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MRG-1, an autosome-associated protein, silences X-linked genes and protects germline immortality in *Caenorhabditis elegans*

Teruaki Takasaki[1,*], **Zheng Liu**[2,*], **Yasuaki Habara**[1,†], **Kiyoji Nishiwaki**[3], **Jun-ichi Nakayama**[3], **Kunio Inoue**[1], **Hiroshi Sakamoto**[1], and **Susan Strome**[2,‡]

1*Department of Biology, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodaicho, Nadaku, Kobe 657-8501, Japan*

2*Department of Biology, Indiana University, Bloomington, IN 47405, USA*

3*RIKEN Center for Developmental Biology, Chuoku, Kobe 650-0047, Japan*

Abstract

MRG15, a mammalian protein related to the mortality factor MORF4, is required for cell proliferation and embryo survival. Our genetic analysis has revealed that the *Caenorhabditis elegans* ortholog MRG-1 serves similar roles. Maternal MRG-1 promotes embryo survival and is required for proliferation and immortality of the primordial germ cells (PGCs). As expected of a chromodomain protein, MRG-1 associates with chromatin. Unexpectedly, it is concentrated on the autosomes and not detectable on the X chromosomes. This association is not dependent on the autosome-enriched protein MES-4. Focusing on possible roles of MRG-1 in regulating gene expression, we determined that MRG-1 is required to maintain repression in the maternal germ line of transgenes on extrachromosomal arrays, and of several X-linked genes previously shown to depend on MES-4 for repression. MRG-1 is not required for PGCs to acquire transcriptional competence or for the turn-on of expression of several PGC-expressed genes (*pgl-1*, *glh-1*, *glh-4* and *nos-1*). By contrast to this result in PGCs, MRG-1 is required for ectopic expression of those germline genes in somatic cells lacking the NuRD complex component MEP-1. We discuss how an autosome-enriched protein might repress genes on the X chromosome, promote PGC proliferation and survival, and influence the germ versus soma distinction.

Keywords

C. elegans; MRG-1; Germ line; X chromosome silencing

INTRODUCTION

In most sexually reproducing animals, germ cells are the only cells that can give rise to the next generation, and thus are responsible for perpetuation of species. To serve this role, germ cells must preserve the properties of totipotency and immortality. Understanding the molecular mechanisms that ensure the special properties of germ cells remains a central issue in biology.

Studies in the nematode *Caenorhabditis elegans* have identified numerous types of factors required during embryogenesis and early larval stages for the primordial germ cells (PGCs) to develop properly (reviewed by Strome, 2005). The maternally provided factor PIE-1 plays a

‡Author for correspondence (e-mail: sstrome@indiana.edu).
*These authors contributed equally to this work
†Present address: Department of Pediatrics, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuoku, Kobe, 650-0017 Japan

key role, by blocking RNA polymerase II-mediated transcription in the germline blastomeres and protecting those cells from following somatic fates (Mello et al., 1992; Seydoux et al., 1996; Batchelder et al., 1999). The *C. elegans* Nanos homologs NOS-1 and NOS-2 and several Pumilio-related proteins, probably operating as translational regulators, ensure that the PGCs become incorporated into the somatic gonad primordium, remain mitotically quiescent at early stages, and survive at later stages (Subramaniam and Seydoux, 1999). The 'maternal-effect sterile' proteins MES-2, MES-3, MES-4 and MES-6 operate at the level of histone tail modifications to regulate chromatin organization and gene expression in the germ line; MES-4 cooperates with MES-2, MES-3 and MES-6 to repress the X chromosomes in the germ line (Capowski et al., 1991; Fong et al., 2002; Bender et al., 2004; Bender et al., 2006). Their function is required for PGC proliferation and survival.

The *C. elegans* mrg-1 gene was previously identified by RNAi as being required for PGC proliferation (Fujita et al., 2002). The predicted *C. elegans* MRG-1 protein is related to three human proteins: mortality factor MORF4 and two mortality factor-related proteins MRG15 and MRGX. MORF4 induces senescence in human tumor cell lines and therefore appears to oppose immortality (Bertram et al., 1999). Based on analysis of MRG knockout mice, MRG15 promotes cell proliferation and is essential for embryo survival, whereas MRGX is not required for viability or fertility (Tominaga et al., 2005a; Tominaga et al., 2005b). *Caenorhabditis elegans* MRG-1 is considered to be an ortholog of MRG15, although MRG-1 shows lower sequence similarity (26% identity, 50% similarity) to human MRG15 than do the homologs in the other 17 species examined (Bertram and Pereira-Smith, 2001). Notably, MRG-1, like MRG15, possesses a chromodomain.

The presence of a chromodomain in MRG-1 suggests that it associates with chromatin, specifically with methylated histone tails, as has been demonstrated for several chromodomain-containing proteins. For example, heterochromatin protein 1 (HP1) binds H3 tails methylated on Lys9 (H3K9), Polycomb (Pc) binds methylated H3K27, and Eaf3 binds methylated H3K36 (Bannister et al., 2001; Lachner et al., 2001; Cao et al., 2002; Czermin et al., 2002; Carrozza et al., 2005; Keogh et al., 2005). Among the candidate *C. elegans* proteins for creating the methyl marks that recruit MRG-1 are the MES proteins. MES-2 operates in a complex with MES-3 and MES-6 to methylate H3K27 (Bender et al., 2004; Ketel et al., 2005), and MES-4 methylates H3K36 (Bender et al., 2006).

To further understand the role of MRG-1 in cell proliferation and development, we isolated and analyzed three *mrg-1* deletion mutants. Loss of maternal MRG-1, like loss of mouse MRG15, leads to significant levels of embryonic lethality. Surviving embryos develop into apparently healthy adults that lack a germ line; the latter is a result of failure of PGCs to proliferate and also PGC degeneration. As predicted, MRG-1 is associated with chromatin. Intriguingly, it is only detected on the autosomes and not on the X chromosomes. This pattern resembles that of MES-4, and yet neither MRG-1 nor MES-4 depends on the other for its chromosomal association. Studies of gene expression patterns suggest that MRG-1 is not essential for activation of germline-expressed genes in *mrg-1* mutant larvae but is needed for gene silencing in the germ lines of their mothers. Specifically, transgenes and genes on the X are de-repressed in mutant mothers. This finding, and the differential sensitivity of XX and XO worms to loss of MRG-1 function, points to the X chromosome as a likely target of MRG-1 regulation during germline development. MRG-1 also can serve an important role in somatic cells, as loss of MRG-1 function suppresses the ectopic expression of several germline genes and the larval lethality caused by loss of the chromatin regulator MEP-1. Our results provide insights into chromatin-level regulation of germline potential and immortality.

MATERIALS AND METHODS

C. elegans strains

Wild type was *C. elegans* Bristol strain N2. The following mutations, deficiencies and balancers were used: LGI, *dpy-5*(*e61*), *lin-6*(*e1466*), *hT2*[*bli-4*(*e937*)*let*(*q782*)*qIs48*](*I;III*); LGII, *mes-2(bn11), unc-4(e120), mnC1[dpy-10(e128)unc-52(e444)*]; LGIII, *ced-4(n1162), dpy-18(e364), glp-1(q339*), *mrg-1(ok1262, qa6200, tm1227*), *unc-46(e177), unc-64(e246*), *unc-119(ed3), eT1(III;V), hT2[bli-4(e937)let(q782)qIs48](I;III), qC1[dpy-19(e1259)glp-1 (q339)qIs26(lag-2::GFP)](III:V)*; LGIV, *ced-3(n717), pgl-1(bn101, bn102), DnT1[unc(n754) let qIs50](IV;V)*; LGV, *dpy-11(e224), mes-4(bn67), sDf26, sDf46, eT1 (III;V), DnT1[unc (n754)let qIs50](IV;V)*.

Isolation of *mrg-1* deletion alleles

The deletion allele *mrg-1(qa6200)* was isolated by screening a library of UV/TMP-mutagenized worms by two sequential rounds of PCR. First round primers were 5′-AAGGGCATTCTTCACTGTTGGA-3′ and 5′-GATAAATGGCCGCTGAAACTTG-3′. Second round primers were 5′-TTGTTCACAACTTTCACCGGCT-3′ and 5′-CTCGGCCATGGGCTAGAAAC-3′. The *tm1227* and *ok1262* alleles were isolated using similar PCR screens by Dr Shohei Mitani at the National BioResource Project and by the *C. elegans* Gene Knockout Consortium, respectively. Each *mrg-1* allele was backcrossed to wild type ten times and then balanced with either *eT1* or *qC1[qIs26]*.

Sequencing *mrg-1* mutations

DNA was prepared from sterile homozygous *mrg-1(ok1262)* worms. PCR primers 5′-GAAGATCGTCTGGGATGGAA-3′ and 5′-AGCGATGGCAAGGAACTCTA-3′ amplified a ~1.5 kb product. Sequencing primers 5′-GAGCAAATGAGAACGGTCGATGATCTGC-3′ and 5′-GTTCGA-TGGAGCGCGCTTGCATTATTTTC-3′ were used in standard ABI BigDye sequencing reactions. For *mrg-1(qa6200)* and *mrg-1(tm1227)*, DNA was prepared from heterozygous worms. From both, PCR primers 5′-AAGGGCATTCTTCACTGTTGGA-3′ and 5′-GATAAATGGCCGCTGAAACTTG-3′ amplified a ~1.3 kb product and a ~0.9 kb product. Both DNA bands were gel purified and sequenced using the primers used for PCR.

Immunofluorescence analysis

Worms and embryos were fixed using a paraformaldehyde and methanol procedure (Subramaniam and Seydoux, 1999) or a methanol and acetone procedure (Strome and Wood, 1983). Primary antibodies used were: affinity-purified rabbit anti-MRG-1 fusion protein at 1:400–1:1000 (Fujita et al., 2002), affinity-purified rat anti-MRG-1 peptide (raised against the C-terminal 24 amino acids conjugated to keyhole limpet hemocyanin) at 1:5, affinity-purified rabbit anti-MES-4 at 1:100 (Bender et al., 2006), mouse monoclonal antibody H5 to RNA Pol II pSer2 at 1:25 (Covance), mouse monoclonal antibody PA3 at 1:500 [from M. Monestier (Monestier et al., 1994)], rabbit anti-H3K27me3 at 1:1000 [from Y. Zhang (Plath et al., 2003)], rabbit anti-H3K36me2 at 1:200 [from Y. Zhang (Tsukada et al., 2006)], rabbit anti-PGL-1 at 1:100,000 (Kawasaki et al., 1998), mouse monoclonal anti-PGL-1 antibody K76 (Strome and Wood, 1983) and affinity-purified rabbit anti-GLH-1 at 1:300 (Gruidl et al., 1996). Secondary antibodies used were Alexa Fluor 488, 546, 594, 647 and TRITC goat anti-mouse, anti-rabbit and anti-rat IgGs (from Molecular Probes). Samples were mounted in Gelutol or SlowFade (Molecular Probes) and examined by fluorescence microscopy and Nomarski optics on an Olympus BX51 microscope, a Nikon Eclipse TE200 microscope with an UltraVIEW LCI spinning-disk confocal laser and UltraVIEW software (Perkin-Elmer), or a Nikon Eclipse E800 microscope with Metamorph software (Universal Imaging Corp.). Images were assembled using Adobe Photoshop 7.0 and Illustrator 10.0.3.

Generation of transgenic animals containing a *pie-1 promoter::gfp::mrg-1* transgene

mrg-1 coding sequence, PCR amplified from a *mrg-1* cDNA clone, was transferred using Gateway technology into the vector pID3.01, which contains the *unc-119*(+) gene [from G. Seydoux (Poteryaev et al., 2005)]. The final clone was bombarded into *unc-119* worms (Praitis et al., 2001). Transgenic lines were identified by rescue of the Unc phenotype and by observing GFP expression in the germ line.

Testing for expression of a repetitive extrachromosomal array in the germ line

The extrachromosomal array pBK48.1, which contains many copies of GFP-tagged *let-858* driven by its own promoter (Kelly et al., 1997; Kelly and Fire, 1998), was introduced via genetic crosses into *dpy-18 mrg-1(ok1262)/eT1*. Expression of the *let-858::gfp* array in *dpy-18 mrg-1* M+Z− adult hermaphrodites was scored at 20°C by fluorescence microscopy.

Quantification of mRNA levels in larvae and dissected adult gonads

For the analysis shown in Fig. 7, total RNA was isolated from ~300 L1 and L2 larvae and reverse transcribed using oligo dT primer and High-Capacity cDNA Archive Kit (Applied Biosystems). RT-PCR was performed in triplicate using SYBR Green PCR Mastermix (Applied Biosystems) and an Applied Biosystems 7300 Real-Time PCR system. Primer Express 3.0 software (Applied Biosystems) was used to design primers for *pgl-1*, *glh-1*, *nos-1*, *myo-2* and *rpa-1*. All data were normalized to *rpa-1*, which encodes ribosomal subunit protein P1. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative fold changes, as described in an Applied Biosystems user bulletin.

For the analysis shown in Fig. 6, wild-type and *mrg-1(qa6200)* M+Z− hermaphrodites were incubated in a drop of M9 buffer overnight. Approximately 3000 of their wild-type and *mrg-1 (qa6200)* M−Z− L1 larvae were collected and fed for 6 hours. cDNA preparation, RT-PCR performed in triplicate and Pfaffl analysis were done as described in Bender et al. (Bender et al., 2006), but using a Stratagene MX3000p QPCR system.

For the analysis shown in Fig. 8, 50 gonad arms were dissected from wild-type and *mrg-1 (qa6200)* M+Z− young adult hermaphrodites and total RNA isolated as previously described (Chi and Reinke, 2006;Bender et al., 2006). cDNA preparation, primer design, RT-PCR performed in triplicate and Pfaffl analysis were done as described in Bender et al. (Bender et al., 2006), but using a Stratagene MX3000p QPCR system.

Western blot analysis

Approximately 100 N2, *dpy-18 mrg-1(ok1262)* M+Z−, and *mrg-1(tm1227)* M+Z− homozygous worms boiled in SDS sample buffer were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Antibodies used were affinity-purified rabbit anti-MRG-1 (1:500), mouse monoclonal anti-α-tubulin (1:2000, Sigma) and horseradish peroxidase-conjugated goat secondaries (1:10,000, Jackson Laboratories). Horseradish peroxidase was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

Isolation of *mrg-1* mutant alleles

Based on previous RNAi analysis, loss of MRG-1 activity results in germ cell proliferation defects and sterility (Fujita et al., 2002). To gain insights into the zygotic and maternal requirements for MRG-1 function and to enable in-depth analysis, we obtained three deletion alleles of *mrg-1* (Fig. 1A). The *tm1227* allele lacks 401 bp surrounding the initiation codon.

The *qa6200* allele lacks 450 bp of the first two exons and first intron of the gene and is predicted to shift the translational reading frame. The *ok1262* allele lacks 1443 bp extending from the middle of intron 2 to the middle of intron 3. After immunostaining using anti-MRG-1 antibody, nuclear signal was detected in wild-type embryos, but not in *mrg-1* mutant embryos (Fig. 1B and data not shown). By western blot analysis using anti-MRG-1 antibody, a protein of the predicted size (37 kD) was observed in wild-type worm extracts; in the two mutant alleles tested, signal at 37 kD was reduced to <5% of wild-type level, which may be residual maternal product in the worms analyzed (Fig. 1C). These results suggest that all three *mrg-1* alleles are strong loss-of-function and perhaps null alleles.

Mutations in *mrg-1* cause maternal-effect lethality and sterility

To investigate the contribution of zygotic and maternal MRG-1 to development, we first examined homozygous *mrg-1* hermaphrodites from heterozygous mothers. For all three alleles, such M+Z− (M, maternal load; Z, zygotic synthesis) *mrg-1* hermaphrodites developed into healthy appearing adults, about 10–15% of which were sterile (Table 1). The fertile M+Z− worms produced a substantial number of dead embryos (~30–50%); all surviving embryos developed into sterile M−Z− adults. Thus, a maternal supply or maternal function of *mrg-1* gene product is important for proper embryo development and especially crucial for the germ line.

Zygotic expression of MRG-1 can restore fertility to M−Z+ worms (Table 1). Although the percentage of fertile M−Z+ hermaphrodites was low (7–15%), the extent of germline rescue was dramatic: fertile M−Z+ hermaphrodites contained a fully proliferated germ line with gametes and viable embryos, while sterile M−Z− hermaphrodites contained 0–7 germ cells and no gametes (see below). Interestingly, zygotic rescue was more dramatic for *mrg-1* males: whereas most M−Z− *mrg-1* males were sterile, all of the M−Z+ *mrg-1* males examined were fertile. Enhanced zygotic rescue of males compared to hermaphrodites may be due to their different X chromosome composition (one X in males versus two Xs in hermaphrodites) or to their different sexual phenotype or germline program.

In *mrg-1* mutants the PGCs fail to proliferate and degenerate

In wild-type worms, the two PGCs (Z2 and Z3) present in newly hatched L1 larvae divide to generate over 1000 germ nuclei. In *mrg-1* M−Z− L1s, two PGCs are present, and P granules appear to have been inherited normally (data not shown). However, the PGCs do not proliferate normally (Fig. 2A and see Table S1 in the supplementary material). The average number of germ nuclei in *mrg-1(qa6200)* larvae increased slightly to 6 (range 2–8) in L2 larvae and then decreased to 1 (range 0–3) by the L4 stage. The number of nuclei in *mrg-1(tm1227)* was 2–6 in L2/L3 and 0–7 by L4. These results indicate that *mrg-1* sterility results from failure of the PGCs to proliferate, and from death of germ cells in at least some worms.

Two types of cell death have been observed in the *C. elegans* germ line: programmed cell death (apoptosis), as observed in *nos-1; nos-2* mutants (Subramaniam and Seydoux, 1999), and degenerative cell death (necrosis), as observed in *mes-2*, *mes-3*, *mes-4* and *mes-6* mutants (Paulsen et al., 1995; Garvin, 1998). Loss of *ced-3* or *ced-4*, two genes required for programmed cell death (Ellis and Horvitz, 1986), did not suppress the germline proliferation defect and sterility of *mrg-1(RNAi)* worms (data not shown), suggesting that programmed cell death is not responsible for the tiny germ lines in *mrg-1* mutants. Instead, it is likely that necrosis-like death contributes. Consistent with this, *mrg-1* mutant germ lines contain enlarged nuclei (Fig. 2B,C), similar to those observed in degenerating somatic cells (Chalfie and Wolinsky, 1990; Hall et al., 1997) and in *mes* mutant germ lines (Paulsen et al., 1995).

MRG-1 accumulates in nuclei and is enriched in the germ line

To investigate when and where MRG-1 might function, we examined the subcellular localization of MRG-1 by immunofluorescence staining. We obtained similar results using two different affinity-purified antibodies: rabbit antibody raised against the entire MRG-1 protein fused to 6xHis (Fujita et al., 2002), and rat antibody raised against the C-terminal 24 amino acids of MRG-1. Both antibodies are specific for MRG-1 in embryonic stages (e.g. see Fig. 1B). In adult stages, the rabbit antibody shows some nonspecific staining of nucleoli in *mrg-1* mutant adults (data not shown).

MRG-1 is highly enriched in nuclei and concentrated on chromatin. In early embryos, MRG-1 is present in the nuclei of all blastomeres (Fig. 3A–F). In late embryos and young larvae, MRG-1 staining is higher in the nuclei of the two PGCs, Z2 and Z3, than in somatic blastomeres (Fig. 3G–L). In larvae and adults, MRG-1 staining is seen primarily in the nuclei of germ cells, although it is also faintly visible in the nuclei of several somatic cell types, including intestinal cells. In the adult germ line, all germ nuclei in the mitotic and meiotic regions are stained (Fig. 3M–O). These results demonstrate that MRG-1 is present in the germ line at all stages of development and is maternally loaded into embryos. In addition, zygotically expressed MRG-1 is produced in all cells by at least the 100-cell stage; it accumulates to higher levels in the PGCs than in somatic tissues (see Fig. S1 in the supplementary material).

MRG-1 associates preferentially with the autosomes in a MES-independent manner

Based on immunofluorescence staining, MRG-1 appears to be absent from some chromatin in each nucleus (Fig. 3M–O, Fig. 4E–G). Because the relatively high level of nucleoplasmic MRG-1 sometimes makes this difficult to image, we generated a transgenic strain that expresses GFP-tagged MRG-1 in the germ line under the control of the *pie-1* promoter. In one-cell embryos at pronuclear meeting, we observed that GFP::MRG-1 associates with only five of the six chromosomes in each pronucleus. Co-staining for MES-4, known to selectively associate with the autosomes and to be absent from most of the length of the X (Fong et al., 2002; Bender et al., 2006), revealed that GFP::MRG-1 associates with the same five chromosomes as MES-4 (Fig. 4A-D). Thus, the chromosome lacking MRG-1 is the X.

The observation that both MES-4 and MRG-1 are concentrated on autosomes and the finding in yeast that the MRG-1-related chromodomain protein Eaf3 binds preferentially to histone H3 tails methylated on Lys36 (Carrozza et al., 2005; Keogh et al., 2005) suggested the attractive possibility that MRG-1 association with chromatin requires MES-4 and its H3K36 methylation activity. To test this, we imaged MRG-1 in early *mes-4* M−Z− null mutant embryos, which lack detectable MES-4 and H3K36me2 (Bender et al., 2006). MRG-1 showed robust chromosomal association in *mes-4* null early embryos (Fig. 4K–M). MRG-1 also appeared to be associated with chromosomes in the PGCs of *mes-4* M−Z− L1s, but the small size of those nuclei, the high level of nucleoplasmic MRG-1 present even in wild type (see above), and the poor staining by anti-MRG-1 of samples fixed for optimal preservation of chromosomes made it difficult to assess with confidence the dependence of MRG-1 on MES-4 in those cells. In other epistasis experiments, we observed a normal-appearing pattern of MRG-1 in *mes-2* M −Z− embryos (Fig. 4H–J), of MES-4 and H3K36me2 in *mrg-1* M−Z− embryos (Fig. 4Q–S, and see Fig. S2A-F in the supplementary material), and of MES-2-catalyzed H3K27me3 in *mrg-1* M−Z−embryos (see Fig. S2G-J in the supplementary material). Thus, the results of molecular epistasis tests do not support the notion that the recruitment of MRG-1 to chromatin depends on the MES system, or vice versa.

MRG-1 is not required for PGCs to initiate expression of several germline genes

Because MRG-1 binds to chromosomes, and the Mrg-1 sterile phenotype reflects defective development of the PGCs, we sought to determine whether the gene expression capabilities of

the PGCs are impaired. Transcription is repressed in the germline blastomeres, and is thought to commence shortly after P4 divides into Z2 and Z3, at about the 100-cell stage (Seydoux et al., 1996; Seydoux and Dunn, 1997). One type of evidence for transcriptional turn-on in Z2 and Z3 is the appearance of the elongating form of RNA polymerase II (Pol II), assessed by staining with the H5 antibody to Pol II phosphorylated on Ser2 in the C-terminal domain (Seydoux and Dunn, 1997). In early *mrg-1* embryos, as in wild-type embryos, H5 staining is detected in the nuclei of somatic blastomeres but not of the germline blastomere (Fig. 5A–D). In 100-cell and older *mrg-1* embryos, as in wild type, H5 staining is detected in the nuclei of Z2 and Z3 (Fig. 5E–H). Thus, MRG-1 is not required for the appearance of elongating Pol II in PGCs, suggesting that transcriptional turn-on occurs, at least for some genes.

To assess whether MRG-1 is needed for activation of germline-expressed genes, we examined the accumulation in L1s of several transcripts whose zygotic synthesis is known to commence during embryogenesis in Z2 and Z3. The *pgl-1* and *glh-1* genes encode protein components of germline-specific P granules (Kawasaki et al., 1998; Gruidl et al., 1996). *nos-1* encodes a Nanos homolog that participates in regulation of PGC development (Subramaniam and Seydoux, 1999). For all three genes, transcript levels drop to undetectable in early-mid stages of embryogenesis and then increase specifically in Z2 and Z3 in late-stage embryos (Kawasaki et al., 2004; Subramaniam and Seydoux, 1999) (Y. Kohara, personal communication). Transcript levels were measured in wild-type L1s and *mrg-1* M−Z− L1s by RT-PCR. As shown in Fig. 6A, although the expression levels varied somewhat, all three transcripts accumulated to wild-type levels in *mrg-1* mutant L1s.

As another test of zygotic turn-on of gene expression, we assessed appearance of newly synthesized PGL-1, GLH-1 and GLH-4 proteins in L1s (Fig. 6B–F). To assess new synthesis of PGL-1, it was necessary to eliminate the maternal load of PGL-1, which persists into larval stages (Kawasaki et al., 1998). We mated *pgl-1* mutant hermaphrodites (lacking maternal *pgl-1* mRNA and protein) with *pgl-1*(+) males bearing a GFP transgene, and determined whether GFP+ outcross L1s (*pgl-1*/+) could express the paternal *pgl-1*(+) allele. When *pgl-1* mothers were treated with *mrg-1* dsRNA, PGL-1 was observed in nine of 22 L1s analyzed (Fig. 6C); all L1s that were allowed to grow up developed into sterile adults, confirming the effectiveness of *mrg-1* RNAi. Thus, at least some *mrg-1(RNAi)* larvae activated zygotic expression of *pgl-1*. The absence of detectable PGL-1 in the remaining *mrg-1(RNAi)* L1s may reflect impairment in some larvae of transcription activation or of post-transcriptional accumulation of PGL-1 protein. Appearance of newly synthesized GLH-1 and GLH-4 could be assessed more directly. In wild type, the maternal load of both GLHs and (of *glh-1* mRNA) drops to undetectable during embryogenesis (Z.L., T.T. and S.S., unpublished; Y. Kohara, personal communication). Synthesis from newly transcribed mRNA is presumed to be responsible for the restored levels of both GLHs in newly hatched L1s. *mrg-1* M−Z− embryos and larvae showed the same pattern of GLH-1 and GLH-4 staining as wild type, disappearance in mid-late embryos and reappearance in L1s (Fig. 6E,F and data not shown), revealing that MRG-1 is not required for turn-on of these two *glh* genes. These protein-staining results are consistent with the mRNA quantification results in *mrg-1* mutants.

The above results demonstrate that *mrg-1* mutant PGCs resemble wild-type PGCs in their acquisition of transcriptional competence in ~100-cell embryos and their ability to activate expression of at least some germline genes in late embryos and early larvae. Thus, MRG-1 does not appear to be required for correct specification of PGC identity or for initiation of the germline program of gene expression.

MRG-1 is required for somatic cells to misexpress several germline genes in *mep-1* mutant larvae

As MRG-1 does not appear to be required to activate expression of germline genes in the PGCs, we investigated its involvement in the aberrant activation of germline genes in the somatic cells of synMuv B mutants. A subset of synMuv B mutants (e.g. *mep-1*, *lin-35/Rb*) display ectopic expression of *pgl-1* in the somatic cells of young larvae (Unhavaithaya et al., 2002; Wang et al., 2005; Andersen et al., 2006). Strikingly, simultaneous loss of MES-4 function suppresses this 'ectopic PGL-1 in the soma' phenotype, and also suppresses the larval lethality of *mep-1* (Unhavaithaya et al., 2002; Wang et al., 2005). We tested whether loss of MRG-1 also suppresses these dramatic Mep-1 mutant phenotypes. RNAi of *mrg-1* suppressed the ectopic expression of PGL-1 protein and the overexpression of *pgl-1* RNA in *mep-1(RNAi)* larvae (Fig. 7A-C,G), as recently observed by Cui et al. (Cui et al., 2006). Analysis of two other germline genes yielded similar results – depletion of MRG-1 reduced the ectopic expression of GLH-1 (Fig. 7D-F) and the overexpression of *glh-1* and *nos-1* transcripts (Fig. 7G) caused by MEP-1 loss. mRNA levels for *myo-2*, a gene normally expressed in somatic cells, were not increased by *mep-1* RNAi or decreased by simultaneous RNAi of *mep-1* and *mrg-1* (Fig. 7G). Thus, MRG-1(+) function is required for aberrant expression of germline genes in *mep-1* larvae.

MRG-1(+) function is also required for the larval arrest phenotype of *mep-1*. Only 1.6% of the progeny from *mep-1(RNAi)* mothers grew to adult worms, whereas 100% of the progeny from *mep-1(RNAi); mrg-1(RNAi)* mothers grew to adult, albeit sterile, worms. Our findings and similar findings of Cui et al. (Cui et al., 2006) show that loss of MRG-1 suppresses multiple *mep-1* mutant defects, including aberrant patterns of gene expression and larval arrest.

MRG-1 is required for silencing of transgenes and X-linked genes in the maternal germ line

The *mes* mutants display gene expression defects in the germ line of fertile M+Z− mothers (Kelly and Fire, 1998; Bender et al., 2006). The similar phenotypes displayed by *mrg-1* and *mes* mutants prompted us to test whether gene expression defects in the mother's (M+Z−) germ line might contribute to the Mrg-1 maternal-effect sterile phenotype. As one test, we examined gene expression from an extrachromosomal array containing many copies of a GFP-tagged ubiquitously expressed gene, *let-858*. In wild-type germ lines, such arrays are silenced (Kelly et al., 1997) (Fig. 8A); in fertile *mes-4* and *mes-3* M+Z− germ lines, they are desilenced (Kelly and Fire, 1998). We observed desilencing also in the germ lines of fertile *mrg-1* M+Z− mutants (Fig. 8C). Cui et al. (Cui et al., 2006) observed similar transgene desilencing after RNAi depletion of *mrg-1*. These results demonstrate that MRG-1 is required for transgene silencing in the germ line.

Recent microarray analysis of the mRNA accumulation profile in *mes-4* M+Z− dissected gonads revealed selective de-repression of genes on the X chromosome: among the 71 genes whose expression was significantly (>1.8-fold) affected in *mes-4* germ lines, 67 genes were upregulated in *mes-4* compared with wild type, and 61 of those genes are located on the X (Bender et al., 2006). RT-PCR analysis confirmed the microarray results for six upregulated X-linked genes, three upregulated autosomal genes and five unaffected genes (Bender et al., 2006). We dissected 50 gonads from *mrg-1(qa6200)* M+Z− and from wild-type adult hermaphrodites and used RT-PCR to examine the levels of nine of the mRNAs examined by RT-PCR in *mes-4* mutant germ lines. In two independent experiments (Fig. 8E), five X-linked genes upregulated in *mes-4* were also upregulated at least twofold in *mrg-1* compared with wild type; four genes unaffected in *mes-4* were also unaffected (less than twofold difference) in *mrg-1* compared to wild type. These and the transgene results suggest that MRG-1 cooperates with the MES system to achieve transcriptional repression, and that the X chromosome is a target for repression.

DISCUSSION

Mrg-1 mutant phenotypes and an X chromosome connection

Based on genetic analysis of three deletion alleles of *mrg-1*, a maternal load of MRG-1 enables most M+Z− worms to develop into fertile adults. Absence of a maternal load in the next generation causes many M−Z− embryos to die and causes all surviving M−Z−hermaphrodites to develop into sterile adults, containing no or a tiny number of germ cells, compared with >1000 germ cells in wild type. In the absence of maternal MRG-1, zygotic expression of *mrg-1* (+) can rescue germline proliferation and survival in many M−Z+ hermaphrodites and enabled up to 15% to be fertile. Interestingly, *mrg-1* M−Z− males are less severely affected than hermaphrodites and all M–Z+ males examined were restored to fertility. We speculate that the differential sensitivity of hermaphrodites and males to loss of MRG-1 is due to their different X chromosome composition (hermaphrodites are XX and males are XO) and to a role for MRG-1 in silencing of genes on the X (see below).

The causes of sterility in *mrg-1* M−Z− hermaphrodites appear to be failure of PGCs to proliferate combined with degeneration of the few germ cells present. Among other characterized mutants that display maternal-effect ‘tiny germline’ phenotypes, *mrg-1* most closely resembles the *mes* mutants, *mes-2*, *mes-3*, *mes-4* and *mes-6* (Capowski et al., 1991; Paulsen et al., 1995). This resemblance extends to the differential sensitivity of XX and XO animals to *mrg-1* and *mes* mutations (Garvin et al., 1998).

MRG-1 associates with autosomes and does so independently of autosomal MES-4

MRG-1 joins MES-4 in showing the unique property of associating selectively with autosomes (Bender et al., 2006). This along with the similar maternal-effect sterile phenotypes of *mrg-1* and *mes-4* mutants suggested that they might function together. Based on the finding that *Saccharomyces cerevisiae* Set2-catalyzed methylation of H3K36 is required for association of the MRG-1 homolog Eaf3 with nucleosomes (Keogh et al., 2005), we predicted that MES-4-catalyzed methylation of H3K36 would be required for MRG-1 association with chromosomes. This requirement was not observed, arguing against a model in which MRG-1 associates with MES-4-deposited methyl marks and serves as a downstream effector of MES-4. Other tests of MES-MRG relationships gave similar negative results: MES-2 function is not required for the chromosomal association of MRG-1, and MRG-1 is not required for the chromosomal association of MES-4 or for MES-mediated methylation of H3K36 or H3K27. It remains possible that MES-4 functions redundantly with other chromatin regulators to recruit MRG-1 to autosomes and/or that MES-mediated methylation regulates the *activity* of MRG-1.

MRG-1 and activation of expression of germline genes

PGCs in *mrg-1* mutants appeared normal in their acquisition of the elongating form of RNA Pol II at the ~100-cell stage of embryogenesis and in their ability to turn on expression of the germline genes *pgl-1*, *glh-1*, *glh-4* and *nos-1* in late embryos and L1s. We conclude that *mrg-1* PGCs have germline identity and are competent to express at least some genes characteristic of the early germline program.

Important insights into possible roles for MRG-1 have emerged from genetic interactions between *mrg-1* and *mep-1*. MEP-1, known to exist in a complex with the NuRD subunits LET-418 and HDA-1, is required to prevent expression of germ cell traits in somatic cells; in larvae depleted of MEP-1 or LET-418, somatic cells express germline genes such as *pgl-1* (Unhavaithaya et al., 2002). We and Cui et al. (Cui et al., 2006) have found that loss of MRG-1 suppresses the ectopic expression of germline genes in *mep-1* mutant larvae and suppresses *mep-1* larval arrest. Loss of MES function also suppresses these *mep-1* phenotypes (Unhavaithaya et al., 2002). These findings lead to a model in which the MES proteins and

MRG-1 confer germline competence on all cells; in somatic cells the NuRD complex antagonizes the functions of the MES proteins and MRG-1 to protect somatic cells from expressing germline traits (Unhavaithaya et al., 2002; Shin and Mello, 2003; Strome, 2005) (this study). It is interesting that MRG-1 is needed for *mep-1* somatic cells, but not for wild-type PGCs, to express germline genes. One possibility is that a single MRG-1-requiring mechanism causes *mep-1* somatic cells to inappropriately express germline genes, but that redundant mechanisms ensure the germline fate of PGCs and launch their gene expression program. Another possibility is that MRG-1 has different functions in somatic cells versus PGCs. It has been reported that the MRG-1 homologs yeast Eaf3 and human MRG15 exist in both histone acetyltransferase (HAT)-containing and histone deacetylase (HDAC)-containing complexes (Gavin et al., 2002; Doyon et al., 2004). By analogy, MRG-1 may exist in different complexes and regulate different target genes in somatic versus germline cells.

MRG-1 and silencing transgenes and the X chromosomes

Tests for whether gene-silencing mechanisms are operating properly in early PGCs have not been developed. Consequently, to investigate if *mrg-1* mutants are defective in silencing genes in the germ line, we analyzed gene expression in fertile *mrg-1* M+Z−mothers, as done previously for fertile *mes* M+Z− mothers (Kelly and Fire, 1998; Bender et al., 2006). Known targets of silencing in the germ line are transgenes present in repetitive extrachromosomal arrays (Kelly et al., 1997), and the X chromosomes (Kelly et al., 2002; Fong et al., 2002; Bender et al., 2006). Evidence for X silencing comes from microarray analysis comparing mRNA accumulation in germline-containing versus germline-lacking hermaphrodites (Reinke et al., 2000; Reinke et al., 2004) and from staining germline chromosomes with antibodies that recognize marks of actively expressed chromatin (Kelly et al., 2002; Fong et al., 2002). The X chromosomes lack marks of active chromatin, and X-linked genes are significantly under-represented in the germline mRNA pool.

We observed that *mrg-1* M+Z− germ lines are defective in silencing both types of targets. Similar to *mes-3* and *mes-4* M+Z−germ lines (Kelly and Fire, 1998), *mrg-1* M+Z− germ lines display expression of a repetitive GFP transgene. Similar to *mes-4* M+Z−germ lines (Bender et al., 2006), *mrg-1* M+Z− germ lines upregulate at least some genes on the X chromosome. We specifically sampled X-linked genes known to be upregulated in *mes-4* mutant germ lines; all five of those genes were also upregulated in *mrg-1* mutant germ lines. Importantly, four autosomal genes that were not upregulated in *mes-4* were also not upregulated in *mrg-1*. Future microarray analysis will reveal whether the profile of up- and downregulated genes in *mrg-1* overlaps extensively or not with the profile in *mes-4*.

How can autosomally concentrated MRG-1 participate in repressing genes on the X chromosomes? Two models were proposed for MES-4 (Bender et al., 2006). One model invokes that MES-4 activates expression of an autosomal gene that encodes a repressor of the Xs and repetitive arrays. The other model proposes that autosomally concentrated MES-4 or its H3K36me mark repels a repressor, and focuses its action on the Xs and repetitive arrays. Similar models can be proposed for MRG-1. In fact, MRG-1 and MES-4 may operate together, even though they do not display dependence on one another for recruitment to chromosomes. We speculate that desilencing of genes on the X may underlie the PGC defects and death observed in *mrg-1* M−Z− worms. Desilencing of genes on the X may also contribute to suppression of *mep-1* mutant phenotypes in somatic cells (P. Raghavan and T. H. Shin, personal communication). Elucidating the gene targets of MRG-1 in PGCs and in somatic cells are important future directions.

The biochemical activities of MRG-1 are currently unknown. The dosage compensation complex (DCC) in *Drosophila*, the NuA4 complex in *S. cerevisiae* and the NuA4-related Tip60 complex in humans all contain a MRG-1 homolog (fly MSL3, yeast Eaf3 and human MRG15)

and also a HAT subunit (Smith et al., 2000; Eisen et al., 2001; Akhtar, 2003; Cai et al., 2003). We speculate that MRG-1 may exist in a complex similar to the DCC and NuA4 HAT complexes and may regulate chromatin organization and gene expression through regulation of histone acetylation. Consistent with this, MRG-1 interacts with ZK1127.3 (Li et al., 2004), the predicted *C. elegans* homolog of the yeast NuA4 subunit Eaf7, and recent studies have revealed that RHA-1, a *C. elegans* homolog of the *Drosophila* DCC component MLE, is required for germline gene silencing (Walstrom et al., 2005), as is MRG-1. Either of the two models proposed above for how MRG-1 participates in repressing genes on the X could involve histone acetylation by autosomally concentrated MRG-1-containing HAT complexes.

Temporal requirement for MRG-1 in PGC development

We hypothesize that a chromatin state induced by MRG-1 in the maternal germ line is crucial for the normal early program of PGC development and to ensure germline immortality. Relevant to this, we noticed that in *mrg-1* M–Z+ worms, some germ lines showed normal proliferation, whereas others showed severe underproliferation. This ‘all or nothing’ effect suggests that MRG-1 participates in some stochastic event or decision, and that the outcome is normal proliferation and fertility or underproliferation and sterility. An attractive model is that MRG-1 acts epigenetically to induce a ‘germline’ chromatin state that is passed from generation to generation of germ cells. Normally, that state must be inherited from the maternal germ line, but at low frequency it can be induced de novo (i.e. in *mrg-1* M–Z+ worms). Resolving the temporal requirement for MRG-1 function will shed light on the important issue of how germline properties are maintained in perpetuity.

Supplementary material

Refer to Web version on PubMed Central for supplementary material.

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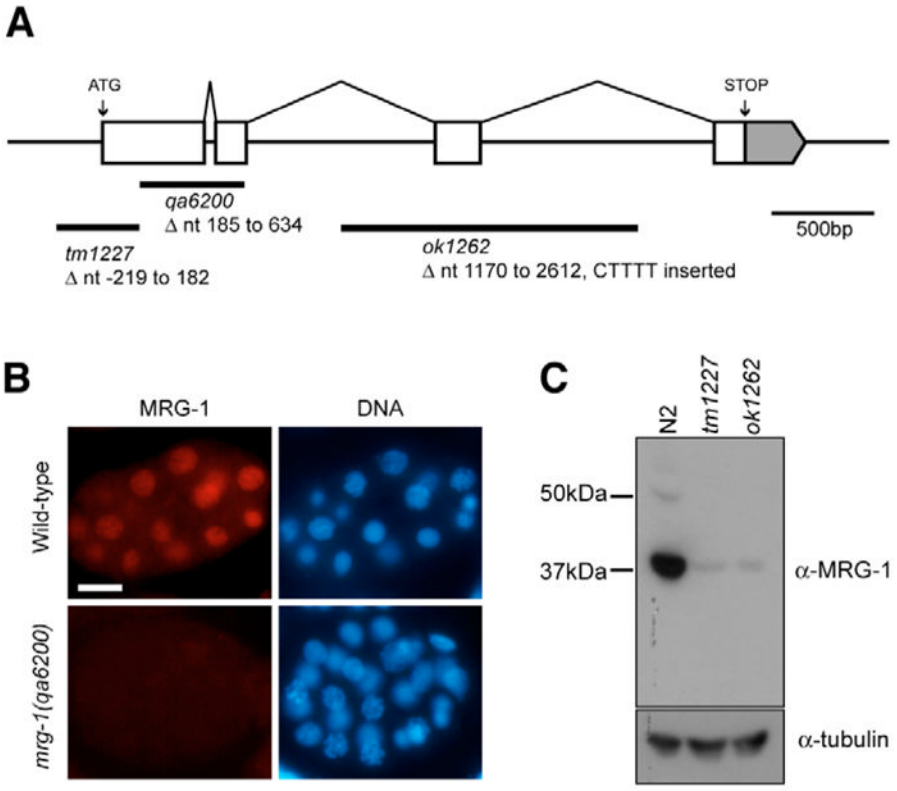


Fig. 1. Deletion alleles of *mrg-1*

(**A**) The *mrg-1* gene structure with exons (boxes), introns (joining lines), and the 3′UTR (gray). Black bars below the gene indicate regions deleted by each allele. (**B**) Wild-type and *mrg-1 (qa6200)* M–Z− embryos co-stained with rabbit anti-MRG-1 antibody (red) and DAPI (blue). Scale bar: 10 μm. (**C**) Western blot analysis of 100 N2, *mrg-1(tm1227)* and *mrg-1(ok1262)* M +Z− adult hermaphrodites reacted with rabbit anti-MRG-1 (upper panel) and mouse anti-tubulin as a loading control (lower panel).

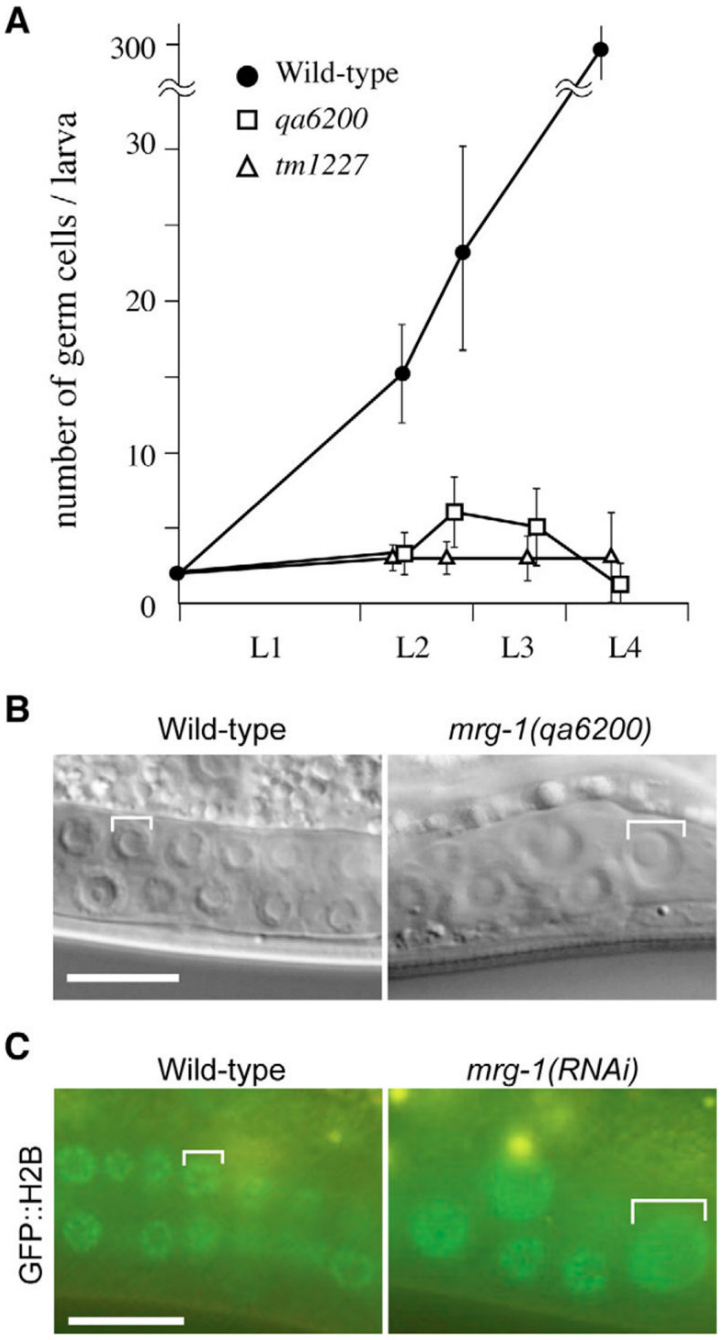


Fig. 2. Germ cell proliferation and degeneration in *mrg-1* mutants

(**A**) Number of germ cells in *mrg-1*(M−Z−) mutants. Each data point represents the average of 3–18 worms; see Table S1 in the supplementary material for values. Error bars show standard deviations. (**B**) Nomarski photomicrographs of live L3 larvae. (**C**) Germ nuclei containing GFP-tagged histone H2B. Enlarged nuclei were observed in the gonads of *mrg-1* mutant and RNAi larvae (compare brackets). Scale bars: 10 μm.

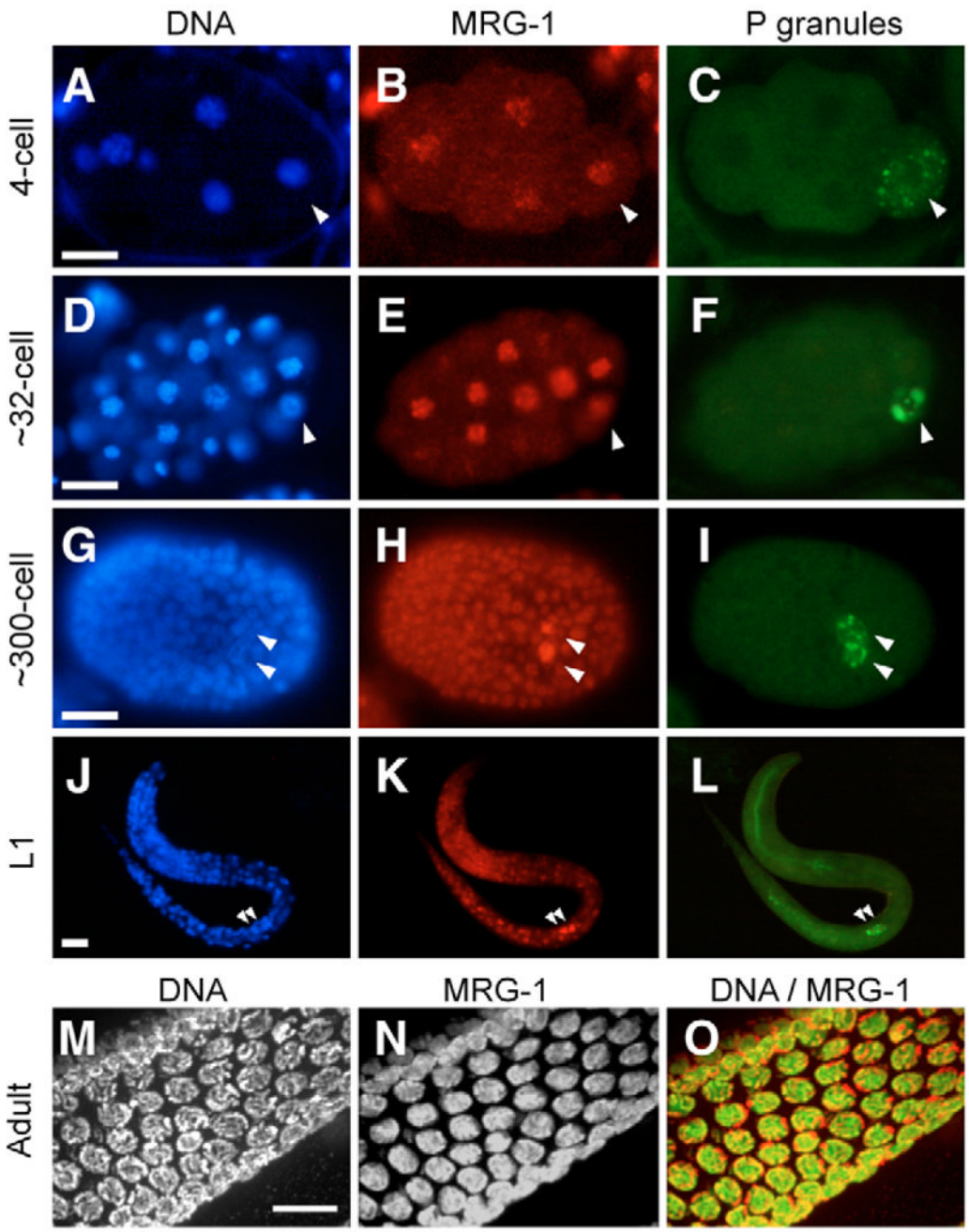


Fig. 3. Distribution of MRG-1 protein

(**A**–**L**) Wild-type embryos and L1 larva triply stained with DAPI (A,D,G,J), rabbit anti-MRG-1 antibody (B,E,H,K) and mouse anti-P-granule antibody K76 (C,F,I,L). (**M**–**O**) Extruded gonad co-stained with mouse PA3 to stain DNA (M and red in merge) and rabbit anti-MRG-1 (N and green in merge). Scale bars: 10 μm.

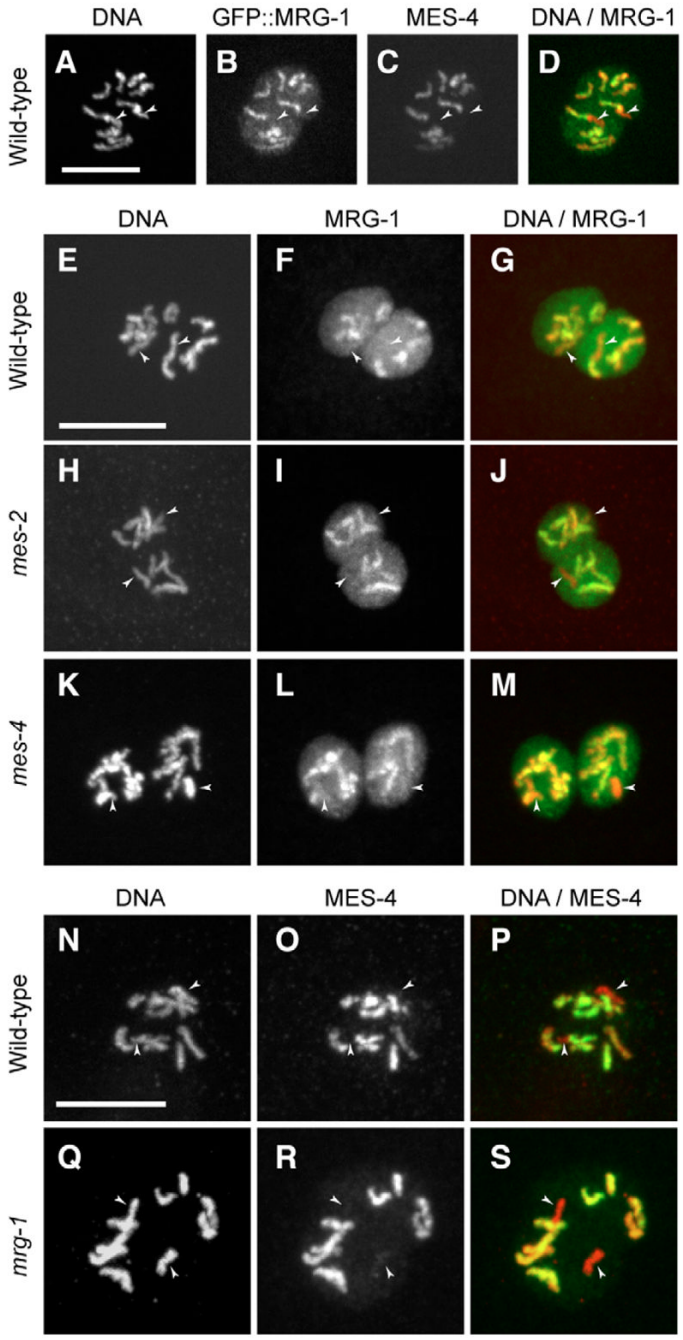


Fig. 4. MRG-1 associates with autosomes independently of the MES proteins

(**A**–**D**) One-cell wild-type embryo at pronuclear meeting doubly stained with mouse PA3 to stain DNA (A) and rabbit anti-MES-4 (C) and imaged for GFP::MRG-1 (B). (D) Merged image of DNA (red) and GFP::MRG-1 (green). Arrows point to chromatin lacking both MRG-1 and MES-4. (**E**–**M**) One-cell embryos at pronuclear meeting stained with mouse PA3 (E,H,K and red in merge) and rabbit anti-MRG-1 (F,I,L and green in merge). (E–G) Wild-type embryo. (H-J) *mes-2(bn76)rol-1* M−Z− embryo. (K-M) *mes-4(bn73)* M−Z− embryo. Arrows point to presumptive X chromosomes. (**N**–**S**) One-cell embryos at pronuclear meeting stained with mouse PA3 (N,Q and red in merge) and rabbit anti-MES-4 (O,R and green in merge). (N-P)

Wild-type embryo. (Q–S) *mrg-1(qa6200)* M−Z− embryo. Arrows point to X chromosomes. Scale bars: 10 μm.

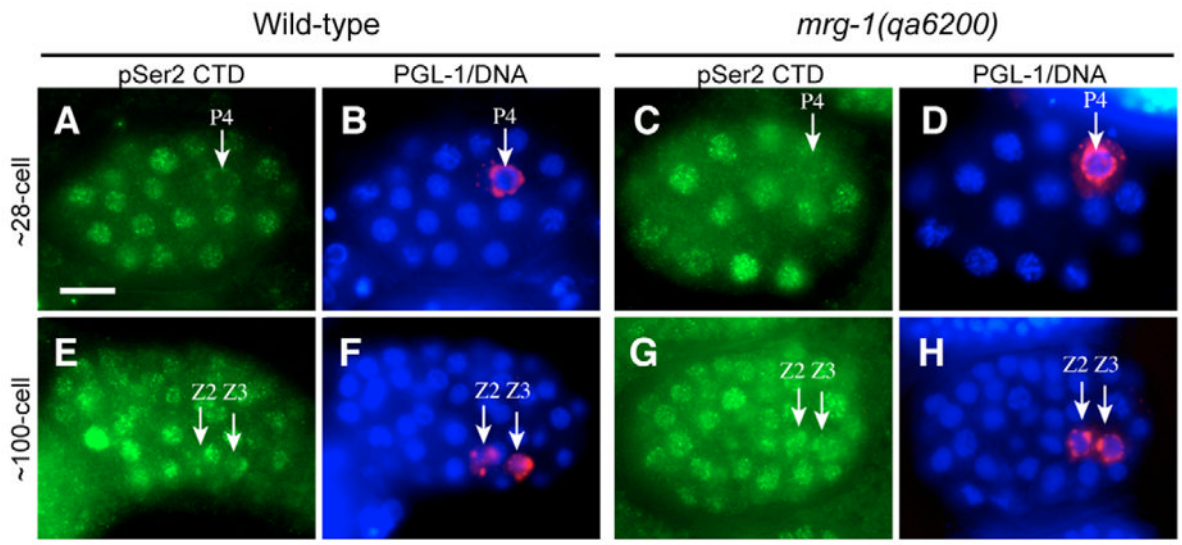


Fig. 5. MRG-1 is not required for the appearance of elongating RNA polymerase II in early PGCs

(**A**–**H**) Embryos (~28-cell and ~100-cell) triply stained with antibodies to RNA Pol II CTD phosphorylated on Ser2 (green) and PGL-1 (red) and with DAPI (blue). In both wild-type and *mrg-1(qa6200)* M−Z− embryos, phosphorylated Pol II was not detected in the germline blastomere P4 (A,C), but appeared in Z2 and Z3 (E,G). Scale bar: 10 μm.

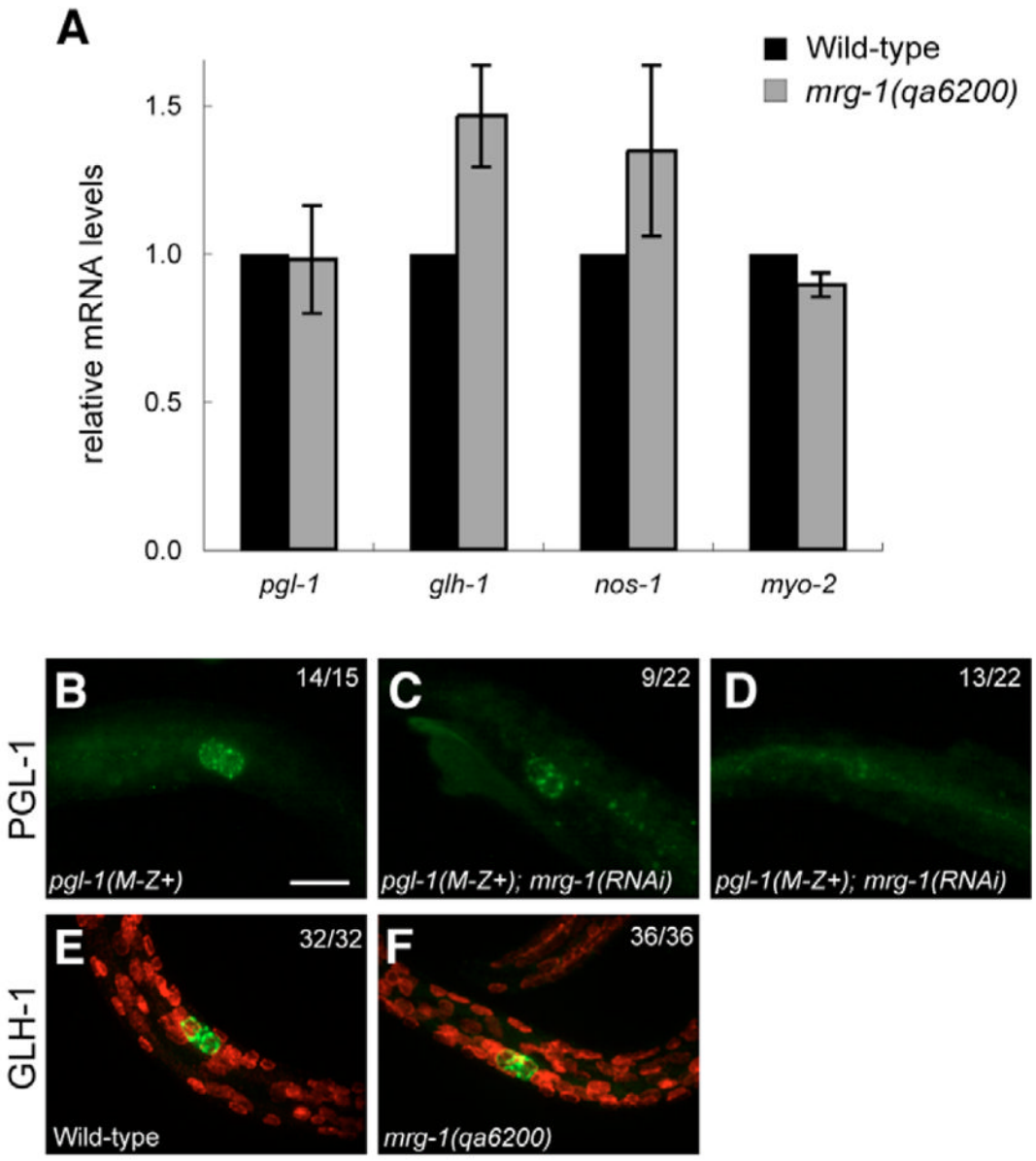


Fig. 6. MRG-1 is not required for activation of transcription of at least some germline genes in PGCs

(**A**) RT-PCR comparison of mRNA levels in wild-type and *mrg-1(qa6200)* M−Z− L1 larvae. All transcript levels were normalized to *ama-1* mRNA. Histograms show mean±s.e. of three independent experiments. (**B–D**) Expression of a paternally contributed *pgl-1*(+) allele in *pgl-1*/+ heterozygous L1s derived from mating *pgl-1/pgl-1* mutant hermaphrodites, untreated (B) or treated with *mrg-1* RNAi (C,D), with wild-type males. L1s were stained with anti-PGL-1. (**E**,**F**) Appearance of newly synthesized GLH-1 in wild-type and *mrg-1* M−Z− L1s, seen by staining with anti-GLH-1 (green). PA3 stain of DNA is in red. Values in the upper right corners are numbers of L1s that displayed that staining pattern. Scale bar: 10 μm.

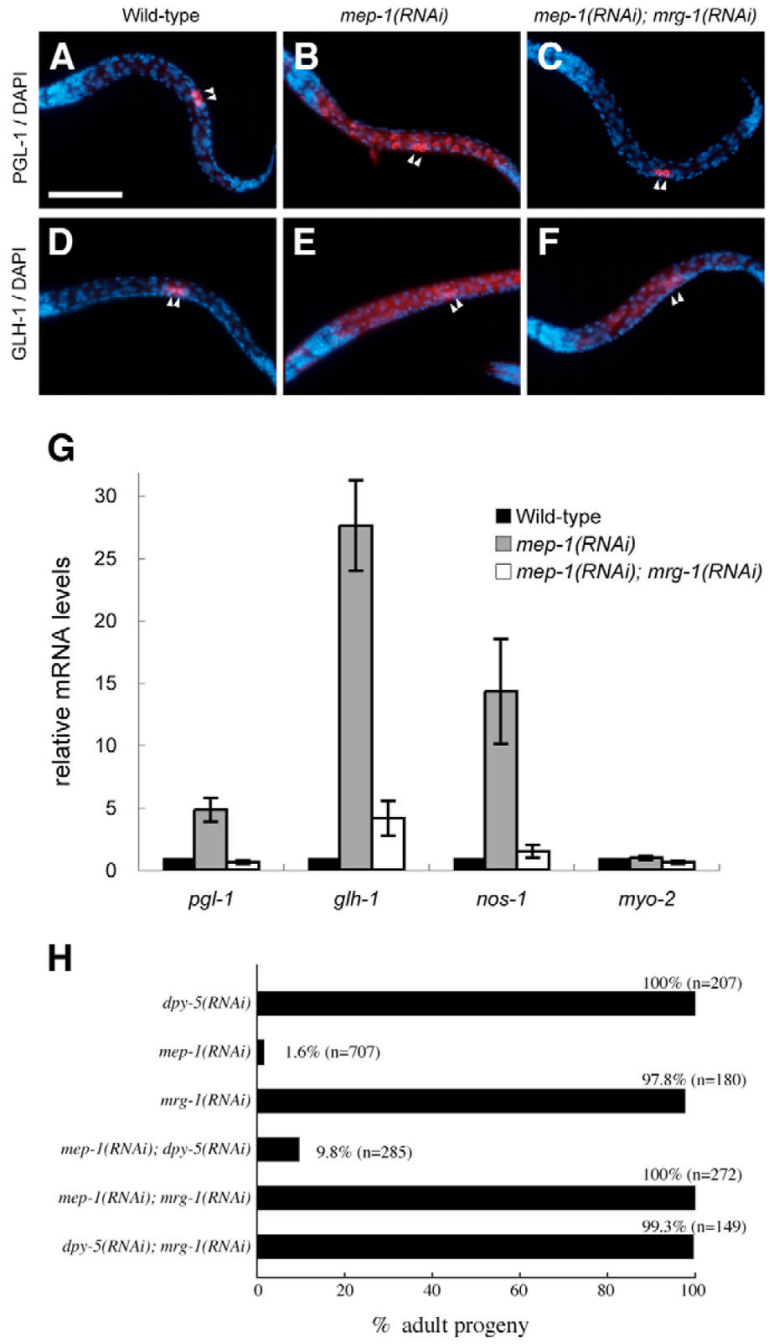
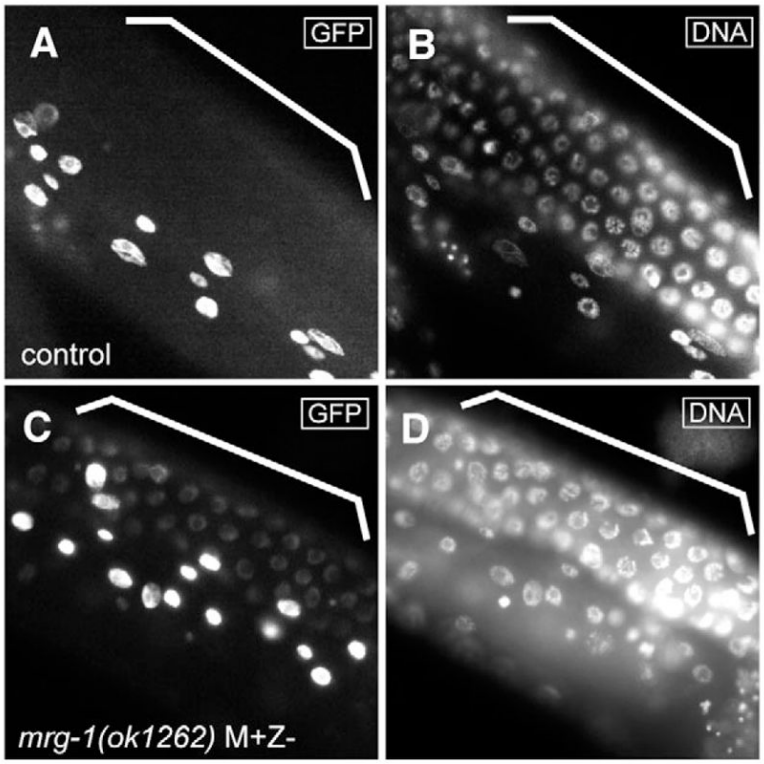


Fig. 7. MRG-1 is required for several *mep-1* mutant phenotypes

(**A–F**) L1 larvae stained with DAPI (blue) and anti-PGL-1 (red) (A–C) or anti-GLH-1 (red) (D–F). *mep-1(RNAi)* larvae displayed ectopic expression of PGL-1 and GLH-1 in somatic cells (B,E). Ectopic expression was reduced in *mep-1(RNAi); mrg-1(RNAi)* larvae (C,F). Arrowheads point to the position of PGCs. Scale bar: 50 μm. (**G**) RT-PCR comparison of *pgl-1*, *glh-1*, *nos-1* and *myo-2* mRNA levels in L1 and L2 larvae with the genotype indicated. *ama-1* was used as the internal reference. Histograms show mean±s.e. of four independent experiments. (**H**) Percent of larvae that developed into adults after treatment of their mothers with the RNAi reagents shown. RNAi of *mep-1* induced larval arrest. RNAi of *mrg-1* but not *dpy-5* strongly suppressed the *mep-1(RNAi)* larval arrest phenotype.



E

Gene	Chromosome	Real-time PCR relative to ZK381.1	
		mrg-1/WT #1	*mrg-1*/WT #2
C09B8.7	X	5.85	8.11
T13C5.6	X	7.08	6.14
F08F1.7	X	5.64	5.08
C25A11.1	X	8.13	8.45
H11L12.1	X	2.22	2.95
F14B4.2	I	0.85	0.55
F49D11.8	I	0.79	1.05
B0361.8	III	1.03	1.40
K07C11.2	V	1.01	0.76

Fig. 8. MRG-1 is required for silencing of a transgene and several X-linked genes in the germ line of *mrg-1* M+Z− mutants

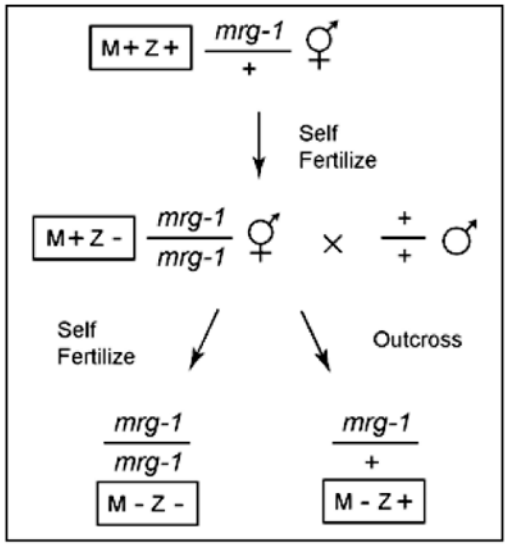
(**A**,**B**) Control adult hermaphrodite stained with Hoechst 33342 [as done in Garvin et al. (Garvin et al., 1998); B] and displaying expression of LET-858::GFP fluorescence in somatic nuclei but not in the germ line. None of 27 worms analyzed displayed GFP in the germ line. (**C**,**D**) *mrg-1* (M+Z−) adult hermaphrodite stained with Hoechst 33342 (D) and displaying LET-858::GFP fluorescence in germ nuclei as well as somatic nuclei (C). Of 26 worms analyzed, 25 displayed GFP in the germ line. Brackets indicate the germ line. (**E**) RT-PCR comparison of mRNA levels from five X-linked and four autosomal genes in dissected wild-type and *mrg-1(qa6200)* M+Z− gonads. #1 and #2 show the results from two independent sets

of dissected gonads. For each gene, RT-PCR was performed in triplicate. Transcript levels are relative to the reference gene ZK381.1/*him-3* (Bender et al., 2006). The five X-linked genes are upregulated in *mes-4* mutant gonads compared with wild type (Bender et al., 2006). Upregulation of those X-linked genes was also observed in *mrg-1* gonads. The four autosomal genes are expressed at approximately equivalent levels in *mes-4* and wild-type gonads (Bender et al., 2006). *mrg-1* and wild-type gonads also accumulate similar levels of those genes.

Table 1

mrg-1 mutants display maternal-effect lethality and sterility

	% sterile hermaphrodites[a] (total number scored)				% sterile males[b] (total number scored)		% inviable embryos[c] (total number scored)
g-1 allele	M+Z+	M+Z−	M−Z−	M−Z+	M−Z−[d]	M−Z+	M−Z−
1227	0.0 (139)	9.4 (110)	100.0 (106)	84.7 (59)	69 (45)	0.0 (8)	29.4 (323)
6200	0.0 (196)	15.8 (114)	100.0 (32)	93.2 (44)	nd	nd	37.0 (359)
1262	nd	nd	100.0 (100)	87.0 (41)	80 (15)	0.0 (10)	52.5 (162)



lermaphrodites were scored by visual examination on a dissecting microscope. Adult hermaphrodites that lacked embryos in their uterus were scored as ‘sterile’.

Males were scored by fixing and staining them with Hoechst. Adult males that lacked a well-proliferated germ line or lacked sperm were scored as ‘sterile’.

Control embryos (wild type and *dpy-18*) display very low levels of embryonic lethality.

Generating M−Z− males required mating *dpy-18 mrg-1/dpy-18 mrg-1* hermaphrodites with *dpy-18 mrg-1*/++ males, and scoring the Dpy offspring. These values are estimates of the % sterile males, because they may include some recombinant M−Z+ *dpy-18 mrg-1/dpy-18* + males, which show 100% fertility. nd, not determined.