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Targeted engineering of the *Caenorhabditis elegans* genome following *Mos1*-triggered chromosomal breaks

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The *Drosophila* element *Mos1* is a class II transposon, which moves by a 'cut-and-paste' mechanism and can be experimentally mobilized in the *Caenorhabditis elegans* germ line. Here, we triggered the excision of identified *Mos1* insertions to create chromosomal breaks at given sites and further manipulate the broken loci. Double-strand break (DSB) repair could be achieved by gene conversion using a transgene containing sequences homologous to the broken chromosomal region as a repair template. Consequently, mutations engineered in the transgene could be copied to a specific locus at high frequency. This pathway was further characterized to develop an efficient tool—called *Mos*TIC—to manipulate the *C. elegans* genome. Analysis of DSB repair during *Mos*TIC experiments demonstrated that DSBs could also be sealed by end-joining in the germ line, independently from the evolutionarily conserved Ku80 and ligase IV factors. In conjunction with a publicly available *Mos1* insertion library currently being generated, *Mos*TIC will provide a general tool to customize the *C. elegans* genome.
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Introduction

Gene knockout and knock-in techniques have emerged as powerful tools to study gene function in eukaryotic model organisms such as yeast (Scherer and Davis, 1979), mouse (Doetschman *et al*, 1987; Thomas and Capecchi, 1987) and more recently *Drosophila* (Rong and Golic, 2000; Bibikova *et al*, 2002; Bibikova *et al*, 2003; Beumer *et al*, 2006). These techniques rely on recombination between an engineered DNA fragment and the locus to target via regions of homology provided in the DNA fragment. The engineered DNA is introduced into cells by transformation, usually as a linear fragment, or produced *in vivo* as for *Drosophila*. In the nematode *Caenorhabditis elegans*, spontaneous recombination between the genome and exogenous sequences is inefficient. This might be due to the ability of *C. elegans* germline cells to rapidly concatemerize DNA fragments introduced in the gonad and build stable extrachromosomal transgenes (Stinchcomb *et al*, 1985), a process that might outcompete recombination with chromosomal DNA. Very few examples of genome engineering by homologous recombination have been documented so far in *C. elegans* (Plasterk and Groenen, 1992; Broverman *et al*, 1993; Barrett *et al*, 2004; Berezikov *et al*, 2004; Jantsch *et al*, 2004).

One strategy to increase the recombination rate at a specific locus consists of generating a DNA double-strand break (DSB) at the locus. DSBs are very deleterious lesions, which are repaired by the cellular machinery using a number of different mechanisms conserved among eukaryotes (Haber, 2000) (Figure 1A). DSB repair mechanisms can be split in two classes: non-homologous end-joining (NHEJ) (for reviews see Dudasova *et al*, 2004; Daley *et al*, 2005; Hefferin and Tomkinson, 2005), which involves the rejoining of DNA ends by ligation, and homologous recombination (Paques and Haber, 1999). NHEJ can be achieved by several pathways (Lieber *et al*, 2003; Hefferin and Tomkinson, 2005). The canonical pathway depends on the evolutionarily conserved Ku and ligase IV proteins, which ensure protection from exonucleolytic degradation of broken ends and their sealing by ligation, respectively. This process will restore the initial sequence if the ends are cohesive, or introduce small footprints if ends are not complementary. In this paper, repair processes where little or no processing of the DSB is observed will be referred to as conservative. Recombination pathways are initiated by exonucleolytic processing of the DSB end by a 5′-to-3′ exonuclease, which exposes a single-stranded region of DNA that is engaged in a search for homology. If complementary strands of homologous regions flanking the DSB can anneal within the same chromosome, a process called single-strand annealing is initiated. Non-homologous 3′ end tails will be removed, new DNA synthesis and ligation occur, and the intervening sequence is lost, resulting in deletions at the DSB site. Alternatively, the single-stranded DNA can invade a homologous donor sequence and act as a primer for new DNA synthesis. One subsequent pathway, among others, can lead to non-reciprocal transfer of DNA from the donor to the recipient broken allele, a process called gene conversion. Various homologous sequences can potentially be used as repair templates, including sister chromatids and homologous chromosomes, or transgenes containing sequences homologous to the DSB flanking regions. If the transgenic fragments have been modified, these modifications will be copied in the broken locus during gene conversion, thus providing the possibility of engineering custom alleles.

The feasibility of transgene-instructed DSB repair in the *C. elegans* germ line was demonstrated by Plasterk and

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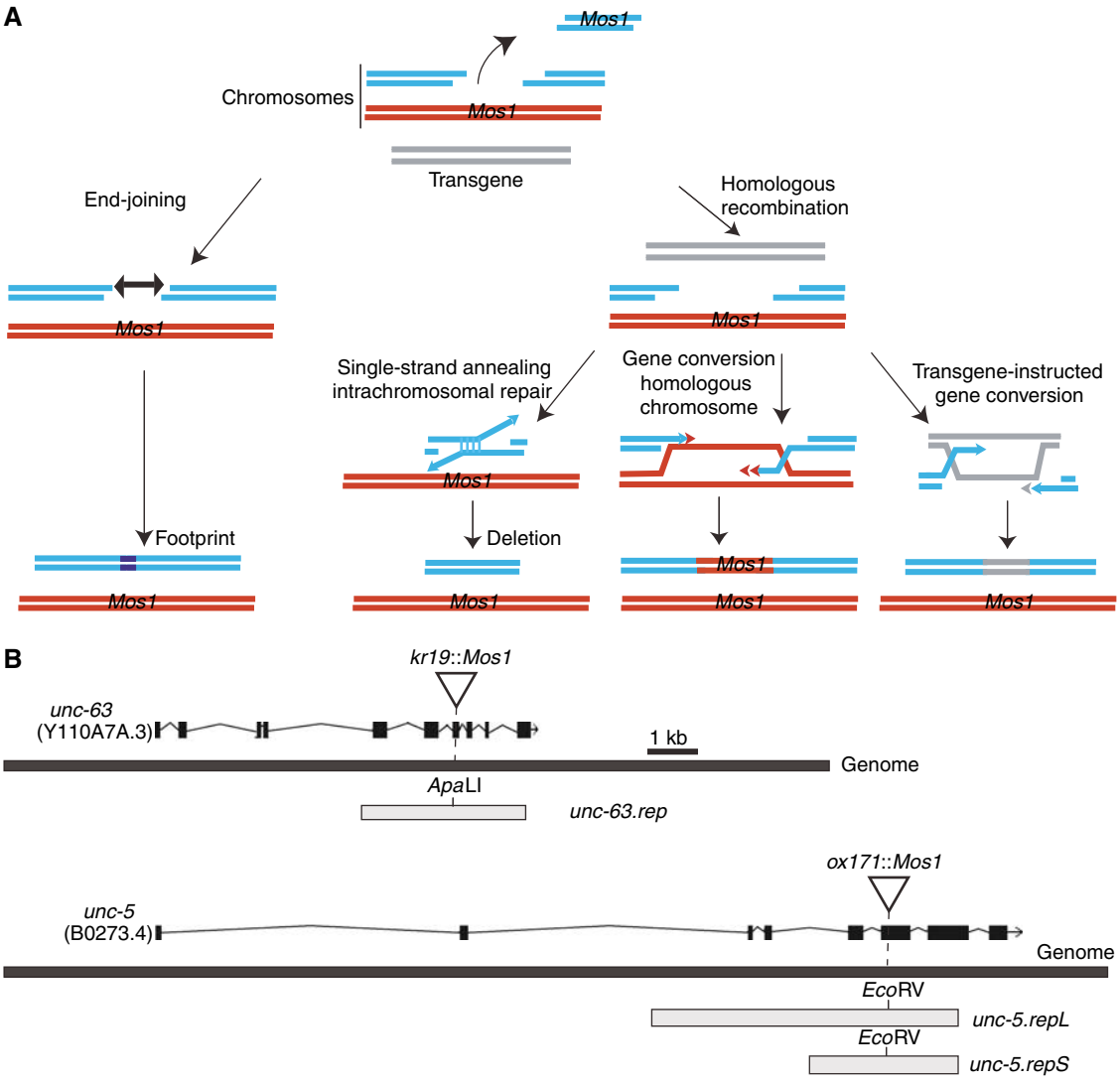


C

Allele	Repair template	Phenotypic revertants	Converted by *Mos*TIC	*n*
unc-63(kr19::Mos1)	*unc-63.rep*	6×10^{-5} (2/33 000)	6×10^{-5} (2/33 000)	5
unc-5(ox171::Mos1)	*unc-5.repL*	5×10^{-4} (5/10 000)	10^{-4} (1/10 000)	5
unc-5(ox171::Mos1)	*unc-5.repS*	1.4×10^{-3} (18/12 500)	7×10^{-4} (9/12 500)	4

Figure 1 *Mos*TIC strategy and efficiency. (**A**) Main pathways potentially used to repair a *Mos1* excision-induced DSB during *Mos*TIC experiments. In this example, homologous chromosomes (in red and blue) carry the same *Mos1* insertion and a homologous repair template is provided on a transgene (in gray). Repair of the broken chromosome (in blue) is based either on end-joining or homologous recombination (single-strand annealing or gene conversion) (see Introduction). (**B**) Schematic representation of the exon/intron structure of the *Mos1*-containing alleles *unc-63(kr19::Mos1)* and *unc-5(ox171::Mos1)* with the repair templates (*unc-63.rep*, *unc-5.repL* and *unc-5.repS*) used in *Mos*TIC experiments. *Mos1* elements are indicated by triangles. The restriction sites *Apa*LI and *Eco*RV were introduced into the repair templates to identify *Mos*TIC events. The repair templates did not contain enough sequences to rescue the mutant phenotypes. However, copying these sequences into the *Mos1*-mutated genomic loci would restore functional genes. (**C**) *Mos*TIC efficiency at the *unc-63* and *unc-5* loci. Frequencies correspond to the number of phenotypic revertants in the progeny of transgenic animals where *Mos1* excision was triggered by heat-shock. *Mos*TIC events were identified among the phenotypic revertants by the presence of an *Apa*LI site (*unc-63* locus) or an *Eco*RV site (*unc-5* locus) copied into their genome. *n*, number of independent experiments.

Groenen (1992), who remobilized a copy of the endogenous DNA transposon Tc1 out of the *unc-22* gene. When Tc1 excision was triggered in the presence of a transgene carrying a *unc-22* fragment with silent polymorphisms, in rare instances repair was achieved using the transgene as a repair template and point mutations were copied in the *unc-22*

locus. Similar experiments were recently performed to show that, apart from introducing point mutations, a similar strategy can be used to engineer deletions and insertions at a Tc1 excision site (Barrett *et al*, 2004). However, a major drawback of using endogenous *C. elegans* transposons to introduce DSBs at given loci is inability to control transposition. One haploid genome of the wild-type *C. elegans* N2 strain contains at least 70 copies of active DNA transposons. Transposition can be observed in somatic cells but is completely repressed in the germ line. Derepression of germline transposition is only achieved in some mutant backgrounds, the 'mutator' strains, causing a high rate of spontaneous mutations and resulting in the accumulation of transposon copies in the genome (Bessereau, 2006). Besides problems related to the morbidity of these mutant strains, there is a significant probability of recovering uncontrolled mutations tightly linked to the locus that is to be engineered when performing transgene-instructed gene conversion in mutator backgrounds.

To circumvent these problems, the controlled excision of a heterologous transposon could be used to generate DSBs at loci to be engineered. The transposon *Mos1* was first isolated in *Drosophila* (Jacobson and Hartl, 1985; Jacobson *et al*, 1986) and experimentally introduced in *C. elegans* (Bessereau *et al*, 2001). *Mos1* is a 1280 bp DNA transposon of the Tc1/mariner family and transposes via a 'cut-and-paste' mechanism (Benjamin and Kleckner, 1992; van Luenen *et al*, 1994; Lampe *et al*, 1996). The Mos transposase is the only factor required to achieve transposition. It binds the terminal inverted repeats present at each site of the transposon and catalyzes *Mos1* excision and reinsertion. Excision leaves behind a DSB, which must be repaired by the cellular machinery. *Mos1* was initially used in *C. elegans* to perform insertional mutagenesis and rapidly identify mutated genes (Bessereau *et al*, 2001; Granger *et al*, 2004; Williams *et al*, 2005). *Mos1* transposition could be achieved by driving the expression of the Mos transposase under the control of a heat-shock-inducible promoter. The Mos transposase proved unable to mobilize endogenous transposable elements. Therefore, *Mos1* transposition in the *C. elegans* germ line is controlled and specific.

In the present study, we triggered *Mos1* excision from specific loci to generate localized chromosomal breaks and analyzed DSB repair. We observed that repair could be achieved by multiple pathways. Specifically, we demonstrated that DSBs could be efficiently repaired by transgene-instructed gene conversion in the germ line. In addition, DSBs could also be repaired by conservative NHEJ. Although this process required the Ku80 and ligase IV in somatic cells, conservative repair could be achieved in the germ line in the absence of these proteins. Therefore, controlled excision of *Mos1* gives access to DSB repair mechanisms and provides an efficient way to engineer the *C. elegans* genome.

Results

Transgene-instructed gene conversion can be triggered by Mos1 excision

To investigate the feasibility of engineering the *C. elegans* genome using transgene-instructed gene conversion triggered by *Mos1* excision, we designed a strategy that would enable the detection of the gene conversion events based on the reversion of a mutant phenotype. For this purpose, we first selected *Mos1* insertions causing strong visible phenotypes. Second, we constructed repair templates containing wild-type genomic sequences that flanked the *Mos1* insertion point. Third, point mutations were introduced into the repair templates to generate silent restriction sites close to the *Mos1* insertion point. These repair templates did not contain enough sequences to rescue the mutant phenotypes by themselves. However, copying these sequences into the *Mos1*-mutated genomic locus would restore a functional gene. Therefore, transgene-instructed gene conversion events could be easily identified by (i) scoring for phenotypic revertants among the progeny of mutant animals and (ii) testing the genome of these revertants for the presence of the restriction site contained in the repair template.

We first used a *Mos1* insertion in the *unc-63* gene, which encodes an α-subunit of nicotinic acetylcholine receptors present at neuromuscular junctions (Culetto *et al*, 2004) (Figure 1B). *unc-63(kr19*::*Mos1)* mutants display severely impaired locomotion (Williams *et al*, 2005). We designed a repair template, *unc-63.rep*, containing 1.8 and 1.4 kb of genomic sequence flanking the left and right side of the *Mos1* insertion point, respectively. Two point mutations were introduced to create a silent *Apa*LI restriction site at the *Mos1* insertion point. The repair template was injected into *unc-63(kr19*::*Mos1)* homozygous mutants together with a vector enabling the expression of the Mos transposase under the control of the heat-shock promoter *hsp-16.48* (Bessereau *et al*, 2001). Transgenic lines were heat-shocked and screened at the next generation for animals with improved locomotion. Two phenotypic revertants were identified out of 33 000 scored progeny ($n = 5$ experiments) (Figure 1C). PCR amplification of the genomic region initially containing *Mos1* indicated that *Mos1* was no longer present in *unc-63*. Furthermore, restriction analysis and sequencing of the PCR products demonstrated that the *Apa*LI restriction site that was initially contained in the repair template had been copied into the *unc-63* genomic locus. Based on these results, we concluded that these revertants resulted from transgene-instructed gene conversion after excision of the *Mos1* element out of the *unc-63* locus.

We subsequently performed similar experiments with a *Mos1* insertion in the *unc-5* gene (kind gift from W Davis and E Jorgensen), which encodes a netrin receptor (Hedgecock *et al*, 1990; Leung-Hagesteijn *et al*, 1992). *unc-5(ox171*::*Mos1)* homozygous mutants display strong axonal outgrowth defects and are severely paralyzed. As the length of homologous sequence contained in the repair template might influence the efficiency of the gene conversion process (Barrett *et al*, 2004), we designed two repair templates. Both contained a silent *Eco*RV restriction site at the *Mos1* insertion site. *unc-5.repL* (for *unc-5.repair Long template)* contained 4.5 kb plus 1.4 kb genomic fragments flanking the left and right side of the *Mos1* insertion point, respectively; *unc-5.repS* (*Short*) contained 1.5 kb plus 1.4 kb genomic fragments. Transgenes carrying one repair template and the Mos transposase expression vector were introduced into *unc-5(ox171*::*Mos1)* homozygous mutants. Transgenic lines were heat-shocked and screened at the next generation for wild-type moving worms (Figure 1C). Reversion events were recovered at a frequency of 5×10^{-4} using *unc-5.repL* (5 in 10 000 scored progeny, $n = 5$ experiments) and 1.4×10^{-3}

using *unc-5.repS* (18 in 12 500, $n=4$). PCR analysis demonstrated that all revertants lost the *Mos1* element from one *unc-5* locus. However, only a fraction of these revertants contained the *Eco*RV site provided in the repair templates (one out of five derived from *unc-5.repL* and nine out of 18 from *unc-5.repS*). Analysis of additional revertants confirmed that transgene-instructed repair only accounted for a fraction of the reversion events (*unc-5.repL*: 3/15 revertants analyzed; *unc-5.repS*: 12/27). Further characterization of the revertants that were generated by other mechanisms is presented below.

Together, these results demonstrated that we were able to use transgene-instructed gene conversion following *Mos1* excision to introduce point mutations in the *C. elegans* genome at two different loci, with frequencies varying from 6×10^{-5} to 7×10^{-4} events per generation. We called this technique *Mos*TIC for *Mos1* excision-induced transgene-instructed gene conversion.

Analysis of the conversion tract

During the repair of a DSB by gene conversion, sequences adjacent to the DSB site are copied from the repair template to the broken chromosome (Gloor *et al*, 1991; Plasterk and Groenen, 1992). To characterize the gene conversion tracts during *Mos*TIC experiments, we introduced silent mutations in *unc-5.repL* to generate *unc-5.repP* (*Polymorphic*) (Figure 2A). The *unc-5.repP* repair template contains nine polymorphisms distributed over the *unc-5* genomic fragment, in exons and introns, which can be detected by restriction analysis or DNA sequencing. An extrachromosomal array carrying *unc-5.repP* and the Mos transposase expression vector was generated in mutant animals homozygous for

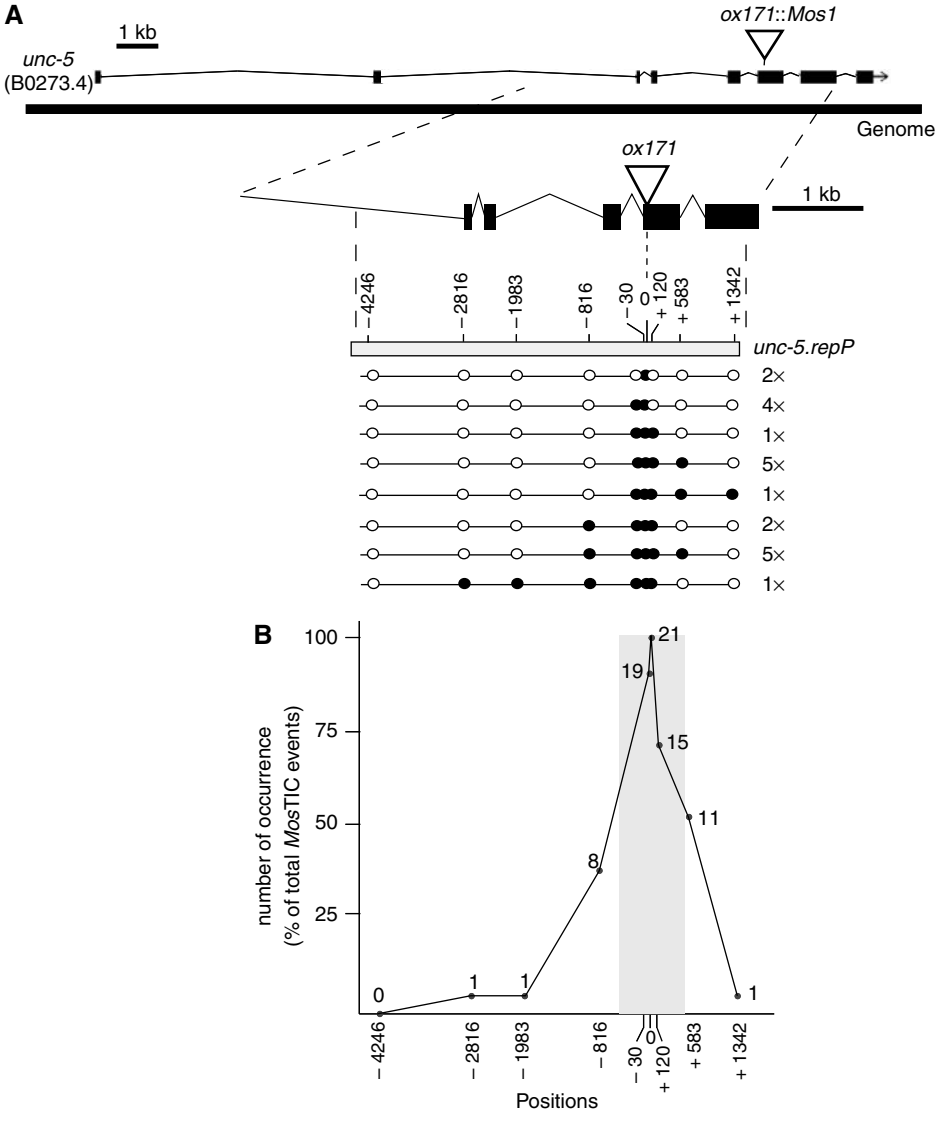


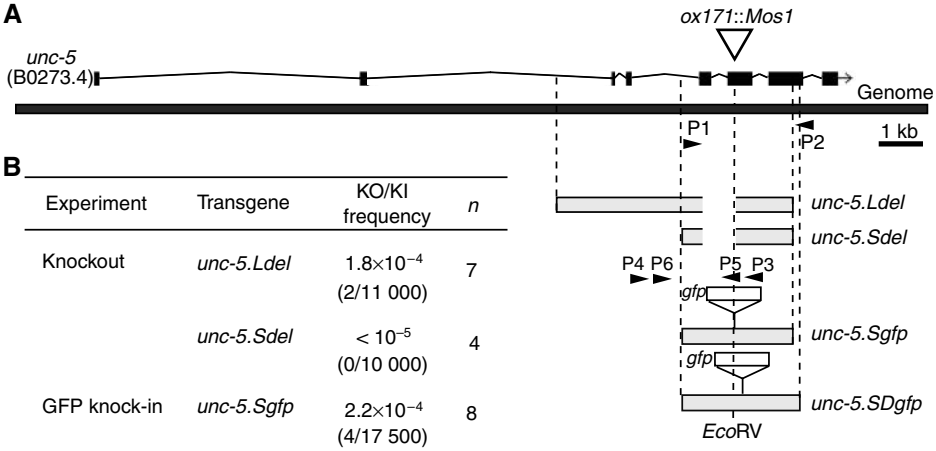
Figure 2 Analysis of *Mos*TIC conversion tract. (**A**) Repair template engineered to monitor the *Mos*TIC conversion tract. The restriction sites and SNPs introduced into the repair template are designated by their position relative to the *Mos1* insertion point and are as follows: −4246 nt = *Acl*I, −2816 = *Hin*dIII, −1983 = *Nhe*I, −816 = *Acc*65I, −30 = SNP1, 0 = *Eco*RV, +120 = SNP2, +583 = *Sac*I and +1342 = *Sma*I. The conversion tracts analyzed are represented below the repair template scheme. Black and white dots show sites that were present and absent, respectively, in the chromosome after gene conversion. Numbers to the right of conversion tracts indicate the occurrence of each tract (out of 21). (**B**) The number of occurrences per site was plotted against their position in the repair template. The numbers close to the dots are the raw data. The gray zone corresponds to the 1 kb region centered at the *Mos1* insertion point.

unc-5(ox171::Mos1). *Mos*TIC experiments were performed as described above. Phenotypic revertants were observed at a frequency of 7×10^{-4} events per generation. Twenty-one out of 48 revertants had copied the *Eco*RV site, which overlaps the *Mos1* insertion point in *unc-5.repP*, into their genome and were further analyzed for the presence of additional polymorphisms (Figure 2A). The size of the conversion tracts ranged from fewer than 30 bp (two events) to up to 3 kb (one event). In one-third of the events (7/21), more than 1 kb of sequence was copied from the repair template into the genome. No discontinuous conversion tract was observed. When the frequency of conversion at a given site was plotted against the distance from the *Mos1* insertion point, the curve described a bell shape centered at the *Mos1* insertion point (Figure 2B), which is characteristic of a gene conversion repair mechanism (Gloor *et al*, 1991; Nassif and Engels, 1993; Nassif *et al*, 1994). These data indicate that a mutation engineered in a 1 kb region centered around the DSB site would be copied in the genome in about half of the *Mos*TIC events, at frequencies above 10^{-4} events per generation.

Engineering gene knockouts and gene knock-ins with MosTIC

Apart from creating point mutations in the *C. elegans* genome, the *Mos*TIC technique could be used to generate deletions and introduce exogenous sequences into the chromosomes. First, we attempted to generate a deletion in the *unc-5* locus. Two templates, *unc-5.Ldel* and *unc-5.Sdel*, carrying 800 nt deletions, were derived from *unc-5.repL* and *unc-5.repS*, respectively (see Materials and methods). *unc-5.Ldel* and *unc-5.Sdel* were introduced into a homozygous background for *unc-5(ox171::Mos1)* together with the Mos transposase expression vector. As we could not rely on phenotypic reversion to detect *Mos*TIC events, the progeny of heat-shocked animals were screened by PCR for the presence of the *unc-5* deletion using primers on each side of the deleted region. One primer was present in both the genome and the transgene sequence, whereas the second primer was present in the genome but absent from the repair template. According to this strategy, a specific 1.6 kb PCR fragment would be amplified only if the deletion had been introduced into the genome by *Mos*TIC. However, we could amplify such a 1.6 kb PCR fragment from every tested pool of transgenic animals, including pools of non-heat-shocked control animals (data not shown). We hypothesized that amplification of this fragment resulted from a process described as 'PCR jumping' or 'PCR bridging' (Paabo *et al*, 1990; Liu *et al*, 2002): during PCR, 'bridging' occurs between single-stranded DNA molecules elongated from the genome and from the transgene because these two templates contain a region of sequence similarity. This generates a fusion fragment that is efficiently amplified at the next cycles. In our experiments, the amplification of the PCR bridging product could be minimized by decreasing the annealing time and increasing the annealing temperature. Using optimized PCR conditions, we screened pools of 100 animals. Sibs from positive pools were further analyzed to isolate strains homozygous for the deletions initially designed in the repair templates. Using three independent transgenic lines containing *unc-5.Ldel,* we isolated two deleted lines out of 11 000 screened animals (Figure 3B). Sequencing analysis of the *unc-5* locus demonstrated the presence of a deletion identical to the one as in the repair template. No *Mos*TIC event was detected in 10 000 screened F1 animals using *unc-5.Sdel*, suggesting that the regions of homology left in this repair template might be too short to drive efficient gene conversion.

Second, we used *Mos*TIC to introduce the GFP coding sequence into the *unc-5* locus. The repair template *unc-5.Sgfp*, carrying the *gfp* sequence at the *Eco*RV site, was derived from *unc-5.repS* (Figure 3A). *Mos*TIC experiments were performed as described above and recombinants containing chromosomal insertion of the GFP in the *unc-5* locus were screened by PCR (Figure 3A). Out of 175 pools of 100 F1 animals, we isolated four lines containing genomic insertion of the GFP sequence (Figure 3B). *gfp* insertion was confirmed



B

Experiment	Transgene	KO/KI frequency	*n*
Knockout	*unc-5.Ldel*	1.8×10^{-4} (2/11 000)	7
	unc-5.Sdel	$< 10^{-5}$ (0/10 000)	4
GFP knock-in	*unc-5.Sgfp*	2.2×10^{-4} (4/17 500)	8
	unc-5.SDgfp	4.2×10^{-4} (8/19 200)	2

Figure 3 Knockout and knock-in by *Mos*TIC at the *unc-5* locus. (**A**) Scheme of the repair templates designed to engineer deletions and insertions in the *unc-5* gene. The primers used to screen by PCR for *Mos*TIC-engineered animals are indicated by arrows. Limits of the repair templates are indicated by dotted lines. The *Eco*RV site present in *unc-5.SDgfp* is shown. (**B**) Frequencies of the different events. *n*, number of independent experiments.

by direct sequencing of the *unc-5* engineered loci. Despite the fact that GFP was fused in-frame with the UNC-5 coding sequence, insertion of the GFP in the middle of the protein likely disrupts the function of the UNC-5 receptor as demonstrated by the absence of mutant phenotype rescue and the absence of GFP detectable by fluorescence microscopy (data not shown). Although we demonstrated that *Mos*TIC was efficient at introducing point mutations within a 1 kb region surrounding the *Mos1*-triggered DSB, we wondered whether the presence of a region of non-homology, such as *gfp*, at a distant site from the transposon in the repair template would modify recombination efficiency. To answer this question, we inserted the *gfp* sequence in-frame with the UNC-5 coding sequence 180 nt away from the DSB site (*unc-5.SDgfp* repair template; Figure 3A). Animals containing full-length *gfp* in the *unc-5* locus were recovered with a frequency of 4.2×10^{-4} (8 in 19 200 scored progeny, $n = 2$; Figure 3B). Such frequency is similar to what was obtained when introducing SNPs at a similar distance from the *Mos1* insertion (Figure 2) and suggests that the presence of heterologous sequence in the donor template does not significantly affect the gene conversion process.

Together, these results demonstrate that *Mos*TIC can be used to engineer gene knockouts and gene knock-ins at frequencies higher than 2×10^{-4} *Mos*TIC events per F1 animal.

Mos1-triggered DSB can be repaired by end-joining in the germ line

Over the course of *Mos*TIC experiments conducted to modify the *unc-5* locus, it was systematically possible to identify wild-type moving animals that did not copy the polymorphisms contained in the repair templates (Table I). These events accounted for a significant fraction of the revertant animal population: for instance, they represented 80% of revertants generated using the repair template *unc-5.repL* (12/15), 56% of the revertants isolated while using *unc-5.repS* (15/27) and 54% of the revertants analyzed in the *Mos*TIC experiments performed with *unc-5.repP* (27/48). PCR and sequencing analysis demonstrated that these revertants had lost the *Mos1* insertion from the *unc-5* locus. However, the absence of the *Eco*RV restriction site contained in the repair template suggested that these events did not result from repair by *Mos*TIC. At the position that corresponded to the *Mos1* insertion site, we observed footprints that introduced frame-preserving mutations, deletions or insertions (Table I), indicating that the corresponding region in the UNC-5 protein can tolerate significant changes without altering the overall functionality of UNC-5. These data demonstrated that apart from transgene-instructed repair, a DSB caused by *Mos1* excision could be efficiently repaired by an additional process in the *C. elegans* germ line. The lack of recovery of similar events while performing *Mos*TIC experiments at the *unc-63* locus is likely explained by the insertion of *Mos1* in a *unc-63* exon encoding a highly conserved region among AChR subunits, which probably cannot tolerate mutations without inactivating the AChR.

Mos1 excision leaves non-complementary 3′ protruding single strands (3′-PSS) that are 3 nt long at each side of the break. To gain further insight into possible DSB repair mechanisms, we analyzed the DNA footprints left at the *Mos1* excision site. They could be classified into three categories. Class I contained small footprints ranging from a few base-pair insertions contained in *Mos1*-derived 3′-PSS to small deletions (less than 10 nt). Among these events, we could further distinguish between footprints where both TA dinucleotides that flank the 3′-PSS were intact (class Ia) and footprints where at least one nucleotide in the 3′-PSS flanking sequence had been deleted (class Ib). These footprints represented 72% (55/76) of the analyzed events. Such footprints can be considered conservative and are usually found after repair by a canonical NHEJ mechanism. Class II footprints (3/76) consisted of larger deletions. Remarkably, a 633 bp deletion was able to reconstitute an in-frame sequence encoding a functional UNC-5 protein. Class III footprints (18/76) contained insertions that could be up to 36 nt long. The inserted sequences always corresponded to small direct duplications of sequences adjacent to the DSB point. In every case, it was possible to find microhomologies between one broken end, usually in the 3′-PSS, and the sequence immediately 5′ to the duplicated region, suggesting that this end was used to prime DNA synthesis. In most cases, nucleotides immediately 3′ to the duplication could pair with the other broken end, suggesting that sealing the two ends might use a microhomology-based repair mechanism involving the *de novo*-synthesized strand and one broken end (see Discussion and Supplementary Figure S1).

These results demonstrate that during *Mos*TIC experiments, DSB caused by *Mos1* excision can be repaired by various mechanisms including end-joining, in addition to transgene-instructed gene conversion.

Ku–ligase IV-dependent NHEJ mechanisms are preferentially used in *C. elegans* somatic cells but are dispensable in the germ line for DSB repair after Mos1 excision

The data presented above demonstrated that both end-joining repair and transgene-instructed gene conversion operate in the germ line to heal *Mos1*-triggered DSBs, hence raising the possibility that they might compete during *Mos*TIC experiments. Therefore, we tested whether *Mos*TIC efficiency would be increased in genetic backgrounds defective for end-joining repair. Specifically, we used mutants expected to be defective for the Ku80 and ligase IV, two factors highly conserved from yeast to humans that define a canonical NHEJ pathway (Hefferin and Tomkinson, 2005). These factors were recently shown to affect the sensitivity to ionizing radiations in somatic cells but not in the *C. elegans* germ line (Clejan *et al*, 2006). Yet, DSB repair was not analyzed at the sequence level.

To characterize the involvement of CKU-80 and LIG-4 in the repair of *Mos1*-triggered DSBs, we first used a PCR-based assay that could identify most end-joining repair events, including those generating sequences encoding non-functional proteins and those that occur in somatic cells. A transgene carrying the Mos transposase-encoding gene under the control of a heat-shock promoter was introduced into animals homozygous for the *unc-5*(*ox171*::*Mos1*) allele. As opposed to the previous experiments in which DSB repair events were analyzed in the progeny of heat-shocked animals, transgenic animals were harvested 18 h after heat-shock and directly analyzed by PCR using primers in the *unc-5* sequence flanking the *Mos1* insertion (Figure 4). In every experiment, we could amplify two PCR products: a predicted 2.6 kb fragment, which contained *Mos1*, and a

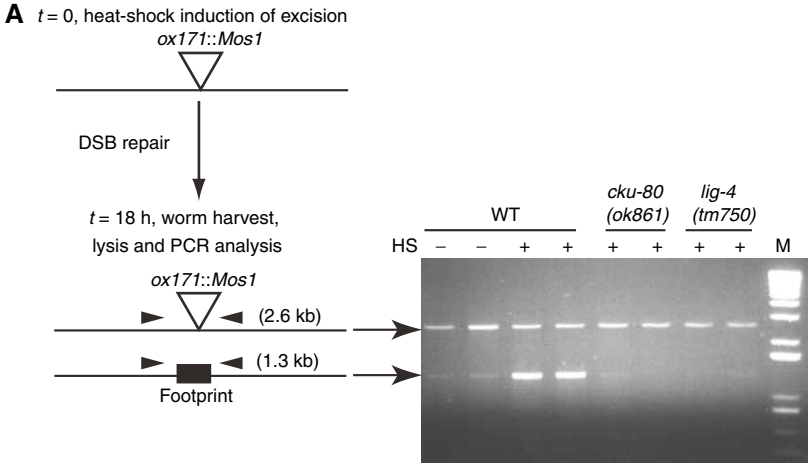
Table I Footprints generated in the germ line during *Mos*TIC experiments at the *unc-5* locus[a]

unc-5 wild-type locus	
	…GCGGACTCCTCTCGGATAGTAATCAGTGATACTGCTGTTTATGG…
unc-5(ox171::Mos1)	
	…GCGGACTCCTCTCGGATAGTATCAGGT……*Mos1*……ACCTGGTAATCAGTGATACTGCTGTTTATGG…
	-1 +1
Mos1 excision induced DSB	-▼ ▼
	…GCGGACTCCTCTCGGATAGTATCA TAATCAGTGATACTGCTGTTTATGG…
	…CGCCTGAGGAGAGCCTATCAT ACCATTAGTCACTATGACGACAAATACC…
Class Ia[b,c] (16)	
1X	…GCGGACTCCTCTCGGATAGTATTAATCAGTGATACTGCTGTTTATGG…[1Sg]
2X	…GCGGACTCCTCTCGGATAGTAGTAATCAGTGATACTGCTGTTTATGG…[2S]
8X	…GCGGACTCCTCTCGGATAGTATCGGTAATCAGTGATACTGCTGTTTATGG…[1L, 6S, 1Sg]
2X	…GCGGACTCCTCTCGGATAGTATTGGTAATCAGTGATACTGCTGTTTATGG…[2Ld]
1X	…GCGGACTCCTCTCGGATAGTATCAGTAATCAGTGATACTGCTGTTTATGG…[1Ld]
1X	…GCGGACTCCTCTCGGATAGTATATGTAATCAGTGATACTGCTGTTTATGG…[1S]
1X	…GCGGACTCCTCTCGGATAGTATCAATGGTAATCAGTGATACTGCTGTTTATGG…[1S]
Class Ib[b,c] (39)	
13X	…GCGGACTCCTCTCGGATAGTAATCAGTGATACTGCTGTTTATGG…[3L, 11S, 7Ld, 2Sg]
9X	…GCGGACTCCTCTCGGATAGTATCAATCAGTGATACTGCTGTTTATGG…[1L, 3S, 3Ld, 1Sg, 1 SDg]
7X	…GCGGACTCCTCTCGGATAGTGGTAATCAGTGATACTGCTGTTTATGG…[1L, 3S, 1Ld, 2Sg]
4X	…GCGGACTCCTCTCGGATGGTAATCAGTGATACTGCTGTTTATGG…[3S, 1Sg]
1X	…GCGGACTCCTCTCGGATAGTAT---GTGATACTGCTGTTTATGG…[1S]
2X	…GCGGACTCCTCTCGGATAGT---CAGTGATACTGCTGTTTATGG…[1S, 1Ld]
1X	…GCGGACTCCTCTC------TAATCAGTGATACTGCTGTTTATGG…[1S]
2X	…GCGGACTCCTCTCGGATA---ATCAGTGATACTGCTGTTTATGG…[1L, 1S]
Class II[b,c,d] (3)	
1X	d33 (-40/+2) …TTTTTACATCTT/GTAATCAGTGAT…[1Ld]
1X	d633 (-632/+4) …GTAAACTTGATG/GTGATACTGCTG…[1Ld]
1X	d42 (-30/+21) …ATCTTTTCAGGC/TTTATGGATCAG…[1S]
Class III[b,c,e] (18)	
1X	…GCGGACTCCTCTCGGATAGTATCAgtgATCAgtgATACTGCTGTTTATGG…[1S]
1X	…GCGGACTCCTCTCGGATAGTATCAgtgaTGGTAATCAgtgaTACTGCTGTTTATGG…[1S]
1X	…GCGGACTCCTCTCGGATAGTATCAAtgaCGGTAATCAGtgaTACTGCTGTTTATGG…[1S]
1X	…GCGGACTCCTCTCGGATAGTATCAgtgatactAGTGGTAATCAgtgatactGCTGTTTATGG…[1Sg]
1X	…GCGGACTCCTCTCGGATAGTATCAgtgatactgcTGGTAATCAgtgatactgcTGTTTATGGATCAGTTGC…[1Ld]
1X	…GCGGACTCCTCTCGGATAGTATCAgtaatcagtgatactgctgtttatggatcagtT(G)gtaatcagtgatactgctgtttatggatcagtGC…[1Ld]
1X	…GCGGACTCCTCTCggatAGTATCggatAGTGATACTGCTGTTTATGG…[1S]
1X	…GCGGACTCCTCTCGGataGTATataTGGTAATCAGTGATACTGCTGTTTATGG…[1S]
2X	…GCGGACTCCTCtcggaTAGTATCtcggaTGGTAATCAGTGATACTGCTGTTTATGG…[2S]
2X	…GCGGACTCCTCTCggatagtaTCggatagtaTGGTAATCAGTGATACTGCTGTTTATGG…[1Sg, 1L]
1X	…GCGGACTCctctcggaTAGTATCctctcggaTGGTAATCAGTGATACTGCTGTTTATGG…[1L]
1X	…GCGGactcctctcggaTAGTATactcctctcggaTGGTAATCAGTGATACTGCTGTTTATGG…[1SDg]
1X	…GcggActcctctcggaTAGTACcggTctcctctcggaTGGTAATCAGTGATACTGCTGTTTATGG…[1S]
1X	…GCGGActcctctcggatagTATCActcctctcggatagtaTGGTAATCAGTGATACTGCTGTTTATGG…[1L]
1X	…GCGGACTCCTCTcggaTAGTcagtcggaTAATcagtGATACTGCTGTTTATGG…[1Sg]
1X	…GCGGACTCCTCTCGGATAGtaTCAtataTGGtaATCAGTGATACTGCTGTTTATGG…[1S]

[a]The duplicated TA is in blue and the *Mos1* sequence in red.
[b]The footprints presented in this table were obtained when performing *Mos*TIC experimentswith repair templates *unc-5.repS* (S), *unc-5. repL* (L), *unc-5.Ldel*(Ld), *unc-5.Sgfp*(Sg) and *unc-5.SDgfp*(SDg).
[c]The nucleotides that could have been used to stabilize junctions by microhomology during the repair processes are highlighted. For class I and II footprints, we chose to restrict the search for such nucleotides to the 5 bp stretches flanking the junction point.
[d]Class II events are designated by 'dx', where x represents the number of deleted nucleotides. The junction point is represented by a '/'.
[e]Extra nucleotides (in purple) copied from the DSB flanking regions and present in the class III footprints are in lower case, as are their genomic templates.

1.3 kb fragment corresponding to end-joining products (Figure 4A). Subcloning the total PCR products and sequencing 1.3 kb inserts confirmed that *Mos1* was absent from these fragments. The small amount of end-joining products in non-heat-shocked animals most likely reflects leaky expression of the Mos transposase in somatic tissues from the heat-shock promoter, as observed with an *hsp*::*gfp* transgene (data not shown). We analyzed footprints generated at the excision site (Figure 4B). They were mainly class Ia (12/14), suggesting that the broken ends were efficiently protected against exonucleolytic degradation as observed during repair by a conservative NHEJ mechanism. In addition, six class II deletions were identified as well as one deletion containing a remaining *Mos1* fragment at the undeleted break point. Such events are frequently observed after transposon excision repair and might result from a mechanism called synthesis-dependent strand annealing (SDSA) (Nassif *et al*, 1994; Adams *et al*, 2003; Izsvak *et al*, 2004). Class III duplications were under-represented as compared to what we observed previously in the germ line during *Mos*TIC experiments. This suggests that DSB repair products detected by the PCR-based assay might occur mostly in somatic cells where canonical NHEJ might predominate. Accordingly, similar results were obtained using early L2 larvae in which the germ line was not yet developed (data not shown).

To test the involvement of Ku80 and ligase IV in the conservative repair process detected by PCR, we performed similar experiments in *lig-4*(*tm750*) and *cku-80*(*ok861*)



B

WT (21)

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Class I a (13)
2X …GCG GACTC CTCTC GGA TA G TA TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGATA G TA G TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGA TA G TA T TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGATA G TGG TA AT CAGTG ATACT GCTGT TTATG G…
2X …GCG GACTC CTCTC GGATA G TA TC TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGA TA G TA GG TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGATA G TA T CA TA AT CAGTG ATACT GCTGT TTATG G…
3X …GCG GACTC CTCTC GGA TA G TA T GG TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGATA G TA TC AGG TA AT CAGTG ATACT GCTGT TTATG G…
Class I b (1)
1X …GCG GACTC CTCTC GGATA G TA A AT CAGTG ATACT GCTGT TTATG G…
Class I I (6)
1X d618 (-62 4/+3) …T TTCAA AAC TGA/ TA AT CA GTGAT A…
1X d215 (-2/ +218) …CTC GGA TA GTA T/T GCTCG GAGAG A…
1X d356 ( -1/+3 59) …T CGGAT AG TA TC /T CAAGA ATGTA G…
1X d651 ( +1/+6 54) …C GGATA G TA TC A/A CGTGA GTGTA A…
1X d537 (-36 9/+17 5) …A CTTCC GA TA T H/A GTTCG AGTAG T…
1X d696 (-33 1/+37 4) …T CTAAT TTTTAT /AGAA A CG CTGGC …
SDSA event (1)
1X …TAA ACAAG GAA- d219 - /AAC TA T GGTGG -123n t Mbs1 TG GTA AT CAGTG ATACT GCTGT TTATG G …
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cku-80(*ok861*) (17)

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Class I b (8)
1X …GCG GACTC CTCTC GGATA G TA AT CAGTG ATACT GCTGT TTATG G…
1X …GCG GACTC CTCTC GGATA GG TA AT CA GTGAT ACTGC TGTTT ATGG…
1X …GCG GACTC CTCTC GGATA G TA TC A AT CA GTGAT ACTGC TGTTT ATGG…
1X …GCG GACTC CTCTC GGATA G TA TC CA GTGAT ACTGC TGTTT ATGG…
1X …GCG GACTC CTCTC GGATA G TA -T CA GTGAT ACTGC TGTTT ATGG…
1X …GCG GACTC CTCTC GGATA GC -A TCA GTGAT ACTGC TGTTT ATGG…
1X …GCG GACTC CTCTC GGAT AG T- - - - - - -GA TACTG CTGTT TATGG …
1X …GCG GACTC CTCTC G GA T A- - - - - - - - - - TACTG CTGTT TATGG …
Class I I (7)
1X d680 ( -3/+6 83) …T CTCGG A TA G TA /A TTTAC ATTTC A…
1X d211 (-15 1/+69 ) …AG AGAAC CG TT G/ CA /C AATTC TTGCA A…
1X d691 (-37 6/+32 5) … AA TTCTA AT TTT/C T TCA ACAAG AA…
2X d101 3(-39 0/+63 5) …A GAAAC AGCAAA /AA /AC ACC TTAAC AG…
1X d101 5(-50 2/+52 2) …C TGATG ACACAA /G G CA GATTG AT…
1X d101 8(-39 0/+63 8) …A GAAAC AGCAAA /AAA CG / CC TTAAC AGATC …
1X d101 8(-39 0/+63 8) …A GAAAC AGCAAA /AAA CT / CC TTAAC AGATC …
Class I II (1)
1X …GCG GACTC CTCTC GGata g ta TC GT AGGAa tagta GG TA AT CAGTG ATACT GCTGT TTATG GATCA G…
SDSA event (1)
1X …GCG GACTC CTCTC GGATA G TA TCA 484 nt Mbs1 TTT/ C TG CTGTT TATGG
```

lig-4(*tm750*) (19)

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Class I a (1)
1X …GCG GACTC CTCTC GGATA G TA T GG TA AT CAGTG ATACT GCTGT TTATG G…
Class I I (13)
1X d42 (-48/ 0) …T TTTAT ACATT T/ T GG TA AT CAGTG …
1X d315 (-32 2/0) …ATTT AGTAA TAT/ AA AAAAA /TGG TA AT CAGTG …
1X d520 (-52 5/+3) …AGC CATGT TT TGG/ TA AT CAGTG AT…
1X d457 ( -4/+4 62) …C TCGGA TA G TA T/T A T G ATTAT ATG …
1X d615 ( -3/+6 22) …T CGGAT AG TA TC /C /GGCTGT TTCGG A…
1X d667 ( 0/+67 3) …C GGATA G TA T CA/A AAAAT GTGAG T…
1X d15 (-8/+ 15) … ACTCC TC H CGGA /CT C CT GTTTA TG…
1X d729 (-47 7/+25 9) …A GTGAG TTCAAA /TT TTTTT TTTG /G AGCCT CCTCC T…
1X d789 (-22 3/+57 5) …G AAACA TCGAAA /T TA AT TGTAC CT…
4X d101 3(-39 0/+63 5) …A GAAAC AGCAAA /AA /AC ACC TTAAC AG…
Class I II (3)
1X …GCG GACTC CTCTC GGATA G TA TC A tg ata TGG TA AT CA Gt gataC TGCTG TTTAT GGATC AGT…
1X …GCG GACTC CTCTC GGATA G TA TC ca gtgat acTGG TA AT c ag tgata ctgCT GTTTA TGGAT CAGT…
1X …GCG GACTC CTCTC GGATA G TA TC A At gatac tgctg tttat ggatc agGTA A AT CA Gt gatac tgctg tttat ggatc agT…
SDSA events (2)
1X …GCG GACTC CTCTC GGATA G TA TCA 66 nt Mbs1 / AA T CA GT GATAC TGCTG TTTAT GGATC AGT…
1X …GCG GACTC CTCTC GGATA G TA TCA 195 nt Mbs1 AC AAA /TGG TA AT CAGTG ATACT GCTGT TTATG G…
```

Figure 4 PCR analysis of DSB repair in *lig-4*/*cku-80*-defective backgrounds. (**A**) Details of the experimental procedure used to study DSB repair by PCR. *unc-5*(*ox171*::*Mos1*) adults were heat-shocked ($t = 0$) to induce *Mos1* excision and DSB repair was analyzed by PCR with primers flanking the *Mos1* insertion point ($t = 18$ h). PCR products were analyzed on an agarose gel: (−) non-heat-shocked, (+) heat-shocked samples, M = size marker (1 kb Plus DNA Ladder, Invitrogen). (**B**) Sequence analysis of footprints generated in wild-type and *cku-80* and *lig-4* mutants. See Table I footnotes for legends. Numbers in parentheses represent the numbers of footprints analyzed per genotype.

mutant backgrounds. The abundance of the shorter PCR fragment was strongly decreased in both mutants with respect to the *Mos1*-containing product (Figure 4A). As NHEJ factors were previously reported to regulate Sleeping Beauty transposition in mammalian cells (Izsvak *et al*, 2004), we cannot rule out that this decrease might reflect a change of *Mos1* excision frequency in this assay. To further characterize a potential impairment of NHEJ in *cku-80/lig-4* mutants, we analyzed the sequence of the footprints generated in those backgrounds (Figure 4B). In *cku-80(ok861)* mutants, conservative repair was still detected. However, no class Ia event could be isolated (0/8), in contrast to the class I events recovered in the wild type. This suggests that in the absence of CKU-80, the broken ends are no longer efficiently protected against exonucleolytic degradation. In *lig-4* mutants, class I events were almost absent (only one event was recovered, which might have been generated in the germ line; see below). In contrast, class II footprints were not significantly affected by the absence of either CKU-80 or LIG-4. These results indicated that in *C. elegans* somatic cells, at least two end-joining mechanisms coexist. One prominent conservative mechanism depends on LIG-4 and partially on CKU-80 and generated small footprints in which the genomic sequences flanking the DSB are intact, whereas a second mechanism, which was mostly CKU-80- and LIG-4-independent, generated deletions of the flanking genomic sequences and could also be used to generate small direct duplications.

As the results described above demonstrated that *cku-80* and *lig-4* mutations impaired NHEJ, we tested whether the relative frequency of end-joining repair events versus trans-gene-instructed gene conversion would be affected in *cku-80* and *lig-4* mutant backgrounds during *Mos*TIC experiments. *Mos*TIC experiments were performed as described previously at the *unc-5* locus in *cku-80* and *lig-4* mutants. Phenotypic reversion and *Mos*TIC events were recovered at frequencies similar to those obtained in a wild-type background (Table II). Similarly, we were not able to distinguish any qualitative difference between the repair events generated in wild-type or mutant backgrounds by analyzing the sequences of the repair events. Specifically, the class I and III footprints that we observed in *cku-80* and *lig-4* mutants were similar to that observed in the wild-type background (Table I). Therefore, these results indicated that repair of DSBs induced by *Mos1* excision in the germ line, including conservative end-joining, can be achieved in the absence of CKU-80 and LIG-4.

Discussion

A by-product of type II transposon mobilization is the generation of a DSB at the transposon excision site. In this study, we took advantage of the ability to control *Mos1* transposition in the *C. elegans* germ line to introduce chromosomal breaks at given loci. Analysis of the repair process indicated that *Mos1*-triggered DSB can be repaired by end-joining and homologous-recombination mechanisms. First, we demonstrated that the well-conserved canonical Ku80–ligase IV-dependent end-joining pathway is also present in *C. elegans* where it is mostly used in somatic cells, whereas a second mechanism is Ku80- and ligase IV-independent and functions

Table II Footprints generated in the germ line of *lig-4/cku-80*-defective animals during *Mos*TIC experiments[a]

cku-80 (ok861) (reversion rate = 5.7 X 10^{-4} (9/15 600), *Mos*TIC events = 40% (10/25))[b]
Class Ia
1X …GCGGACTCCTCTCGGATAGTATCGGTAATCAGTGATACTGCTGTTTATGG
Class Ib
1X …GCGGACTCCTCTCGGATAGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGAATGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATATATGGAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCCG---GTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAG---------ATACTGCTGTTTATGG…
Class III
1X …GCGGACTCCTCTCGGATAGTATCAGgtgTGGTAATCAgtgATACTGCTGTTTATGG…
1X …GCGGACTCCTCTtcggatAgtaTCggatGgtaTGGTAATCAGTGATACTGCTGTTTATGG…
lig-4 (tm750) (reversion rate = 7.6 X 10^{-4} (10/13 100), *Mos*TIC events = 43% (10/23))[b]
Class Ia
1X …GCGGACTCCTCTCGGATAGTATCGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCAATAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCATTGGTAATCAGTGATACTGCTGTTTATGG…
Class Ib
4X …GCGGACTCCTCTCGGATAGTAATCAGTGATACTGCTGTTTATGG…
4X …GCGGACTCCTCTCGGATGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTGGTAATCAGTGATACTGCTGTTTATGG…
2X …GCGGACTCCTCTCGGATAGTATCAATCAGTGATACTGCTGTTTATGG…
lig-4 (ok716) (reversion rate = 1.3 X 10^{-3} (22/15 700), *Mos*TIC events = 44% (16/36))[b]
Class Ia
1X …GCGGACTCCTCTCGGATAGTATTGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCATTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCAGTGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCATTGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCAGTAGTAATCAGTGATACTGCTGTTTATGG…
Class Ib
6X …GCGGACTCCTCTCGGATAGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTGGTAATCAGTGATACTGCTGTTTATGG…
1X …GCGGACTCCTCTCGGATAGTATCAATCAGTGATACTGCTGTTTATGG…
Class III
1X …GCGGACTCCtctcggatagtaTATCAAAtctcggatagtaTGGTAATCAGTGATACTGCTGTTTATGG…

[a]See Table I for legends.
[b]In a wild-type background, reversion rate is 9.6×10^{-4} (31/32 200) and the fraction of *Mos*TIC events is 40% (29/73). There are no significant differences between wild-type and mutant backgrounds (χ^2 test).

in germline cells to achieve conservative end-joining. Second, we showed that a homologous recombination repair pathway can be used to achieve transgene-instructed gene conversion. Based on this observation, we developed a novel technique, which we named *Mos*TIC. It allows the engineering of custom alleles and provides an efficient tool to manipulate the *C. elegans* genome.

Mos1 excision repair generates diverse footprints, including direct duplications

During DSB repair, the structure of the broken ends affects the end-joining process. Complementary and blunt termini can be rapidly repaired by a single ligation event, whereas complex substrates require multiple processing steps to achieve break repair (Pfeiffer and Vielmetter, 1988). The *Mos1* transposase catalyzes DSBs at each end of the *Mos1* element. The 5′ cut of the non-transferred strand is made 3 bases within the transposon, whereas the 3′ cut is made exactly at the end of the transposon. As a result, *Mos1* excision leaves non-complementary 3′-PSS that are 3 nt long at each side of the break (Dawson and Finnegan, 2003). In our experiments, end-joining repair generated several categories of footprints.

The first class of events generated small footprints ranging from the insertion of few bases contained in the 3′-PSS to the deletion of fewer than 10 bases immediately flanking the break point. Such footprints are very similar to those usually observed after DNA transposon excision (see, for instance, examples reported for *Mos1* in *Drosophila* (Bryan *et al*, 1990), *Tc1* and *Tc3* in *C. elegans* (Ruan and Emmons, 1987; Plasterk, 1991; Plasterk and Groenen, 1992; van Luenen *et al*, 1994) or *Sleeping Beauty* in mammalian cells (Fischer *et al*, 2001; Izsvak *et al*, 2004)), and are typical of repair by canonical NHEJ. These events were prominent in somatic cells, where they required Ku80 and ligase IV activities.

A second class of repair events was Ku–ligase IV-independent and generated deletions from one hundred to one thousand base pairs long, which were mostly asymmetrical. This class of footprints was Ku80- and ligase IV-independent. The generation of similar deletions after *Tc1* excision has been widely used to inactivate genes in *C. elegans*. As short direct repeats were sometimes found at the end points of these deletions, it was proposed that the two broken DNA ends scan each other's flank until a match is found, after which the break is sealed and the intervening sequence is lost (Zwaal *et al*, 1993). In our experiments, careful analysis of the sequences flanking deletion points could only identify microhomologies limited to one or a few nucleotides. An alternative pathway called SDSA has been proposed for transposition-induced DSB repair based on the observation that repair often generates a deleted version of the transposon (Nassif *et al*, 1994; Adams *et al*, 2003). In this scenario, DSB repair uses a homologous template, which in our case would be the homologous chromosome or the sister chromatid, which contains a *Mos1* copy, and possibly the repair template during *Mos*TIC experiments. If this mechanism is processive enough, it regenerates a transposon copy at the excision site and is therefore not detected. If the process aborts before the whole template is copied, a Ku–ligase IV-independent, microhomology-mediated end-joining pathway is used to seal the broken ends and often generates deleted versions of transposable elements (Verkaik *et al*, 2002; Adams *et al*, 2003). SDSA was shown to be used in different organisms during transposon excision repair (Nassif *et al*, 1994; Adams *et al*, 2003; Yant and Kay, 2003; Izsvak *et al*, 2004; McVey *et al*, 2004). In our experiments, footprints containing fragments of the transposon were found in the PCR approach. Similarly, a *unc-5* revertant was found over the course of *Mos*TIC experiments to contain a fragment of the GFP tag copied from the repair template together with deletion of the adjacent genomic sequences (Supplementary Figure S2). These footprints suggest that SDSA is active in *C. elegans*. Finally, besides SDSA, direct end-joining might occur independently of Ku–ligase IV activities via microhomology-dependent processes after one or both broken ends have been subjected to exonucleolytic processing (Ma *et al*, 2003).

The third class of footprints consists of small direct duplications. Sparse examples of such repair events after transposon excision can be found in the literature. Repaired DSB sites containing duplications have been described in human cells (Roth *et al*, 1985) and *Drosophila* (Adams *et al*, 2003; McVey *et al*, 2004), and were also found at the site of chromosomal translocations in follicular lymphomas (Jager *et al*, 2000) and promyelocytic leukemias (Welzel *et al*, 2001). Several mechanistic hypotheses have been put forward to account for these duplications. In human cells, slipped mispairing was proposed (Roth *et al*, 1985). In *Drosophila*, footprints containing templated nucleotides were thought to be 'characteristic of aberrant end-joining after aborted homologous recombination' (Adams *et al*, 2003). The existence of an error-prone polymerase involved in the generation of those events was also postulated (McVey *et al*, 2004). In our experimental situation, the duplication events that we recovered might result from DNA synthesis primed at the 3′-PSS. In most cases, we could identify microhomology between one of the 3′-PSS and the region immediately 5′ to the duplicated sequence. After pairing with the opposite chromosomal broken end, eight bases were copied on average (from 3 to 32), then followed by microhomology-directed end-joining between the *de novo*-synthesized sequence and the broken end (Supplementary Figure S1). It would be interesting to test whether such a mechanism is only observed in the repair of DSB with 3′-PSS ends or if it is a more general mechanism able to generate small direct duplications.

A conservative NHEJ mechanism might function independently of Ku–ligase IV in the C. elegans germ line

Analysis of DSB repair after *Mos1* excision was performed in adult *C. elegans* hermaphrodites, which contain 959 somatic cells and more than 1000 germline nuclei. Therefore, detection of repair products using PCR on whole animal lysates potentially identifies events that might occur in the soma and in the germ line. Using this strategy, we confirmed the expected function of *cku-80* and *lig-4* genes in NHEJ in *C. elegans*. The Ku80 and Ku70 proteins are widely conserved among phyla, from yeast to human (for review see Hefferin and Tomkinson, 2005). They form heterodimers that bind broken ends, protect them from degradation and might serve as alignment factors. In *cku-80* mutants, the detected amount of NHEJ products was dramatically reduced in our PCR assay. Some end-joining products could be identified but in every case we observed limited exonucleolytic degradation of at least one of the broken ends, including the 3′-PSS and a few

adjacent nucleotides, and repair occurred at sites of microhomologies. In vertebrate cells, the loss of Ku80 causes the disappearance of the Ku70 protein (Errami *et al*, 1996; Gu *et al*, 1997; Singleton *et al*, 1997). Repair of DSBs in the absence of Ku80 mostly generates large deletions or SDSA repair products containing partial sequences of the transposon (Feldmann *et al*, 2000; Guirouilh-Barbat *et al*, 2004; Izsvak *et al*, 2004), as also observed in this study. The occurrence of imperfect NHEJ events suggests that, even in the absence of Ku activity, the ligase IV can achieve end-joining before the broken ends have been subjected to extensive degradation. However, we cannot totally exclude that a residual Ku70 activity functions in *C. elegans* in the absence of Ku80 and still protects broken ends, but less efficiently than a Ku70–Ku80 dimer.

During conservative end-joining, the ligase IV provides the ligase activity required to seal broken ends. In contrast to mouse *Lig4* mutants that die during development due to early neurodegeneration (Barnes *et al*, 1998; Frank *et al*, 2000), *C. elegans lig-4* mutants are viable and fertile and do not exhibit gross phenotypes, as in *Drosophila* (Gorski *et al*, 2003; McVey *et al*, 2004; Romeijn *et al*, 2005). However, we demonstrated that NHEJ repair was heavily impaired in the absence of ligase IV. PCR amplification of the *unc-5* locus after *Mos1* excision hardly detected any amplification products corresponding to precise repair of the broken ends. Out of 19 events that were sequenced, only one corresponded to precise end-joining (and might actually have been generated in the germ line; see below). Most events were deletions, either symmetrical or asymmetrical, with occasional insertions at the repair site. These data are very similar to what was observed after repair of transposon excision in other systems such as *Drosophila* in the absence of ligase IV (Romeijn *et al*, 2005) or of its cofactor XRCC4 in mammalian cells (Izsvak *et al*, 2004). Altogether, our results demonstrate that a canonical Ku–ligase IV-dependent NHEJ pathway is at work in *C. elegans* to perform conservative end-joining of DSBs.

To test whether impairing NHEJ would favor transgene-instructed repair in the germ line after *Mos1* excision, we performed *Mos*TIC experiments in *cku-80* and *lig-4* mutants. DSB repair was detected based on reversion of the uncoordinated phenotype of *unc-5(ox171*::*Mos1)* mutants. By contrast with the results obtained in PCR-based experiments, the frequency and the nature of the repair events were unchanged in *cku-80* and *lig-4* mutants as compared to wild type. Specifically, class I events that derive from conservative end-joining of broken chromosome ends were recovered at the same frequency in mutant and wild-type backgrounds. Several hypotheses can be proposed to explain how these footprints are generated. First, they might result from an SDSA process that would abort immediately. Testing this hypothesis would require the analysis of *Mos1*-induced DSB repair in homologous recombination-defective mutants, such as *rad-51*, which mediates strand invasion required for SDSA. Unfortunately, *rad-51* is an essential gene in *C. elegans*, which precludes analysis of DSB repair in the germ line. Second, conservative end-joining observed in the absence of Ku80–ligase IV could be reminiscent of microhomology-dependent end-joining processes that have been described in yeast and *Drosophila*. In yeast, microhomology-mediated end-joining was shown to be independent of Ku and partially independent of ligase IV (Ma *et al*, 2003), leading to small deletions at the repair site. In *Drosophila*, DSB repair could still be performed in the germ line in the absence of the ligase IV, even after the homologous recombination machinery was genetically disrupted (McVey *et al*, 2004). However, it is important to note that microhomologies were not often found at the site of repair after *Mos1*-induced DSB repair, as opposed to what was observed in cases described above. Altogether, our results revealed that two processes coexist in *C. elegans* to generate conservative end-joining. One process, which resembles well-characterized canonical NHEJ repair pathways, depends on Ku and ligase IV and seems to be mostly active in somatic cells. A second process is capable of achieving conservative repair of broken chromosomes in the absence of Ku80 and ligase IV, in agreement with recent results indicating that Ku80 and ligase IV are dispensable in the germ line for DNA repair after exposure to ionizing radiations (Clejan *et al*, 2006).

MosTIC, a novel technique to engineer the C. elegans genome

Apart from end-joining, mechanisms based on homologous recombination are able to repair DSBs in the *C. elegans* germ line. Because this study was performed in animals homozygous for *Mos1* insertions, repair events based on homologous recombination using the intact chromosome as a repair template could not be identified. Repair by homologous recombination-based mechanisms was only observed when providing homologous transgenes that could be used as repair templates. During this process, sequences contained in the transgene were copied into the genome, resulting in gene conversion. The length of the gene conversion tract was investigated using an asymmetric repair template carrying 1.4 and 4.5 kb of homologous sequences with silent mutations distributed along both arms of the repair template. Mutations were copied in the chromosome at frequencies distributed along a bell-shaped curve centered at the *Mos1* excision site. The median length of the conversion tract was roughly 500 bp on each side of the DSB and in one example conversion extended up to 2.8 kb from the *Mos1* excision site. This distribution is highly reminiscent of the data obtained in the *Drosophila* germ line while performing targeted gene replacement via P element-induced gap repair (Gloor *et al*, 1991). Similar conversion tracts are also observed in yeast (Borts and Haber, 1989; Palmer *et al*, 2003) and mammalian cells (Taghian and Nickoloff, 1997; Elliott *et al*, 1998), although they tend to be shorter. Interestingly, the presence of non-homologous sequence in the donor template, such as *gfp*, does not cause abrupt termination of the conversion tract. Therefore, the *Mos*TIC technique provides a means to modify a sequence at distance from a given *Mos1* insertion.

Transgene-instructed gene conversion triggered by *Mos1* excision, a technique that we called *Mos*TIC, provides an efficient way to engineer custom alleles in the *C. elegans* genome. *Mos*TIC events were recovered at frequencies ranging from 10^{-5} to 10^{-4} per generation. This efficiency is much higher than the initial gene-conversion experiments performed after Tc1 mobilization in a *mut-6* mutator background (Plasterk and Groenen, 1992) and comparable to what was reported more recently after repeating those experiments in *mut-2* and *mut-7* backgrounds (Barrett *et al*, 2004). Several hypotheses were raised to account for the improved efficiency in the most recent study. First, the use of specific

mutator backgrounds, which were needed to derepress Tc1 transposition, was proposed to improve transposition excision rate. However, apart from global derepression of all endogenous transposable elements, *mut-2* and *mut-7* also participate in other regulatory pathways such as RNA interference and germline co-suppression (Ketting *et al*, 1999; Tabara *et al*, 1999; Dernburg *et al*, 2000; Ketting and Plasterk, 2000; Vastenhouw *et al*, 2003; Robert *et al*, 2005) that could have modified gene-conversion efficiency. In our system, *Mos1* transposition was achieved in a wild-type background. To test if a mutator background would modify *Mos*TIC efficiency, some *Mos*TIC experiments were repeated in a *mut-7(pk204)* background. Gene conversion events were recovered at similar frequencies as in the wild type (data not shown). This indicates that *Mos*TIC experiments can be performed in a wild-type background without losing efficiency. Second, the use of long asymmetric templates containing about 7–9 kb of homologous sequence was proposed to be more efficient than the symmetrical 3 kb template used by Plasterk and Groenen (1992). In *Mos*TIC experiments performed to target the *unc-5* locus, we obtained the same efficiency with 6 kb asymmetrical templates and 2.9 kb symmetrical templates. Interestingly, similar *Mos*TIC frequencies were obtained when the region of homology was interrupted by a deletion or by the presence of a GFP-coding fragment. These results suggest that the search for homology to engage recombination is restricted to the regions adjacent to the DSB. As a practical correlate, it is possible to mutate a *C. elegans* gene by *Mos*TIC starting with a fragment of less than 3 kb that contains the modification to be introduced in the genome.

In conclusion, *Mos*TIC provides an efficient way to manipulate the *C. elegans* genome that relies on the mobilization of *Mos1* at a specific locus. When *Mos1* transposes in the *C. elegans* germ line, an average of 2.6 insertions occur per haploid genome (Williams *et al*, 2005). These insertions are unlinked and can easily be separated by out-crossing. Therefore, *Mos*TIC experiments can be performed in animals where a single *Mos1* insertion is present, hence minimizing the risk of introducing uncontrolled mutations at other loci. In addition, the only requirement to achieve efficient germline *Mos1* excision is a transgenic Mos transposase source, which can be easily eliminated once the conversion experiment is performed without further genetic manipulation of the *Mos*TIC-engineered strains. The prerequisite of this technique is to have a *Mos1* element inserted into the genomic region to be engineered. Such insertions can be recovered in genetic screens using *Mos1*-mediated mutagenesis (Williams *et al*, 2005). In addition, a European effort (http://elegans.imbb.forth.gr/nemagenetag/) is in progress to generate a comprehensive *Mos1* insertion library. To date, around 5000 *Mos1* insertions have been isolated. They are annotated in Wormbase (http://www.wormbase.org/) and can be freely obtained on simple request. As the distribution of *Mos1* insertions is relatively uniform in the genome, *Mos*TIC potentially provides an efficient means to manipulate a large proportion of the *C. elegans* genes.

Materials and methods

C. elegans strains and alleles

Mos*TIC lines*: Microinjections (Stinchcomb *et al*, 1985) were performed in EN19 (*unc-63(kr19*::*Mos1)* I) or EN59 (*unc-5* (*ox171*::*Mos1*) IV) strains with DNA mix containing pJL44 [*hsp-16.48*::*transposase*] (50 ng/µl), repair template (50 ng/µl) and pPB118.33[*myo-2:gfp*] (5 ng/µl). Transgenic F1 animals were selected based on GFP expression in the pharynx and transgenic lines were cultivated at 25°C.

NHEJ-defective strains: FX750 (*lig-4*(*tm750*) III) was generated by the National Bioresource Project for the Experimental Animal 'Nematode C. elegans' (Japan) and RB873 (*lig-4*(*ok716*) III) and RB964 (*cku-80*(*ok861*) III) by the OMRF Knockout Group (Canada).

Plasmid construction

unc-63.rep: Genomic sequences between positions 5 145 834 and 5 149 076 of chromosome I were cloned into pBS KSII + (Stratagene) digested by *Xho*I and *Eco*RV. Positions 5 147 665 and 5 147 668 were respectively changed into a 'g' and a 'c' to create an *Apa*LI site.

unc-5.repS and *unc-5.repL*: Genomic sequences between positions 5 497 225 and 5 500 133 and positions 5 497 225 and 5 503 280 of chromosome IV were respectively cloned into pBS KSII + . Positions 5 498 637–5 498 643 were replaced by 'gatatc' to create the *Eco*RV site.

unc-5.repP: *unc-5.repL* was mutagenized using the QuickChange® II XL site-directed mutagenesis and QuickChange® multi-site-directed mutagenesis kits (Stratagene). Introduced polymorphisms are described Figure 2.

unc-5.Ldel and *unc-5.Sdel*: *unc-5.Ldel* and *unc-5.Sdel* were constructed starting respectively from *unc-5.repL* and *unc-5.repS*, which were both digested with *Eco*RV and *Sal*I. After filling the *Sal*I site with the klenow enzyme, the plasmids were religated. The *Eco*RV–*Sal*I junctions were sequenced in *unc-5.Ldel* (…tcagattc gacg…) and *unc-5.Sdel* (…tcagatcgacg…).

unc-5.Sgfp: *unc-5.Sgfp* was obtained by cloning a *gfp* PCR-amplified (Phusion, Finnzyme) fragment in-frame into the *Eco*RV site of *unc-5.repS*.

unc-5.SDgfp: *unc-5.SDgfp* was obtained in two steps: first, a *gfp* PCR-amplified (Phusion, Finnzyme) fragment was cloned in-frame into the *Bst*BI site of *unc-5.repS*. Second, a 275-bp-long *unc-5* genomic fragment (primers: gcgaggttaatgctagctgg and ggggtacc catcgcatgagaatccagg) was added between *Nhe*I and *Kpn*I sites of this construct.

Transgene instructed gene repair experiments

Heat-shock treatments were performed on mixed populations of transgenic and non-transgenic animals from *Mos*TIC lines for 1 h at 33°C, followed by 1 h at 15°C and 1 h at 33°C. Heat-shocked animals were allowed to recover overnight at 20°C and transgenic individuals were transferred as pools on fresh plates. When working with repair templates *unc-63.rep*, *unc-5.repL*, *unc-5.repS* or *unc-5.repP*, pools of heat-shocked animals were grown for 5 days at 20°C and their progeny were scored for wild-type moving animals. During scoring, counting was performed to estimate the size of the scored populations. When working with repair template *unc-5.Ldel*, *unc-5.Sdel* or *unc-5.Sgfp*, pools of heat-shocked animals were grown at 20°C for 1 week and PCR screens were performed on populations collected from starved plates. We estimated that approximately 100 F1 animals were generated from five heat-shocked P0 animals. Lysis was performed for 3 h at 65°C followed by 20 min at 95°C in 50 µl of buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin) complemented with proteinase K (1 mg/ml). Lysates were diluted 10 or 100 times before performing PCR. *MosTIC* events possibly generated with repair templates *unc-5.Ldel* and *unc-5.Sdel* were scored using primers CGAATGGT CCCCGTGGATCG (P1 on Figure 3) and CCATACACTTTCCATTGCTG (P2) (2.5 mM $MgCl_2$; annealing temperature: 55°C). *Mos*TIC events possibly generated using *unc-5.Sgfp* were screened using primers AAAGGGCAGATTGTGTGGAC (P3) and TCAGATCCGAAAGCA GAGGT (P4) for the first PCR (2.5 mM $MgCl_2$; annealing time: 15 s; annealing temperature: 68°C) and primers TCACCTTCACCCTCTC CACT (P5) and GCGGCACATTTCAAAAGAAT (P6) for the nested PCR (2.5 mM $MgCl_2$; annealing time: 30 s; annealing temperature: 65°C).

Detection of end-joining events by PCR

Heat-shock treatments were performed for 1 h at 33°C, followed by 1 h at 15°C and 1 h at 33°C on wild-type or mutant young

hermaphrodites containing a Mos transposase-expressing vector. Eighteen hours after heat-shock, lysis was performed on single animals for 1 h. Alternatively, heat-shock treatments were performed on L2 stage larvae which were lysed 9 h after heat shock. Primers AGTCATGTACCGTTCCACCTC and ATCGCAATGAAGTCC GCTATT were used to detect end-joining repair events. Subsequent TA cloning of the total PCR product was performed with the pGEM-T® Vector System I (Promega, Madison, WI) before sequencing.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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