Targeted Gene Knockout Reveals a Role in Meiotic Recombination for ZHP-3, a Zip3-Related Protein in Caenorhabditis elegans

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The meiotically expressed Zip3 protein is found conserved from Saccharomyces cerevisiae to humans. In baker’s yeast, Zip3p has been implicated in synaptonemal complex (SC) formation, while little is known about the protein’s function in multicellular organisms. We report here the successful targeted gene disruption of zhp-3 (K02B12.8), the ZIP3 homolog in the nematode Caenorhabditis elegans. Homozygous zhp-3 knockout worms show normal homologue pairing and SC formation. Also, the timing of appearance and the nuclear localization of the recombination protein Rad-51 seem normal in these animals, suggesting proper initiation of meiotic recombination by DNA double-strand breaks. However, the occurrence of univalents during diplo-tene indicates that C. elegans ZHP-3 protein is essential for reciprocal recombination between homologous chromosomes and thus chiasma formation. In the absence of ZHP-3, reciprocal recombination is abolished and double-strand breaks seem to be repaired via alternative pathways, leading to achiasmic chromosomes and the occurrence of univalents during meiosis I. Green fluorescent protein-tagged C. elegans ZHP-3 forms lines between synapsed chromosomes and requires the SC for its proper localization.

Most meiotic recombination is likely to depend on recombinational repair of programmed meiotic DNA double-strand breaks (DSBs), which are induced by Spo11p (6, 19). In the budding yeast Saccharomyces cerevisiae, the fungus Coprinus cinereus, the mouse, and the flowering plant Arabidopsis thaliana, it was shown that this or an additional function of Spo11p is also required for the formation of the synaptonemal complex (SC), the proteinaceous structure that intimately links homologous chromosomes in meiotic prophase (2, 17, 25, 28, 34). C. elegans and Drosophila melanogaster are different in that Spo11p is dispensable for the initiation of SC formation (12, 26). For the budding yeast S. cerevisiae, it was proposed that synopsis initiates at DSB sites (9), specifically those which are destined to become crossovers (1, 18). The protein Zip3 would mark these sites and recruit Zip2 and Zip1, the latter being the major component of the SC’s central region (1, 16).

In S. cerevisiae, a null mutation in ZIP3 leads to a two- to threefold reduction in crossovers (1). Moreover, DSBs accumulate in this mutant, indicating a defect in the normal progression of recombination (7). ZIP3 is a member of the group of ZMM genes, which all confer similar mutant phenotypes. The ZMM genes have been implicated in the progression of meiosis from crossover-destined DSBs to subsequent steps (single-end invasion, double Holliday junction) on the way to crossing over and SC nucleation, possibly by coordinating the biochemical processes with the formation of underlying chromosome structures (7).

A C. elegans protein homologous to Zip3p (Cst9p), K02B12.8p (www.wormbase.org), was assigned a meiotic func-

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Gene disruption. The construct for the disruption of the K02B12.8 open reading frame was generated by inserting two genomic regions (fragment 1 and fragment 2 in Fig. 1a) of about 1.25 kb each of the K02B12.8 gene into pPD#MM016 (24), a Bluescript vector containing the 
*unc-119*

rescuing locus (of about 5.7 kb) cloned into the XbaI and HindIII sites. Fragment 1 was amplified with primers 5′/H11032-TCC CCG CGG CTC GTT AGG TAA AGG CGA CAA-3′/H11032 and 5′/H11032-ATA AGA ATG CGG CCG CAT GAA GAA TCC ATC CGG TGG T-3′/H11032 and cloned into the SacII and NotI sites. Fragment 2 was amplified with 5′/H11032-CCC AAG CTT GAA ATG CGC GAA AGC AGG TAC-3′/H11032 and 5′/H11032-CGG GGT ACC TGA ACA CGT ATC GCA GAA CCG-3′/H11032 and cloned into the KpnI and HindIII sites. Prior to transformation, the disruption construct was digested with SpeI and ScaI (Fig. 1a).

*unc-119*(ed3) mutant worms were transformed with the above construct by microparticle bombardment (5, 31) with a Bio-Rad (Hercules, Calif.) biolistic particle gun. In 25 transformation experiments, 100 lines carrying the *unc-119* gene were obtained, of which 6 segregated dead embryos. These were scored for the *zhp-3* mutant phenotype (see Results). Candidate mutant lines were subjected to diagnostic PCR. Worms were lysed in lysis buffer (38); proteinase K was inactivated by incubation at 95°C. An aliquot of 0.5 μl of the lysed material was added to the PCRs. Primer pairs were designed to amplify a segment of wild-type *zhp-3* (PP1, 5′/H11032-CGT TTT TCC GGC AAA TTC AGC-3′/H11032 and 5′/H11032-TAC GGT GAC AAT CAC ACG CTA-3′/H11032) and to amplify a segment when the disruption cassette was integrated (PP2, 5′/H11032-GCC GGA AAT TTT CAG TTC TGG-3′/H11032 and 5′/H11032-TAC GGT GAC AAT CAC ACG CTA-3′/H11032). One mutant line, UV1 *zhp3*(jf61), was recovered, for which PCR with PP2 produced a product of the expected size and none with PP1 (Fig. 1b). The PCR fragment of PP2 was sequenced, and the expected sequence for a homologous integration, consisting of *unc-119* adjacent to K02B12.8, was obtained.

Reverse transcription-PCR. cDNA was generated from about 100 mutant or wild-type worms, as described (22). For the amplification of the *zhp-3* message, the following primer pairs were used: outer primers 5′-CCATCGCGTAATAATGGGAG-3′/H11032 and 5′-AATGAAGCTCCGACCATTGATC-3′/H11032; nested (inner) primers 5′-CTACGGCAAATCAGACAATCATG-3′/H11032 and 5′-GGTTTCCTTTG TGCGAGGTATC-3′/H11032. For the *spo-11* message (as a control for the quality of cDNA), the following primer pairs were used: outer primers 5′-CATTGCGTAATAAA TATGGGAG-3′ and 5′-AATGAAGCTCAGGACCATTGATC-3′; nested (inner) primers 5′-CTACCGCAAATCAGACAATCATG-3′ and 5′-GGTTTCCTTTG TGCGAGGTATC-3′.
nested (inner) primers 5'-GTOAACCTTGGATAGAAGTGCG-3' and 5'- CATTTCCGAGTCGAAATCGCGG-3'. Amplification with the outer primers pair resulted in a 501-bp product and with the nested pair in a 296-bp product.

Construction and expression of a GFP-tagged K02B12.8p. The K02B12.8::green fluorescent protein (GFP) fusion construct was made by inserting the cDNA at the N terminus of the GFP open reading frame in a modified pie-1 expression vector (see below) into a BamHI site. The cDNA was amplified with primers 5'-CGG GAT CCG ATT TTG TTC ACT GTA ATA AAT GC-3' and 5'-CGG GAT CCA TCG GCG GGT CCA ATG AAG CTC CG-3' (H11032/H11032). The 3'-end of the K02B12.8p have a similar domain structure, with an N-terminus of 27 amino acids, while Zip3p and ZHP-3 are 18.5% identical to each other. Moreover, Zip3p and human sequences might be missing significant parts from their C termini.

Immunohistochemistry. Standard cytological squash preparations or spread preparations fixed with formaldehyde (30) were postfixed in a series of methanol, methanol-acetone (1:1), and acetone for 5 min each at −20°C and immediately transferred to 1% phosphate-buffered saline and washed three times for 5 min in this buffer. Primary antibodies were applied for 1 h at 4°C in a humid chamber. After repeated washes in 1% phosphate-buffered saline, secondary antibodies were applied for 1 h at room temperature. For details, see reference 30.

RESULTS

K02B12.8, a homolog of budding yeast Zip3p. A BLAST search of the C. elegans genome performed with budding yeast Zip3p (Cst9p) protein revealed the translation product of open reading frame K02B12.8 as the only C. elegans homolog of yeast Zip3p (see also www.wormbase.org). Related proteins can also be found in Drosophila melanogaster and humans (Fig. 2). The translation product of K02B12.8 is 387 amino acids in length, while Zip3 is a protein of 482 residues. The two proteins are 18.5% identical to each other. Moreover, Zip3p and K02B12.8p have a similar domain structure, with an N-terminal RING finger, followed at a 50-amino-acid distance by a PHD finger, and association with pairing chromosomes (see below), of the K02B12.8 will be referred to as C. elegans ZHP-3 (Zip3-homologous protein).

Depletion of ZHP-3 protein and disruption of zhp-3. K02B12.8p (C. elegans ZHP-3) was originally assigned a meiotic function by RNAi producing a Him phenotype (15). RNAi yielded a reproducible but not very penetrant cytological phe-
notype in our hands. Therefore, the consequences of ZHP-3 depletion were studied by two different approaches. First, K02B12.8p was depleted by transgene-mediated cosuppression (see Materials and Methods). Second, the zhp-3 gene was disrupted by homologous integration of a marker gene with biolistic transformation (5). To this aim, unc-119 worms were transformed with a construct containing the unc-119/-H11001 marker gene, inserted into the first exon of the zhp-3 gene, 57 bp downstream of the ATG. The insertion deleted 27 bp of exon 1 (Fig. 1). Reverse transcription-PCR showed the absence of a transcript of K02B12.8 (Fig. 1c). The mutant allele zhp-3 (jf61) can therefore be regarded as a null mutation. The mutant displayed the expected meiotic phenotype (see below) and a Mendelian segregation pattern (a ratio of 1:2 homozygous mutants versus heterozygous animals within the non-Unc offspring), indicating full somatic viability.

Since gene disruption and protein depletion by cosuppression resulted in identical phenotypes, the observations made with the two approaches will be treated jointly. The data shown below are from zhp-3(jf61) animals.

Homologous pairing and SC formation in zhp-3. Homozygous zhp-3(jf61) animals showed no apparent defect in homologous chromosome alignment, pairing, and SC formation. Immunostaining of HIM-3 (Fig. 3a) and REC-8 (Fig. 3b), both being components of axial/lateral elements of the SC (11, 30, 39), as well as of SYP-1 (Fig. 3c), a component of the central element (23), revealed linear structures in pachytene, identical to those seen in the wild type. Moreover, SYP-1 was found to localize in the gap between paired chromosomes, as visualized by DAPI (Fig. 4a). Also, the relative size and position of the pachytene zone within the gonad were not notably different between the zhp-3 mutant and the wild type, and pachytene SCs extended all along the DAPI-positive bivalents. This suggests that SC formation is normal in the zhp-3 mutant except for possible ultrastructural anomalies which might escape detection by light microscopy.

To confirm that the SC indeed forms between homologous chromosome regions, two chromosomal loci, one (5S rRNA) near the right end of chromosome V, and the other near the left end of chromosome I, were delineated by FISH, and the nuclear distribution of the homologous FISH signals was scored. In 92 of 93 pachytene nuclei, both pairs of homologous FISH signals were closely associated or fused into one, indicating that synopsis took place between homologous chromosomes (Fig. 4b). While pairing was normal in the mutant, there was no chiasma formation at diplotene and diakinesis. Instead
of six bivalents, 12 well-condensed univalents were present (Fig. 3d to f) in a total of 153 diakineses scored.

**Localization of RAD-51 in zhp-3.** To determine if meiotic DSBs are induced in the mutant, we performed immunostaining of the recombination protein RAD-51. Rad-51 is believed to be an indicator of the presence of DSBs because it forms foci in wild-type *C. elegans* meiosis but not in the *spo-11* mutant (4), which is unable to initiate DSBs (12). In the *zhp-3* mutant and in the wild type, RAD-51 foci appeared in similar regions within the gonad (Fig. 5). Although in the mutant the RAD-51-positive region seemed to be somewhat extended, RAD-51 disappeared well before the end of the pachytene zone. The disappearance of RAD-51 foci was not gradual but sudden and simultaneous (Fig. 4c). The number of foci per nucleus was more than twice as high in the mutant (5.2 ± 3.4 [n = 170], 5.9 ± 3.2 [n = 101], 6.6 ± 3.5 [n = 200]; three individuals) than in wild type (2.2 ± 1.2 [n = 68], 2.6 ± 1.5 [n = 147], 2.7 ± 1.5 [n = 130], 2.9 ± 1.8 [n = 122]; four individuals) (Fig. 4c and d). The disappearance of RAD-51 foci suggests either that, in the mutant, RAD-51 detaches from unrepaired DSBs or that DSBs are repaired by a pathway that does not lead to chiasmata. As the relatively high viability of offspring is not compatible with the persistence of unrepaired DNA (see below and Discussion), it must be assumed that DSBs are repaired at a high rate by noncrossover processes.

**Reduced recombination and fertility of zhp-3 animals.** *zhp-3(jf61)* hermaphrodites produced a reduced number of viable offspring. Of 522 embryos laid by homozygous *zhp-3(jf61)* mothers, 22 hatched, but most of them died prematurely. Nine embryos (~2%) developed beyond the L3 stage; two out of the nine survivors were males. This is a relatively high rate of survival considering that only offspring which received a fairly normal chromosome complement by random segregation of all

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**FIG. 4.** Pairing and localization of recombination protein in the mutant. (a) SYP-1 localizes to the gap between paired chromosomes as visualized by DAPI, as can be seen in the merged image. (b) FISH of the 5S rDNA (red) and a locus on chromosome I left arm (green) in pachytene nuclei of the *zhp-3* mutant. The presence of a single FISH signal per nucleus for each of the two loci tested is evidence for homologous pairing. Sectors with late pachytene nuclei of gonads of (c) the *zhp-3* mutant and (d) the wild type immunostained for the Rad-51 recombination protein (red). Note the larger number of Rad-51 foci and their sudden disappearance before the end of pachytene in the mutant (distal-proximal indicates the orientation of the sector shown within the gonad). Chromatin is stained blue by DAPI. Bar, 5 µm.
homologous univalents in both male and female meiosis can survive (see Discussion). However, the ~2% viable offspring could be explained if zhp-3(jf61) animals produced normal sperm. To test if both female and male meiosis was equally affected by the mutation, surviving mutant hermaphrodites were crossed to wild-type males. The offspring from these crosses showed higher viability (8 out of 168 eggs laid, 4.7%) than by selection of 2% viable offspring (Fig. 6d). However, the viable offspring could be explained if zhp-3(jf61) animals produced normal sperm. To test if both female and male meiosis was equally affected by the mutation, surviving mutant hermaphrodites were crossed to wild-type males. The offspring from these crosses showed higher viability (8 out of 168 eggs laid, 4.7%) than by selection of 2% viable offspring (Fig. 6d).

Cytological localization of ZHP-3 protein. We generated a fully viable transgenic line expressing GFP-tagged ZHP-3. The transgene was confirmed to be functional because transformation of mutant animals with this construct resulted in the rescue of embryonic lethality (not shown). Immunodetection of ZHP-3::GFP showed a dot-like nuclear distribution in transition zone nuclei (Fig. 6a) (for a classification of meiotic stages in the gonad, see references 23 and 29). Major ZHP-3::GFP spots seemed to colocalize with early SYP-1 structures at this stage (Fig. 6a). In pachytene nuclei of ZHP-3 became organized as lines or linear arrays of dots which occupied the space between synapsed chromosomes (23) (Fig. 6b). Since the absence of the ZHP-3 protein does not affect SC formation, it is evident that ZHP-3, in spite of its localization in the bivalent regions where the SC also resides, is not an essential component of this structure. In diakinesis, ZHP-3 was still present in cells but no longer associated with chromosomes (Fig. 6c).

We next wanted to determine which structures are required for ZHP-3 localization along pachytene chromosomes. To this aim, we studied the distribution of ZHP-3::GFP in nuclei which were depleted of HIM-3 by RNAi. HIM-3 is a homologue of budding yeast Hop1p. It is associated with the lateral elements of the SC, and in its absence presynaptic alignment and synopsis do not take place (11, 39). In him-3(RNAi) nuclei no linear ZHP-3 structures but one to several spots were detected (Fig. 6d). These spots possibly delineate sites of residual synopsis (caused by the incomplete deletion of HIM-3 by RNAi) or aggregates of unutilized SC material (polycomplexes), since they also contained the central element protein SYP-1 (Fig. 6d).

To confirm that C. elegans ZHP-3 localizes only to bivalents with mature SCs, we depleted gonads of SYP-1 by RNAi. SYP-1 is homologous to the S. cerevisiae transversal filament protein Zip1p, and in its absence the axially oriented elements of the SC are not connected (23). In SYP-1-depleted nuclei of the pachytene zone, ZHP-3 did not form lines (Fig. 6e), whereas REC-8 delineated the unsynapsed chromosome axes (Fig. 6f). This demonstrates that ZHP-3 depends on SYP-1 for wild-type localization and confirms that it fails to associate with unsynapsed axes. To further test the localization of ZHP-3 to the synapsed portions of homologous chromosomes, we prepared nuclei by an SC spreading technique. Immunostaining of ZHP-3 produced dotted arrays along the SYP-1 lines (Fig. 6g). In no case did we observe ZHP-3 signals outside SYP-1-positive lines. This confirms that ZHP-3 occupies only the synapsed portions of bivalents.

To study if the chromosomal localization of ZHP-3 depends on the creation of meiotic DSBs, the C. elegans zhp-3::GFP line was crossed to a spo-11 mutant and selected for homozygous spo-11 individuals expressing ZHP-3::GFP. In C. elegans, spo-11 mutants do synapse their chromosomes but fail to induce DSBs (12). spo-11; zhp-3::GFP individuals exhibited linear ZHP-3 structures (Fig. 6h) indistinguishable from those of the wild type, indicating that ZHP-3 expression and localization to SCs do not depend on DSBs.

DISCUSSION

Disruption of zhp-3 by homologous targeting. Here we report the first successful application of a novel technique (5) with homologous integration to study the function of a previ-
ously uncharacterized gene in *C. elegans*. While we did not attempt to quantitate the efficiency of the technique, we can state that out of 25 biolistic transformation experiments, ~100 lines carrying the *unc-119* gene were obtained, of which a single line could be verified as carrying the desired gene disruption. In our experiment, the length of homology available for homologous integration (2.5 kb) was notably smaller than the 4 to 6 kb described by Berezikov et al. (5). It thus appears as if knockout lines could be generated with a reasonable effort, representing a good alternative to current gene silencing and RNAi techniques.

*C. elegans* ZHP-3 localizes along synapsed bivalents but does not function in synapsis. In the budding yeast (and most other organisms), SC formation depends on the presence of meiotic

FIG. 6. Immunostaining of GFP-tagged ZHP-3 (green) in the wild type and various mutants. In the wild type in nuclei of the transition zone (a), ZHP-3 forms spots (with major spots colocalizing with SYP-1, shown in red), while in pachytene nuclei (b) it appears as lines. In diakinesis (c) it is dispersed between the chromosomes. (d) Upon RNAi depletion of the SC-associated protein HIM-3, the linear organization of ZHP-3 is lost, and it colocalizes with the synopsis protein SYP-1 to one to several spots (orange, resulting from the mixing of green ZHP-3 and red SYP-1 markers). (e) No ZHP-3 lines are present in nuclei devoid of SYP-1, whereas unsynapsed axial elements in SYP-1-depleted nuclei are delineated by staining of REC-8 (f, red). (g) In spread SC preparations of the wild type, ZHP-3 (green) colocalizes with SYP-1 (red) on SCs, but the ZHP-3 pattern is discontinuous and faint, possibly due to an only weak association of ZHP-3 with SCs. Chromatin is stained blue by DAPI in all pictures. (h) In a spo-11 mutant, the pachytene linear organization of ZHP-3 is not affected. Bars, 5 μm.
DSBs. The transversal filaments of the SC connect homologous chromosomes all along their length in pachytene. Starting at a single or a few sites of a prealigned homologous chromosome pair, the transverse filaments establish the connection from which the SC progresses in a zipper-like fashion. In the budding yeast, Zip3p and another protein, Zip2p, were shown to interact and overlap with recombination proteins and hence proposed to localize to sites of meiotic interhomologue recombination (1, 9). Interhomologue recombination occurs by two alternative pathways, conversion and crossing over, upon which is decided soon after the formation of DSBs (3, 7). Since Zip3p foci appear to show interference (as do crossovers) (16) and since the effects of spo-11 mutations correlate better with effects on crossover frequencies than with effects on total DSB levels (18), it seems likely that Zip3p localizes to the crossover-destined subset of recombination intermediates. At these sites, Zip3p and Zip2p would lead to the assembly of Zip1p, the major component of transverse filaments (35) and zipping up of the SC. In the absence of Zip3p, synopsis was found to be substantially delayed and incomplete. Taking all the evidence together, it was proposed that Zip3p is a component of recombination nodules and serves to link synopsis to meiotic recombination (1, 7).

In spite of its sequence homology, *C. elegans* ZHP-3 cannot share the role proposed for budding yeast Zip3p because initiation of recombination is not required for SC formation in *C. elegans* (12). A homologue of yeast Zip2p, one of Zip3p’s putative partners, has not been detected in the worm genome, which supports the idea that *C. elegans* ZHP-3 works in a different context. Furthermore, as we show here, the localization of the SC components HIM-3, REC-8, and SYP-1 is normal in zhp-3(/f61) nuclei, which indicates that wild-type SC formation does not require ZHP-3. Finally, the distribution of *C. elegans* ZHP-3 all along synapsed chromosome axes suggests that its localization to chromosomes requires rather than allows synopsis.

*C. elegans* ZHP-3 has the interesting property of being either part of the SC structure or associated with it without being essential for its formation. *C. elegans* ZHP-3 has no function in homology recognition either, since we showed that in the mutant the SC was formed between homologous chromosomes. It is noteworthy, however, that proper localization of ZHP-3 is dependent on the formation of a mature SC with incorporated central elements, therefore allowing ZHP-3 to reside at the SC. Recently, it was shown that the central element of the SC is essential for proper completion of recombination in *C. elegans* (10). ZHP-3, by depending on the presence of the central element, might thereby play an essential role in this process.

**ZHP-3 is essential for crossing over and chiasma formation.** The recombination protein RAD-51 is believed to serve as a marker for DSBs. It transiently localizes as spots to chromatin in wild-type meiotic prophase nuclei but fails to do so in *spo-11* meiocytes, where no DSBs are generated. In strains where DSB repair is impaired, RAD-51 foci show extended persistence (4, 10). In the zhp-3 mutant, the dynamics of formation and disappearance of RAD-51 foci was not dramatically different from that of the wild type, but DSBs were not repaired in a way that produced chiasmata. It might be argued that RAD-51 disappeared from chromatin, while DSBs remained unrepaired. This possibility is invalidated by the relatively high viability of offspring (≈2%) by zhp-3 compared to rad-51 null mutants (which produce zero viable offspring due to inefficient repair of DSBs [4]). It suggests that no additional factor contributes to lethality other than the disturbed segregation of univalents and that DSBs are efficiently repaired in the mutant.

Since in a total of 93 progeny only one putative recombinant was found (see Results), we can rule out the possibility that crossovers were formed but not maintained. Therefore, it can be assumed that DSBs were repaired by a noncrossover mechanism (gene conversion, sister chromatid recombination, or nonhomologous end joining). The experimental system does not allow us to distinguish among these possibilities. It is, however, tempting to speculate that *C. elegans* ZHP-3 may have a function in the SC-dependent processing of DSBs and their conversion into chiasmata. It might well play a role analogous to budding yeast Red1 and Hop1 by preventing sister chromatid recombination (21, 33, 36), which was recently also proposed for *C. elegans* HIM-3 (11, 33, 36). Thus, the function of ZHP-3 and its yeast paralogue, although working within very different settings, could be similar, namely, communication between recombination sites (DSBs) and SC components. While in budding yeast Zip3 seems to recruit SC initiation factors to the sites of DSBs (1), the flow of information could be in the opposite direction in *C. elegans*. Here ZHP-3 could sense the presence of the SC and direct DSB repair toward an SC-dependent (crossover) pathway.

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