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PNAS published online Oct 23, 2006;
doi:10.1073/pnas.0605633103

Induction and repair of zinc-finger nuclease-targeted double-strand breaks in *Caenorhabditis elegans* somatic cells

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Edited by Martin Chalfie, Columbia University, New York, NY, and approved September 13, 2006 (received for review July 5, 2006)

Zinc-finger nucleases are chimeric proteins consisting of engineered zinc-finger DNA-binding motifs attached to an endonuclease domain. These proteins can induce site-specific DNA double-strand breaks in genomic DNA, which are then substrates for cellular repair mechanisms. Here, we demonstrate that engineered zinc-finger nucleases function effectively in somatic cells of the nematode *Caenorhabditis elegans*. Although gene-conversion events were indistinguishable from uncut DNA in our assay, nonhomologous end joining resulted in mutations at the target site. A synthetic target on an extrachromosomal array was targeted with a previously characterized nuclease, and an endogenous genomic sequence was targeted with a pair of specifically designed nucleases. In both cases, ≈20% of the target sites were mutated after induction of the corresponding nucleases. Alterations in the extrachromosomal targets were largely products of end-filling and blunt ligation. By contrast, alterations in the chromosomal target were mostly deletions. We interpret these differences to reflect the abundance of homologous templates present in the extrachromosomal arrays versus the paucity of such templates for repair of chromosomal breaks. In addition, we find evidence for the involvement of error-prone DNA synthesis in both homologous and nonhomologous pathways of repair. DNA ligase IV is required for efficient end joining, particularly of blunt ends. In its absence, a secondary end-joining pathway relies more heavily on microhomologies in producing deletions.

DNA repair | gene targeting | nematodes | nonhomologous end joining

The ability to make targeted double-strand breaks in chromosomal DNA has several important uses. It allows the detailed study of DNA repair mechanisms; it leads to localized mutagenesis at the break site; and it enhances the efficiency of targeted gene replacement through homologous recombination. We have been exploring the capabilities of one class of targetable cleavage reagents, the zinc-finger nucleases (ZFNs).

ZFNs are chimeric proteins composed of DNA-binding Cys_2His_2 zinc fingers fused to the nonspecific nuclease domain of the restriction enzyme FokI (1). Each finger makes contact primarily with a separate DNA triplet (2, 3). Natural and artificial zinc fingers have been characterized that bind to all 5′-GNN-3′, many ANN and CNN, and some TNN triplets (4–7). Furthermore, the modular nature of the zinc fingers allows them to be joined in essentially arbitrary combinations. Typically, three zinc fingers are combined to bind to a specific 9-bp DNA sequence with the nanomolar affinity required to be biologically useful, but additional fingers can be incorporated to confer increased specificity (8–11). Zinc-finger fusions to various functional domains have been used to create artificial transcription factors and DNA-modifying proteins (12, 13).

When attached to the FokI nuclease domain, zinc fingers can direct cleavage to specific DNA sequences. The nuclease domain must dimerize to cleave DNA (14), and because the dimer interface is weak (15), two nuclease domains are typically brought into close proximity by pairs of zinc-finger sets binding to neighboring sites. A pair of inverted 9-bp zinc-finger-binding sites, spaced 6 bp apart, yields optimal DNA cleavage (16). In this configuration (Fig. 1), the engineered nucleases recognize a specific 18-bp sequence, which is long enough to be unique even in a large genome.

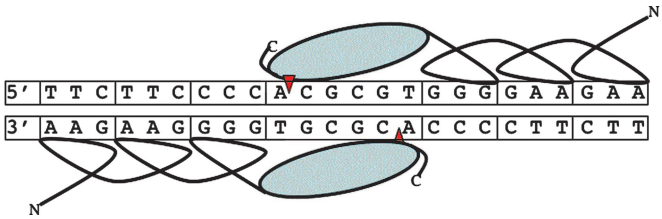

Fig. 1. Diagram showing a pair of ZFNs bound to DNA. The sequence shown is that of the synthetic QQR target. Each finger is represented by a loop shown contacting 3 bp of DNA; cleavage domains are represented by filled ovals; the N and C termini of the protein are indicated. The expected cut sites on the DNA strands are indicated with triangles.

Compared with other methods for generating unique double-strand breaks, ZFNs provide unprecedented targeting flexibility, because they do not depend on prior manipulation of the target. Transposon excision creates a double-strand break (17, 18), but an element must exist or be inserted in the desired location. Meganucleases such as I-SceI are valuable cleavage reagents because of their long recognition sites (19–21), but their sites must be introduced before they can be attacked. Because zinc fingers exist for many of the DNA triplets, nucleases of this type can theoretically be created to target almost any preexisting locus.

Studies performed in *Drosophila* and in human cells have shown that ZFNs can be designed to effectively cleave specific sequences (22–28). Additional studies with synthetic targets have demonstrated the efficiency of ZFN-directed cleavage in *Xenopus*, in mammalian cells and in plants (16, 29–31). When repaired by nonhomologous end joining, these double-strand breaks result in localized mutagenesis. In the presence of a homologous donor DNA, the nucleases stimulate targeted gene replacement at high levels. Because DNA repair mechanisms are quite universal, this approach should be applicable to most organisms.

We have begun to apply the ZFN technology to the nematode *Caenorhabditis elegans*, for which no efficient gene targeting procedure exists. Previous efforts, some based on transposon excision, indicate that the relevant repair machinery is present, if stimulatory breaks can be made (18, 32–35). We demonstrate that engineered

Author contributions: J.M., M.W.D., E.M.J., and D.C. designed research; J.M. and M.W.D. performed research; J.M., M.W.D., E.M.J., and D.C. analyzed data; and J.M., M.W.D., E.M.J., and D.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: Nw, nowhere; ZFN, zinc-finger nuclease.

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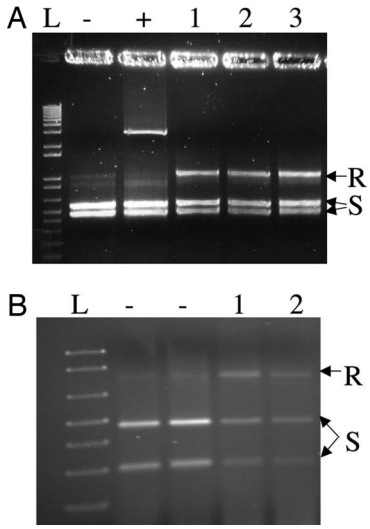

Fig. 2. Specific DNA targeting by ZFNs in *C. elegans*. The locations of PCR products resistant (R) and sensitive (S) to the diagnostic enzymes in both panels are indicated. (*A*) Alteration of a synthetic, extrachromosomal target by induction of the QQR nuclease. A 1-kb region around the QQR target was amplified by PCR and digested with MluI. L, linear size standards; −, a nematode not heat-shocked; +, plasmid positive control; 1–3, individual heat-shocked nematodes. (*B*) Alteration of the genomic Nw target with specifically engineered nucleases. A 752-bp genomic segment containing the Nw target was amplified and digested with HindIII. L, linear size standards; −, two nematodes that did not carry the NwA and NwB nucleases; 1,2, two heat-shocked nematodes carrying NwA, NwB.

nucleases cleave extrachromosomal and chromosomal targets, and we have used this approach to study the nonhomologous end joining pathway for the repair of double-strand breaks. We find that DNA ligase IV is required for efficient end joining, particularly of blunt ends, whereas a ligase IV-independent pathway relies more heavily on microhomologies.

Results

ZFN Cleavage in *C. elegans*. As a preliminary test of the efficacy of engineered nuclease technology in *C. elegans* somatic cells, we used a previously constructed nuclease, QQR, which has been shown to be active in *Xenopus* oocytes, human cells and *Arabidopsis thaliana* (16, 29, 30). The zinc fingers of this protein bind the DNA sequence 5′-GGG GAA GAA-3′. We created a synthetic target for QQR, consisting of two inverted binding sites separated by the 6-bp MluI restriction endonuclease recognition site (Fig. 1). An expression construct for the QQR nuclease was prepared with the coding sequence under the control of the well-characterized *C. elegans* 16-48 heat-shock promoter (36).

The QQR target and expression plasmids were coinjected into the germ line to generate an extrachromosomal, multicopy transgene array (37). Such arrays typically consist of tens to hundreds of copies of the injected plasmids. L2–L3 larvae from a stable line were heat-shocked to induce expression of the nuclease. Larvae were used rather than adult animals to minimize the contribution of the germ line, because transgenes on arrays are effectively silenced in worm germ tissue (38). Because 10–100 germ cells are present even in these larval stages [Kimble, J. and Crittenden, S. L. (2005) in *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.13.1, www.wormbook.org.], the frequencies of nuclease-induced events are modestly underestimated. After recovery for several hours, DNA was isolated and the QQR target was amplified by PCR in a 1-kb fragment. The PCR products were treated with MluI to identify targets that were altered by inaccurate repair after cleavage. As shown in Fig. 2*A*, a substantial portion of the QQR targets had become resistant to MluI digestion in the heat-shocked nematodes, but not in controls.

Table 1. Sequences of ZFN-induced mutations in the QQR target

Target after cleavage	TTCTTCCCCA CGCGTGGGGAAGAA AAGAAGGGGTGCGC ACCCCTTCTT	
Insertions	A**CGCG**CGCGT	(25)
	ACGCG-**GCG**T	
	A**CGC**-CGCGT	
	A**CG**--CGCGT	
	ACGCG---**G**T	
	ACG---**G**CGT	
Deletions	A-----GCGT	(2)
	<--374 bp-----239 bp-->	
	<--256 bp------18 bp-->	
	<--243 bp-----223 bp-->	
	<---62 bp-----403 bp-->	
Substitutions	**t**CGCG----T	
Complex	<--490 bp-----109 bp-->**a**	(3)
	T-CCCCACGCG----126 bp->**a**	
	tCCA**CGCG**CGCG**a**	
	CC-A**CGC**-CGCGT	

The target sequence after ZFN cleavage with the expected 4-nt 5′ overhangs is shown in the top section. Lower sections show sequences of MluI-resistant products recovered after nuclease induction. Deletions are indicated with dashes, insertions are indicated with bold upper case letters, and substitutions are indicated with bold lower case letters. Microhomologies at the junctions are underlined. The lengths of the long deletions on either side of the cleavage site are given. In cases where a mutation was recovered more than once, the number of instances is given at the right in parentheses.

To get a quantitative measurement of the frequency of MluI site loss, PCR products were cloned without prior digestion and assayed individually. QQR-induced mutations occurred in 26% (43 of 167 clones from four individual worms) of the targets examined. This is a minimum estimate, because some repair events would restore the MluI site, and some deletions may have been large enough to escape detection. By comparison, animals not subjected to heat shock exhibited no mutations at the QQR target, indicating that the observed mutations did not arise independently, and that uninduced QQR expression was minimal.

The MluI-resistant cloned PCR products were sequenced to determine the nature of the QQR-induced mutations. As shown in Table 1, the majority were simple insertions at the cleavage site. 58% (25 of 43) of the products had a duplication of the 4-bp sequence at the center of the MluI recognition site, which very likely arose by fill-in and blunt-end ligation of the 5′ overhangs created by QQR cleavage (14). Five products corresponded to partial fill-ins, and two showed fill-ins with an additional nearby alteration; thus, the new junction was likely created by blunt-end ligation in 74% of the cases. Among the remaining products, eight had rather large deletions (>100 bp), two had single base pair deletions, and one had a simple substitution in the MluI site. All together, five of the cloned products contained untemplated substitutions very close to the new junction, whereas none had substitutions elsewhere in the sequenced region, which extended for several hundred base pairs on either side of the cleavage site. Although the 25 identical 4-bp fill-ins were derived from only four nematodes, it is likely that they arose independently, because there are thousands of targets in each worm that should all be amplified with comparable efficiency, and most other alterations were recovered only once. The observed mutations all fall within the range expected for inaccurate repair after QQR cleavage.

Cleavage of a Genomic Target. Having demonstrated that one engineered nuclease works in worms, we set out to target a

Table 2. ZFN-induced mutations

	Long PCR products*		QQR, short PCR*	
Alteration	QQR	Nw†	WT†	*lig-4*†
Fill-in				
4 bp	25 (4)‡	6 (4)	89 (17)	0
3 bp	2 (2)	1	10 (8)	0
2 bp	1 (1)	0	3 (3)	0
1 bp	2 (2)	0	0	0
Complex	2 (2)	0	3 (2)	0
Deletions				
<10 bp	2 (2)	17 (14)	11 (11)	7 (7)
>10 bp	8 (7)	27 (17)	0	8 (8)
Substitutions	1 (1)	3 (3)	11 (10)	61 (51)

*Long PCR products (from WT) were 1,006 bp for QQR and 752 bp for Nw; short QQR PCR products were 207 bp.

†Sequences of these mutations are presented in Tables 4, 5, and 6, which are published as supporting information on the PNAS web site.

‡The numbers of alterations in each category are shown. The numbers in parentheses represent independent observations that are either different sequences or the same sequence recovered from different worms. The numbers of worms in each category are QQR-long, 4; Nw, 4; QQR-short WT, 18; QQR-short *lig-4*, 21.

nematode genomic locus with newly designed ZFNs. To facilitate analysis, we searched for sites in the form 5′-$(NNC)_3N_6(GNN)_3$-3′ in which the 6-bp spacer contained a convenient restriction site. We limited the search to GNN triplets, because the corresponding fingers have been most extensively tested. Among many such sites in the *C. elegans* genome, we chose a target at bases 3008453–3008476 of the X chromosome, which contains a HindIII site in the spacer (Wormbase, www.wormbase.org, release WS160, July 12, 2006). The sequence is shown below.

5′-ACC CAC TCC AAGCTT **GGG GTG GCT**-3′

3′-**TGG GTG AGG** TTCGAA CCC CAC CGA-5′

Because this target is located more than 1 kb away from the nearest predicted gene, we named it Nowhere (Nw). We constructed a new pair of ZFNs designed to bind the component 9-mers of this site, NwA for GGG GTG GCT, NwB for GGA GTG GGT, shown in bold above, and cloned them behind the 16-48 heat-shock promoter. Transgenic larvae were heat-shocked to express the nucleases recognizing each half site. The target region was amplified by PCR, and nuclease-induced mutations were identified by HindIII restriction enzyme digest of the 752-bp products. A substantial portion (≈20%) of the DNA was resistant to HindIII cleavage in the products from several individual nematodes (Fig. 2*B*); thus, cleavage at a chromosomal site by engineered nucleases is efficient.

In contrast to nuclease-induced double-strand breaks in the extrachromosomal arrays, repair of many of the breaks in the genomic target resulted in deletions. The frequency of induced mutations at Nw was quite similar to that for the QQR array: 18% (27 of 154 unselected clones) were HindIII-resistant ($P = 0.10$ for comparison of the QQR and Nw results). DNA sequence analysis of these and additional clones (Table 2) revealed relatively few simple fill-ins (13%, 7 of 54 sequences), whereas a much higher proportion was represented by small and large deletions. A small number of single-base pair substitutions was observed. A possible reason for the differences between the repair products at the genomic and extrachromosomal loci is that homologous repair is more frequent with the abundance of templates on the array, and this biases the outcome of end joining (see *Discussion*).

DNA Ligase IV Is Required for Blunt-End Joining. The mutations generated by ZFN expression apparently resulted from inaccurate

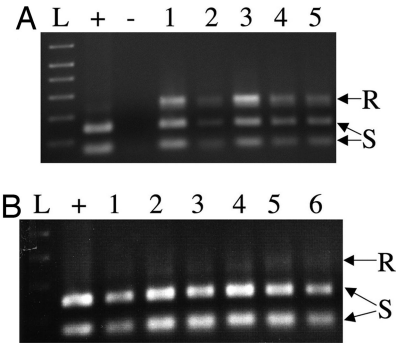

Fig. 3. PCR products from the QQR target after ZFN induction in wild-type (*A*) and *lig-4* (*B*) nematodes. The unmodified product is 207 bp long. The format is as in Fig. 2.

DNA repair via nonhomologous end joining. To test this hypothesis, we examined repair in mutants lacking DNA ligase IV, which is required for this process (39). An array carrying the QQR target and expression construct was established in a *lig-4(ok716)* strain. Nuclease induction and target analysis were performed as described above, except that the PCR products were considerably shorter. Fig. 3 shows a comparison of MluI digests of 207-bp PCR products from wild-type and *lig-4* nematodes obtained after QQR induction. As observed before, a substantial proportion of the PCR products were MluI-resistant in the wild type. By contrast, the *lig-4* worms yielded a very low level of such products. When unselected cloned products were characterized, 11% (25 of 239 clones) from the wild type were MluI-resistant, whereas only 0.4% (1 of 232 clones) were resistant in the *lig-4* case. This difference is highly significant ($P = 5 \times 10^{-6}$). No MluI-resistant products were recovered from the wild type without heat shock (0 of 112 clones). As expected, ligase IV was required for efficient end joining; the remaining ends were presumably either lost or repaired by homologous recombination.

Sequence analysis of MluI-resistant products showed that the spectrum of alterations was dramatically affected by the *lig-4* mutation (Table 2). As before, repair products from wild-type worms were dominated by small insertions at the cleavage site: full (89 of 127 sequences) and partial (16 of 127) fill-ins represented 83% of the products. The remainder were small deletions (9%) and localized substitutions (9%). (Larger deletions were presumably absent because of the limited size of the PCR product.) No insertions were seen in the *lig-4* background (Table 2), indicating that DNA ligase IV is absolutely required for blunt-end joining. Deletions comprised 20% of the total (15 of 76); however, the most common alterations were single-base pair substitutions in the MluI site (80%, 61 of 76). Several of the deletions and substitutions (8 cases total) showed additional replacements close to the junction. These are not simply artifacts of inaccurate *Taq* polymerase amplification, because they were very rarely seen in conjunction with insertions in the wild-type PCR products or at sites more than 30 bp from the new junction. Rather, these substitutions likely arose during repair via error-prone DNA synthesis (see *Discussion*).

Repair of Double-Strand Breaks in the Germ Line. Because expression of transgenes on extrachromosomal arrays is largely suppressed in the nematode germ line (38), we could only analyze repair of nuclease-induced double-strand breaks in somatic cells in the above experiments. To determine whether similar repair mechanisms heal breaks in the germ line, we investigated the joining of ends of exogenous DNA injected into the syncytial gonad of both wild-type and *lig-4* nematodes. Repair products were recovered by PCR. Plasmid DNA was cut with two different restriction enzymes, leaving incompatible four-base 5′ overhangs. This configuration eliminates repair by homologous recombination with uncut tem-

Table 3. Sequences of junctions formed in the germ line

Injected DNA	GCTACGTAATACGACTCACTAGTGGGCA AGCTTCCCATGGTGACGTCACCGGTTCT CGATGCATTATGCTGAGTGATCACCCGTCTAG AGGGTACCACTGCAGTGGCCAAGA	
Products from WT	AG--- --CTT	(3)
	AGATC ---TT	
	tGTGGGCAGATC -----gC	
	C----- -GCTT	
	AGA-- -GCTT	
	AGA-- AGCTT	
	A---- AGCTT	
Products from *lig-4*	AG--- --CTT	(2)
	AGA-- ----TCC	(2)
	gGA-- ----TCC	
	CA---- ---------T	
	ACGT---------- ----------CA	
	AGA-- ----------CT	

Substrate ends generated by BglII and HindIII digestion are shown in the top section. Symbols for deletions, insertions, substitutions and microhomologies are as in Table 1.

plates and by simple religation, both of which were possible with the breaks generated by the induction of the ZFNs. Thus, in addition to examining the germ-line function of the nonhomologous end joining machinery, it does so in the absence of competition by gene conversion.

Products recovered from wild-type animals consisted largely of partial fill-ins of the 5′ overhang (Table 3). In only two of nine products were the ends resected past the single-stranded 5′ overhang, and then by only one or two bases. Only four of nine products showed possible microhomologies at the junctions, and these were only one or two bases long. These repair products are consistent with previously demonstrated *lig-4*-dependent ligation of blunt ends and resembled those recovered from somatic cells after nuclease cleavage.

By contrast, almost all products recovered from *lig-4* mutants exhibited resection into double-stranded DNA. Three of eight had large deletions, and seven of eight had some loss of sequence from double-stranded regions. Furthermore, seven of eight showed junctional microhomologies of two or four bases. Again these products resemble those produced in somatic cells in *lig*-4 mutants.

Very similar results were obtained with an injected DNA having blunt ends (Table 7, which is published as supporting information on the PNAS web site). In the wild type, blunt joins were recovered, along with very small deletions, some of which had short microhomologies. One particular 9-bp deletion with a 4-bp microhomology was recovered multiple times. In *lig-4* worms, this deletion was the most common product (7 of 16 clones). Another favored junction (5 of 16) had a 266-bp deletion based on a microhomology in which 8 of 9 bp matched. In contrast to the wild type, all junctions formed in the *lig-4* mutants were deletions bounded by microhomologies of at least 3 bp. Thus, *lig-4*-independent repair processes in both the soma and germ line rely on a microhomology-based mechanism of end joining.

Discussion

The ability to create DSBs at unique and arbitrary points in the genome of *C. elegans* is a powerful new tool with many potential uses. This study demonstrates that ZFNs can induce specific breaks in both an extrachromosomal and a genomic target. Of particular importance is the demonstration that novel zinc-finger combinations can be designed to target a preexisting chromosomal sequence (Nw) with good efficiency. Furthermore, the analysis of repaired DNA demonstrates that the broken ends are often rejoined inaccurately to produce mutations in the target at high frequency.

Double-strand breaks were repaired differently in extrachromosomal and genomic targets. Breaks in the extrachromosomal array were repaired largely by fill-in and blunt-end ligation, whereas the majority of nuclease-induced genomic mutations were deletions. We offer a molecular model that can account for these findings (Fig. 4). We imagine two possible early fates of molecular ends at the break: fill-in followed by blunt joining or 5′-3′ exonucleolytic resection. The 3′-ending single-stranded tails generated by resection are substrates for gene conversion by homologous recombination. Fill-in products cannot be used for homologous recombination, and resected products cannot participate directly in blunt-end joining, although the tails might be removed allowing blunt ligation.

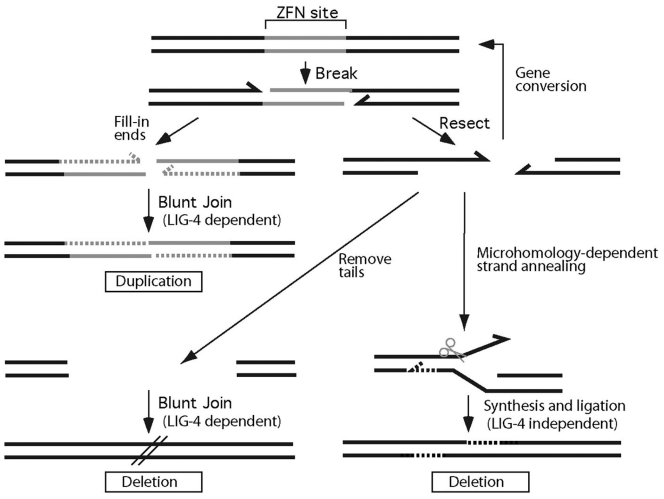

Fig. 4. Models of repair pathways after ZFN cleavage. The double-strand break has 4-bp 5′ overhangs that can be filled in completely or partially by DNA polymerase and the resulting blunt ends ligated. This process depends on DNA ligase IV. Alternatively, 5′->3′ resection at the ends leaves single-stranded 3′ tails that can be substrates for either template-dependent gene conversion or for a nonhomologous end-joining process leading to deletions. Gene conversion would restore the original sequence, which could then be recut and reprocessed. End joining could occur by removal of the single-stranded tails and blunt joining, relying on DNA ligase IV. An alternative end-joining process, independent of ligase IV, relies on microhomologies; the one illustrated envisions extension of a transient microhomology-based primer–template complex by DNA polymerase (41).

Why do fill-ins predominate in the repair of extrachromosomal targets? We propose that resection, not fill-in, is the preferred fate for breaks at both chromosomal and extrachromosomal targets. After resection, the balance between homologous and nonhomologous repair depends on the availability of homologous templates. In the case of the extrachromosomal target, there are many copies on the array, and gene conversion with an uncut sequence may be the most likely outcome. This regenerates the original sequence, which can then be recut and subjected to fill-in or resection. When fill-ins occur the nuclease target is destroyed, the mutation becomes fixed, and it may even serve as a template for subsequent gene conversion. In contrast, breaks at the chromosomal site have only the homologue and sister chromatid as possible repair templates, and if all of the Nw sites in a single cell are cleaved, no intact template remains. For these reasons, resected ends may more frequently be repaired by nonhomologous end joining after removal of the 3′ tails, with the result that deletions are common. In summary, the paucity of homologous templates drives the formation of deletions at the chromosomal target.

DNA ligase IV is required for blunt-end joining both in nematode somatic cells and in the germ line, consistent with findings in other systems (40). In the absence of ligase IV, ends are resected and repaired by an alternative nonhomologous end-joining mechanism that depends on microhomologies, again consistent with observations in other organisms. We illustrate this in Fig. 4 as DNA synthesis initiated at a transient primer-template complex (41), but microhomology-mediated direct joining is also possible, perhaps by using DNA ligase I. In *lig-4* nematodes, resected ends are most frequently repaired by homologous recombination. Only when this process creates sequence changes that eliminate the MluI recognition site do we identify them in our screen. Thus, the simple substitutions that we recover are probably generated during gene conversion by error-prone DNA synthesis, perhaps by DNA polymerase η (42, 43). We also found substitutions associated with some deletion products, particularly in *lig-4* worms, so error-prone synthesis may be characteristic of the alternative end joining pathway as well (44). The substitutions all occurred within ≈30 bp of the cleavage site, indicating that synthesis tracts during repair are rather short.

In a very recent study, V. Robert and J. L. Bessereau (personal communication) examined repair products generated in *C. elegans* after transposon excision from a chromosomal locus, with results broadly similar to ours. In somatic tissues, in agreement with our findings, they observed both deletions and insertions at the join point. Many of the latter appeared to be partial fill-ins. This is remarkable, because the ends left by MosI excision are 3-nt 3′ (not 5′) overhangs, and therefore provide no recessed 3′ end to prime DNA synthesis. In a *lig-4* background, repair products were much less abundant, insertions were largely gone, and deletions supported by microhomologies were common.

In summary, our study shows that custom-engineered ZFNs are capable of generating targeted double-strand breaks in nematode somatic DNA at high frequency. Repair of these breaks by nonhomologous end joining often generates mutations at the targeted locus. This procedure has relatively little practical value as a genetic tool at this stage, unless essentially complete cleavage could be obtained in specific cell lineages and transient effects investigated. Clearly the next step is to produce cleavage and mutagenesis in the nematode germ line. Although reiterated transgenes are rather effectively suppressed in germ-line cells (38), several potential solutions to this problem are available. These include driving nuclease expression with a natural germ-line promoter (45), reducing the number of transgenes through the use of bombardment (46), eliminating one or more components of the apparatus responsible for suppression (47), and direct protein or mRNA injection. Once germ-line expression is achieved, it is quite likely that deletion alleles and targeted gene replacements can be generated by using engineered ZFNs.

Materials and Methods

Nematode Strains. The wild-type N2 was the parent strain for all others. The DNA ligase IV mutant strain was RB873 *lig-4(ok716) III*, which harbors a deletion of more than half the gene, including most of the ligase consensus sequences, that almost certainly abrogates ligase function.

Plasmid Constructions. Plasmid pJM1-ZFN carries a ZFN coding sequence under heat-shock promoter control, in which the zinc fingers are easily replaced for attack of alternative targets. The pJL44.2 vector, containing the 16-48 heat-shock promoter driving MosI transposase expression (36), was modified by PCR-directed mutagenesis of the SpeI and NdeI sites. The transposase coding sequence was excised by MluI + NheI digestion and replaced with a DNA fragment containing a four-adenosine translational initiation consensus sequence, an ATG codon, codons for a nuclear localization signal (DPKKKRKV), and the coding sequence for the ben-1A ZFN (J.M. and D.C., unpublished results). The latter has a very short linker between the binding and cleavage domains (L = 0) (16), and the zinc-finger region is bounded by NdeI and SpeI sites.

The coding sequence for the QQR zinc fingers was excised from the Zif-QQR-F_N (L0) plasmid (16) by NdeI + SpeI digestion and placed in the pJM1-ZFN backbone to create pJM1-QQR. Coding sequences for the NwA and NwB zinc fingers were synthesized from long oligonucleotides (48) in a Zif268 framework (2). The resulting PCR products were cloned in the pJM1-ZFN backbone to create pJM1-NwA and pJM1-NwB. The amino acid sequences of the specificity-determining residues (6) of the three zinc fingers in NwA are: finger 1, QSSDLTR (recognizes GCT); finger 2, RSDALTR (GTG); finger 3, RSDHLSR (GGG). In NwB they are: finger 1, TSGHLVR (GGT); finger 2, RSDALTR (GTG); finger 3, QSGHLQR (GGA).

The QQR target was created by hybridizing the complementary oligonucleotides (5′-CTAGCTTCTTCCCCACGCGTGGGGAAGAA-3′ and 5′-AGCTTTCTTCCCCACGCGTGGGGAAGAAG-3′). The annealed fragment was ligated into HindIII + NheI-digested pL4440 (gift from Susan Mango, University of Utah) to create pJM2-QQRt.

Transgenic Arrays. N2 nematodes were transformed with pJM1-QQR (5 ng/μl), pJM2-QQRt (15 ng/μl), pPD118.33 (*Pmyo-2*::GFP; 1 ng/μl) and 1-kb ladder (80 ng/μl; Invitrogen, Carlsbad, CA) to produce strain EG3526 *oxEx654* that carries both the QQR target and the QQR expression construct. The RB873 *lig-4(ok716) III* strain was transformed with the same mixture, creating strain EG3521 *lig-4(ok716) III; oxEx653*. Strain EG3839 *oxEx706*, with the NwA and NwB ZFNs, was constructed by injecting N2 worms with pJM1-NwA (5 ng/μl), pJM1-NwB (5 ng/μl), pPD118.33 (1 ng/μl) and 1-kb ladder (100 ng/μl). Plasmid DNAs were linearized by ScaI digestion. Injections were performed on young adult N2 and RB873 nematodes by using standard techniques (37).

ZFN Induction. Plates containing L2–L3 nematodes from transgenic strains were wrapped in Parafilm and heat-shocked in a water bath at 35°C for 1 h on two consecutive days, then allowed to recover at room temperature for several hours.

DNA Isolation and Analysis. Heat-shocked worms were frozen at −80°C in single-worm lysis buffer [50 mM KCl/10 mM Tris·HCl (pH 8.3)/2.5 mM $MgCl_2$/0.45% Nonidet P-40/0.45% Tween-20/0.01% gelatin/200 μg/ml Proteinase K], lysed at 65°C for 1 h, followed by 95°C for 15 min. The QQR target DNA was amplified by PCR with *Taq* polymerase (New England Biolabs, Beverly, MA) in two different formats. To produce a 207-bp target fragment, the primers 5′-CCTGGCTTATCGAAATTAATACGA-3′ and 5′-

CTATAGGGCGAATTGGGTACC-3′ were used. An alternative pair of primers, 5′-AACCGGCCCGGGTGAGATACCTACAGCGTGAGC-3′ and 5′-AACCGGCCCGGGGTCGAGGTGCCGTAAAGCAC-3′, gave a 1,006-bp product. The Nw target was amplified by using primers 5′-AACCGGGAATTCCAATCTATTTTTCGTGTAACGTG-3′ and 5′-AATTCCGGATCCACAAAATTGGCTTTCTTGTAACC-3′ to give a 752-bp product. After amplification, DNA was purified on MinElute columns (Qiagen, Valencia, CA), digested with MluI (QQR target) or HindIII (Nw target) and examined by electrophoresis in 1.5% or 2% agarose gels. DNA resistant to digestion was excised from the gels, purified by using Qiagen MinElute columns, and reamplified by PCR using *Taq* polymerase. When determining the mutation frequency, this initial restriction digest and analysis by gel electrophoresis were not performed. For sequence analysis, the short QQR product was digested with NotI and XhoI, which produces an 88-bp fragment from the unmodified target, and ligated into a similarly digested pBluescript backbone. The 1-kb QQR product was ligated directly into the pGEM-T vector backbone according to the manufacturer's instructions (Promega, Madison, WI). The Nw target was digested at the EcoRI and BamHI sites introduced on the PCR primers, and ligated into digested pBluescript. Ligation mixtures were used to transform *Escherichia coli* SS320 electrocompetent cells, which were allowed to grow overnight at 37°C on plates containing 100 μg/ml ampicillin. Individual transformants were screened by colony PCR (49) and digestion with MluI (QQR) or HindIII (Nw). Transformants yielding resistant PCR products were cultured, DNA was purified with Qiagen columns, and automated DNA sequences obtained at the University of Utah Core Sequencing Center. Sequence analysis was performed by using DNA Strider.

Germ-Line Injection. For incompatible 5′ overhangs, pLitmus28 plasmid (New England Biolabs) was digested with BglII and HindIII, purified by using a Qiaquick spin column, dried in a SpeedVac concentrator and resuspended at a concentration of 100 ng/μl, and injected by using standard techniques (37). After 1–4 h, worms were frozen at −80°C in single-worm lysis buffer as described above. PCR was performed with primers 5′-GCCCCCGATTTAGAGCTTG-3′ and 5′-ACGTTGTTGCCATTGCTACAGG-3′, followed by gel purification of the resulting bands and DNA sequencing by using the primer 5′-TGTAAAACGACGGCCAGT-3′ (M13 forward). For blunt DNA fragments, plasmid pWD97 was used. This plasmid contains coding sequences for mRFP (50), with a PvuII-SnaBI fragment from pLitmus28 inserted into a PvuII site. This construct provides the multiple cloning site from pLitmus, flanked by mRFP sequences. The plasmid was digested with EcoRV and PvuII and purified, concentrated, and injected as described above. The worms were frozen, lysed and their DNA amplified as before, except the primers 5′-TGCTAGTTATTGCTCAGCGGT-3′ and 5′-TGCTAGTTATTGCTCAGCGGT-3′ were used. Bands were purified from an agarose gel and TA-cloned by using the pGEM-T kit from Promega. Plasmids were purified from single colonies and sequenced by using a standard SP6 primer 5′-ATTTAGGTGACACTATAG-3′.

We thank Josh Checketts and Erin Fuchs for technical assistance and Susan Mango, Kelly Beumer, Jon Trautman, and Ana Bozas for advice. The Mango Lab generously allowed J.M. to make use of their nematode facilities. We thank Sofia Robb for performing the search that identified the Nw target. This work was supported by National Institutes of Health (NIH) Research Grant GM065173 (to D.C.) and Training Grant in Genetics T32 GM07464 (to J.M.), National Science Foundation research Grant 0516815 (to E.M.J.), a seed grant from the University of Utah (to D.C.), and core facilities funded in part by the University of Utah Cancer Center Support Grant. The *lig-4* strain was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources.

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Table 4

Sequences of ZFN-induced mutations at the Nw (Nowhere) target

Target after cleavage	ACCCACTCCA AGCTTGGGGTGGCT TGGGTGAGGTTCGA ACCCCACCGA	
Insertions	CA**AGCT**AGCTTG	(6)
	CAAGCT-**GCT**TG	
Deletions	CA-----GCTTG	(7)
	CAAG------TG	(2)
	CAAGC-----TG	(3)
	CA-------TTG	(2)
	------AGCTTG	
	CAAG--------	
	<---16bp----------	
	CAAGCT-----53bp----->	
	----------55bp----->	
	<-----55bp------CTTG	
	<-----65bp----AGCTTG	
	<------------97bp---------->	
	<-----------127bp---------->	
	<----210bp-------TTG	
	CA--------237bp----->	(3)
	CAAGCT----248bp----->	
	CAAGC----->252bp----->	
	<--275bp------AGCTTG	
	<--296bp------AGCTTG	(10)
	<--325bp---------TTG	
	<--389bp--------CTTG	
	<--403bp--------CTTG	
Substitutions	CA----AGC**c**TG	(2)
	CA----AGCT**c**G	
Complex	CAAG------TGG**a**	

Format as in Table 1.

Table 5

Sequences of mutations recovered in short QQR PCR products from wild type

Target after cleavage	TTCTTCCCCA CGCGTGGGGAAGAA AAGAAGGGGTGCGC ACCCCTTCTT	
Insertions	A**CGCG**CGCGT	(89)
	A**CG-G**CGCGT	(5)
	A**C-CG**CGCGT	(3)
	A**CG--**CGCGT	(3)
	A**CA-G**CGCGT	
Deletions	-----CGCGT	(3)
	ACGC-----T	(3)
	ACGCG -	
	------GCGT	
	---------T	
	----------T	
Substitutions	ACGCG----**c**	(3)
	gCGCG----T	(2)
	A**t**GCG----T	
	Ac**a**CG----T	
	ACGC**a**----T	
Complex	--------**c**T	
	CC**t**A**C-CG**CGCGT	

Format as in Table 1.

Table 6

Sequences of QQR mutations from *lig-4* worms

Target after cleavage	TTCTTCCCCA CGCGTGGGGAAGAA AAGAAGGGGTGCGC ACCCCTTCTT	
Insertions	(none observed)	
Deletions	ACGCG-------- ------GCGT -------GCGT ------------CGT ------------------------ <---------19bp------- ---22bp------------> ---------24bp------------> -27bp------------> <---------31bp------------> <---------32bp------------>	
Substitutions	**g**CGCG----T **t**CGCG----T **c**CGCG----T A**t**GCG----T Ac**a**CG----T ACG**t**G----T ACG**a**G----T ACGC**a**----T ACGC**c**----T ACGCG----**c** ACGCG----**a** ACGCG----**g**	(9) (2) (4) (7) (4) (8) (6) (2)
Complex	TTtTT----21bp------------> ACGCG----**g**GGGGAAG**g**A ACGCG----**c**GGGG**g**A ACG**t**G----TGGGG**g**A ACGCc----TGGGGA**g**G T**c**CCC--------CGT **C**TCCCCACGCG----**c** ----------**c**GG ----------**g**	

Format as in Table 1.

Table 7

Sequences of junctions formed from blunt ends in the germline

Injected DNA	CGCACCGTACGTCTCGAGGAATTCCTGCAGGAT CTGCCCGGCGCCTACAAGACCGACATCA GCGTGGCATGCAGAGCTCCTTAAGGACGTCCTA GACGGGCCGCGGATGTTCTGGCTGTAGT	
Products From wt	GGAT CTGC GGAT --GC GGAT ----CCG TT---------- ---CCCG TTC--------- CTGCCCG	(3) (3)
Products From lig-4	GGAT CTGC TTC--------- CTGCCCG TTC--------- C**c**GCCCG TT---------- ----------CCTAC <----------171bp---------- -----CGGCGCCTA <----------266bp---------- CTGCCCCGCGC <----------333bp---------- -----CGGCGCC	 (7) (5)*

Substrate ends generated by *Eco*RV and *Pvu*II digestion are shown in the top section. Symbols for deletions, insertions, substitutions and microhomologies are as in Table 1. The asterisk indicates that one of the 5 sequences in this group had an untemplated substitution 3 bp from the microhomology at the junction.