Visualization and Quantification of Transposon Activity in Caenorhabditis elegans RNAi Pathway Mutants

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ABSTRACT RNA silencing pathways play critical roles in maintaining quiescence of transposons in germ cells to promote genome integrity. However, the precise mechanism by which different types of transposons are recognized by these pathways is not fully understood. Furthermore, the location in the germline where this transposition occurs after disruption of transposon silencing was previously unknown. Here we utilize the spatial and temporal organization of the Caenorhabditis elegans germline to demonstrate that transposition of DNA transposons in RNA silencing pathway mutants occur in all stages of adult germ cells. We further demonstrate that the double-strand breaks generated by transposons can restore homologous recombination in a mutant defective for the generation of meiosis-specific double-strand breaks. Finally, we detected clear differences in transposase expression and transposon excision between distinct branches of the RNA silencing pathway, emphasizing that there are multiple mechanisms by which transposons can be recognized and routed for small-RNA-mediated silencing.

KEYWORDS transposons double-strand breaks RNAi germline C. elegans

Transposons are discrete segments of DNA that are capable of excising themselves from one locus and reintegrating themselves at another genomic location. Movement of transposons in and out of genes can alter their expression and function, making transposons a major source of deleterious mutations as well as a driving force of evolution. In many organisms, transposons have also been co-opted by researchers for mapping, random and site-directed mutagenesis, and gene tagging (Williams et al. 1992; Barrett et al. 2004; Williams et al. 2005; Robert and Bessereau 2007; Frøkjær-Jensen et al. 2008; Frøkjær-Jensen et al. 2010). Because transposons utilize their host’s cellular machinery for their mobilization, they are considered to be selfish DNA parasites, similar to viruses.

There are two major classes of transposable elements – retrotransposons (Class I), which contain an open reading frame coding for a retroviral-like reverse transcriptase and transpose through an RNA intermediate, and DNA transposons (Class II), which move via a DNA-based “cut-and-paste” mechanism. DNA transposons usually contain a transposase sequence flanked by Terminal Inverted Repeats (TIRs). The transposase recognizes the specific sequence of its TIRs and catalyzes a cleavage reaction that releases the transposon ends. The transposase also recognizes a preferred target site, and inserts the transposon at the chosen location (Bessereau 2006). At the site of excision, a DNA transposon leaves behind a double-strand break (DSB), which must be repaired by the host’s cellular machinery, either through homologous recombination or non-homologous end joining. The mechanism of repair is determined primarily based on cell type – somatic cells favor end joining pathways whereas germ cells often repair breaks via homologous recombination, and a subset of these events are resolved as interhomolog crossovers (Plasterk 1991; Robert et al. 2008).

There are numerous retrotransposons in the genome, which, until recently, were thought to be inactive. However, a study published in 2012 demonstrated that CER1, Gypsy-like retrotransposon, is transcriptionally active and produces viral-like particles in wild-type C. elegans germlines (Dennis et al. 2012). More recently, it has been demonstrated that several other retrotransposons, including CER3, are targets of the nuclear RNA interference (RNAi) pathway and H3K9 methylation.
(Ni et al. 2014; 2016; Zeller et al. 2016; Ni et al. 2018). It is not yet known whether any of these retrotransposons are capable of transposition in C. elegans. In contrast, transposition has been detected for at least seven distinct families of DNA transposons (Tc1-Tc5, Tc7, CemaT1), though there are many more transposons present that have not been well studied (Eide and Anderson 1985; Collins et al. 1989; Levitt and Emmons 1989; Yuan et al. 1991; Collins and Anderson 1994; Rezsohazy et al. 1997; Brownlie and Whyard 2004; Besseureau 2006). The most well characterized DNA transposon family in C. elegans is Tc1, of which there are 31 intact copies present in the genome (Fischer et al. 2003). Tc1 is not normally active in germ cells, however, gene mutations that result in activation of Tc1 were identified from a forward genetic screen and are referred to as mutator (mut) class genes (Ketting et al. 1999). Around the same time, a screen for mutations that result in defects in RNAi identified a largely overlapping panel of genes, suggesting that the silencing of transposons is an endogenous function of the RNAi pathway (Tabara et al. 1999).

Many of the mutator pathway genes have been identified as components of the small RNA-mediated silencing pathways, including the nucleotidytransferase (mut-2/rde-3), the 3′-5′ exonuclease (mut-7), the DEAD box RNA helicase (mut-14), the glutamine/asparagine (Q/N)-rich protein (mut-16/rde-6), two proteins of unknown function (mut-8/rde-2 and mut-15), (Ketting et al. 1999; Tijsman et al. 2002; Vestenhouw et al. 2003; Tops et al. 2005; Chen et al. 2005; Gu et al. 2009). C. elegans with mutations in these genes not only have active transposons and defects in response to exogenous RNAi, but also have temperature-sensitive sterility and defects in endogenous siRNA production (Gu et al. 2009; Zhang et al. 2011; Phillips et al. 2014). All of proteins encoded by these mutator pathway genes, along with the RNA-dependent RNA polymerase RRF-1, associate to form a protein complex that synthesizes highly abundant secondary 22G-siRNAs (22 nucleotides starting with a 5′ guanosine) that function downstream of primary Argaonute proteins (Pak and Fire 2007; Sijen et al. 2007; Gu et al. 2009; Gent et al. 2010; Phillips et al. 2012). This complex forms nuclear pore-associated perinuclear condensates in germ cells, referred to as Mutator foci, where it is thought to play a key role in surveillance and silencing of deleterious transcripts, including transposon-derived RNAs, as they exit the nucleus (Phillips et al. 2012; Uebel et al. 2018).

In addition to endogenous siRNAs, PIWI-associated small RNAs (piRNAs) also have roles in transposition silencing (Bastiata et al. 2008; Das et al. 2008). In C. elegans, piRNAs (also referred to as 21U-RNAs) are bound and stabilized by the PIWI protein PRG-1 and trigger production of secondary 22G-siRNAs dependent on the mutator pathway (Ruby et al. 2006; Wang and Reineke 2008; Bastiata et al. 2008; Das et al. 2008; Lee et al. 2012; Bagijn et al. 2012). Only a single transposon family, Tc3, has been demonstrated to transpose upon loss of the piRNA machinery (Das et al. 2008), however, multiple other DNA transposons are up-regulated transcriptionally or lose mutator pathway-dependent 22G-siRNAs (Bagijn et al. 2012; McMurchy et al. 2017).

Here we demonstrate that DSBs generated by transposition of DNA transposons in mutator pathway or piRNA pathway mutants can be visualized throughout the germline of adult C. elegans, allowing us to determine both temporally and spatially where these transposons are active. Furthermore, in mutator pathway mutants these transposon-mediated DSBs can, at some frequency, be repaired by homologous recombination. Thus mutator pathway mutants can partially rescue the meiotic defects of spo-11 mutants, which fail to initiate meiotic recombination through the generation of DSBs. Finally, we observe distinct differences in transposon mRNA expression and frequency of DSBs generated by transposition between mutator pathway and piRNA pathway mutants, highlighting the distinct roles these two pathways play in transposon silencing.

### MATERIALS AND METHODS

#### C. elegans strains

Unless otherwise stated, worms were grown at 20°C according to standard conditions (Brenner 1974). Strains used in this study include:

- **N2** – wild-type
- **AV157** – spo-11(me44)/nT1 I; +/nT1 V
- **GR1833** – dpy-3(e27) unc-3(e151) X
- **GR1922** – mut-7(pk204) III; spo-11(me44)/nT1 IV; +/nT1 V
- **GR1923** – mut-16(pk710) I; spo-11(me44)/nT1 IV; +/nT1 V
- **NL1810** – mut-16(pk710) I
- **PD4792** – mls11 IV
- **SX922** – prg-1(n4357) I
- **USC22** – mls11 IV; dpy-3(e27) unc-3(e151) X
- **USC223** – mut-16(pk710) I; mls11 IV; dpy-3(e27) unc-3(e151) X
- **USC313** – prg-1(n4357) I; spo-11(me44)/nT1 IV; +/nT1 V

#### RNA isolation and quantitative RT-PCR

RNA was isolated from synchronized adult C. elegans (66-68 h after L1 arrest) using Trizol, followed by chloroform extraction and isopropyl precipitation. qRT-PCR was performed using transposon-specific primer pairs and rpl-32 for normalization (Table 1). Data were analyzed using the 2-ΔΔCt method and P-values were calculated in R using the t-test function in the package ‘pca’ (Ahmed and Kim 2018).

#### Fluorescent microscopy

**C. elegans** were picked as L4s and dissected the following day for immunofluorescence for most experiments. For diakinesis imaging and scoring, animals were picked as L4 and kept for three days at 15°C prior to dissection. All strains carrying the spo-11 mutation were selected as L4s from the progeny of synchronized spo-11/nT1 animals. Gonads were immunostained according to previously described protocol with rabbit anti-RAD-51 (SIDX, 2948.00.02), guinea pig anti-HTTP-3 (MacQueen et al. 2005), and Alexa Fluor secondary antibodies purchased from Thermofisher (Phillips et al. 2009). Imaging was performed on an Axio Imager Z1 microscope with ApoTome running Axiovision software (Zeiss) or a DeltaVision Elite microscope running SoftWoRx (GE Healthcare). Images were collected as

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**Table 1 Primers used in this study**

| Name       | Sequence                          |
|------------|-----------------------------------|
| Tc1 - F    | TGGGCTAACACATCGTGGTC              |
| Tc1 - R    | CGGTTGGCCATTGATCTTTG              |
| Tc2 - F    | AGTTATGGAGTTGATGTTGCC             |
| Tc2 - R    | AGTATTGAGCTATGGCG                 |
| Tc3 - F    | GTCCTGTATGTTGTAGCTAG              |
| Tc3 - R    | AATTAGCCTCCAAACGCGTCAAGG          |
| Tc4 - F    | GTTAAGCTGCTAACAAAAGAC             |
| Tc4 - R    | GTGTCGTTATGCCACCGCC               |
| Tc5 - F    | AGTGTACCGTGTCTTTG                 |
| Tc5 - R    | GAGTTGCTCCTACTTGCAGTTGG           |
| RTE1 - F   | CCCCAGGAGATGAGTTAATGG             |
| RTE1 - R   | GTGAGTTGCTTGGAGACATTGG            |
| CER1/Gypsy - F | CCGGAGAATCTGATCTACTTAC | |
| CER1/Gypsy - R | TCAGTACAGCAGAGGACGTTC        |
| Mirage - F | AGAACTGTAACGACACAGTTG             |
| Mirage - R | TCAGAGAACACGACACAGTTG             |
| rpl-32 - F | CAAAGTCTGTCAGAGAAGAGC             |
| rpl-32 - R | GGCTACACGACGGTATCTG               |
three-dimensional data stacks, displayed as maximum intensity projections, and pseudocolored using Adobe Photoshop.

**RAD-51 quantification**
Age-matched (one day post-L4) hermaphrodite gonads were immunostained for RAD-51 and imaged. Gonads were divided into six zones of equal length, starting at the distal tip through the end of pachytene and the number of foci per nucleus were scored for each zone. Three gonads were scored for each genotype.

**Brood size analysis**
Hermaphrodites of the indicated genotypes were placed on individual plates as L4-stage larvae. They were moved to fresh plates approximately every 24 hr until egg laying was complete. At the time the animal was removed from the plate, the total number of embryos and hatched larvae was counted. Approximately three days later, the total number of hermaphrodite and male progeny on the plate was scored. Total number of broods scored was 11 broods for wild-type, 29 broods for spo-11, 13 broods for mut-16; spo-11, 15 broods for mut-16; spo-11, and 21 broods for prg-1; spo-11.

**Recombination analysis**
Hermaphrodites heterozygous for dpy-3 unc-3 and homozygous for spo-11 were generated by mating balanced spo-11/mIs11 males to mls11; dpy-3 unc-3 hermaphrodites. The transgene mls11 is located on chromosome IV near the spo-11 locus; it can be identified by a pharyngeal GFP signal and was used to balance the spo-11 mutant. The spo-11/mLs11; dpy-3 unc-3 F2 hermaphrodites were then mated to spo-11/mLs11 males to generate the spo-11; dpy-3 unc-3/+ strain used for recombination analysis. The cross was performed similarly using mut-16/+; spo-11/mLs11 males and mut-16; spo-11; dpy-3 unc-3 hermaphrodites to generate the mut-16; spo-11; dpy-3 unc-3/+ and the mut-16/+; spo-11; dpy-3 unc-3/+ strains, which were identified as homozygous or heterozygous for mut-16 by genotyping after egg-laying was completed. Progeny from each cross were scored as wild-type, Unc Dpy, Unc non-Dpy, or Dpy non-Unc, and as hermaphroditic or male.

Recombination frequency (p) for hermaphrodites was calculated as

\[
p = \frac{\text{[# of hermaphrodites]} + \text{[# of males]} - \text{[# of hermaphrodites]} \times \text{[# of males]}}{\text{[# of hermaphrodites]} \times \text{[# of males],}}
\]

where R is the fraction of recombinant progeny, scored as two times the number of Unc non-Dpy hermaphrodites to account for the possibility that Dpy non-Unc animals are non-recombinant triplo-X animals (Brenner 1974). For males, recombination frequency is calculated as \( P = R \). The total recombination frequency is calculated as

\[
\frac{(2 \times \text{[mutant]} + \text{[pharmpodite]} + \text{[# of hermaphrodites]})}{(2 \times \text{[# of hermaphrodites]} + \text{[# of males])}}.
\]

Data Availability
All strains are available either at the Caenorhabditis Genetics Center (CGC) or upon request from the Phillips lab. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

**RESULTS**

**Transposon mRNA expression profiles of RNAi pathway mutants**

It is well known that RNAi pathways regulate DNA transposon activity in the *C. elegans* germline (Billi et al. 2014). However, the specific temporal and spatial region of the germline where this transposition occurs has not previously been studied. To address this question, we sought to visualize transposon activity by utilizing the DSBs left behind by the DNA transposons when they transpose, which are then repaired by cellular DNA repair machinery. First, however, we sought to determine how transposon activity differs between distinct branches of the RNAi pathway. It was previously reported that mutants in the *mutator* pathway and the piRNA pathway have different rates of transposon mobilization depending on the transposon being examined. For example, *T. c1, T. c3, and *T. c4 transposons are active in a *mut-7* mutant, whereas only the *T. c3* transposon is active in a *prg-1* mutant (Das et al. 2008). As a preliminary analysis of which transposons may mobilize in mutants from either the *mutator* pathway or the piRNA pathway, we performed qRT-PCR analysis of transposon mRNA expression in *mut-16* and *prg-1* mutants (Figure 1). Specifically, we examined the mRNA expression from several known DNA transposons (*T. c1, T. c2, T. c3, T. c4v, T. c5, and MIRAGE1) and two retrotransposons (RTE1 and CER1/Gypsy) and found that, of the DNA transposons, *T. c1, T. c4v and T. c5 had significantly increased mRNA expression in *mut-16* but not *prg-1* mutants, whereas *T. c2, T. c3, and MIRAGE1 had increased expression in both mutants. Interestingly, *T. c2 was significantly higher in *prg-1 (170-fold)* compared to *mut-16* (eightfold), which is surprising because piRNA-mediated silencing is generally thought to be upstream of and to require the *mutator* pathway (Das et al. 2008; Lee et al. 2012; Bagijn et al. 2012). It is important to note that this analysis is only indicative of transposon mRNA expression in the RNAi pathway mutants relative to wild-type animals, and is not direct evidence of transposon mobilization rates. Furthermore, while this analysis does not distinguish between somatic and germline transposon activity, it does suggest that *T. c2* transposon silencing may be mediated, at least in part, by a piRNA pathway that is independent of the *mutator* pathway and WAGO 22G-siRNAs.
Visualization and quantification of transposon mobilization

To visualize transposon activity specifically in the *C. elegans* germline, we chose to examine the expression of RAD-51, a homolog of the bacterial RecA protein and a key protein in DSB repair pathways (Ogawa et al. 1993). In wild-type *C. elegans*, RAD-51 can be visualized as distinct, punctate foci in the zygotene/pachytene stages of meiosis (Figure 2A) (Alpi et al. 2003). We initially examined multiple mutants in the RNAi pathway for increased RAD-51 foci in germ cells, however, the presence of programmed DSBs generated for meiotic recombination complicated the analysis. To alleviate this problem, we crossed the RNAi pathway mutants into a *spo-11* mutant. *SPO-11* is the type-II...
topoisomerase that is required to initiate meiotic recombination through the generation of DSBs (Keeney et al. 1997; Dernburg et al. 1998). In the spo-11 mutant, RAD-51 foci are virtually eliminated (Figure 2A-B) (Alpi et al. 2003), providing us a background where we can examine spo-11-independent DSBs generated due to transposon mobilization. We first examined the germline of mutator mutants (mut-7 or mut-16) in the spo-11 background. In these strains we could visualize numerous DSBs throughout the germline, starting in the mitotic proliferation zone, and extending through the meiotic stages of leptotene, zygotene, pachytene, and diplotene (Figure 2A-B). Because the number of foci increases as the nuclei progress through stages of leptotene, zygotene, pachytene, and diplotene (Figure 2A-B). These results are consistent with a role for the piRNA pathway in silencing only a subset of transposons, in contrast to the mutator pathway, which is more broadly required for transposon silencing.

Transposon-induced DSBs can partially rescue meiotic defects of spo-11 mutants

We next sought to examine whether transposon-induced DSBs are competent to rescue the meiotic phenotypes of the spo-11 mutant. In the absence of functional SPO-11 protein, chromosomes fail to undergo meiotic recombination (Keeney et al. 1997; Dernburg et al. 1998). Failure to undergo meiotic recombination causes errors in segregation of chromosomes at the meiosis I division, ultimately resulting in aneuploidy and embryonic lethality. The few progeny surviving to adulthood from spo-11 mutants are frequently males (Dernburg et al. 1998). This “High incidence of males” or Him phenotype is also indicative of a chromosome segregation defect; male C. elegans have a single sex chromosome (XO) and thus mis-segregation of the X chromosomes in an XX hermaphrodite results in an increased production of males (Hodgkin et al. 1979). In contrast to the spo-11 null mutants, which largely produce inviable embryos that fail to survive to adulthood (2.8% viable), mut-7; spo-11 and mut-16; spo-11 produce 12.1% and 10.7% viable embryos that survive to adulthood, respectively (Table 2).

Similarly, mut-7; spo-11 and mut-16; spo-11 produce fewer male progeny (14.9% males for mut-7; spo-11 and 21.4% males for mut-16; spo-11) than the spo-11 mutant alone (40.9% males) (Table 2). Unlike the mutator pathway mutants, the piRNA pathway mutant prg-1 failed to rescue embryonic viability or the production of male progeny. These data indicate that the DSBs generated by transposon mobilization in the

| Table 2 Mutator pathway mutations increase progeny viability and reduce the number of self-progeny males in a spo-11 mutant |
|---|---|
| Genotype | % Viable Embryos | % Male Progeny |
| wild-type | 100.00 (n = 3035) | 0.07 (n = 3035) |
| spo-11 | 2.80 (n = 5328) | 40.94 (n = 149) |
| mut-7; spo-11 | 12.11 (n = 1882) | 14.91 (n = 228) |
| mut-16; spo-11 | 10.68 (n = 2144) | 21.40 (n = 229) |
| prg-1; spo-11 | 2.12 (n = 4105) | 51.72 (n = 87) |

a) Total number of embryos scored to calculate % viable embryos indicated in parentheses.
b) Total number of adults scored to calculate % male self-progeny indicated in parentheses.

Figure 3 Mutations in the mutator pathway can restore crossover formation in the spo-11 mutant. (A) Representative wild-type and mutant diakinesis oocytes stained with HTP-3 (white) and DAPI (red) to allow for counting of the number of chromosomes connected by chiasmata or univalents in each strain. Yellow arrows in mut-7; spo-11 and mut-16; spo-11 point to a single pair of chromosomes in diakinesis oocytes of each genotype. Wild-type oocytes display six DAPI-stained bivalents, representing the six pairs of homologous chromosomes connected by chiasmata while spo-11, which fails to make double-strand breaks for recombination, displays 12 DAPI-stained univalents. Mutations in the mutator pathway but not the piRNA pathway can partially rescue the spo-11 phenotype. Occasionally, two bivalents lie too close together to be visually resolved, resulting in a modest underestimation of the number of DAPI-stained bodies. (B) Mean number of DAPI-stained bodies scored for each of the genotypes in (A). Total number of oocytes scored is indicated in parentheses.
mutator pathway mutants, but not piRNA pathway mutants, can compensate for the lack of SPO-11 protein and increase the frequency of proper chromosome segregation, presumably by promoting the formation of crossovers.

To test directly whether DSBs generated by transposon mobilization can promote the formation of crossovers, we examined diakinesis stage of meiosis for the presence of recombinant chromosomes. In wild-type C. elegans, six bivalents (pairs of recombinant chromosomes) are present, whereas, in spo-11, 12 non-recombinant univalents can be observed (Villeneuve 1994; Dernburg et al. 1998). The mutator pathway mutants, mut-7 and mut-16, were able to partially rescue the spo-11 diakinesis phenotype, averaging approximately eight DAPI-staining bodies, but with a range of six to 11 DAPI-staining bodies (Figure 3A-C). In contrast, the piRNA pathway mutant, prg-1, was indistinguishable from the spo-11 mutant alone with 12 univalents (Figure 3A-C). We also analyzed the frequency of recombination between two genetic markers, dpy-3 and unc-3, which lie on opposite ends of the X chromosome, a distance of ~38 cM in wild-type animals (Villeneuve 1994; Dernburg et al. 1998; Kelly et al. 2000). In spo-11 mutants, recombination is undetectable in this region (Dernburg et al. 1998) whereas mut-16; spo-11 mutants we calculated the map distance between dpy-3 and unc-3 as 26.6 cM (~70% of wild-type) (Table 3). These data indicate that, in each germline nucleus of a mutator pathway mutant, most chromosomes have at least one mobilized transposon generating a DSB that is subsequently repaired by homologous recombination. Because some DSBs are occurring well before or after the stage at which nuclei are competent for homologous recombination and because many DSBs may be repaired by other mechanisms, these figures significantly underestimate the total number of mobilized transposons per nucleus.

**DISCUSSION**

By visualizing transposon-derived DSBs as RAD-51 foci present in a spo-11 mutant background, we can provide quantification of transposon-hopping levels in the mutator pathway and piRNA pathway mutant backgrounds. Furthermore, in mutator mutants, transposon-derived DSBs can rescue spo-11 mutant phenotypes, including recombination frequency, chiasma formation, viability, and male production. The assays described could be extended to examine new mutants in the transposon-silencing pathway, to screen for new mutants by taking advantage of the increased fertility of spo-11 mutants when combined with mutations in the transposon silencing pathway, or to probe more deeply into which classes of transposons are mobilized and the frequency using ChIP-seq of RAD-51.

Interestingly, we observe clear differences in the expression of transposon mRNAs by qRT-PCR and in the rates of transposition assayed by frequency of DSBs, demonstrating that these two pathways do not have fully overlapping roles in transposon silencing. This result, along with previously reported differences in Tc1 and Tc4 mobilization between the two pathways (Das et al. 2008), is somewhat surprising because piRNA pathways are thought to be the primary mediator of transposon silencing in many organisms (Czech and Hannon 2016).

Table 3 A mutation in the mutator pathway restores recombination in the spo-11 mutant

| Genotype | Unc non-Dpy hermaphrodites | Dpy non-Unc hermaphrodites | Total hermaphrodites | Unc non-Dpy males | Dpy non-Unc males | Total males | Map distance (cM)* |
|----------|----------------------------|----------------------------|---------------------|------------------|------------------|------------|------------------|
| +/+ or mut-16/+; spo-11; dpy-3 unc-3/+ | 0 | 0 | 134 | 0 | 0 | 131 | 0.0 |
| mut-16; spo-11; dpy-3 unc-3/+ | 27 | 29 | 239 | 9 | 10 | 60 | 26.6 |

*Map distance was calculated as described in Materials and Methods.

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