Supplementary Figure Legends

Figure S1. Extrachromosomal array behavior in presence of Flp

A) Diagram of the cross used to create the *bqSi640 js1449 dpy-7p::FRT::GFP-his-58* (abbreviated as *js1449*) transgene with a single *FRT* site. A schematic diagram of the approximate molecular structure (*his-58* not shown) of the two single copy insertions is shown above the cross (mCh= *mCherry*, mNG = mNeonGreen, f=*FRT*, blue arrow indicated direction of transcription). Germline excision of the *FRT* flanked *mCherry* sequences was confirmed by scoring progeny for a hypodermal GFP signal. Crosses in which single hermaphrodites were self-crossed are shown as arrows.

B, C) Injection procedures used to assess if *FRT* containing arrays are stable in the presences of germline expression of Flp recombinase. B). 18-40 ng/μl of NMp3632 vector containing both an *FRT* site and *sqt-1(d)* was injected into *js1449; bqSi711* animals. F1 progeny of the injected animals were screened for Rol and pooled 5 per plate to screen for F2 transmission. C) A mixture of NMp89 (50ng/μl), NMp141(100 ng/μl), NMp3622 (2 ng/μl) and NMp3623 (2 ng/μl) was injected into *js1449*. Two transmitting *ExRol-FRT* arrays were isolated. PCR was used to confirm the arrays contained *FRT* sequences.  *him-8* males were crossed to *mIs12* [*myo-2p::GFP, pes-10p::GFP, F22B7.9p::GFP*] II, and *mIs12/+* males were crossed to *bqSi711* to isolate *bqSi711/+*; *mIs12/+* males. *mIs12* was used because it is closely linked to *js1449* and displays a very strong pharyngeal GFP fluorescent signal that was used to identify cross progeny. Cross progeny were screened for germline mNG fluorescence to distinguish progeny that did and did not carry *bqSi711.* Rol array bearing cross progeny were cloned, and their progeny screened for transmission of the Rol extrachromosomal array. Of 12 Rol *js1449/mIs12; bqSi711/+; ExRol-FRT* animals analyzed, 6 segregated no Rol progeny and 6 segregated 1-5 Rol progeny none of which transmitted to the F3 generation.

D) Additional injection used to assess if *FRT* containing arrays can be efficiently integrated. *jsTi1453* (*landing site I*) animals were injected with a mixture of NMp3623 (5 ng/µl), NMp3632 (10 ng/µl), NMp3658 (60 ng/µl) and NMp3663 (60 ng/µl) and F1 progeny were screened for F2 transmission to identify the *jsTi1453; jsEx1533* line. *jsTi1453; him-8* males were crossed to *jsTi1453; bqSi711* animals to produce *jsTi1453; bqSi711/him-8* males. *jsTi1453; bqSi711/+; jsEx1533* and *jsTi1453; jsEx1533* animals were distinguished by screening for germline mNG fluorescence in late L4 animals.

Figure S2. Chromosomal location of RMCE landing sites

The insertion position of six different *miniMos*-derived landings sites is shown. *jsTi1453* is derived from insertion of plasmid NMp3649 and does not contain a *mex-5* promoter *FLP* cassette. The other five are derived from insertion of NMp3689 and contain a *mex-5* promoter *FLP* cassette (though not all insertions express the *FLP* cassette). The insertion is represented as a triangle 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. Genes are shown with 5’ and 3’ non-coding sequences in brown, coding exons in blue or pink depending on the direction of transcription. Small non-coding RNAs and pseudogenes are shown in gray. Only a single isoform of each gene is represented. Only non-coding RNAs near the insertion site are included in the diagram. The nucleotide position of the region within the chromosome is shown below the line representing the chromosome. Site of Insertions:  *jsTi1453* Chr I 11,933,068 (13.1 m.u.) ccctactatcaacgcaaaaactatttggcttttactTAaacataacgttttgaatttgaaaatcaaaaag. *jsTi1490* Chr IV at 7,310,985 (3.32 m.u.) gtacataaattataccaaatattgaTAaaagctacgaaaattccactgatat. *jsTi1492* Chr II at ~3,160,571 (-8.14 m.u.) ttttttgcaaaaaagtgcagtcataTAtgtatgtaaaaaattaattgaagac. Insertion is in a repeat and the exact site is ambiguous but likely near the edge of *sri-34* side of the repeat region. *jsTi1493* Chr IV at 9,197,338 (4.11 m.u.) gttcgcaaaccgtctgcgtctctTAttctcttgcaattccgcgcacacac. *jsTi1509* Chr III at position 13,031,845 (20.75) aaattatggtatgttttctgtaatgTAaataatttttttaaaattagttcct.  *jsTi1510* Chr X at 15,253,021 (22.29) taacggagtaagtttattttttaatTAaaaattgattttaaacgattatttt.

Figure S3. Molecular Characterization of landing sites and insertions

Agarose gels of DNA fragments spanning RMCE insertions amplified from genomic DNA or control plasmid DNA. See method for details of primers and PCR conditions used in amplifications. All digests yielded expected banding patterns except for the *jsIs1528* insertion (H). Markers: 100, 200, 300, 400, 500. 650, 850, 1000, 1650 bp and, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 kb. Invitrogen kb+ ladder, Cat. 10488-085.

A) Analysis of RMCE insertions. Acc65I (A) and SalI (S) restriction endonuclease digests of DNA fragments spanning RMCE landing insertion sites obtained by long range PCR of genomic DNA. Digests of *jsTi1453 (landing site I)*, *jsTI1490 (landing site IVb)*, *jsTi1492 (landing site II)*, and *jsTi1493 (landing site IVa)* is shown. Amplification of sequences from *jsTi1492*\* (a germline silenced derivative of *jsTi1492*) shows no change in the structure of the insertion.

B) Analysis of *jsTi1453* insertions. Undigested DNA fragments spanning *jsTi1453* and RMCE insertions at *jsTi1453* PCR amplified from genomic DNA using long range PCR.

C) Restriction digest analysis of *jsTi1453* insertions. Insert template DNAs and DNA fragments spanning RMCE insertions were amplified from plasmid and genomic DNA, respectively, and digested with HindIII (or EcoRI for *jsSi1500* and *jsSi1501*) restriction endonuclease.

D-G) Restriction digest analysis of additional RMCE insertions. DNA fragments spanning RMCE insertions were PCR amplified from genomic DNA and digested with XhoI restriction endonuclease. D) *jsTi1453* insertions, E) *jsTi1490* insertions, F) *jsTi1492* insertions and G) *jsTi1493* insertions.

H) Restriction digest analysis of the complex *jsIs1528* insertion, a functional *mec-4p GAL4SK-QFAD* driver line.  *jsIs1528* was derived from an injection (Table S1, Injection 163) of both NMp3734 (*mec-4p GAL4SK-QFAD tbb-2* ‘3) and NMp3741 (*mec-4p rtetR-QF tbb-2* 3’). Amplification of *jsIs1528* genomic sequences with primers pairs that amplify the left site (*mos* arm *mec-4p GAL4SK-QFAD tbb-2* ‘3, expected size: 2719 bp), the right side (*mec-4p GAL4SK-QFAD tbb-2* ‘3 *mos* arm, expected size: 2662 bp), the entire insert (expected size: 3519 bp), and from *rtetR* sequences to the *tbb-2* ‘3 (expected size if *rtetR-QFAD tbb-2* 3’ was inserted as found in NMp3741: 1332 bp). Only the right-side PCR shows an amplification product of expected size. The large size of the amplification product of the entire insert indicates additional sequences are integrated. The ability to amplify *rtetR* sequences indicates that portions of both plasmids in the injection mix were integrated at the site. The exact structure of the insert remains unknown.

I) PCR analysis of the unusual *js1503* and *js1504* events. *js1503* and *js1540* candidate *tetO 7X GFP-C1* insertions at *jsTI1492* do not express GFP. PCR analysis of insertion strains with primers designed to amplify across the insertion site and to amplify neighboring genetic loci. j*s1503* and *js1540* ‘insertions’ fail to amplify with the oligonucleotides used to characterize *jsTi1492* insertions (see F). In addition, both *js1503* and *js1504* fail to amplify *sri-34* sequences, the adjacent locus on the left side of *jsTi1492*, and *js1503* also fails to amplify *fbxc-55*, the adjacent locus to on the right side of the *jsTi1492* landing site.

Figure S4. GFP fluorescence in various developmental stages of *jsTi1493* animals

Shown are representative L1, L3, L4 and adult *landing site IVa* (*jsTi1493)* animals imaged using widefield epi-fluorescence microscopy. The ubiquitous *rpl-28* promoter drives GFP-HIS-58 expression, which accumulates in all somatic and germline nuclei. This signal becomes much weaker in adult animals. The germline *mex-5* promoter drives mNeonGreen which accumulates at low levels in the developing gonad and becomes much brighter in the gonad and developing eggs of adult animals. Scale bar 100 µm.

Figure S5. Diagrams of reporter constructs

Organization of the inserts of reporter plasmids integrated using RMCE. All inserts of NMp plasmids are in the pLF3FShC (MNp3643) integration plasmid. Shown is the entire region which remains at the insertion site after heat shock mediated SEC excision. Inserts are shown to scale with the distinct regions of the insert labeled above the first, but not subsequent appearances of the region. A) Four bipartite expression system *GFP* reporters, and a direct *mec-4p GFP* reporter. B) Modifications made to the NMp3724 *tetO* reporter to dissect the contribution of various insert regions to background levels observed in absence of a driver.

Figure S6. Diagrams of driver constructs

Organization of the inserts of driver plasmids integrated using RMCE. All inserts of NMp plasmids are in the pLF3FShC (MNp3643) integration plasmid. Shown is the entire region which remains at the insertion site after heat shock mediated SEC excision. Inserts are shown to scale with the distinct regions of the insert labeled above the first, but not subsequent appearances of the region. Insertion regions shaded with diagonal bars are synthetic coding regions, and those fully shaded are from native sources. A) Functional driver constructs that were integrated and whose activity is assayed in Figure 3 and 4. B) Non-functional driver constructs that were integrated (Table S1) and described in supplemental methods.

Figure S7. Kinetics of GFP expression in tet-OFF driver reporter lines

Quantification of GFP expression in TRN cell bodies after addition of doxycycline to  *jsSi1519 [tetO 7x GFP-C1 tbb-2 3’]*; *jsSi1560 [mec-4p tetR-L-QFAD act-4 3’]* animals. Recently hatched starved L1 animals were transferred to *E. coli* plates with or without 1ng/ml doxycycline and grown at 22.5°C for 24 or 48 hr. In addition, doxycycline was added to a set of plates after 24 hrs. of growth. Animals at 24 hrs. were in the L3 stage, and animals at 48hr were young adults.

Figure S8. Robust induction of tetO 7X promoter in doxycycline

Epi-fluorescence images of the head region of L4 hermaphrodites including the entire pharynx in the red channel to visualize mCherry and mKate, and green channel to visualize a GFP-RAB-3 fusion. A-B) *jsSi1486,* an insertion of the *phat-5* ventral g1 gland cell promoter driving a bi-cistronic *mCherry::V2A::GFP-rab-3* cassette. C-D) *jsSi1487*, an insertion of a *tetO 7X ∆pes-10* promoter driving a bi-cistronic *mCherry::V2A::GFP-rab-3* cassette, which shows essentially no expression. E-F) *jsTi1485*, a *miniMos* insertion of a *phat-5* promoter driving a tri-cistronic *rTetR-QF::P2A::mKate::T2A::tetR-pie1* (*tTS*) cassette (Mao et al., 2019). *tetR-pie* is used by Mao et al. (2019) to reduce background in the absence of doxycycline. However, neither *mKate* or *tetR-pie* are used in the mec-4 promoter RMCE tet ON or tet OFF constructs created in the paper. mKate is detected in the g1 gland cells at similar levels to that seen in *jsSi1486*. G-J) *jsSi1487; jsTi1485* double transgenic animals in G-H) the absence, and (I-J) presence of 1 ng/ml of doxycycline. *rtetR-QF* drives robust expression of the bicistronic *mCherry::V2A::GFP-rab-3* cassette in the presence of doxycycline. Scale Bar 40 µm.

Figure S9. Comparison of background in the head region of RMCE reporter lines

Widefield epi-fluorescence images of L4 animals taken using a 40X air lens. A diagram of the inserted sequences appears above each image. A-K) Animals homozygous for RMCE insertions of four distinct reporter constructs at *landing site I* and *II* as well as a wild type control, a *QUAS* integrant isolated using MosSCI (Wei et al., 2012), and a multi-copy *UAS* 15X integrated array (Wang et al., 2017). Signal in the intestine is background autofluorescence. L-R) Background levels in RMCE insertions at *landing site I* in cases where the fluorescent protein, the 3’ UTR sequences or the basal promoter of the *tet0 7X ∆pes-10 GFP-C1 tbb-2 3’* reporter construct have been replaced. P-Q) Comparison of *tetO* reporter background levels in presence and absence of a *mec-4* driver showing that background in not amplified by the presence of doxycycline. All images are taken under identical conditions (500 ms exposure). Insert diagrams are not to scale. See Figures S5 and S6 for more detailed diagrams. Detailed tail images are presented in Figures S10. Scale bar: 40 µm.

Figure S10. Comparison of background in the tail region of RMCE reporter lines

Widefield epi-fluorescence images of L4 animals taken using a 40X air lens. A diagram of the inserted sequences appears above each image. A-K) Animals homozygous for RMCE insertions of four distinct reporter constructs at *landing site I* and *II* as well as a wild type control, a *QUAS* integrant isolated using MosSCI (Wei et al., 2012), and a multi-copy *UAS* 15X integrated array (Wang et al., 2017). Signal in the intestine is background autofluorescence (except for panel I). L-R) Background levels in RMCE insertions at *landing site I* in cases where the fluorescent protein, the 3’ UTR sequences or the basal promoter of the *tet0 7X ∆pes-10 GFP-C1 tbb-2 3’* reporter construct have been replaced. P-Q) Comparison of *tetO* reporter background levels in presence and absence of a *mec-4* driver showing that background in not amplified by the presence of doxycycline. All images are taken under identical conditions (500 ms exposure). Insert diagrams are not to scale. See Figures S5 and S6 for more detailed diagrams. Detailed head images are presented in Figures S9. Scale bar: 40 µm.

Figure S11. Attempts at multi-plasmid insertion using RMCE

Schematic diagrams illustrating an attempt to integrate multiple plasmids into an RMCE landing site. A) The integration plasmid pLF3FShC was modified by moving the *loxP* site from adjacent to the *FRT3* site to before the vector sequences creating pLAOF3FShC. B) Worms were co-injected with mixtures of pLAOF3FShC and two pBluescript plasmids containing inserts at a ratio of 1:5:5. C) I speculate that at some frequency homologous recombination events during array formation yield larger plasmids that incorporate the insert plasmids in between the *loxP* and *FRT3* site. These ‘plasmids’ are likely to be elements in a much larger array that formed by random homologous recombination between input plasmids (see Figure 7). D) These larger insert containing plasmids integrate by RMCE yielding multi-plasmid inserts. Not shown is the final heat shock Cre loop out of the *mex-5p::FLP::mNG* operon and *hygr sqt-1* SEC sequences.

Figure S12. Behavior of oligonucleotides and plasmids in the *C. elegans* germline

Images of adult wild type *C. elegans* one hour after a mixture of FITC-labeled 80 mer oligonucleotide and Cy3-labelled pRF4 plasmid DNA were injected into the rachis of the distal part of the gonad arm. A) a focal plane through the center of the rachis and B) a focal plane through the edge of the gonad showing the syncytial nuclei. Scale Bar: 40 µm.

Figure S13. Effects of DNA concentration on integration frequency

A) A scatter plot of data from 23 injection sessions which were performed at different DNA concentrations using only integration plasmid DNA plotting integration frequency vs. DNA concentration (blue), and 7 injections which were performed using a combination of integration plasmid DNA and other ‘carrier’ plasmid DNA plotted both as integration frequency vs. integration vector DNA concentration (orange) and integration frequency vs. total DNA concentration (green). A gray line connects the matched pairs of plotted orange and green data points. B) A scatter plot of data from 30 injection sessions into 4 distinct integration strains. The data is plotted as integration frequency vs. DNA concentration with a different label used for each strain as shown in the legend. C) A scatter plot of data from 30 injection sessions plotting integration frequency vs. size of the insertion. On the X axis, the final size of the insert is in large font, and the size of the insert as isolated before SEC excision is in smaller font. All data from Table S1.

Figure S14. Two step RMCE insertion at landing sites

Schematic diagram of the likely two step RMCE integration mechanism. A) A Flp-mediated recombination event at the *FRT* site yields loop in integration of the targeting vector containing the insert creating an unstable intermediate that contains two *FRT* sites and two *FRT3* sites. Flp-mediated excision resolves this intermediate to a stable state, either returning to the initial genomic structure if the excision event occurs between *FRT* sites, or a cassette replacement if the excision event occurs between *FRT3* sties. B) Flp-mediated integration events initially occurring at the *FRT3* site are equally capable of resolving to yield a cassette replacement. However, in the case of recombination at the *FRT3* site, the intermediate would not yield expression of *hygr*, which could influence the efficacy of the RMCE procedure if performed using a hygromycin selection step.