A Targeted RNAi Screen for Genes Involved in Chromosome Morphogenesis and Nuclear Organization in the *Caenorhabditis elegans* Germline

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ABSTRACT

We have implemented a functional genomics strategy to identify genes involved in chromosome morphogenesis and nuclear organization during meiotic prophase in the *Caenorhabditis elegans* germline. This approach took advantage of a gene-expression survey that used DNA microarray technology to identify genes preferentially expressed in the germline. We defined a subset of 192 germline-enriched genes whose expression profiles were similar to those of previously identified meiosis genes and designed a screen to identify genes for which inhibition by RNA interference (RNAi) elicited defects in function or development of the germline. We obtained strong germline phenotypes for 27% of the genes tested, indicating that this targeted approach greatly enriched for genes that function in the germline. In addition to genes involved in key meiotic prophase events, we identified genes involved in meiotic progression, germline proliferation, and chromosome organization and/or segregation during mitotic growth.

IN metazoans, genetic information is transmitted from one generation to the next via a specialized cell lineage known as the germline. The germ cell lineage is set aside early during development, and its chromosomes are insulated from events that can occur in somatic lineages that disturb or interfere with genomic integrity such as programmed gene rearrangements, chromatin diminution, and telomere shortening. Further, most animals reproduce sexually, so after germ cells exit a proliferative state they undergo a specialized nuclear division program called meiosis, which enables diploid germ cells to generate haploid gametes.

The nematode *Caenorhabditis elegans* is an especially favorable system for investigating the mechanisms that govern the faithful transmission of the genome through the germline (Hubbard and Greenstein 2000). In the adult hermaphrodite, the germline accounts for >50% of the cell nuclei in the organism. Proliferating premeiotic nuclei and nuclei at all stages of meiotic prophase are present simultaneously in a temporal/spatial gradient along the distal-proximal axis of the gonad, such that each germline represents a complete time course of meiotic prophase. Moreover, chromosome morphology and nuclear organization can be readily visualized in whole-mount cytological preparations that preserve this temporal/spatial context (Dernburg *et al.* 1998). Further, traditional genetic and molecular approaches, coupled with the accessible cytology, have been extremely fruitful for investigating mechanisms underlying various aspects of the development and function of the germline. An integration of molecular, genetic, and cytological methods has led to the identification of numerous genes involved in germline specification, growth and maintenance of the germline, entry into and progression through the meiotic program, and behavior and inheritance of chromosomes during meiosis (for reviews see Albertson *et al.* 1997; Hubbard and Greenstein 2000; Seydoux and Schedl 2001; Villeneuve and Hillers 2001).

Further advances in the identification of germline genes have been made possible by the completion of the *C. elegans* genome sequence (*C. elegans* Sequencing Consortium 1998) and the development of DNA microarrays that can be used to survey gene expression on a genome-wide scale (Reinke *et al.* 2000). The nematode germline is particularly well suited for exploiting this type of approach, both because it represents such a large fraction of the mass of the organism and because of the availability of robust temperature-sensitive (ts) mutations affecting germline development and function (Hubbard and Greenstein 2000). Reinke *et al.* (2000) used a ts mutant that lacks a germline when grown under restrictive conditions (Beanan and Strome 1992) to conduct time course experiments comparing gene expression from worms with and without germlines at various developmental stages and further compared gene expression in hermaphrodite worms producing only sperm with that in worms producing only oocytes. Utilizing DNA microarrays containing 11,917 genes (63% of the total predicted genes in the genome), they identified 1416 genes preferentially expressed in

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the germline of *C. elegans*. These comprised three groups: 650 spermatocyte-enriched genes, 258 oocyte-enriched genes, and 508 "germline-intrinsic" genes [genes that exhibited a highly reproducible enrichment in germline (+) *vs.* germline (−) worms but whose expression was not significantly different in the spermatocyte *vs.* oocyte comparison]. Comparison of this data set with lists of previously known germline genes provided a compelling validation of the experimental approach and suggested that these global expression profiles would be useful for identifying genes involved in important germline functions. For example, most known meiosis genes had been shown previously to exhibit germline-specific or germline-enriched gene expression, and these genes behaved precisely as expected in the microarray experiments.

Here we report the use of a targeted functional genomics strategy using RNA-mediated interference (RNAi) to investigate the function of a selected pool of the germline-intrinsic genes identified by Reinke *et al.* (2000). Our approach differed from that of several other recently reported RNAi screens (Fraser *et al.* 2000; Gönczy *et al.* 2000; Maeda *et al.* 2001) in focusing on a restricted set of candidate genes defined by their expression profiles and in employing an in-depth analysis of phenotype over several generations. This targeted strategy was successful in demonstrating germline roles for at least 27% of the genes tested, indicating that this approach greatly enriched for genes that function in the germline.

MATERIALS AND METHODS

Worm strains: Only wild-type *C. elegans* from the Bristol N2 strain were utilized. Worms were cultured at 20° under standard conditions as described by Brenner (1974).

Primer pair design and dsRNA synthesis: For 185 of the genes tested, PCR products amplified from genomic DNA were used as templates for dsRNA synthesis. The primers used were adapted from those used by Reinke *et al.* (2000) in their microarray experiments, allowing us to relate our results directly to the gene-expression profiles previously reported. The genomic locations of these original Genepairs primer pairs (Research Genetics, Carlsbad, CA) and the regions they amplify are indicated on Wormbase Sequence Reports under "PCR Assay." The relevant amplified regions are labeled "sjj-<gene name>." Each primer pair amplified ~1 kb of coding sequence from the corresponding gene. For most primer pairs, T7 promoter sequences (TAATACGACTCACTATAG) were added to the 5′ ends of each primer, allowing subsequent transcription and annealing steps to be performed as single-tube reactions. For three genes, C08F8.3, C49C3.7, and T07C4.3, one primer of the pair included the T7 promoter sequence while the other included a T3 promoter sequence (ATTAACCCTCACTAAAG). Comparison of these reactions with the yield obtained from the same reactions where both primers carried a T7 promoter and were synthesized as single-tube reactions indicated no difference in yield.

Primary PCR reactions (50 μl) were done in 96-well plates using ~0.1 μg template genomic DNA and 0.5 μM primers. To optimize the yield of products with full-length T7 promoters, we used 2 μl of the primary PCR reaction as template for a secondary amplification using T7 primers (0.5 μM). Both primary and secondary PCR products were assessed on 1% agarose gels to confirm size and yield. Products were purified with a 96-well QIAGEN (Valencia, CA) PCR purification kit and eluted in 30 μl. All the eluted DNA was used in a 100-μl transcription reaction with T7 RNA polymerase (Promega, Madison, WI), followed by DNAse I treatment. RNA samples were then purified (QIAGEN) and eluted in 30 μl; an aliquot (ssRNA) was removed prior to annealing samples 10 min at 68° followed by 30 min at 37°. dsRNA concentrations ranged from 1 to 5 μg/μl. To score for shifts in mobility for the dsRNA, ssRNA was run parallel to dsRNA on 1.4% agarose gels.

dsRNA synthesis for seven additional genes (F57B10.4, R12B2.4, T06E4.1, ZK1055.1, F26D2.2, F39H2.4, and F56A3.4) tested in our pilot screen and included in this article was done following the protocol described by Fire *et al.* (1998) using cDNA clones yk176b1, yk428e9, yk204f9, yk362d7, yk414g2, yk252b7, and yk295b7, respectively, provided by Dr. Yuji Kohara, NIG (Japan).

Injections and phenotypic analysis: Each dsRNA was injected into one or both gonad arms of 15 young adult hermaphrodites (P_0's), which were plated individually and transferred serially to fresh plates to collect broods of F_1's (Figure 1). In most cases, four F_1's per injected P_0 (arising from embryos laid 12–48 hr post-injection) were picked to individual plates, left to lay eggs for 24–36 hr, and transferred to new plates. P_0's were kept for cytological analysis. In some cases, RNAi led to death of a substantial fraction of F_1's, and <60 viable F_1's were available. Because our primary goal was to identify genes involved in meiosis, plates containing progeny produced by P_0's (during 12–48 hr post-injection) and the initial plates containing progeny of F_1's were scored for (1) a *h*igh *i*ncidence of *m*ales (or Him) phenotype (Hodgkin *et al.* 1979), indicative of X chromosome missegregation, and (2) embryonic lethality, which might be caused by aneuploidy resulting from meiotic chromosome missegregation. F_1 animals were also scored for (3) sterility (failure to lay any eggs), which can result from an inability to complete the meiotic program. Levels of dead eggs on plates were ranked as low (5–30%), medium (30–70%), or high (>70%) on the basis of the total number of unhatched eggs scored over the total number of eggs laid. Levels of males on plates were ranked as low (<5%), medium (5–20%), or high (>20%) on the basis of the total number of males scored over the total number of viable progeny produced.

P_0's or F_1's that displayed any of the above phenotypes were subjected to cytological analysis. Undissected whole worms were fixed with Carnoy's fixative and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described in Villeneuve (1994) and examined for alterations in the appearance or distribution of germline nuclei. The Carnoy's method was used for screening purposes since it can be performed on intact worms, thereby allowing rapid processing of large numbers of worms with minimal manipulations. Chromosomes were visualized with a Zeiss Axiophot 2 microscope equipped for fluorescence microscopy; images were either captured on photographic film (Kodak Elite Chrome 100) and converted to digital images or collected using a CCD camera.

List of genes that elicited no defect upon RNAi: 3R5.1, B0001.2, B0001.3, C01G5.8, C08C3.2, C08F8.3, C13F10.6, C16A11.3, C16A11.4, C16C8.4, C18H2.2, C29H12.5, C34G6.5, C50C3.8, C56A3.5, D1081.7, D2030.8, F01G4.4, F07H5.10, F10B5.5, F13G3.6, F14D2.8, F23B12.8 (*klp-14*), F26A1.1, F26H9.4, F28F8.6, F30F8.3, F32E10.2, F33H2.1, F35G12.12, F38A5.13, F38B7.7 a.k.a. H12C20.2a (*pms-2*), F39H2.1, F45E4.10 (*gfi-4*), F49E8.7, F52C9.7, F53F4.14, F54D5.9,

F57A10.4, F57B10.4, F57B10.6, K07A1.1, K07H8.10, K08F4.2, K08F4.3, K10D2.1, K12D12.5, M03E7.5, R05D11.5, R05D3.11, R06C7.2, R06C7.9, R07B7.2, R09B3.1 (*exo-3*), R10D12.14, R11A8.2, R74.8, T01G9.4 (*kup-2*), T05H10.2 (*apn-1*), T06E4.1 (*hcp-2*), T07C12.3, T07C4.3a and b, T08B2.11, T09A5.8, T13F2.6, T19B10.8, T23B12.4, T24A6.1, T24D1.3, T24G10.2, T25G3.3, T26A5.2, T26A5.5, W02D3.10, W05F2.2a and b, Y102E9.2, Y17G7A.1 (*hmg-12*), Y32B12B.2, ZC155.3, ZC410.3, ZK1055.1 (*hcp-1*), ZK1307.9, ZK856.12.

Statistical analysis: Statistical analyses (Fisher's exact test, chi-square test for independence, and Mann-Whitney test) were performed using the InStat software package (GraphPad.com).

RESULTS AND DISCUSSION

Rationale of the RNAi screen: A major goal of our research program is to investigate the mechanisms responsible for faithful segregation of homologous chromosomes during meiosis. Because previously identified components of the meiotic machinery are expressed preferentially in the germline, we reasoned that it should be possible to discover additional components by screening among genes exhibiting germline-enriched gene expression to identify those for which inhibition by RNAi elicits defects in meiotic chromosome morphology or behavior. Meiotic defects can be detected indirectly by examining the products of meiosis through progeny testing of affected individuals; in *C. elegans*, defects in meiotic chromosome segregation are manifested by a high frequency of XO male progeny and inviable aneuploid embryos among the self-progeny of XX hermaphrodites (the Him phenotype). Alternatively, meiotic defects can be detected more directly, by cytological examination of DAPI-stained meiotic prophase chromosomes in the germlines of affected individuals (Figure 2). Any defects leading to an absence or reduced frequency of crossover recombination are readily detected at diakinesis, the last stage of meiotic prophase: whereas wild-type nuclei at this stage contain six discrete DAPI-stained bodies, each corresponding to a pair of homologs attached by a chiasma, a deficit in crossing over results in the presence of up to 12 univalent chromosomes that are unattached to their homologous partners. Further, failure of chromosomes to reorganize or align lengthwise with their homologs at earlier stages of meiotic prophase can be detected by an altered appearance of the DAPI-stained chromatin in more distal parts of the germline.

Because RNAi effects are often most pronounced in the F_1 progeny of treated animals, the former means of assessment of meiotic defects requires examination of progeny two generations after dsRNA administration, whereas the latter requires microscopic imaging of the germlines of F_1 animals after they have reached adulthood. Thus since an effective screening procedure for identifying meiotic defects would involve a nontrivial investment of effort for each gene tested, we also chose to document the effects of RNAi on several additional aspects of germline development, organization, and function.

Selection of 192 candidate genes: A subset of genes from the germline-intrinsic list of Reinke *et al.* (2000) was selected for targeting by RNAi on the basis of several criteria. In an initial small-scale screen, we targeted 14 genes that were predicted to contain extended coiled-coil domains by the Paircoil (Berger *et al.* 1995) and/or COILS (Lupas *et al.* 1991) prediction programs. We chose these genes because one of our goals was to identify structural components of the synaptonemal complex (SC), the highly ordered proteinaceous structure that forms at the interface between paired and aligned homologous chromosomes during meiotic prophase. SC structural proteins are notoriously poorly conserved, and a predicted coiled-coil domain is the only feature shared in common between SC central region structural proteins identified in rodents and budding yeast (Zickler and Kleckner 1999). We then used the 2 meiosis genes identified from this set together with 8 other previously known meiosis genes as an entrainment set to define parameters for a meiotic prophase expression profile. This analysis indicated that candidate genes should exhibit germline-enriched expression in at least the L4 and adult stages and might exhibit germline-enriched expression in the L3 (and less frequently the L2) stage, in agreement with the timing of entry into meiosis during development. Further analysis of the entrainment set also indicated that the ratio of expression in worms producing only oocytes to worms producing only sperm should be <2.2. In addition to candidate genes meeting these criteria, we also targeted two other genes (B0414.3 and C18G1.5) with oocyte/sperm expression ratios that were >2.2; these genes encode histone H1 variants and were included because a meiosis-specific H1 variant from Lilium had been suggested to be important for meiosis (Riggs 1997).

To a large extent, we excluded from our list genes for which the biological role was already known or which we knew to be under investigation by other laboratories. We also used sequence information to make strategic decisions to exclude genes we thought unlikely to play a direct or specific role in meiotic chromosome behavior (*e.g.*, RNA polymerase, ribosomal proteins). Including both the initial small-scale screen and the main screen, we screened a total of 192 genes.

Design and validation of the screening strategy: To develop a screening strategy that would be successful in identifying genes involved in diverse meiotic prophase events, we conducted pilot RNAi experiments using six genes previously implicated in various aspects of the meiotic prophase program. We used the following genes: *him-3*, which encodes a meiosis-specific component of the chromosome axis similar to Hop1, an axial element/lateral element protein from budding yeast (Zetka *et al.* 1999); F41H10.10 and F57C9.5, which encode two HIM-3/Hop1 related proteins; *spo-11*, re-

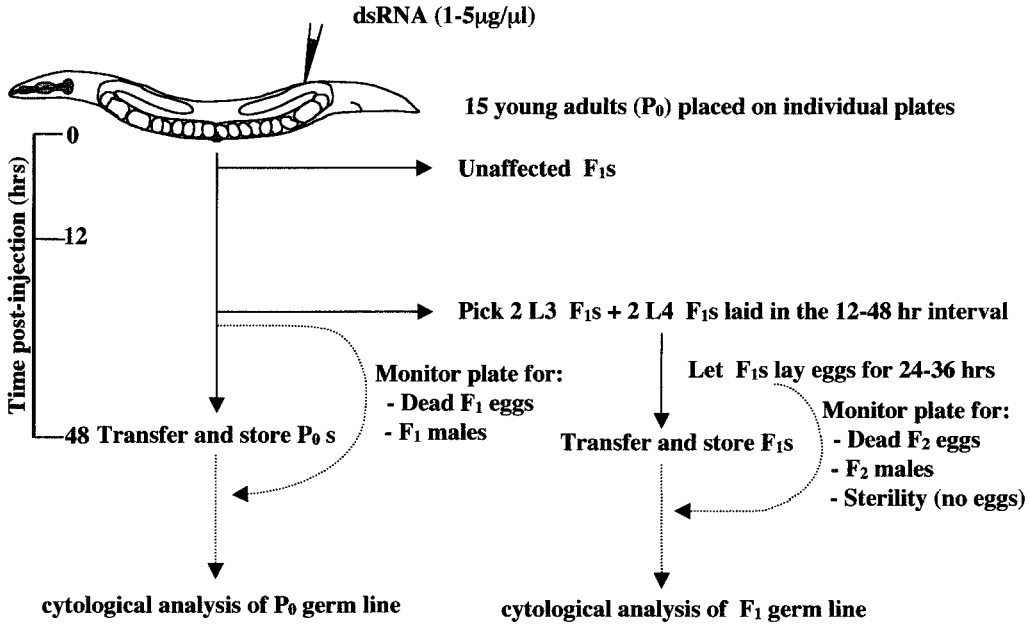

FIGURE 1.—Flowchart for screen. See text for description.

quired for initiation of meiotic recombination (DERNBURG *et al.* 1998); *him-6*, required for normal levels of recombination (ZETKA and ROSE 1995; C. WICKY, personal communication); and *msh-5*, which encodes a protein that acts downstream of the initiation step of recombination to promote formation of crossover products (KELLY *et al.* 2000). For each of these genes, mutant alleles, RNAi, and/or transgene-mediated cosuppression were shown previously to cause (1) a Him phenotype, reflecting a defect in meiotic chromosome segregation, and/or (2) a high frequency of achiasmate chromosomes at the diakinesis stage of meiotic prophase (Figure 2k), reflecting an absence or reduced frequency of crossover recombination between homologs. We succeeded in eliciting both the plate and cytological phenotypes using the RNAi regimen outlined in Figure 1.

In our initial experiments with the above-described positive control genes, we injected dsRNA at concentrations of ~50–250 ng/μl. Although this concentration elicited a robust meiotic-defective phenotype at high frequency for some genes, for other genes this concentration either elicited only weak phenotypes or produced a strong phenotype only at a low frequency. By increasing the concentration of the dsRNA to 1–5 μg/μl (as in GRISHOK *et al.* 2000), we were able to obtain strong phenotypes at high frequency for most of the positive control genes. Specifically, for nearly 100% of injectees, most F_1 progeny produced during a specified time window (see below) exhibited robust meiotic defects, producing both high levels of dead embryos and males in the F_2 generation. In some instances RNAi also generated a late-onset phenotype in the germline of the injected P_0, resulting in dead embryos and males in their late broods. The *msh-5* gene was an exception: even at high concentrations, we observed only a modest Him phenotype (40–50% dead embryos, 1% males) among only 23% of the F_1's. Thus, to maximize our chances of identifying genes that function in many different meiotic prophase events, we chose to examine the germline phenotypes of 4 F_1's from each of 15 P_0 injectees as well as those of the injectees themselves.

In addition to defining an appropriate dsRNA concentration and the numbers of worms we would be handling during the screen, it was important to define a time window during which the F_1 progeny produced by the injected P_0 animals would be most likely to exhibit germline defects. By transferring the injected P_0's to new plates at 12-hr intervals, we were able to monitor F_1's generated at successive intervals both for their plate phenotypes (frequencies of males and dead embryos produced) and for their cytological phenotypes (altered chromosome morphology or organization in germline nuclei). We observed the strongest effects of RNAi in F_1's derived from embryos laid between 24–48 hr postinjection, while F_1's laid before or after that time period exhibited a weaker RNAi effect for some of the targeted genes.

For the genes used in our design and validation phase, reduction or loss of gene function leads to chromosome missegregation but does not prevent completion of the meiotic program, so embryos are produced (albeit many are aneuploid and inviable). We also wished to identify genes that are crucial for initiating or completing the meiotic program, so in addition to the Him and cytological phenotypes described above, in the screening phase F_1's were also scored for sterility (failure to produce any embryos).

Classification of genes: For 57% of the 192 genes tested, phenotypic defects were observed following RNAi.

These fall into four broad classes, as summarized in Table 1. We have used various descriptor terms to indicate the phenotypes observed. For many of the genes, RNAi elicited a combination of phenotypes, so more than one descriptor term is applicable. Where we have used multiple descriptors, the first descriptor generally denotes what we considered to be either the primary defect or that which most directly reflects the germline function for the gene in question. This may indicate a phenotype that preceded all others observed and/or the phenotype most frequently observed among the adult F_1's. In some cases, additional descriptors refer to phenotypes present among subpopulations of the affected F_1's. In other cases more general descriptors (*e.g.*, sterile) were also applied after more specific primary descriptors (*e.g.*, tumorous germline) both to indicate a phenotype that could be assessed at lower resolution and to facilitate searching the database containing a compilation of our results (the full database is available by ftp at http://villeneuveRNAi.stanford.edu; user ID and password: villeneuveRNAi; see CONCLUSION).

Strong germline class:

Fifty-one genes (27%) were classified as "strong germline" genes because RNAi elicited clear defects in function and/or development of the germline. Genes in this class were listed under the following primary descriptors.

Meiotic: This descriptor was applied when RNAi elicited defects in meiotic chromosome segregation generating anywhere from medium to high levels of the Him phenotype (see MATERIALS AND METHODS) and/or resulted in the presence of achiasmate chromosomes at the diakinesis stage of meiotic prophase. In some cases the appearance of nuclei at earlier stages of meiotic prophase was also affected.

Included under this primary descriptor are three genes (*syp-1*, *syp-2*, and *syp-3*) encoding proteins predicted to have extended coiled-coil domains. RNAi for each of these genes led to a high frequency of univalent chromosomes at the diakinesis stage of meiotic prophase (Figure 2k) and defects in chromosomal organization earlier in prophase, including a persistence of the polarized nuclear organization normally seen only at the onset of homologous chromosome pairing in early meiotic prophase (extended transition zone phenotype; Figure 2f). All three genes have now been matched with meiotic mutants identified in our genetic screens (A. J. MACQUEEN, M. P. COLAIÁCOVO, J. ENGEBRECHT, K. C. REDDY and A. M. VILLENEUVE, unpublished data), and an in-depth analysis of their functional roles will be reported elsewhere.

The *rad-50* gene encodes a conserved protein whose fungal orthologs play important roles in both meiotic recombination and DNA repair, acting in a complex together with the Mre11 protein (HABER 1998). On the basis of this orthology and our previous work demonstrating that *C. elegans* MRE-11 is essential for meiotic recombination (CHIN and VILLENEUVE 2001), we anticipated that RNAi for *rad-50* would elicit a meiotic defect. It was included in the screen to serve as a positive control for our ability to detect genes of this functional class. As expected, RNAi for *rad-50* led to a high frequency of univalents at diakinesis, presumably reflecting a defect in meiotic recombination.

Chromosome segregation defects were also accompanied by variable numbers of achiasmate chromosomes in diakinesis-stage oocytes for several other genes tested. RNAi for one gene, T09E8.2, appeared to preferentially affect the segregation of the X chromosomes; we have since found that T09E8.2 corresponds to the *him-17* gene (defined by multiple mutant alleles; K. REDDY, J. HODGKIN and A. M. VILLENEUVE, unpublished data) and are currently investigating its function in more detail. For F59A1.7, affected worms exhibiting a high frequency of achiasmate chromosomes at diakinesis also had abnormally large nuclei at the pachytene stage, earlier in meiotic prophase. For C05D2.5, a medium Him phenotype in affected F_1's was accompanied by defects in meiotic progression (see below) and achiasmate chromosomes at diakinesis in a subset of animals; a Him phenotype had been seen previously for C05D2.5 when gene function was inhibited by transgene-mediated cosuppression or by RNAi (L. KUERVERS and D. BAILLIE, personal communication).

Meiotic defects were also observed following RNAi for M04F3.1, which encodes the *C. elegans* ortholog of RPA2/Rfa2, a subunit of the eukaryotic single-stranded DNA binding protein shown in other systems to function in DNA replication, repair, and recombination (FLORES-ROZAS and KOLODNER 2000; KOWALCZYKOWSKI 2000). Some F_1's were sterile and exhibited phenotypes characteristic of defects in postembryonic cell proliferation (see below), consistent with a role in DNA replication or repair. In other F_1's the defects were restricted to the mature germline: in addition to the presence of achiasmate chromosomes at diakinesis, nuclei in early meiotic prophase appeared abnormal, suggesting the possibility that homolog alignment might be defective. Whether this cytological phenotype was a reflection of a meiotic role *per se* or was a consequence of defects during prior mitotic cell cycles is not clear. There also appeared to be reduced numbers of nuclei representing both mitotic and meiotic stages.

For all but one of the genes to which the "meiotic" descriptor was applied, defects were evident at one or more stage(s) of meiotic prophase in at least a subset of affected animals. The exception was *klp-16*, which encodes the *C. elegans* ortholog of kinesin-related motor proteins *Drosophila melanogaster* Ncd and *Saccharomyces cerevisiae* Kar3 (ENDOW *et al.* 1990; MCDONALD and GOLDSTEIN 1990; MELUH and ROSE 1990). While meiotic prophase appeared to be normal, a Him phenotype

TABLE 1

RNAi phenotypes

Gene	Functional annotation[a]	Descriptor	Phenotypes from other screens
Strong germline class			
C24G6.1 *syp-1*	uk, coiled-coil protein	Meiotic (univalents at diakinesis; persistent polarized organization)	el[b]
F26D2.2 *syp1*	uk, coiled-coil protein	Meiotic (univalents at diakinesis; persistent polarized organization)	el[b]
F39H2.4 *syp-3*	uk, coiled-coil protein	Meiotic (univalents at diakinesis; persistent polarized organization)	el;[b] none[c]
F59A1.7	F-box containing protein; wsf, wp	Meiotic (univalents at diakinesis; pachytene nuclei enlarged)	
T09E8.2 *him-17*	uk, nc	Meiotic (occasional univalents; X preferentially affected)	none[b]
T04H1.4 *rad-50*	Rad50 subunit of DSB repair complex	Meiotic (univalents at diakinesis)	
C41G7.2 *klp-16*	Kinesin-like, Ncd/Kar3 subfamily; wp	Meiotic (postprophase segregation defect)	el,[c,d] Him,[c] Mei[c]
K12H4.8 *dcr-1*	Helicase and RNAse involved in RNAi	Meiotic[e] + sterile	None[f]
M04F3.1	RPA2/Rfa2 (replication protein A2); wp	Meiotic + mitotic + sterile	None[c]
C30G12.6a or b	uk, nc	Weak meiotic	None[b]
C05D2.5	uk, nc, AT hook motif	Meiotic progression + meiotic	Ste (meiotic progression),[g] none[f]
ZC317.7	uk, nc; wp	Meiotic progression + sterile	
ZK1127.4	Weak similarity to CDK2 regulator BCCIP	Meiotic progression + sterile	
C48E7.3	bZIP transcription factor domain	Abnormal gonad structure + meiotic progression	Lva,[b] Lvl,[b] Gro[c]
C18G1.5 (H1.4)	Histone H1 variant; wp	Abnormal gonad structure + meiotic progression	
C32F10.5 *hmg-3*	HMG box protein; wp	Abnormal gonad structure + meiotic progression + embryonic lethal	
B0414.3	Histone H1 variant; wp	Abnormal gonad structure + meiotic progression + low frequency meiotic	None[c]
C16A3.8	ScRlr (transcription coupled recombination)	Abnormal gonad structure + mitotic + meiotic	el,[b] Bmd,[b] Stp,[b] Dpy,[f] Unc,[f] Rol,[f] Abv[f]
B0464.2	Ctr9/Tpr1; TPR-containing protein	Abnormal gonad structure + embryonic lethal + variable	el[b,f]
F43G9.10	Associated microfibril protein 1 (AMF)	Mitotic	el,[b,c] Gro,[c] Led,[c] Lva[b]
F56A3.4 *spd-5*	uk, nc, coiled-coil protein	Mitotic	el,[b] Spd[b]
T10B5.6	uk, nc	Mitotic	
W07E6.1	NOL1/NOP2 nucleolar protein family	Mitotic + larval arrest	
C25A1.9	uk, EF hand motif	Mitotic + meiotic + variable	None[c]
C27A2.3	uk, nc	Mitotic + embryonic lethal	
K08E3.6 *cyk-4*	GTPase-activator for Rho-like GTPases	Mitotic + embryonic lethal	el,[b,f] bmd[b]
R12B2.4 *him-10*	Nuf2 related kinetochore component	Mitotic + embryonic lethal	el[f]
T23H2.1 *npp-12*	nup210 nuclear pore complex protein; wp	Mitotic + embryonic lethal	el,[b] Lva,[b] none[c]
W02D9.1 *pri-2*	DNA polymerase α primase subunit C	Mitotic + embryonic lethal	el,[b,c] Nmo,[c] Pvl[c]
T26A8.4	CCCH-type Zn finger domains	Mitotic + embryonic lethal + sterile	
W01B6.9	Tid3/Ndc10 kinetochore complex	Mitotic + embryonic lethal + sterile	

(*continued*)

TABLE 1

(Continued)

Gene	Functional annotation[a]	Descriptor	Phenotypes from other screens
F56A3.1	uk, nc	Mitotic + embryonic lethal + sterile	None[b,c]
F10C2.4	DNA polymerase δ1	Mitotic + embryonic lethal + larval lethal	
R06C7.8	Bub1 spindle checkpoint kinase	Mitotic + embryonic lethal + larval lethal	el,[c] Lvl,[c] Mul,[c] Pvl,[c] Rup[c]
F32H2.3	uk, nc	Mitotic + embryonic lethal + sterile + meiotic progression	el,[c] Spd[c]
F57C2.1	uk, BTB/POZ domain; wp	Mitotic + embryonic lethal + sterile + variable	
F26H11.1	uk, nc	Mitotic + embryonic lethal + sterile + rudimentary gonad	
C54G10.2 *rfc-1*	Replication factor C1	Mitotic + rudimentary gonad + embryonic lethal + sterile	
F23F12.2	uk, nc	Mitotic + meiotic progression + embryonic lethal + sterile	Ste[f]
K07F5.13 *npp-1*	Nucleoporin	Mitotic + sterile + rudimentary gonad + embryonic lethal	
T22D1.10	RuvB2-like RNA/DNA helicase+B50	Rudimentary gonad + sterile	
ZK1248.10	Rab-like GTPase activator and PH domains	Rudimentary gonad + sterile	
ZK742.2	uk, c	Rudimentary gonad + sterile	
T12F5.2	uk, nc; wp	Rudimentary gonad + sterile	None[c]
F30A10.10	Ubiquitin C-terminal hydrolase domain	Tumorous germline + sterile	None[c]
T23B12.7 *dnj-22*	DnaJ domain	Tumorous germline + sterile	
T07F8.3a	Weak similarity to Dm BicC	Tumorous germline + sterile + embryonic lethal	el[b]
F35G12.10 *asb-1*	ATP synthase B chain; wp	Embryonic lethal + sterile + meiotic progression + variable	el,[f] Ste,[g] pachytene arrest[g]
F58A4.8 *tbg-1*	γ-Tubulin	Sterile + embryonic lethal	el[f]
B0025.2	Subunit of COP9 (signalosome)	Variable	el,[c] Gro[c]
C31H1.8	uk, nc	Feminization of germ line (Fog)	
Low-frequency class			
F20C5.1	Poly ADP ribose glycohydrolase domain; wp	Low-frequency meiotic	None[b]
C17G10.1	20G and Fe(II)-dependent oxygenase family	Low-frequency meiotic	
F42H10.7	uk, c	Low-frequency meiotic	None[b,f]
F25G6.9	uk, nc	Low-frequency meiotic	
F11A10.1	AAA family ATPase	Low-frequency meiotic	
C05C8.6	BTB/POZ domain, kelch motifs	Low-frequency meiotic	None[b]
F13D12.5	uk, nc; wp	Low-frequency meiotic	
F26B1.2a	Poly (rc) RNA binding protein, c	Low-frequency meiotic	None[c]
C08B11.6	Actin family member	Low-frequency meiotic	
C26E6.9a *set-2*	3 SET domains and 2 RNA recognition motifs	Low-frequency meiotic	None[b,f]
F52H3.4	uk, nc; wp	Low-frequency meiotic	None[b]
Y37D8A.13 *adm-1*	A disintegrin and metalloprotease	Low-frequency meiotic	None[f]

(*continued*)

TABLE 1

(Continued)

Gene	Functional annotation[a]	Descriptor	Phenotypes from other screens
C30A5.3	uk, c	Low-frequency meiotic	None[h,f]
C33H5.15	uk, nc	Low-frequency meiotic	
ZK328.4	uk, c	Low-frequency meiotic	None[f]
F12F6.5	Rho GAP domain	Low-frequency meiotic	
Y53C12B.1	Ylr222C ortholog, WD repeats	Low-frequency meiotic	
Y43E12A.3	BTB/POZ domain; wp	Low-frequency meiotic	
W02D9.3	HMG-box protein	Low-frequency sterile	None[c]
ZK945.3	Pumilio-repeat family protein	Low-frequency sterile	
T07D4.3 *rha-1*	DEAD-box RNA helicase family	Low-frequency sterile	None[b]
F32A5.1	Myb-like DNA binding domain	Low-frequency sterile	el,[b] Stp[b] (escapers: Ste)
D2096.11	Actin binding domain and GAS2 domain	Low-frequency sterile	None[b]
C24F3.2	Dual specificity phosphatase catalytic domain, c	Low-frequency sterile	
F01F1.11	uk, nc; wp	Low-frequency sterile	None[f]
F52E1.10	Vacuolar H(+)-ATPase subunit	Low-frequency sterile	
ZK418.8	2 KH domains	Low-frequency sterile + rudimentary gonad	None[h,f]
C49C3.7	uk, nc; wp	Low-frequency embryonic lethal + meiotic progression	
C56C10.1	Sec1 family member	Low-frequency Fog	None[b]
Nongermline class			
T19B10.6	uk, c	Larval arrest	Ste (P_0s),[b] Stp (escapers: F_1 Ste)[b]
F55F8.5	G-protein β WD-40 repeat family	Larval arrest	Lva[c]
C16C10.2	uk, c	Larval arrest	None[f]
F28D1.1	ScYER2p like; G-protein β WD-40 repeats, c	Larval arrest	
K12H4.3	Ortholog of ScBrx1 nucleolar protein	Larval arrest	Lva[f]
T04A8.6	RNP-1 motif	Larval arrest	Lva[f]
C37H5.5	Noc3 (nucleolar complex associated protein)	Larval arrest	
K06A5.4	uk, nc	Larval arrest	el,[c] Nmo,[c] dMBS[c]
F54D5.14	Rhc18/Rad18 DNA repair homolog; wp	Embryonic lethal	None[b]
F33E11.2	uk, nc	Embryonic lethal	None[b]
M7.2 *klc-1*	Kinesin light chain; wp	Embryonic lethal	
T01H3.4	uk, nc	Embryonic lethal	
F55A3.3	FACTP140/Spt16-like transcription factor	Embryonic lethal	el,[c,d] Led[c]
K02F6.7	uk, nc; wp	Embryonic lethal + mitotic-emb[h]	
Y23H5A.3	uk, nc	Embryonic lethal + mitotic-emb	el,[c] Led[c]
C38D4.3	AT-hook motif	Embryonic lethal + mitotic-emb	el[f]
F28C6.2	Transcription factor AP-2 like	Embryonic lethal + larval arrest	
C45G3.1	2 IQ calmodulin-binding domains	Embryonic lethal + larval arrest	el,[c] Mei,[c] Unc[c]
ZC434.4	uk, nc	Embryonic lethal + larval arrest + mitotic-emb	
C27B7.4	RAD54/SNF2 family member	Backwards Unc	

(*continued*)

TABLE 1

(Continued)

Gene	Functional annotation[a]	Descriptor	Phenotypes from other screens
Embryonic lethal + low-frequency germline class			
D1054.14	uk, c	Embryonic lethal + low-frequency meiotic	
F49E11.1	DYRK family dual-specificity protein kinase	Embryonic lethal + low-frequency meiotic	
C33H5.4 *klp-10*	Kinesin-like protein; wp	Embryonic lethal + low-frequency meiotic	
R08D7.1	uk, c	Embryonic lethal + low-frequency meiotic	el[h,f]
F33H1.3	uk, c	Embryonic lethal + low-frequency meiotic	el (escapers: Lvl)[b]
F46A9.4 *skr-2*	Skp1 family; wp	Embryonic lethal + low-frequency meiotic	el,[c] Gro,[c] Pvl,[c] Spn,[c] Unc[c]
F08H9.1 *coh-3*	Cohesin related	Embryonic lethal + low-frequency meiotic	
C27A2.1	SMC protein family	Embryonic lethal + low-frequency mitotic	
Y75B12A.1		Embryonic lethal + low-frequency sterile	

The 109 genes for which an RNAi phenotype was observed are listed under four major classes. Descriptors were applied as indicated in the text. In addition to the sequence names, official three-letter gene designations were included where available. uk, unknown protein/function; c, conserved across species; nc, nonconserved; wsf, protein is member of a *w*orm *s*pecific protein *f*amily; wp, protein has a *C. elegans* paralog; Abv, aberrant vulva; Bmd, body morphology defect; dMBS, decreased maternal brood size; Dpy, dumpy; el, embryonic lethal; Gro, slow growth; Led, late embryo defect; Lva, larval arrest; Lvl, larval lethal; Mei, defective meiosis; Mul, multiple nuclei in early embryo; Nmo, nuclear morphology alteration in early embryo; Pvl, protruding vulva; Rol, roller; Rup, exploded; Spd, Spindle, abnormal embryonic; Spn, abnormal spindle orientation; Ste, sterile; Stp, sterile progeny; Unc, uncoordinated.

Screen(s) in which the indicated phenotype was observed:

[a] Includes predicted orthologies, protein families, or domains based on information from Wormbase and WormPD.

[b] Screen(s) in which the indicated phenotype was observed: Maeda *et al.* (2001).

[c] Screen(s) in which the indicated phenotype was observed: Fraser *et al.* (2000).

[d] Screen(s) in which the indicated phenotype was observed: Piano *et al.* (2000).

[e] The meiotic phenotype was not reproduced upon retesting.

[f] Screen(s) in which the indicated phenotype was observed: Gonczy *et al.* (2000).

[g] Screen(s) in which the indicated phenotype was observed: Hanazawa *et al.* (2001).

[h] mitotic-emb: DAPI-stained embryos showed evidence of abnormal embryonic cell divisions, such as visible chromatin bridges between nuclei.

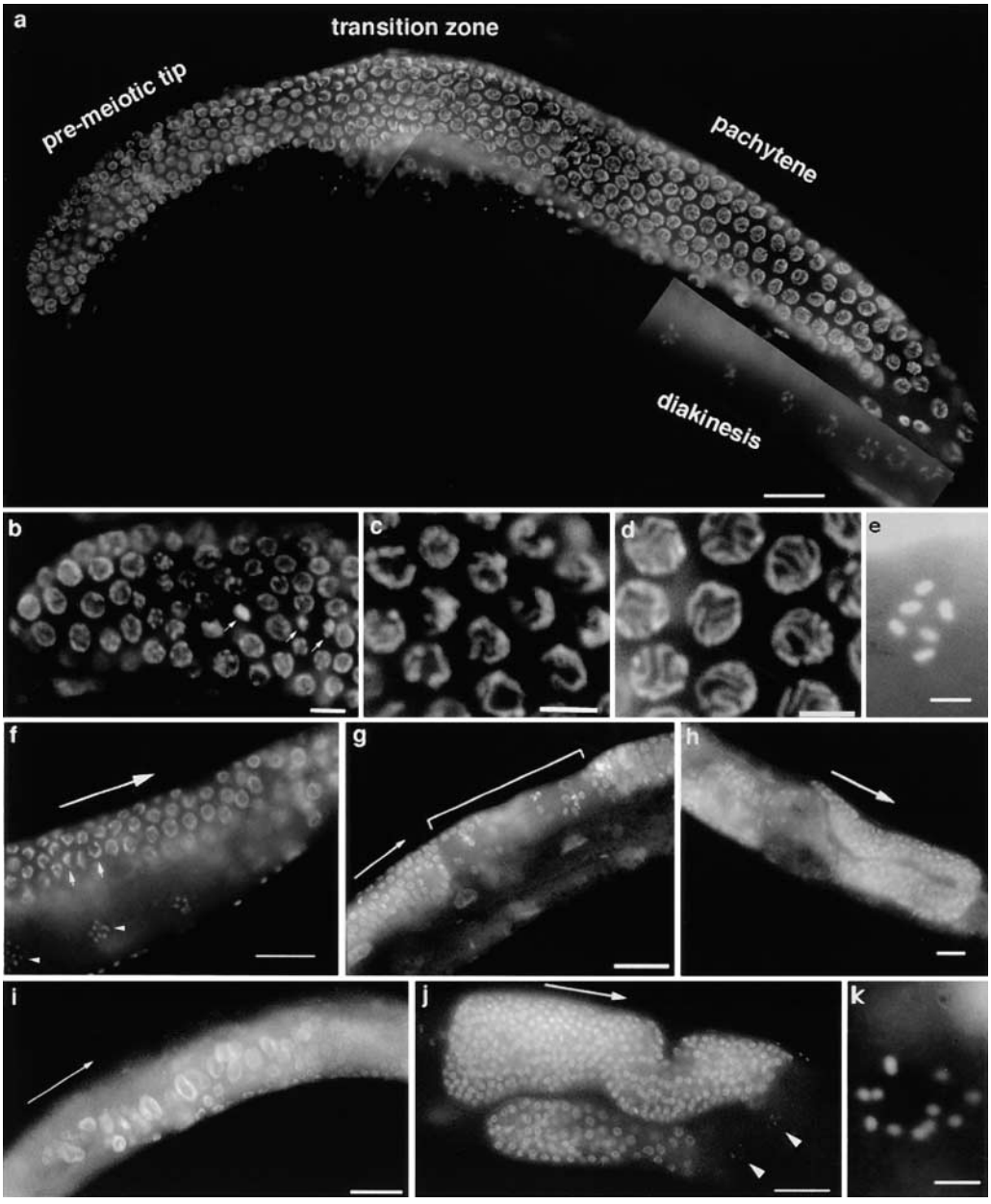

FIGURE 2.—Organization and appearance of DAPI-stained chromosomes in germline nuclei. All images show portions of germlines in whole, undissected worms fixed with Carnoy's fixative and stained with DAPI, viewed with conventional fluorescence microscopy. (a–e) Images from wild-type germlines, indicating features of germline and nuclear organization that were examined in the screen. (a) Composite image of a whole gonad arm shows organization along the distal/proximal axis of the germline. The uterus-proximal region of the germline extends along the ventral side of the animal, and then the germline reflexes and the distal portion extends back along the dorsal side. Nuclei are organized in a temporal/spatial gradient, ranging from nuclei undergoing mitotic proliferation in the most distal region (premeiotic tip) to nuclei in early meiotic prophase (transition zone) to nuclei at progressively later stages of meiotic prophase (pachytene prior to the bend in the gonad arm, diakinesis after the bend). (b–e) Higher-magnification images from each of the above regions. (b) In the premeiotic region, with the exception of mitotic figures (indicated by small white arrows), nuclei tend to be homogeneous in size, with chromatin dispersed to impart a round appearance to the DAPI signal. (c) In the transition zone, as nuclei enter meiotic prophase and chromosomes begin to pair, chromosomes cluster to one side of the nucleus, imparting an asymmetric crescent-shaped appearance to the DAPI signal. (d) Pachytene nuclei. Chromosomes have redispersed about the nuclear periphery, and DAPI signals are visible as distinct strands corresponding to the fully paired and aligned homologous chromosomes. The synaptonemal complex is present at this stage, and meiotic recombination is completed within this context. (e) A single diakinesis-stage nucleus. Upon exit from the pachytene stage, the SC disassembles and the chromosomes continue to condense as the nuclear volume increases; homologs lose their side-by-side associations but remain attached by chiasmata, temporary connections that form as a consequence of crossing over. Six discrete DAPI-stained bodies, each corresponding to a pair of homologous chromosomes attached by a chiasma, are visible at this stage. (f–j) Examples of phenotypes elicited by RNAi. Long white arrows indicate orientation from distal toward proximal regions of the gonads. (f) Meiotic defects elicited by RNAi for C24G6.1 (*syp-2*). The phenotype included persistence of a transition zone-like organization of chromosomes in nuclei that should be at the pachytene stage (small white arrows) and univalent chromosomes in nuclei at the diakinesis stage (white arrowheads). (g) Defect in meiotic progression elicited by RNAi for C05D2.5. In this example, a normal-appearing transition zone is followed by a large gap (indicated by brackets) with a greatly reduced density of nuclei; the gap is followed by some nuclei at the pachytene and diakinesis stages. Whether such gaps resulted from altered kinetics of progression or from degeneration of a substantial fraction of nuclei was not investigated further. (h) Abnormal gonad structure/meiotic progression elicited by RNAi for C32F10.5. This gonad is misshapen, spacing between nuclei is abnormal, and no nuclei at the diakinesis stage are present. (i) Mitotic defect elicited by RNAi for R12B2.4 (*him-10*). Many nuclei in the premeiotic tip are enlarged, and nuclei vary widely in size and DAPI-staining intensity. (j) Example of the variable sterile phenotype elicited by RNAi for F35G12.10. This enlarged gonad contains densely packed nuclei in the distal portion, likely reflecting overproliferation; nuclei with apparent transition zone-like organization are infrequent, and several compact DAPI-bright signals that may correspond to mitotic figures are observed in ectopic positions distant from the distal tip. There is an abrupt transition to two diakinesis-stage nuclei (arrowheads), followed by a proximal region of smaller nuclei that may represent a zone of proximal proliferation and/or reversion to earlier prophase-like stages. (k) Meiotic defect elicited by RNAi for *syp-2*. Twelve univalent chromosomes are visible in this diakinesis-stage nucleus, indicating an absence of chiasmata. Bars (a and f–j), 20 μm; (b–e and k), 5 μm.

apparently resulted from a postprophase defect in chromosome segregation. This result is consistent with the well-characterized role for Ncd in the Drosophila female meiotic spindle (Matthies *et al.* 1996) and our previous observation that inhibition of *klp-16* function by transgene-mediated cosuppression resulted in defective assembly of the oocyte meiotic spindle (Dernburg *et al.* 2000). Thus *klp-16* served as a positive control indicating that genes of this class could be identified.

We also detected a meiotic phenotype for K12H4.8, recently named *dcr-1* (Grishok *et al.* 2001; Ketting *et al.* 2001; Knight and Bass 2001). K12H4.8/*dcr-1* encodes a dsRNAse that has been implicated in the processing of input dsRNAs into small interfering RNAs (siRNAs) that direct degradation of cognate mRNAs during RNAi. *dcr-1* is also required for processing of small temporal RNAs that regulate developmental timing; RNAi of *dcr-1* caused reiterations of larval cell fates in the epidermis that resulted in bursting of animals at the vulva following the larval/adult molt. Consistent with the previous reports, we also found that most F_1 animals burst at the vulva, but in addition, cytological analysis of both P_0's and the few surviving F_1 animals (2/55) revealed an extended transition zone and 6–12 DAPI-stained bodies at diakinesis. The intriguing possibility that small regulatory RNAs might be involved in the meiotic program prompted us to investigate the meiotic phenotype more extensively, but several attempts to reproduce the meiotic phenotype failed despite successfully generating the bursting vulva phenotype. The fact that *dcr-1* is apparently involved in the mechanism of RNAi has significantly complicated analyses of its biological roles using RNAi, since the very mechanism being used to interfere with gene function is itself impaired by the treatment. It seems likely that this complicating factor may lead to variability in phenotypic outcome following RNAi treatment.

Meiotic progression: For several genes the spatial/temporal gradient of meiotic prophase was altered by RNAi, as evidenced either by a pachytene arrest or by the presence of reduced numbers of nuclei representing particular substages in meiotic prophase as if meiotic progression were accelerated (see Figure 2g). While affected P_0's laid low-to-medium levels of dead eggs, F_1's were sterile. The pachytene arrest and accompanying sterility phenotype are similar to phenotypes reported for mutations in genes encoding components of the mitogen-activated protein kinase (MAPK) signaling pathway required for exit from pachytene (Church *et al.* 1995). Meanwhile, an advancement of meiotic progression has been previously seen following RNAi for a gene encoding caveolin-1, a protein associated with cholesterol-enriched membrane microdomains that appears to interact with Ras/MAPK signaling in the germline (Scheel *et al.* 1999). Further analysis of these genes will be necessary to determine whether any do play roles in Ras/MAPK signaling events that govern meiotic progression.

Abnormal gonad structure: For several genes, RNAi led to a variety of abnormalities in the shape or size of the gonads in the affected individuals (Figure 2h). In addition, we observed intermixing of nuclei at different stages of meiotic prophase rather than a clear spatial/temporal gradient of meiotic nuclei. In such cases it was not possible to discern from our analysis whether the abnormality in gonad structure was the cause of the aberrant organization of meiotic stages or whether these two phenotypes represent separate effects of the RNAi. Further, in some cases a subset of nuclei appeared to have degenerated or fragmented. Such phenotypes were sometimes associated with embryonic lethality or sterility in the F_1.

Mitotic: This descriptor was applied to a broad spectrum of phenotypes in which the appearance of nuclei was abnormal in the premeiotic region of the germline. (This region includes both mitotically proliferating nuclei as well as nuclei in the G_1 and S phases immediately preceding meiotic prophase.) For many genes in this class, we observed variation in the size, shape, and brightness of the DAPI signals in germline nuclei of affected adults, likely indicating defects in chromosome segregation during mitotic growth (Figure 2i). Examples of genes in this subclass include R12B2.4, recently reported by Howe *et al.* (2001) to correspond to *him-10*, which encodes a conserved protein that plays a crucial role in kinetochore structure and function; R06C7.8, which encodes the *C. elegans* ortholog of spindle assembly checkpoint protein Bub1 (Roberts *et al.* 1994), recently shown to localize to nematode kinetochores (Oegema *et al.* 2001); and K08E3.6/*cyk-4*, previously shown to be required for cytokinesis during embryonic cell divisions (Jantsch-Plunger *et al.* 2000). Further, this subclass identifies the W01B6.9 gene product (which has weak similarity to the Ndc80/Tid3 component of the yeast kinetochore complex; Wigge *et al.* 1998) as a likely kinetochore component. For other genes in this class, nuclei in the premeiotic region were substantially enlarged and sometimes misshapen but more uniform in size. The appearance of nuclei in these cases was similar to that seen either when the DNA damage checkpoint is triggered by exposure to ionizing radiation (Gartner *et al.* 2000; MacQueen and Villeneuve 2001) or when proliferation is arrested following exposure to hydroxyurea (an inhibitor of DNA replication) (MacQueen and Villeneuve 2001). Consistent with the idea that this cytological phenotype reflects defects in DNA metabolism, genes in this subclass included F10C2.4, which encodes the catalytic subunit of DNA polymerase δ (involved in both replication and repair), and C54G10.2/*rfc-1*, which encodes the large subunit of replication factor C (the clamp-loading protein; O'Donnell *et al.* 2001).

For most genes in the mitotic class, the majority of F_1's produced by treated P_0's died as embryos, arrested as larvae, or became thin, sterile uncoordinated adults

(a syndrome that reflects defects in postembryonic cell divisions; Albertson *et al.* 1978). The germline phenotypes described above were seen as late-onset phenotypes in the injected P_0's themselves and/or in surviving F_1's produced in a time window that allowed them to escape early effects of RNAi. Clearly, our comprehensive multigenerational screening strategy has allowed us to detect germline roles for genes that function in essential cellular processes.

Rudimentary gonads: For some genes, the treated P_0 animals produced sterile F_1 progeny that had very few or no germline nuclei and gonad arms that were very small or missing. In most cases, this likely reflected a defect in early proliferation of the germline, as seen in mutants such as *glp-4* (Beanan and Strome 1992). It is possible that, at least in some cases, this rudimentary gonad phenotype reflected problems in cell division or DNA metabolism. However, since either no nuclei were observed or those few that were present did not have an overtly abnormal appearance, the cytological criteria necessary for applying the "mitotic" descriptor were not satisfied. It is also possible that this phenotype arose from a defect in the specification of gonadal tissues (*e.g.*, like that of *gon-4*; Friedman *et al.* 2000) or from a defect in gonad morphogenesis (*e.g.*, like that of *gon-1*; Blelloch and Kimble 1999).

Tumorous germline: RNAi for some genes generated a tumorous germline phenotype. For these genes, affected F_1's were usually sterile, and occasional escapers that presumably avoided the full effect of RNAi laid very small broods. Cytologically, the gonad arms of most affected F_1's had normally shaped, mitotically dividing nuclei throughout their entire lengths, with no evidence of entry into meiotic prophase. In a few cases some apparent meiotic prophase nuclei were also observed, but no diakinesis nuclei were evident. These tumorous germline phenotypes are reminiscent of those observed in loss-of-function *gld-1* and *gld-1*; *gld-2* double mutants and in *glp-1* gain-of-function mutants, which are defective in regulating exit from the mitotic cell cycle and entrance into meiosis (Francis *et al.* 1995; Berry *et al.* 1997; Kadyk and Kimble 1998).

Low-frequency phenotypes

For a significant fraction of genes tested in our screen (19%), we observed F_1 animals exhibiting robust germline-defective phenotypes only at very low frequencies (1–7 of 60 F_1's scored were affected). In the rare affected animals, however, the phenotypes observed were quite strong. For example, for most of the genes in the "low-frequency meiotic" subclass, a high frequency of achiasmate chromosomes was seen in all oocytes of the affected animals.

For nine genes for which low-frequency germline phenotypes were detected, essentially all of the F_1's produced during the later broods of the injected P_0's died as embryos, indicating that these genes provide essential functions. In these cases, it seems likely that most of the F_1's that survived to adulthood were in fact escapers from the effects of RNAi. In such cases, detection of a small subset of the viable F_1's that have strong germline-defective phenotypes may reflect a small "window of opportunity" in which reduction of gene activity by RNAi was not sufficient (either in time or in amount) to preclude viability but was sufficient to interfere with germ cell function. This category includes several genes encoding proteins with orthologs or paralogs that have been implicated in chromosome segregation or cell-cycle progression: F08H9.1/*coh-3*, which encodes a protein related to the Scc1 subunit of cohesins (Cohen-Fix 2001; Pasierbek *et al.* 2001); C33H5.4/*klp-10*, which encodes a kinesin-like protein; C27A2.1, which encodes a putative structural maintenance of chromosomes (SMC) protein; and F46A9.4/*skr-2*, which encodes a worm ortholog of Skp1, a ubiquitin-protein ligase that regulates cell-cycle transitions (Bai *et al.* 1996).

For 29 genes, a "low-frequency" phenotype was the only phenotype observed following RNAi. For 28 of these genes, the defects observed were in the germlines of affected F_1's, while for one (C49C3.7), the phenotype was embryonic lethality in the progeny of the rare affected F_1's. For the largest single subclass (17 genes), affected F_1's exhibited a high frequency of achiasmate chromosomes in diakinesis-stage oocytes, a phenotype that is normally a reliable diagnostic of defects in meiotic prophase. It was unexpected that we would see a very strong germline phenotype in only a few F_1's for such a large fraction of the genes tested. It is possible that these low-frequency phenotypes reflect a nonspecific effect of the RNAi procedure or a baseline spontaneous occurrence of these phenotypes detected because of the large numbers of animals analyzed. If either of these were the case, then it would be appropriate to group the "low-frequency-only" genes into a single class together with the genes for which no defect was observed following RNAi. While the basis for these low-frequency phenotypes remains unclear, several considerations argue for a specific effect of RNAi. First, we found that the low-frequency-only and "no defect" classes differed significantly with respect to the fraction of genes in the class having probable orthologs in other species (see below); this finding is inconsistent with the idea that these two groups constitute a single class. Second, a further prediction of a "single-class" model (in which the incidence of the phenotype reflects random occurrence of a low-frequency event) is that the proportions of genes for which zero, one, two, or more F_1's exhibit a particular low-frequency phenotype should follow the Poisson distribution. This expectation is not borne out by the existing data set. We examined the distribution of worms exhibiting the "achiasmate diakinesis" phenotype among genes in the consolidated low-frequency-only or no defect class and found that it dif-

fered significantly from the Poisson distribution ($P < 0.04$).

Thus we infer that for at least a subset of genes falling into the low-frequency-only category, the phenotype observed was not merely random or nonspecific, but rather provided a clue about the biological role for the gene tested. Some of these genes may be refractory to RNAi and, for some genes, a more reliable phenotype might be obtained by delivering dsRNA by another means or at a different time or by targeting a different part of the gene. Further, although RNAi may not prove to be an effective approach to investigate their functions, the low-frequency class of genes may serve as useful candidates to facilitate molecular identification of genes identified by mutational analysis.

While we have just argued that at least a subset of genes in the low-frequency class probably have legitimate functions in meiosis, we do not exclude the possibility that some of these low-frequency events could have been a nonspecific consequence of the RNAi treatment. It has been previously shown that loss of function of *ego-1*, which encodes an RNA-dependent RNA polymerase involved in the mechanism of RNAi in the germline, also causes pleiotropic effects including defective oogenesis, altered meiotic progression, and some achiasmate chromosomes (Smardon *et al.* 2000). Although the basis of these pleiotropies is not understood, it seems plausible that overloading the RNAi machinery with input dsRNA could mimic loss of function of one or more RNAi machinery components. It should be noted, however, that similar pleiotropies are not observed in other classes of RNAi-defective mutants.

Nongermline class

Of the genes tested, 10% showed a "nongermline" phenotype such as larval arrest, embryonic lethality, or unco-ordinated movement (Unc) without any evident germline-associated phenotype. For such genes it is possible there is no role for the gene product in the germline *per se* and that the germline expression of the gene reflects transcription solely for the purpose of deposition of the corresponding mRNA or protein in the embryo. Alternatively, it is possible that some of these gene products also function in the germline, but that the early arrest or lethality elicited by RNAi precluded our ability to uncover such roles in F_1 animals. In some cases the embryonic lethal descriptor was accompanied by the secondary descriptor "mitotic-emb"; this designation indicated that DAPI staining revealed evidence of abnormal cell or nuclear divisions in the embryos, such as nuclei with abnormal DNA content and/or chromatin bridges visible between nuclei.

Sequence conservation and detection of RNAi phenotypes: Genetic redundancy is regularly cited as a possible explanation for failure to detect a phenotype following targeting of a gene. The simplest form of redundancy is the presence in the genome of a second gene whose sequence is closely related to that of the gene in question. We assessed whether this type of redundancy might have contributed significantly to cases where no defect was detected or where phenotypes were detected only at low frequency. Specifically, we asked whether genes with close paralogs were overrepresented among genes in the no defect or low-frequency-only classes compared with genes for which RNAi elicited strong phenotypes. For this analysis we considered a gene to have a close worm paralog when one or more additional *C. elegans* genes encoded a protein with an overall level of extended similarity greater than or equal to that of the closest homologs from other species. Using these criteria, we found that genes with close paralogs accounted for 27% of no defect genes, 21% of low-frequency-only genes, 22% of "strong germline" genes, and 23% of "all strong phenotype" genes; there were no significant differences between any of these groups regarding the proportion of genes with and without close paralogs (pairwise comparisons were conducted using Fisher's exact test).

We then took into account whether paralog genes were likely to be subject to co-RNAi in our experiments. For this analysis a paralog gene was considered "likely" to be subject to co-RNAi if it contained multiple stretches considerably greater than 23 nucleotides (nt) in length with 100% nucleotide identity with the input dsRNA; co-RNAi was considered "plausible" (but uncertain) for paralog genes that contained only one to three stretches of identity between 23 and 35 nt in length or for one short gene that had multiple close paralogs with significant stretches of identity. (These criteria were based on experimental data from M. Montgomery, personal communication). We discovered that genes for which co-RNAi was likely or plausible were significantly overrepresented among the paralogs of strong germline genes and all strong phenotype genes compared with those of no defect genes ($P = 0.013$ for the strong germline *vs.* no defect comparison and $P = 0.007$ for the all strong phenotype *vs.* no defect comparison using chi-square test for independence). When we then examined the whole gene list with regard to whether genes had a *C. elegans* paralog that would not be subject to co-RNAi, we found that such genes were significantly overrepresented among the no defect genes (23%) compared with the strong germline genes (8%) and all strong phenotype genes (9%) ($P = 0.033$ for the strong germline *vs.* no defect comparison and $P = 0.018$ for the all strong phenotype *vs.* no defect comparison using Fisher's exact test). These analyses suggest that genetic redundancy probably does account for a subset of the cases in which no phenotype was elicited by RNAi.

We next considered whether there might be differences among the classes with respect to the fraction of genes with probable orthologs in other species. We

found that RNAi was significantly more likely to elicit a detectable phenotype for genes with apparent functional conservation across species than for genes lacking probable orthologs. The proportion of genes with probable orthologs in one or more of the species referenced in the Proteome database (WormPD Bioknowledge Library from Incyte Genomics; Costanzo *et al.* 2001; *S. cerevisiae, Schizosaccharomyces pombe, D. melanogaster, Mus musculus, Homo sapiens*) did not differ significantly in pairwise comparisons between strong germline genes (50%), a consolidated class containing both nongermline and "embryonic lethal + low-frequency" genes (54%), all strong phenotype genes (51%), and low-frequency-only genes (61%). In contrast, genes with probable orthologs in other species were significantly underrepresented in the no defect class (33%) compared with each of these classes; *P* values for pairwise comparisons conducted using Fisher's exact test were 0.039, 0.044, 0.014, and 0.009, respectively. Our observation that required roles were detected more frequently for conserved gene products than for nonconserved products is consistent with the findings of other RNAi screens, in which conserved genes were overrepresented among genes for which an RNAi phenotype was detected (Fraser *et al.* 2000; Gönczy *et al.* 2000; Piano *et al.* 2000).

Gene-expression profiles and RNAi phenotypes: The genes tested in our screen were chosen from a list of genes exhibiting germline-enriched expression in the microarray analysis of Reinke *et al.* (2000). Although all of these genes exhibited reproducibly higher levels of expression in normal worms compared with worms that lacked a germline, there was nevertheless considerable variability among the tested genes with respect to (1) the actual level of expression, (2) the degree of induction, and (3) the ratio of expression in worms making only oocytes compared with worms making only sperm. Thus we wondered whether any features of the expression profiles of the genes tested in our screen might be useful predictors of the likelihood that RNAi would elicit a strong germline phenotype, a low-frequency germline phenotype, a nongermline phenotype, or no defect.

To ask whether there might be any correlation between the expression level of a gene and the phenotypic class to which it belonged, we compared the distributions of mean gene expression (MGE) values between the different classes. The MGE value for a given gene is a normalized representation of its expression level. For each gene in a given experiment, we calculated the ratio of the raw expression level for the individual gene to the average per gene-expression level for all genes on the microarray; MGE values for each gene were derived from the published data set (Reinke *et al.* 2000), representing an average of the ratios from eight independent RNA samples (four from wild-type L4 hermaphrodites and four from wild-type adult hermaphrodites). We did not find any significant differences between any of the classes using the two-tailed Mann-Whitney rank-sum test. Thus within the set of expressed genes included in our screen, the level of expression of a gene does not serve as a useful predictor of the phenotypic class. It is important to keep in mind that to be included in the screen at all, a gene had to be expressed in wild-type worms at a level that could be reproducibly detected; genes expressed at very low levels would have been systematically excluded from consideration.

When a nongermline phenotype (particularly embryonic lethality) was the major or only phenotype detected for a gene, it might be the case that transcripts and/or protein products expressed in the germline were there primarily for deposition in the oocyte to support development of the embryo. It seemed plausible that the ratio of expression in worms producing only oocytes to worms producing only sperm might tend to be higher for such genes compared with genes for which strong phenotypes were observed in the germline itself, but we failed to find any statistical support for this possibility. Comparison of the distributions of oocyte/sperm ratios between the strong germline and nongermline classes did not reveal any significant difference between these classes (Mann-Whitney test).

Finally, we asked whether the level of induction of a gene's expression in the germline might be a useful predictor of a gene's phenotypic class. We conducted pairwise comparisons of the distributions of "fold-induction" values between the strong germline class and the low-frequency, nongermline, and no defect classes, but once again found no significant differences. Thus for the set of genes tested in our screen, we failed to find any expression criteria that could be used to further subdivide the genes in a way that would be predictive of biological function.

Comparison with previous RNAi screens: The results of several RNAi screens have been reported to date. In two of these screens, the set of genes tested was defined by chromosomal position; screens of 96% of the predicted genes on chromosome III (Gönczy *et al.* 2000) and 87.3% of the genes on chromosome I (Fraser *et al.* 2000) led to detectable RNAi phenotypes for 12.9 and 13.9% of those genes, respectively. Another screen targeted a set of 2500 genes (defined by their representation in the expressed sequence tag cDNA library) and detected phenotypes for 27% of genes tested (Maeda *et al.* 2001). This increased success rate in identifying phenotypes suggests that genes for which expression has been verified may be more likely to exhibit phenotypes when targeted by RNAi. Two smaller screens focused specifically either on genes expressed in the ovary (Piano *et al.* 2000) or on genes identified as germline enriched by cDNA subtraction and differential hybridization (Hanazawa *et al.* 2001). In the former screen, RNAi elicited defects in embryogenesis for a large fraction of cDNA clones tested (101/350), identifying 81 genes (because only positive clones were sequenced, the num-

ber of genes tested represented in the tested pool is unknown). In the latter screen, phenotypes were detected for 22% of 168 germline-enriched genes tested; roles in the germline *per se* or in embryogenesis were detected for 9 and 8%, respectively.

In our screen of 192 genes representing a defined subset of germline-enriched genes, we detected some type of phenotype for 57% of genes tested. Even if we eliminate from consideration those genes for which a phenotype was detected only at low frequency, we still saw phenotypes for 42% of genes tested, a "hit rate" significantly higher than that for any of the previously reported screens. Moreover, we detected strong germline phenotypes for 27% of tested genes, indicating that our focused strategy was highly effective in identifying genes required for normal development and function of the germline. We attribute our high success rate in identifying germline phenotypes to at least two key factors. First, we focused on genes expressed in the relevant tissue at the relevant time. Second, our multigenerational screening strategy allowed us both to identify phenotypes revealed preferentially in later generations and/or later time points than those examined in some screens and to uncover germline phenotypes that may have been masked by embryonic lethality among F_1 animals.

The efficacy of our screening strategy is emphasized when we examine the 105 genes that were tested both in our screen and in one or more of the other screens mentioned above (Table 1). For 69 of these genes, similar phenotypes were detected in our screen and the other screens. However, we detected RNAi phenotypes for a total of 27 genes that were classified as "no phenotype" in the other screens; most of these were in the low-frequency-only class, but 12 showed strong phenotypes. Further, we also observed additional phenotypes associated with embryonic lethality for 9 genes identified only as embryonic lethal in the other screens; for 7 of these, RNAi elicited strong germline phenotypes in our hands. In contrast, there were only 3 genes among the 38 for which a phenotype that was not detected in our screen was detected in other screens.

CONCLUSION

Our work has shown that the combination of a preselected pool of target genes and a multigenerational screening strategy has a high success rate for identifying genes that function in the *C. elegans* germline. We achieved our initial goal of identifying several new genes involved in key meiotic prophase events. Moreover, these efforts, in synergy with other work in the laboratory, have greatly expedited the molecular identification of genes we had previously defined by mutational analysis (A. J. MacQueen, M. P. Colaiácovo, J. Engebrecht, K. C. Reddy and A. M. Villeneuve, unpublished data). We also identified genes involved in many other aspects of germline development and function, including meiotic progression, germline proliferation, and chromosome organization and/or segregation during mitotic growth. Thus the information obtained in our screen will be useful not only for understanding germline function *per se*, but also for understanding chromosome metabolism and cell division in general. To provide broad access to detailed information about each of the genes analyzed in the screen, a FilemakerPro file containing our full database has been made available by ftp at ftp://villeneuveRNAi.stanford.edu (user ID and password: villeneuveRNAi). The database includes extensive descriptions of our phenotypic observations, sample images of DAPI-stained worms, and links to Wormbase, WormPD, and the germline microarray data from Reinke *et al.* (2000).

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