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Regulation of Heterochromatin Assembly on Unpaired Chromosomes during *Caenorhabditis elegans* Meiosis by Components of a Small RNA-Mediated Pathway

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Abstract

Many organisms have a mechanism for down regulating the expression of non-synapsed chromosomes and chromosomal regions during meiosis. This phenomenon is thought to function in genome defense. During early meiosis in *Caenorhabditis elegans*, unpaired chromosomes (e.g., the male X chromosome) become enriched for amodification associated with heterochromatin and transcriptional repression, dimethylation of histone H3 on lysine 9 (H3K9me2). This enrichment requires activity of the cellular RNA-directed RNA polymerase, EGO-1. Here we use genetic mutation, RNA interference, immunofluorescence microscopy, fluorescence in situ hybridization, and molecular cloning methods to identify and analyze three additional regulators of meiotic H3K9me2 distribution: CSR-1 (a Piwi/PAZ/Argonaute protein), EKL-1 (a Tudor domain protein), and DRH-3 (a DEAH/D-box helicase). In *csr-1*, *ekl-1*, and *drh-3* mutant males, we observed a reduction in H3K9me2 accumulation on the unpaired X chromosome and an increase in H3K9me2 accumulation on paired autosomes relative to controls. We observed a similar shift in H3K9me2 pattern in hermaphrodites that carry unpaired chromosomes. Based on several assays, we conclude that ectopic H3K9me2 accumulates on paired and synapsed chromosomes in these mutants. We propose alternative models for how a small RNA-mediated pathway may regulate H3K9me2 accumulation during meiosis. We also describe the germline phenotypes of *csr-1*, *ekl-1*, and *drh-3* mutants. Our genetic data suggest that these factors, together with EGO-1, participate in a regulatory network to promote diverse aspects of development.


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Introduction

During sexual reproduction, mutations existing in the gametes will be inherited by the offspring. Therefore, it is essential that gametes contain accurate copies of the genetic information. Through evolution, multiple mechanisms have been developed to safeguard gamete quality. One such mechanism may be a process referred to as meiotic silencing of unpaired chromatin (MSUC) whereby genes located on unpaired chromatin are silenced during first meiotic prophase. This is a widespread phenomenon that has been described in fungi, nematodes, and mammals (for reviews, see [1–3]). While it naturally involves sex chromosomes in the heterogametic sex, the machinery may involve small RNA, e.g., small interfering (si) RNA, as activity of the RNA-directed RNA polymerase (RdRP), EGO-1, is required for H3K9me2 enrichment on unpaired regions [9]. In mouse, as in *C. elegans*, histone modifications correlate H3K9me2 accumulation with repression of gene expression in males. However, transcription of X-linked oogenesis-specific genes decreases dramatically in sexually transformed XO hermaphrodites, suggesting that the H3K9me2 marks indeed correlate with gene silencing [8]. The *C. elegans* meiotic silencing machinery may involve small RNA, e.g., small interfering (si) RNA, as activity of the RNA-directed RNA polymerase (RdRP), EGO-1, is required for H3K9me2 enrichment on unpaired regions [9]. In mouse, as in *C. elegans*, histone modifications associated with gene silencing accumulate on regions of unpaired chromatin, e.g., the male X and Y chromosomes and chromosomal translocations in both XX and XO germ lines. These regions also accumulate histone variants, e.g., macroH2A1.2 and γH2AX [5,10–13,[ see also [3]]. Mammalian meiotic silencing is known to

MSUC in nematodes and mammals occurs at the transcriptional level. In *C. elegans*, regions of unpaired chromatin, e.g., the male X chromosome, accumulate a histone modification associated with transcriptional silencing, H3K9me2 [4]. High levels of H3K9me2 also accumulate on free chromosomal duplications and chromosomes that fail to synapse due to mutations in both the XX and XO germ line [4]. Few X-linked genes are expressed in the male germ line, therefore it is difficult to correlate H3K9me2 accumulation with repression of gene expression in males. However, transcription of X-linked oogenesis-specific genes decreases dramatically in sexually transformed XO hermaphrodites, suggesting that the H3K9me2 marks indeed correlate with gene silencing [8]. The *C. elegans* meiotic silencing machinery may involve small RNA, e.g., small interfering (si) RNA, as activity of the RNA-directed RNA polymerase (RdRP), EGO-1, is required for H3K9me2 enrichment on unpaired regions [9]. In mouse, as in *C. elegans*, histone modifications associated with gene silencing accumulate on regions of unpaired chromatin, e.g., the male X and Y chromosomes and chromosomal translocations in both XX and XO germ lines. These regions also accumulate histone variants, e.g., macroH2A1.2 and γH2AX [5,10–13,[ see also [3]]. Mammalian meiotic silencing is known to
require machinery closely related to the DNA repair pathways [1]. In the filamentous fungus, *Neurospora crassa*, MSUC (also called meiotic silencing by unpaired DNA, MSUD) requires the activity of: an RNA-directed RNA polymerase called EGO-1. Here, we identify three more regulators of meiotic chromatin, the proteins CSR-1, EKL-1, and DRH-3. Our data suggest that these proteins collaborate with EGO-1 to ensure that paired chromosomes (autosomes and hermaphrodite X chromosomes) are regulated correctly and in a manner distinct from the male X chromosome. Our findings suggest that these four proteins participate in a mechanism to ensure proper gene expression for gamete formation.

Dicer activity does not appear to be required for meiotic H3K9me2 enrichment on unpaired chromatin in *C. elegans*, suggesting that microRNAs and other Dicer-dependent RNA products do not participate in the regulatory process [9].

To identify additional components of the MSUC machinery in *C. elegans*, we surveyed candidate genes to identify those whose loss of function altered the pattern of H3K9me2 accumulation during meiosis. Here, we report the identification of CSR-1, EKL-1, and DRH-3 as additional regulators of meiotic H3K9me2 accumulation. These proteins function in RNAi, and DRH-3 (like EGO-1) is implicated in the biogenesis of endogenous siRNAs [34,35]. Here, we provide evidence that H3K9me2 does not accumulate properly on unpaired chromatin in *csr-1*, *ekl-1*, and *dbh-3* mutants and is mis-targeted to correctly paired and synapsed chromatin. Moreover, the germline phenotypes of *csr-1*, *ekl-1*, and *dbh-3* mutants are complex and share some features with the ego-1 phenotype. As previously shown for *ego-1* [36,37], *csr-1*, *ekl-1*, and *dbh-3* interact genetically with *glp-1*, which encodes the germline Notch-type receptor required for germ cell proliferation. We discuss alternative models for how these factors may participate in the regulation of meiotic chromatin.

**Results**

**Identification of new regulators of meiotic H3K9me2 distribution**

We used two approaches to identify candidate genes whose products might participate in meiotic silencing: (i) we compiled a list of factors that had been implicated in small RNA-mediated processes, including Argonaute proteins and putative chromatin-associated proteins [38–52] (Table 1); and (ii) we surveyed a set of ego mutants, previously isolated in our screens for genetic enhancers of *glp-1* (ego mutations), whose phenotypes resemble that of *ego-1* [36] [J. Spoerke and E. Maine, unpublished data]. *ego-l* mutants have a specific developmental phenotype that is not commonly observed, but is characteristic of some other mutants isolated in our ego screens.

We subjected these candidates to two tests. First, we used indirect immunofluorescence to evaluate the meiotic H3K9me2 staining pattern in available mutants or after depletion via RNAi (see Materials and Methods). Our RNAi assays were performed using *him-8* mutants, as the hermaphrodite X chromosomes remain unpaired/unsynapsed and therefore become enriched for H3K9me2. Second, we tested type (i) candidates genes for an Ego phenotype using RNAi-mediated knockdown in animals with a weak *glp-1* loss of function mutation, *glp-1(bn18ts)* at 20°C (see Materials and Methods).

We identified four genes from the candidate gene list whose activities influenced meiotic H3K9me2 distribution: *csr-1*, *ekl-1*, *dbh-3*, and *sin-3* (Table 1). Three of these genes were also identified as Ego: *csr-1*, *ekl-1*, and *dbh-3* (Table 1). We also identified three ego mutants with altered H3K9me2 distribution, *ego(om55)*, *ego(om56)*, and *ego(om83)*. The three ego mutations mapped close to *ekl-1* and *dbh-3* (see Materials and Methods); we cloned them in order to determine whether they represented alleles of *ekl-1*, *dbh-3*, and/or other genes whose products function in meiotic chromatin regulation. Our data indicate that *ego(om56)* and *ego(om83)* are alleles of *ekl-1*, and *ego(om55)* is an allele of *dbh-3* (Figure 1) (see Materials and Methods). Intriguingly, CSR-1 (an Argonaute protein), DRH-3 (a Dicer-related DEAH/D-box helicase), and EKL-1 (a Tudor domain protein) all, like EGO-1, promote RNAi (Table 1). Hence, we hypothesize that these factors may work together to regulate meiotic H3K9me2 accumulation via a mechanism that involves small RNA, e.g., endogenous siRNA.
### Table 1. Candidate genes surveyed for H3K9me2 and Ego phenotypes.

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Gene</th>
<th>Protein family/domain</th>
<th>Small RNA processes [ref]</th>
<th>Δ H3K9me2</th>
<th>Ego</th>
</tr>
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<tr>
<td>C01G5.2</td>
<td>prg-2</td>
<td>Piwi-AGO Family</td>
<td>Tc3 silence [41]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C04F12.1</td>
<td></td>
<td>Piwi-AGO Family</td>
<td>Soma RNAi [39,42]</td>
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<td>--</td>
</tr>
<tr>
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<td>--</td>
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<tr>
<td>C18E3.7</td>
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<td>Piwi-AGO Family</td>
<td>Gln RNAi, T silence [42,43]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>D2030.6</td>
<td>prg-1</td>
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<td>Tc3 silence, 21U-RNA [41,43–45]</td>
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<td>--</td>
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<tr>
<td>F20D12.1</td>
<td>csr-1</td>
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<td>Co-sup, RNAi, Slicer [40,42,46]</td>
<td>+</td>
<td>+</td>
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<tr>
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</tr>
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<td>ppw-2</td>
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<td>dhr-2</td>
<td>DEXH-box helicase</td>
<td>RNAi [50]</td>
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<tr>
<td>D2005.5</td>
<td>dhr-3</td>
<td>DEAH/D-box helicase</td>
<td>RNAi, siRNA biogenesis [34,46]</td>
<td>+</td>
<td>+</td>
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<tr>
<td>F15810.2</td>
<td>dhr-1</td>
<td>DEAH-box helicase</td>
<td>RNAi [39,50]</td>
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<td>--</td>
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<tr>
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<td>D2096.8</td>
<td></td>
<td>NAP-related</td>
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<tr>
<td>F02E9.4</td>
<td>sin-3</td>
<td>SIN3 HDAC</td>
<td>RNAi [39]</td>
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<td>--</td>
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<tr>
<td>F22D6.6</td>
<td>eki-1</td>
<td>Tudor domains</td>
<td>Co-sup, RNAi [39,40]</td>
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<td>+</td>
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<tr>
<td>F45E4.9</td>
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<td>hmg-5</td>
<td>HMG box</td>
<td>Co-sup [36]</td>
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<tr>
<td>F54F2.2</td>
<td></td>
<td>zfp-1</td>
<td>Various1</td>
<td>RNAi [39,51]</td>
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<td>K04G7.3</td>
<td>ogt-1</td>
<td>See footnote2</td>
<td>Predicted SIN-3 interactor [52]</td>
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<tr>
<td>M04B2.3</td>
<td>gfi-1</td>
<td>See footnote2</td>
<td>RNAi [39,51]</td>
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<tr>
<td>T22B7.1</td>
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<td>W02D9.8</td>
<td></td>
<td>HMG box</td>
<td>Co-sup [40]</td>
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<td>ZK1127.7</td>
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<td>DNA topo IIa</td>
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<td>--</td>
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<tr>
<td>T09E8.1</td>
<td></td>
<td>Novel5</td>
<td>Co-sup [40]</td>
<td>--</td>
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</table>

All genes were tested using a deletion mutant (see Materials and Methods) except for the following, for which we knocked down the protein using RNAi: C08B11.2/hda-2, C10E2.8/hda-4, C35A5.9, K04G7.3/ogt-1, T22B7.1/egl-13/cog-2, and ZK1127.7/cin-4. Ego phenotype means all germ cells prematurely exited mitosis, entered meiosis, and underwent gametogenesis. Δ H3K9me2 means a change in the distribution and/or level of H3K9me2 in the meiotic germ line. We previously reported that mutations in the Argonaute genes rde-1 and alg-1 did not visibly alter the pattern of H3K9me2 accumulation during meiosis [9]. Additional putative Piwi/Argonaute genes, previously reported in the literature and now thought to be pseudogenes, are not listed here (http://www.wormbase.org). Gln, germ line; Co-sup, co-suppression; T silence, transposon silencing; Tc3 silence, Tc3 silencing; 21U-RNA, 21U-RNA regulation; HDAC, histone deacetylase; NAP, nucleosome assembly protein. NR, none reported.

1 Contains leucine zipper, Zinc finger, and PHD/LAP domains; homologous to human AF10.
2 Co-linked N-acetylgluconosamine transferase.
3 Homology to human glioma-amplified sequence 41, yeast transcription factor AF-9, and human transcription factor ENL.
4 Related to transcription factor SOX5.
5 DNA topoisomerase, type IIa.
6 Loss of gene function causes sterility.
7 Contains leucine zipper, PHD/LAP domains; homologous to human AF10.
8 Homology to human glioma-amplified sequence 41, yeast transcription factor AF-9, and human transcription factor ENL.
9 Related to transcription factor SOX5.
10 Loss of gene function causes sterility.

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We will focus this report on the role of CSR-1, EKL-1, and DRH-3 in meiotic silencing and will discuss SIN-3 in a future report.

Meiotic H3K9me2 distribution in csr-1, ekl-1, and dru-3 mutants

In wild type males, the X chromosome preferentially becomes enriched for H3K9me2 during early pachytene stage and maintains this enrichment until germ cells become primary spermatocytes [4] (Figure 2A). Other chromosomes exhibit a relatively low level of H3K9me2. In ego-1 null mutant males (hereafter designated ego-1 males), X chromosome enrichment fails to occur, and all chromosomes accumulate a variable and low level of H3K9me2 [9] (Figure 2C). In csr-1(tm892), ekl-1(om83), and dru-3(tm1217) males, H3K9me2 was distributed more broadly across the chromosomes than in controls, and a single focus was rarely observed (Figure 2B, 2D, 2E). Several foci were visible in some nuclei, which also tended to have a higher overall level of the mark. We quantified the relative proportion of nuclei with each labeling pattern, and the proportion of nuclei with normal meiotic versus abnormal chromosomal morphology (Table 2). It was difficult to quantify the H3K9me2 level since the labeling intensity varied even among control preparations. However, we obtained a general measure of labeling intensity by comparing images captured at equivalent exposures. The majority of nuclei with normal pachytene morphology lacked a strong focus of H3K9me2 labeling when compared with wild type (ranging from 57% of pachytene nuclei in euchromatic regions that failed to accumulate H3K4me2 and were presumably the X chromosomes (Figure 3). We consistently observed a chromosome without H3K4me2, which presumably corresponds to the X chromosome (Figure 3). We observed a variable level of H3K9me2 associated with this chromosome; in many nuclei, the level was substantially reduced compared with controls. We also observed frequent H3K9me2 enrichment co-localizing with H3K4me2 (Figure 3). We interpreted this phenotype to reflect enrichment for H3K9me2 on autosomal sites in the absence of CSR-1, EKL-1, or DRH-3 activity.

To identify the X chromosome, we co-labeled H3K9me2 and a histone “activating” mark that is present on autosomes but absent from the X chromosomes in germ cells, H3K4me2 [4, 53]. We consistently observed a chromosome without H3K4me2, which presumably corresponds to the X chromosome (Figure 3). We observed a variable level of H3K9me2 associated with this chromosome; in many nuclei, the level was substantially reduced compared with controls. We also observed frequent H3K9me2 enrichment co-localizing with H3K4me2 (Figure 3). We interpreted this phenotype to reflect enrichment for H3K9me2 on autosomal sites in the absence of CSR-1, EKL-1, or DRH-3 activity.

In contrast to the male (XO) germ line, we observed no obvious defect in H3K9me2 accumulation in mutant hermaphrodite (XX) germ lines (data not shown). We considered two possibilities for why this might be the case: CSR-1, EKL-1, and DRH-3 function might affect chromatin assembly only in male germ cells or, alternatively, only in germ cells with significant unpaired chromatin (e.g., unpaired chromosomes or a chromosomal duplication). To distinguish between these hypotheses, we examined H3K9me2 accumulation in mutant hermaphrodites where the X chromosomes did not pair or synapse (genotype ekl-1(om83); him-8 and dru-3(tm1276); him-8) and in hermaphrodites carrying a free chromosomal duplication (genotype ekl-1;Dp3, and dru-3;Dp5). In these five strains, H3K9me2 foci were reduced in intensity relative to controls, and there appeared to be a mild increase in H3K9me2 levels on other chromatin (Figure 4). To distinguish between autosomes and X chromosomes in dru-3;him-8 and ekl-1;him-8 hermaphrodites, we co-labeled H3K4me2 and H3K9me2 marks. In both control and experimental animals, we consistently identified chromosomal regions that failed to accumulate H3K4me2 and were presumably the X chromosomes (Figure S1). Consistent with the data presented in Figure 4, these H3K4me2-negative regions were highly enriched for H3K9me2 in him-8 controls, but much less so in the mutants. These results suggest that unpaired regions are not as highly targeted for H3K9me2 in carceral and ekl-1, and dru-3 hermaphrodites as they are in wildtype hermaphrodites.
**csr-1, ekl-1, and drh-3 mutants have multiple germline developmental defects**

We compared the developmental phenotypes of *ego-1, csr-1, ekl-1* and *drh-3* mutants in order to address further the functional relationship among the four gene products (see Materials and Methods). As discussed above, loss-of-function mutations in each gene enhanced a mild GLP-1/Notch defect in the germ line. We observed additional germline defects in young adult hermaphrodites and males of each genotype, as follows: a moderately reduced number of germ cells; a larger than normal proportion of...
Table 2. Meiotic H3K9me2 distribution patterns in csr-1, ekl-1, and drh-3 mutant males.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nuclear morphology</th>
<th>H3K9me2 label:</th>
<th>Elevated</th>
<th>Dispersed</th>
<th>1 focus</th>
<th>N</th>
</tr>
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<td>csr-1</td>
<td>pachytene</td>
<td>34%</td>
<td>57%</td>
<td>9%</td>
<td></td>
<td>164</td>
</tr>
<tr>
<td></td>
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<td>78%</td>
<td>22%</td>
<td>0%</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>ekl-1;him-8</td>
<td>pachytene</td>
<td>14%</td>
<td>83%</td>
<td>3%</td>
<td></td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>abnormal</td>
<td>85%</td>
<td>15%</td>
<td>0%</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>drh-3;him-8</td>
<td>pachytene</td>
<td>23%</td>
<td>65%</td>
<td>11%</td>
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<td>126</td>
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<tr>
<td></td>
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<td>5</td>
</tr>
</tbody>
</table>

See Results and Figures 2 and 3 for descriptions and representative images of the three H3K9me2 distribution patterns (elevated, dispersed, and single focus). “1 focus” indicates that a single strong focus of H3K9me2 labeling was observed. N, number of nuclei counted.

1These nuclei have recognizable pachytene morphology.
2These abnormal nuclei are large and have a diffuse chromosomal morphology not typical of meiosis. The percent of nuclei within the pachytene zone with this morphology was: 10% for csr-1; 6% for ekl-1;him-8; and 4% for drh-3;him-8.

doi:10.1371/journal.pgen.1000624.t002

Figure 3. Relative distribution of H3K4me2 and H3K9me2 marks during XO meiosis. Each panel shows meiotic nuclei co-labeled with DAPI to visualize DNA, polyclonal antibody against H3K4me2, and monoclonal antibody against H3K9me2. In wildtype germ cells, the X chromosome (arrow) lacks H3K4me2 (*) and becomes highly enriched for H3K9me2 (arrow). In contrast, autosomes become highly enriched for H3K4me2 and accumulate a very low level of H3K9me2. In csr-1, ekl-1, and drh-3 nuclei, one chromosomal region lacks H3K4me2 (*) and contains a variable level of H3K9me2 (arrow); this is presumably the X chromosome. In some cases, the X chromosome lacks detectable H3K9me2 altogether (*). Other chromosomes contain substantial H3K4me2 and a variable level of H3K9me2 (arrowheads). Circled nuclei are examples of the “elevated” H3K9me2 pattern referred to in Table 2; most other nuclei have the “dispersed” H3K9me2 pattern referred to in Table 2. See Results. Images were captured on a Zeiss Axioscope at the same exposure and processed in a similar manner.

doi:10.1371/journal.pgen.1000624.g003
leptotene-zygotene nuclei; a smaller than normal proportion of pachytene nuclei; a delay in the sperm-oocyte switch; and abnormal oogenesis (Figure 5D, 5G, 5H, Table 3, data not shown).

Ekl-1, Csr-1, and Drh-3 germ lines contained some large nuclei with a diffuse chromosomal morphology quite distinct from pachytene and diplotene nuclei; we previously observed morphologically similar nuclei in ego-1 mutants [35,54] (see Figures 2 and 3, Table 2). 100% of hermaphrodites produced abnormal, small oocytes and 100% of their progeny died as embryos. As adults aged, oocytes tended to back up around the loop and there was a reduction in the proportion of the germ line in mitosis and first meiotic prophase. These observations are consistent with previous reports of sterility in csr-1 mutants [40,42,55] and ekl-1 mutants [40] and an oogenesis defect in dbh-3 mutants [34,56].

Wild type oocytes arrest at diakinesis with six pairs of bivalents visible per nucleus (Figure 5A). In csr-1, ekl-1 and dbh-3 mutants, a subset of oocyte nuclei appeared to contain unpaired homologous chromosomes (univalents), as previously observed for ego-1 (Figure 5H, and data not shown) [37]. The penetrance of this phenotype was variable with respect to the number of univalents per nucleus and the proportion of diakinesis nuclei with this abnormal morphology. The penetrance was more penetrant in ekl-1 mutants (50% of gonad arms contained at least one oocyte with univalents) than in ego-1, csr-1, or dbh-3 mutants (≤18% of gonad arms contained oocytes with univalents) (Table 3) [37]. The presence of univalents was rarely fully penetrant within any single oocyte; instead, for individual mutants, the number of univalents per nucleus and the proportion of diakinesis nuclei with this abnormal morphology was consistent with previous reports of sterility in csr-1 mutants [40,42,55] and ekl-1 mutants [40] and an oogenesis defect in dbh-3 mutants [34,56].

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Analysis of double mutant phenotypes suggested a complex relationship among ego-1, csr-1, ekl-1, and dbh-3 with respect to germline development. (See Materials and Methods for generation of double mutants.) Several aspects of the phenotype were more severe in at least a subset of double mutants. For example, the frequency of animals with univalents at diakinesis was higher among ekl-1 dbh-3, ego-1 dbh-3, and ekl-1 ego-1 double mutants than in ekl-1, ego-1, and dbh-3 single mutants reflecting either a...
synergistic or additive effect (Table 3). Interestingly, although the frequency of animals showing the phenotype increased, the degree of asynapsis in individual nuclei was not significantly higher in double mutants compared with single mutants (Table 3). In contrast, the univalent frequency in *ego-1;csr-1* double mutants was similar to that observed in *csr-1* and *ego-1* single mutants (Table 3). We observed the sperm condensation defect in *ekl-1 ego-1* and *ego-1 drh-3* double mutants, indicating it is epistatic to the more normal sperm morphology present in *ego-1* single mutants (Figure 5E and 5F'). Interestingly, we observed a similar, although less severe, condensation defect in a subset of *ego-1;csr-1* double mutants (Table 3). The implications of these double mutant phenotypes are considered in the Discussion.

Pairing, synapsis, and meiotic H3K9me2 levels

The presence of univalent chromosomes in *csr-1*, *ekl-1*, and *drh-3* diakinesis nuclei was particularly relevant to the H3K9me2 defect. In *C. elegans*, univalents can result from defective homolog pairing, synapsis, and/or double-strand break (DSB) formation [64,65]. Both pairing and synapsis have been implicated as important in the process by which meiotic silencing is triggered, whereas DSB formation/repair has not: H3K9me2 enrichment is observed on autosomes in XO mutants with pairing and/or synapsis defects, but not in mutants defective only in double-strand break formation (A. Fedotov and W. Kelly, manuscript in preparation). Therefore, we considered that autosomal H3K9me2 levels might be elevated.

Figure 5. Examples of gametogenesis defects associated with loss of *csr-1*, *drh-3*, and *ekl-1* function. Each panel shows a portion of a dissected gonad arm stained with DAPI to visualize DNA. Known or putative null alleles are used in all cases. (A) Wildtype (N2) hermaphrodite with oocyte nuclei (open arrowheads) and sperm (Sp, line) are indicated. Note that sperm are essentially uniform in size. Oocyte nuclei at diakinesis have six bivalent chromosomes. (B) *ego-1* sperm are essentially uniform in size. (C) *ekl-1drh-3*, (D) *ego-1drh-3*, and (H) *ekl-1* germ lines contain small, crowded oocytes with univalent chromosomes (closed arrowheads). (C) *ekl-1drh-3*, (D) *drh-3*, (E) *ego-1drh-3*, and (F) (some) *ekl-1 ego-1* germ lines contain variably sized sperm (arrows). (F) Some *ekl-1 ego-1* germ lines contain sperm of essentially uniform size. (E and G) Morphologically abnormal nuclei are visible amidst diakinesis (oocyte) nuclei in *ego-1drh-3* and *csr-1* germ lines (fat arrows). In addition, *csr-1* meiotic nuclei are often clumped together, leaving gaps without nuclei (bracket). doi:10.1371/journal.pgen.1000624.g005
Table 3. Gametogenesis defects associated with csr-1, ekl-1, drh-3, and ego-1 single and double mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% No oocytes (N)</th>
<th>% Irregular sperm (N)</th>
<th>% Univalents (N)</th>
<th>#Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>0 (&gt;100)</td>
<td>0 (&gt;100)</td>
<td>0 (&gt;100)</td>
<td>NA</td>
</tr>
<tr>
<td>ego-1</td>
<td>9 (33)</td>
<td>0 (33)</td>
<td>18 (11)</td>
<td>7.7±0.2</td>
</tr>
<tr>
<td>ekl-1</td>
<td>0 (34)</td>
<td>0 (34)</td>
<td>18 (11)</td>
<td>7.3±0.3</td>
</tr>
<tr>
<td>drh-3</td>
<td>100 (43)</td>
<td>9 (11)</td>
<td>8.4±0.4</td>
<td>7.11</td>
</tr>
<tr>
<td>ekl-1 drh-3</td>
<td>100 (50)</td>
<td>50 (32)</td>
<td>9.7±0.2</td>
<td>9.10</td>
</tr>
<tr>
<td>ekl-1 ego-1</td>
<td>100 (36)</td>
<td>69 (13)</td>
<td>9.6±0.3</td>
<td>7.11</td>
</tr>
<tr>
<td>ego-1; csr-1</td>
<td>56 (34)</td>
<td>17 (18)</td>
<td>8.8±0.5</td>
<td>7.12</td>
</tr>
<tr>
<td>ekl-1; drh-3</td>
<td>27 (59)</td>
<td>92 (26)</td>
<td>9.0±0.4</td>
<td>7.11</td>
</tr>
<tr>
<td>ekl-1; csr-1</td>
<td>7 (42)</td>
<td>100 (21)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Alleles used were ego-1(om84), ekl-1(om83), drh-3(tm1276), and csr-1(tm892). Animals were grown at 20°C and characterized at 24 hr post-L4 stage. At a slightly later time point (66–72 hr post-L1 stage), most animals contain oocytes, hence the absence of oocytes indicates a delay in the sperm-to-oocyte switch. (N), number of germ lines counted.

1Percent of germ lines where oocytes were absent at the time of assay; note that wildtype germ lines all contain oocytes at this stage.
2Percent of germ lines where sperm nuclear morphology was highly irregular.
3Abnormal sperm morphology was less severe than for the other genotypes (see Results).
4Number of chromosomes at diakinesis in nuclei with at least one set of univalent chromosomes. Nuclei with 6 bivalents are not included in the calculation. Standard error of the mean (±) is indicated. The range of observed values is indicated in brackets; e.g., among ego-1 nuclei with univalent chromosomes, a range of 7–9 chromosomes were observed. NA, not applicable. ND, univalents were observed, but not counted.

We also evaluated whether H3K9me2 accumulates at synapsed chromatin in csr-1, ekl-1, and drh-3 mutant males. To do so, we co-labeled H3K9me2 and SYP-1 (see Materials and Methods). In wild type males, we consistently observed a single chromosomal region that failed to accumulate SYP-1 and was highly enriched for H3K9me2 (Figure 7). In csr-1, ekl-1, and drh-3 mutant males, we typically observed a single SYP-1(-) region that accumulated a variable level of H3K9me2. In addition, we observed H3K9me2 at other chromosomal regions that contained SYP-1 (Figure 7). At the limit of sensitivity of our data, these results are consistent with the hypothesis that elevated H3K9me2 accumulation occurs at synapsed regions in these germ cells.

EGO-1 is not required for the elevated autosomal H3K9me2 observed in csr-1, ekl-1, and drh-3 mutants

Our previous work indicated that the loss of EGO-1 activity prevents H3K9me2 accumulation on unpaired chromatin [9]. Here, we tested whether EGO-1 activity is required for ectopic H3K9me2 accumulation by determining the H3K9me2 distribution in eko-1;csr-1, eko-1;drh-3, and eko-1;ego-1 double mutant males. The H3K9me2 distribution in all three double mutants resembled the distribution we observed in csr-1, eko-1, and drh-3 single mutant males (Figure 2F, 2G, 2H). Therefore, EGO-1 activity is not necessary for ectopic H3K9me2 to accumulate on autosomes. Moreover, since EGO-1 is required for the H3K9me2 accumulation diagnostic of meiotic silencing, this result strengthens our conclusion that the autosomal H3K9me2 in csr-1, eko-1, and drh-3 males is mis-targeted to paired chromatin. We also note that, in double mutants such as eko-1;drh-3, the H3K9me2 distribution resembled that observed in the two corresponding single mutants (Figure 2I, and data not shown).

Loss of HIM-17 function combined with CSR-1, EKL-1, or DRH-3 function produces severely abnormal germ lines

We sought to determine whether the pattern of elevated autosomal H3K9me2 in csr-1, eko-1, and drh-3 mutants depended...
on HIM-17 activity. HIM-17 is a chromatin-associated protein reported to be required for normal accumulation of H3K9me2 per se in both XX and XO germ lines [69]. We constructed him-17;csr-1, ekl-1;him-17, and drh-3;him-17 double mutants and found that they have severe germline defects similar to those previously described for ego-1;him-17 double mutants [9] (data not shown). Unfortunately, nuclear morphology was abnormal throughout the severely impaired germ line, prohibiting meaningful interpretation of the H3K9me2 labeling pattern.

Discussion

Here, we demonstrate that CSR-1, EKL-1, and DRH-3 activities promote the normal accumulation of a chromatin silencing modification, H3K9me2, during meiosis. Our data suggest that, in csr-1, ekl-1, and drh-3 mutants, H3K9me2 fails to accumulate to normal levels on chromatin that is unpaired and unsynapsed (e.g., the single male X and the two him-8 hermaphrodite X chromosomes) and accumulates inappropriately on chromatin that is both paired and synapsed (e.g., autosomes). We interpret these findings to mean that the normal targeting mechanism is disrupted in csr-1, ekl-1, and drh-3 mutants. Therefore, CSR-1, EKL-1, and DRH-3 act, directly or indirectly, to target H3K9me2 to appropriate (unpaired) sites and/or prevent accumulation at inappropriate (paired) sites.

Alternative models for the regulation of H3K9me2 accumulation

Biochemical analysis of CSR-1 and DRH-3 has provided direct insight into their functions. AGO proteins are known to localize to target RNAs via interaction with a siRNA “guide” molecule [38]. Using in vitro assays, Aoki et al. [46] demonstrated that CSR-1 has Slicer endonuclease activity and binds to secondary (2') siRNAs that are produced as a consequence of RdRP activity on target mRNA during the RNAi process. DRH-3 activity promotes the formation of diverse classes of small RNAs [34,35]. In vitro, DRH-3 interacts physically with the somatic RdRP, RRF-1, and is required for 2' siRNA production [46]. By analogy, we hypothesize that DRH-3 may promote EGO-1 activity in the germ line.

Although little is known about the biochemical function of EKL-1, we hypothesize that it may bind methylated proteins via its Tudor domains [70]. Tudor domains from several mammalian proteins have been shown to bind methylated peptides in vitro, specifically peptides corresponding to histone H3 tails methylated at either lysine 4 or 9 and histone H4 tail methylated at lysine 20 [71]. Similarly, the DNA repair function of Saccharomyces cerevisiae RAD9 apparently requires binding to methylated H3 lysine 79 via its Tudor domain [72].

We consider two general models for how an EGO-1/CSR-1/EKL-1/DRH-3 pathway might function in meiotic chromatin regulation. One model is that these factors directly target the chromatin-modifying machinery to unpaired regions, perhaps via a mechanism similar to that which directs H3K9me2 to centromeric repeats in S. pombe. There is increasing evidence that siRNAs and other small RNAs participate in transcriptional silencing in many organisms, although thus far the mechanisms are poorly understood [19]. Ultimately, this pathway may establish a self-amplifying loop to attract histone methyltransferase (HMTase) to unpaired chromatin and/or exclude HMTase from paired regions.
activity from paired chromatin. We speculate that chromatin-associated RNA pol II transcripts [73–76] act as templates for EGO-1 RdRP activity and are essential for establishment of the self-amplifying loop. One possible scenario is that all or a subset of these proteins are initially recruited to unpaired chromatin via interaction with a factor that is lost or masked by successful pairing and/or synapsis. As an amplification loop is established, the HMTase is preferentially recruited to unpaired regions. In the absence of EGO-1 activity, the HMTase may be recruited to specific sites but be unable to methylate chromatin effectively. In the absence of CSR-1, EKL-1, or DRH-3 activity, the HMTase may not be properly recruited or retained and therefore be free to modify chromatin in an unregulated manner, perhaps through enhanced interaction with another competing complex.

As an alternative model, EGO-1, CSR-1, EKL-1, and DRH-3 may influence H3K9me2 by participating in post-transcriptional and/or transcriptional silencing mechanisms that ultimately regulate the expression of genes whose products discriminate between paired and unpaired chromatin. This model is complicated by the fact that we would expect direct targets of such a hypothetical silencing mechanism to be up-regulated upon loss of silencing activity. Therefore, we propose that loss of silencing activity would indirectly down-regulate the discriminatory factors, perhaps by allowing over-expression of a negative regulator. EGO-1 might regulate a different constellation of genes than do CSR-1, EKL-1, and DRH-3, resulting in the different H3K9me2 patterns in ego-1 versus csr-1, drh-3, and ekl-1 mutants. Identification of specific sites on unpaired chromatin that are targeted for H3K9me2 accumulation, and investigation of whether EGO-1, CSR-1, EKL-1, and/or DRH-3 associate with those sites will help to distinguish between these alternative models.

A larger regulatory framework for EGO-1 activity

Our phenotypic analysis of CSR-1, EKL-1, and DRH-3 suggests that they participate in a complex regulatory network to promote development of the germ line. Their activity is critical for maintenance of germline proliferation, meiotic progression, spermatogenesis, and oogenesis. Previous reports in the literature have indicated that EGO-1, EKL-1, DRH-3, and CSR-1 may promote other aspects of development, including embryonic viability and proper chromosome segregation [34,42,58]. Most strikingly, Rocheleau et al. [63] demonstrated that reduction in function of each of these four genes enhanced the lethality of a weak ksr-1 (kinase suppressor of ras) mutation. ksr-1 lethality results from a defect in excretory duct formation due to impaired Ras signaling [77]. Rocheleau et al. proposed that EGO-1, CSR-1, DRH-3, and EKL-1 may affect the development of the excretory duct cell by promoting the biogenesis/activity of a set of germline small RNAs whose activity ultimately regulates expression of factors important for the KSR-1/KSR-2 Ras-ERK signaling pathway. We have now demonstrated the importance of this non-coding RNA pathway in meiotic chromatin regulation and other aspects of germine development.

Our genetic data suggest that EGO-1, CSR-1, EKL-1, and DRH-3 participate in a complex regulatory network. Based on strict epistasis criteria, EGO-1 and CSR-1 act in a common genetic pathway to promote bivalent stability at diakinesis, and this pathway works in parallel with DRH-3 and EKL-1 pathways. Given what is known about the biochemical functions of these proteins, perhaps the simplest way to think about these genetic pathways is that they may involve distinct classes of small RNAs (e.g., [35,45,78]). The EGO-1/CSR-1/EKL-1/DRH-3 pathway
may be responsible for biogenesis/function of one class of small RNA, while other classes of small RNA may require EKL-1 and/or DRH-3, but rely on a different RdRP and/or AGO protein in place of EGO-1 and CSR-1. Indeed, DRH-3 is required for production of many classes of small RNAs, while individual RdRPs function in biogenesis of a subset of such RNAs [34,35]. Analysis of sperm nuclear morphological defects also suggested a complex pattern of regulation by multiple small RNA-mediated pathways. In this case, the most important pathway(s) require(s) DRH-3 and EKL-1 activity while EGO-1 and CSR-1 activity appear to play only a minor role in this process. Hence, analysis of mutant phenotypes can provide insight into the relationships among different small RNA-mediated pathways.

**Materials and Methods**

**Nematode strains and culture**

*C. elegans* strains were cultured using standard methods as described [79]. *C. elegans* var Bristol (N2) is the wild-type parent strain of all of the mutants used in this study. The following mutations, chromosomal deficiencies, duplications, and reciprocal translocations were used: LG (linkage group) I: C04F12.1 tm1217, dbh-3(tm1217), dbh-3(tm53) (this report), ego-1(tm84), ekl-1(tm56, om53) (this report), F55A12.1 ok1078, R06C7.1 ok1074, ppc-1(p1425), ppc-2(tm1120), pgf-1(tm672), sgo-2(tm904), sin-3(tm1276), T23D8.7 tm1163, unc-13(e51), unc-13(e73), unc-53(e402), sDf5, nd25; LG II: alg-2(ok304), C06A1.4 tm807, F56G1.1 tm1019, Y4965.1 tm127, ZK1428.7 tm1155; LG III: C16C10.3 tm1290, tag-76(ok4114), unc-32(e189), zfp-1(ok554), sDf125, sDf3 (III), kT2(bli-4(e357) let-3702) qD89(III); LG IV: cws-1(tm892), dhr-1(tm1329), dhr-2(tm270), gfl-1(gk321), him-8(e1489), him-17(e2006), ok424, M03D4.6 tm1144, pgf-2(tm1094), T22B3.2 tm1155, nT1(qg57)/IV; LG V: ego-1(tm1800), sgo-1(tm1953), T22H9.3 tm1156, ZK218.5 tm1524, LG X: RHA9.2 tm1116. The tm, ok, and gk alleles are deletions and therefore likely to be null or extreme loss-of-function. ego-1(tm84) is a protein null [54]. ekl-1(ok304) is a deletion allele (this report). An integrated transgenic array, ckl-1(3my-3;Nglp-lacZ:myo-3;Mglp), was used as an LGI marker. Information on specific genes and alleles can be found at Wormbase (http://www.wormbase.org) unless otherwise noted.

Multiple mutant strains were generated using standard genetic strategies. PCR analysis was routinely used to confirm the presence of deletion mutations. The following strategy was used to build cis-doubles. To generate ego-1(tm84) dhr-3(tm1217) double mutants, we generated an ego-1(tm84) unc-53(e402)/unc-13(e51) dhr-3(tm1217) male/hermaphrodite strain and mated non-Unc males with unc-13(e51) unc-53(e402) hermaphrodites. Non-Unc-13, non-Unc-53 progeny were recovered; PCR analysis was used to identify the lines carrying both ego-1(tm84) and dhr-3(tm1217) deletions (i.e., ego-1 dhr-3/unc-13 unc-53). The ego-1(tm84) dhr-3(tm1217) chromosome was then balanced with kT2(bli-4(e357) let-3702) qD89(III), ekl-1(ok304), ego-1(tm84) and ekl-1(tm56) dhr-3(tm1217) double mutants were constructed by the same general strategy (using different marker mutations in one case).

**RNAi**

RNAi was done by the feeding method as described [80] except that double strand RNA production was sometimes induced by 0.2% lactose rather than 1 mM IPTG. Multiple L4 N2 and gfp-1(bn18) hermaphrodites were placed onto each bacterial "feeding" strain at 25°C and 20°C, respectively. Adult F1 progeny were scored for sterility using a dissecting microscope. Steriles were examined at high magnification as described [81] to determine whether they had a Glp-1 sterile phenotype (premature meiotic death of all germ cells).

**Single nucleotide polymorphism mapping and DNA sequencing**

ego mutations om55, om56, and om83 were recovered in genetic screens for enhancers of gfp-1(bn18) as previously described [36] using either ethylmethylene sulfonate (EMS) (om55, om56) or trimethylpsoralein/UV irradiation (om83) as the mutagen. Three-factor and deletion mapping placed the three mutations on the right arm of LGI. Based on complementation tests, om56 and om83 comprise a single complementation group while om53 comprises another.

Three-factor mapping placed om56 and om83 between dpy-3 and unc-13. We subsequently mapped om56 relative to single nucleotide polymorphisms (SNPs), ultimately localizing it between SNPs at nucleotide position ~7050 K and 7120 K. This interval was predicted to encode 19 genes, including ekl-1 (see www.wormbase.org). DNA from the ekl-1 gene region was amplified from ego(om83) and ego(om56) mutants and sequenced. In ego(om83) animals, the ekl-1 open reading frame (ORF) contained a 110 nucleotide deletion and concomitant single nucleotide insertion (at the deletion site); the net 109 nucleotide deletion is predicted to shift the ORF, resulting in production of a truncated product comprising 314 amino acids (Figure 1A). In ego(om56) mutants, the ekl-1 ORF contained a single nucleotide substitution, inserting a stop codon for tryptophan 319 (Figure 1A). Primers used to sequence the ekl-1 region were: 5’→3’; ekl-1r cgctgggagagagctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctgatgctg atagctgaagcttgccacgg; ekl1-4f cgttcatttccaacagattg. DNA from the om55 strain at 25°C was cultured using standard methods as described [36]. ok1078 was used as the wild-type strain at 25°C. The double mutant strain at 25°C was used as the wild-type strain at 25°C.

**Indirect immunofluorescence**

H3K9me2 single labeling was carried out as described [9] using polyclonal anti-H3K9me2 (gift of C. D. Allis) at 1/500 dilution and Alexa488-labeled secondary antibody (Invitrogen) at 1/200 dilution. H3K9me2/SYP-1 double labeling was performed as follows. Gonads were dissected in 8 μL of 0.25 mM levamisole/PBS on a poly-lysine treated slide. 8 μL of 6% paraformaldehyde (PFA)/2X EGG buffer were added to the dissected tissue and a Super-Frost slide (Fisher) immediately placed on top. The slide sandwich was placed on dry ice for 15 minutes, cracked open, and immediately washed with PBST. After a total of 3X 5 min washes in PBST (1X PBS/0.1% Tween-20), the sample was blocked for 30 minutes in 30% goat serum (GS)/PBST, stained for H3K9me2 (1:200 dilution, STD143 gift of A. Villeneuve) were added. H3K9me2 was visualized using either ethylmethane sulfonate (EMS) (om55, om56) or trimethylpsoralein/UV irradiation (om83) as the mutagen. Three-factor and deletion mapping placed the three mutations on the right arm of LGI. Based on complementation tests, om56 and om83 comprise a single complementation group while om53 comprises another.
Fluorescent in situ hybridization

The 5S rDNA probe was generated by amplification of a 1 kb region of the 5S rDNA locus using published primers [82]. The probe was labeled with DIG-11-dUTP using the DIG-Nick Translation Kit (Roche Applied Science). FISH was carried out as described [8]. A 1:200 dilution of anti-Digoxigenin-Fluorescein antibody (Roche Applied Science) was used for probe detection. Samples were examined using a DRMXA fluorescent microscope (Leica); the images were acquired using a CCD camera (Q Imaging) and processed using SimplePCI (Hamamatsu Corporation) software.

Phenotypic characterization

DAPI staining was used to characterize the germ line developmental phenotypes. To avoid variations in germ line morphology caused by aging, animals were harvested at a consistent developmental stage (24 hours post-L4 stage at 20°C). Animals were then dissected to expose the gonad. Fixation and staining were performed as described [83]. Nuclei in mitosis and different stages of meiosis were identified based on nuclear morphology as described [37,83].

Supporting Information

Figure S1 Relative distribution of H3K9me2 and H3K4me2 in him-8 XX germlines. Panels show meiotic nuclei co-labeled with DAPI to visualize DNA and with polyclonal antisera to visualize HIM-3 and SYP-1. HIM-3 associates with all chromosomes. A single region fails to accumulate SYP-1 (arrowheads), which is presumably the X chromosome. The arrow in the csr-1 image indicates an example of the large abnormal nuclei we also observe in ekl-1, drh-3, and ego-1 mutants. See Text S1 for discussion. N, the number of pachytene zone nuclei that were counted.

Table S1 Distribution of LGV FISH signals in csr-1, ekl-1, and drh-3 mutants. In csr-1, ekl-1, and drh-3 mutants, nuclei with abnormal chromosomal morphology are scattered within the pachytene zone as discussed in Text S1. The number of FISH foci was counted in each nucleus within the pachytene zone regardless of chromosomal morphology. Independent values are given for XX and XO germ lines. N, the number of pachytene zone nuclei that were counted.

Table S2 Single LGV FISH signals in morphologically pachytene nuclei. The number of FISH foci was counted in nuclei with recognizable pachytene morphology. Independent values are given for XX and XO germ lines. See Text S1 for discussion. N, number of nuclei counted.

Table S3 The majority of large, diffuse nuclei within the pachytene zone contained multiple FISH foci. The percent of abnormal, large nuclei containing multiple FISH foci is indicated. Independent values are given for XX and XO germ lines. Note that some abnormal nuclei contained only a single FISH signal. See Text S1 for discussion. N, number of nuclei counted. NA, not applicable.

Figure S2 HIM-3 and SYP-1 distribution in csr-1, ekl-1, and drh-3 XO mutants. Each panel shows pachytene nuclei from an XO germ line co-labeled with DAPI to visualize DNA and with polyclonal antisera to visualize HIM-3 and SYP-1. HIM-3 associates with all chromosomes. A single region fails to accumulate SYP-1 (arrowheads), which is presumably the X chromosome. The arrow in the csr-1 image indicates an example of the large abnormal nuclei we also observe in ekl-1, drh-3, and ego-1 mutants. See Text S1 for discussion. N, the number of pachytene zone nuclei that were counted.

Figure S3 Co-localization of HIM-3 and SYP-1 on pachytene chromosomes in XX csr-1 and ekl-1 mutants. Each panel shows pachytene nuclei from an XX germ line co-labeled with DAPI (A,E,I) to visualize DNA and with polyclonal antisera to visualize HIM-3 (B,F,J) and SYP-1 (C,G,K). (D,H,L) Merged SYP-1 and HIM-3 images. (A–D,I–L) HIM-3 and SYP-1 labeling is co-linear in N2 wildtype and ekl-1 nuclei. (E–H) Some csr-1 nuclei contain chromosomal regions with only HIM-3 or only SYP-1 (arrows). (I–J) csr-1 image contains an example of a large, putative “polyploid” nucleus (arrow). Images were captured on a Zeiss LSM 710 confocal microscope.

Figure S4 Co-localization of HIM-3 and SYP-1 on pachytene chromosomes in XX drh-3;him-8 mutants. Each panel shows pachytene nuclei from an XX germ line co-labeled with DAPI (A,E,I) to visualize DNA and with polyclonal antisera to visualize HIM-3 (B,F,J) and SYP-1 (C,G,K). (D,H,L) Merged SYP-1 and HIM-3 images. (A–D,I–L) HIM-3 and SYP-1 labeling is co-linear in N2 wildtype and ekl-1 nuclei. (E–H) Some csr-1 nuclei contain chromosomal regions with only HIM-3 or only SYP-1 (arrows), which presumably correspond to the X chromosomes. Images were captured on a Zeiss LSM 710 confocal microscope.
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Author Contributions

Conceived and designed the experiments: XS WKG EMM. Performed the experiments: XS AXF EMM. Analyzed the data: XS AXF WKG EMM. Contributed reagents/materials/analysis tools: WKG EMM. Wrote the paper: XS EMM.

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