A C. elegans eIF4E-family member upregulates translation at elevated temperatures of mRNAs encoding MSH-5 and other meiotic crossover proteins

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Summary
Caenorhabditis elegans expresses five family members of the translation initiation factor eIF4E whose individual physiological roles are only partially understood. We report a specific role for IFE-2 in a conserved temperature-sensitive meiotic process. ife-2 deletion mutants have severe temperature-sensitive chromosome-segregation defects. Mutant germ cells contain the normal six bivalents at diakinesis at 20°C but 12 univalents at 25°C, indicating a defect in crossover formation. Analysis of chromosome pairing in ife-2 mutants at the permissive and restrictive temperatures reveals no defects. The presence of RAD-51-marked early recombination intermediates and 12 well condensed univalents indicate that IFE-2 is not essential for formation of meiotic double-strand breaks or their repair through homologous recombination but is required for crossover formation. However, RAD-51 foci in ife-2 mutants persist into inappropriately late stages of meiotic prophase at 25°C but not in ife-2 mutants, suggesting that IFE-2 translationally upregulates synthesis of MSH-4/HIM-14 and MSH-5 at elevated temperatures to stabilize Holliday junctions. This is confirmed by an IFE-2-dependent increase in MSH-5 protein levels.

Key words: Chiasmata, Homologous recombination, eIF4E, Polysomal RNA, Translational initiation

Introduction
Initiation of translation in eukaryotes requires the action of at least 33 polypeptides constituting the canonical initiation factors, which act to assemble a series of complexes of increasing size: 43S, 48S and 80S (Kapp and Lorsch, 2004). The rate-limiting step under normal conditions is binding of mRNA to the 43S pre-initiation complex to form the 48S pre-initiation complex. Efficient recruitment of mRNA requires a 5’-terminal 7-methylguanosine-containing cap, which inserts into a narrow pocket in eukaryotic initiation factor (eIF) 4E (Marcotrigiano et al., 1997; Matsuo et al., 1997). For cap binding to lead to productive translational initiation, eIF4E must also bind to eIF4G, which occurs with high affinity on the opposite side of eIF4E (Marcotrigiano et al., 1999).

eIF4E can also bind to proteins other than eIF4G, the first discovered being the 4E-BPs: 4E-BP-1, -2 and -3 (Gingras et al., 2004). eIF4G and the 4E-BPs contain a consensus sequence motif necessary for their interaction with eIF4E, YxxxxLΦ, where X is any amino acid and Φ is L, M or F. Because their binding is mutually exclusive, the 4E-BPs sequester eIF4E from eIF4G and the 48S pre-initiation complex, thereby inhibiting translation. The number of known eIF4E-binding partners that utilize the YxxxxLΦ motif has now grown to at least 15 (Rhoads, 2009). Interaction with some of these binding partners has revealed new physiological roles for eIF4E. For instance, an eIF4E-binding protein, Maskin, participates in mRNA-specific translational repression through interaction with the cytoplasmic polyadenylation element-binding protein (Richter, 2008).

Nearly all eukaryotes examined to date express multiple eIF4E-family members (Joshi et al., 2005). Physiological roles for the different eIF4E-family members within a single organism are beginning to emerge. For instance, 4EHP, one of seven eIF4E-family members expressed in Drosophila, specifically interacts with Bicoid to suppress caudal mRNA translation in the anterior region of the embryo (Cho et al., 2005). Translation of hunchback mRNA also is regulated by 4EHP, but its binding partner is Brat (Cho et al., 2006). The Xenopus oocyte-specific family member eIF4E-1B represses translation early in oogenesis when Maskin is absent by binding 4E-T and regulating the timing of maturation (Evskov and Marin de Evsikova, 2009; Minshall et al., 2007).

Five eIF4E-family members are expressed in Caenorhabditis elegans, IFE-1 to IFE-5 (Jankowska-Anyszka et al., 1998; Keiper et al., 2000). RNAi experiments revealed that only IFE-3 is essential for viability. Three closely related family members, IFE-1, -2 and -5, are partially redundant since at least one is required for viability, whereas IFE-4 is not essential for viability in any combination of IFE knockdown (Keiper et al., 2000). IFE-1 is enriched in the germ line and binds to PGL-1, which localizes IFE-1 to P granules (Amiri et al., 2001). Depletion of IFE-1 by RNA interference (RNAi) shows it is required for spermatogenesis in both hermaphrodites and males (Amiri et al., 2001). Disruption of the ife-1 gene causes spermatocytes...
to fail in cytokinesis and produces a moderate defect in oocyte development (Henderson et al., 2009). Disruption of the ife-2 gene reduces global protein synthesis in somatic cells, protects cells from oxidative stress, and extends lifespan (Hansen et al., 2007; Syntichaki et al., 2007). Disruption of the ife-4 gene produces a pleiotropic phenotype that includes defects in egg-laying and serotonin signaling (Dinkova et al., 2005). These findings begin to reveal how specific elf4E-family members function in protein synthesis to promote specific developmental programs.

In the current work, we studied the phenotype of an ife-2 deletion mutant at normal (20°C) and elevated (25°C) temperatures. Low brood size, severe embryonic lethality, and a high incidence of males (Him) at the elevated temperature suggested chromosome non-disjunction. We found that chiasmata fail to form at the elevated temperature, but this is not due to defects in meiotic chromosome alignment, synopsis, meiotic double-strand break (DSB) formation, or repair of meiotic DSBs by homologous recombination (HR). Rather, the ife-2 mutants fail to convert HR intermediates into crossovers. Translation of mRNAs encoding several meiotic crossover proteins is upregulated in wild-type worms at 25°C, but translation of these mRNAs is diminished at 25°C in the ife-2 deletion mutant.

Results
Disruption of the ife-2 gene
We previously observed no phenotype when dsRNA for ife-2 was injected into the gonads of worms and the progeny were monitored at 20°C (Keiper et al., 2000). We requested creation of a ife-2 gene disruption mutant from the C. elegans Knockout Consortium and backcrossed it to wild-type worms to generate strain KX15 [ife-2(ok306)]. We sequenced the breakpoints and found a deletion of 1628 bp of chromosome X that included the fourth exon of ife-2 and the first two exons of the neighboring gene R04A9.3 (Fig. 1A). The deletion is detectable by single-worm genomic PCR (supplementary material Fig. S1); the same primers amplify a 3504-bp product from wild-type worms (supplementary material Fig. S1, lanes 2-4) but a 1876-bp product from ife-2(ok306) (supplementary material Fig. S1, lanes 6-8). Deletion of exon 4 of ife-2 removes sequences that correspond in mouse elf4E to helix-3, β-sheet 7, helix-4 and β-sheet 8, which make up half of the cap-binding pocket (Marcotrigiano et al., 1997). IFE-2 is detectable by affinity chromatography on m7GTP-Sepharose in extracts of wild-type worms (Jankowska-Anyzskza et al., 1998), but not in extracts of ife-2(ok306) (D. Chiluza and R.E.R., unpublished data). These findings indicate that ife-2(ok306) is a null mutant.

Growth of ife-2(ok306) at 25°C causes embryonic lethality and a Him phenotype
ife-2(Ok306) animals develop normally and at a similar pace as the wild type at both 20°C and 25°C, suggesting that ife-2(ok306) has no major defects in cell-cycle progression or larval development during embryogenesis. At 20°C, ife-2(ok306) worms produce a normal brood size in comparison with wild-type worms (Fig. 1B, upper panel, bars 1 and 2). Nearly all wild-type and ife-2(ok306) progeny hatch (middle panel, bars 1 and 2), and the percentage of males is 0.1% in both strains (lower panel, bars 1 and 2). At 25°C, by contrast, ife-2(ok306) worms have a smaller brood size (174±14) than wild type (268±36; upper panel, bar 6 vs. 5). Only 13% of ife-2(ok306) embryos hatch compared with 85% for the wild type (middle panel). The terminal morphology of ife-2(ok306) embryos indicates an arrest during blastula development (supplementary material Fig. S2). Of the ife-2(ok306) embryos that hatch at 25°C, 20% are males compared with 0.38% for wild-type worms (Fig. 1B, lower panel, bar 6 vs. 5). Between 22°C and 24°C, there is a dramatic decrease in hatching from 83.6% to 12.5% (Fig. 1C).

Since not only the fourth exon of ife-2 but also the first two exons of the neighboring gene R04A9.3 (of unknown function) are deleted in ife-2(ok306), we verified that the observed phenotypic traits were entirely due to the absence of ife-2 expression by conducting ife-2 RNAi experiments, employing the feeding method to introduce dsRNA into wild-type worms (Kamath et al., 2001; Timmons and Fire, 1998). At 20°C, ife-2 RNAi had no effect on brood size, embryonic lethality or percentage of males compared with control RNAi (Fig. 1B, bars 3 vs. 4). At 25°C, by contrast, ife-2 RNAi decreased brood size, decreased hatching and increased male production (bars 7 vs. 8). These results indicate the absence of ife-2 rather than R04A9.3 expression is responsible for the observed phenotypes.

Both oogenesis and spermatogenesis are impaired in ife-2(ok306) at 25°C
Mutations in several meiosis-specific genes such as spo-11 (Dernburg et al., 1998), msh-4/him-14 (Zalevsky et al., 1999),...
msh-5 (Kelly et al., 2000), and zhp-3 (Jantsch et al., 2004) also lead to high embryonic lethality and a Him phenotype, suggesting that ife-2 may play a role in meiosis. Adult C. elegans hermaphrodites generate both spermatocytes and oocytes from the same pool of germ cells. Meiotic divisions lead to spermatogenesis during the early L4 stage and oogenesis at the end of L4 and during early adulthood (Kimble and Crittenden, 2007). The high embryonic lethality in ife-2(ok306) at 25°C could conceivably result from defects in either spermatogenesis or oogenesis.

To test this, we carried out mating experiments at 25°C (Fig. 1D). Embryonic viability was similar for wild-type hermaphrodites crossed with wild-type males (upper panel, bar 2) and hermaphrodites undergoing self-fertilization (bar 1). When crosses were conducted with ife-2(ok306) males, however, hatching decreased from 88.7% to 48.1% (bar 2 vs. 3), indicating that male ife-2(ok306) sperm are defective. Embryonic viability also decreased from 88.7% to 31.8% when ife-2(ok306) hermaphrodites were crossed with wild-type males (bar 2 vs. 4), indicating that ife-2(ok306) oocytes are also defective. Combining defective male ife-2(ok306) sperm with defective ife-2(ok306) oocytes produced the greatest decrease in viability, from 88.7% to 9.4% (bar 2 vs. 5). A similar degree of embryonic lethality was seen in self-fertilized ife-2(ok306) hermaphrodites (bar 6). The fact that there were approximately 50% males in all mating experiments (lower panel, bars 2-5) indicates the progeny resulted from crosses.

Since both ife-2(ok306) oocytes and sperm produce defective embryos at 25°C, the temperature-sensitive step could conceivably occur during mitotic division of germ cell lineages (L1 and L2 stages), meiotic division (L3), sperm development (early L4), or oocyte development (late L4 and young adult). We performed temperature shift-down and shift-up experiments to identify the temperature-critical period (TCP; Table 1). In shift-down experiments, ife-2(ok306) worms were maintained at 25°C until the beginning of a given larval stage and then shifted to 20°C. Embryonic viability in ife-2(ok306) remained high (~90%) if the shift-down occurred at or before L4 but decreased to 11% if it occurred at the young adult stage. Therefore, the temperature-sensitive step occurs after the beginning of L4. In shift-up experiments, worms were maintained at 20°C until the beginning of a given larval stage and then shifted to 25°C. Embryonic viability was ~3% if the shift occurred at or before the beginning of L4 and rose to only ~15% if it occurred at the beginning of the young adult stage. Therefore, the TCP occurs between the beginning of L4 and the end of young adulthood. This

Table 1. Determination of the temperature-critical period (TCP) for embryonic viability in ife-2(ok306) worms

<table>
<thead>
<tr>
<th>Stage of temperature shift</th>
<th>Shift down</th>
<th>Shift up</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ife-2(ok306)</td>
<td>ife-2(ok306)</td>
</tr>
<tr>
<td>L2</td>
<td>92.4±3.0</td>
<td>95.2±1.1</td>
</tr>
<tr>
<td>L3</td>
<td>92.1±1.5</td>
<td>90.1±2.4</td>
</tr>
<tr>
<td>L4</td>
<td>91.8±1.3</td>
<td>87.3±6.3</td>
</tr>
<tr>
<td>Young adult</td>
<td>89.0±2.5</td>
<td>11.1±4.4</td>
</tr>
</tbody>
</table>

Embryonic viability (% hatching) measured for each temperature-shift condition (15 worms (~4500 embryos) were monitored).
germline cells (Fig. 2G, left panels). In ife-2(ok306), only background immunostaining is observed (Fig. 2G, right panels). The finding that ife-2 is expressed in germline cells is consistent with the idea that IFE-2 has a role in meiosis.

**IFE-2 is required for chiasmata formation at 25°C**

The Him phenotype is characteristic of *C. elegans* mutants defective in chromosome segregation (Hodgkin et al., 1979). To determine the basis of the ife-2(ok306) phenotype at 25°C, we examined cells in meiotic prophase by DAPI staining. In the pachytene stage, the appearance of chromosomes is the same in wild type and ife-2(ok306) at both 20 and 25°C (supplementary material Fig. S3). Paired and synapsed homologs exhibiting discrete DAPI-stained tracks are observed in all cases. At diakinesis, six DAPI-stained bodies are observed in wild-type nuclei at 20°C (Fig. 3A), corresponding to six pairs of homologs physically connected by chiasmata (bivalents) (Dernburg et al., 1998; Villeneuve, 1994). The same six bivalents are observed in wild-type nuclei at 25°C and ife-2(ok306) oocytes at 20°C (Fig. 3B and C). By contrast, 12 DAPI-stained bodies appear in ife-2(ok306) nuclei at 25°C (Fig. 3D), which correspond to chromosomes unattached by chiasmata (univalents). Examination of 135 ife-2(ok306) nuclei in diakinesis at 25°C revealed an average of 11.5 DAPI-stained bodies.

**ife-2(ok306) mutants are proficient in pairing and synapsis at 25°C**

The presence of 12 univalents in diakinesis in ife-2(ok306) nuclei suggests a defect in chiasma formation. A lack of chiasmata can be due to a failure in the recombination process per se or to defective pre-synaptic alignment and/or synapsis. To investigate the nature of the defect, a time-course analysis of pairing was performed at 25°C using fluorescence in situ hybridization (FISH). In wild-type gonads, homolog alignment initiates in leptotene-zygotene (Dernburg et al., 1998) and is represented by one (paired) and two (unpaired) FISH signals in zone II (Fig. 4A); pairing is then stabilized by synapsis in pachytene (MacQueen et al., 2002), corresponding to the paired FISH signals observed in the early, mid-, and late pachytene (zone III-V) nuclei of wild-type germ cells.

**Fig. 3. Chiasmata are not formed in ife-2(ok306) mutants at 25°C.** (A-D) DAPI-stained single oocyte nuclei at diakinesis. Six DAPI-stained bodies, corresponding to six pairs of homologs connected by chiasmata, are present in wild-type nuclei at 20°C (A) and 25°C (B), and in ife-2(ok306) nuclei at 20°C (C). Twelve DAPI-stained bodies, corresponding to twelve univalents, are present in ife-2(ok306) nuclei at 25°C (D), indicating chiasmata have not formed. Images shown are projections through the whole nucleus. Scale bar: 5 μm.

**Fig. 4. (A) Time-course analysis of pairing levels in ife-2(ok306) mutant germ cells.** Hermaphrodite gonads were divided into five equivalently sized zones along the distal-proximal axis (45-50 μm each). Each zone contains a population of nuclei corresponding predominantly to the following stages: mitotic/premeiotic (I), leptotene-zygotene (II), and early, mid- and late pachytene (III-V). The number of nuclei in each zone in wild-type and mutant worms, respectively, were: I, 249, 254; II, 340, 363; III, 334, 368; IV, 278, 282; V, 180, 201. A 5S rDNA repetitive sequence probe was used to follow the pairing of the right arm of chromosome V (LG V) by FISH. The histogram shows the level of pairing for ife-2(ok306) compared with wild type. Homolog pairing initiates in leptotene-zygotene (zone II) in wild type and rapidly increases and persists throughout pachytene (zone III-V). In ife-2(ok306), pairing levels are not significantly different from wild type [(I) P=0.1, (II) P=0.09, (III) P=0.28, (IV) P=0.26, (V) P=0.28, considered not significant for each zone], indicative that the pairing process takes place correctly in the mutant. (B) Immunofluorescence micrograph of mid-pachytene nuclei from wild-type and ife-2(ok306) mutant germ lines showing colocalization of the meiotic chromosome axis component HTP-3 (red) and the synaptonemal complex component SYP-1 (green). Scale bars: 5 μm.
lines. In *ife-2(ok306)* germ cells, the level of pairing is not significantly different from that in wild type, indicating that the mutant is proficient for chromosome pairing. Furthermore, *ife-2(ok306)* mutants show no defects in chromosome morphogenesis or synopsis as ascertained by the loading of the axis component HTP-3 (Goodyer et al., 2008) and SYP-1, a component of the central region of the synaptonemal complex (MacQueen et al., 2002). Similar to wild-type germ cells, HTP-3 localizes to chromosomes in the transition zone nuclei of *ife-2(ok306)* mutants, colocalizes with the synapsed chromosomes at pachytene, and remains associated with the axes of diakinesis chromosomes following desynapsis (Fig. 4B, and data not shown). SYP-1 localizes to the interface between paired homologs in *ife-2(ok306)* pachytene nuclei germ cells as it does in wild type (Fig. 4B), indicating that pairing is appropriately stabilized by synopsis in the absence of IFE-2. In *C. elegans*, the formation of a crossover triggers asymmetrical synaptonemal complex disassembly at late pachytene-diplotene, and SYP-1 is retained on the short arm of the diakinesis bivalent. Mutants defective in crossover formation fail to asymmetrically remodel the chromosome and instead exhibit univalents at diakinesis that either lack any SYP-1 at their axes or exhibit SYP-1 along the entire length (Nabeshima et al., 2005). *ife-2(ok306)* mutants similarly exhibit diakinesis nuclei in which some univalents have lost all SYP-1 staining, whereas others have retained SYP-1 along their lengths (data not shown), consistent with the interpretation that IFE-2 is not required for meiotic chromosome pairing or synopsis but is essential for crossover formation.

**ife-2(ok306) mutants are defective in converting recombination intermediates into crossovers**

Given that chromosome pairing takes place normally in *ife-2(ok306)* mutants, we asked if the lack of chiasmata originates in a failure to initiate recombination. We monitored, at 25°C, the formation of recombination intermediates marked by RAD-51, which is required for repair of meiotic DSBs during HR (Colaiacovo et al., 2003). Time-course analyses of appearance and disappearance of RAD-51 in wild-type germ cells revealed that the number of RAD-51 foci per nucleus peaks in early pachytene and virtually disappears by late pachytene (Fig. 4, upper panel), consistent with the repair of early recombination intermediates over time. In contrast, RAD-51-marked intermediates in *ife-2(ok306)* germ cells are observed at higher levels in early pachytene, peak in mid-pachytene, and persist at inappropriately high levels in late pachytene (lower panel). Since *ife-2(ok306)* mutants do not exhibit the chromatin decondensation defect observed in the diakinesis nuclei of HR mutants unable to repair DSBs (Alpi et al., 2003), our results indicate that *ife-2(ok306)* mutants are proficient in the formation and repair of DSBs through HR, but are unable to do so using the crossover pathway. The accumulation of RAD-51 foci into inappropriately late stages of pachytene has been previously observed in mutants specifically defective in the components required for the formation of the crossover intermediate, including MSH-4/HIM-14 and MSH-5 (Colaiacovo et al., 2003).

**γ-irradiation-induced DSBs cannot bypass the requirement for IFE-2**

Meiotic recombination is initiated in *C. elegans* by programmed induction of DSBs by the enzyme SPO-11 (Dernburg et al., 1998). Like *ife-2(ok306) at 25°C*, *spo-11* mutants have 12 univalents at diakinesis, but γ-irradiation artificially introduces DSBs, bypassing the need for SPO-11, and restores chiasmata. Mutations in *msh-5* and *mre-11* also produce 12 univalents at diakinesis, but irradiation does not restore chiasmata (Chin and Villeneuve, 2001; Kelly et al., 2000). The appearance of chiasmata after irradiation therefore serves as a test for a *spo-11*-like mutation.

We exposed wild-type and *ife-2(ok306)* hermaphrodites to 5 krad of irradiation at either 20 or 25°C and analyzed oocyte chromosomes at diakinesis 18 hours later by DAPI staining; we included the *spo-11(ok79)* mutant as a positive control (Fig. 6). Three different outcomes were possible: (1) restored chiasmata, as with *spo-11* mutants (Dernburg et al., 1998); (2) chromosome fragments or gross chromosomal abnormalities, as with *mre-11* mutants (Chin and Villeneuve, 2001); and (3) 12 univalents without chromosome fragments, as with *msh-5* mutants (Kelly et al., 2000). Irradiation had no effect in *ife-2(ok306)* at 20°C (Fig. 6G vs. H) or in wild-type worms at either temperature (Fig. 6A vs. B). But in *ife-2(ok306)* at 25°C, chiasmata were not induced and 12 univalents remained, the third outcome (Fig. 6I vs. J). The appearance of the chromosome was indistinguishable from that of the unirradiated controls, suggesting that *ife-2(ok306)* mutants are able to repair irradiation-induced DSBs. DAPI-stained bodies were quantified in nuclei at diakinesis for *spo-11(ok79), ife-2(ok306)* and wild-type worms (supplementary material Table S1). At 20°C, only 2% of *ife-2(ok306)* nuclei
Fig. 6. γ-irradiation (IR)-induced DSBs cannot bypass the requirement for IFE-2 at 25°C. Late L4 spo-11(ok79), wild-type and ife-2(ok306) mutant worms were exposed to 5 krad of IR at the indicated temperatures as described by Dernburg et al. (Dernburg et al., 1998). Images show DAPI-stained nuclei at diakinesis 18 hours after IR. Irradiated wild-type nuclei at 20°C and 25°C, contain 12 univalents (A) and six bivalents (B) whereas unirradiated controls contain 12 univalents (C). ife-2(ok306) nuclei at 25°C contain 12 univalents (D) and this is unchanged by IR (E). All images are projections through the whole nucleus. Scale bar: 5 μm. Quantification of the DAPI-stained chromosomes is provided in supplementary material Table S1.

The translational efficiency of msh-4/him-14 and msh-5 mRNA is enhanced at 25°C in an ife-2-dependent manner

IFE-2 is a translation initiation factor, so it is reasonable to postulate that its absence affects the expression of certain genes at the translational level. The data presented above suggest that deficiency of IFE-2 impairs chiasma formation at 25°C while homolog pairing, synopsis, recombination initiation and meiotic DNA repair are normal. Proteins involved in the latter processes are therefore unlikely to be affected by the absence of IFE-2. These include proteins involved in homologous pairing and synopsis such as HIM-8 and members of the ZIM, HTP and SYP families (Colaiacovo et al., 2003; Couteau and Zetka, 2005; MacQueen et al., 2002; Phillips and Dernburg, 2006; Phillips et al., 2005; Severson et al., 2009; Smolikov et al., 2007), proteins involved in meiotic recombination initiation such as SPO-11, HIM-17 and MRE-11 (Chin and Villeneuve, 2001; Dernburg et al., 1998; Reddy and Villeneuve, 2004), and proteins involved in meiotic DNA repair such as RAD-51 (Alpi et al., 2003). However, MSH-4/HIM-14, MSH-5 and ZHP-3 act after DSB formation but before double Holliday junction resolution (Bhalia et al., 2008; Jantsch et al., 2004; Kelly et al., 2000; Winand et al., 1998; Zalevsky et al., 1999). We therefore investigated whether the absence of IFE-2 affects the expression of msh-4/him-14, msh-5 and/or zhp-3 genes at 25°C but not 20°C.

To test the hypothesis that IFE-2 promotes expression of these genes at the translational level, we analyzed the polysomal distribution of their mRNAs by quantitative real-time PCR (qRT-PCR). More efficient initiation of translation increases the ribosomal loading of an mRNA and increases its sedimentation rate during ultracentrifugation (Lodish, 1974). An mRNA that is efficiently initiated appears in monosome and polysome fractions (Fig. 7A, right of dashed line) rather than free messenger ribonucleoprotein (mRNP) fractions (left of dashed line). We compared the polysomal distribution of several mRNAs in late L4 wild-type and ife-2(ok306) worms at 20°C and 25°C (Fig. 7B).

In wild-type worms, gpd-3 (encoding GAPDH) and zhp-3 mRNAs are very efficiently translated at 20°C and do not shift to larger polysomes when the growth temperature is raised to 25°C (left panels). Similar results are obtained with syq-1, syq-2 and tim-1 (data not shown). However, the translational efficiency of several meiosis-related mRNAs is increased at 25°C. For instance, 48.4±1.0% of msh-5 mRNA is polysomal at 20°C but this increases to 70.2±0.7% at 25°C (P=0.00005). The mRNAs for msh-4/him-14 and spo-11 are also shifted to larger polysomes at 25°C (see legend to Fig. 7 for quantification). Since Holliday junctions are less stable at elevated temperatures (Shah et al., 1994), we propose a temperature-dependent translational upregulation of MSH-4/HIM-14 and MSH-5 expression, proteins that stabilize Holliday junctions (see Discussion).

In ife-2(ok306) worms, the opposite is observed: the fraction of these mRNAs in polysomes decreases when the growth temperature is raised to 25°C. For instance, 49.7±1.6% of msh-4/him-14 mRNA is polysomal in ife-2(ok306) at 20°C compared with only 20.1±1.8% at 25°C (P=0.0003). Similarly, 73.8±3.8% of msh-5 mRNAs is polysomal at 20°C compared with 40.6±3.4% at 25°C (P=0.003). The temperature-dependent increase of spo-11 mRNA in polysomes observed for wild-type worms also does not occur in ife-2(ok306). There is also a small shift of zhp-3 mRNA from polysomal to non-polysomal fractions in ife-2(ok306) worms at 25°C. The same data for ife-2(ok306) and wild-type worms at 25°C are re-plotted in the right panels of Fig. 7 to facilitate comparison. These results indicate that efficient translation of spo-11, msh-4/him-14, msh-5 and zhp-3 mRNAs at 25°C depends on the presence of IFE-2.

Although IFE-2 is a translation initiation factor, it could conceivably affect total mRNA levels by an indirect effect. We therefore tested whether the levels of mRNAs encoding these proteins are altered in the ife-2(ok306) mutant at 25°C. We analyzed the total levels of these mRNAs in late L4 stage worms by qRT-PCR (Fig. 7C). We found that there are no significant changes in the total mRNA levels of gpd-3, spo-11, zhp-3, msh-4/him-14 or msh-5 in either wild-type or ife-2(ok306) worms when comparing growth at 20°C vs. 25°C or when comparing wild type vs. ife-2(ok306) at either temperature (P>0.05), the only exception being that msh-5 mRNA increases by about 1.5-fold in wild-type worms when the temperature is raised (P=0.028).

Steady-state levels of MSH-5 are increased at 25°C in an ife-2-dependent manner

The polysome shift results (Fig. 7B) suggest that synthesis of MSH-4/HIM-14 and MSH-5 is impaired in ife-2(ok306) at 25°C. If there is no change in the degradation of these proteins, this result would predict that they are present at lower steady-state levels at 25°C.
in intracellular levels in ife-2(ok306) at 25°C. We were unable to find antibodies against these proteins from either commercial sources or the published literature, so we developed antisera to a peptide derived from the MSH-5 sequence. MSH-5 is predicted to be a polypeptide of 153 kDa (WormBase). We detected a band peptide derived from the MSH-5 sequence. MSH-5 is predicted sources or the published literature, so we developed antisera to the anti-MSH-5 antisera before immunoblotting, resulting in the disappearance of the ~150-kDa band (data not shown). Based on immunoreactivity, electrophoretic mobility, and molecular genetics, we conclude that the ~150-kDa band represents MSH-5.

MSH-5 increases when wild-type worms are grown at 25°C compared with 20°C (Fig. 7D, upper panel, lane 5 vs. 6), suggesting an upregulation at elevated temperatures and agreeing with the temperature-dependent upregulation.

Discussion
IFE-2 has both germline and somatic roles
The only publications on IFE-2 since its original identification as a member of the eIF4E family (Jankowska-Anyszka et al., 1998) and the failure of injected ife-2 RNAi to affect viability of the progeny (Keiper et al., 2000) have focused on somatic roles for IFE-2. It was reported that deletion of ife-2 reduces global protein synthesis, increases tolerance to oxidative stress and extends life span (Hansen et al., 2007; Syntichaki et al., 2007). Syntichaki et al. (Syntichaki et al., 2007) also reported that ife-2 disruption increases embryonic lethality. Our observation of a Him phenotype raised the possibility that IFE-2 may also function in the germ line.
Embryonic lethality and a Him phenotype are hallmarks of mutations in meiotic genes (Chin and Villeneuve, 2001; Dernburg et al., 1998; Jantsch et al., 2004; Kelly et al., 2000; Zalevsky et al., 1999). We find that IFE-2 is expressed in both somatic and germ line cells and is required for the formation of crossovers in a temperature-dependent manner. Three other eIF4E-family members, IFE-1, IFE-3 and IFE-5, are expressed in the germ line as well, based on their absence in glp-1 mutants (Amiri et al., 2001) and expression of ife-1::GFP and ife-3::GFP (B.D.K. and R.E.R., unpublished data).

IFE-2 functions as a translation factor

eIF4E, an mRNA cap-binding protein, is a well-characterized translational initiation factor. The best studied role of eIF4E is that of translation enhancer involved in the recruitment of mRNAs to the protein synthesis initiation machinery, but eIF4E can also be component of complexes that repress translation (see Introduction). We believe IFE-2 functions as a translation enhancer for the following reasons. First, IFE-2 can be detected immunologically in 48S initiation complexes (N.L.K. and R.E.R., unpublished data). Second, depletion of IFE-2 reduces global translation in adult worms (Hansen et al., 2007; Syntichaki et al., 2007). Third, in the absence of IFE-2, there is a shift of at least four mRNAs (spo-11, msh-4/him-14, msh-5 and zhp-3) from polysomes to the free mRNP fraction at 25°C. The latter behavior is indicative of mRNAs whose rate of initiation decreases relative to elongation (Lodish, 1974). The data, therefore, support the idea that the phenotypic effects of ife-2 disruption are due to the absence of an eIF4E-family member acting as an enhancer of specific mRNA translation.

What is the nature of the defect at 25°C?

We observe that ife-2(ok306) worms fail to form chiasmata at 25°C but not at 20°C. We propose that this failure results from a deficiency of MSH-4/HIM-14 and MSH-5. These two proteins make up a heterodimer that binds to Holliday junctions and forms sliding clamps on pairs of duplex DNA arms emanating from the Holliday junction, stabilizing it until endonucleases are recruited to resolve the Holliday junction intermediates (Snowden et al., 2004). Our proposal is supported by three lines of evidence. First, the phenotype of ife-2(ok306) worms at 25°C exactly mimics that of msh-4/him-14 and msh-5 mutants (Colaiacovo et al., 2003; Kelly et al., 2000; Zalevsky et al., 1999): high embryonic lethality, a Him phenotype, normal pairing and alignment of homologs, generation of meiotic DSBS, inability of \( \gamma \)-irradiation to rescue to crossover failure, a persistence in the number of RAD-51 foci as pachytene progresses, and appearance of 12 well condensed univalents at diakinesis. Second, there is a significant decrease in crossing over at 25°C. On the contrary, translational efficiency of msh-4/him-14 and msh-5 mRNAs in ife-2(ok306) worms at 25°C. The ife-2(ok306) mutation does not result in lower steady-state levels of msh-4/him-14 or msh-5 mRNA at either temperature. Third, steady-state intracellular protein levels of MSH-5 are lower in ife-2(ok306) worms at 25°C than at 20°C. These findings indicate that IFE-2 is specifically required for enhanced translation of msh-4/him-14 and msh-5 mRNAs at elevated temperatures.

Why is translation of msh-4/him-14 and msh-5 mRNAs impaired in ife-2(ok306) worms at 25°C?

Injection of dsRNA against either ife-1 or ife-2 at 20°C does not cause embryonic lethality, but simultaneous injection of ife-1 and ife-2 dsRNA causes 75% lethality, and injection of dsRNA against ife-1, ife-2 and ife-5 causes 99% lethality, indicating that IFE-1, IFE-2 and IFE-5 have some functional redundancy (Keiper et al., 2000). IFE-3 is expressed in germ line cells and may also be functionally redundant with IFE-2, but this has not been tested because injecting ife-3 dsRNA is lethal to embryos (Keiper et al., 2000). To explain the striking reduction of msh-4/him-14 and msh-5 mRNA translation over a narrow temperature range, we propose that both IFE-2 and one of the other germ line IFEs facilitate the translation of these mRNAs at 20°C, but only IFE-2 can function at 25°C. This raises the question of whether IFE-2 may have unique properties not shared by the other germ line IFEs. In C. elegans, there are two kinds of mRNAs, those capped with m7GTP and those capped with m2,2,7GTP, the latter resulting from trans-splicing (Liou and Blumenthal, 1990; Van Doren and Hirsh, 1990). Ife-1, -2 and -5 can bind both m7GTP-containing and m2,2,7GTP-containing caps in vitro whereas IFE-3 and -4 have a strong preference for m7GTP-containing caps (Jankowska-Anyzskia et al., 1998; Keiper et al., 2000; Stachelska et al., 2002). Both msh-4/him-14 and msh-5 mRNAs are predicted to contain the m2,2,7GTP-type cap (WormBase). If IFE-3 supplies the redundant activity at 20°C, it may lose the ability to recognize m2,2,7GTP-containing mRNAs at 25°C, making IFE-2 dominant. Absence of IFE-2 in ife-2(ok306) mutants would then severely impair the translation of these mRNAs. It is less likely that IFE-1 supplies the redundant activity at 20°C. Ife-1 disruption also causes smaller broods at 25°C, but this has been traced to an arrest in secondary spermatocyte cytokinesis that prevents formation of mature sperm, rather than embryonic lethality (Henderson et al., 2009). Furthermore, ife-1(bn127) oocyte nuclei contain a normal complement of six bivalents at diakinesis rather than the 12 univalents in ife-2(ok306) mutants. Rather, it appears that IFE-1 and IFE-2 regulate different steps in the developmental progression of germ cells. As most of developmental progression in oocytes and spermatocytes is accomplished under transcriptional silence (Kelly and Fire, 1998), translational control of gene expression by eIF4E-family members may provide an important mode of regulation.

Selective translation of mRNAs by eIF4E-family members

The data presented indicate that the presence of IFE-2 enhances translation at 25°C of all mRNAs tested, but some mRNAs (spo-11, msh-4/him-14, and msh-5) are much more affected than others (gpd-3, zhp-3, syp-1, syp-2 and tim-1). Among the other C. elegans eIF4E-family members, IFE-1 selectively enhances the translation of the P-granule-resident mRNAs mex-1 and pos-1, and non-resident pal-1, all of which are required for embryonic differentiation, as well as oma-1 and glp-1, which are required for late oogenesis, but has no effect on gpd-3 mRNA translation (Henderson et al., 2009). IFE-4, which is expressed in nerve and muscle cells, is required for efficient translation of a small subset of mRNAs required for egg laying and response to food cues that include egl-3, egl-5 and daf-12, but has no effect on act-5 and gpd-3 (Dinkova et al., 2005). Thus, the translation of subsets of functionally related mRNAs requires specific eIF4E-family members and has been shown to have major developmental consequences for at least three cell differentiation events.
completion of meiotic chromosome resolution being added in the present study. The mechanisms linking translational repression of a specific mRNA to a specific eIF4E-family member are beginning to be understood (see Introduction), but how a specific eIF4E-family member might enhance translation of an mRNA is an intriguing, yet currently unanswered, question.

Materials and Methods

Strains

Worms were grown at 20°C unless otherwise indicated on NGM plates seeded with *Esherichia coli* strain OP50 (Brenner, 1974). The following *C. elegans* strains were used in this study: N2 var. Bristol, used as the wild type (WT); A V106, the deletion allele were shifted from 20 to 25°C or from 25 to 20°C.

Construction of the *ife-2::GFP* strain

*C. elegans* genomic DNA was used to amplify two portions of the *ife-2* gene for subcloning to flank the nematode-optimized GFP gene in plasmid pPD117.01 (Mello and Fire, 1995). The 5′-portion of *ife-2* was amplified using the Boehringer Long Range PCR system (Boehringer Mannheim) (supplementary material Table S2). The resulting 3128-bp product was digested with *Sall* and *KpnI* and ligated into the corresponding sites in pPD117.01 upstream and from the GFP gene. The 3′-portion of *ife-2* (noncoding only) was similarly amplified (supplementary material Table S2). The 3382-bp PCR product was digested with *Nhel* and *NarI* and ligated into the corresponding sites of the same plasmid downstream of the GFP gene. The resulting plasmid was sequenced to verify the correct orientation. In addition, wild-type worms were generated by outcrossing RB579, obtained from the Knockout Consortium, ten times to wild-type worms. Deletion breakpoints were confirmed by PCR using the primers corresponding to the two ends of the deletion and amplifying these portions from genomic DNA.

Phenotype characterization

For broad size, embryonic lethality (percentage hatching), and male production assays, synchronized L1 worms were cultured at the indicated temperatures for the entire duration of the experiment. Three L4 hermaphrodites were transferred to each of five new plates. Worms were transferred to new plates every 12 hours until no eggs were laid for 12 hours. The number of eggs was counted immediately, and 24 hours later the unhatched embryos were counted for the calculation of percentage hatching. For mating experiments, one L4 hermaphrodite was transferred to a plate along with five males. In temperature-shift experiments, synchronized L1 worms were placed on NGM plates, and at the beginning of each larval stage, some plates were shifted from 20 to 25°C or from 25 to 20°C.

Imaging methods

DAPI staining was performed as described by Shaham (Shaham, 2006). GFP fluorescence was detected as described previously (Dinkova et al., 2005). FISH, image acquisition and time-course analysis of pairing and recombination, and IHC were performed as described by Couteau et al. (Couteau et al., 2004). For IHC, primary antibodies were incubated overnight at room temperature at the following dilutions: guinea pig anti-SYP-1, 1:800; rabbit anti-HTP-3, 1:200; rabbit anti-RAD-51, 1:100; and rabbit anti-IFE-2 (Jankowska-Anyszka et al., 1998), 1:200. The following day, after washing with PBS, secondary antibodies were added: Alexa Fluor 488 goat anti-guinea pig, Alexa Fluor 555 goat anti-rabbit, and Alexa Fluor 488 goat anti-rabbit, all at 1:1000 (Molecular Probes).

qRT-PCR analysis of mRNA

Polyosomal mRNAs were prepared from 0.25 g of late L4 stage worms homogenized with a motor-driven Tetlon homogenizer, and their distribution after sucrose gradient ultracentrifugation was analyzed by qRT-PCR (supplementary material Table S2) as described by Dinkova et al. (Dinkova et al., 2005). For total mRNA analysis, late L4 worms (0.1 g) were homogenized for 2 minutes in 700 µl TRK buffer (Omega Bio-Tek) with a hand-held pestle using an Eppendorf tube. The lysate was centrifuged at 17,000 g for 10 minutes and the supernatant purified with an E.Z.N.A. total RNA kit (Omega Bio-Tek), following the manufacturer’s protocol. qRT-PCR was carried out as described above. The concentrations of individual mRNAs were calculated using the 2^(-ΔΔCt) method (User Bulletin No. 2 for the ABI Prism 7700) relative to 18S rRNA (*rnl-1.1*) (supplementary material Table S2). Three qRT-PCR determinations were performed for each RNA sample, each in duplicate. The presumption that these mRNAs are present in polysomes is based on their sedimentation rates, resolution into discrete peaks, and distribution patterns characteristic of the mRNA size.

Western blotting

Rabbit anti-MSH-5 antibodies were generated against the peptide (N-terminal to C-terminal) CGLLPYPSNVACVYYP, which is derived from an amino acid sequence in the middle portion of MSH-5 (aa 479-493), by GL Biochem, (Shanghai). For western blotting, worm lysate (60 µg protein) was mixed with one-tenth volume of 10× SDS-PAGE loading buffer and heated at 95°C for 5 minutes. Proteins were separated by SDS-PAGE (5% gel for detection of MSH-5, 8% gel for detection of β-actin). Proteins were transferred to PVDF membranes (Bio-Rad) in a Mini Trans-Blot cell (Bio-Rad) at 90 V for 2 hours at 4°C. Membranes were blocked in buffer A [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20] containing 5% Phosphoblocker Blocking Reagent (Cell Biosabs, Inc.), washed four times (30 minutes each) with buffer A, and incubated in buffer A containing 5% Phosphoblocker and the primary antibody overnight at 4°C (1:500 dilution for anti-MSH-5 and 1:10,000 for mouse anti-β-actin (C4) (MP Biomedicals)). Membranes were then washed four times (30 minutes each) with buffer A and incubated in buffer A containing 5% Phosphoblocker and a 1:10,000 dilution of either peroxidaselabeled goat anti-rabbit IgG (H+L) or horse anti-mouse IgG (H+L; Vector Laboratories) for 2 hours at room temperature. Membranes were then washed three times (15 minutes each) with buffer A, developed with SuperSignal West Dura Extended Duration Substrate (Pierce), and exposed to HyBlot CL autoradiography film (Denville Scientific).

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/13/2228/DC1

References


Role for IFE-2 in crossover formation


Song_Fig S1
**Table S1.** Analysis DAPI-stained germline nuclei at diakinesis as a function of γ-irradiation<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temp. (°C)</th>
<th>γ-Irradiation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>5 Krad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bivalents</td>
<td>Univalents</td>
<td>Bivalents</td>
<td>Univalents</td>
<td>Bivalents</td>
<td>Univalents</td>
</tr>
<tr>
<td>spo-11(ok79)</td>
<td>20</td>
<td>30</td>
<td>58</td>
<td>68</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>20</td>
<td>63</td>
<td>0</td>
<td>54</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>25</td>
<td>52</td>
<td>0</td>
<td>61</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ife-2(ok306)</td>
<td>20</td>
<td>48</td>
<td>1</td>
<td>90</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ife-2(ok306)</td>
<td>25</td>
<td>2</td>
<td>61</td>
<td>1</td>
<td>54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>L4 worms were subjected to either no irradiation or 5 krad of γ-irradiation at the indicated temperatures. After 18 hours, nuclei were stained with DAPI as in Fig. 6. The number of nuclei at diakinesis containing either 6 DAPI-stained bodies (bivalents) or 12 DAPI-stained bodies (univalents) were recorded.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ife-2(ok306) (breakpoint)</td>
<td>ATCGGTTTTTTGCGTGTCTCT</td>
<td>CGGGTGTTCACGTTGTCTCTCT</td>
</tr>
<tr>
<td>ife-2 (5′-portion)</td>
<td>CCCCGTCGACGCGCGGAAT-ACATTTTTTGTAATG</td>
<td>GGAAGGTACCTCGGT-TGAGACTGGAACCTG</td>
</tr>
<tr>
<td>ife-2 (3′-portion)</td>
<td>CCCCGCTAGCCTTAA-ATTTCTTTTTATGCG</td>
<td>CCCCGGCCGCGGGGTGTT-TGGTTTAACACTGG</td>
</tr>
<tr>
<td>gpd-3</td>
<td>CAGCACAAAGATC-AAGGTCCTACAAC</td>
<td>GAAGACTCCCGGTGGACCTCAAC</td>
</tr>
<tr>
<td>spo-11</td>
<td>TCGGATTCTGATGATGATGAC</td>
<td>TCCTTTCTCTTGCTGTATAACG</td>
</tr>
<tr>
<td>zhp-3</td>
<td>AACGGCACCATCTCTTGTTC</td>
<td>CTGTCCACTGCTCATTTGTATTG</td>
</tr>
<tr>
<td>msh-4/him-14</td>
<td>GACTGAAGAAAGGAAATCGCCATC</td>
<td>TGTGTAGCCAGAAGGATAGGC</td>
</tr>
<tr>
<td>msh-5</td>
<td>ACTCTAATGCGGGGTGTCTC</td>
<td>CGTCGTCTAATCTTTATGTTGTTG</td>
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<tr>
<td>rrm-1.1</td>
<td>AGTAGCAAGGAGAGGGGCAAGTC</td>
<td>ACCGCAGCAATAACGAGATACAC</td>
</tr>
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</table>

\(^a\)Primer sequences are written 5′ to 3′ from left to right.