Differential Regulation of Germline Apoptosis in Response to Meiotic Checkpoint Activation

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ABSTRACT In *Caenorhabditis elegans*, germline apoptosis is promoted by *egl-1* and *ced-13* in response to meiotic checkpoint activation. We report that the requirement for these two factors depends on which checkpoints are active. We also identify a regulatory region of *egl-1* required to inhibit germline apoptosis in response to DNA damage incurred during meiotic recombination.

FOR chromosomes to properly segregate during meiosis, homologous chromosomes must pair, synapse, and recombine (Bhalla and Dernburg 2008). Defects in these processes result in birth defects and infertility; thus, checkpoints monitor meiotic events to ensure they occur properly (MacQueen and Hochwagen 2011). In the *Caenorhabditis elegans* germline, two distinct checkpoints exist: the DNA damage checkpoint monitors the proper repair of double-strand breaks (DSBs) during meiotic recombination (Gartner *et al.* 2000; Bhalla and Dernburg 2005) and the synapsis checkpoint ensures homologous chromosomes are synapsed (Bhalla and Dernburg 2005). Checkpoint-induced apoptosis removes defective meiotic nuclei to prevent aneuploidy and defective gametes. EGL-1 and CED-13 promote DNA damage checkpoint-induced germline apoptosis (Hofmann *et al.* 2002; Schumacher *et al.* 2005), but their relative contributions and potential roles in the synapsis checkpoint have been unclear (Nehme and Conradt 2008).

Pairing and synapsis of *C. elegans* homologs are promoted in *cis* by sequences near the ends of chromosomes called pairing centers (PCs) (MacQueen *et al.* 2005). When these sequences are deleted from a single chromosome, such as in *meDf2* mutants that remove the *X* chromosome PC (Villeneuve 1994; MacQueen *et al.* 2005), asynapsis results and meiotic checkpoints are activated (Bhalla and Dernburg 2005). Animals homozygous for *meDf2* only activate the DNA damage checkpoint and animals heterozygous for *meDf2* only activate the synapsis checkpoint (see Figure 1A) (Bhalla and Dernburg 2005). An unsynapsed PC is required for the synapsis checkpoint signal, explaining why *meDf2* homozygotes fail to activate it (Bhalla and Dernburg 2005). However, it is unknown why the DNA damage checkpoint is not active in *meDf2* heterozygotes. We tested whether either checkpoint had different genetic requirements for activating germline apoptosis. Loss of *egl-1* in both the *meDf2* homozygote and heterozygote mutant backgrounds reduced apoptosis to physiological levels (Figure 1B). This background level of physiological apoptosis in wild-type hermaphrodites is independent of *egl-1* and *ced-13* (Gumienny *et al.* 1999; Schumacher *et al.* 2005). Mutation of *ced-13* in both checkpoint backgrounds did not significantly affect germline apoptosis (Figure 1B). We observed similar results when *egl-1* or *ced-13* was inactivated by RNA interference (RNAi) in *meDf2* homozygotes and heterozygotes, indicating that these are not allele-specific phenomena (Figure 1C). To test if this was a general feature of meiotic checkpoint activation, we assessed the requirement for *egl-1* and *ced-13* in *rad-54* mutants, which fail at all meiotic DSB repair (Mets and Meyer 2009), and observed a similar dependence on *egl-1* but not *ced-13* (Figure 1D). Therefore, *egl-1*, but not *ced-13*, is required for both checkpoints when each is activated individually.

We determined the role of *egl-1* in promoting germline apoptosis in mutants that activate both checkpoints. SYP-1 is a component of the synaptonemal complex (MacQueen *et al.* 2002) and loss of *syp-1* activates both meiotic checkpoints (see Figure 2A) (Bhalla and Dernburg 2005). In contrast to our studies with *meDf2* homozygotes and heterozygotes, loss of *egl-1* in *syp-1* mutants revealed a role specific to the synapsis checkpoint (Figure 2B). Deletion of *egl-1* in the *syp-1* background reduced apoptosis to intermediate

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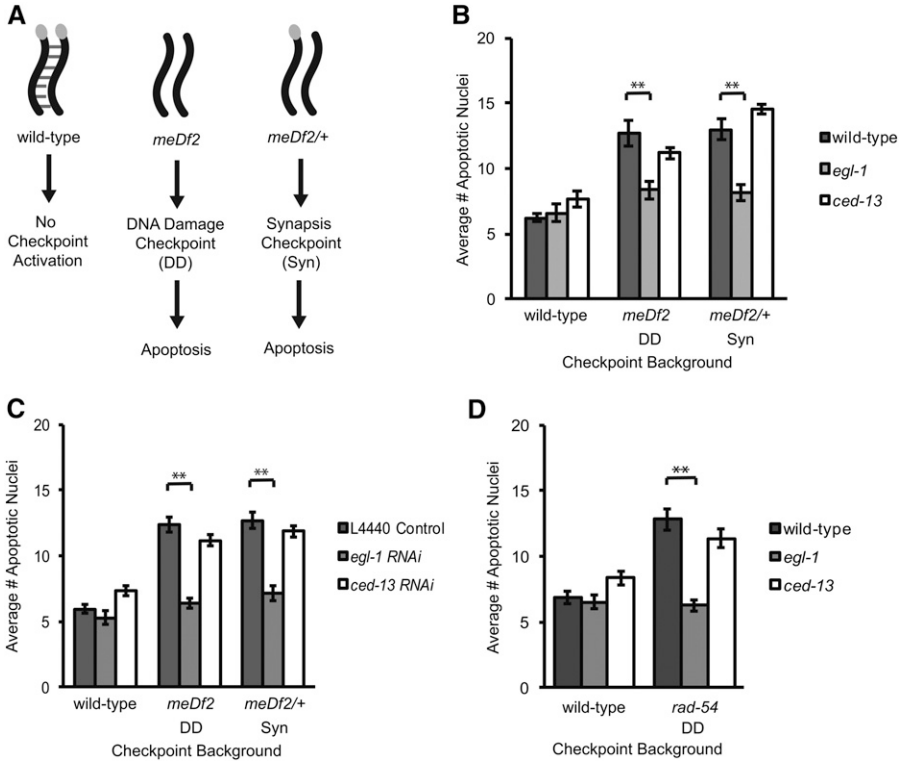

Figure 1 *egl-1* is required for the DNA damage and the synapsis checkpoint when each is activated individually. (A) Checkpoint activation during meiotic prophase in strains homozygous or heterozygous for *meDf2*. Homozygotes activate the DNA damage checkpoint (DD) and heterozygotes activate the synapsis checkpoint (Syn). In most graphs, we indicate which checkpoint (DD, Syn, or both) is activated under the relevant mutant background. (B) Mutation of *egl-1* but not *ced-13* reduces germline apoptosis in both *meDf2* homozygous (DD) and heterozygous mutants (Syn). (C) RNAi of *egl-1*, but not *ced-13*, reduces germline apoptosis in *meDf2* homozygotes (DD) and heterozygotes (Syn). (D) Mutation of *egl-1* but not *ced-13* reduces germline apoptosis in *rad-54* mutants (DD). Except where indicated, *egl-1(n1084n3082)* (Conradt and Horvitz 1998) and *ced-13*(*tm536)* (Schumacher *et al.* 2005) were used to inactivate respective gene function in all experiments. Germline apoptosis was assayed as in Bhalla and Dernburg (2005). Error bars in all graphs represent 2× SEM. $^{**}P < 0.01$. Significance was assessed by performing a paired *t*-test.

levels compared to *syp-1* alone, corresponding to loss of one checkpoint but not both. We prevented activation of the DNA damage checkpoint by mutating *spo-11* (Dernburg *et al.* 1998), the enzyme responsible for generating DSBs during meiosis, in the *egl-1 syp-1* mutant background. These triple mutants (*spo-11;egl-1 syp-1*) exhibited physiological levels of apoptosis, demonstrating that *egl-1* is required for the synapsis checkpoint. We also inactivated the synapsis checkpoint by mutating *pch-2* in *egl-1 syp-1* mutants (Bhalla and Dernburg 2005) and observed intermediate levels of apoptosis in these triple mutants (*pch-2;egl-1 syp-1*), establishing that *egl-1* is not required for the DNA damage checkpoint even when the synapsis checkpoint is abrogated. These data show that when both the synapsis checkpoint and the DNA damage checkpoint are activated, *egl-1* promotes germline apoptosis specifically in response to the synapsis checkpoint.

We then interrogated the role of *ced-13* in promoting checkpoint-induced apoptosis in *syp-1* mutants. Loss of *ced-13* reduced the average number of apoptotic nuclei in *syp-1* mutants to intermediate levels, indicating its requirement for one checkpoint but not both (Figure 2C). In *spo-11;syp-1* mutants, loss of *ced-13* did not further reduce apoptosis. However, *pch-2;syp-1;ced-13* triple mutants had fewer average apoptotic nuclei than both *pch-2;syp-1* and *spo-11;syp-1;ced-13* mutants (Figure 2C). Thus, *ced-13* activates apoptosis in response to DNA damage in *syp-1* mutants, consistent with previous data illustrating a proapoptotic role for *ced-13* in response to genotoxic stress (Schumacher *et al.* 2005). We observed similar results when *egl-1* or *ced-13* was inactivated by RNAi in *syp-1*, *spo-11;syp-1*, and *pch-2;syp-1* mutants (Figure 2D).

The reduction in germline apoptosis in *syp-1;ced-13* double and *pch-2;syp-1;ced-13* triple mutants was less severe than when *egl-1* was inactivated in the same mutant backgrounds (Figure 2, B and C), leading us to wonder if *egl-1* might be contributing to germline apoptosis when *ced-13* function is compromised. To test this possibility, we assayed germline apoptosis in *egl-1 syp-1;ced-13* triple mutants. Deletion of both *egl-1* and *ced-13* in the *syp-1* mutant further reduced apoptosis below the levels observed in *egl-1 syp-1* double mutants but did not rescue apoptosis to physiological levels (Figure 2E). Therefore, germline apoptosis can be elevated even in the absence of two characterized proapoptotic factors, suggesting that either another proapoptotic factor promotes checkpoint-induced apoptosis or that physiological apoptosis can be upregulated in response to meiotic checkpoint activation. We also monitored germline apoptosis in *pch-2;egl-1 syp-1;ced-13* mutants (Figure 2E) and did not observe any further reduction in apoptosis from the levels observed in *pch-2;syp-1;ced-13* triple mutants (Figure 2C). These data allow us to conclude that the increase in germline apoptosis in *pch-2;syp-1;ced-13* triple mutants is not due to *egl-1* function compensating for the absence of *ced-13* during DNA damage checkpoint activation.

We determined whether checkpoint activation affected transcription of *egl-1* and *ced-13* by performing quantitative RT-PCR. In *meDf2*, *meDf2*/+, and *rad-54* strains, *egl-1* mRNA was present at higher relative levels when compared to wild-type worms (Figure 3, A and B), consistent with *egl-1*'s requirement for checkpoint-induced apoptosis in all of these mutant backgrounds (Figure 1, B and C). In *syp-1* and *spo-11*; *syp-1* mutant worms, *egl-1* was also transcriptionally

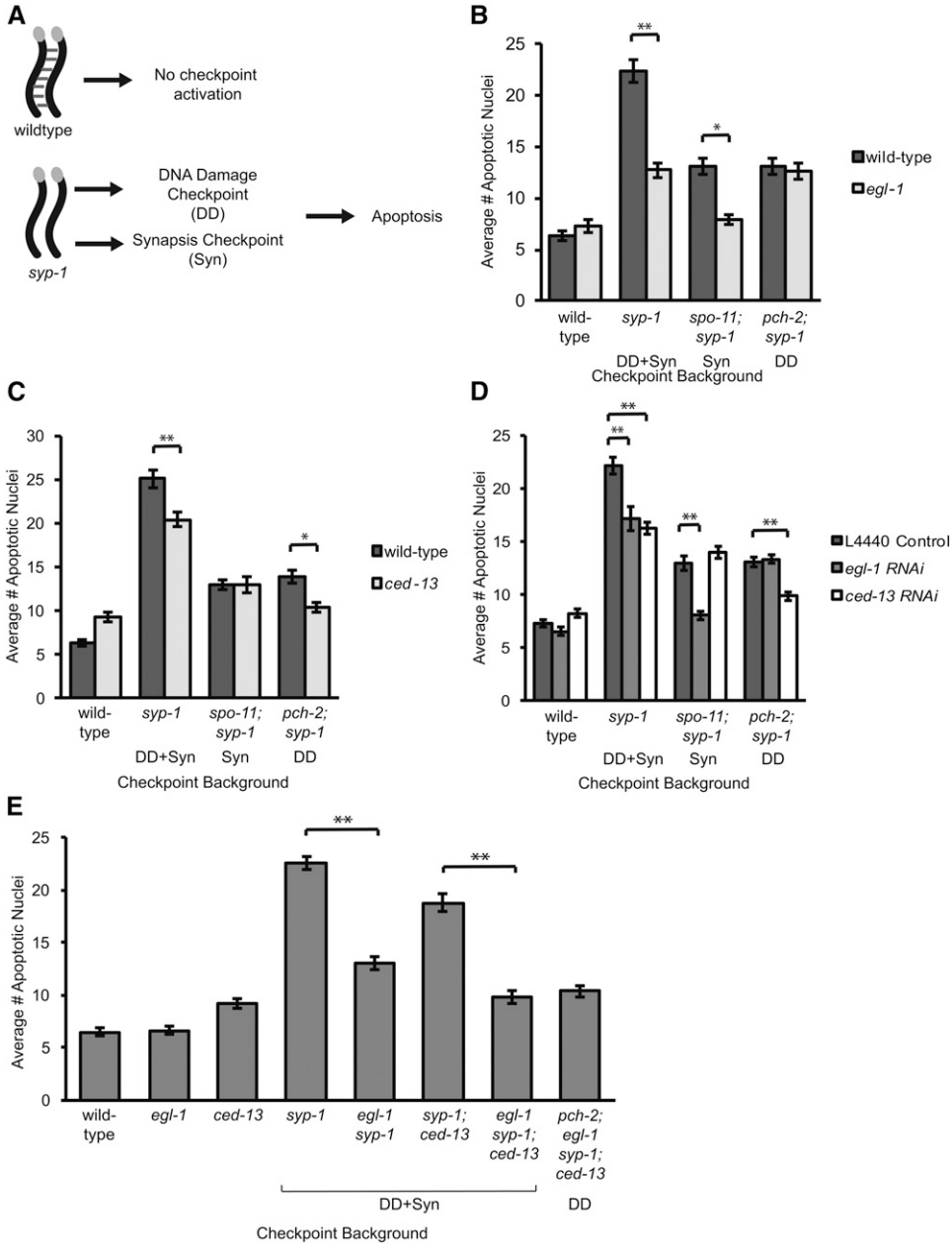

Figure 2 *egl-1* and *ced-13* promote germline apoptosis in response to different checkpoints in *syp-1* mutants. (A) *syp-1* mutants activate both checkpoints (DD + Syn) during meiotic prophase. (B) *egl-1* is required for the synapsis checkpoint in *syp-1(me17)* mutants. Mutation of *egl-1* reduces germline apoptosis in *syp-1 (me17)* (DD + Syn) and *spo-11(ok79);syp-1(me17)* (Syn) mutants but not in *pch-2 (tm1458);syp-1(me17)* mutants (DD). (C) *ced-13* is required for the DNA damage checkpoint in *syp-1(me17)* mutants. Mutation of *ced-13* reduces germline apoptosis in *syp-1(me17)* (DD + Syn) and *pch-2 (tm1458);syp-1(me17)* (DD) mutants but not in *spo-11(ok79);syp-1(me17)* mutants (Syn). (D) RNAi analysis of *egl-1* and *ced-13* recapitulates our mutant analysis. RNAi of *egl-1* reduces apoptosis in *syp-1(me17)* (DD + Syn) and *spo-11(ok79);syp-1(me17)* (Syn) mutants and RNAi of *ced-13* reduces apoptosis in *syp-1(me17)* (DD + Syn) and *pch-2(tm1458);syp-1(me17)* (DD) mutants. (E) Mutation of both *egl-1* and *ced-13* in *syp-1(me17)* (DD + Syn) and *pch-2 (tm1458);syp-1(me17)* (DD) mutants does not reduce apoptosis to physiological levels. $*P < 0.05$ and $**P < 0.01$. Significance was assessed by performing a paired *t*-test.

induced (Figure 3C). However, *egl-1* was not transcriptionally upregulated in *pch-2;syp-1* double mutants (Figure 3C), validating our genetic data placing *egl-1* in the synapsis checkpoint pathway in *syp-1* mutants (Figure 2B). By contrast, *ced-13* mRNA was present at higher relative levels in *syp-1* mutants and *pch-2;syp-1* double mutants when compared to wild-type and *spo-11;syp-1* double mutants (Figure 3D), lending support to our finding that *ced-13* is required for the DNA damage checkpoint in *syp-1* mutants (Figure 2C).

During *C. elegans* development, *egl-1* is transcriptionally regulated to limit somatic apoptosis to specific tissues in response to developmental cues (Nehme and Conradt 2008). Much of this regulation occurs at the *egl-1* locus, where *cis*-acting regulatory sites are the downstream targets of well-characterized developmental pathways (Conradt and Horvitz 1999; Thellmann *et al.* 2003; Liu *et al.* 2006; Potts *et al.* 2009; Hirose *et al.* 2010; Hirose and Horvitz 2013). We wondered whether *egl-1* transcription was similarly regulated in response to events during meiotic prophase. We identified a sequence downstream of *egl-1* that is required to limit *egl-1*'s contribution to germline apoptosis during checkpoint activation. The *egl-1(bc274)* allele removes a section of DNA ~1.6–3 kb downstream of the *egl-1* stop codon (see Figure 4A). Deletion of this region elevated apoptosis in wild-type and *meDf2/+* mutant worms in a *spo-11*-dependent manner (Figure 4, B and C), indicating that this region is specifically required to inhibit germline apoptosis in response to DNA damage incurred during meiotic recombination. In support of this interpretation, apoptosis was also enhanced in *syp-1* and *pch-2;syp-1* mutants but not in *spo-11;syp-1* mutants

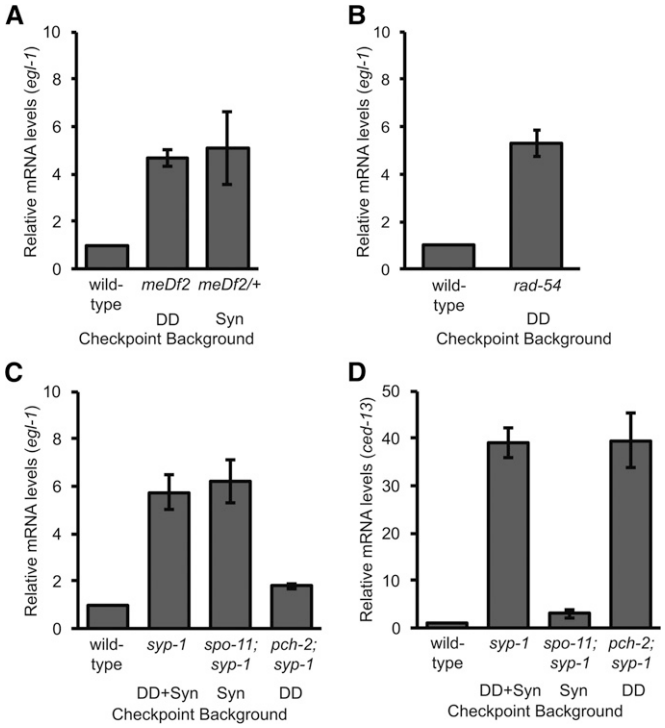

Figure 3 *egl-1* and *ced-13* are transcriptionally induced in response to different checkpoints in *syp-1* mutants. (A) *egl-1* transcription is induced when either the DNA damage checkpoint (*meDf2*) or the synapsis checkpoint (*meDf2/+*) is active. Relative transcript levels of *egl-1* mRNA are shown in wild type, *meDf2* homozygous (DD), and *meDf2* heterozygous (Syn) mutants. (B) *egl-1* transcription is induced when only the DNA damage checkpoint is active. Relative transcript levels of *egl-1* mRNA are shown in wild type and *rad-54* mutants (DD). (C) *egl-1* transcription is induced when the synapsis checkpoint is active (*syp-1[me17]*) and *spo-11[ok79];syp-1[me17]* mutants). Relative transcript levels of *egl-1* mRNA are shown in wild type, *syp-1(me17)* (DD + Syn), *spo-11(ok79);syp-1 (me17)* (Syn), and *pch-2(tm1458);syp-1(me17)* (DD) mutants. (D) Transcription of *ced-13* is induced when the DNA damage checkpoint is active (*syp-1[me17])* and *pch-2[tm1458];syp-1[me17]* mutants). Relative transcript levels of *ced-13* mRNA are shown in *syp-1(me17)* (DD + Syn), *spo-11(ok79);syp-1(me17)* (Syn), and *pch-2(tm1458);syp-1(me17)* (DD). RNA was extracted from 100 worms of each genotype using TRIzol (Life Technologies) according to the manufacturer's directions. RNA was converted to cDNA using the Superscript III First Strand Synthesis System (Life Technologies) and quantitative RT-PCR was performed to determine relative mRNA levels using the following primers: *egl-1* in A and B, 5′-tactcctcgtctcaggactt-3′ and 5′-catcgaagtcatcgcacat-3′; *ced-13* in C, 5′-acggtgtttgagttgcaagc-3′ and 5′-gtcgtacaagcgtgatggat-3′; and *tbg-1* (reference mRNA used to normalize the quantitative results) in A–C, 5′-cgtcatcagcctggtagaaca-3′ and 5′-tgatgactgtccacgttgga-3′. Thermoprofile was as follows: 95° for 30 sec, 52° for 30 sec, 72° for 45 sec for 45 cycles. Reactions were run using Power SYBR Green PCR Master Mix (Life Technologies), and fold enrichment was calculated using the ddCt method (Livak and Schmittgen 2001). Average of three experiments is shown.

(Figure 4D). Quantitative RT-PCR in *egl-1(bc274)* mutants indicated that this regulatory region was required to inhibit *egl-1* transcription (Figure 4E).

The region deleted in *egl-1(bc274)* removes the first exon of the gene *F23B12.1* (Figure 4A). To determine whether the elevation in apoptosis in *egl-1(bc274)* was due to inactivation of this gene, we inactivated *F23B12.1* by feeding RNAi in wild-type worms and did not observe any increase in germline apoptosis (Figure 4F). We performed qPCR with *F23B12.1*-specific primers to determine whether our RNAi was successful. However, we had difficulty detecting transcripts in both wild-type adult hermaphrodites and *syp-1* adult hermaphrodites (data not shown), suggesting that this gene may not normally be transcribed in wild-type adult hermaphrodites or during meiotic checkpoint activation. Therefore, it is unlikely to contribute to the phenomena we observe in *egl-1(bc274)* mutants. Consistent with this interpretation, *F23B12.1* is among a group of genes identified as spermatogenesis enriched by microarray analysis (Reinke *et al.* 2004) and RNA-Seq analysis indicates that the transcript is enriched among L4 hermaphrodites (which undergo spermatogenesis) and males (Hillier *et al.* 2009; Lamm *et al.* 2011; Thomas *et al.* 2012). Meiotic nuclei in male germlines do not undergo apoptosis (Gumienny *et al.* 1999).

We tested which transcription factors might be regulating *egl-1* through *egl-1(bc274)*. *egl-1(bc274)* includes binding sites for transcription factors that regulate *egl-1* in the soma, namely *ces-1*, *hlh-2*, *and hlh-3* (Thellmann *et al.* 2003). HLH-2 and HLH-3 act as a heterodimer and mutation of one phenocopies loss of the other in the context of regulating apoptosis (Thellmann *et al.* 2003). We inactivated *ces-1* and *hlh-2* by feeding RNAi in wild-type worms and did not observe any elevation of germline apoptosis (Figure 4F). A similar phenotype has been reported in *ces-1* mutants (Gumienny *et al.* 1999). To verify that RNAi was effective, we evaluated the progeny of hermaphrodites that were exposed to RNAi and observed 10–20% adult progeny compared to the empty vector control, indicating that RNAi produced embryonic lethality and/or larval arrest (Krause *et al.* 1997; Thellmann *et al.* 2003).

Altogether, our experiments clarify the relative contributions of *ced-13* and *egl-1* in checkpoint-induced germline apoptosis in *C. elegans* (Figure 1, Figure 2, and Figure 3). Moreover, our results provide an explanation for why the DNA damage checkpoint-induced apoptosis is not observed in *meDf2* heterozygotes: *egl-1* transcription is negatively regulated in response to DNA damage incurred during meiotic recombination (Figure 4). This inhibition of *egl-1*-mediated apoptosis may be a mechanism to promote repair of DNA damage over the removal of defective nuclei during meiosis. Future investigations will focus on identifying the factor(s) that contributes to this negative regulation.

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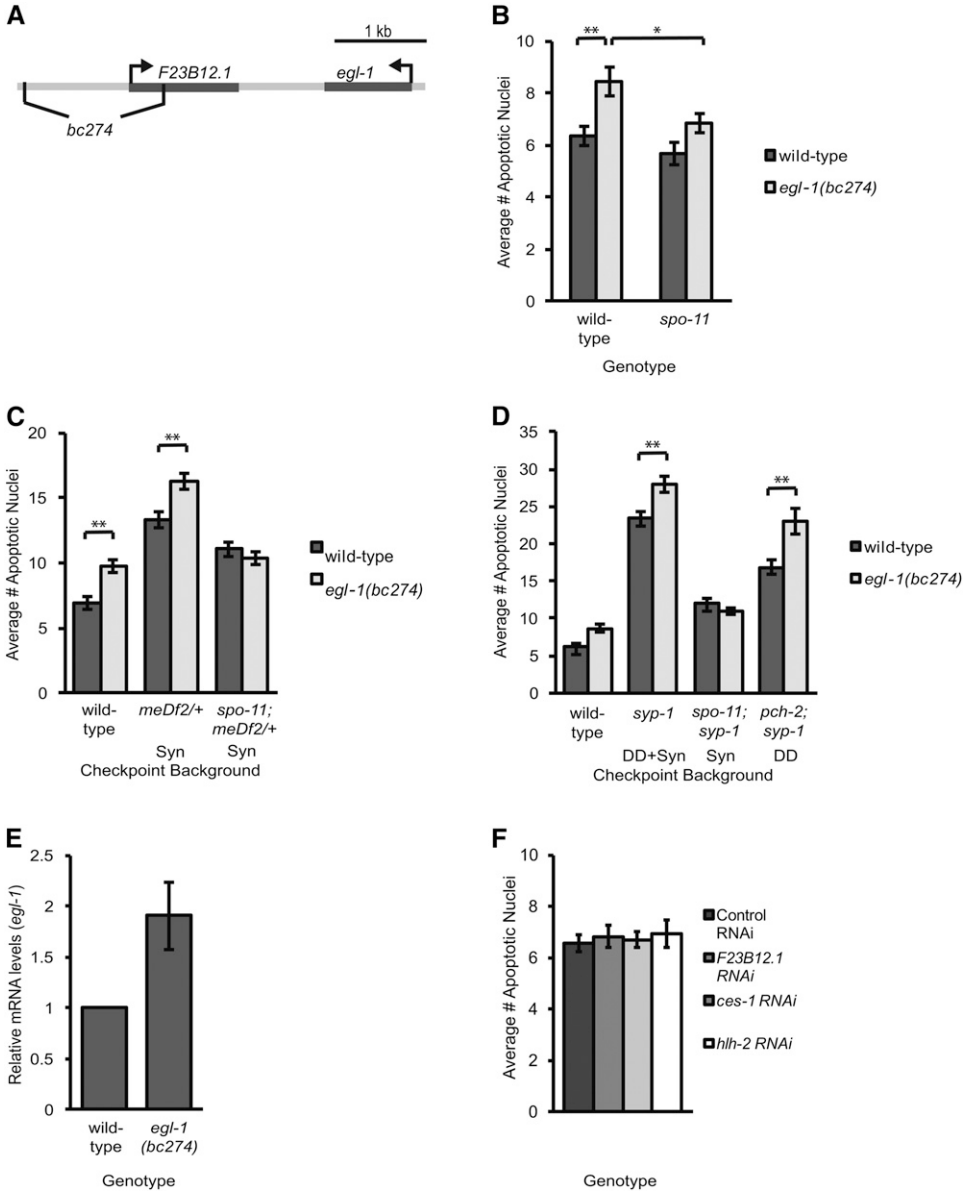

Figure 4 Identification of a negative regulatory element of *egl-1* required for germline apoptosis in response to double strand breaks. (A) Schematic of the *egl-1* locus. Coding sequence is indicated by darker gray rectangles (*egl-1* and *F23B12.1*). The *bc274* allele removes a 1.4-kb region starting 1.6 kb downstream of the *egl-1* coding sequence. (B) The *egl-1(bc274)* allele results in *spo-11*-dependent elevation of germline apoptosis. (C) *egl-1(bc274);meDf2/+* double mutants exhibit *spo-11*-dependent elevation of apoptosis in the germline. (D) *egl-1(bc274)* mutants elevate DNA damage checkpoint-induced apoptosis in the germline. Germline apoptosis is elevated when *egl-1(bc274)* is combined with mutants that activate the DNA damage checkpoint (*syp-1[me17]* and *pch-2 [tm1458];syp-1[me17]* mutants). (E) Relative transcript levels of *egl-1* mRNA are shown in wild type and *egl-1(bc274)*. *egl-1* transcription is slightly induced in *egl-1(bc274)* mutants. (F) RNAi of *F23B12.1*, *ces-1*, or *hlh-2* does not affect apoptosis in wild-type hermaphrodite animals. For *ces-1* and *hlh-2* RNAi, RNAi was performed postembryonically. $*P < 0.05$ and $**P < 0.01$. Significance was assessed by performing a paired *t*-test.

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