Functional redundancy of two *C. elegans* homologs of the histone chaperone Asf1 in germline DNA replication

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**Abstract**

Eukaryotic genomes contain either one or two genes encoding homologs of the highly conserved histone chaperone Asf1, however, little is known of their in vivo roles in animal development. UNC-85 is one of the two *Caenorhabditis elegans* Asf1 homologs and functions in post-embryonic replication in neuroblasts. Although UNC-85 is broadly expressed in replicating cells, the specificity of the mutant phenotype suggested possible redundancy with the second *C. elegans* Asf1 homolog, ASFL-1. The asf-1 mRNA is expressed in the meiotic region of the germline, and mutants in either Asf1 genes have reduced brood sizes and low penetrance defects in gametogenesis. The asf-1, unc-85 double mutants are sterile, displaying defects in oogenesis and spermatogenesis, and analysis of DNA synthesis revealed that DNA replication in the germline is blocked. Analysis of somatic phenotypes previously observed in unc-85 mutants revealed that they are neither observed in asf-1 mutants, nor enhanced in the double mutants, with the exception of enhanced male tail abnormalities in the double mutants. These results suggest that the two Asf1 homologs have partially overlapping functions in the germline, while UNC-85 is primarily responsible for several Asf1 functions in somatic cells, and is more generally involved in replication throughout development.

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**Introduction**

Histone chaperones are chromatin-associated proteins that assist in nucleosome formation and help mediate the post-translational modification of chromatin. These functions are important for replication, DNA repair and regulation of gene expression (Cheung et al., 2008). Among the most highly conserved histone chaperones are the Anti-silencing function 1 (Asf1) proteins. ASF1 was originally isolated in budding yeast as a gene that derepresses the silent mating type locus when overexpressed (Le et al., 1997). Homologs from various organisms were subsequently demonstrated to function in critical cellular processes including chromatin assembly and disassembly (Adkins et al., 2004, 2007b; Galvani et al., 2008; Moshkin et al., 2002; Tyler et al., 1999), histone acetylation (Adkins et al., 2004, 2007a; Recht et al., 2006; Tsubota et al., 2007), DNA replication and repair (Chen et al., 2008; Franco et al., 2005; Grigsby and Finger, 2008; Groth et al., 2005, 2007; Le et al., 1997; Mello et al., 2002; Sanematsu et al., 2006; Schulz and Tyler, 2006; Sen and De Benedetti, 2006; Tyler et al., 1999), transcriptional regulation (; Adkins et al., 2004, 2007a; Sharp et al., 2001; Sutton et al., 2001; Zabaronick and Tyler, 2005), and cell cycle progression (Chen et al., 2008; Sanematsu et al., 2006; Tyler et al., 1999).

Although Asf1 is one of the most highly conserved histone chaperones, with either one or two isoforms found in eukaryotic genomes, little information is available as to their in vivo functions in animals, their expression patterns, and the roles of the different isoforms in those organisms with two Asf1 isoforms encoded in their genomes. The *Drosophila* genome contains a single *ASF1* gene, and the dASF1 protein localizes to replicating embryonic nuclei, indicative of its role in DNA replication (Bonnefoy et al., 2007; Moshkin et al., 2002). *Drosophila* ASF1 also participates in transcriptional regulation through interactions with the Brahma (SWI/SNF) chromatin-remodeling complex (Moshkin et al., 2002) and the Su(H)/H DNA binding protein complex (Goodfellow et al., 2007). There is evidence that the two human ASF1 isoforms have distinct, but overlapping roles in vitro and when expressed in yeast (Tamburini et al., 2005). Both human ASF1s are implicated in both DNA-dependent and DNA-independent histone H3 assembly pathways for DNA synthesis (Galvani et al., 2008; Tagami et al., 2004). However, only ASF1A is proposed to regulate the quiescence pathway in the presence of histone repression A factor (HIRA) (Zhang et al., 2005), while ASF1B is the sole Asf1 in the regulatory pathway directed by the transcription factor E2F during cell cycle progression (Hayashi et al., 2007).

The nematode *Caenorhabditis elegans* has two Asf1 homologs encoded in its genome (Grigsby and Finger, 2008). We previously found that unc-85 encodes one of two worm Asf1 homologs (Grigsby and Finger, 2008). Mutations in unc-85 cause variable post-embryonic cell division failures, primarily in terminal neuronal lineages, which result in defective locomotion and a reduced ability to mate and lay...
embryos (Sulston and Horvitz, 1981). UNC-85 is widely expressed in replicating cells, where it localizes to nuclei, and in unc-85 mutants, post-embryonic lineage failures in ventral cord neuroblasts arise during DNA replication (Grigsby and Finger, 2008). However, the contrast between the broad expression of unc-85 in replicating cells and the specificity of the mutant phenotypes suggested that unc-85 might be functionally redundant with the second C. elegans Asf1 homolog.

In the present study, we investigated the developmental functions of the second C. elegans Asf1 homolog, asf-1 (asf-1-like), corresponding to sequence CO3De5. The asf-1 gene is highly expressed in the meiotic region of the hermaphroditic germline. In contrast to unc-85 mutants, no obvious phenotypes were observed in asf-1 mutants, except for a modest reduction in brood size. However, the asf-1; unc-85 double mutants were completely sterile, suggesting that UNC-85 and ASFL-1 have overlapping roles in the germline. Additionally, knockdown of unc-85 in an asf-1 mutant background results in embryonic lethality. The sterility of the Asf1 double mutants results from germline replication failures, which result in abnormal chromatin and DNA damage, but not in apoptosis. Several somatic unc-85 mutant phenotypes, including uncoordinated locomotion, are neither shared by nor modified in strains bearing mutations in asf-1. The exception is the additional morphological defects of the male tail sensory rays observed in double mutants, but not in unc-85 males. These results suggest that both of the C. elegans Asf1 homologs function to promote DNA replication in the germline, while UNC-85 is primarily responsible for several Asf1 functions in somatic cells, and is more generally involved in replication throughout development.

Materials and methods

Nematode strains and maintenance

All strains used in this study were derived from the Bristol strain N2 and were cultured using standard techniques on nematode growth medium (NGM) plates seeded with E. coli strain OP50 (Brenner, 1974).

The following strains were used in this work: FX874 asf-1(tm874) I; FX1625 asf-1(tm1625) I; FX2812 unc-85(tm2812) II; MT319 unc-85 (n319) II; MT1605 unc-85(n417) II; CB1414 unc-85(e1414) II; NG3124 dsh-2(e302)/mIn1I(dpy-10(e128) mIs14) II; FN57 [asf-1(tm874)]; unc-85(n319)/mlnIII]. Strains FX874, FX1625 and FX2812 were kindly provided by S. Mitani and the National Bioresource Project, Tokyo Women’s Medical University School of Medicine, Tokyo, Japan. Strains MT319 and MT1605 were kind gifts from H. R. Horvitz, Massachusetts Institute of Technology. Strain FN57 was created for this study (see below). All other strains were obtained from the Caenorhabditis Genetics Center. All mutant strains were backcrossed to N2 at least three times before use.

To obtain unc-85(n319); asf-1(tm874) double mutants, strain FN57 [asf-1(tm874); unc-85(n319)/mlnI] was constructed as follows: FX874 males were crossed to NG3124 hermaphrodites, and their GFP-expressing male progeny were subsequently crossed with MT319 hermaphrodites. The F1, hermaphrodite progeny from this second cross that expressed GFP, but appeared otherwise wild-type, were individually cultured. Their uncoordinated non-GFP-expressing progeny were scored and used as templates for single worm PCR assays (Fay, 2006) to confirm homozygosity of the tm874 allele. FN57 hermaphrodites were heat shocked at 30 °C for 4 h, and the resulting male progeny were mated to FN57 hermaphrodites to obtain unc-85 (n319); asf-1(tm874) males for use in analysis of male germlines and male tails.

Two alleles of asf-1 were used in this work: tm874 deletes a 623 bp genomic region including the entire first exon and part of the second exon, and is predicted to be null; tm1625 deletes 605 bp, including the entire first intron and the second exon (Fig. 1A), and is predicted to result in a frameshift resulting in a premature stop codon at residue 41. A third allele of asf-1, ok2060, became available after we completed phenotypic characterization of the other two asf-1 alleles, and displayed very similar phenotypes (data not shown). Four alleles of unc-85 were used in these studies. The e1414 allele is a deletion of 142 nucleotides, resulting in a frameshift that produces a 132 residue truncated protein containing only the first 115 amino acids of the anti-silencing functional domain followed by 17 novel residues. The n319 and n471 alleles contain nonsense mutations at codons W41 and W154, respectively. The e1414, n319 and n471 alleles were previously characterized and shown to have significantly decreased unc-85 mRNA expression (Grigsby and Finger, 2008). The tm2812 allele deletes a genomic region spanning 206 bp in the second exon of unc-85 and is predicted to be null. MT319 hermaphrodites were crossed to N2 males, and the heterozygous male progeny were mated to MT319 hermaphrodites to obtain unc-85(n319) males for analysis of the male germline and tail.

Confirmation of the exon–intron structure and 5′ UTR of asf-1

Total RNAs from mixed developmental stages of N2s were prepared as previously described (Grigsby and Finger, 2008). SuperScript™ III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) was used to generate first strand cDNAs using asf-1-specific primers, according to the manufacturer’s instructions. The first strand cDNAs were incubated with E. coli RNaseH (Invitrogen) at 37 °C for 20 min, then used as templates for PCR reactions with iProof high-fidelity DNA polymerase (Bio-Rad Laboratories, Hercules, CA). PCR products were purified using a PCR Cleanup Kit (Eppendorf AG, Hamburg, Germany), then sequenced (Macrogen, Rockville, MD). Sequences of primer sets are available upon request.

Brood size, egg-laying, and embryonic lethality assays

To determine brood sizes, ten worms from each strain analyzed (N2, MT319, FX2812, FX874, FX1625 and FN57) were individually plated, then transferred every 24 h to new plates. Each worm’s progeny were counted daily until no further progeny were produced. We analyzed more unc-85(tm2812) worms (n = 97) than worms of the other strains because a small proportion (3%) die as later stage larvae, a phenotype not observed with the other three unc-85 alleles. To determine whether animals lay embryos efficiently, ten L4 hermaphrodites of each strain (N2, FX2812, FX874, FX1625, MT319 and FN57) were individually plated, and the number of embryos laid was counted 24 h later.

Because unc-85 mutants are defective in egg-laying (Sulston and Horvitz, 1981), the following method was used to analyze embryonic lethality: Gravid young adult hermaphrodites were placed in egg buffer (118 mM NaCl, 48 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM Hepes, pH 7.3) and cut open at the vulva to release embryos, which were transferred onto NGM plates and counted. Embryo counting was repeated 24 h later to determine the number of un Hatchet embryos. At least 300 N2, MT319, FX874 and FX1625, and NG3124 embryos were analyzed. The progeny of +/mlnIII hermaphrodites, asf-1 (tm874); +/mlnIII hermaphrodites, unc-85(n319)/mlnIII hermaphrodites, and asf-1(tm874); unc-85(n319)/mlnIII hermaphrodites were similarly analyzed.

To assess whether the maternal Asf1 contribution was responsible for embryonic viability of the Asf1 mutants, RNAi of the Asf1 homolog was performed. Double stranded RNAs for asf-1 and unc-85 were prepared as follows: A 345 bp Salt– HindIII restriction fragment of the asf-1 CDNA, and a 343 bp HincII– HindIII restriction fragment of the unc-85 CDNA were each ligated into the dsRNA expression vector L4440 to create plasmids pCM75 and pCM90, respectively. These plasmids were then used as templates for in vitro production of dsRNAs using T7 RNA polymerase (New England Biolabs, Ipswich, MA) according to the supplier’s recommendations.
The dsRNAs were purified by phenol–chloroform extraction and ethanol precipitation, then resuspended in TE. One gonad arm of N2, FX874, and MT319 young adult hermaphrodites were injected with 425 ng/μl asfl-1 dsRNA and/or 612 ng/μl unc-85 in DEPC-treated water (Invitrogen) water, or with DEPC-water alone (injection control). The numbers of worms injected for each experiment are: eight N2 injection control worms, nine N2s injected with asfl-1 dsRNA, ten N2s injected with unc-85 dsRNA, eight N2s injected with both asfl-1 dsRNA and unc-85 dsRNA, ten FX874 worms injected with asfl-1 dsRNA, twelve FX874 worms injected with unc-85 dsRNA, eleven MT319 worms injected with asfl-1 dsRNA, and eight MT319 worms injected with unc-85 dsRNA. Injected worms were moved to new plates every 24 h, and the number of dead embryos and viable worms was determined for each plate 24 h after the mother was shifted to a new plate.

**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Brood size</th>
<th>Embryos laid 24 h post-L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>328 ± 5.1</td>
<td>46 ± 1.3</td>
</tr>
<tr>
<td>asfl-1(tm874)</td>
<td>273 ± 3.5&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>35 ± 0.6&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>asfl-1(tm1625)</td>
<td>264 ± 1.6&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>38 ± 0.9&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>unc-85(n319)</td>
<td>92 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>unc-85(tm1625)</td>
<td>61 ± 22.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22 ± 6.0&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>asfl-1(tm874); unc-85(n319)</td>
<td>0</td>
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</tr>
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</table>

Data are averages ± SEM. For unc-85(tm1625), n = 97. For all other strains, n = 10.

<sup>a</sup> Significantly different from wild-type, P < 0.05.

<sup>b</sup> Significantly different from unc-85(n319), P < 0.05.

<sup>c</sup> Significantly different from unc-85(tm1625), P < 0.05.

**Quantitative RT-PCR**

Embryos and staged worms (L1/L2, L3, L4, and young adults) were collected and total RNAs were extracted as described (Grigsby and Finger, 2008). For each sample, 100 ng of total RNA were used for reverse transcription and PCR using the SuperScript<sup>™</sup> III Platinum<sup>®</sup> SYBR<sup>®</sup> Green One-Step qRT-PCR Kit (Invitrogen). ama-1, the gene encoding the largest subunit of C. elegans RNA polymerase II (Johnstone and Barry, 1996), was used as an endogenous standard. The asfl-1 primers used in the real-time PCR reaction were 5′-ATGATGCGCAGGATGATGAC and 5′-TCATGTGCATTCGCCATTCGTTCGT, located in the fourth exon of asfl-1. The reverse primer, 5′-ACGGCCTACCTAUCTCCT; ama-1 primers were 5′-ACGGCCTACCTAUCTCCT and 5′-TACGGCCTACCTAUCTCCT. Experiments and data analysis were performed with a LightCycler<sup>®</sup> 480 Real-Time PCR Instrument and LightCycler<sup>®</sup> 480 Basic Software (Roche Applied Science, Indianapolis, IN).

**RNA in situ hybridization**

A reverse primer, 5′ TCATGGCATCGGCGGATCCTGC, located in the fourth exon of asfl-1 was used to synthesize the first strand cDNA with the SuperScript<sup>™</sup> III Reverse Transcriptase (Invitrogen) as described above. The cDNA then served as a PCR template using Taq polymerase (Invitrogen) with a forward primer, 5′ TCTCGAATGG-GAATTGGTACG, located in the second exon and a reverse primer.
located in the fourth exon of asfl-1. The PCR products were cloned into the PCR2.1 vector (Invitrogen) to create pFlB67, and used as templates for preparation of digoxigenin (DIG) labeled anti-sense and sense DNA probes with DIG DNA Labeling Mix (Roche Applied Science, Indianapolis, IN). Probe synthesis and RNA in situ hybridization of dissected gonads and embryos were performed as described (Grigsby and Finger, 2008; Lee and Schedl).

Terminal deoxynucleotidyl transferase-mediated dNTP nick end-labeling (TUNEL) and SYTO-12 apoptosis assays

L4 worms were synchronized and cultured at 20 °C. Young adult worms were dissected 12 h later in PBS (200 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl) containing 0.25 mM levamisole (Sigma-Aldrich Co., St. Louis, MO), and extruded gonads were fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature. The TUNEL assay was performed as described (Parusel et al., 2006).

Apoptosis assays on 25 hermaphrodites of each genotype were performed using SYTO-12 (Invitrogen) as described (Gumienny et al., 1999) at 12 h and at 36 h post-L4.

Germ cells, sperm counts and fluorescence microscopy

To examine germ cell morphology, young adult hermaphrodites (12 h post-L4) were dissected and fixed as described above. For counting sperm, whole worms were fixed with Bouin’s fixative as described (Nonet et al., 1997). After fixation, dissected gonads and whole animals were washed three times in PBSTx (PBS containing 0.5% Triton X-100) and stained with 1 μg/ml 4′-6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich Co.), for 10 min, washed twice in PBSTx, and mounted on slides (Duerk, 2006). A Zeiss LSM510 META confocal microscope equipped with C-Apochromat 40×/1.2W Corr and plan-Apochromat 63×/1.4 N.A oil immersion objectives and a Zeiss Axiovert 200M inverted microscope equipped with differential interference contrast optics and with a plan-Apochromat 63×/1.4 N.A. oil immersion objective. The total number of cells that incorporated Cy3-dUTP was determined for each dissected germine.

In situ detection of germline DNA synthesis

Direct incorporation of Cy3-dUTP into germline nuclei was performed as described (Jaramillo-Lambert et al., 2007), except that the injection mix consisted of 50 μM Cy3-dUTP (Amersham Biosciences, Piscataway, NJ) in PBS, pH 7.2. After ~2.5 h of exposure to the Cy3-dUTP, gonads were dissected, fixed and DAPI stained as described (Jaramillo-Lambert et al., 2007). Optical sections of gonad arms were collected using a Zeiss LSM510 META confocal microscope equipped with a C-Apochromat 40×/0.75 N.A, 63×/1.4 N.A oil immersion objectives and a Zeiss AxioCam HRm digital CCD camera at 1× zoom, was used to collect 0.3 μm optical sections. The nuclear morphology of dissected germine and the number of germ cells and sperm were determined from three-dimensional reconstructions created with the Zeiss LSM 510 software. Transmitted light differential interference contrast (DIC) images of dissected gonads or animals were obtained using a Zeiss Axiovert 200M inverted microscope equipped with plan-Apochromat 40×/0.75 N.A, 63×/1.4 N.A oil immersion objectives and a Zeiss AxioCam HRm digital CCD camera. At least 10 animals of each strain were analyzed.

Analysis of male tail morphology, alae, and locomotory behavior

Ten young adult males of wild-type, asfl-1(tm874), unc-85(tm319) and unc-85(n319); asfl-1(tm874) genotypes were observed using the Zeiss Axiovert 200M microscope equipped with differential interference contrast optics and with a plan-Apochromat 63×/1.4 N.A oil immersion objective. Adult hermaphrodites of the same genotypes were observed in the same manner to determine the presence and appearance of the alae.

Asfl-1 mRNA is expressed at relatively high levels in the germline and in early embryos

To determine the expression levels of asfl-1, quantitative RT-PCR (qRT-PCR) was performed on embryos and four different developmental stages of wild-type (N2) and asfl-1 mutant worms. The qRT-PCR results were normalized to the expression level of wild-type L1/L2 larvae. In wild-type worms, relative asfl-1 mRNA expression levels were high in embryos (16-fold, Fig. 1B), increased dramatically at the L4 (10-fold, Fig. 1B) and young adult stages (30-fold, Fig. 1B), indicating that asfl-1 expression is developmentally regulated. In worms homozygous for either of the two asfl-1 mutant alleles, overall expression remained low, reaching only ~7–20% of the wild-type levels, and no relative increases in embryonic expression levels were observed (Fig. 1B). Because its mRNA expression level was the lower of the two asfl-1 alleles tested (Fig. 1B), and the deletion eliminates the entire first exon including the start codon, the tm874 allele was selected for more detailed analysis.

The slightly reduced brood size of the mutants and the asfl-1 mRNA expression peak in young adults suggested that asfl-1 might be expressed and function in the germline. The C. elegans gonad contains...
Fig. 2. Asf1 homologs are required in hermaphrodites for oocyte production and normal gonad morphology. (A) Schematic of an adult hermaphrodite gonad arm. (B–E) DIC images of gonad arms from young adult hermaphrodites. Panels B and C show focal planes at the surface of the germline in the pachytene region. (B) Wild-type. Nuclei in the pachytene region are uniformly sized. After passing through loop area, developing oocytes gradually enlarge and are arranged as a single row of cuboidal cells. (C) asf-1(tm874) has germline morphology similar to wild-type. (D) A focal plane through the middle of an unc-85(n319) gonad arm to visualize the rachis with two rows of nuclei aligned along the periphery of the gonad arm (arrow). No abnormalities are observed. (E) asf-1(tm874); unc-85(n319). Abnormally enlarged germ cell nuclei are often observed (arrowheads), and oocytes are rounded, rather than the normal cuboidal shape (arrow). A vacuole is indicated (white arrow). (F, G) Wild-type gonad arms. (F) The distal tip cells (DTCs, arrowheads) are indicated and embryos (black arrow) are located within the uterus, near the vulva (white arrow). (G) A wild-type, U-shaped gonad arm. The direction of distal tip cell migration, inferred from the shape of the gonad arm, is indicated. (H) A gonad arm of an asf-1(tm874); unc-85(n319) double mutant (similar focal plane as in A, arrowhead indicates DTC, white arrow indicates the vulva). No embryos are present. (I) An abnormally shaped gonad arm of an asf-1(tm874); unc-85(n319) double mutant, with inferred distal tip cell migration path indicated. Scale bars are 10 μm.
two (anterior and posterior) symmetrical U-shaped arms which share a common uterus, diverging at their spermathecae, where sperm are stored (Hirsh et al., 1976; McCarter et al., 1999). Extension of the gonad arms begins in the L2 stage, and is led by distal tip cells (DTCs; Hall et al., 1999; Hedgcock et al., 1987). Germline nuclei exhibiting mitotic features can often be found in approximately the first 20 rows from the distal end (Crittenden et al., 1994). Upon exiting this region, germ cells progress through a narrow transition zone, where nuclei take on a characteristic crescent-shape as their chromatin begins to condense and early prophase I of meiosis is completed (Crittenden et al., 1994). Germ cells are in the pachytene stage of meiotic prophase from when they exit the transition zone until the loop area of the gonad arm, where oogenesis begins (Hirsh et al., 1976). To address whether asf-1 is expressed in the germline, in situ hybridizations to sense and antisense DNA probes derived from the asf-1 cDNA were performed on dissected gonad arms (Figs. 1C–E). We found that the asf-1 mRNA expression level was relatively low in the distal end of the germline in wild-type worms, increased gradually through the transition zone, and peaked in the pachytene region (Fig. 1C). Oocytes at diakinesis were also observed to express the asf-1 mRNA (Fig. 1C arrowhead). Expression of the asf-1 mRNA in embryos was also examined (Figs. 1F–I). Weak signals were detected in early embryos (Fig. 1F), but no signals were detectable past the 1.5-fold stage (data not shown). No mRNA expression was detected in asf-1(tm874) dissected gonad arms (Fig. 1E) and embryos (Fig. 1G), consistent with the findings from qRT-PCR experiments that asf-1 mRNA expression was dramatically decreased in the mutants compared to wild-type young adults and embryos. In conclusion, the two Asf1 homologs are required for fertility and normal gonad morphology.

Although Asf1 histone chaperones are very highly conserved and function in critical processes, including replication, DNA repair, and regulation of transcription, the phenotypes of asf-1 and unc-85 mutants are relatively mild, which led us to suspect that they might be partially functionally redundant. Double mutants were therefore constructed with the asf-1(tm874) and unc-85(n319) alleles, as these are putative null alleles. We first tried to construct the double mutant by directly crossing asf-1(tm874) and unc-85(n319) hermaphrodites with asf-1(tm874) males, but the resulting double mutants were completely sterile, as well as uncoordinated. We then balanced unc-85(n319) with mnh1 (Edgley and Riddle, 2001), crossed in asf-1(tm874), and again found that the asf-1(tm874); unc-85(n319) progeny were viable, but uncoordinated and sterile, phenotypes commonly observed in mutants that are defective in post-embryonic and germline cell divisions (Albertson et al., 1978; Horvitz and Sulston, 1980; O’Connell et al., 1998; Sulston and Horvitz, 1981). The reproductive systems of the asf-1(tm874) and unc-85(n319) single mutants and of the double mutant were analyzed to determine the cause of the sterility. Hermaphrodite gonads from each single mutant appeared normal in morphology (Figs. 2C, D). However, asf-1(tm874); unc-85(n319) hermaphrodites were often observed to contain unequally sized germ cell nuclei (Fig. 2E, arrowheads), and abnormal oocytes (Fig. 2E, black arrow), and never contained embryos (Fig. 2H). Normal, U-shaped gonad arms were observed in wild-type (Figs. 2B, F, G), asf-1(tm874) (Fig. 2C), and unc-85(n319) hermaphrodites (Fig. 2D). In contrast, asf-1(tm874); unc-85(n319) hermaphrodite gonad arms were generally small and variably misshapen (Fig. 2I). Both C. elegans Asf1 homologs are required for normal oogenesis.

The small size of the hermaphrodite germlines from the asf-1(tm874); unc-85(n319) double mutants (Figs. 2I and 3D) suggested that germ cell proliferation might be defective. To determine if this were the case, gonad arms from wild-type, asf-1(tm874), unc-85(n319) and asf-1(tm874); unc-85(n319) young adult hermaphrodites were dissected, DAPI-stained, and the number of germ cells in individual gonad arms were counted (Fig. 3E). The asf-1(tm874) germlines (Figs. 3B, E) contained 27% fewer germ cells than wild-type worms (Figs. 3A, E). Although there was considerable variability within this genotype, the unc-85(n319) mutant germlines (Figs. 3C, E) contained an average of 36% fewer germ cells than wild-type germlines. The reductions in germ cell number observed with the individual Asf1 mutants were enhanced in the asf-1(tm874); unc-85(n319) germlines (Figs. 3D, E), which contained 63% fewer germ cells than wild-type germlines. These results suggest that the two C. elegans Asf1 homologs have partially overlapping functions in germ cell proliferation.

To further understand the cause of the germ cell proliferation defect in the Asf1 mutants, germline chromatatin was analyzed in DAPI-stained germlines dissected from wild-type, both single mutants, and the double mutant worms. In the loop region of the C. elegans germline, sister chromosomes are condensed and arranged at the periphery of the spermathecae.
numbers of sperm are observed in the occasionally observed to not be fully condensed. Scale bars are 10

the nuclei as the germ cells progress from the late pachytene to the diplotene stages of meiosis I prophase (Hirsh et al., 1976). Between the loop region and the proximal region of the germ line, germ cells proceed through the diplotene stage to diakinesis, with developing oocytes arranged in a single row and gradually enlarging as they move toward the proximal end of the germ line (Fig. 2A) (Hirsh et al., 1976). Oocytes remain arrested in diakinesis of meiotic prophase I and do not complete meiosis until after fertilization (Hirsh et al., 1976). In wild-

type worms (Figs. 4A and 5A, insets) and in unc-85(n319) mutants (Fig. 4C, inset), condensed chromosomes localized to the periphery of the germ cells, and six bivalents were present in oocytes, however the chromosomes in unc-85(n319) were not always fully condensed (Fig 4C, inset). In contrast, developing oocytes from asfl-1(tm874) mutants occasionally (13%, n = 15) contained chromosomes with abnormal morphology (Fig 4B, inset). Oocytes from asfl-1(tm874); unc-85(n319) double mutants rarely contained six bivalents, instead containing clumps of chromosomes, some of which appeared to be fragmented (Fig. 4D, inset). Germ cells containing disorganized chromosomes were always observed in the asfl-1(tm874); unc-85(n319) double mutants, particularly towards the proximal end of the pachytene region (Figs. 4D and 5J inset).

Because the germ cell chromatin often appeared abnormal in the double mutants, TUNEL assays were performed on dissected gonad arms to determine whether these abnormalities resulted in increased germ cell apoptosis. In contrast to wild-type (Fig. 5B) and to the single mutants (Figs. 5E, H), strong TUNEL signals were detected in the asfl-1(tm874); unc-85(n319) double mutant hermaphrodites. We found that at 12 h post-L4, the time at which we detected TUNEL staining of nuclei in the double-mutant germ lines, there was essentially no SYTO-12 staining of the germ lines of any of the tested strains. We repeated the SYTO-12 staining on worms at 36 h post-L4, the time at which this assay is generally performed, and found no significant differences between any of the tested strains. These results suggest that although the germ cell DNA of the double mutants contains numerous double-stranded breaks, the damage does not result in and is not caused by programmed cell death.

**The C. elegans Asf1 homologs are required for spermatogenesis**

After determining that oogenesis was defective in the C. elegans Asf1 double mutant hermaphrodites, we next investigated whether the Asf1 homologs also are required for spermatogenesis. To determine if there were defects in hermaphrodite spermatogenesis, the total number of spermatozoa within the spermathecae of wild-type, unc-85(n319), asfl-1(tm874); unc-85(n319) double mutant hermaphrodites were counted (Figs. 4A–D arrows and E). In each of the single mutants, the number of sperm was reduced by approximately 30–35% compared to wild-type. More severe effects were observed with asfl-1(tm874); unc-85(n319) hermaphrodites, which have only approximately 17% of the wild-type number of sperm, suggesting that the two Asf1 homologs function synergetically in hermaphrodite spermatogenesis.

While only approximately the first forty germ cells that enter meiosis in each gonad arm differentiate into hermaphrodite sperm, males generate sperm throughout their adult lives (L'Hernault, 1997). To determine if the C. elegans Asf1 homologs also function in male
spermatogenesis, testes from wild-type, \textit{unc-85(n319)}, \textit{asf-1(tm874)}, and double mutant males were examined. In wild type males, the proximal part of the germline contains pachytene nuclei, primary spermatocytes, secondary spermatocytes, and spermatids arrayed sequentially with each stage from pachytene to secondary spermatocytes progressively reduced in DNA content (Figs. 6A and B). Primary spermatocytes (4n) separate from the rachis, a central core of syncytial germline cytoplasm, prior to meiosis I and immediately undergo meiosis I, giving rise to secondary spermatocytes (2n), which then initiate meiosis II, producing spermatids (n) (Lints and Hall, 2005) (Fig. 6A). We used DAPI staining to distinguish between these stages and counted the primary spermatocytes and spermatids in the wild-type male germline, primary spermatocytes, secondary spermatocytes and sperm are sequentially arrayed, and can be distinguished by their relative DNA contents (B). Fewer spermatocytes and sperm are present in the germlines of \textit{asf-1(tm874)} (C), \textit{unc-85(n319)} (D), and \textit{asf-1(tm874); unc-85(n319)} males (E). Reduced numbers of primary spermatocytes (F) and sperm (G) are observed in the \textit{asf-1(tm874)}, \textit{unc-85(n319)}, and \textit{asf-1(tm874); unc-85(n319)} males, compared to wild-type males. Additionally, the sperm nuclei are irregular in morphology, suggesting a possible chromatin condensation defect. Means±SEM are shown. *Significantly different from wild-type, \(P<0.001\). Ten animals of each genotype were examined.

![Diagram](image.png)
Fig. 7. Replication is blocked in asf-1(tm874); unc-85(n319) germlines. Germlines from wild-type (A–C), asf-1(tm874) (D–F), unc-85(n319) (G–I), and from asf-1(tm874); unc-85(n319) (J–L) young adult hermaphrodites were directly injected with Cy3-dUTP, then allowed to recover for 2.5 h, before germlines were dissected, DAPI stained, and observed by confocal microscopy. (A, D, G, J) DAPI. (B, E, H, K) Cy3-dUTP incorporation. (C, F, I, L) Merge. Scale bars are 10 μm. (M) Quantification of Cy3-dUTP incorporation. The means of labeled nuclei per germline ± SEM are shown. *Significantly different from wild-type, P< 0.001, N = 7 for asf-1(tm874) and asf-1(tm874); unc-85(n319), and N = 8 for wild-type and unc-85(n319).
tests from wild-type, asfl-1(tm874), unc-85(n319), and asfl-1 (tm874); unc-85(n319) double mutant young adult males. Although its organization appeared normal, only nine primary spermatocytes on average were present in asfl-1(tm874) tests (Figs. 6C, F), a 73% decrease from the average of 33 primary spermatocytes present in wild-type tests (Figs. 6B, F). Only 12 primary spermatocytes on average were observed in the unc-85(n319) mutant tests, (Figs. 6D, F), 64% fewer than in wild-type tests. Testes from double mutant males resembled those from asfl-1(tm874) mutants, with an average of only nine primary spermatocytes (Figs. 6E, F). Furthermore, about 50% fewer spermatids were present in asfl-1(tm874), unc-85(n319) and asfl-1(tm874); unc-85(n319) testes than in wild-type tests (Fig. 6G), possibly because of the reduced number of primary spermatocytes. Germlines from unc-85(n319) males were occasionally disorganized, with sperm located in proximity to primary spermatocytes. A possible explanation for this phenotype is that copulatory apparatus defects of unc-85 males (Sulston and Horvitz, 1981) (Fig. 6C) may result in sperm accumulation and germline disorganization. In addition to the decreased numbers of sperm in the double mutant, the few sperm have irregular nuclear morphology (Fig. 6E), suggesting that chromatin may not be properly condensed. Taken together, these results indicate that the Asf1 homologs participate in spermatogenesis in both sexes.

The Asf1 homologs function redundantly in germline DNA replication

Based on the known roles of Asf1s in other organisms (Franco et al., 2005; Groth et al., 2005; Kats et al., 2006; Le et al., 1997; Sanematsu et al., 2006; Schulz and Tyler, 2006; Tyler et al., 1999), and our previous finding that unc-85 mutants are defective in post-embryonic replication (Grigsby and Finger, 2008), we hypothesized that the most critical function of the Asf1 homologs in the C. elegans germline might be in DNA replication. To test this hypothesis, we directly assessed incorporation of deoxyribonucleotides into germline DNA (Jaramillo-Lambert et al., 2007). Following direct injection of Cy3-dUTP into young adult hermaphrodite germlines, worms were allowed to recover for 2.5 h, and then their germlines were dissected, fixed, DAPI-stained, observed by confocal microscopy, and the number of nuclei that had incorporated Cy3-dUTP was quantified for each germline. There were no significant differences in the number of nuclei that incorporated Cy3-dUTP in germlines from wild-type (Figs. 7A–C, M), asfl-1(tm874) (Figs. 7D–F, M), and unc-85(n319) (Figs. 7G–I, M) hermaphrodites. In contrast, little to no incorporation of Cy3-dUTP (Figs. 7J–M) occurred in asfl-1(tm874); unc-85(n319) hermaphrodite germlines. The double mutant germlines clearly contained Cy3-dUTP, and although it surrounded germ cell nuclei in the distal end of the germlines, confirming that the injections were successful, there was little colocalization with the DAPI staining. The DAPI-stained nuclei in the distal end of the asfl-1 (tm874); unc-85(n319) germlines sometimes appeared as enlarged, possibly interconnected masses of varying sizes, rather than the smaller, more uniformly-sized nuclei observed in the other strains analyzed, consistent with replication defects. These results demonstrate that germline DNA replication is blocked in the asfl-1(tm874); unc-85(n319) double mutants.

Asf1 function is required for embryo viability

Because the qRT-PCR results indicated that both genes are expressed at relatively high levels in embryos (Grigsby and Finger, 2008) (Fig. 1B), we examined whether mutations in either of the two Asf1 homologs affected embryonic viability (Table 2). Both of the asfl-1 alleles and all four of the unc-85 alleles tested conferred less than 6% embryonic lethality, indicating that the loss of each Asf1 homolog individually does not severely affect viability. Because the asfl-1 (tm874); unc-85(n319) double mutants are sterile, the viability of their progeny could not be assessed; however no synergistic effect on embryonic lethality was observed in the progeny of asfl-1(tm874); unc-85(n319)/+; mnl1 hermaphrodites from the FN57 balanced strain compared to control asfl-1(tm874); +/mnl1 or unc-85(n319)/+; mnl1 worms (Table 2). The viability of the asfl-1(tm874); unc-85(n319) double mutants may be due to maternal contributions from the balanced hermaphrodites, which is commonly observed in other C. elegans sterile coordinated mutants (O’Connell et al., 1998). Alternatively, the viability of the double mutants may be due to functional redundancy with other genes.

To determine if maternal Asf1 is necessary for embryo viability, dsRNA corresponding to asfl-1 and/or to unc-85 was injected into gonad arms of wild-type, asfl-1(tm874), unc-85(n319), and the numbers of viable larvae and dead embryos from each injected mother were quantified (Table 2). Simultaneous RNAi of both Asf1s in a wild-type background did not reveal any increased embryonic lethality; however the efficiency of knockdown is known to be reduced when multiple genes are simultaneously targeted (Gonczy et al., 2000). Injection of asfl-1 dsRNA into unc-85 germlines also did not result in any increased embryonic lethality. The converse experiment, injection of unc-85 dsRNA was injected into asfl-1 (tm874) germlines did result in a significant increase in embryonic lethality, consistent with the maternal contribution of Asf1 being necessary for embryonic development.

Table 2

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<th>Genotype</th>
<th>Embryonic lethality (%)</th>
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<tr>
<td>Wild-type</td>
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<td>711</td>
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<tr>
<td>asfl-1(tm874)I</td>
<td>5.6 a</td>
<td>304</td>
</tr>
<tr>
<td>asfl-1(tm1625)I</td>
<td>5.1 a</td>
<td>331</td>
</tr>
<tr>
<td>unc-85(e1414)I</td>
<td>3.9 a</td>
<td>356</td>
</tr>
<tr>
<td>unc-85(n319)I</td>
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<td>325</td>
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<td>unc-85(g711)I</td>
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<td>unc-85(m2812)I</td>
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<td>+/mnl1I</td>
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<th>Genotype</th>
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<tr>
<td>asfl-1(RNAi)</td>
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<tr>
<td>unc-85(RNAi)</td>
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<td>asfl-1(RNAi); unc-85(RNAi)</td>
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<tr>
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<td>asfl-1(tm874I); unc-85(RNAi)</td>
<td>4.3±2.8 d</td>
<td>1234</td>
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<td>asfl-1(RNAi); unc-85(n319)I</td>
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<td>unc-85(n319)I; unc-85(RNAi)</td>
<td>5.8±1.5</td>
<td>889</td>
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Strains above the double line were compared to wild-type and those below the double line were compared to +/mnl1I. Groups below the triple line were compared to the wild-type injection control.

*Significantly different from wild-type, P ≤ 0.002.

*Significantly different from +/mnl1I, P ≤ 0.007.

*Significantly different from asfl-1(tm874I); asfl-1(RNAi), P ≤ 0.05.

*Significantly different from all other groups below triple line, P ≤ 0.002.

<table>
<thead>
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<th>Genotype</th>
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<td>asfl-1(tm1625)I</td>
<td>151±7.0</td>
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<tr>
<td>unc-85(e1414)I</td>
<td>102±17.7</td>
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<td>asfl-1(tm874); unc-85(n319)</td>
<td>116±5.7 a</td>
</tr>
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Data are averages ± SEM. For all strains, n = 10.

* Significantly different from wild-type, asfl-1(tm874) and asfl-1(tm1625), P < 0.01.
Requirements for Asf1 homologs in egg-laying, male tail development, and locomotion

All of the previously characterized unc-85 mutant phenotypes arise from cell division failures during post-embryonic development and manifest during larval or adult life. These phenotypes include a reduced ability to lay embryos, morphological defects affecting the male copulatory apparatus, and uncoordinated locomotion (Sulston and Horvitz, 1981). We therefore examined these phenotypes in the Asf1 single and double mutant worms. Two unc-85 mutants were found to retain embryos (Table 1), consistent with previous studies of unc-85(e1414) (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981).

Both of the asfl-1 mutants tested also laid significantly fewer embryos than wild-type hermaphrodites, consistent with the reduced fertility of these worms (Table 1).

The C. elegans male tail contains a cuticular fan, in which nine pairs of sensory rays are embedded (Sulston et al., 1980). The ray neurons enable the male to respond to contact with the hermaphrodite and to turn when it encounters the end of the hermaphrodite prior to reaching the vulva (Liu and Sternberg, 1995). Examination of the tails of wild-type worms confirmed that all nine pairs of rays were present (Fig. 8A). The tails of asfl-1(tm874) males (Fig. 8B) resembled those of wild-type males, with all of the sensory rays generally present. Consistent with previous observations of missing rays in the tails of unc-85(e1414) males (Sulston and Horvitz, 1981), fewer rays were present in unc-85(n319) male tails (Fig. 8C). The unc-85(n319); asfl-1(tm874) male tails also had reduced numbers of sensory rays (Fig. 8D), resembling the tails of unc-85 mutant males in ray number, however sensory rays often were observed to be wider than normal, suggesting that in addition to lineage defects, morphogenetic or cell identity defects resulting in ray fusions may have occurred. These results suggest that unc-85 is the sole Asf1 homolog essential for normal egg-laying, and that both Asf1s may contribute to normal male tail morphology.

We previously determined that DNA replication failures are responsible for cell division failures in the post-embryonic ventral cord neuroblasts of unc-85 mutants (Grigsby and Finger, 2008). To determine if UNC-85 and ASFL-1 function in other aspects of nervous system development or function, we assayed the locomotory behavior of newly hatched larvae (L1s). Post-embryonic neuroblast cell divisions do not begin until several hours post-hatching, allowing defects that occur as a result of post-embryonic cell division failures to be distinguished from those arising earlier in development. To assay locomotory behavior controlled by the ventral cord motor neurons, worms were placed in low-viscosity media, where they exhibit characteristic rapid, sequential thrashing (alternating body bends) (Chalfie and White, 1988). Determination of the number of times that a worm thrashes within a set time period allows quantification of locomotory defects (Miller et al., 1996). Thrashing assays of newly hatched L1s revealed that from 20–33% (depending on the allele) of unc-85 mutant larvae are uncoordinated (Table 3). In contrast, asfl-1 mutant larvae exhibited normal locomotion (Table 3). The unc-85(n319); asfl-1(tm874) double mutant L1s were uncoordinated to a similar degree as unc-85(n319) mutants (Table 3), suggesting that only unc-85 contributes to normal locomotion in newly hatched larvae.

Seam cells are lateral epidermal cells that produce the alae, longitudinal ridges of the lateral cuticle that are present in C. elegans in the L1, dauer, and adult stages. New seam cells are produced before each molt, and defects in seam cell division result in abnormalities, such as gaps in the alae (Sulston and Horvitz, 1981).
divisions in unc-85(e1414) were previously reported to occur normally, and no alae defects were observed (Sulston and Horvitz, 1981). To determine if either or both of the C. elegans Asf1 homologs function in the seam cell divisions, the adult alae of wild-type, asf-1(tm874), unc-85(n319) and unc-85(n319); asf-1(tm874) double mutant hemaphrodites were observed by DIC optics. The alae appeared to be normal in all of the strains (data not shown), suggesting that the Asf1 homologs may not be required for seam cell divisions.

Discussion

The genomes of many multicellular organisms, including C. elegans, Homo sapiens, Mus musculus, Rattus norvegicus, and Arabidopsis thaliana (Haas et al., 2002; Kawai et al., 2001; Munakata et al., 2000; The C. elegans Sequencing Consortium, 1998), contain two Asf1-encoding genes. All predicted Asf1 proteins are highly homologous, with most of the variation occurring in the carboxyl-terminal domain (Daganzo et al., 2003), and it is difficult to associate specific roles with primary amino acid sequence differences between Asf1 isoforms. Although much is known about the cellular functions of yeast Asf1, little is known about in vivo developmental functions for Asf1 homologs in more complex organisms, including whether (in those organisms with two Asf1s) each homolog has distinct and/or redundant roles. In this study, partially redundant functions in germline replication were elucidated for the two C. elegans Asf1 homologs, UNC-85 and ASFL-1, and several somatic functions were demonstrated to solely require UNC-85.

ASFL-1 and UNC-85 have partially redundant functions in germline DNA replication

The data presented here demonstrate that the two C. elegans Asf1 homologs have partially overlapping functions in germline replication. Mutations in either asf-1 or in unc-85 result in reduced numbers of progeny compared to wild-type worms, the double mutants are completely sterile (Table 1), and injection of asf-1(tm874) hemaphrodites with dsRNA for unc-85 results in embryonic lethality in the progeny (Table 2). Germlines in each of the single mutants and in the double mutants contained fewer germ cells (Fig. 3E), and oogenesis, as well as both male and hermaphrodite spermatogenesis, was also defective in the mutants (Figs. 2–4 and 6). Moreover, abnormal gonad morphologies were observed only in the double mutants, and are likely to reflect defects in germ cell proliferation, although it is also possible that the two Asf1 homologs also function in cells of the somatic gonad, such as the distal tip cells (Hall et al., 1999; Hedgecock et al., 1987). Furthermore, TUNEL staining revealed extensive double-stranded breaks in DNA in the asf-1(tm874); unc-85(n319) double mutants (Fig. 5K), although apoptosis assays were negative (Fig. 5M). A similar phenotype of extensive germline DNA breakage in the absence of apoptosis was previously reported for C. elegans him-6(e1104);top3Δ3α(RNAi), defective in a RecQ DNA helicase and in a topoisomerase III (Kim et al., 2002). The germ cells in him-6(e1104);top3Δ3α(RNAi) germlines were arrested in mitosis, and failed to enter into meiosis (Kim et al., 2002). Cognate pairs of RecQ DNA helicas and topoisomerase III are involved in a number of pathways important for genome stability, including in checkpoint signaling and as a checkpoint effector in response to DNA damage, and as a “dissolver-some”, resolving structures created by homologous recombination (Mankouri and Hickson, 2007). This latter function is important for repair of ssDNA gaps, double-stranded DNA breaks, and stalled replication forks (Mankouri and Hickson, 2007). The similarity between the phenotypes of the Asf1 double mutant and loss-of-function in both him-6 and top-3α may reflect their involvement in related functions in the germline.

The absence of apoptosis in the presence of extensive DNA damage in C. elegans Asf1 double mutants is somewhat surprising, as studies in other systems suggest a link between loss of Asf1 function and apoptosis. Cell death resembling apoptosis at G2/M was previously reported in a yeast asf1 deletion mutant (Yamaki et al., 2001), and a recent study suggests that cells lacking asf1 do not properly exit the DNA damage checkpoint after repair of damaged DNA is completed (Chen et al., 2008). Apoptosis also occurs in Drosophila loss-of-function mutants in the Tousled-like kinase, tkl (Carrera et al., 2003), and Asf1s from several organisms (Ehsan et al., 2004; Silijje and Nigg, 2001), including Drosophila (Carrera et al., 2003) and C. elegans (Han et al., 2005), are in vitro targets for TTK phosphorylation. However, our previous studies of replication failures in the post-embryonic ventral cord neuroblasts of C. elegans suggested that the replication failures do not always result in cell cycle arrest (Grigsby and Finger, 2008), which would be consistent with the failure to invoke a cell-cycle checkpoint leading to apoptosis.

Direct monitoring of germline DNA replication with microinjected Cy3-dUTP revealed little to no DNA synthesis in the asf-1(tm874); unc-85(n319) double mutants (Fig. 7). Cy3 fluorescence was observed within the germlines of the double mutant, but only rarely coincided with the DAPI-stained germ cell DNA. These results indicate that replication in the germline is blocked, the most likely cause of the observed sterility of the asf-1(tm874); unc-85(n319) double mutants (Table 1, Fig. 2H). DNA replication failures during the mitotic and/or meiotic cell cycles may result in daughter germ cells with unequal DNA content and chromosomes that would be unable to pair properly, leading to mitotic and/or meiotic cell division failures (Fig. 5K). These results are consistent with our previous findings that UNC-85 functions in DNA replication in post-embryonic neuroblast cell divisions (Grigsby and Finger, 2008). Furthermore, Asf1 proteins play critical roles during S phase in a variety of other organisms (Franco et al., 2005; Groth et al., 2005; Kats et al., 2006; Le et al., 1997; Sanematsu et al., 2006; Schulz and Tyler, 2006; Tyler et al., 1999). Additionally, RNA-mediated interference of the expression of both human Asf1 genes (hASF1B and hASF1B) in cultured human cells results in a much greater delay in S-phase than the knockdown of either single gene (Groth et al., 2005), suggesting that it may be a general phenomenon in organisms with two Asf1 homologs encoded in their genomes, that the two proteins have partially redundant functions in promoting DNA replication.

The precise roles for the worm Asf1 homologs in germline replication are not yet known, however there are clues from studies of Asf1 in other organisms. Yeast and Drosophila Asf1 homologs facilitate mitotic and meiotic S-phase-associated acetyl transferases of histone H3 (Adkins et al., 2007a; Driscoll et al., 2007; Fillingham et al., 2008; Han et al., 2007; Recht et al., 2006), and the C. elegans Asf1 homologs may similarly function to present histones to histone acetyltransferases. Yeast Asf1 also physically interacts in vitro with a replication fork component, replication factor C, and ASF1 deletion mutants are unable to maintain several replication factors at stalled replication forks (Franco et al., 2005). Drosophila ASF1 is detected in replicating embryonic nuclei (Bonnefoy et al., 2007), and localizes to replication forks in vivo (Schulz and Tyler, 2006), while in cultured chicken cells, Asf1s are required for replication-coupled chromatin assembly and cell cycle progression (Sanematsu et al., 2006). Finally, RNAi depletion of human ASF1 blocks DNA unwinding at replication forks (Groth et al., 2007). These results suggest that Asf1 proteins play a direct role in replication, perhaps by helping to remove nucleosomes ahead of the replication fork, or by facilitating replication-coupled histone deposition.

Non-overlapping roles for the C. elegans Asf1 homologs in the germline

While our results demonstrate that Asf1 function is necessary for germline replication, the reduced brood sizes in each of the single mutants (Table 1) indicate that unc-85 and asf-1 are not fully redundant in the germline. By qRT-PCR, asf-1 mRNA is primarily
expressed in adults and embryos (Fig. 1B), and results of in situ hybridization show that asf-1 mRNA is expressed at low levels in the distal end of the germline, where mitotic and meiotic DNA replication occurs, and is expressed more strongly in the pachytene region (Fig. 1C). In contrast, unc-85 mRNA is highly expressed throughout the germline, and is expressed in many replicating cells throughout development (Grigsby and Finger, 2008). The expression patterns observed in C. elegans parallel observations of the mRNA expression patterns of the mouse Asf1 homologs, CIA and CIA-II. CIA is universally expressed in adult tissues, while CIA-II expression is largely restricted to testicular germ cells and the thymus, with lower expression levels in the small intestine and colon (Umehara and Horikoshi, 2003). These results suggest that the different expression patterns of the two C. elegans Asf1 homologs could underlie the phenotypic differences in their respective mutants. An alternative explanation is that their divergent C-terminal tails (Grigsby and Finger, 2008) provide functional specificity to UNC-85 and ASF-1. There is also precedence for this in functional studies of the human Asf1s. Specialization of the two human Asf1s was observed when they each were expressed in a yeast Asf1 null mutant under the control of the yeast ASF1 promoter to determine which yeast Asf1 functions could be complemented by each of the human genes (Tamburini et al., 2005). The hASF1A gene conferred resistance to DNA-damaging reagents, while hASF1B provided maintenance of genome stability during replication and transcriptional regulation (Tamburini et al., 2005). Other studies indicate that while hASF1A functions with HRIR in the G1 phase (Zhang et al., 2005), only hASF1B is regulated by the transcription factor E2F in human cell lines (Hayashi et al., 2007). However, these studies did not address developmental stage-specificity or tissue-specificity for the two human Asf1 homologs. Additionally, a functional proteomics study of substrates phosphorylated by the C. elegans mitogen activated protein kinase, mpk-1, identified ASF-1, but not UNC-85, as a potential MPK-1 target (Lin and Reinke, 2008). MPK-1 functions in chromosome condensation and segregation through the pachytene stage of meiosis I (Church et al., 1995), and ASF-1 may function downstream of MPK-1 to regulate either or both of these functions.

In the unc-85 mutants, somatic defects, rather than germline defects, may also contribute to the decreased brood size. The unc-85 mutants are fertile, but display egg-laying defects (Sulston and Horvitz, 1981; Table 1) that result in the death of approximately 2/3 of hermaphrodites within 2–3 days post-L4, a time when wild-type hermaphrodites remain viable and continue reproduction. The high expression of asf-1 in the pachytene region (Fig. 1C) suggests that asf-1 may function to regulate gene expression necessary for progression through meiotic prophase. The absence of asf-1 would cause deregulated gene expression, resulting in delayed progression through meiosis, and in fewer germ cells.

Roles of the C. elegans Asf1 homologs during somatic development

In addition to the differences in germline expression patterns of the two Asf1 genes, and in the phenotypes of the mutants, our studies also suggest that the two C. elegans Asf1 homologs differ in their roles in somatic development. Previously identified somatic phenotypes of unc-85 mutants (Grigsby and Finger, 2008; Sulston and Horvitz, 1981) including defective egg-laying, abnormal male tail morphology, and uncoordinated locomotion, are not observed in asf-1 mutants, and the double mutants resemble unc-85 mutants (Tables 1 and 3, Fig. 8). We conclude that UNC-85 is the primary Asf1 involved in several somatic functions, including development and function of the ventral nerve cord and egg-laying. There are also missing sensory rays in unc-85(n319), but not asf-1(m874) male tails. The defective egg-laying and male tail phenotypes are likely to result, at least in part, from cell division failures, as blocked ray and vulval lineages were previously observed in unc-85(e1414) mutants (Sulston and Horvitz, 1981). Although rays were also missing from the double mutant male tails, their abnormal morphologies suggest that ray fusions or other morphogenetic defects may have occurred in addition to lineage failures, and suggest the possibility of effects of Asf1 loss on gene expression. Some of the uncoordinated observed in unc-85 mutants is also likely to result from post-embryonic ventral nerve cord lineage failures (Grigsby and Finger, 2008; Sulston and Horvitz, 1981); however the impaired locomotory behavior observed in some newly hatched unc-85 mutant larvae (Table 3) suggests that unc-85 loss may also affect gene expression required for normal locomotion.

Observations of the alae in the C. elegans Asf1 single and double mutants suggest that some cell divisions may not require Asf1 function. The seam cells, specialized cells of the lateral epidermis, are born during embryonic development, and undergo post-embryonic cell divisions during each larval stage before fusing to form a syncytium in the mid-fourth larval stage. Alae are specializations of the lateral cuticle produced by the seam cells in the L1, dauer, and adult stages. Failed seam cell divisions result in defects in the alae, such as gaps (Sulston and Horvitz, 1981). No alae defects were observed in Asf1 single or double mutant adult hermaphrodites, suggesting that the seam cell divisions are not impaired. Since the double mutants are produced from a balanced line, maternally supplied Asf1 may be sufficient to support their seam cell divisions, although this would require the perudrance of maternally contributed Asf1 through four rounds of post-embryonic cell divisions occurring over 3.5 days. Alternatively, Asf1 function may not be required for successful replication in the seam cell lineages. Other classes of histone chaperones are encoded in the C. elegans genome, and may function in the seam cell lineages to load newly synthesized DNA with histones and regulate gene expression.

In conclusion, the expression pattern of asf-1 in the meiotic region of the germline, together with the phenotypic characterization of asf-1 single mutants and double mutants with unc-85, suggests that ASF-1 may function primarily in the germline. In contrast, UNC-85 appears to be more ubiquitously involved in replication throughout development (Grigsby and Finger, 2008), although it is possible that some cell divisions may be Asf1-independent. The relatively weak germline phenotypes of the asf-1 and unc-85 single mutants, in comparison to the strong germline replication defects and complete sterility of the double mutant, indicate that the two Asf1 homologs have partially overlapping functions in replication in the C. elegans germline. It will be interesting to learn if a similar division of roles between two Asf1 homologs is conserved in other organisms.

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