Random and targeted transgene insertion in *Caenorhabditis elegans* using a modified *Mos1* transposon

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We have generated a recombinant *Mos1* transposon that can insert up to 45-kb transgenes into the *Caenorhabditis elegans* genome. The minimal *Mos1* transposon (*miniMos*) is 550 bp long and inserts DNA into the genome at high frequency (~60% of injected animals). Genetic and antibiotic markers can be used for selection, and the transposon is active in *C. elegans* isolates and *Caenorhabditis briggsae*. We used the *miniMos* transposon to generate six universal *Mos1*-mediated single-copy insertion (mosSCI) landing sites that allow targeted transgene insertion with a single targeting vector into permissive expression sites on all autosomes. We also generated two collections of strains: a set of bright fluorescent insertions that are useful as dominant, genetic balancers and a set of *lacO* insertions to track genome position.

Some DNA transposons can carry nontransposon DNA and still retain the ability to insert themselves randomly into chromosomal DNA. For example, the P element is used extensively to insert transgenes into the fruit fly *Drosophila melanogaster*[1]. The P element has also been used in the fly to generate large-scale gene knockout libraries, to drive tissue-specific expression using the Gal4 enhancer trap, to study genomic position effects and to generate targeted transgene insertion sites[2–5]. Similarly, other DNA-based transposons (such as *Sleeping Beauty*, *piggyBac* and *Tol2*) have successfully been used for transgenesis in a variety of genetically tractable systems including human tissue culture cells, mice, zebrafish, frogs and flies[6].

In *C. elegans*, transgenic animals are most frequently generated by DNA injection into the syncytial germ line to generate extrachromosomal arrays[7]. Biolistic transformation can be used for stable, but random, genomic integration of a single or a small number of plasmids[8]. The fly transposon *Mos1* is active in *C. elegans* but has limited cargo capacity (~500 bp) and is therefore not used directly for transgenesis[9]. Instead, excisions of *Mos1* inserts are used to generate double-strand DNA breaks, which are repaired from injected template DNA[10]. Through the use of positive and negative selection markers, a single copy of a transgene can be inserted into the genome directly via injection of mosSCI[11,12]. An alternative method to modify genomes that does not rely on transposons but on the bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 system[13] has recently been adapted for *C. elegans* to allow genome editing at endogenous loci[14–16].

Here we demonstrate that a modified *Mos1* transposon *miniMos* can carry large fragments of DNA, even 45-kb fosmids, into the *C. elegans* genome. We show that insertions can be selected using either genetic or antibiotic markers and that the transposon can be mobilized in wild isolates of *C. elegans* and *C. briggsae*. We have used *miniMos* to generate a set of strains with fluorescent markers that can be used as genetic balancers and *lacO* insertions that can track genome position in the nucleus. Furthermore, we have used the *miniMos* transposon to generate six universal mosSCI landing sites that allow insertion of a single transgene construct into permissive sites on all autosomes.

RESULTS

A recombinant *Mos1* element transposes with exogenous DNA

The requirements for transposition of mariner elements (*Mos1* and the closely related Peach transposon) vary depending on whether the transposon is embedded in chromatin or is contained within injected plasmid DNA. Mariner transposons within chromosomes require internal sequences to transpose[17] and can carry cargo only if the cargo is flanked by intact transposons[18]. By contrast, transposons injected as plasmids can transpose efficiently even if they contain internal deletions and carry cargo[19]. Experiments *in vitro* have further demonstrated that modifications to the inverted terminal repeats improve transposition frequency[20]. We tested whether modified *Mos1* elements and plasmid injection protocols[11] could overcome previously described limitations for *Mos1* transposition in *C. elegans*[9]. We generated a composite

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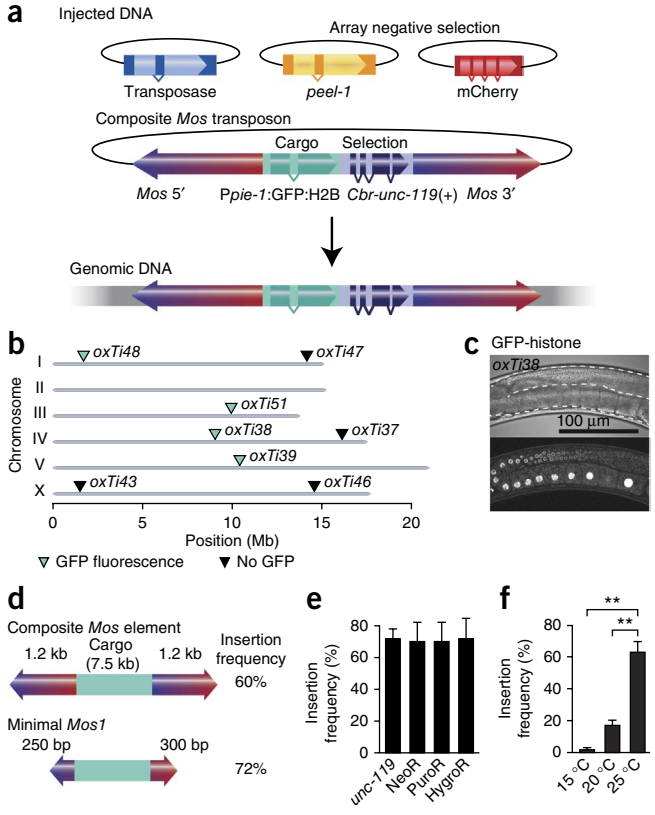

Figure 1 | A modified *Mos1* transposon can carry cargo. (**a**) Schematic of the recombinant *Mos1* insertion protocol. Transposon DNA is co-injected with a helper plasmid expressing the transposase (P*eft-3*:*Mos1* transposase). Negative selection markers (P*hsp-16.41*:*peel-1*, P*myo-2*:mCherry, P*rab-3*:mCherry and P*myo-3*:mCherry) were used to select against array-bearing transgenic animals. (**b**) Genomic locations of insertions identified by *Cbr-unc-119*(+) rescue of *unc-119* mutants. All insertions rescued *unc-119*, but not all strains expressed GFP-histone in the germ line. Germline fluorescence is indicated with turquoise (GFP positive) or black (no fluorescence) triangles. (**c**) Fluorescence image of germline expression. Transposon insertion *oxTi38* expressed GFP-histone in the germ line (P*pie-1*:GFP:H2B). Top, differential interference contrast; bottom, confocal fluorescence image. (**d**) Schematic of the minimal *Mos1* transposon (*miniMos*). 550 bp was enough to retain full insertion frequency. (**e**) Insertion frequencies with the genetic marker *unc-119*(+) and antibiotic selection markers G418 (NeoR), puromycin (PuroR) or hygromycin B (HygroR). Each antibiotic was tested on animals injected on two different days. Values show the average from all injections (n = 45–122 animals), and error bars show the 95% confidence interval (modified Wald method). (**f**) Insertion frequencies at different temperatures. Values shown are averages of three independent replicates (injections), and error bars represent s.e.m. Statistics: repeated measures ANOVA (P = 0.0017) with Bonferroni *post hoc* comparison; $^{**}P < 0.01$.

Mos1 transposon with a 7.5 kb transgene (containing P*pie-1*:GFP:histone and *Cbr-unc-119*(+)) and tested transposition by plasmid injection (**Fig. 1a** and **Supplementary Fig. 1**). We co-injected the composite *Mos1* transposon with a helper plasmid expressing the transposase and fluorescent extrachromosomal array markers. We injected 27 *unc-119* animals and identified 17 independent lines with recombinant *Mos1* insertions (62% P_0 insertion frequency). 47% (8 of 17) of the strains expressed GFP in the germ line (**Fig. 1c**). We mapped four GFP expressors and four non-expressors by inverse PCR[21] to unique insertion sites. Nonfluorescent insertions were found on autosomal arms, which have high levels of repressive chromatin marks[22], or the X chromosome, which is inactivated in the germ line[23] (**Fig. 1**). It is likely that these P*pie-1*:GFP:histone insertions are silenced through a combination of small RNAs that detect foreign DNAs and protect endogenous gene expression in the germ line[24–26] and subsequent modifications to the chromatin environment. We are currently characterizing germline and somatic position effects in detail (C.F.-J. and E.M.J., unpublished data).

The composite *Mos1* element was flanked by two essentially full-length *Mos1* elements. To identify a *miniMos* we tested transposition of truncated composite elements. Only 250–300 bp on either side was required for transposition with comparable efficiency to that of the composite transposon (**Supplementary Fig. 1**).

The composite transposon could also be mobilized from extrachromosomal arrays containing the transposon and the transposase under the control of a heat-shock promoter. From one extrachromosomal line (EG6346) we isolated two insertions from 300 heat-shocked animals (0.7%), and from a second line (EG6347) we isolated 12 insertions from 410 heat-shocked animals (2.9%). All insertions generated by mobilization from arrays were independent and mapped to unique genomic locations. It might be possible to generate large-scale transposon collections using a heat-shock protocol that are similar to the genome-wide collection of wild-type *Mos1* inserts[27]. However, it is currently more efficient to generate insertions directly by plasmid injection.

To determine whether composite *Mos1* insertions can be remobilized from genomic locations, we tried to remobilize the *oxTi51* insert by injection of the transposase gene and use of selection markers to detect germline excision and repair (**Supplementary Note**). We were unable to detect remobilization from 48 injections.

Thus, in agreement with experiments in flies[18–20,28]: (i) composite *Mos1* elements were able to transpose at high efficiency from injected plasmids and did not require most internal *Mos1* sequences, (ii) composite *Mos1* elements transposed at lower efficiency from extrachromosomal arrays and (iii) genomic insertions were not easily remobilized.

Insertion into natural isolates and *C. briggsae*

We tested other genetic and antibiotic constructs as selectable markers for *miniMos* insertion. We generated insertions of otherwise identical constructs using *unc-119*(+)[29], G418 (NeoR)[30], puromycin (PuroR)[31] and hygromycin B (HygroR)[32] selection at similar frequencies (**Fig. 1e**). The genetic marker *unc-18*(+) was also as efficient as *unc-119*(+) selection (*unc-18*(+), 38%, n = 13; *unc-119*(+), 34%, n = 32) for a different construct. We were unable to generate insertions with two temperature-sensitive selection markers, *lin-5* and *spd-1*, that are necessary in the germ line. Insertions were probably not recovered because *miniMos* transposition was strongly temperature sensitive, with insertions occurring only at low frequency at 15 °C but at high frequency at 25 °C (2% at 15 °C, n = 114; 62% at 25 °C, n = 102) (**Fig. 1f**). Extrachromosomal arrays are generally silenced in the germ line[33], and injected DNA therefore cannot rescue *lin-5* and *spd-1* animals at 25 °C. Excision of the native *Mos1* element for mosSCI transgenesis at *ttTi5605* showed no temperature

Table 1 | Recombinant *Mos1* transposon inserts at high frequency

Injected P_0 animal no.	1	2	3	4	5	Total
Singled F_1 animals (rescued)	24	45	40	18	29	156
Insertions from rescued F_1 animals	5	5	1	1	6	18
Insertions from nonrescued F_1 animals	0	1	0	0	1	2
Single fluorophore	5	6	1	1	7	20
Multiple fluorophores	0	0	0	0	0	0
Fluorescence of insertions						
P*eft-3*:GFP:H2B	1	1	1	0	2	5
P*eft-3*:mCherry	2	3	0	1	2	8
P*eft-3*:tdTomato:H2B	2	2	0	0	3	7

Five *unc-119* animals were injected with a mix containing three *miniMos* elements carrying *Cbr-unc-119* and either P*eft-3*:GFP:H2B, P*eft-3*:mCherry or P*eft-3*:tdTomato:H2B transgenes. Three days later, a single F_1-rescued animal was picked to a new plate. One week later, plates were heat shocked to express PEEL-1 and kill array-bearing animals, and insertions from rescued F_1 animals were screened for the presence of single ("single fluorophore") or multiple ("multiple fluorophores") transgenes. All seven insertions from strain no. 5 mapped to independent genomic locations.

dependence (15% at 15 °C, n = 71; 13% at 20 °C, n = 75; 15% at 25 °C, n = 71). It may be possible to use temperature-sensitive genetic markers such as *lin-5* or *spd-1* by injecting DNA into balanced strains that can be maintained at 25 °C.

We tested the P_0 insertion frequency into three highly diverged natural *C. elegans* isolates with NeoR selection: CB4856 (Hawaii), ED3040 (South Africa) and JU345 (France)[34]. The *miniMos* element was active in all strains although with variable insertion frequencies (6%, CB4856, n = 17; 68%, ED3040, n = 22; 16%, JU345, n = 19). This variation might be due to differences in genetic backgrounds or differences in susceptibility to antibiotics[30]. *miniMos* could also be mobilized in other species. We successfully inserted a P*pie-1*:GFP:histone construct into a *C. briggsae* strain (6%, n = 90) that was mutant for *Cbr-unc-119* (ref. 35); two of five animals showed stable GFP expression in the germ line. In an attempt to improve transposition efficiency in *C. briggsae*, we generated *cbr*-P*eft-3*:*Mos1* transposase and *cbr*-P*pie-1*:*Mos1* transposase constructs; however, the insertion frequency did not improve with either construct (0%, *cbr*-P*eft-3*, n = 137 and 5%, *cbr*-P*pie-1*, n = 43).

Each strain contains a single *miniMos* insertion

To determine the insertion frequency in F_1 animals and the transgene copy number in each strain, we injected a mix of three different *miniMos* elements that could be distinguished by color (red or green) and cellular localization (cytosolic or nuclear) (**Table 1**). We injected five P_0 animals, picked 156 *unc-119* rescued F_1 animals to individual plates and recovered 20 independent insertions (11.5% F_1 insertion frequency). This frequency is comparable to the frequency of generating semistable transgenic animals by simple array injection (10%)[7]. All 20 insertions were fluorescent and expressed only one of the fluorophores from the injection mix (**Table 1**). Insertions from the same injected animal were independent; we determined all seven insertion sites from animal no. 5 by inverse PCR and all mapped to unique positions in the genome (*oxTi306*–*oxTi312*; **Supplementary Table 1**).

We also confirmed that insertion strains contain a single insertion by segregation in crosses (**Supplementary Note**). How can a single injection generate several independent insertions and yet each strain contain only a single insertion? We determined that this is possible because insertions were generated at relatively low frequency but occurred in the F_1 generation when the population expanded (**Supplementary Fig. 2**).

To facilitate identification of transposon insertion sites, we added new symmetric restriction sites to the *miniMos* vectors and optimized the inverse PCR protocols (**Supplementary Fig. 3** and **Supplementary Protocol**). We tested the optimized protocol in individual reactions and 96-well reactions on a collection of bright fluorescent P*eft-3*:tdTomato:H2B inserts (where tdTomato is tandem dimer Tomato and H2B is histone H2B), which will be useful as dominant chromosome balancers for *C. elegans* crosses (**Supplementary Fig. 4**).

12% of the inverse PCR reactions contained sequences from the injected plasmid backbone, a result indicating that some transpositions included two adjacent *miniMos* elements ('composite transposition'; **Supplementary Fig. 1**). Sequencing showed that the entire backbone of the injected plasmid had inserted. Incorporating the negative *peel-1* selection marker[11], which is heat-shock inducible, into the backbone of injected *miniMos* plasmids effectively selected against these types of complex insertions.

P-element transgenesis has been used to generate loss-of-function mutants in *Drosophila*[3]. Although we did not directly

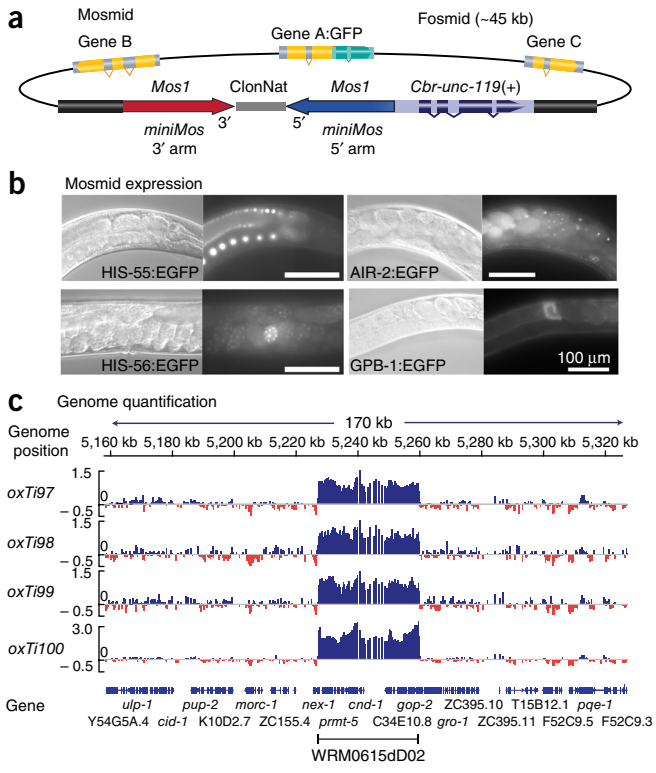

Figure 2 | Fosmid insertions are intact. (**a**) Schematic of *Mos1*-based fosmids (Mosmids). *Mos1* and *Cbr-unc-119*(+) selection recombineered into the backbone of a fosmid carrying a GFP-tagged gene. (**b**) Fluorescence microscopy of Mosmid insertions. Four different Mosmid insertions with GFP show expression from the tagged genes. (**c**) Comparative genome hybridization (CGH) of genomic DNA from four independent insertions of the Mosmid WRM0615dD02 containing tagged *cnd-1*. CGH is based on dense oligonucleotide arrays tiled against a genome of interest and labeling of sample DNA and control DNA with different fluorophores. Genomic regions that differ between sample and control will show a difference in the ratio between the two color intensities. The Mosmid with *cnd-1*:EGFP contained an error rendering the fusion protein nonfluorescent.

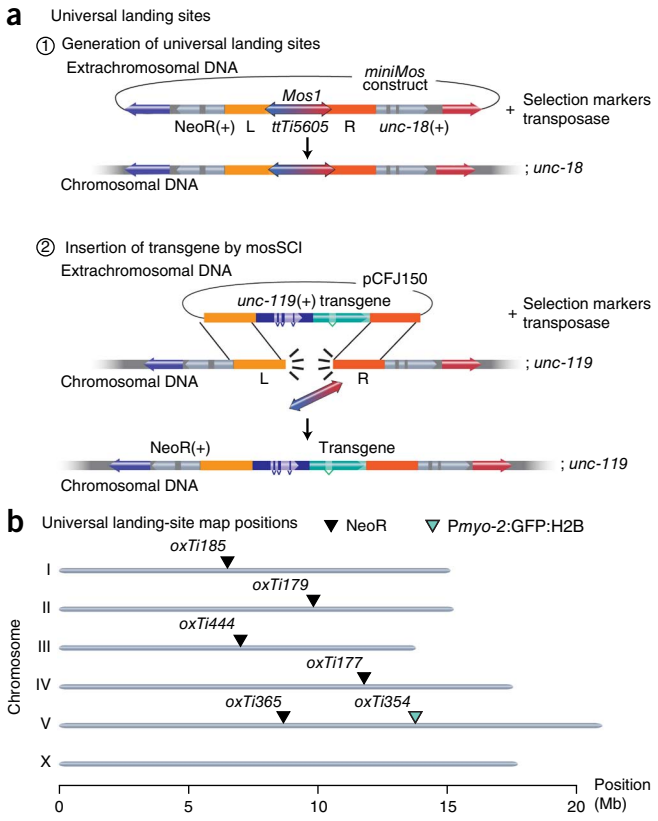

Figure 3 | Using *miniMos* to generate universal mosSCI insertion sites. (**a**) Schematic of the method to generate universal mosSCI insertion sites. Step (1): insert *miniMos* with the *ttTi5605* genomic region (including the native *Mos1* element) into *unc-18* mutants. Cross inserts to *unc-119*. Step (2): inject the pCFJ150-based targeting vector to insert the transgene by mosSCI. All insertions were verified as functional, single-copy insertions. L, left, and R, right, recombination region from the *ttTi5605* mosSCI insertion site. (**b**) Genomic location of universal mosSCI insertion sites with verified germline expression. NeoR, neomycin-resistance gene.

screen for mutant phenotypes, we noted that several of the P*eft-3*:tdTomato:H2B insertions were inserted into introns and exons of genes with obvious phenotypes: *unc-13* I, *unc-22* IV and *him-4* X. All three insertions showed the phenotypes expected from loss-of-function alleles.

To test whether expression of insertions was affected by neighboring promoters, we generated strains with promoters driving GFP expression in pharyngeal muscles (P*myo-2*, $n = 3$) and body-wall muscle (P*unc-54*, $n = 3$). In this relatively small sample, we were unable to detect misexpression in other tissues (**Supplementary Fig. 5**). The insertion frequency and fidelity of insertions is robust enough that *miniMos* transposition could be a convenient alternative to extrachromosomal arrays in cases in which the unstable and multicopy nature of arrays is undesirable (**Supplementary Note** and **Supplementary Fig. 6**).

Mos1 can transpose with fosmids and *lacO* repeats

To determine the maximum cargo capacity of recombinant *Mos1* elements, we generated *Mos1*-based fosmids (Mosmids) by recombineering[36]. We inserted a cassette with a 1-kb recombinant *Mos1* element and *Cbr-unc-119*(+) into the backbone of several fosmids with GFP-tagged genes (**Fig. 2**). We injected five different Mosmids into *unc-119* animals and obtained stable integrated lines at P_0 frequencies ranging from 2% to 14% (5% ± 2%; mean ± s.e.m.) of all constructs. The drop-in insertion frequency was likely caused by two effects: larger cargo may inhibit transposition, and Mosmid injections only inefficiently form extrachromosomal arrays. Inserted Mosmids expressed EGFP in the expected tissues, including the germ line (**Fig. 2b**).

From one Mosmid (*air-2*:EGFP) we obtained 18 independent insertions that were all fluorescent, which suggests that Mosmid insertions were generally intact. We verified the integrity of the inserted fosmids by comparative genome hybridization (CGH); this method can detect deletions, insertions and even single-base-pair mutations with high sensitivity[37,38] (**Fig. 2c** and **Supplementary Fig. 7**). In the four lines generated from a tagged *cnd-1* gene, either a single, fully intact copy or two full copies (into a single location) of the Mosmid were inserted. We observed similar full-length insertions by CGH on lines from *gpb-1*, *his-55* and *air-2* inserts (**Supplementary Fig. 7**).

lacO repeats can be used to visualize chromosome position when they are bound to a fluorescently tagged LacI repressor[39]. We tested whether a recombinant *Mos1* element could insert a large repetitive transgene containing 256× *lacO* repeats and selection markers. We generated 20 independent insertions (**Supplementary Fig. 8**). These strains showed two distinct fluorescent dots in embryos when crossed into a line expressing LacI:GFP, corresponding to the two homologous chromosomes containing the *lacO* repeats (P. Meister, University of Bern, personal communication).

These experiments showed that the *miniMos* element is compatible with a wide variety of transgenic cargo and selection markers. We have generated a set of 16 standardized *miniMos* cloning vectors to facilitate use of the technique (**Supplementary Fig. 9**).

A set of universal mosSCI insertion sites

The ΦC31 recombinase has been used in flies to develop universal insertion sites that are compatible with a single targeting vector[4,40]. We unsuccessfully attempted to adapt the ΦC31 system for *C. elegans* (M.S. and C.F.-J., unpublished observations). As an alternative, we developed a *miniMos* system that achieves the same goal. We generated a *miniMos* element containing the *ttTi5605* mosSCI site and flanked it with two selection markers, *unc-18* and either NeoR or P*myo-2*:GFP:H2B (**Fig. 3**). The embedded *ttTi5605* Mos element within the *miniMos* transposon can be used as a landing site for single-copy insertion using mosSCI[12] and is compatible with previously published targeting vectors (pCFJ150 or pCFJ350) (**Fig. 3**). Furthermore, mosSCI insertions can be followed in crosses by the adjacent selection marker (NeoR or P*myo-2*:GFP:H2B). We generated a set of validated single-copy, full-length mosSCI universal insertion sites that were permissive for germline expression (**Fig. 3**). Additionally, we targeted the insertion of a universal landing site into the *ttTi25545 Mos1* site at the center of chromosome III by mosSCI because no insertion site on chromosome III was compatible with germline expression (data not shown). All universal landing sites were validated: we could generate single-copy inserts at frequencies similar to those for insertions into the native *ttTi5605* site, and a P*dpy-30*:GFP:H2B transgene was expressed in the germ line (**Supplementary Table 1**).

DISCUSSION

Random insertion of transgenes with the *miniMos* element has several advantages relative to biolistic transformation[8]. First, the exact insertion site can be determined by PCR. Knowledge of the exact insertion site ensures that mutations caused by *miniMos* insertion, or effects on expression of the transgene by the genomic environment, can be assessed. Second, a single intact copy of the transgene with well-defined end points in the genome is inserted. Third, the *miniMos* element can insert intact fosmids[41] and is active in other species and natural *C. elegans* isolates[42]. Finally, the insertion frequency of the *miniMos* element is high enough that several insertions are frequently generated from a single injection. Redundant inserts improve the chance of identifying insertions that do not disrupt endogenous genes and that are appropriately expressed.

We imagine *miniMos* transgenesis will mostly be used to insert single copies of transgenes, but there are at least four additional uses for the *miniMos* resources described here. (i) The set of dominant chromosome balancers is composed of 158 inserts that express red or green fluorescent proteins in somatic nuclei spaced about every 2–5 map units (**Supplementary Fig. 4**). These balancers can be used to generate strains with complicated genotypes. (ii) We generated two mapping strains that contain three distinguishable fluorescent markers that cover all six chromosomes in high incidence of male (*him*) mutant backgrounds. These strains are useful for mapping new mutations to chromosomes. (iii) The *lacO* insertions mark 20 different genomic sites and can be used to locate chromosome positions in the nucleus: for example, during meiosis or differentiation[43]. (iv) We generated a set of universal mosSCI insertion sites that are compatible with a single targeting vector. These strains can be used to insert single-copy transgenes at multiple positions in the genome.

In the future, two compelling uses for *miniMos* will be to probe the genome on a global scale for chromatin effects and to determine expression patterns using gene-trap constructs. First, the preliminary experiments with the composite *Mos* inserts demonstrate that transgene expression in both the soma and germ line of *C. elegans* is position dependent, with high degrees of silencing on the X chromosome and on autosomal arms. For example, almost all of the nonfluorescent P*pie-1*:GFP insertions were inserted into the X chromosome, which is inactivated in the germ line[23], or into autosomal arms containing a high incidence of repressive histone marks[22]. Second, *miniMos* constructs can be used to generate enhancer-trap and gene-trap constructs. For determining the expression pattern of a single gene, it will be much more efficient to specifically target the gene with the CRISPR-Cas9 system[15,16,44]. But for determining the expression patterns of all genes, random insertions with *miniMos* will be preferable, as has been done in *Drosophila* using P elements[2]. The *miniMos* element could be combined with the Q system[45] to generate strong, inducible driver lines for most tissues. In particular, it may be possible to identify promoters or enhancers that target expression individually to many of the 302 neurons of the adult nervous system.

Protocols, annotated plasmid sequences and a searchable list of strains are available at the Wormbuilder web page (http://www.wormbuilder.org/).

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.F.-J. designed experiments under the supervision of E.M.J. and M.W.D. C.F.-J., M.S., A.P., J.T., M.L. and S.F. performed the research. C.F.-J. performed molecular biology, injections, imaging and genetics; M.L. generated mapping strains; M.S. and A.P. performed fosmid recombineering; and J.T., S.F. and D.G.M. performed comparative genome hybridization. C.F.-J. and E.M.J. wrote the paper with input from all coauthors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. Please see the web page http://www.wormbuilder.org/ for annotated plasmid sequences, protocols and a searchable lists of strains. Plasmids are available from Addgene as a single kit (#1000000031; https://www.addgene.org/minimos/) or as individual plasmids. Strains were maintained using standard methods[46]. Temperature-sensitive strains *lin-5* and *spd-1* were grown at 15 °C. All other strains were grown at room temperature on OP50 or HB101 bacteria. Fluorescent balancer strains, including the two mapping strains, have been deposited with the *Caenorhabditis* Genetics Center (CGC).

Molecular biology. Plasmids were designed with ApE (A plasmid Editor, M.W. Davis), which is freely available at http://www.biology.utah.edu/jorgensen/wayned/ape/.

All plasmids were generated by standard molecular techniques, including isothermal assembly[47] and three-fragment Gateway cloning (Life Technologies). PCR amplification was performed using a high-quality DNA polymerase, Phusion (New England BioLabs).

Please see **Supplementary Table 1** for GenBank-formatted plasmid sequences of all plasmids used in this study.

Reproducibility. All injections were performed at least in duplicate and usually in triplicate on different days. Only injections with DNA isolated by the same preparation method were compared. The number of injections and the sample size were selected to reach statistical significance in tests that correct for multiple comparisons. Overall, the reproducibility on different days was high. This is particular apparent in the experiment to identify the minimal *Mos1* element (*miniMos*), where all truncated constructs larger than the *miniMos* transposon show reproducible insertion frequencies (**Supplementary Fig. 1**).

Exclusion criteria. Plates that did not contain any transgenic F_1 progeny as determined by phenotypic rescue (*unc-119* injections) or the presence of fluorescent co-injection markers (antibiotic injections) were not counted toward the number of injected animals. This exclusion criteria excluded approximately 5–10% of all injected animals and served to reduce the variability caused by differences in injection needles between separate injections.

Blinding and randomization. No blinding or randomization was performed.

Recombinant *Mos1* insertions. *miniMos insertions*. Insertions were generated and mapped as described in detail in the **Supplementary Protocol**. In brief, injection strains were maintained on HB101 bacteria at 15–20 °C. An injection mix containing the *miniMos* transgene at 10–15 ng/µl, red fluorescent co-injection markers pGH8 at 10 ng/µl, pCFJ90 at 2.5 ng/µl and pCFJ104 at 10 ng/µl, a helper plasmid expressing the *Mos1* transposase pCFJ601 at 50 ng/µl and the negative, heat shock–inducible *peel-1* selection marker pMA122 at 10 ng/µl. The remaining volume was made up of milliQ purified water. Injected worms were placed at room temperature for 1–2 h, transferred to individual plates and incubated at 25 °C until starvation (approximately 1 week). For experiments aimed at quantifying insertion frequency, plates were screened for F_1 rescue 3 d after injection, and plates with no F_1 rescue were discarded. Once starved, plates were heat shocked for 2 h at 34 °C or for 1 h at 37 °C in an air incubator to kill animals with extrachromosomal arrays. All plates were screened for *miniMos* insertions the day after heat shock on a fluorescence microscope on the basis of rescue and the absence of red co-injection markers. Because of obvious visual differences (state of animals at 25 °C vs. 15 °C or the fluorescence of injected plasmids), the investigator was not systematically blinded to the injected constructs. A single animal from each plate containing insertions was picked for further analysis. The location of *miniMos* elements was determined by an inverse PCR protocol modified from Boulin and Bessereau[21] on genomic DNA isolated with the kits "ZR Tissue and Insect DNA miniprep" or "ZR-96 Genomic DNA Tissue miniprep" (Zymo Research). The DNA was digested with restriction enzymes (New England BioLabs) for 3 h to overnight, ligated with T4 ligase (Enzymatics) and PCR amplified twice with oligos that anneal in the *miniMos* transposon with Phusion DNA Polymerase. The PCR product was electrophoresed on a 1% agarose gel, and single bands were gel purified with the "Zymoclean Gel DNA Recovery Kit" (Zymo Research). The gel-purified product was Sanger sequenced at the University of Utah Sequencing Core.

We performed two or three independent injections for each set of conditions tested (for example, temperature or length of composite *miniMos* transposon) to minimize effects of a single bad injection needle. Generally, we observed very little variability between independent injections. Following advice from M. Maduro (UC Riverside), we determined that the largest source of variability was in the quality of injected DNA. We isolated DNA with Spin Miniprep (cat. no. 27106) and Plasmid Plus Midiprep (cat. no. 12943) kits from Qiagen and with a PureLink HQ Mini Plasmid kit from Invitrogen (cat. no. K2100-01). The higher-quality DNA kits (Qiagen Midi and Invitrogen Mini kits) resulted in a fourfold increase in F_1-rescued animals (20 vs. 5 rescued animals per injection) and a 50% (Qiagen Midi) to 100% (Invitrogen mini) increase in mosSCI insertion frequency (**Supplementary Fig. 6**). Although we have not tested the effect of DNA purity on *miniMos* insertion frequency, we generally recommend using DNA of higher purity for injection than what is isolated with the standard Qiagen Miniprep Kit. At the time of injections performed to quantify the insertion frequency of the *miniMos* transposon, we were not aware of the increased frequency resulting from higher DNA quality, and these injections were therefore all done with the Qiagen miniprep kit.

Quantification of insertions per injection (**Table 1**). We injected a mix of three different *miniMos* plasmids carrying P*eft-3*:GFP:H2B, P*eft-3*:mCherry or P*eft-3*:tdTomato:H2B with the *Cbr-unc-119*(+) selection together with the *Mos1* transposase and the negative PEEL-1 selection plasmid into *unc-119* mutant animals. We picked rescued animals in the F_1 generation to individual plates and allowed the animals on these plates to starve out at 25 °C. We heat-shocked plates with rescued F_2 or F_3 animals to kill animals with extrachromosomal arrays and screened for insertions the following day. We screened each plate containing an insertion for the presence of multiple different fluorescent patterns and picked a single animal from each plate for further analysis. We isolated genomic DNA and performed inverse PCR on all seven different insertions (*oxTi306*–*oxTi312*) that originated from injection into P_0 animal no. 5. All seven insertions mapped to different genomic locations.

Universal insertion sites. The universal insertion sites were generated by injection into *unc-18*(*md299*) animals following the protocol for *miniMos* insertions. The internal *Mos1* element depressed *miniMos* insertion frequency from approximately 60% to 12% (n = 180) and resulted in a high frequency of complex insertions (56%, n = 23). Strains with a putative insertion were tested for antibiotic resistance to G418 (NeoR). Genomic DNA was isolated from homozygous, G418-resistant strains and tested by PCR for the presence of the *ttTi5605 Mos1* element and the absence of backbone fragments from the cloning vector. Inverse PCR was performed on strains with intact universal insertion sites with oligos that specifically detect the *miniMos* element and not the wild-type (internal) *Mos1* element. The genomic location was determined by Sanger sequencing and verified by oligos designed for each individual insertion (**Supplementary Table 1**). Strains with universal insertion sites were outcrossed five times against an 11× outcrossed *unc-119*(*ed3*) strain, EG6207, derived from PS6038 (a kind gift from A. Sapir and P. Sternberg (Caltech)) by following neomycin resistance. We verified homozygosity of the universal insertion sites in the *unc-119* background after out-crossing by PCR. The ability to insert transgenes into all universal landing sites was verified by insertion of pCFJ150-derived constructs with P*eft-3*:GFP:H2B:*tbb-2* UTR, P*dpy-30*:GFP:H2B:*tbb-2* UTR or P*pie-1*:GFP:H2B:*pie-1* UTR transgenes.

In one case, *oxTi444*, a universal insertion site was generated by targeted insertion of the universal landing site into a preexisting mosSCI site, *ttTi25545*. In this case, the *miniMos* element was exchanged for left and right homology regions adjacent to *ttTi25545* and inserted by the standard mosSCI protocol[11].

Antibiotic selection protocol. We used antibiotic selection protocols modified from Giordano-Santini *et al.*[30], Semple *et al.*[31] and Radman *et al.*[32]. For G418 selection, we made a 25 mg/ml (Gold Biotechnology) solution in water and filter-sterilized the solution with a 0.2-µm filter. For puromycin selection we purchased a 10 mg/ml solution (InvivoGen) and added 0.1% Triton X-100 (Sigma). For hygromycin B we made a 20 mg/ml (Gold Biotechnology) solution in water and filter-sterilized the solution with a 0.2-µm filter. For use in antibiotic selection, 500 µl of the stock solutions were added directly to plates containing wild-type worms that had been injected 1 or 2 d before. Plates were allowed to dry with the lid off. Dry plates were returned to the 25 °C incubator, and worms were allowed to starve. The animals were heat shocked to remove those with extrachromosomal arrays and were screened for insertions the next day on the basis of survival on antibiotic plates, lack of fluorescent co-injection markers and fluorescence from the *miniMos* construct carrying P*eft-3*:GFP(NLS). At least ten animals from each antibiotic selection were propagated and homozygosed by fluorescence to verify true insertions. We note that the antibiotic selection markers are very convenient for injecting into healthier strains, such as wild-type animals, but suffer from the disadvantage that they are harder to homozygose, especially in the absence of a fluorescent insertion marker. In our hands, G418 and hygromycin B killed almost all nontransgenic animals within 2 d, whereas puromycin typically took 3–4 d to kill nontransgenic animals.

Composite Mos1 *remobilization.* To determine whether composite *Mos1* insertions can be remobilized from genomic locations, we generated a strain carrying an insertion (*oxTi51*; **Fig. 1b**) and a mutation in the *unc-18* gene. A rescuing template containing *unc-18*(+) was constructed so that a double-strand break generated by transposon excision would be repaired by homologous recombination and copy *unc-18*(+) into the excision site. From 48 injected animals we did not recover any targeted *unc-18*(+) insertions. This result is in agreement with similar experiments in *Drosophila*, where the insertion frequency was intact but genome mobilization was reduced by two orders of magnitude for modified transposons of the same family as *Mos*[18].

Bioinformatic analysis of recombinant Mos1 *insertions.* The locations of transposons were determined by inverse PCR. Genomic location was determined by identifying the junction between the transposon and genomic DNA. A BLAST search at http://www.wormbase.org/ against genome version WS190 (ce6) was used to determine the genomic position. Generally only uniquely identified insertions were used; however, some insertions that map to several position within a small genomic interval (~10 kb) were included in some figures.

Comparative genome hybridization. Genomic DNA from worms was isolated with the ZR Tissue & Insect DNA MiniPrep kit (Zymo Research) following the manufacturer's protocol. DNA labeling, sample hybridization, image acquisition and determination of fluorescence were all performed as previously described[37,38]. We used a 3× high-density (HD) chip divided into three whole-genome sections with 720,000 different oligos for all experiments. The chip design was based on our original whole-genome chip containing 385,000 different oligos. All microarrays were manufactured by Roche-NimbleGen with oligonucleotides synthesized at random positions on the arrays. The chip design name is 90420_Cele_RZ_CGH_HX3. Quantile normalization was performed on the intensity ratios for all experiments. Seven strains—EG7784 (*oxTi97*), EG7785 (*oxTi98*), EG7786 (*oxTi99*), EG7787 (*oxTi100*), EG6840(*oxTi109*), EG6731 (*oxTi114*) and EG6788 (*oxTi118*)—were tested against wild-type DNA. All strains showed a duplication of the full genomic region contained within the recombineered fosmid, except for the strain EG7787, which contains a dual insertion. PCR amplification from EG7787 showed the presence of backbone DNA, which is consistent with a duplicate insertion into the same genomic locus. For all analyzed Mosmid insertions, the end points of genomic duplications identified by CGH closely matched the ends of recombineered fosmids, and no second-site duplications were detected.

Fosmid recombineering. The fosmids were engineered essentially as in ref. 41, except for the fosmid backbone modification step, where the *Mos1* transposon (1,000 bp) with inverted repeats (IR) was added to the *Cbr-unc-119*-Nat cassette (on each side of the NatR marker). To make the fosmid host bacteria EPI300 (Epicentre) proficient for recombineering, we transformed the EPI300 cells with the pRedFlp4 plasmid, which allows for inducible expression of either the λ Red operon+RecA or the Flp recombinase. For gene tagging, a multipurpose tagging cassette that contains the flexible linker peptide TY1, GFP, *FRT*-flanked positive selection (NeoR), counterselection (rpsL) and the affinity tag 3xFlag was PCR amplified. The PCR used gene-specific primer extensions of 50 bp upstream and downstream of the insertion point that serve as homology arms for recombineering. Recombinants were selected for kanamycin resistance in liquid culture. The *rpsl/neo* selection-counterselection marker

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was removed by Flp/FRT recombination. The homology arms targeting the *Cbr-unc-119*/IR NatR IR cassette to the fosmid backbone were the same for all fosmids and were included in the same plasmid (pCFJ496); this cassette was isolated by restriction digest from pCFJ496 and used for recombineering the fosmid containing a EGFP-tagged gene. Both the template for the multipurpose tagging cassette and the template for inserting the *Mos1* and *Cbr-unc-119* genes were cloned in plasmids with the R6K origin of replication, which is nonfunctional in the fosmid host strain, and removal of the plasmid is thus not required before recombineering. The fosmid modification cassette pCFJ496 is available from Addgene (plasmid #44488).

Mosmids generally integrate into the genome at lower frequencies than *miniMos* transposons that can be propagated as high-copy plasmids in bacteria. The lower insertion frequency is likely due to (i) lower transposition frequency of the *miniMos* element with larger cargo, (ii) decreased ability of fosmids to form extrachromosomal arrays owing to reduced homology and (iii) toxic sequences present on the fosmid. Some of the Mosmids that we tested were specifically chosen because integrated lines could not be generated by biolistic transformation despite repeated attempts and appear to be toxic (M.S., unpublished data). For example, we injected 48 and 60 *unc-119* animals with the *his-55*:EGFP and *his-56*:EGFP Mosmids, respectively. From these injections we did not recover a single rescued F_1 animal but were able to isolate one *his-55*:EGFP (2%) and two *his-56*:EGFP (3%) rescued insertion lines in the F_2 progeny. This suggests that these Mosmids are toxic at high copy number and that higher integration efficiencies may be achieved by titrating the Mosmid concentration. In support of this, we did not observe any toxicity from an *air-2*:EGFP Mosmid and recovered 18 independent insertions from 125 injected *unc-119* animals (14%).

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Supplementary Information

Random and targeted transgene insertion in *C. elegans* using a modified Mos1 transposon

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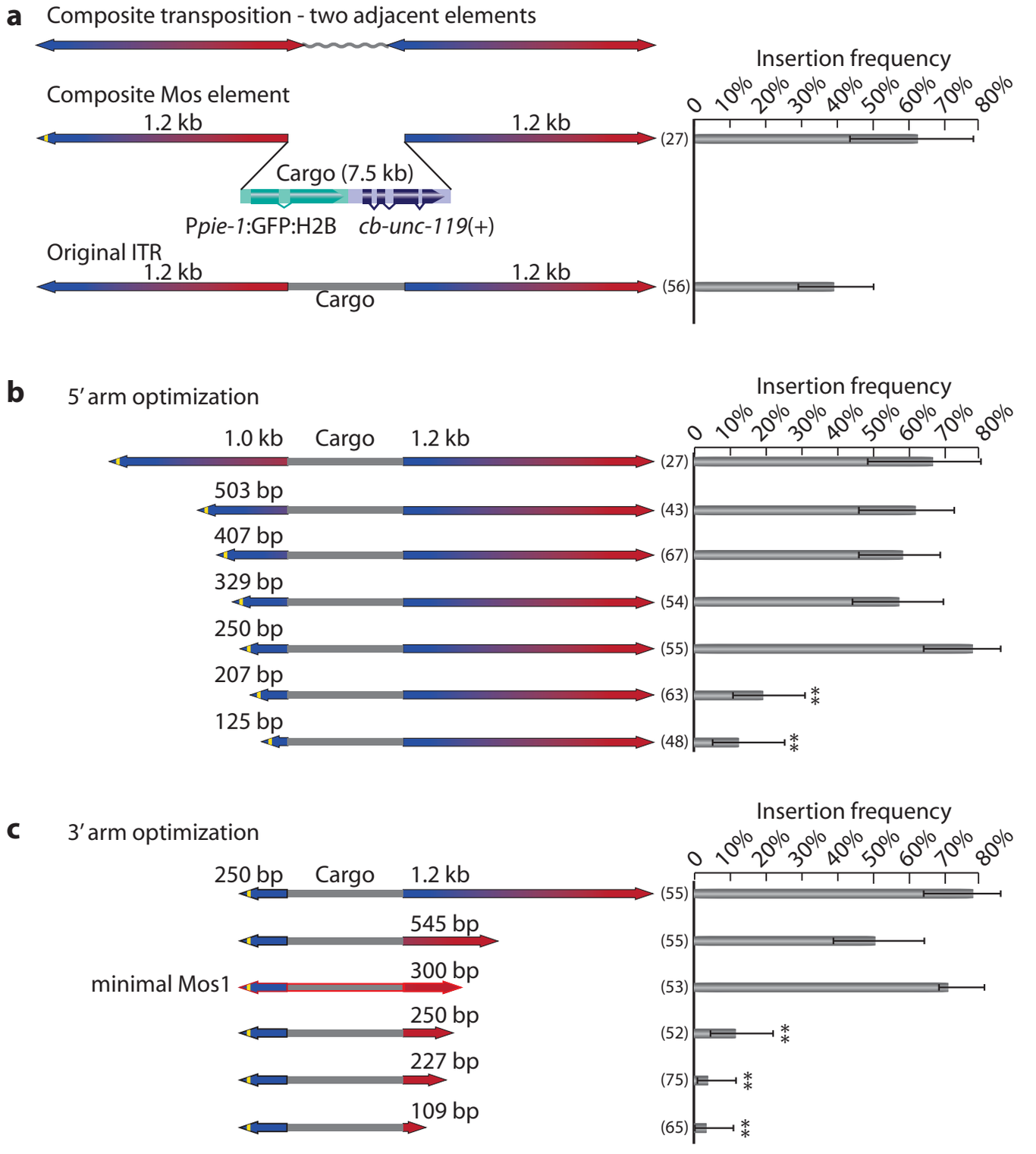
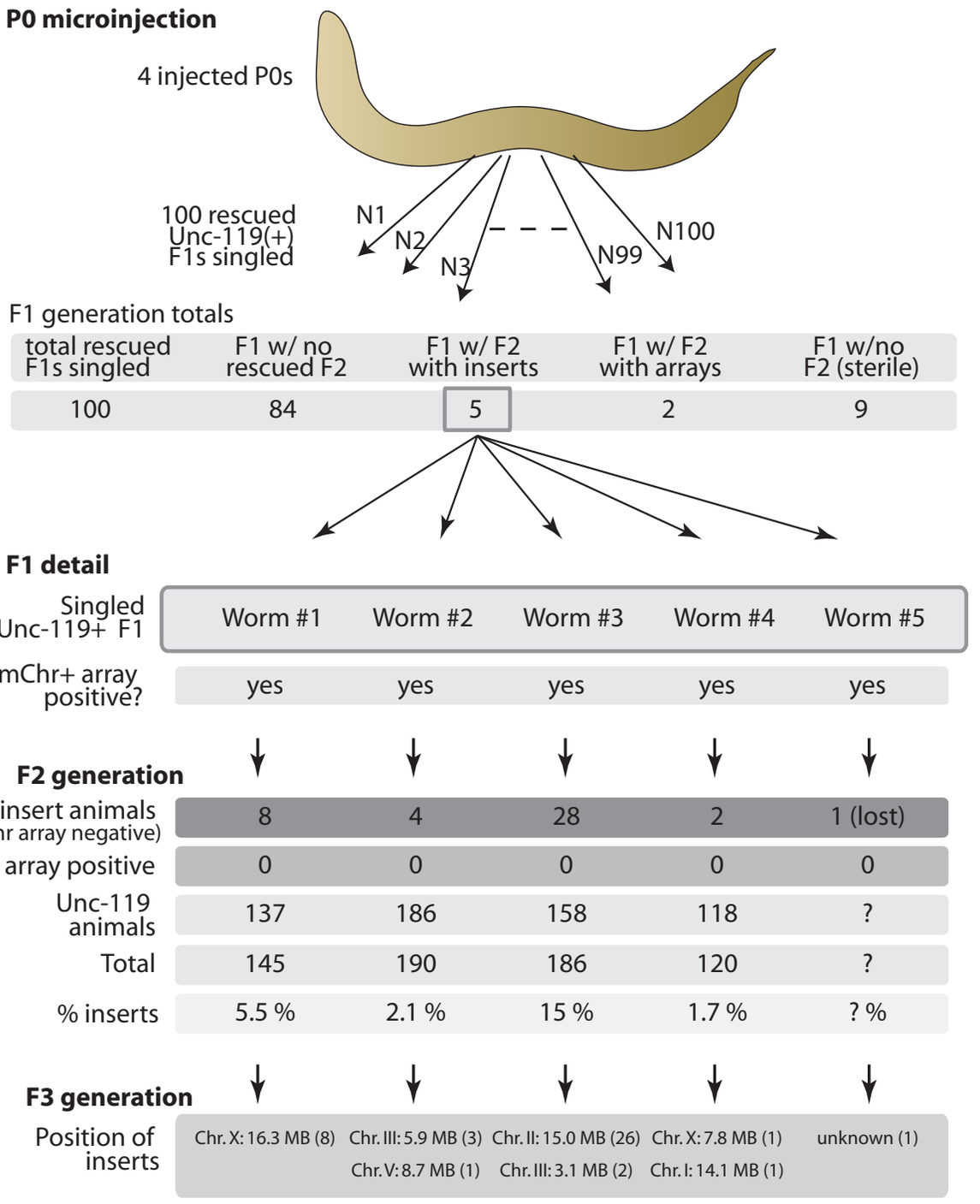
Please see www.wormbuilder.org for strains, protocols and reagents.



Supplementary Figure 1. The minimal Mos1 transposon is 550 bp.

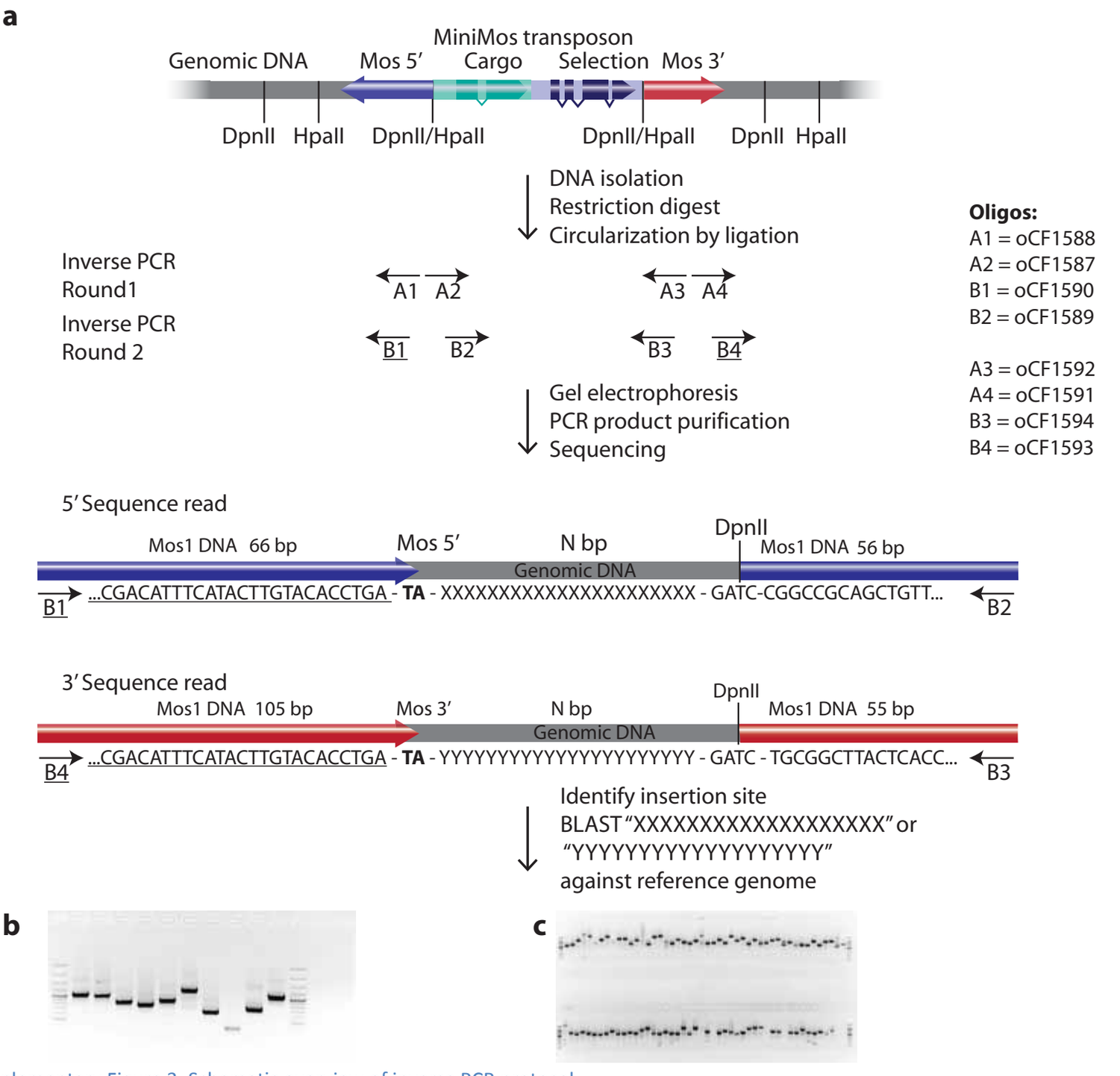
(a) Above, schematic of two full Mos1 transposons. Insertions caused by composite transposition carrying the intervening DNA were occasionally observed (MWD, unpublished), suggesting that composite Mos elements could be an effective method for introduction of exogenous DNA. Below, schematic of composite Mos1 transposon. The cargo is flanked by two complete Mos1 transposons, except the internal inverted repeats were deleted. The 5' end of the Mos1 transposon was modified to increase Mos1 transposase binding (yellow line, top) which moderately increased the transposition frequency compared to the non-modified composite transposon (bottom) (Casteret et al., 2009). The cargo consists of a 7.5 kb P*pie-1*:GFP:H2B:pie-1UTR and *cb-unc-119*(+) fragment. Right, insertion frequency. Insertion frequency is the percentage of successfully injected P0 animals that gave rise to at least one insertion event in the progeny. The number of injected animals is shown in parentheses. Error bar indicates 95% confidence interval. All injections were done as a minimum of two independent replicates on different days. **(b)** Composite elements truncated from the 5' end. **(c)** Composite elements truncated from 3' end. The minimal fully functional Mos1 element (miniMos) is 250 bp at the 5' end and 300 bp at the 3' end.

Statistics: Chi square test for significance. All truncated constructs were compared to full-length composite element with Fischer's exact test and corrected for multiple comparisons (Bonferroni). **, $p < 0.01$.



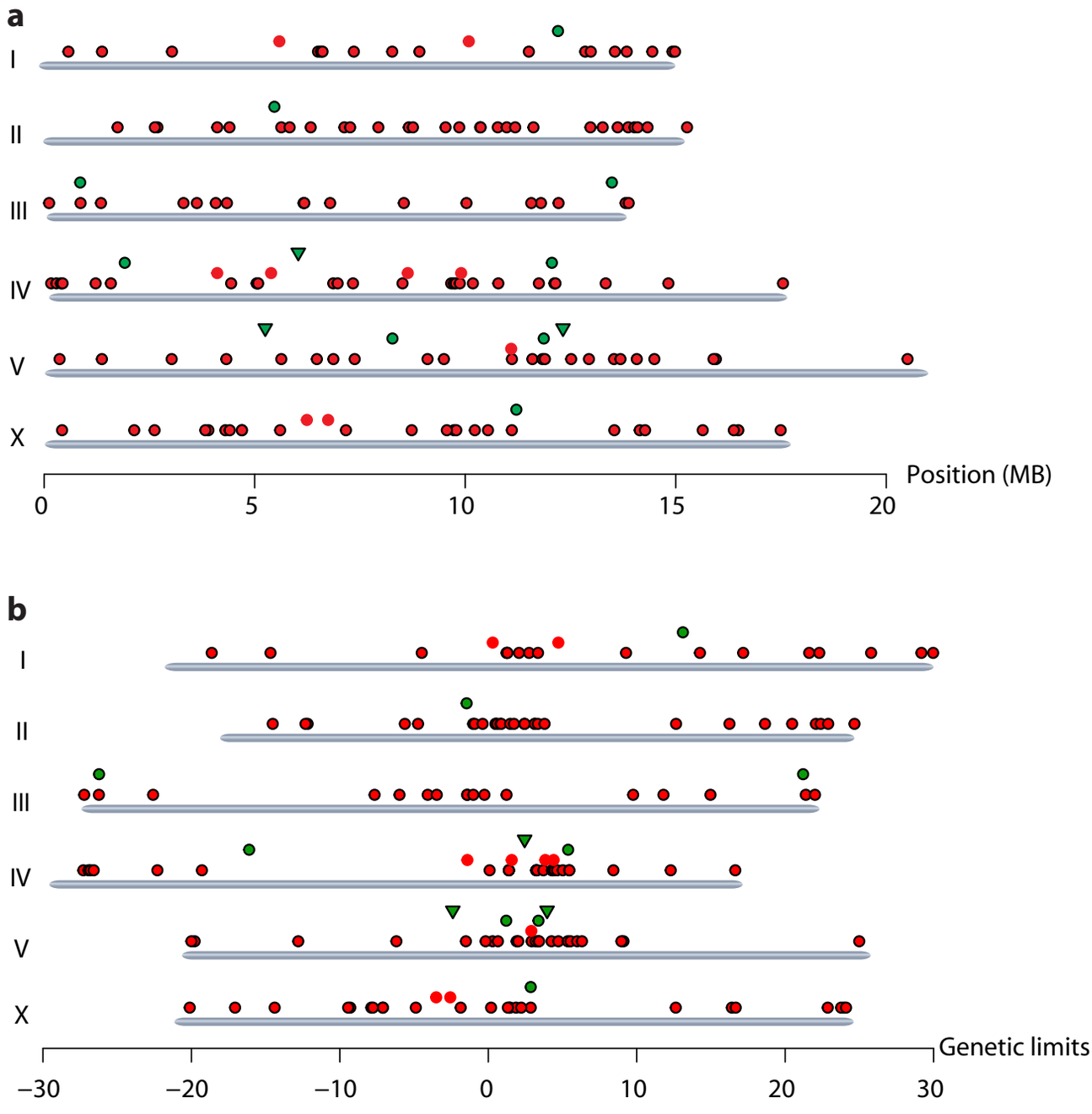
Supplementary Figure 2. miniMos insertions occur in the germline of F1 animals.

Experiment to determine when the miniMos insertion occurs. From 4 injected P0 *unc-119* animals, we singled 100 rescued F1 animals (all mCherry array positive). From these 100 F1 animals, five F1 animals produced a total of 8 independent insertions. Only 2-15% of the F1 progeny carried the insertion, thus mobilization of miniMos must occur late during the proliferation of the F1 germline. Insertion sites were determined by inverse PCR and confirmed with gene-specific primers to identify the presence of a particular insertion.



Supplementary Figure 3. Schematic overview of inverse PCR protocol.

(a) Schematic of the protocol to determine miniMos insertion site. The miniMos vectors have been re-engineered to contain DpnII and HpaII restriction sites (four base recognition sites) flanking the transgene cargo. Purified genomic DNA is digested with either of the enzymes, which will digest the Mos1 transposon at these sites and the flanking genomic sequence at the nearest restriction site. The digested fragments are circularized by ligation followed by two rounds of PCR with nested oligos to amplify Mos1 and the flanking genomic region. For increased probability of successful amplification, the PCR protocol can be done with oligos specific to both ends of the transposon on the same ligation mix. PCR amplified products are isolated (by gel purification or by ExoSAP purification) and submitted for sequencing. Successful sequencing reads contain the Mos1 sequence, the TA dinucleotide that Mos1 inserts into, the flanking genomic region, the DpnII (or HpaII) restriction site, and the other end of the Mos1 transposon. A BLAST search against the reference genome with the flanking genomic region identifies the transposon insertion site. **(b)** Examples of individual inverse PCR reactions on purified genomic DNA. Each bright band corresponds to the single insertion in each strain. **(c)** Example of 96-well inverse PCR, where all steps (genomic DNA isolation, ligation, and two rounds of PCR) were done in a 96-well format. The gels show that most inverse PCR reactions result in a single, unique band that can be sequenced without gel purification (ExoSAP protocol = ExonucleaseI digest of oligos and Shrimp Alkaline Phosphatase removal of nucleotides).

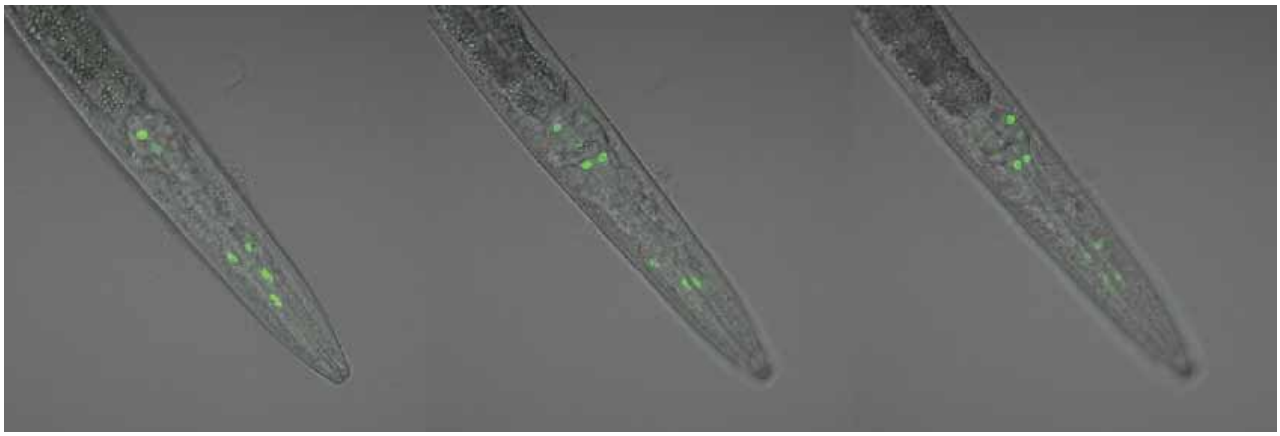


Supplementary Figure 4. Fluorescent marker strains

(a) Physical map of fluorescent balancer chromosomes. Four different constructs were mobilized: Either green (GFP) or red (tdTomato and mCherry) fluorescence can be used to avoid confusion when mapping fluorescent integrations. The *eft-3* promoter is broadly expressed in somatic tissue. Histone H2B fusions express fluorescence in the nucleus. Fluorescence is visible on a fluorescence dissection microscope for all inserts. Strains containing the hsp:*peel-1* transgene can be selected against by heat-shock for ease in generating homozygotes of the original chromosome. **(b)** Genetic map of fluorescent marker strains.

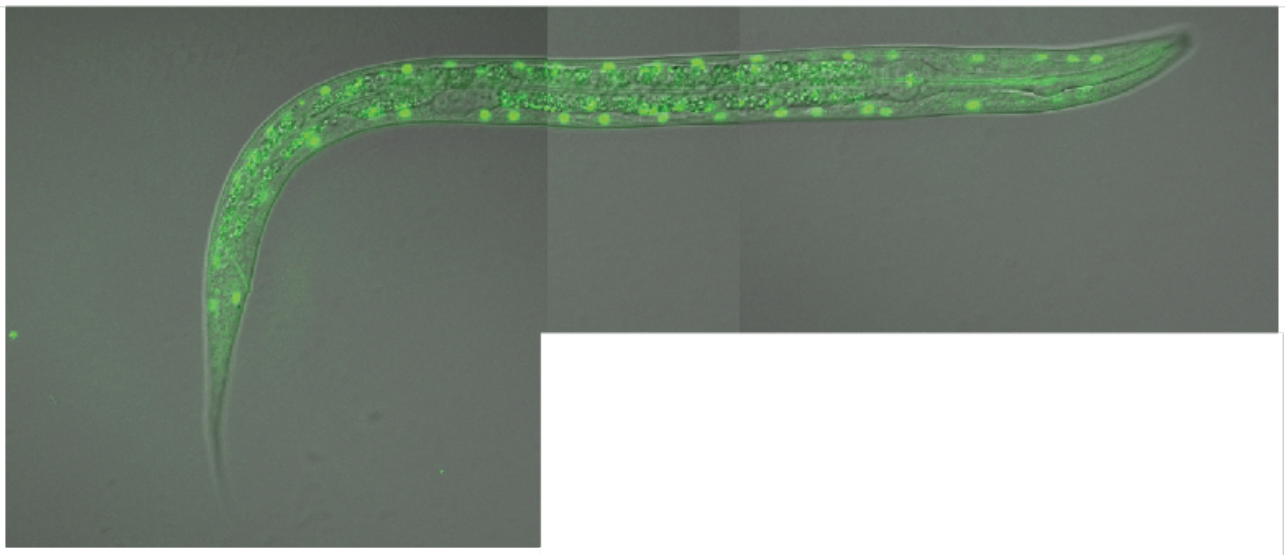
a

P*myo-2*:GFP:H2B:*tbb-2* 3'UTR



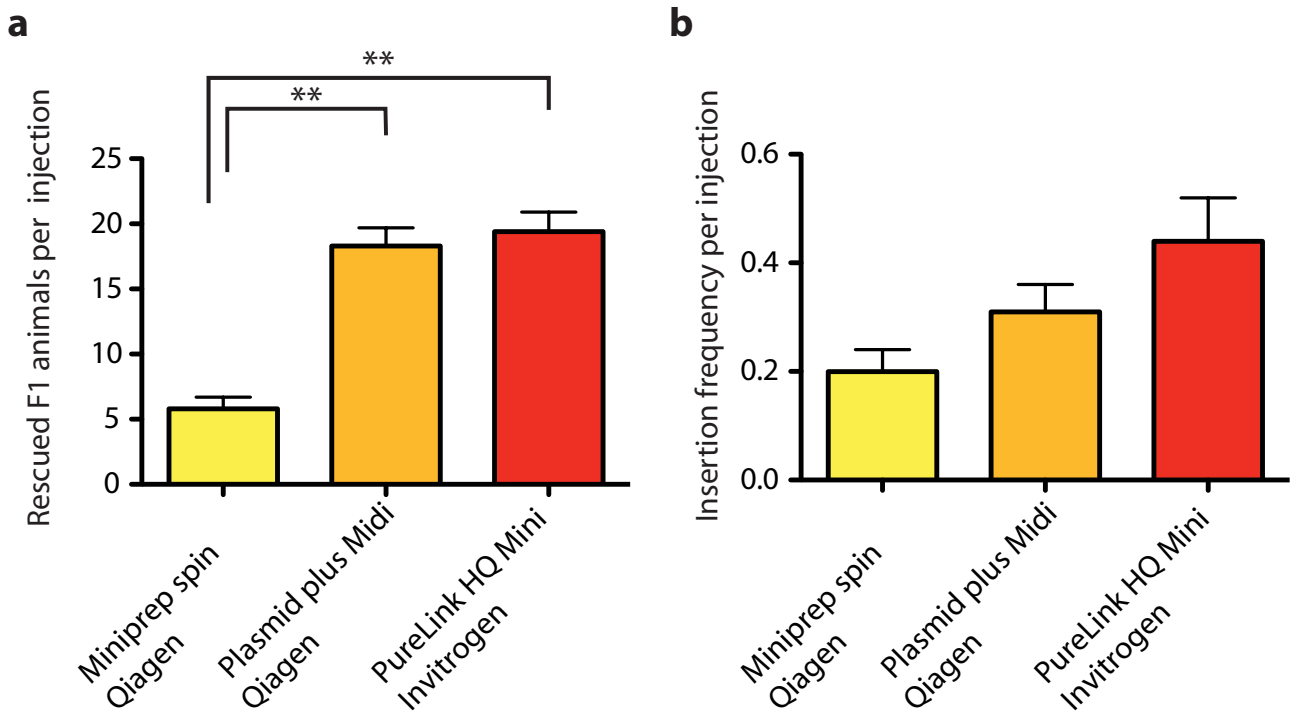
b

P*unc-54*:GFP:H2B:*tbb-2* 3'UTR



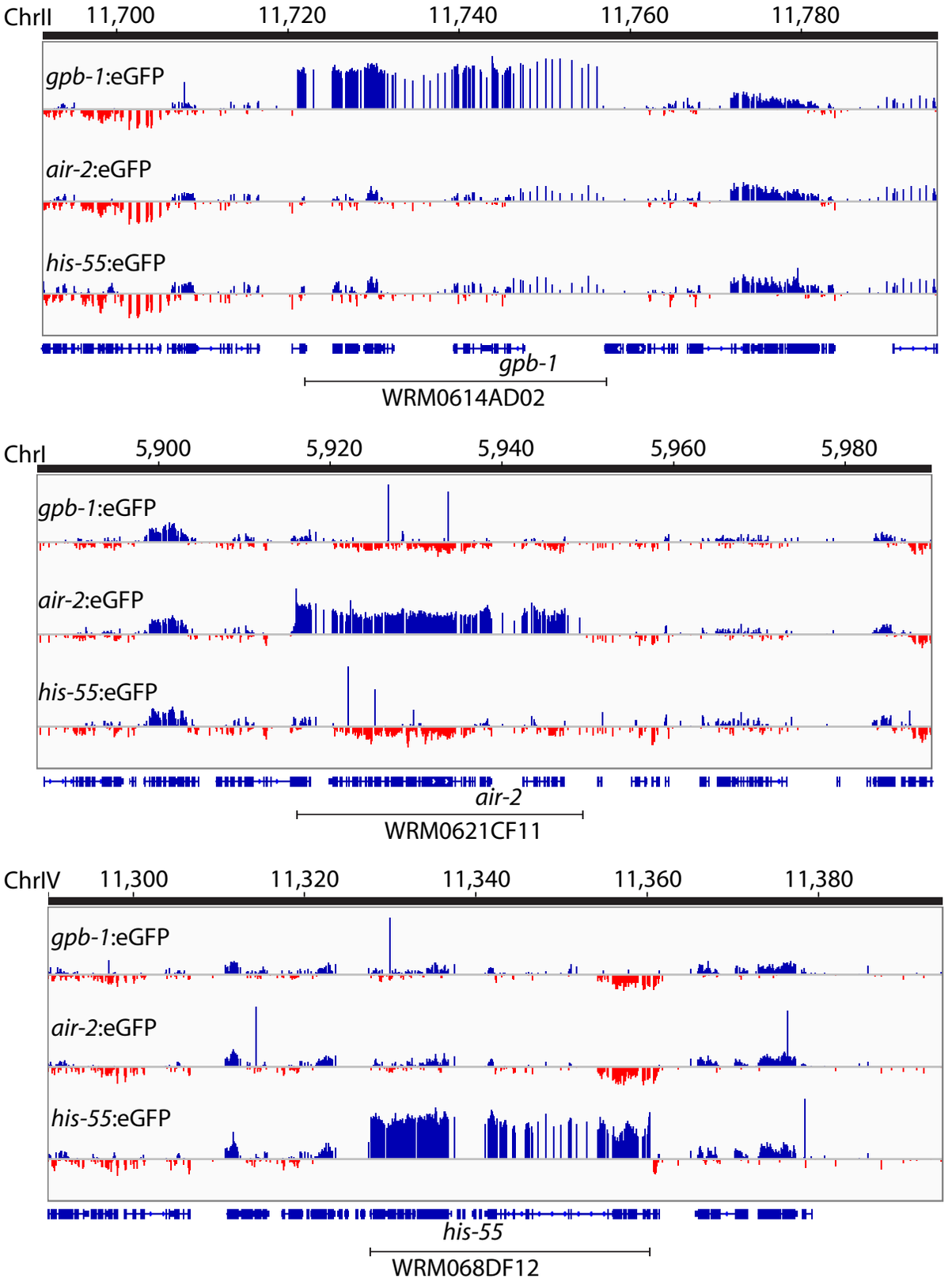
Supplementary Figure 5. GFP expression from miniMos insertions

MiniMos constructs exhibit specific expression in somatic tissues. Combined differential interference contrast (DIC) and GFP fluorescence images do not exhibit broadened or narrowed expression for tissue specific promoters. **(a)** A miniMos insertion carrying a P*myo-2*:GFP:H2B:tbb-2 UTR construct. Three planes are shown with specific expression in pharyngeal muscles. We could not detect any expression outside of the pharyngeal muscles. **(b)** A miniMos insertion carrying a P*unc-54*:GFP:H2B:*tbb-2* UTR insertion. Expression is only detected in body wall muscle. All images: 42x magnification, oil immersion objective.

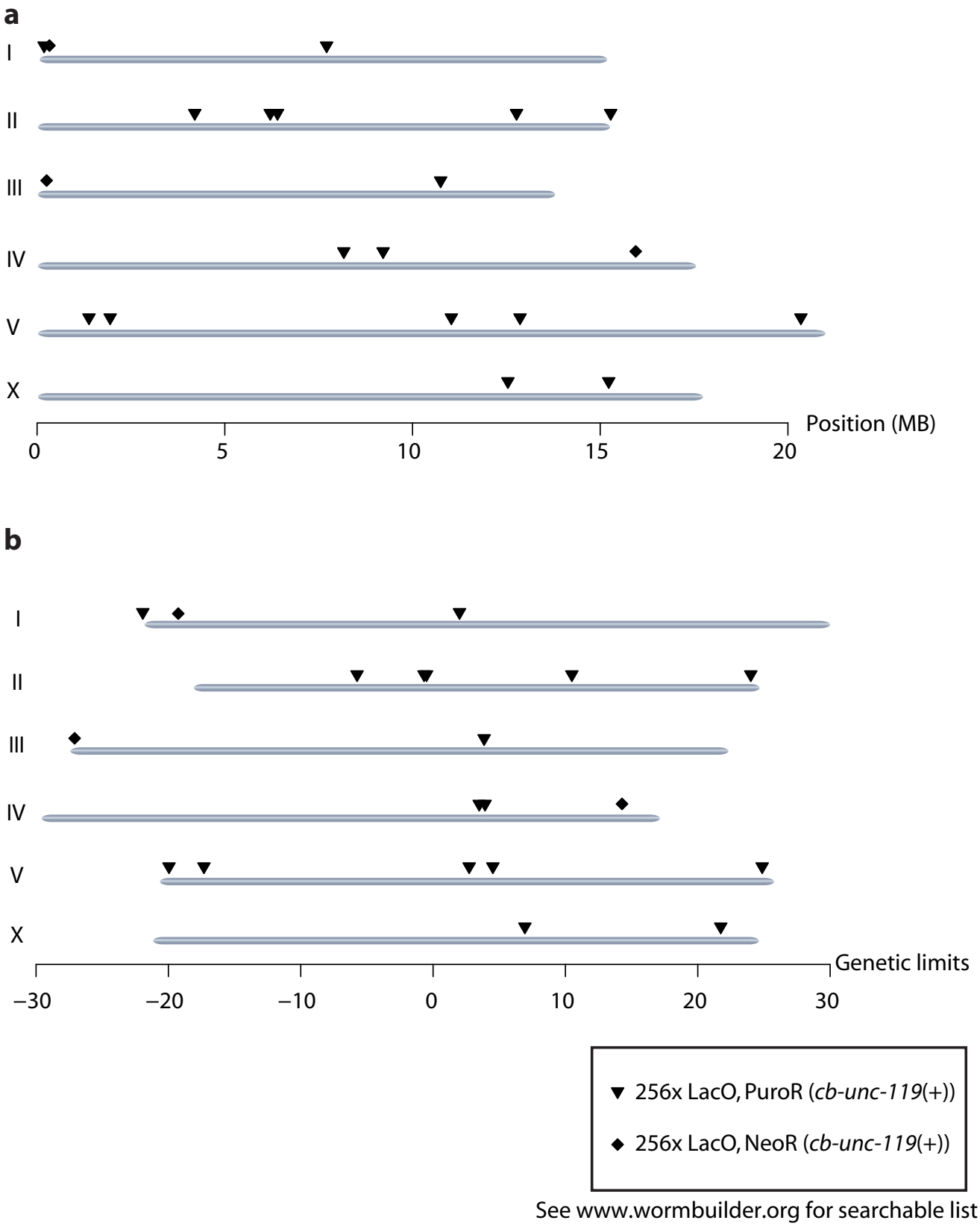


Supplementary Figure 6. MosSCI insertion frequency depends on DNA quality.

(a) Quantification of the number of F1 rescued animals per injected animal. The bar graph shows the insertion frequency at the ttTi5605 site of the same targeting plasmid with *unc-119* selection from DNA isolated with three different kits. Bar height corresponds to the average number of phenotypically rescued F1 animals and the error bar represents the SEM. Three replicates (injections) of each DNA mix were performed with 18 to 21 animals injected. Six plates were selected randomly from each replicate to quantify the number of rescued F1 animals on each plate. All the DNA in the injection mix (co-injection markers, Mos1 transposase and targeting vector) were isolated with each kit in parallel from the same bacterial culture. Statistics: Repeated measures ANOVA. Post-hoc test: Tukey's multiple comparison. **(b)** Quantification of the number of insertions per injected animal. Three replicates (injections) of each DNA mix were performed for a total number of injections: Miniprep (Qiagen): 54 animals injected, 11 insertions, Midiprep (Qiagen): 59 animals injected, 18 insertions and Miniprep (Invitrogen): 55 animals injected, 24 insertions. The overall difference was not statistically significant based on three replicates; however we find it likely that the higher number of rescued animals is biologically significant and will result in increased insertion frequency. Statistics: Repeated measures ANOVA.



Supplementary Figure 7. Mosmid insertions are fully intact as analyzed by Comparative Genome Hybridization (CGH). Comparative Genome Hybridization (CGH) analysis of three independent mosmid insertions containing the genes *gpb-1* (WRM0114AD02), *air-2* (WRM0621CF11) and *his-55* (WRM068DF12) tagged with GFP within fosmids (listed in parenthesis). The signal from all three CGH experiments are shown at all three genomic loci for comparison. The genomic limits of the insertions identified based on the CGH traces closely follow the predicted ends of the fosmids (shown below traces). All CGH data are consistent with insertion of a full-length fosmid. All CGH traces are scaled from [-1 to +2.5].



Supplementary Figure 8. lacO insertion strains

lacO insertions can be used to localize chromosome positions in nuclei because they will bind LacI:GFP fusions. **(a)** Physical map of *lacO* (256x) insertion strains. **(b)** Genetic map of *lacO* (256x) insertion strains.

a

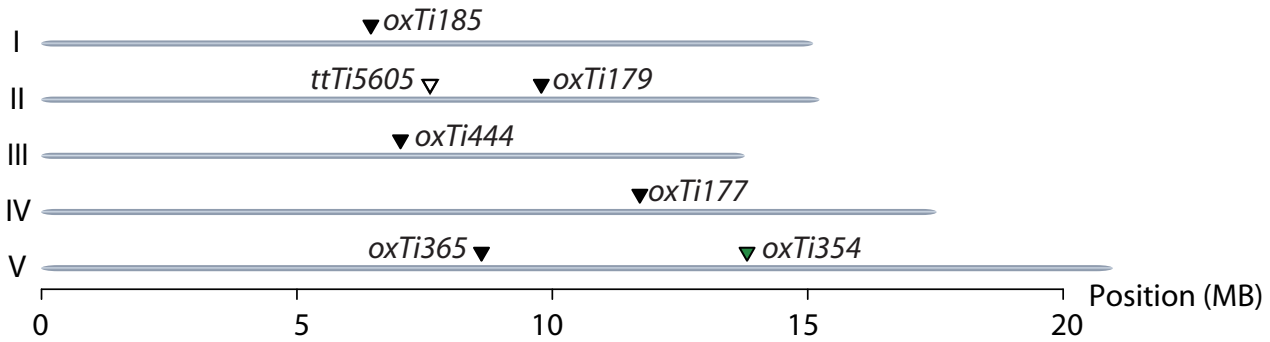
MiniMos cloning vectors

w peel-1 selection

Selection	3-fragment Gateway	MCS vector	3-fragment Gateway	MCS vector
unc-119	pCFJ906	pCFJ909	pCFJ1001	pCFJ1201
NeoR	pCFJ907	pCFJ910	pCFJ1002	pCFJ1202
PuroR	pCFJ908	pCFJ1666	pCFJ1000	pCFJ1200
HygroR	pCFJ1655	pCFJ1662	pCFJ1656	pCFJ1663

b

Standard Universal MosSCI insertion sites



Insertion site	Coinsertion markers	Genetic position	Genomic position (WS190)	Genomic environment	Strain	Germline expression
oxTi185	NeoR *unc-18(+)*	I: 1.17	I: 6,503,678	Intergenic	EG8078	yes
ttTi5605	None	II: 0.77	II: 8,420,108	Intergenic	EG6699	yes
oxTi179	NeoR *unc-18(+)*	II:1.73	II: 9,833,502	In ZK938.3	EG8079	yes
oxTi444	NeoR *unc-18(+)*	III:-0.85	III: 7,014,336	In *lgc-38*	EG8080	yes
oxTi177	NeoR *unc-18(+)*	IV:7.43	IV: 13,048,924	In *scl-10*	EG8081	yes
oxTi365	NeoR *unc-18(+)*	V:1.52	V: 8,643,273	In *asp-13*	EG8082	yes
oxTi354	P*myo-2*:GFP:H2B *unc-18(+)*	V:5.59	V: 13,783,531	In F53C11.3	EG8083	yes

See www.wormbuilder.org for strains and plasmids

Supplementary Figure 9. miniMos cloning vectors and Universal MosSCI insertion sites

(a) The table shows cloning vectors for generating miniMos vectors. All vectors are available from Addgene, either as single vectors or as part of a collection of miniMos vectors. MCS, multiple cloning site. **(b)** Universal MosSCI insertion sites. Top, All universal insertion sites are compatible with targeting vectors for the *ttTi5605* insertion site. Most insertion sites contain a NeoR element adjacent to the insertion site; *oxTi354* on Chr. V contains a P*myo-2*:GFP:H2B insertion instead. Bottom, Table of universal mosSCI insertion sites with their characteristics listed for comparison. All sites are permissive for germline expression as tested by a P*dpy-30*:GFP:H2B transgene insertion at each site.

Supplementary Note

We determined when insertions are generated by examining the progeny from four P0 animals injected with a miniMos transposon (**Supplementary Fig. 2**). We cloned 100 F1 progeny rescued for *unc-119*; all rescued F1 carried an extra-chromosomal array as determined by the presence of a co-injection marker (*mCherry*(+)). Most rescued F1s (84/100) lost the array and did not segregate any rescued F2; only two F1s generated stable arrays. Five F1s generated miniMos insertion lines in the F2, but only a small fraction of the F2 progeny from these five animals contained an insertion (2-15%), and usually represented two independent insertions per F1 animal. These data indicate that miniMos hops from extra-chromosomal DNA into chromosomes in the germline of F1 animals, probably in the last mitotic divisions before meiosis. By contrast, Mos excision from chromosomal DNA occurs in the germline of the injected P0 using the nearly identical MosSCI protocol(Frøkjaer-Jensen et al., 2008).

To improve the inverse PCR protocol for the identification of transposon insertion sites, we incorporated identical restriction sites into both ends of the miniMos transposon and designed a new set of inverse PCR oligos (**Supplementary Fig. 3**). We tested the protocol on a collection of bright fluorescent P*eft-3:tdTomato:H2B* inserts, which are useful as dominant chromosome balancers for *C. elegans* crosses. The method is efficient on moderately pure genomic DNA both in individual reactions (16/20 insertions (80%) identified, first sequencing attempt) and in a 96-well format (63/79 insertions (80%) identified, first sequencing attempt) (**Supplementary Figs. 3, 4** and protocols in **Supplementary Information**).

In some cases, inverse PCR reactions contained sequences from the injected plasmid backbone, indicating that some insertions were generated by transposition of two adjacent miniMos elements from the array into a chromosome ('composite transposition', **Supplementary Fig. 1a**). To determine how often this occurs, we designed oligos to amplify the two junctions between the Mos1 transposon and the plasmid vector, which should not be present in a "clean" single transposon insertion. We used the oligos in a PCR reaction on high quality genomic DNA and detected composite transpositions in 12% of strains (N=95). From five of these strains, we PCR amplified across the composite transposition and determined by sequencing that the full backbone had been co-inserted. Composite elements are therefore likely hopping from an extra-chromosomal array generated by homologous recombination between plasmids. To select against composite insertions, we inserted a negative selection marker into the plasmid backbone. The *peel-1* toxin efficiently kills animals when expressed from a heat-shock promoter(Seidel et al., 2011) and we have used *peel-1* to select against animals with extra-chromosomal arrays(Frøkjær-Jensen et al., 2012). Using a modified transposon carrying P*hsp:peel-*

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1 in the backbone, we were unable to detect the backbone in 82 independent inverse PCR reactions.

P element transgenesis has been used to generate loss of function mutants in *Drosophila*(Spradling et al., 1995). The use of Mos1 has not found widespread use for this purpose, possibly because Mos1 elements mostly insert into introns and is often spliced out of transcripts. Furthermore, the lack of positive selection makes it difficult to recover mutant animals. By contrast, insertion of a miniMos transposon with cargo and strong selection would be expected to disrupt genes by insertion into both introns and exons. We did not directly screen for mutant phenotypes but noted that several of the P*eft-3:tdTomato:H2B* insertions were inserted into introns and exons of genes with obvious phenotypes: *unc-13* I, *unc-22* IV and *him-4* X. All three insertions showed the phenotypes expected from loss of function alleles.

We noted above that some P*pie-1*:GFP:histone insertions were silenced, likely through a combination of small RNAs that detect foreign DNAs and protect endogenous genes in the germline(Seth et al., 2013; Shirayama et al., 2012; Wedeles et al., 2013) and subsequent modifications to the chromatin environment. A related questions is whether neighboring chromatin is able to drive inappropriate somatic expression. To test this, we generated three lines each with promoters specific to pharyngeal muscles (P*myo-2*) and body wall muscle (P*unc-54*). We were unable to detect mis-expression in other tissues in these lines (**Supplementary Fig. 5**). Although the sample size is small, these results suggest that inserted transgenes are not generally mis-expressed by neighboring promoters or by the *cb-unc-119* promoter within the miniMos transposon.

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Supplementary Protocols

Generating miniMos insertions

This protocol describes how to generate miniMos inserts by direct injection. The protocol is very similar to the protocol used to generate MosSCI insertions and most of the necessary reagents are identical.
Please see the webpage **www.wormbuilder.org** for updates to the protocol and a FAQ about common problems.

Reagents

Co-injection plasmids

pGH8	P*rab-3:mCherry:unc-54*UTR
pCFJ90	P*myo-2:mCherry:unc-54*UTR
pCFJ104	P*myo-3:mCherry:unc-54*UTR
pCFJ601	P*eft-3:mos1 transposase:tbb-2*UTR
pMA122	P*hsp16.41:peel-1:tbb-2*UTR

Cloning plasmids (miniMos vectors)
There are different vectors based on *unc-119*, neoR and puroR selection. All vectors are available as three-fragment [4-3] Gateway vectors or as multiple cloning site vectors. We recommend using the vectors with *peel-1* in the backbone for direct insertions and vectors without *peel-1* for heat-shock based insertion from extrachromosomal arrays.

Plasmids can be requested from Addgene.

Strains

EG6207	*unc-119(ed3)*. 11x outcross. Outcrossed by Amir Sapir in Sternberg lab.
Wild type	For NeoR and PuroR selection

Antibiotics
G418 for NeoR selection. We purchase powder from Gold Biotechnology and make up our own solution. Make 25 mg/ml solution in water.
Important: Filter sterilize to avoid contamination. Store working stock in refrigerator, keep stocks in -20C freezer.

Puromycin for PuroR selection. We purchase 10 mg/ml solution from Invivogen. Store working stock in refrigerator and stock in -20C freezer.

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Note: In our hands, G418 selection is more effective and considerably cheaper than puromycin.

Before injection

1. Insert transgene into miniMos vector.

Insert the transgene of interest into the appropriate miniMos vector (*unc-119*, NeoR, PuroR) by your preferred cloning method (for example, Gateway cloning, restriction enzyme cloning or multiple fragment assembly). Or generate a fosmid-based vector by inserting the miniMos-*unc-119* cassette into the backbone of the fosmid by recombineering.

2. Make injection mix.

MiniMos-based vector	10 ng/ul
pGH8	10 ng/ul
pCFJ90	2.5 ng/ul
pCFJ104	10 ng/ul
pCFJ601	50 ng/ul
pMA122	10 ng/ul

Making the injection mix is much easier if you make a 2x stock solution of all the co-injection plasmids. Lower the concentration of the miniMos vector if your transgene is toxic. Omit pMA122 if you are using a miniMos vector with peel-1 selection in backbone of vector. We think the purity of the DNA is important for good success so we suggest using a kit that gives better quality DNA than a miniprep kit or that you do an ethanol precipitation after isolating DNA with the miniprep kit (see Morris Maduro's description in Worm Breeders Gazette).

3. Grow injection strain at 15°C to 20°C on HB101 bacteria.

unc-119 animals are much healthier (and easier to inject) if they are grown at lower temperatures on HB101 bacteria. We generally grow N2 on OP50 at room temperature.

Injection

4. Inject worms.

Inject into the appropriate injection strain. Put 1-3 animals on each NGM plate seeded with HB101 or OP50.

It is difficult to give guidelines for how many injections to perform to generate an insertion. In our hands, the technique is as efficient as generating extra-chromosomal arrays for plasmids and less efficient for fosmids.

After Injection

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4. Place injected worms at 25°C. (Day 1)

Place the plates with injected worms at 25°C.

The insertion frequency is strongly temperature dependent, with more insertions happening at higher temperatures. Although the insertion appears to happen in the F1 generation, we place the injected animals at 25°C within a few hours of injection.

4b. Add antibiotic to the injection plates. (Day 2)

If you are injecting into *unc-119* animals then skip this step. For NeoR selection, add 500 ul of the stock solution (25 mg/ml) directly to the plate the day after injection. For PuroR selection, add 500 ul of the stock solution (10 mg/ml) directly to the plate the day after injection. Let plates dry with the lid off. Keep plates at 25°C.

This is a modified protocol from the protocols described in Giordano-Santini et al. (2010) and Semple et al. (2010). We prefer to add the antibiotic directly to the seeded plates because it requires less planning ahead. In our hands the protocol is efficient but it is quite possible that making NGM plates with antibiotic already added is more efficient. Please see the two references for the standard protocol for antibiotic selection.

The amount of antibiotic added is based on our NGM plates weighing approx. 8 g each. Adjust the volume added based on the weight of plates in your lab.

5. Let worms starve out at 25°C. (Days 2-7)

This takes approximately 1 week. The protocol works best if the worms are fully starved before you proceed to the next step.

We do not pick off individual F1 progeny from each plate but let them starve out as a population. As we show, you can generate several independent insertions if you pick off individual F1 progeny. However, we find that picking F1 progeny takes a lot of time and uses a fair amount of resources so generally we prefer to inject more animals instead.

Can you find insertions before the plate starves out? Yes. But again, it's much harder and usually more work to find these rare early inserts relative to waiting a few days and letting the plate starve fully.

6. Heat-shock animals for two hours at 34°C in air incubator. (Day 7)

This step kills animals that are carrying the extra-chromosomal array by activating the *peel-1* toxin. Wait until the plates are fully starved. Insertions happen relatively long after injection and if you heat-shock too early you will kill the animals with insertions before they can get rid of the extra-chromosomal arrays.

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This works very efficiently if the plates actually heat up relatively fast to 34°C for the duration of the heat-shock. For example, it works well in our incubator that has a fan but is much less effective in a similar incubator without a fan, probably because it takes longer to heat the plates up. Don't heat-shock a full box of plates in a closed box in an air incubator. Separate out plates so they are only stacked one or two high. Can you use a water incubator? Yes. In fact, it is more efficient that way but it is also a lot of work to wrap and un-wrap a lot of plates. So, depending on how many plates you have you should choose the most convenient method.

7. Screen plates for insertions. (Day 8)

Screen at least four hours after heat-shock and preferably the next day. Look for animals that are alive and move well but lack the fluorescent co-injection markers.

We screen the plates on a normal dissection microscope and then secondarily verify on a fluorescence dissection microscope. We typically do not see any false positives. Adjust the heat-shock if you are not killing all the extra-chromosomal array animals.

8. Chunk or pick rescued animals. (Day 8 - 10)

Chunk plates with insertion animals to a seeded NGM plate. Pick off a single, healthy adult animal two days later.

We prefer to chunk animals and then pick a healthy adult animal two days later instead of picking off individual starved animals. The starved L1 animals have a relatively high incidence of sterility so you often have to go back and re-pick. Chunking also often lets you screen visually for the transgene (germline expression, for example) before picking a clonal worm. Since multiple independent insertions are often generated, this can save some work in finding the animal that will work for your experiment.

Can you pick several independent insertions from a single plate? Yes. But you have to be careful to verify that the insertions are independent - most insertions on a plate will not be independent.

8. Determine insertion site. (~ 2 days of molecular biology)

If necessary, use the inverse PCR protocol to determine the insertion site (see Supplementary Protocols 2 and 3). For some experiments this may not be necessary; for other experiments this may be crucial.

Treat the insertions as you would treat different alleles of a gene. It's always nice to have more than one allele. Some insertions will be affected by genomic environment (for example, X chromosome inactivation in the germline). Other insertions will disrupt a genomic locus that is important.

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Inverse PCR protocol on individual inserts

There is a very nice and comprehensive protocol that covers how to map Mos1 insertions by (Boulin and Bessereau, 2007). This protocol is meant as a complement to their protocol because we changed and optimized several parameters which in our hands improve the reliability of inverse PCR reactions. This is the protocol that we currently (December 2013) use in the lab.

Use aerosol resistant tips for all steps!! Contamination is a real problem when doing two sequential PCR reactions on small amounts of template. And it only gets worse with every reaction you do.

Reagents

Molecular Biology Reagents

Genomic DNA isolation kit from Zymo Research. Catalog # D6016
Ligase from Enzymatics: Catalog # L6030-LC-L
DpnII from NEB: Catalog # R0543L
Phusion DNA Polymerase: Catalog #M0530S

Oligos sequences (5' → 3')

5' end

oCF1587	ATAGTTTGGCGCGAATTGAG
oCF1588	GGTGGTTCGACAGTCAAGGT
oCF1589	AGAGCAAACGCGGACAGTAT
oCF1590	CGATAAATATTTACGTTTGCGAGAC

3' end

oCF1591	AAAAATGGCTCGATGAATGG
oCF1592	TAAGAATCGAAGCGCTGCTC
oCF1593	AGCTAGCGACGGCAAATACT
oCF1594	CATCGAAGCGAATAGGTGGT

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1. Isolate genomic DNA

We use the kit from Zymo Research but any method that generates genomic DNA should give similar results. Follow manufacturer's protocol.

The protocol can work, but not as efficiently, on crude genomic DNA lysates generated with freezing and proteinase K digest. It's much easier to get a good inverse PCR product with decent quality DNA.

2. Digest 150 ng of genomic DNA in 25 ul volume for 3 hours.

Digest genomic DNA with the DpnII enzyme.

DpnII cuts the same sequence as MboI but is slightly cheaper and works better over extended digests. It's important to use the DpnII buffer because there is a lot of star activity in the regular NEB buffers. In our hands, DpnII and MboI work well possibly because the enzymes leave a 4 bp overhang after cutting compared to the often 1 bp or blunt ends that most four-cutter enzymes leave. The protocol also works with HpaII - adjust digest conditions.

Component	1x
DNA sample (150 ng - add water to 10ul)	10 ul
Restriction buffer DpnII (10x)	2.5 ul
Restriction enzyme (DpnII 10U/ul)	1.0 ul
H_20	11.5 ul

Reaction conditions: Digest at 37ºC for three hours to overnight.

Heat inactivate the enzyme after restriction digest at 80ºC for 20 min.

3. Ligate the digested DNA for 2 hours at room temperature

Set up ligation in large volume to favor intra-molecular reactions. Use the 10x ligation buffer from Enzymatics.

Set up 25 ul reactions with:

Component	1x
Digested DNA from step 2	2.5 ul
10x ligation buffer	2.5 ul (Enzymatics ligase buffer)
T4 ligase	1.0 ul (Enzymatics ligase)
H_20	19.0 ul

The ligation reactions can be frozen indefinitely before proceeding to the next step.

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4. Do first round of inverse PCR

Set up a **10** ul PCR reaction with the following components:

Component	1x
Ligation mix from step 3	2.0 ul
Primer oCF1587 (10 uM)	1.0 ul
Primer oCF1588 (10 uM)	1.0 ul
dNTPs (10 mM)	0.2 ul
Phusion 5x GC buffer	2.0 ul
NEB Phusion Polymerase	0.1 ul
H_20	3.7 ul

Make master mix of PCR ingredients and add "ligation mix" individually to each tube. It is very difficult (read = impossible) to accurately pipette only 0.2 ul and 0.1 ul.

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **64ºC**
Elongation time: 1 min

If you use another polymerase than the Phusion polymerase, you will probably want use the appropriate PCR buffer and decrease the annealing temperature to 60C. The higher temperature works well for getting specific bands.

5. Second round of inverse PCR.

Dilute the first round of PCR product 100 fold. Transfer 1 ul of PCR product to new PCR tube, add 99 ul of distilled water. Mix with vortexer. Spin down to avoid contamination.

Set up a 25 ul PCR reaction with the following components:

Component	1x (20ul)
PCR from step 4	1.0 ul
Primer oCF1589 (10uM)	2.5 ul
Primer oCF1590 (10uM)	2.5 ul
dNTPs (10 mM)	0.5 ul
Phusion 5x GC buffer	5.0 ul
NEB phusion polymerase	0.2 ul
H_20	13.0 ul

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **64ºC**
Elongation time: 1 min

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If you use another polymerase than the Phusion polymerase, you will probably want use the appropriate PCR buffer and decrease the annealing temperature to 60C.

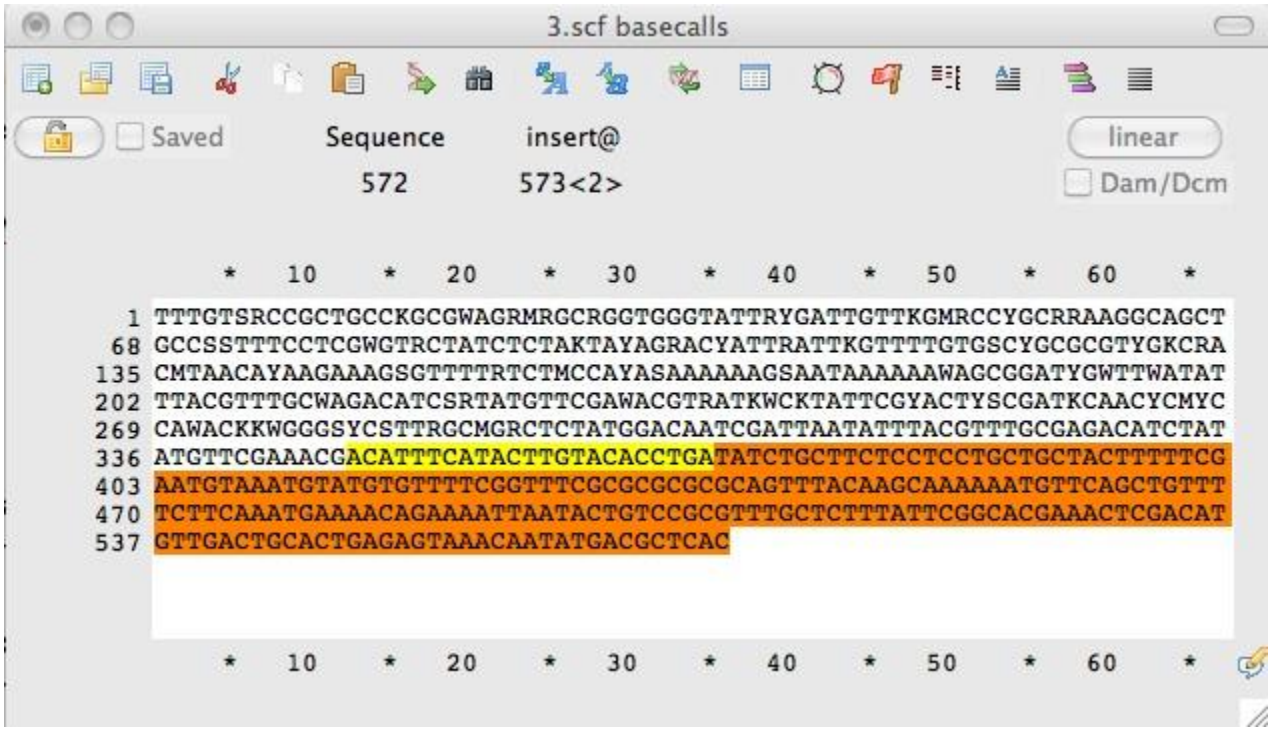
6. Run the PCR products on a 1% agarose gel, excise clear bands from gel and gel purify.

Only excise one band from each reaction. Do not excise bands that are not clearly distinct or when there is a smear. The sequence read will come back garbled. Only excise bands that are larger than 100bp. Send the gel purified product for sequencing with oCF1590.

Alternatively, you can run only 10 ul of the PCR reaction to determine if the band is specific. If there is only a single band, we use the ExoSAP protocol (ExonucleaseI digest to remove oligos and Shrimp Alkaline Phosphatase removal of dNTPs) to purify the PCR reaction and submit for sequencing.

7. Determine insertion site

Once you get the sequence read back, you can determine the insertion site. Search the sequence read for the following sequence: ACATTTCATACTTGTACACCTGA. Allow for two mismatches to accommodate poor sequence calls. This is the end of the Mos1 transposon (in yellow below). The next two nucleotides should be a “TA”, where the Mos1 transposon inserted. The rest of the read is the genomic DNA insertion site (in orange below).



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A) Go to wormbase and blast search.
Change “Query Type” to Nucleotide.
Change “E-value Threshold” to 1E-4
Unclick “Filter”

B) Identify the correct match to your insertion site. Typically it will be the best match but make sure the query match starts at position “1”. Otherwise the read is probably finding part of the *unc-119* rescue gene or the transgene you put in. Some insertions cannot be mapped to unique locations because of repetitive regions in the genome or too short reads.

8. No bands?

Redo the PCR reactions with oligos that anneal at the other end of the transposon. Start with the ligated DNA from step3.

Do first round of inverse PCR

Set up a **10** ul PCR reaction with the following components:

Component	1x
Ligation mix from step 3	2.0 ul
Primer oCF1591 (10 uM)	1.0 ul
Primer oCF1592 (10 uM)	1.0 ul
dNTPs (10 mM)	0.2 ul
Phusion 5x GC buffer	2.0 ul
NEB phusion polymerase	0.1 ul
H_20	3.7 ul

Make master mix of PCR ingredients and add “ligation mix” individually to each tube. It is very difficult (read = impossible) to accurately pipette only 0.2 ul and 0.1 ul.

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **62ºC**
Elongation time: 1 min

Second round of inverse PCR.

Dilute the first round of PCR product 100 fold. Transfer 1 ul of PCR product to new PCR tube, add 99 ul of distilled water. Mix with vortexer. Spin down, so you don’t get contamination.

Set up a 25 ul PCR reaction with the following components:

Component	1x (20ul)
PCR from step 4	1.0 ul

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Primer oCF1593 (10uM)	2.5 ul
Primer oCF1594(10uM)	2.5 ul
dNTPs (10 mM)	0.5 ul
Phusion 5x GC buffer	5.0 ul
NEB phusion polymerase	0.2 ul
H_20	13.0 ul

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **62ºC**
Elongation time: 1 min

Sequence the PCR product with oCF1593.

9. Still no bands?

Repeat protocol with another restriction enzyme, for example HpaII.

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Inverse PCR protocol in 96-well format

There is a very nice and comprehensive protocol that covers how to map Mos1 insertions by Boulin & Bessereau (2007) in *Nature Protocols*. This protocol is meant as a complement to their protocol because we changed and optimized several parameters which in our hands improve the reliability of inverse PCR reactions. It is the protocol that we currently (December 2013) use in the lab.

Use aerosol resistant tips for all steps!! Contamination is a real problem when doing two sequential PCR reactions on small amounts of template. And it only gets worse with every reaction you do.

Reagents

Molecular Biology Reagents

ZR-96 quick gDNA kit from Zymo Research. Catalog # D3011
Ligase from Enzymatics: Catalog # L6030-LC-L
DpnII from NEB: Catalog # R0543L
Proteinase K from NEB (20 mg/ml): Catalogue #P8102S
Phusion DNA Polymerase: Catalog #M0530S

Oligos sequences (5' → 3')

5' end

oCF1587	ATAGTTTGGCGCGAATTGAG
oCF1588	GGTGGTTCGACAGTCAAGGT
oCF1589	AGAGCAAACGCGGACAGTAT
oCF1590	CGATAAATATTTACGTTTGCGAGAC

3' end

oCF1591	AAAAATGGCTCGATGAATGG
oCF1592	TAAGAATCGAAGCGCTGCTC
oCF1593	AGCTAGCGACGGCAAATACT
oCF1594	CATCGAAGCGAATAGGTGGT

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1. Generate insertions by injection or by heat-shock.

See Supplementary Protocol 1 for how to generate insertions. Isolate insertions and let plates with inserts starve out.

2. Chunk starved plates (clean) to seeded OP50 plates. (Day 1)

The downstream steps do not work nearly as well if the plates are contaminated.

3. Wash off worms from each plate. (Day 3-4)

a) Wash off worms from each plate into an Eppendorf tube with water containing 0.05% Tween20.
The detergent prevents worms from sticking to pipette tip and Eppendorf tubes.

b) Place Eppendorf tubes on ice for 10 minutes.
This paralyzes the worms so they sink to the bottom of the tube.

c) Pipette off the bottom 50 ul of water with worms into a new Eppendorf tube using a P200 pipette.
The worms are visible. Check that most of the worms were transferred into the new tube.

d) Freeze worms to crack cuticle.
We use a -80ºC for at least 15 minutes but a -20ºC freezer should also work with longer incubations.

4. Digest worms with Proteinase K in lysis buffer

a) Make lysis solution. We use the GC buffer supplied with the Phusion polymerase buffer but the standard lysis buffer should also work.
For one full 96 well plate mix the following:

5x GC buffer	1040 ul
Proteinase K (20 mg/ml)	520 ul

b) Add 15 ul of lysis solution to each Eppendorf tube with frozen worms.
Digest worms overnight at 50ºC (for example in hybridization oven). Make sure to close the Eppendorf tubes carefully, the heat will make some tubes pop open which can lead to contamination. We invert the Eppendorf tubes a couple of times during the incubation.

c) Inactivate Proteinase K
Inactivate the Proteinase K by 1 hour incubation at 95ºC.

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5. Isolate genomic DNA in 96 well format

We use the kit from Zymo Research but any method that generates genomic DNA in a 96 well format should give similar results. Follow manufacturer's protocol. Elute in 50 ul pre-warmed elution buffer into 96 well plate.

5b. PCR reaction to discard complex insertions

Do 20 ul PCR reaction with the oligos: M13F and oCF1593 on 1 ul of the template. Complex insertions will generate a 173 bp band.

In some cases, two miniMos elements are inserted into the same location. If you use the plasmids without peel-1 selection in the backbone of the miniMos vector this happens in approx. 10% of strains. If you used the peel-1 based miniMos plasmids then you should only very rarely get complex insertions. Although the complex insertions are functional they are difficult to map because the inverse PCR read is often from the backbone. We therefore generally discard complex inserts.

6. Digest 10 ul of genomic DNA in 25 ul volume overnight in 96 well plate.

Digest genomic DNA with the DpnII enzyme.

DpnII cuts the same sequence as MboI but is slightly cheaper and works better over extended digests. It's important to use the DpnII buffer because there is a lot of star activity in the regular NEB buffers. Be sure to close the wells very tight, otherwise most of the solution will evaporate.

Component	1x	100x
DNA sample	10 ul	---
Restriction buffer DpnII (10x)	2.5 ul	250 ul
Restriction enzyme (DpnII 10U/ul)	1.0 ul	100 ul
H_20	11.5 ul	1150 ul

Reaction conditions: Digest at 37ºC overnight.

Heat inactivate the enzyme after restriction digest at 80ºC for 20 min.

7. Ligate the digested DNA for 2 hours at room temperature

Set up ligation in large volume to favor intra-molecular reactions. Use the 10x ligation buffer from Enzymatics.

Set up 25 ul reactions with:

Component	1x	100x
Digested DNA from step 2	2.5 ul	---
10x ligation buffer	2.5 ul	250 ul
T4 ligase	1.0 ul	100 ul
H_20	19.0 ul	1900 ul

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The ligation reactions can be frozen indefinitely before proceeding to the next step.

8. Do first round of inverse PCR

Set up a **10** ul PCR reaction with the following components:

Component	1x	100x
Ligation mix from step 3	2.0 ul	---
Primer oCF1587 (10 uM)	1.0 ul	100 ul
Primer oCF1588 (10 uM)	1.0 ul	100 ul
dNTPs (10 mM)	0.2 ul	20 ul
Phusion 5x GC buffer	2.0 ul	200 ul
NEB Phusion Polymerase	0.1 ul	10 ul
H_20	3.7 ul	370 ul

Make master mix of PCR ingredients and add "ligation mix" individually to each well. It is very difficult (read = impossible) to accurately pipette only 0.2 ul and 0.1 ul.

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **64ºC**
Elongation time: 1 min

If you use another polymerase than the Phusion polymerase, you will probably want use the appropriate PCR buffer and decrease the annealing temperature to 60C. The higher temperature works well for getting specific bands.

9. Second round of inverse PCR.

Add 100 ul of water to each well (1:10 dilution). Use 96-well replicator to transfer 0.2 ul template to next 96 well PCR tray.

Set up a 25 ul PCR reaction with the following components:

Component	1x	100x
PCR from step 4	~0.2 ul	----
Primer oCF1589 (100uM)	0.25 ul	25 ul
Primer oCF1590 (100uM)	0.25 ul	25 ul
dNTPs (10 mM)	0.5 ul	50 ul
Phusion 5x GC buffer	5.0 ul	500 ul
NEB phusion polymerase	0.2 ul	20 ul
H_20	18.8 ul	1880 ul

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x

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Annealing temperature: **70ºC**
Elongation time: 1 min

If you use another polymerase than the Phusion polymerase, you will probably want use the appropriate PCR buffer and decrease the annealing temperature.

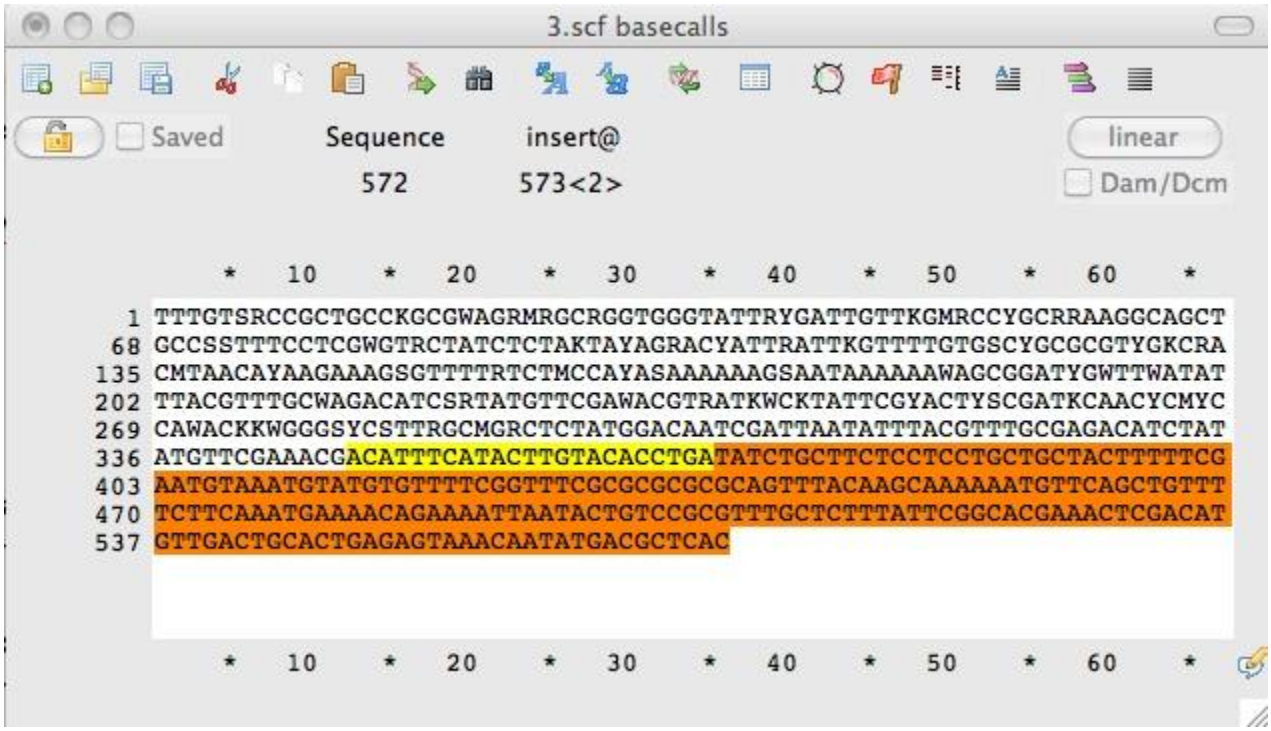
10. Run 10 ul of the PCR products on a 1% agarose gel.

Ideally, Only excise one band from each reaction. Do not excise bands that are not clearly distinct or when there is a smear. The sequence read will come back garbled. Only excise bands that are larger than 100bp. Send the gel purified product for sequencing with oCF1590.

Alternatively, you can run only 10 ul of the PCR reaction to determine if the band is specific. If there is only a single band, we use the ExoSAP protocol (Exonuclease I digest to remove oligos and Shrimp Alkaline Phosphatase removal of dNTPs) to purify the PCR reaction and submit for sequencing.

7. Determine insertion site

Once you get the sequence read back, you can determine the insertion site. Search the sequence read for the following sequence: ACATTTCATACTTGTACACCTGA. Allow for two mismatches to accommodate poor sequence calls. This is the end of the Mos1 transposon (in yellow below). The next two nucleotides should be a “TA”, where the Mos1 transposon inserted. The rest of the read is the genomic DNA insertion site (in orange below).



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A) Go to wormbase and blast search.
Change “Query Type” to Nucleotide.
Change “E-value Threshold” to 1E-4
Unclick “Filter”

B) Identify the correct match to your insertion site. Typically it will be the best match but make sure the query match starts at position “1”. Otherwise the read is probably finding part of the *unc-119* rescue gene or the transgene you put in. Some insertions cannot be mapped to unique locations because of repetitive regions in the genome or too short reads.

8. No bands?

Redo the PCR reactions with oligos that anneal at the other end of the transposon. Start with the ligated DNA from step3.

Do first round of inverse PCR

Set up a **10** ul PCR reaction with the following components:

Component	1x
Ligation mix from step 3	2.0 ul
Primer oCF1591 (10 uM)	1.0 ul
Primer oCF1592 (10 uM)	1.0 ul
dNTPs (10 mM)	0.2 ul
Phusion 5x GC buffer	2.0 ul
NEB phusion polymerase	0.1 ul
H_20	3.7 ul

Make master mix of PCR ingredients and add “ligation mix” individually to each tube. It is very difficult (read = impossible) to accurately pipette only 0.2 ul and 0.1 ul.

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **62ºC**
Elongation time: 1 min

Second round of inverse PCR.

Dilute the first round of PCR product 100 fold. Transfer 1 ul of PCR product to new PCR tube, add 99 ul of distilled water. Mix with vortexer. Spin down, so you don’t get contamination.

Set up a 25 ul PCR reaction with the following components:

Component	1x (20ul)

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PCR from step 4	1.0 ul
Primer oCF1593 (10uM)	2.5 ul
Primer oCF1594(10uM)	2.5 ul
dNTPs (10 mM)	0.5 ul
Phusion 5x GC buffer	5.0 ul
NEB phusion polymerase	0.2 ul
H_20	13.0 ul

PCR settings:
Initial denaturation: 2 minutes @ 98C
PCR cycles: 30x
Annealing temperature: **62ºC**
Elongation time: 1 min

Sequence the PCR product with oCF1593.

9. Still no bands?

Repeat protocol with another restriction enzyme, for example HpaII.

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