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Genome engineering with TALENs and ZFNs: Repair pathways and donor design

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ABSTRACT

Genome engineering with targetable nucleases depends on cellular pathways of DNA repair after target cleavage. Knowledge of how those pathways work, their requirements and their active factors, can guide experimental design and improve outcomes. While many aspects of both homologous recombination (HR) and nonhomologous end joining (NHEJ) are shared by a broad range of cells and organisms, some features are specific to individual situations. This article reviews the influence of repair mechanisms on the results of gene targeting experiments, with an emphasis on lessons learned from experiments with Drosophila.

1. Introduction

The targetable nucleases have created a small revolution in genetics by providing the means to make very specific changes in genomic DNA with high efficiency. First zinc-finger nucleases (ZFNs) [1–3], then transcription activator-like effector nucleases (TALENs) [4,5], and most recently CRISPR/Cas RNA-guided nucleases (CRISPRs) [6,7] have become powerful tools for genome engineering [8–10]. In fact, the only thing these reagents do is make breaks in chromosomal DNA at designed locations. After that, everything is left up to the cells in which the breaks have been made. The reason this works well is that cells detect double-strand breaks (DSBs) as potentially lethal damage and activate mechanisms to repair them (Fig. 1).

Cellular DSB repair pathways fall into two broad categories – ones that depend on extensive sequence homology and ones that do not [11]. The latter are typically classified as nonhomologous end joining (NHEJ). A characteristic of homology-independent repair is that it is frequently inaccurate, since it has no template from which to gather instructions. Genome engineers rely on NHEJ to generate local mutations at sites of nuclease cleavage.

Homology-mediated repair, often simply called homologous recombination (HR), is a more orderly process designed to restore the interrupted sequence precisely. This mechanism is the principal one used during the S and G2 phases of the mitotic cell cycle, and the sister chromatid is the preferred template [11]. Fortunately, cells will also use DNA provided exogenously as a template for repair. Thus, experimenters can introduce desired sequence changes by delivering an engineered donor DNA with sufficient homology flanking a targeted DSB.

It is difficult to measure absolute frequencies of repair by HR and NHEJ in most situations. First, there are repair events – HR with the sister chromatid, accurate religation of the ends at the break – that restore the original sequence and are therefore invisible. These products can, of course, be recut by the targeted nuclease. In many cases, NHEJ products are resistant to recutting, but this is not always true, particularly with TALENs, which allow variable spacer lengths between paired binding sites. Donor DNAs can be engineered to resist recleavage following HR. Any products that are uncuttable constitute a sink that accumulates mutants, and this is what we ultimately measure.

Overall frequencies of mutation stimulated by targetable nucleases can be very high, depending on many parameters. Levels well over 50% have been reported with the best TALENs and CRISPR nucleases [12,13]. When a donor is supplied and HR is desired, cells will still repair a proportion of DSBs by NHEJ. In many cell types, NHEJ is the main pathway of repair, and this can frustrate attempts to make very specific sequence changes at the target.

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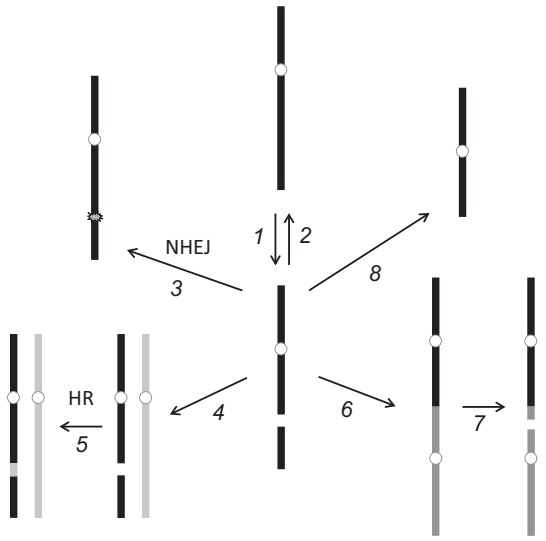

Fig. 1. Fates of double-strand breaks. An intact chromosome (top, middle) may suffer a double-strand break (1). This can be repaired by accurate religation to restore the original sequence (2), or rejoining may be inaccurate and introduce a sequence change (3, NHEJ). Pairing with a sister chromatid or homologous chromosome (4) allows accurate repair by homologous recombination (5, HR). If left unrepaired, the broken end may be inappropriately joined to another chromosome fragment (6). If the product has two centromeres, a new break may be introduced at mitosis due to centromeres being pulled in opposite directions (7). If the unrepaired chromosome enters mitosis, the fragment lacking a centromere may be lost (8).

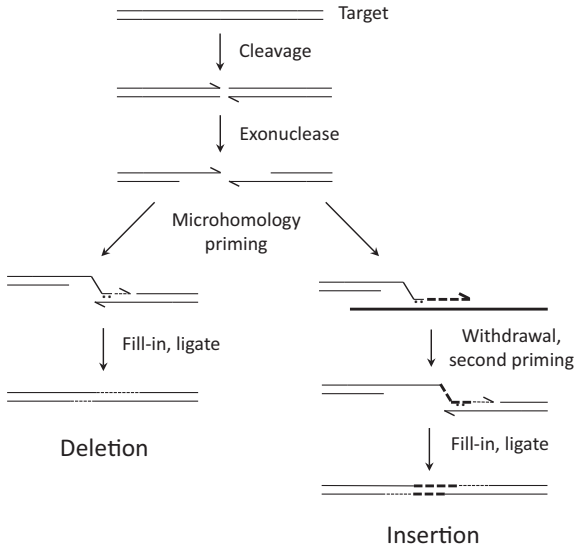

Fig. 2. Possible mechanisms of microhomology-mediated NHEJ. After cleavage, 5′ ends are resected, exposing single-stranded 3′ ends. One such end may pair with the other from the same break using a microhomology (left side). If that very short duplex is captured and extended by DNA polymerase, the junction is extended and stabilized. Filling in on the other side and ligation completes the process, resulting in a deletion. On the right side of the figure, the possibility of using some other template (thick line) is illustrated. The initial microhomology is extended on that template, the extended strand withdraws and pairs with the other end from the original break, perhaps again using a microhomology. The process is completed as on the left, resulting in an insertion of sequences from the first template.

2. Cellular DSB repair mechanisms

2.1. Nonhomologous end joining (NHEJ)

We have an incomplete understanding of all the ways that DNA ends can be rejoined, but it is common practice to specify a canonical pathway and one or more secondary or alternative pathways [14]. Canonical NHEJ in many organisms requires a common set of factors, including a dedicated DNA ligase (ligase IV or Lig4) and its associated Xrcc4 protein, and the end-binding dimer, Ku70/Ku80. When any of these factors is disabled, NHEJ is reduced to a variable extent, depending on the organism or cell type [11]. Genetic requirements for alternative NHEJ are poorly defined, although DNA ligase III is implicated, at least in some situations [15].

Not only do the factors and efficiencies of canonical and alternative NHEJ differ, so do the junctions produced. While both processes yield short sequence insertions and/or deletions at the break site, events occurring in the absence of canonical factors appear to rely more heavily on short sequence matches at or near the break, called microhomologies [14]. The molecular details of microhomology-mediated end joining are not fully worked out, but several plausible models exist. One idea is that the short matches provide a transient primer-template complex that can be captured and extended by DNA polymerase (Fig. 2) [16–18]. DNA ligase I could complete such a process by sealing nicks left after DNA synthesis, obviating a need for Lig4.

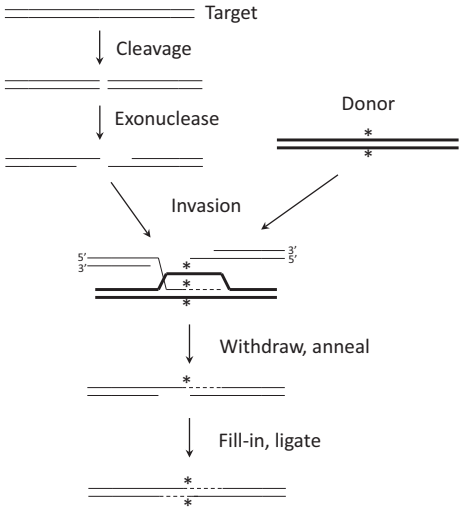

Fig. 3. Synthesis-dependent strand annealing (SDSA) mechanism of homologous recombination. As in Fig. 2 5′ ends are resected after the break. In this case, one 3′ single-stranded tail invades homologous sequence in the double-stranded donor (thick lines), which carries some distinguishing characteristic (*). The invading end is extended by DNA polymerase, using the donor as template. The extended strand withdraws and pairs with the other end from the original break. Polymerase and ligase activities complete the repair.

2.2. Homologous recombination (HR)

Although some aspects may differ, the basic features of break-stimulated HR in mitotically dividing cells (meiosis is special) are shared by essentially all eukaryotic organisms [11]. At the DNA level, each end at the break is resected by 5′ → 3′ nuclease activity, producing a single strand with a free 3′ end (Fig. 3). This end invades homologous sequence and is then extended by DNA polymerase. The extended strand withdraws and pairs with the exposed 3′-ending single strand from the other end of the original break. DNA polymerase and DNA ligase activities restore DNA duplex integrity. Additional protein requirements for the process, which is called synthesis-dependent strand annealing (SDSA), are also known [19]. The Rad51 protein mediates invasion; Rad54 is involved at several steps, including extension of the invading