Additional Landing Sites for Recombination-Mediated Cassette Exchange in *C. elegans*

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**Abstract**

Recombination-mediated cassette exchange (RMCE) is a recently developed alternative method for creating single copy transgenes using recombination rather than repair of double stranded breaks as the mechanism for driving integration into the genome. Two alternative methods for performing RMCE have been developed: a two-component approach using an unlinked source of FLP recombinase, and a one-component approach using a FLP expression cassette within the landing site. Here, I describe new landing sites for performing both types of RMCE. The new landing sites are located within 50 bp of well-vetted MosSCI insertion sites on Chr II and Chr IV.

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**Figure 1. Overview of new RMCE landing sites:**

A) Structure of the miniMos and CRISPR/cas9 mediated single and two-component landing sites. Coding regions and Mos1 transposase arms (thick rectangles), promoters (thin rectangles), and recombinase sites (triangles) are labelled. Unlabeled thick arrows represent 3' UTRs: *unc-54* (yellow), *gpd-2/3* (grey), *his-58* (blue), *glh-2* (pink). The unlabeled grey region between GFP-C1 and *his-58* is a flexible linker. *loxP*, *FRT*, and *FRT3* sites (triangles) not drawn to scale for clarity.

B) Position and structure of the genomic interval of landing sites. *jsSi1579* is a two-component landing site integrated at a cas9 sgRNA site just adjacent to the position of the widely used *ttTi5605* Mos1 insertion on Chr II. *jsSi1691* is a single-component landing site integrated at the same position as *jsSi1579*. *jsSi1669* is a single-component landing site integrated at a cas9 sgRNA site just adjacent to the position of the *cxTi10882* Mos1 insertion on Chr IV. *js1570* is a derivative of *jsTi1453* on Chr I in which the miniMos left arm was deleted using CRISPR/cas9. Mos1 insertions are represented by solid blue triangles. The landing site insertions are represented as triangles colored with a purple gradient oriented such that the dark side of the gradient represents the 5' end of GFP-his-58 coding sequences within the insertion and the light side represents the 3' end. The position of the region on the chromosome (bp) is listed just below the line representing the chromosome. Coding genes are represented in pink and teal and non-coding genes in grey. Analogous schematics for the previously characterized landing sites are also available [See figure S2 of Nonet, (2020)].

C) Comparison of the expression levels of identical insertions at various RMCE landing sites. Expression level of a mec-4p GFP-C1 tbb-2 3' construct integrated at the previously described landing sites (Nonet, 2020) and the new

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C) Comparison of the expression levels of identical insertions at various RMCE landing sites. Expression level of a mec-4p GFP-C1 tbb-2 3' construct integrated at the previously described landing sites (Nonet, 2020) and the new.
Chr I, Chr II and Chr IV landing sites. I assumed that integration into jsSi1579 and jsSi1669 would yield the same expression level as they yield molecularly virtually identical insertions. The Chr II integration was made using jsSi1579. Removing the mosL arm from jsTi453 has only a minor effect on expression of an integrated teto 7X GFP reporter (Rp) when driven by a mec-4p-Tet OFF driver (Dr). Direct GFP fusion and bipartite reporter strains were imaged and quantified under the same conditions and thus can be directly compared. Strains imaged: NM5196, NM5209, NM5228, NM5236, NM5337, NM5467, NM5580, NM5582 and NM5633. n=14-21 for ALM and n=28-42 for PLM. D) Images of the gland cell background GFP expression of transgenic animals carrying teto 7X GFP-CI integrated into jsTi453 and the ∆mosL. js1570 derivative. Strains imaged: NM5264 and NM5327. Scale bar: 20 µm. E) Frequency of insertions obtained at distinct landing sites. All injected animals were counted regardless of perceived quality of injection or survival. In most cases only a single gonad was injected. F1 Rol progeny were typically grouped 6 per plate for identification of integrated lines. Lines represents the number of independent F1 progeny plates that segregated an integrated line. Success is defined as obtaining an integration event at the expected genomic position. The insert size does not include the 7.85 kb vector and SEC sequences excised during heat shock. 3 Injections performed using PB washed DNA.

Description
Transgenic animals are powerful tools in the study of basic biological processes using C. elegans. The recent development of recombination-mediated cassette exchange (RMCE) integration approaches in worms provides a relatively rapid method to create single copy transgenes with inserts up to at least 12 kb (Nonet, 2020; Yang et al., 2021). RMCE is similar to MosSCI (Frokjaer-Jensen et al., 2008) in that it depends on integrating at landing sites that have been engineered. In the case of MosSCI, a transposon is excised, and the excised stranded break is repaired by a template often using synthesis-dependent strand annealing (SDSA) which is error prone (Frokjaer-Jensen et al., 2008). Similar approaches mediated by cell cleavage also have high error rates, approaching 65% in some studies (Au et al., 2019). By contrast, RMCE uses recombination to insert the template into the genome which rarely yield erroneous inserts (Nonet, 2020).

The RMCE approach I developed takes advantage of two distinct recombinases. A plasmid template delivered into the gonad of young adult animals is first integrated into the genome using FLP recombinase. Both the template and the landing site contain two distinct FLP integration sites, 5' FRT and 3' FRT. Recombination between the 5' and 3' FRT sites in the plasmid and the landing site yields replacement of the genomic 5' FRT 3' FRT interval with plasmid 5' FRT 3' FRT interval. This recombination likely occurs in two steps; first a loop in by recombination at one of the sites, followed by excision by recombination at the other. These recombination events typically occur in the F1 germline, though occasionally it occurs in the P0 animal (Nonet, 2020).

Two methods have been developed for performing RMCE. The first method uses a landing site and an unlinked source of FLP recombinase (usually bqSi711). Injection of the plasmid leads to integration of the plasmid at the landing site which is identified as a Rol (or Hygβ) animal. After the initial insertion is made homozygous, the self-excisng marker cassette (SEC) is then excised using a heat shock Cre protocol, leading to the final insertion. The insertion is then outcrossed from bqSi711 using simple crosses. The second method utilizes a landing site which contains a germline FLP expressing transcription unit contained within the landing site. In this case, the FLP expression element is excised using a heat shock Cre protocol. One limitation of the RMCE approach is the lack of landing sites for integration. Here, I describe several new landing sites created using a CRISPR integration approach.

I integrated a two-component landing site using CRISPR/cas9 just adjacent to the position of the tτ5605 Mos1 insertion that has been widely used for creating single copy integrations using MosSCI (Frokjaer-Jensen et al., 2008). I also integrated a single-component landing site at same position on Chr II and another at a site adjacent to ct1108822, another commonly used Mos1 insertion site on Chr IV. In addition, I modified the previously described two-component landing site jsTi4534, deleting the left miniMosarm from that landing site (Fig. 1A, B). I first characterized these novel landing sites by integrating the identical mec-4p GFP-CI tbb-2 3' construct at each site and comparing the expression level of insertions at the new landing sites to identical insertions at the previously described landing sites (Nonet, 2020). The expression of GFP-C1 in touch receptor neurons (TRN) was easily detected in all the new landing site transgenes, though the expression level was slightly lower than that observed in integrations at the four previously described sites (Fig. 1C).

Recently developed bipartite reporters including a tterOffET Tet OFF system exhibit background expression in both the pharynx and the rectal gland cells (Nonet, 2020). Because a similar rectal gland background signal was observed at several different landing sites using distinct bipartite systems, I speculated that the miniMos transposon arm might be contributing to the background. Comparison of the identical teto 7X ∆mec-7p GFP-C1 reporter integrated at both jsTi453 and the js1570 ∆mosL arm derivative confirmed this was the case as expression in the rectal gland cells was undetectable in the js1570 derived transgene (Fig 1D). Despite the reduction in background, the tter Off reporter still robustly expressed GFP in TRNs, when driven by the identical mec-4 promoter tet OFF driver (Fig. 1C).

In developing a new recombination-mediated homolog exchange technique (https://sites.wustl.edu/nonetlab/rmhe/), I have used these new landing sites to create additional RMCE insertions. I collapsed the insertion frequency data from a set of over 90 injection sessions in which I counted the number of Roller F1 animals obtained, and the number of insertions obtained (Fig. 1E). These data demonstrate that js1570, jsSi1579 and jsSi1669 all behave comparably to previously described landing sites, yielding insertions at a rate of approximately one per 1 injected animals. However, the jsSi1669 single component site yielded insertions at a lower frequency (1 per 10 injected animals). I speculate this is due to lower expression of FLP from the jsSi1691 landing site since integration at the same position using jsSi1579 and bqSi711 as a source of FLP yields normal integration frequencies. A recent study indicated that for CRISPR/cas9 genome modifications one could obtain a much higher frequency of integration events using specifically treated miniprep DNA (Huang et al., 2021). A preliminary set of 7 injections performed while this manuscript was under review and presented in Figure 1E suggest that similar benefits are seen for RMCE, raising the insertion frequency to above 1 integration per P0 in this admittedly small sample size.

I previously demonstrated that RMCE yields insertions of expected structure in greater than 95% of cases (Nonet, 2020). I have also characterized most of the transgenes obtained at these additional landing sites either by confirming the presence of an expected fluorescence pattern, recombinase activity, terto reporter activity, or tter reporter activity. In some cases, long range PCR combined with restriction digests and/or sequencing was also performed to confirm the structure of insertions. All of the insertions at js1570 and jsSi1669 have been verified. The analysis of insertions on Chr II is ongoing, but to date only one of over 50 well-characterized insertions is incorrect with that insertion containing a distinct region of Chr II inserted adjacent to the lox site. Three other cases of unexpected outcomes are also worth mentioning. In one case, I obtained a homozygous Rol insertion which still expressed the rpl-28p GBP-his-58 marker from the landing cassette. In addition, in two cases, I was unable to excise the SEC by heat shock. However, in all three cases, an independent sister insertion from the same injection session was used for the excise step to isolate the final insertion. I have not attempted to determine the molecular structure of these unusual “faux” insertions since they are easily identified and discarded.

The new landing sites are now available at the CCGB and should provide additional flexibility in creating RMCE-based transgenic animals. I also plan to create additional landing sites at well-characterized high expressing genomic positions on the remaining chromosomes that currently do not contain landing sites.

Methods
Request a detailed protocol
C. elegans was maintained on NGM agar plates spotted with OP50 at 22.5°C or at 25°C during the RMCE protocol.

RMCE transgenesis
Inserts were cloned into pLF3FShC (Nonet, 2020), pRMHEB or pRMHEP (https://sites.wustl.edu/nonetlab/rmhe_vectors/) and injected at ~50 ng/µl into young adults. In the set of injections summarized in Figure 1E, Qiagen miniprep DNA was prepared using a PB wash as described by Huang et al. (2021) and injected at 40-50 ng/µl. Integrants were identified and isolated as described in detail in Nonet (2020). Performing RMCE at 25°C is critical to obtaining robust integration rates. The criterion for including an injection session in the table (Fig. 1E) was obtaining a mean of at least 1 F1 Rol per injected animal. All injection sessions into js1570, jsSi1669 and jsSi1691 met this criterion. Eleven jsSi1579 injection sessions failed to meet this criterion. They consisted of 5 sessions injecting plasmids with strong ubiquitous promoters (eft-3 or rpl-27) driving tet OFF and 6 sessions injecting plasmids that contained both a tet OFF driver and a terto reporter cassette. In all cases dead eggs were observed on the injection plates. In cases where a fluorescent protein reporter was in the plasmid, the dead eggs were brightly fluorescent. The two jsSi1579 failures included in the table were one rpl-27 session and one dual driver and reporter plasmid session. In some cases, I was able to integrate plasmids containing both a driver and a reporter by injecting animals growing on doxycycline.
js1570 was created using a dpy-10 co-CRISPR strategy. A mix of plasmids NMp3143 (40 ng/ul), NMp3153 (10 ng/ul), NMp3828 (20 ng/ul), NMp3829 (20 ng/ul) and oligonucleotides NMo5238 (0.4 uM) and NMo6761 (0.4 uM) we co-injected into js11452, bqSi712 animals. Rol progeny were screen by PCR (NMp3654/6569) for presence of the deletion and homozygosity. The deletion structure was confirmed by sequence analysis. jsSi1579 was created by injecting unc-T19(exOE); bqs711 animals with a mix of NMp3143 (40ng/ul), NMp3630 (50 ng/ul), NMp3631 (30 ng/ul). Insertions were identified by selection for hygR Rol progeny by adding 25 ul of 100 mg/ml HydroGold™(InvivoGen) to P0 injection plates (3 animals per plate) 3 days after injection. After isolating homozygotes, the hygR sg-I self-excision cassette (SEC) in the insertion was excised by screening for non-Rol progeny after a 20-hr. heat shock at 29° C. The structure of the insertion was validated by a combination of long-range PCR [performed as outlined in Nonet (2020) using NMo3887/3888], restriction digestion and sequence analysis. jsSi1691 was created by injection of N2 (the wild type) with a mixture of plasmids NMp3143 (40 ng/ul), NMp3630 (50 ng/ul), NMp4043 (20 ng/ul), pBluescript KS (50 ng/ul), pGH8 (2 ng/ul) and pCFJ90 (2.5 ng/ul). Insertions were selected for and analyzed as described for jsSi1579. jsSi1669 was created by injection of oxTi1127 animals with a mixture of plasmid NM4055 (35 ng/ul), NM4057 (25 ng/ul), pBluescript KS (+) (50 ng/ul), pGH8 (2 ng/ul) and pCFJ90 (2.5 ng/ul). Insertions were identified by hygromycin selection as described above. The structure of the insertion was validated as described above using oligonucleotides NMo3839/3830. The genomic sequence of the insertions is available at https://sites.wustl.edu/nonetlab/rmce-insertion-strains/.

Microscopy

For quantification of GFP signals, homozygous L4 hermaphrodite animals were mounted on 2% agar pads in a 2 µl drop of 1 mM levamisole in phosphate buffered saline, cover slipped and imaged on an Olympus (Center Valley, PA) BX-60 microscope equipped with a Qimaging (Surrey, BC Canada) Retiga EX1 monochrome CCD camera, a Lumin<n>aur AURA LED light source, Semrock (Rochester, NY) GFP-3035B and mCherry-A-000 filter sets, and a Tofra (Palo Alto, CA) focus drive, run using micro-manager 2.0 software (Schindelin et al., 2012) using a 40X air lens at 20% LED power with 100 ms exposures. PLM soma and ALM soma signals were quantified using the FIJI version of ImageJ software (Edelstein et al., 2014) as described in Nonet (2020). Rectal gland cell images presented were collected using the same conditions.

Oligonucleotides

**NM0 number** | **Sequence 5’ > 3’**
--- | ---
3887 | ACCGGAAACCAAAGGAGCGAGAG
3888 | ACCGCCAGAGAAGCAGTATAG
3889 | CCACACAAGGTCTGGTACCCAG
3890 | CATATCAGCAGCAGACGCC
Novel transgenes

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<td>Chr IIlanding site</td>
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<td>jsSi1571 I</td>
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Worm Strains

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<td>BN711</td>
<td>unc-119(ed3) III; baSi711 m5-5p::FLP::SL2::mNG + unc-119(+) IV</td>
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<td>EG8992</td>
<td>JS1542.9(axT1127[Panex-5::Cas9::tbb-2 3′ UTR, Pspg-16:14::Cre::tbb-2 3′ UTR, Pmyo-2::nls-CyOFP::let-858 3′ UTR + lox2271]) III</td>
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<td>NM5295</td>
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This study

NM5322
js1453 js1570 [∆mosL loxP rpl-28p FRT GFP-his-58 FRT3 mosR] I; bqSi711 IV

NM5327
js1453 js1570 js1571 [loxP tetO 7X ∆mec-7p GFP-C1 tbb-2 3′ FRT3 mosR] I; him-8(e1489) IV

NM3337
js1453 js1570 js1571 [loxP tetO 7X ∆mec-7p GFP-C1 tbb-2 3′ FRT3 mosR] I; jct1493 js1560 [mosL loxP mec-4p tetrR-L-QF act-4 3′ FRT3 mosR] IV

NM5402
js1579 [loxP rpl-28p FRT GFP-his-58 FRT3] I; bqSi711 IV

NM5467
js1453 js1543 [mosL loxP tetO 7X ∆mec-7p GFP-C1 tbb-2 3′ FRT3 mosR] I; jsTi1493 jsSi1560 [mosL loxP mec-4p tetrR-L-QF act-4 3′ FRT3 mosR] IV

NM5471
js11609 [loxP mex-5p FLP D5 si2 mNG glh-2 3′ rpl-28p FRT GFP-his-58 FRT3] IV

NM5500
js11691 [loxP mex-5p FLP D5 si2 mNG glh-2 3′ rpl-28p FRT GFP-his-58 FRT3] II

NM5580
js11579 js1707 [loxP mec-4p GFP-C1 tbb-2 3′ FRT3] II

NM5582
js11669 js1684 [loxP mec-4p GFP-C1 tbb-2 3′ FRT3] IV

NM5633
js1453 js1570 js1734 [∆mosL loxP rpl-28p FRT GFP-C1 tbb-2 3′ FRT3 mosR] I; him-8(e1489) IV

Reagents

Plasmids are available by request from MLN. Strains containing the four new landing sites have been submitted to the Caenorhabditis Genetics Center (CGC). Plasmids and additional strains will be submitted to Addgene or CGC and if demand levels warrant it.

References


Schwartz ML, Davis MW, Rich MS, Jorgensen EM (2021) High-efficiency CRISPR gene editing in C. elegans using Cas9 integrated into the genome. DOI: 10.1101/2021.08.03.454883

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