Supplementary Information

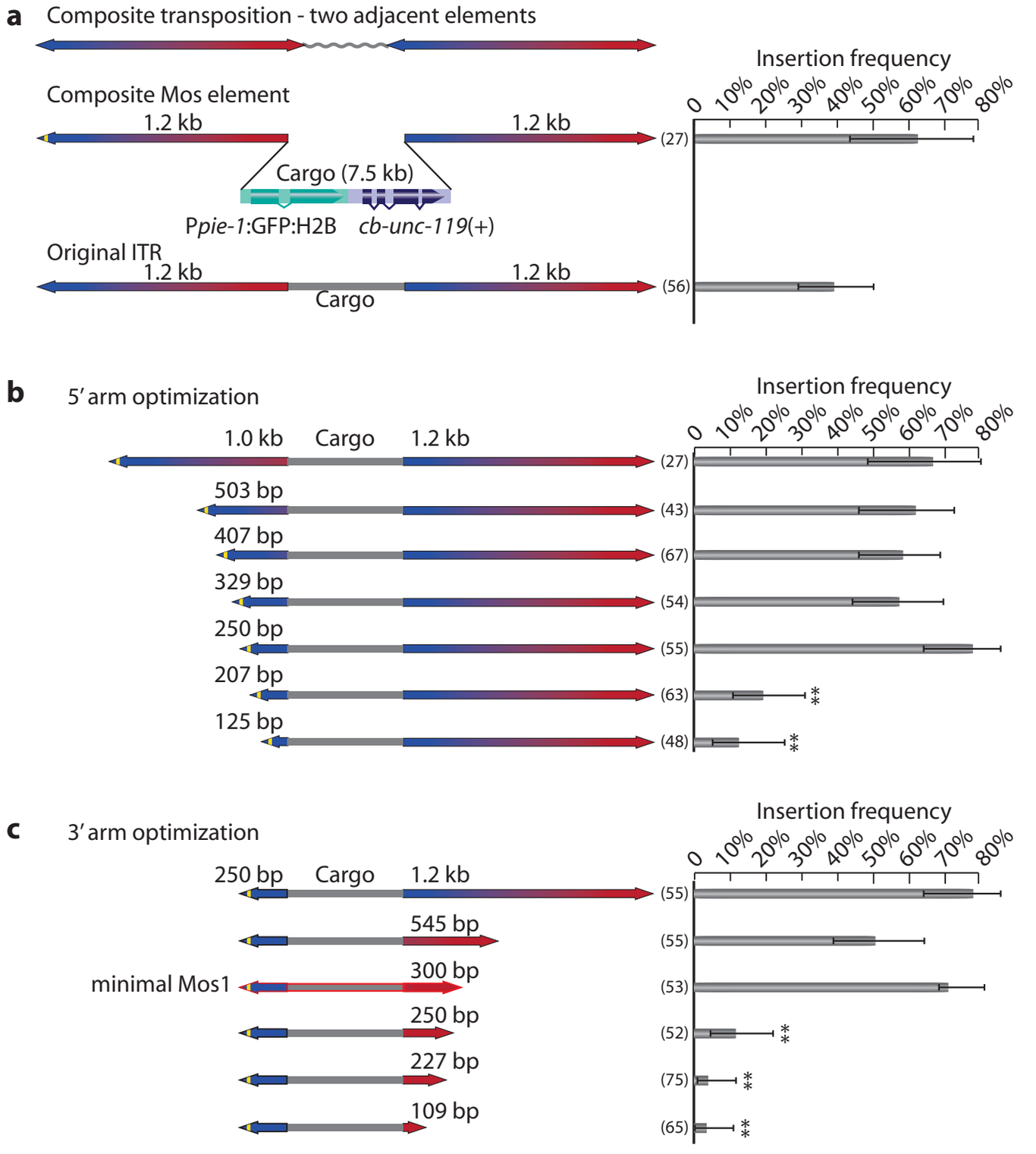
Random and targeted transgene insertion in *C. elegans* using a modified Mos1 transposon

Christian Frøkjær-Jensen, M Wayne Davis, Mihail Sarov, Jon Taylor, Stephane Flibotte, Matthew LaBella, Andrei Pozniakovski, Donald G Moerman, Erik M Jorgensen.
Nature Methods

Corresponding authors
Erik M Jorgensen, Email: jorgensen@biology.utah.edu
Christian Frøkjær-Jensen, Email: christianfj@gmail.com

Supplementary Figure 1 3
Supplementary Figure 2 4
Supplementary Figure 3 5
Supplementary Figure 4 6
Supplementary Figure 5 7
Supplementary Figure 6 8
Supplementary Figure 7 9
Supplementary Figure 8 10
Supplementary Figure 9 11
Supplementary Note 12
Supplementary Protocols 14
  Generating miniMos insertions 14
  Inverse PCR protocol on individual inserts 18
  Inverse PCR protocol in 96-well format 24
References 31

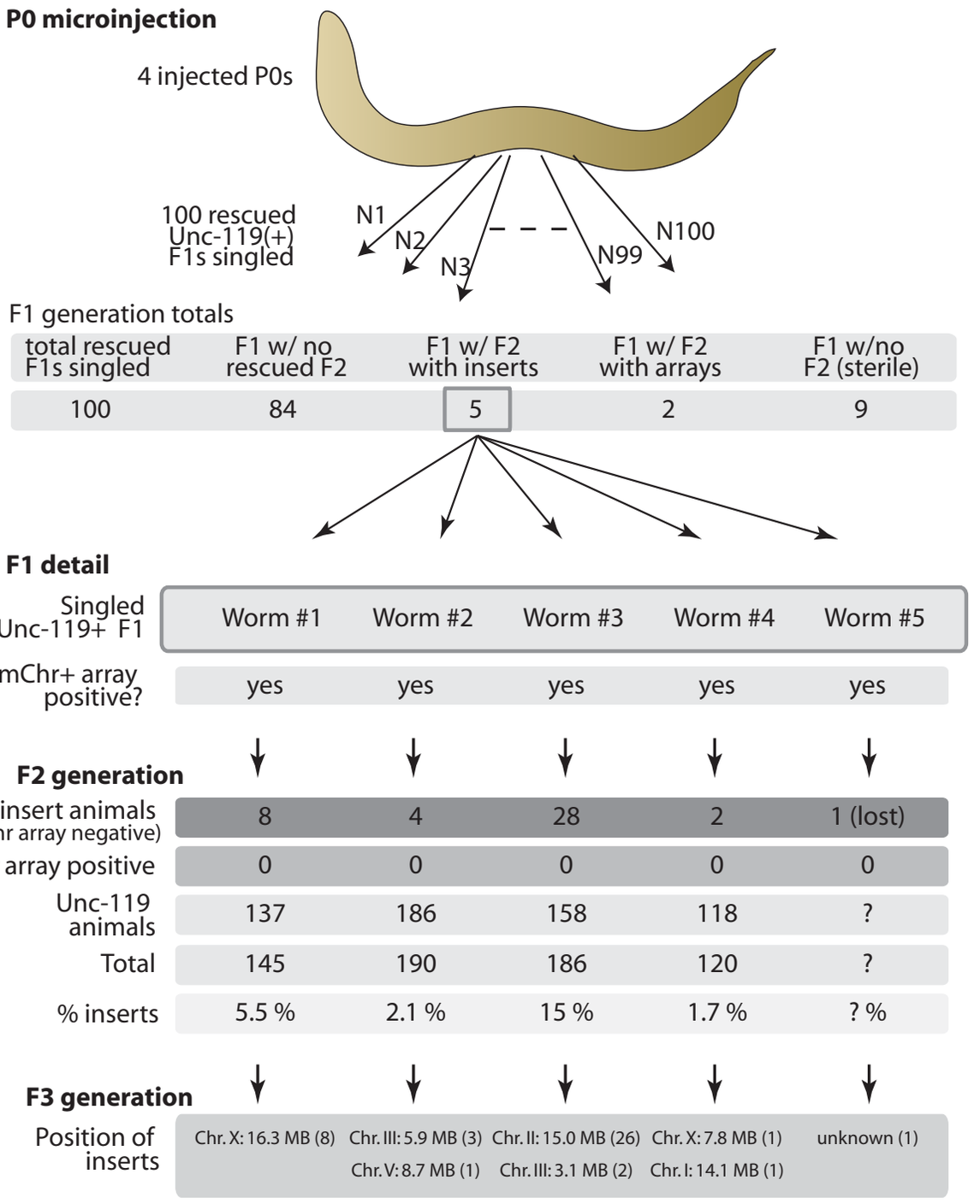
Please see www.wormbuilder.org for strains, protocols and reagents.



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

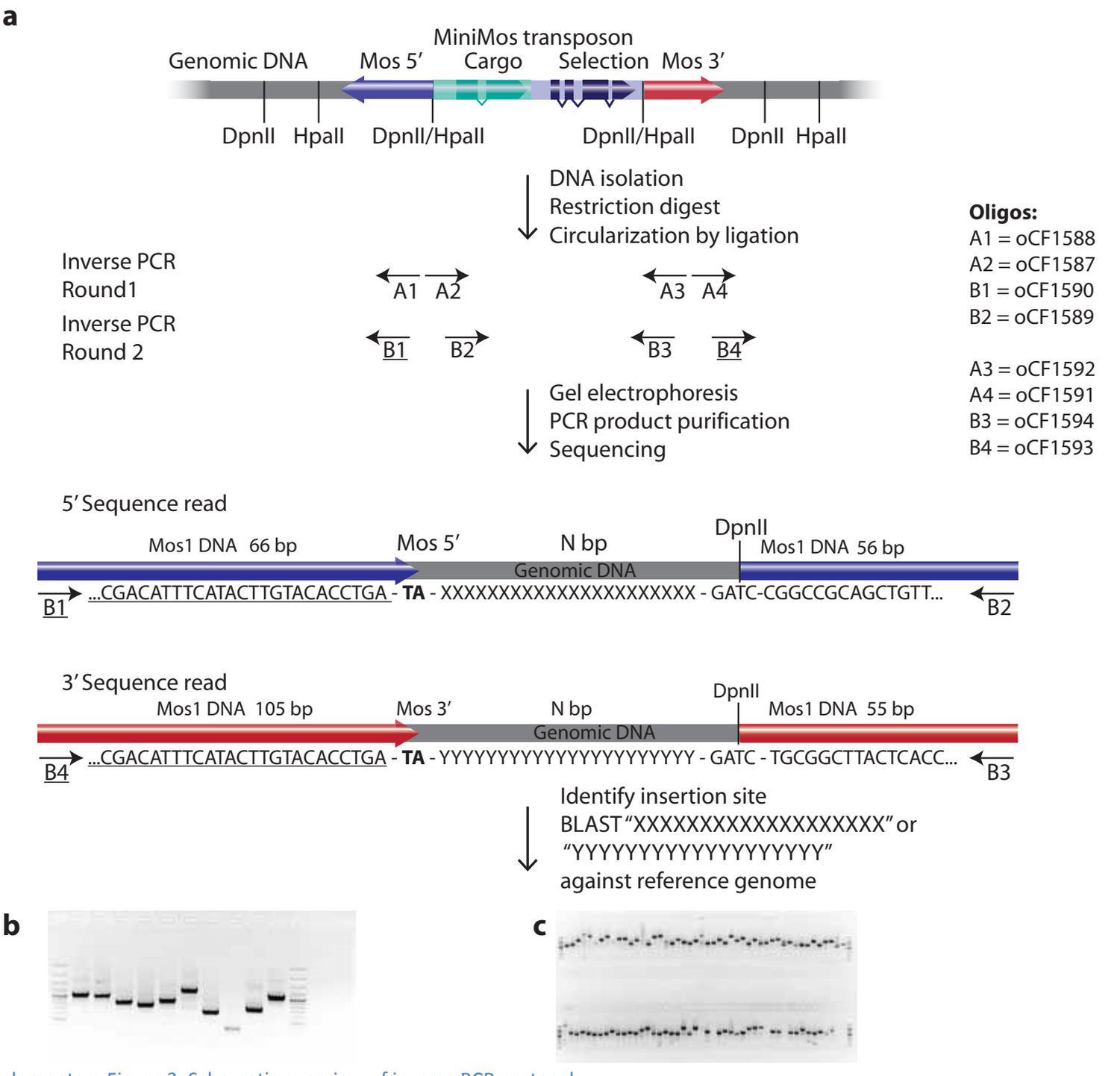
(a) Above, schematic of two full Mos1 transposons. Insertions caused by composite transposition carrying the intervening DNA were occasionally observed (MWD, unpublished), suggesting that composite Mos elements could be an effective method for introduction of exogenous DNA. Below, schematic of composite 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). **, $p < 0.01$.



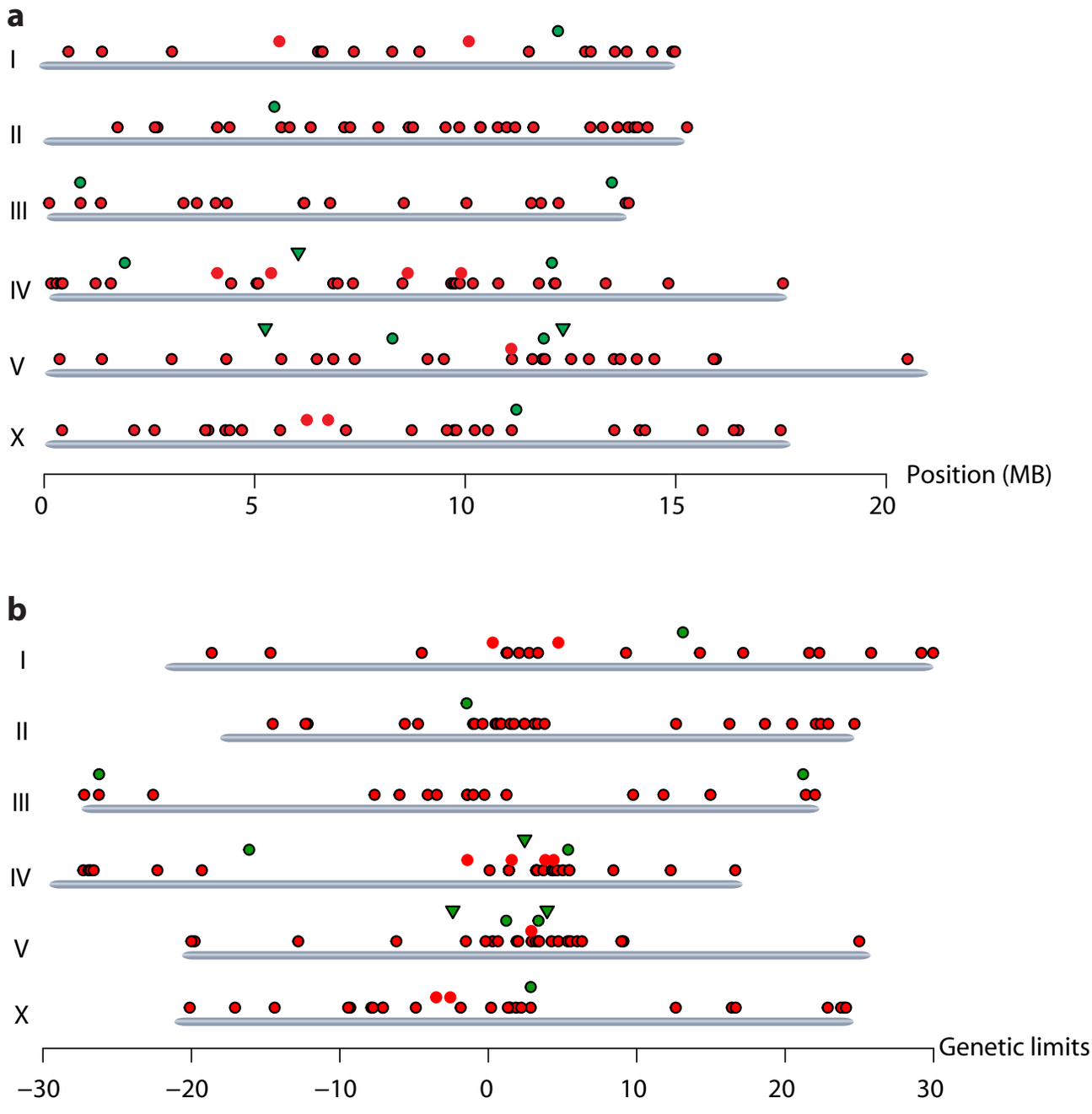
Supplementary Figure 2. miniMos insertions occur in the germline of F1 animals.

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).

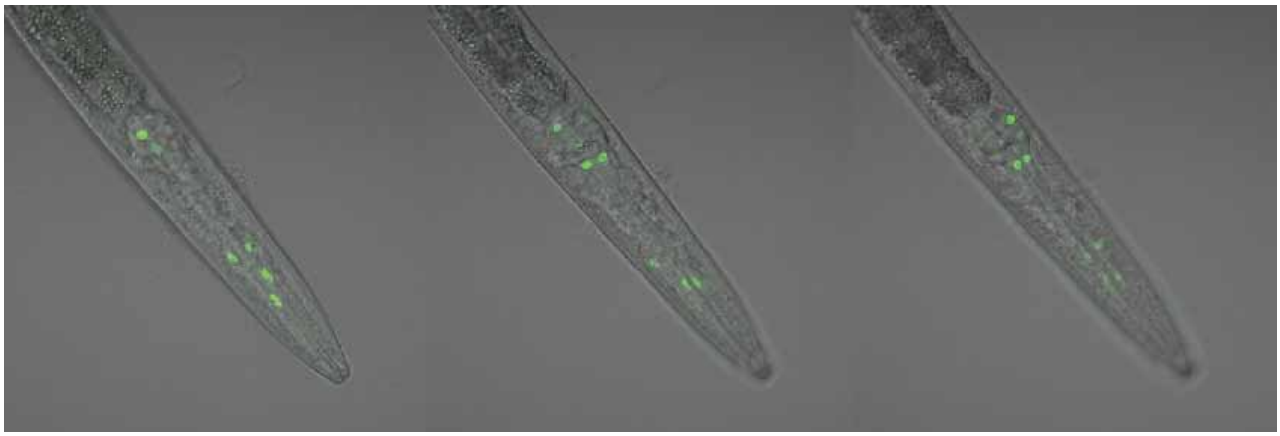


Supplementary Figure 4. Fluorescent marker strains

(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.

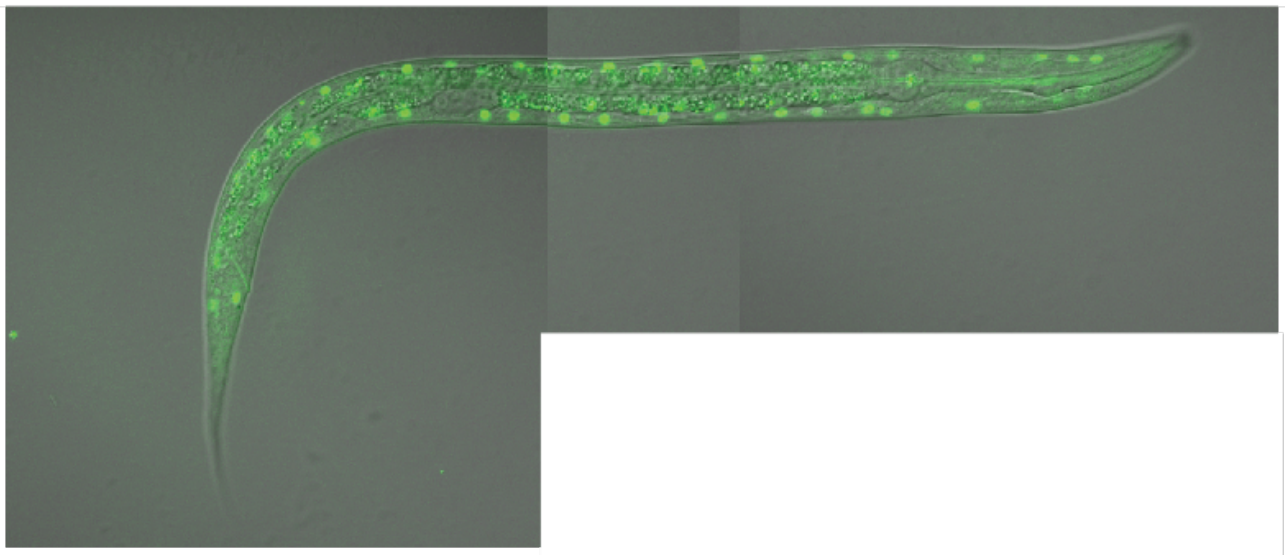
a

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



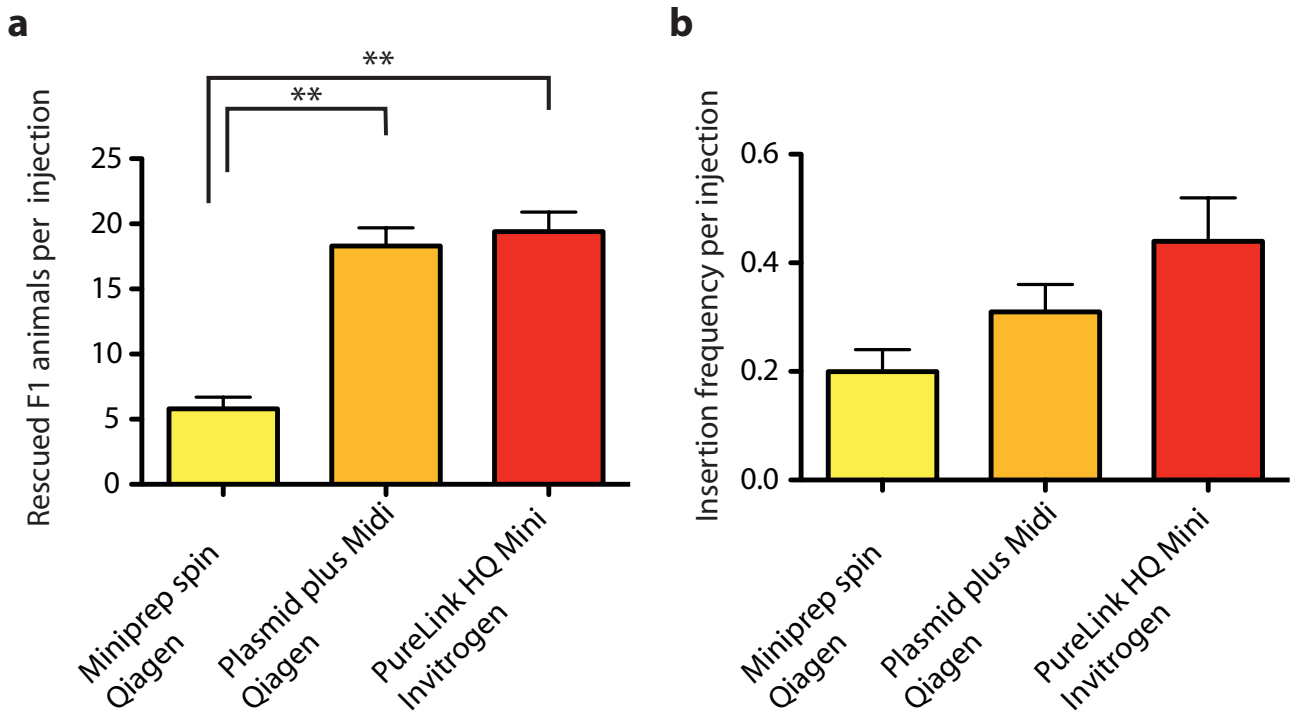
b

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



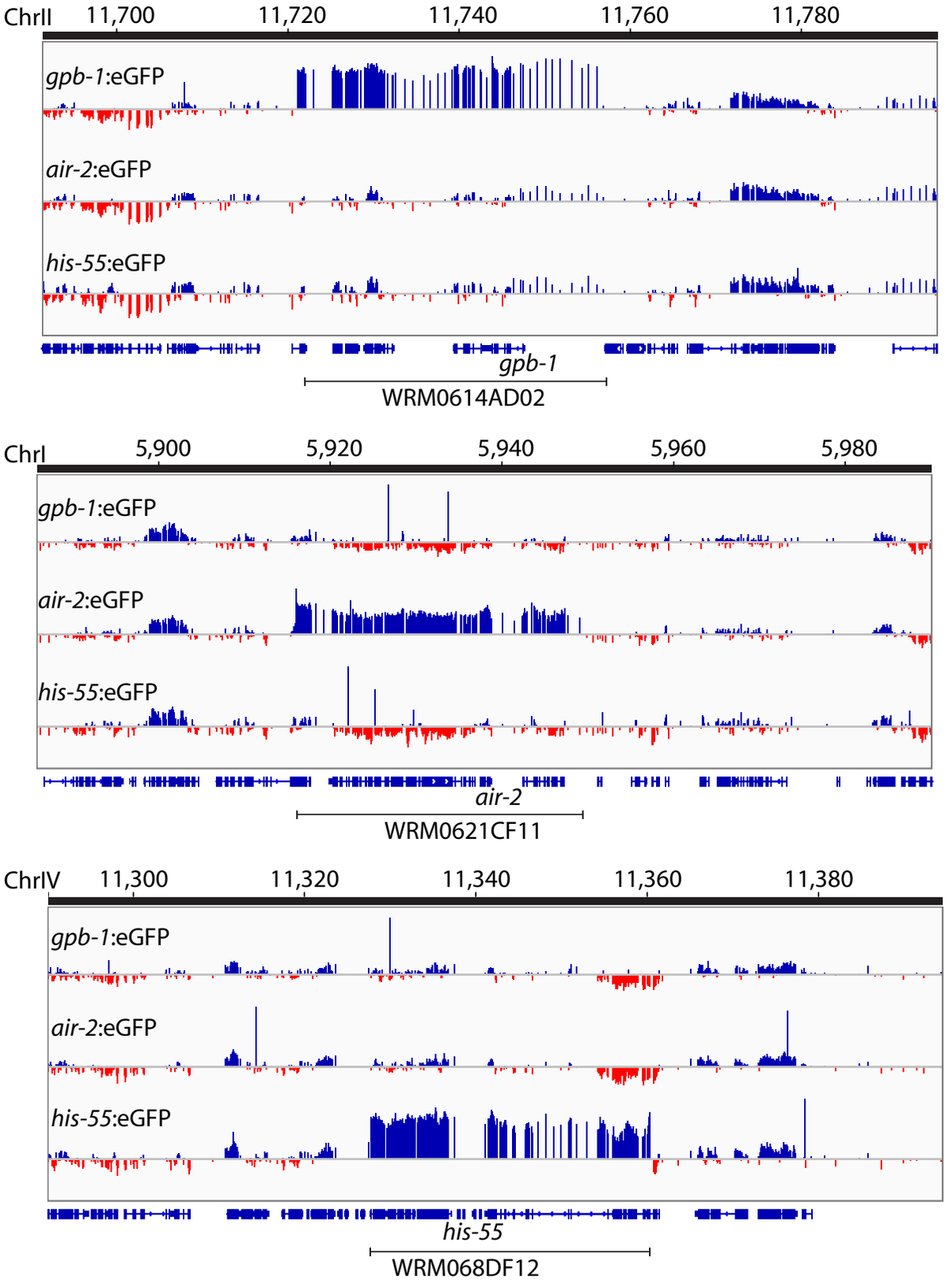
Supplementary Figure 5. GFP expression from miniMos insertions

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.

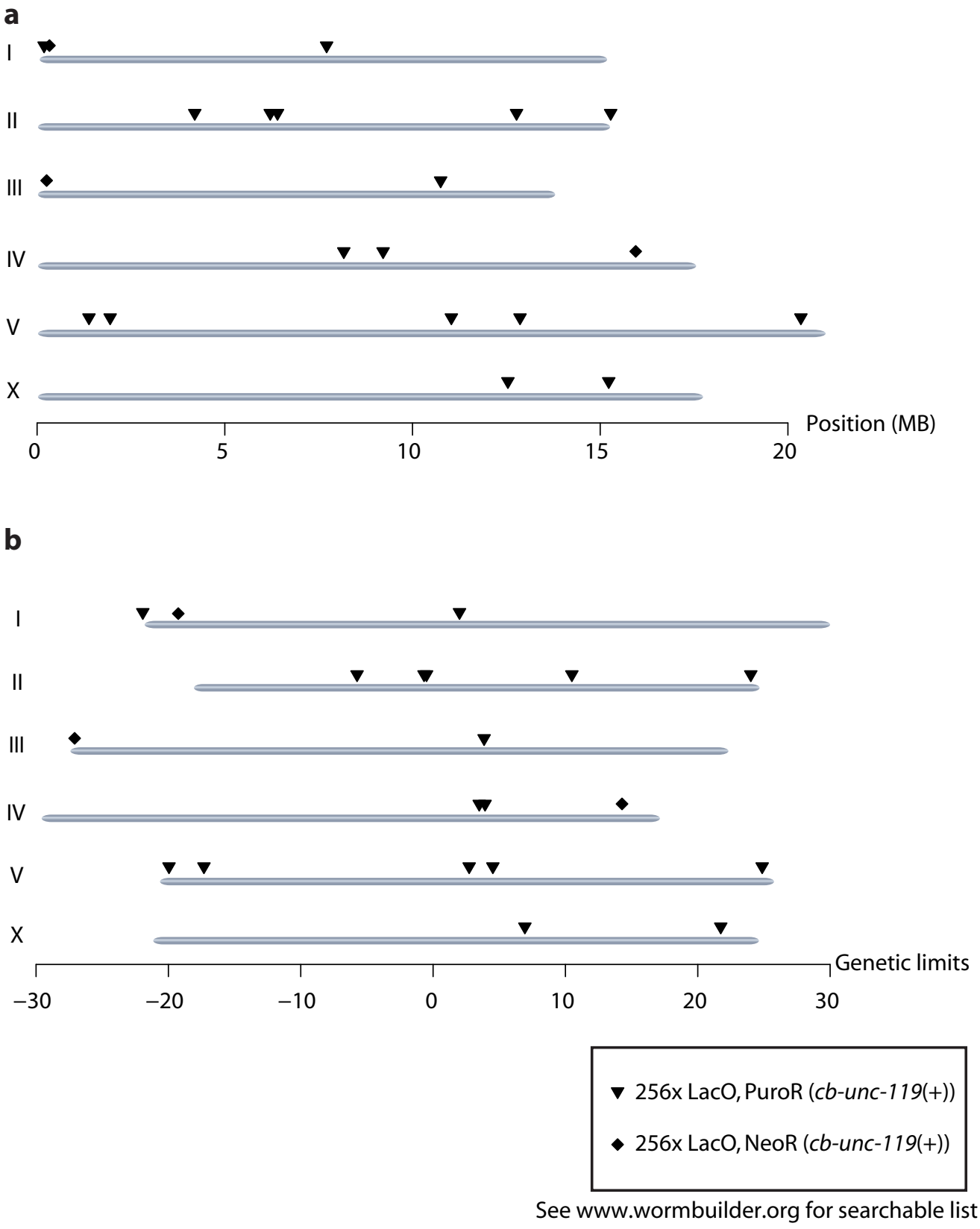


Supplementary Figure 6. MosSCI insertion frequency depends on DNA quality.

(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

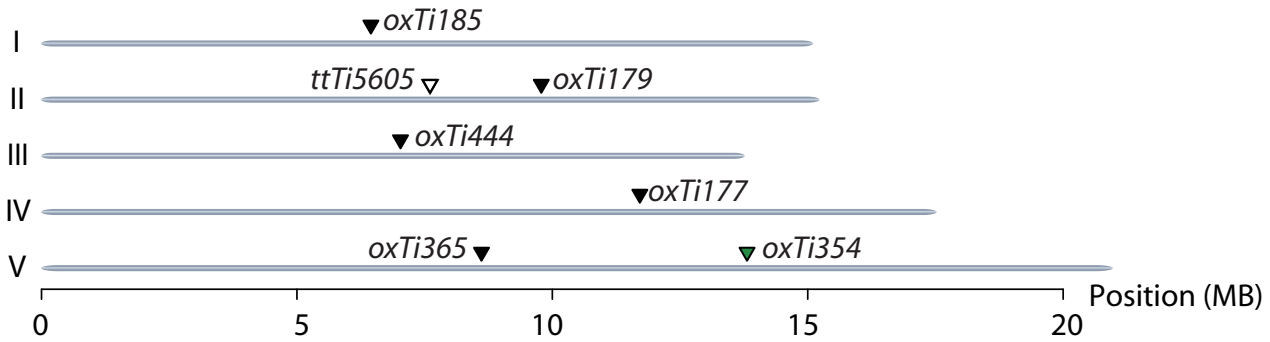
MiniMos cloning vectors

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-*

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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.

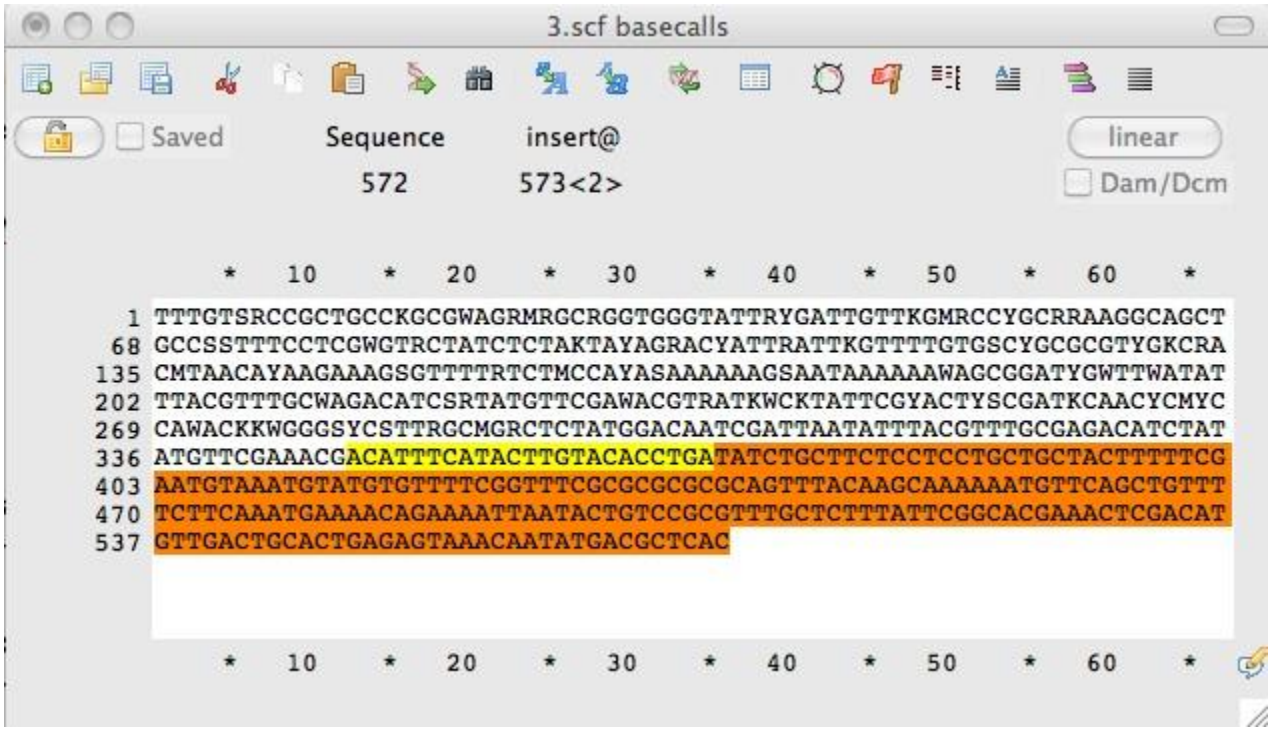
6. Run the PCR products on a 1% agarose gel, excise clear bands from gel and gel purify.

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).



Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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

Nature Methods: doi:10.1038/nmeth.2889

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.

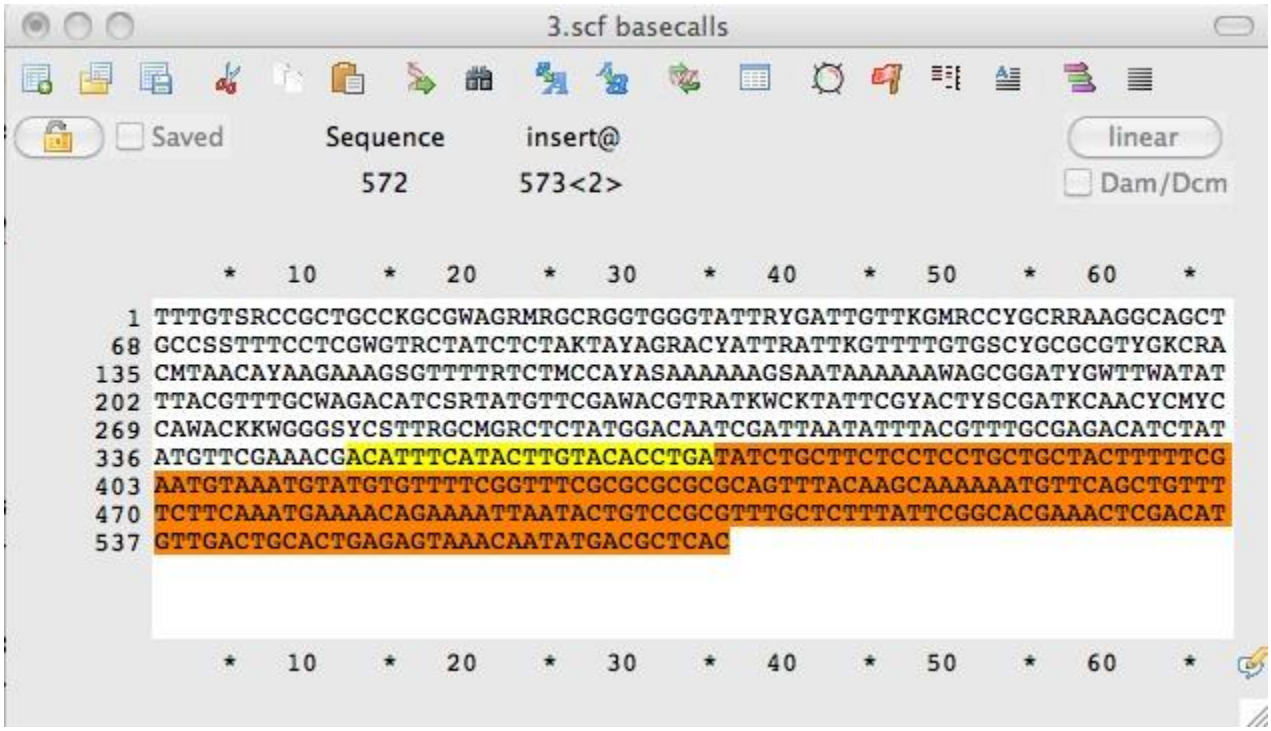
10. Run 10 ul of the PCR products on a 1% agarose gel.

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).



Nature Methods: doi:10.1038/nmeth.2889

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)

Nature Methods: doi:10.1038/nmeth.2889

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.

Nature Methods: doi:10.1038/nmeth.2889

References

Boulin, T., and Bessereau, J.-L. (2007). Mos1-mediated insertional mutagenesis in Caenorhabditis elegans. Nat. Protoc. *2*, 1276–1287.

Frøkjaer-Jensen, C., Davis, M.W., Hopkins, C.E., Newman, B.J., Thummel, J.M., Olesen, S.-P., Grunnet, M., and Jorgensen, E.M. (2008). Single-copy insertion of transgenes in Caenorhabditis elegans. Nat. Genet. *40*, 1375–1383.

Frøkjær-Jensen, C., Davis, M.W., Ailion, M., and Jorgensen, E.M. (2012). Improved Mos1-mediated transgenesis in C. elegans. Nat. Methods *9*, 117–118.

Seidel, H.S., Ailion, M., Li, J., van Oudenaarden, A., Rockman, M.V., and Kruglyak, L. (2011). A novel sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in C. elegans. PLoS Biol. *9*, e1001115.

Seth, M., Shirayama, M., Gu, W., Ishidate, T., Conte, D., Jr, and Mello, C.C. (2013). The C. elegans CSR-1 Argonaute Pathway Counteracts Epigenetic Silencing to Promote Germline Gene Expression. Dev. Cell.

Shirayama, M., Seth, M., Lee, H.-C., Gu, W., Ishidate, T., Conte, D., Jr, and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the C. elegans germline. Cell *150*, 65–77.

Spradling, A.C., Stern, D.M., Kiss, I., Roote, J., Laverty, T., and Rubin, G.M. (1995). Gene disruptions using P transposable elements: an integral component of the Drosophila genome project. Proc. Natl. Acad. Sci. U. S. A. *92*, 10824–10830.

Wedeles, C.J., Wu, M.Z., and Claycomb, J.M. (2013). Protection of Germline Gene Expression by the C. elegans Argonaute CSR-1. Dev. Cell.

Nature Methods: doi:10.1038/nmeth.2889