016). Genome sequencing is not sensitive for most off-target events at a population level but can be a comprehensive check on a single cell derived colony. Methods for comprehensive and rigorous off-target analysis using deep sequencing have been developed (Kim et al. 2016) (Tsai and Joung 2016).

## Concluding Remarks and Future Approaches

RNA guided endonucleases such as CRISPR/Cas9 are revolutionizing the field of biology. The development and discovery of powerful ways to control the formation of DSBs requires an equal effort in DSB repair control. Despite efforts to improve HDR to increase genetic engineering possibilities, highly efficient gene editing by HDR is still challenging. In cycling cells progress has been made to shift the balance of DSB repair towards HDR. Further combination of conditions to suppress NHEJ (via 53BP1, DNA-PKcs or Ku70/80) and activate HDR (via CtIP, Rad51, Rad52, or Exo1) may be identified in future, to shift the balance of NHEJ/HDR repair more efficiently. In addition, negative regulators of the HDR response (reviewed by (Panier and Durocher 2013)) could represent new, yet unexplored targets for HDR enhancement. Improving the local assembly of regulatory molecules at the DSB of interest by e.g. fusion with the Cas9 protein may represent another, focused approach to promote HDR locally and minimize global interference with DSB repair pathways.

. In quiescent or resting cells HDR based changes are dramatically more difficult as the HDR pathway is deactivated in the G0/G1 phase of the cell cycle. Since most cell types relevant for in vivo gene therapy are not cycling, current attempts focus on the subset of genetic defects which can be ameliorated by c-NHEJ mediated deletion of genomic sequences (Cox et al. 2015) (Maeder and Gersbach 2016) (Xue et al. 2016) (Long et al. 2016) (Nelson et al. 2016). The ability to induce HDR in G1 cells with defined factors could support the development of therapeutic applications which can be broadly applied for precise gene correction. The seminal work of Orthwein et al. identified 53BP1 suppression, and CtIP and PALB2 activation as key factors for the reactivation of HDR in G1 cells (Orthwein et al. 2015). Nevertheless, the reactivation of HDR in non-cycling, post-mitotic cells may require the identification and reversal of additional blockades - a field that deserves further exploration.

Methods that rely on DSB repair by c-NHEJ but allow the insertion of genetic sequences may be used in G0/G1 cells. Presently, the most efficient procedure to achieve targeted integration by c-NHEJ into single DSBs has been through the HITI approach (Fig. 1). This enables the integration of a defined DNA segment into a DSB in a preferred orientation, but includes sequence adaptions at the ends and often includes imprecise end joining at the religated junctions. Some regions of the genome may prove to be able to accomodate such mutations.

Alternatively to forming DSBs, the nicking of single strands may provide another precise, HDR-related approach with future potential. Here, ssODNs can be used as templates for the repair of single nicks induced by e.g. the $Cas9^{D10A}$ nickase mutant. Nick repair is a safer alternative to DSBs as c-NHEJ is largely excluded (Kim et al. 2012) (Wang et al. 2012). Maizels and colleagues found that induced nicks are repaired by an alternative HDR pathway which is independent of the canonical HDR key factors Rad51 and BRCA2, which actually

inhibit this alternative pathway (Davis and Maizels 2011) (Davis and Maizels 2014) (Davis and Maizels 2016). This approach is promising and requires further study to increase the frequency of these precise repair events.

Taken together, there are many possibilities to improve these gene editing techniques to make them more efficient, precise, and safe for both *ex vivo* and *in vivo* applications. With the exponential pace of RNA guided nucleases adoption and utilization, this work is critically important and will be widely impactful.

## Tables

| Table 1: Manipulation of DSB repair | | | | |
|---|---|---|---|---|
| | Enhancing HDR | | Inhibiting NHEJ | |
| | Approach | Target | Approach | Target |
| Small molecules | RS-1<br>Brefeldin A<br>L755507 | Rad51<br>ADP ribosylation<br>β3-adrenergic receptor | SCR7<br>KU-0060648<br>W7/ chlorpromazine | DNA Ligase IV<br>DNA-PKcs<br>DNA-PKcs |
| Knockdown | | | shRNA<br>shRNA<br>shRNA<br>shRNA | DNA Ligase IV<br>Ku70<br>DNA-PKcs<br>53BP1 |
| Overexpression | cDNA expression<br>cDNA expression | BRCA1<br>Rad51 | Adenoproteins<br>i53<br>53BP1-Tudor<br>Gene knockout | DNA Ligase IV<br>53BP1<br>53BP1<br>53BP1 |
| Cell cycle regulation | Nocodazol<br>Cas9-Geminin | Reducing Cas9 expression in G1 cells | | |
| | Palb2 (KR)<br>CtiP (T847E) | Prevent deactivation in G1 | i53 | 53BP1 |
| Optimization of HDR template | Assymetric ssODN<br>PTO modified ssODN | Non-target strand 3` end<br>Nuclease resistance | | |
| Alternative approaches | Use of targeted reporter/marker genes (Pop in pop out)<br>PITCh: usage of MMEJ for integration<br>HITI: usage of NHEJ for integration | | | |

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## Figure Legends

### Figure 1 Utilizing DSB repair pathways for gene editing.

The repair of targeted DSBs induced by CRISPR/Cas9 in cycling cells can either religate or resect the DSB ends. End protection and religation is executed by the c-NHEJ repair pathway throughout all phases of the cell cycle. Gene knockout or disruption is achieved by the frequent insertion or deletion of nucleotides (Indel) during c-NHEJ repair. In the c-NHEJ-related HITI approach an exogenously provided DNA fragment is ligated in between the DSB ends in a directional manner. Resection of DSB ends in the S and G2 phase allows homology-directed repair (HDR). The HR pathway uses exogenous double-stranded or single-strand template repair (SST-R). Precise modifications or insertions are flanked by long (HR) or short (SST-R) regions of sequence homologies to the DSB ends and will be copied into the target locus. Alternatively, the PITCh approach utilizes MMEJ repair to achieve insertions with short homology sequences.

### Figure 2 DSB signaling and main repair pathways

DSB repair signaling is initiated by the ATM protein kinase that activates histone H2AX and MDC1. Subsequently the E3 ubiquitin ligases RNF8 and RNF168 set the chromatin mark at K15 of histone H2A that serves to recruit 53BP1. The antagonism between BRCA1 and 53BP1 decides, under control of the cell cycle phase, on the resection or protection of DSB ends. This choice governs repair by the HR or c-NHEJ pathway. Molecules used to bias the outcome of DSB repair are shown (RS-1, i53, NU7441, SCR7).

**Figure 1**

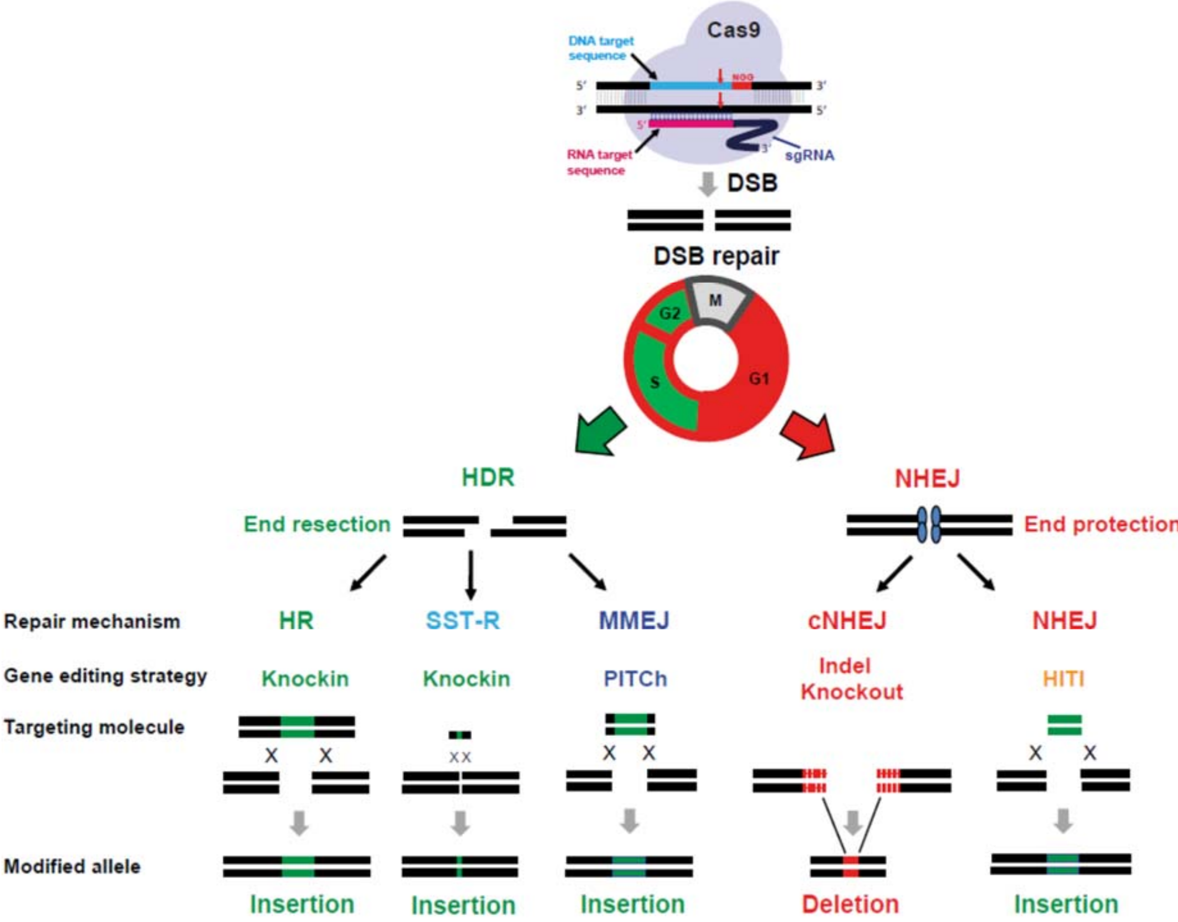

Figure 2