Phenotypic Analysis of Separation-of-Function Alleles of MEI-41, Drosophila ATM/ATR

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ABSTRACT

ATM/ATR kinases act as signal transducers in eukaryotic DNA damage and replication checkpoints. Mutations in ATM/ATR homologs have pleiotropic effects that range from sterility to increased killing by genotoxins in humans, mice, and Drosophila. Here we report the generation of a null allele of *mei-41*, Drosophila ATM/ATR homolog, and the use of it to document a semidominant effect on a larval mitotic checkpoint and methyl methanesulfonate (MMS) sensitivity. We also tested the role of *mei-41* in a recently characterized checkpoint that delays metaphase/anaphase transition after DNA damage in cellular embryos. We then compare five existing *mei-41* alleles to the null with respect to known phenotypes (female sterility, cell cycle checkpoints, and MMS resistance). We find that not all phenotypes are affected equally by each allele, *i.e.*, the functions of MEI-41 in ensuring fertility, cell cycle regulation, and resistance to genotoxins are genetically separable. We propose that MEI-41 acts not in a single rigid signal transduction pathway, but in multiple molecular contexts to carry out its many functions. Sequence analysis identified mutations, which, for most alleles, fall in the poorly characterized region outside the kinase domain; this allowed us to tentatively identify additional functional domains of MEI-41 that could be subjected to future structure-function studies of this key molecule.

IN eukaryotes, genome stability is maintained in part by checkpoint pathways that monitor the state of DNA and regulate the cell division cycle, activate DNA repair, or promote cell death as required. A central place in DNA damage and replication checkpoints in diverse eukaryotes is occupied by homologs of ATM/ATR kinases.

The *Schizosaccharomyces pombe* homolog, *Rad3*, was initially identified in a screen for DNA damage sensitive mutants and later found to act in the DNA damage and replication checkpoints (Al-Khodairy and Carr 1992; Seaton *et al.* 1992). The *Saccharomyces cerevisiae* homolog *Mec1* was isolated as a gene essential for cell cycle progression (Kato and Ogawa 1994; Siede *et al.* 1996). *ATM* was identified by studies of the human disease ataxia telangiectasia (AT; Savitsky *et al.* 1995a). Additional homologs include AtATM and AtRAD3 of *Arabidopsis thaliana*, Ce-atl-1 of *Caenorhabditis elegans* and UVSB of *Aspergillus nidulans*. All are large proteins of >2500 amino acids (aa) that share little similarity outside the kinase domain.

In vitro studies have identified several phosphorylation targets of both ATM and ATR (Kim *et al.* 1999). These include other proteins in the checkpoint pathways such as homologs of CHK1 and CHK2 (Sanchez *et al.* 1996; Cliby *et al.* 1998; Martinho *et al.* 1998; Matsuoka *et al.* 1998, 2000). These studies led to a model in which ATM/ATR homologs act early in the checkpoint pathway to sense the presence of damaged or incompletely replicated DNA and relay this signal to the remainder of the checkpoint pathway via phosphorylation. As such, the kinase domain, which is at the C terminus, has been shown to be required for function of both RAD3 and ATR (Jimenez *et al.* 1992; Savitsky *et al.* 1995a,b; Siede *et al.* 1996). For instance, mutations in the catalytic loop of the kinase affect all functions known for RAD3 and ATR, creating dominant negative activities (Bentley *et al.* 1996; Cliby *et al.* 1998). A temperature-sensitive mutation in the kinase domain of *rad3* disrupts the DNA damage checkpoint response and other functions analyzed (Martinho *et al.* 1998). Despite its importance, the kinase domain by itself is insufficient for function (Morgan *et al.* 1997; Chapman *et al.* 1999).

Relatively little, however, is known about the function of sequences outside the kinase domain in the PI3K-l family. In fission yeast, N-terminal sequences consisting of a leucine zipper and a putative protein-protein interaction site called the P site can confer dominant negative activity when overexpressed (Morgan *et al.* 1997; Chapman *et al.* 1999). A possible explanation for this

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result is that N-terminal sequences facilitate interaction of PI3K-l proteins with their partners and therefore compete with the endogenous protein when overexpressed.

An ATM/ATR homolog in Drosophila is encoded by *mei-41*. *mei-41* is essential for the DNA damage checkpoint in larval imaginal discs and neuroblasts and for the DNA replication checkpoint in the embryo (Hari *et al.* 1995; Brodsky *et al.* 2000; Garner *et al.* 2001). *mei-41* also has an essential role during early nuclear divisions in embryos, where it is required to delay mitosis in response to incomplete DNA replication (Sibon *et al.* 1999). Consistent with these functions, *mei-41* mutants are sensitive to hydroxyurea, an inhibitor of DNA replication, and DNA-damaging agents such as X-ray and alkylating agents (Boyd *et al.* 1976; Sibon *et al.* 1999). MEI-41 also plays an important role during meiosis, where it is proposed to monitor double-strand-break repair during meiotic crossing over, to regulate the progression of prophase I, and to enforce metaphase I delay observed at the end of oogenesis (Ghabrial and Schupbach 1999; McKim *et al.* 2000).

All existing *mei-41* mutant alleles were isolated in screens for mutants with meiotic defects, female sterility, or increased killing by genotoxins. Therefore, only viable alleles would have been recovered (Baker and Carpenter 1972; Smith 1973; Boyd *et al.* 1976; Mohler 1977). One of the strongest of these, $mei\text{-}41^{D3}$, has been described as a null allele on the basis of the absence of detectable protein and complete female sterility in these mutants (Sibon *et al.* 1999). Our recent sequence analysis (see below), however, sheds doubt on whether it is a true null that would be a valuable tool for analysis of MEI-41 function.

Here we report the generation of a null mutant of *mei-41*, which we find to be fully viable in the absence of DNA damage and to show a semidominant effect on a larval mitotic checkpoint and methyl methanesulfonate (MMS) sensitivity. We use the null allele to document the role of *mei-41* in a recently characterized checkpoint that delays metaphase/anaphase transition in response to DNA damage in cellular embryos (Su and Jaklevic 2001). We then compared five existing *mei-41* alleles to the null with respect to known phenotypes (female sterility, G_2/M regulation after DNA damage in larvae, and sensitivity to MMS). Four of these alleles lack meiotic defects; thus, female sterility can be attributed to the failure to regulate syncytial divisions where *mei-41* is required to delay mitosis in response to incomplete DNA replication. Interestingly, we find that not all phenotypes are affected equally by each allele; thus some are separation-of-function alleles. Sequence analysis identified mutations that revealed the importance of N-terminal sequences and identified putative functional domains of MEI-41. Our results support a model in which *mei-41* interacts with different sets of upstream and downstream effectors to carry out its many functions.

MATERIALS AND METHODS

Stocks: Several *mei-41* alleles were tested for lethality: A9 and 12-1483 were homozygous lethal. A16, D18, and 12-3616 were semilethal. In the case of two lethal alleles and one semilethal allele (12-3616), we have been able to separate the lethality from the *mei-41* locus by recombination. Additionally, all heteroallelic combinations of semilethal alleles were fully viable, suggesting that semilethality in $mei\text{-}41^{A16}$ and $mei\text{-}41^{D18}$ mutants is also due to mutations at other loci. Other alleles were fully viable and fell into three classes with regard to female fertility: A8, A11, D1, D2, D3, D4, D7, D8, D9, D10, D11, and D18 were female sterile, with fertility ranging from 0 to 5%. The second class showed fertility ranging from 5 to 15% and was composed of alleles A1, A7, A11, A12, A15, A16, A22, A27, A28, A29, 12-3616, D19, RT1, and RT2. The last class of alleles, A10, D5, D9, D12, D13, D14, and D15, expressed fertility ranging from 50 to 98%. The deficiency Df(1)19 has previously been shown to be *mei-41* deficient and Dp(1;4)r^+f^+, generated by Falk *et al.* (1984), has been shown to contain a wild-type *mei-41* gene (Banga *et al.* 1995). Lethal mutations in genes *l(1)14Ca*, *l(1)14Cc*, *l(1)14Cd*, and *l(1)14Ce* were represented by deficiencies and alleles XR4, XR18, 11b8, $i19e^2$, 4d2-5, and 4a47, which were provided by R. Stanewsky (Stanewsky *et al.* 1993; Banga *et al.* 1995). Genes and chromosomes used in this study but not described in the text can be found in the Drosophila reference works (Lindsley and Zimm 1992; FlyBase 2002).

Transposon mobilization: The screen to recover null alleles was conducted with the two P insertion alleles, $mei\text{-}41^{RT1}$ and $mei\text{-}41^{RT2}$ (Figure 2). Introduction of a *P*-element-encoding transposase (denoted TMS) into these genetic backgrounds should allow a screen for products of imprecise excision that removes flanking coding sequences. However, because MEI-41 is required to repair the damage created by transposon mobilization, combination of a strong *mei-41* mutant and a source of transposase is usually lethal (Banga *et al.* 1991; our unpublished observations). Furthermore, newly induced mutations at the *mei-41* locus might cause lethality in germ cells, in which case products of imprecise excision may not be recovered. To circumvent these difficulties, we used the duplication Dp(1;4)r^+f^+ to provide a copy of the *mei-41* gene on the fourth chromosome. Only $mei\text{-}41^{RT1}$ females survived the imposed genomic context. That is, Dp(1;4)r^+f^+ rescued the viability of *mei-41/Df(1)19; TMS/+; Dp(1;4)*r^+f^+*/+* females but not *mei-41*/Y; *TMS/+*; *Dp(1;4)*r^+f^+*/+* males, for reasons that remain unclear. Therefore, we performed the screen for imprecise excision in the female germline (Figure 2). To increase the frequency of *P*-element losses during the experiment, we provided a deficiency of the region on the homologous chromosome. Without a sister chromatid as a template, repair of the double-strand breaks created by the transposase provided *in trans* is less accurate (Engels *et al.* 1990; Johnson-Schlitz and Engels 1993). From the daughters of *mei-41/Df(1)19; TMS/+; Dp(1;4)*r^+f^+*/+* females, a total of 454 chromosomes were recovered and analyzed for female sterility over $mei\text{-}41^{D3}$ allele. Lethal chromosome recovered (78B) was tested for complementation with lethal mutations in *l(1)14Ca*, *l(1)14Cc*, *l(1)14Cd*, and *l(1)14Ce*.

Measurement of female fertility, recombination, and X chromosome loss: During the screen for a null allele of *mei-41*, female sterility was checked by allowing 10 females to mate with 5–10 FM7a,*f* brothers, and the number of progeny (adult flies) were counted after 10 days. Other fertility experiments as reported in Table 2 were conducted by crossing 5 females to 5–10 wild-type (Canton-S) males. Eggs were collected for 24 hr and scored 24 hr later for hatching to determine the percentage of fertility. To estimate recombination and X chromosome loss frequencies, *w mei-41; net ho dp Sp b*

pr cn/+ virgins were crossed to *net ho dp b pr cn* males. Crosses were scored for net, ho, dp, and b markers for recombination frequencies and chromosome loss was estimated from the number of (w+) males. Flies were raised on standard cornmeal-molasses-agar medium and grown in an incubator at 25°.

PCR and DNA sequencing: The nucleotide numbers refer to the sequence of the 10.5-kb fragment containing the *mei-41* open reading frame (GenBank accession no. U34925). Primer sequences (GIBCO BRL, Gaithersburg, MD) and PCR conditions are available upon request. Location of primers with respect to the *mei-41* sequences shown in Figure 1A. Amplification of genomic DNA from $mei\text{-}41^{RT1}$ and $mei\text{-}41^{RT2}$ flies with primers Pout and Δ2-1 generated fragments of 900 and 400 bp, respectively. Fragments digested with *Eco*RI-*Not*I for $mei\text{-}41^{RT1}$ and *Pst*I-*Not*I for $mei\text{-}41^{RT2}$ were cloned and sequenced. *P*-element insertion creates a direct duplication of 8 bp at its site of insertion (O'Hare and Rubin 1983). These direct duplications have been localized at 3370 bp (GTTCA TAC) and 3870 bp (GGCCAGCT) and share similarity with the described consensus (O'Hare and Rubin 1983).

For mutant flies recovered from the described screen (Figure 2), amplifications were performed with Pout:1964, Pout:Δ2-1, and Pout:2549 primer pairs to determine the presence of the *P* element on the chromosome. With 1964:Δ2-1, we checked for deletion in the vicinity of the insertion site. The 1964-Δ2-1 amplicon was double digested with *Sac*I and *Eco*RI after purification and cloned. DNA from a 29D/78B hybrid fly did not yield any signal with the 31000A:2549 couple. Sequence matching the primer 31000A is located within the 975 bp that is missing in the 29D allele; therefore the deficiency in 78B chromosome includes at least part of the *mei-41* gene. We sequenced the following alleles in entirety: $mei\text{-}41^{D3}$, $mei\text{-}41^{D5}$, $mei\text{-}41^{D9}$, $mei\text{-}41^{D12}$, $mei\text{-}41^{D13}$, $mei\text{-}41^{D14}$, and $mei\text{-}41^{D15}$. PCR products generated from independent vials were used in sequencing. For each allele, we were able to identify single mutations that differ from background; these mutations are shown in Tables 2 and 3.

MMS treatment: MMS sensitivity was quantified as follows. *mei-41*/FM7 virgin females were crossed to *mei-41* males to determine the sensitivity of homozygotes. *mei-41*/FM7a,*f* virgin females were crossed to Canton-S to determine the sensitivity of heterozygous females. Eggs were collected in vials for 24 or 48 hr from 5–10 batches of five females and five males each. After 24 hr at 25°, 250 μl of MMS solution was applied to the food, and the progeny were allowed to develop. Several hundred flies and all classes of progeny were scored for each dose.

Antibody production and Western blotting: To produce a fragment of MEI-41, the largest *Eco*RI fragment of the *mei-41* genomic DNA inserted into the pCasper vector (predicted to produce the last 402 aa) was subcloned into the unique *Eco*RI site of the pET21b expression vector (Novagen, Cambridge, MA). The plasmid was transformed into *Escherichia coli* BL21 (DE3) strain and the protein was induced with isopropyl thiogalactoside according to manufacturer's instructions (Novagen). The expressed protein was gel purified and used to immunize rabbits using a commercial facility (Cocalico Biologicals, Reamstown, PA). Antisera were purified against the antigenic protein fragment immobilized on PDVF membranes (Immobilon-P; Millipore, Bedford, MA). Plasmid maps and detailed protocols for subcloning and antibody purification are available upon request.

For Western blotting, embryos were dechorionated for 2 min in 50% bleach, rinsed in water, and homogenized in HEMG buffer (20 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 1.5 mM EGTA, 10% glycerol) supplemented with protease and phosphatase inhibitors (10 mM β-glycerol phosphate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptin, peptstatin, and aprotinin). An equal volume of 2× SDS loading dye was added to the sample, which was then boiled for 10 min before being subjected to electrophoresis on 5% polyacrylamide gels. The proteins were blotted onto PDVF membranes using a semidry apparatus (Pharmacia Biotech, Piscataway, NJ). The blots were blocked in PBT + 5% milk before being probed with the primary antibody diluted (typically 1:300–500) in blocking solution. The membranes were washed in blocking solution and probed with a secondary antibody (HRP-conjugated anti-rabbit antibody; Pharmacia Biotech) diluted 1:2000 in blocking solution. The membranes were washed in PBT and processed for enhanced chemiluminescence detection according to manufacturer's instructions (Pierce, Rockford, IL). The migration of high-molecular-weight markers (Amersham, Arlington Heights, IL) was used in a semilogarithmic plot of mobility *vs.* molecular weight to estimate the mass of the putative MEI-41 protein in Figure 5.

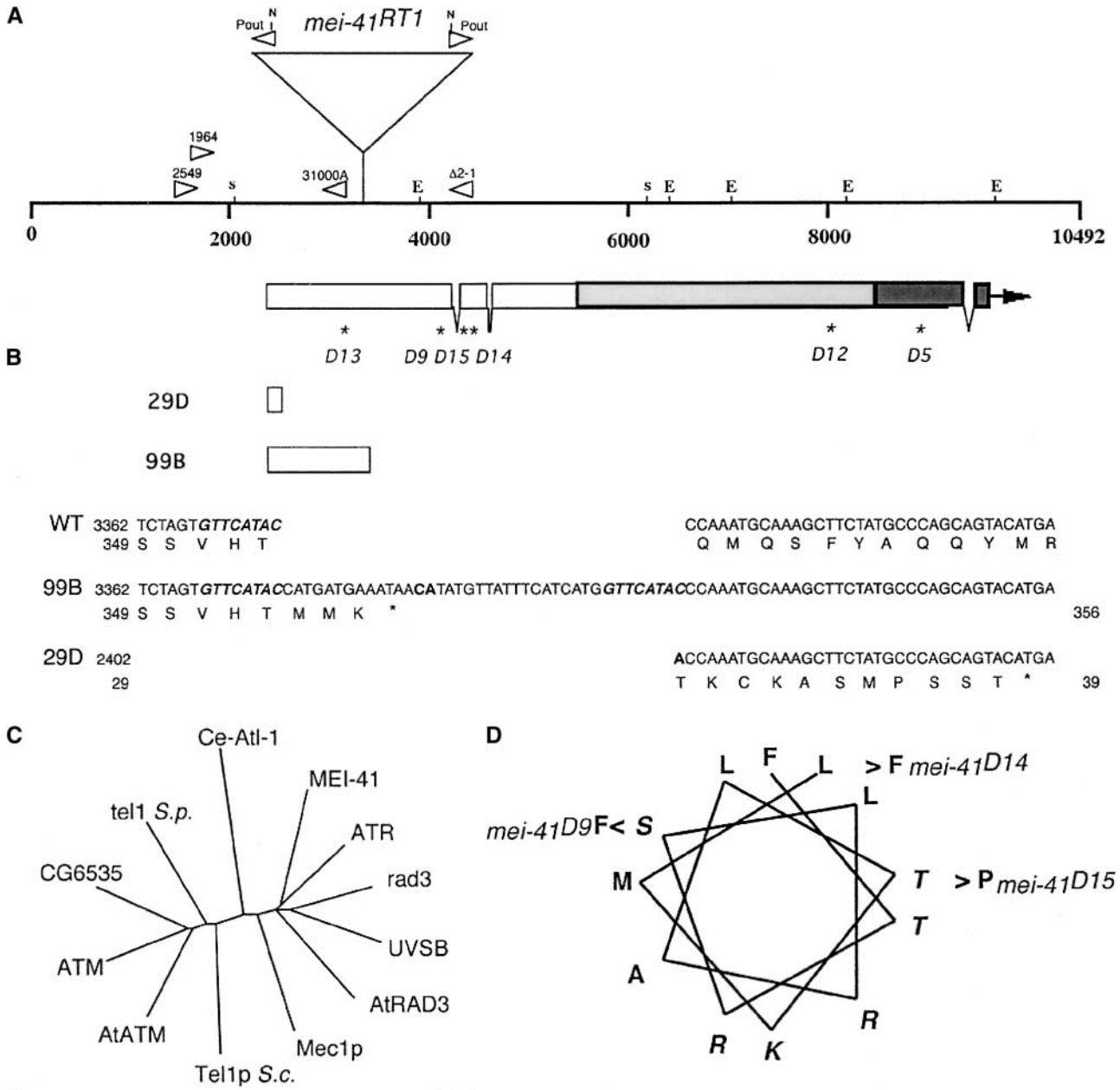
G_2/M checkpoint quantification in imaginal discs: Climbing larvae were collected and sexed. Males from the *mei-41*/C(1)DX/*Y* stocks or from the Canton-S control stock were irradiated on petri dishes with X rays from ^{137}Cs source at 5-Gy dose (Astrophysics Research, Long Beach, CA). Irradiated and nonirradiated larvae were kept on grape agar plates for 1–2 hr at 25° and then dissected in phosphate-buffered saline (PBS; 0.13 M NaCl, 4 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.5). The anterior portions of bisected larvae were inverted to expose the imaginal discs, fixed in 4% paraformaldehyde in PBS for 15 min, and washed in PBT (PBS + 0.3% Triton X-100) for 1 hr. Discs were blocked for 1 hr in 5% bovine serum albumin (albumin fraction V from United States Biochemical, Cleveland) in PBT and incubated for 2 hr (at room temperature) or 12 hr (at 4°) in anti-phosphohistone H3 antibody (1:1000 in PBT; Upstate Biotechnology, Lake Placid, NY). After another washing and blocking step, discs were incubated for 2 hr (at room temperature) or 12 hr (at 4°) with HRP-conjugated secondary antibody (goat anti-rabbit at 1:500 in blocking solution; Zymed, S. San Francisco). After another washing step, 3,3′diaminobenzidine tetrahydrochloride solution in NiCl was applied according to manufacturer's recommendation. The reaction was stopped by several rapid washes in PBT. Discs were stained with a DNA dye, 6-diamidino-2-phenylindole dihydrochloride at 0.05 μg/ml. The discs were then further dissected to remove extraneous tissues and mounted in PBT solution. Slides were observed under visible and UV light on an axioplan Zeiss microscope. Ten or more eye discs were scored to estimate the checkpoint defects, and data are presented ±SE. Chemicals were purchased from Sigma (St. Louis) unless stated otherwise.

Mitotic checkpoints in the embryo: To detect the G_2/M delay, embryos were collected for 10 min, aged for 325 min at 25° to reach interphase of embryonic cycle 16 (Foe *et al.* 1993), irradiated, and allowed to rest for 20 min before fixing in 37% formaldehyde for 5 min. To detect the metaphase/anaphase delay, embryos were collected for 60 min and aged for 330 min at 25° before fixing (for unirradiated controls) or aged for 310 min, irradiated, and allowed to rest for 40 min before fixing. Embryos were irradiated with 5.7 Gy of X rays in a TORREX X-ray generator (Astrophysics Research) set at 115 kV and 5 mA (producing 1.32 Gy/min). Fixed embryos were blocked in PBT containing 3% normal goat serum and stained with the antiphosphohistone H3 antibody to visualize mitotic cells. Embryos were also counterstained with the DNA dye, Hoechst 33258, at 10 μg/ml in PBT.

RESULTS

Generation of null alleles of *mei-41* via internal deletions: The $mei\text{-}41^{RT1}$ allele contains a single nonautonomous *P*-element transposon inserted in the *mei-41* cod-

ing region (Figure 1A; YAMAMOTO *et al.* 1990). Following mobilization of this transposon (MATERIALS AND METHODS; Figure 2), 454 chromosomes were recovered and tested for female sterility when heterozygous with the *mei-41*^{D3} allele. A total of 392 recovered chromosomes caused reduced female fertility when heterozygous with the *mei-41*^{D3} allele, similar to *mei-41*^{D3}/*mei-41*^{RT1} control females. Thirty-three chromosomes were female fertile when heterozygous with the *mei-41*^{D3} allele and thus may represent revertants. Twenty-nine exhibited female sterility when heterozygous with *mei-41*^{D3}. One of these 29, 78B, carries a homozygous lethal mutation and was rescued



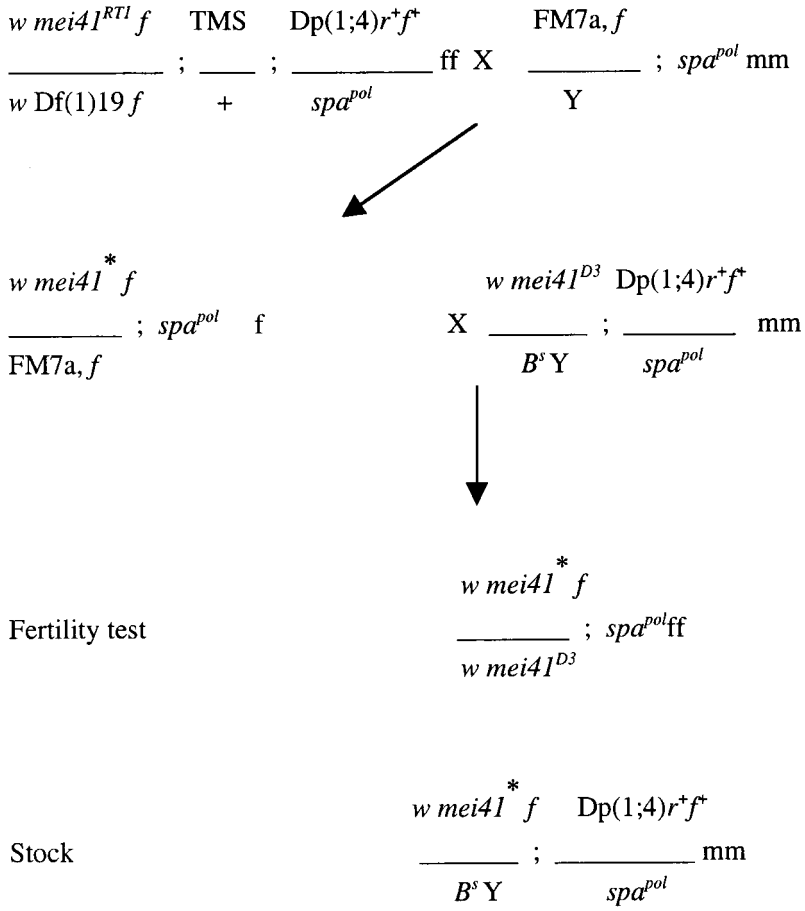


FIGURE 2.—The mating scheme to recover null alleles of *mei-41*. The *mei-41*^{RT1} allele was combined with a stable transposase source on the third chromosome, TMS. These females also carry Df(1)19, uncovering the *mei-41* region, and a duplication of the same region on the fourth chromosome, *Dp(1;4)r*$^+$*f*$^+$. Two females were mated with males carrying the *FM7a,f* balancer chromosome in 180 crosses. The minute phenotype associated with the Df allowed us to monitor X chromosomes in the progeny and the dominant *Sb* mutation on TMS, the third chromosome. To rescue any lethal chromosome generated, single virgin *f, pol* females were mated individually to *mei-41*^{D3} males carrying the *Dp(1;4)r*$^+$*f*$^+$ chromosome. Each cross is tagged to keep track of cluster events. The sterility of 10–20 *w, pol* daughters was analyzed with their *Dp(1;4)r*$^+$*f*$^+$*/pol* sisters as a control. From the same brood, males carrying the mutagenized chromosome were saved to raise stocks. Lines showing no *pol* males were carrying an X lethal mutation. *, mutagenized chromosome; mm, male; ff, female. See MATERIALS AND METHODS for stock and genotype information.

by a chromosomal duplication, Dp(1;4)*r*$^+$*f*$^+$, that includes *mei-41*. However, 78B fails to complement lethal mutations at *l(1)14Ce* locus (alleles 4d25 and 4a27) and therefore is likely to carry a deficiency (MATERIALS AND METHODS).

Genomic DNA from the above 29 mutants was amplified by PCR and analyzed. Three mutants, 29D, 99B, and deficiency-bearing 78B, were found to lack the *P* element. Further sequence analysis showed that the 29D chromosome is missing 975 bp of *mei-41* coding sequence. Moreover, transposon excision was accompanied by the insertion of an extra A, creating a frameshift in the remaining coding sequence (Figure 1B). Consequently, the 40th codon is expected to encode a "stop," leaving a truncated protein of 39 aa; the native *mei-41* gene encodes 2347 aa. The 99B line presents an 8-bp direct duplication with most of the *P*-element sequence deleted, leaving behind 13 bp of 5′ inverted repeat (IR)

FIGURE 1.—Sequence analysis of *mei-41* alleles. (A) In the map of the *mei-41* locus, the insertion site of the *P*-element responsible for the *mei-41*^{RT1} mutation is shown along with the primers used to screen for null alleles (MATERIALS AND METHODS). The protein is highlighted in three shades: white for the N terminus, gray for the rad3 domain, and black for the kinase domain. Asterisks indicate the site of unique mutations affecting D13, D9, D15, D14, D12, and D5. The nucleotide numbers refer to the sequence of the 10.5-kb fragment containing the *mei-41* open reading frame (GenBank accession no. U34925). (B) Comparison of *mei-41*^{29D}, *mei-41*^{99B}, and wild-type sequences. Predicted proteins are depicted at the same scale as full-length MEI-41 protein in A. Wild-type (WT) nucleotide sequence is shown at the top. The 8-bp target site that was duplicated in 99B is italicized. Boldface nucleotides are filler sequences that became added upon transposon excision. Nucleotide numbers correspond to the genomic clone presented in A. The 29D allele contains up to nucleotide 2401 of the wild-type sequence (encoding the first 28 amino acids), followed by a 975-bp deletion. Wild-type sequence resumes at nucleotide 3376, but insertion of an A (in bold) results in a frameshift that produces a stop codon after 11 additional amino acids. (C) Phylogenic tree as calculated from C-terminal 360 amino acids, which correspond to the kinase domain, of the following proteins (GenBank accession numbers in parentheses): Ce-atl-1 (AB018598), UVSB (AF176575), RAD3 (CAA70297), ATR (U76308), MEI-41 (U34925), CG6535 (AE003708), MEC-1/ESR1 (Z36005), ATM (U33841), AtATR (BAA92828), AtATM (AJ250248), Tel1S.p. (T41243), and Tel1S.c. (S45416). Maximum parsimony and neighbor-joining methods were used to investigate protein relationships. (D) Mutations in *mei-41*^{D9}, *mei-41*^{D14}, and *mei-41*^{D15} alleles. Helical wheel of the predicted α-helix in which these mutations are located is shown. Hydrophobic amino acids are italicized. (E) Alignment of a part of the rad3/TRAPP homologous domain in which the *mei-41*^{D12} mutation is located. Amino acids of similar hydrophobicity or structure across species are denoted with a triangle.

and 17 bp of the 3′ IR that are separated by nucleotides, CA. This rearrangement creates a stop codon downstream of the insertion site and is predicted to encode a truncated protein of 340 aa (Figure 1B). 29D and 99B are thus null mutants; we refer to these new alleles as *mei-41^{29D}* and *mei-41^{99B}*.

Despite the severe nature of lesions, homozygous females and hemizygous males bearing *mei-41^{29D}* or *mei-41^{99B}* alleles are fully viable. Thus null alleles of *mei-41* do not appear to act as zygotic lethal mutations. Note that the recovery of the 78B deficiency indicates that lethal alleles of *mei-41* would have been recovered had they been induced. In mammals, ATR is an essential gene while ATM is not (Barlow *et al.* 1996; Xu and Baltimore 1996; Brown and Baltimore 2000). The Genome Project identified a second ATM/ATR homolog in the Drosophila genome (CG6535), which is more closely related to ATM; MEI-41 is more closely related to ATR (Figure 1C). It is therefore interesting that *mei-41* appears to be nonessential while ATR is.

The phenotype of null alleles: Prior to further analysis, we first confirmed that newly generated null alleles exhibit phenotypes expected of strong *mei-41* alleles. In assays for sensitivity to a genotoxin, MMS, doses as low as 0.01% killed 100% of homozygotes for the *mei-41^{29D}* and *mei-41^{99B}* alleles. Moreover, *mei-41^{29D}* homozygous females are semisterile (97.3% of embryos fail to hatch at 25° and 92% at 20°) and display meiotic defects such as reduction of recombination (13% of normal between *net* and *ho* markers) and increased chromosome losses and nondisjunction (10% X chromosome loss; control shows <1%). Likewise, the ability to delay the entry into mitosis upon DNA damage is disrupted in homozygous *mei-41^{29D}* mutant larvae (Figure 3). These phenotypes are identical to those caused by the strongest *mei-41* alleles isolated to date, namely D1, D3, and 195.

Boyd *et al.* (1976) reported a semidominant effect on MMS sensitivity for at least one strong allele of *mei-41*. We find that *mei-41^{29D}* and *mei-41^{99B}* also exhibited a semidominant effect on larval MMS sensitivity and the ability to block mitosis after DNA damage in larval discs (Figure 3). However, female sterility does not appear to be dose dependent since one copy of the *mei-41* genomic DNA can rescue embryonic lethality to wild-type levels (Sibon *et al.* 1999).

The role of *mei-41* in mitotic metaphase/anaphase delay: We next used *mei-41^{29D}* mutants to determine the role of *mei-41* in a recently defined metaphase checkpoint in embryos (Su and Jaklevic 2001). In embryonic cell cycles, DNA damage due to ionizing radiation or MMS causes a delay in the entry into mitosis. After this delay, cells recover but subsequently delay in metaphase/anaphase transition (Su and Jaklevic 2001). This is seen in live measurement of mitotic timing, where duration of metaphase increased about threefold, as well as in quantification of mitotic phases in fixed embryos; the ratio of metaphase to {anaphase + telophase} increases by about threefold in irradiated embryos (Su and Jaklevic 2001; Figure 4). Metaphase/anaphase delay appears to be due to stabilization of a mitotic cyclin, but the role of checkpoint genes in this response has not been addressed.

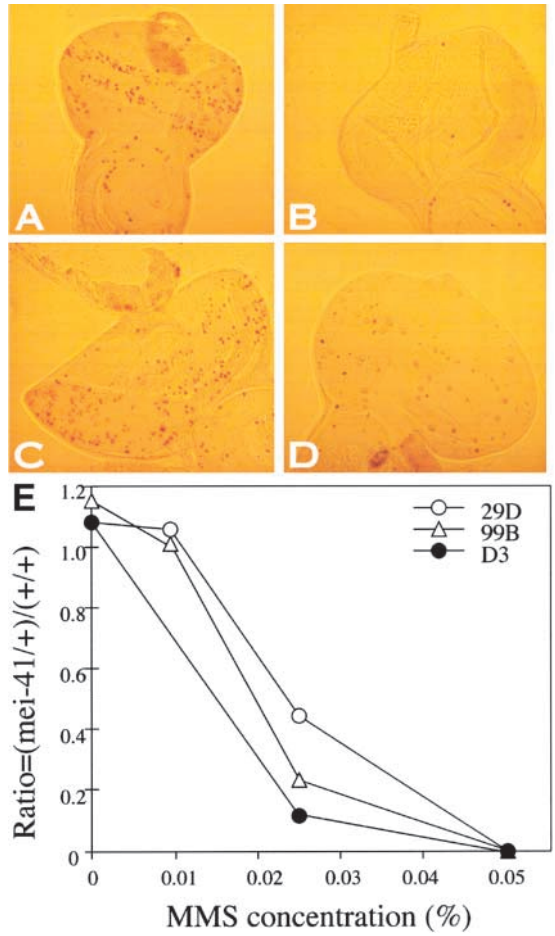
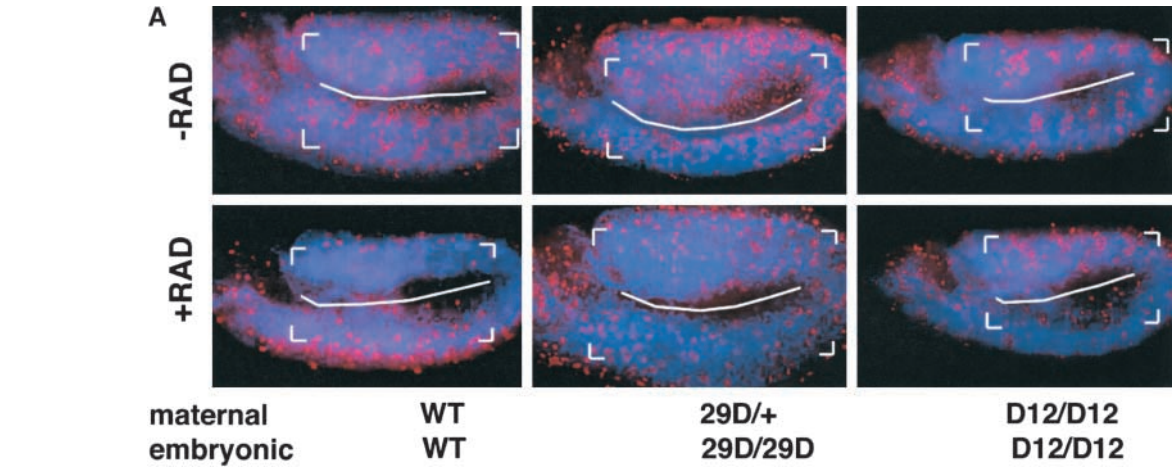


Figure 3.—DNA damage checkpoint and MMS sensitivity in *mei-41* mutant larvae. (A–D) Mitotic cells are visualized by PH3 staining in larval eye discs. In wild-type (Canton-S) larvae, each disc contains 121 ± 11.1 mitotic cells (A) while this number decreased >10-fold to 9.2 ± 1.4 after irradiation (B), indicating a delay in entry to mitosis in the latter. In *mei-41^{29D}* homozygotes, each eye disc contains 156 ± 10 mitotic cells before irradiation (data not shown) and 127 ± 21.3 mitotic cells after irradiation (C), indicating that these larvae are unable to block mitosis after irradiation. In *mei-41^{29D}* heterozygotes, each eye disc contains 170.3 ± 14.4 mitotic cells before irradiation and this number decreased only 2-fold to 69.3 ± 13.3 after irradiation (D), indicating a partial loss of the ability to block mitosis. (E) MMS sensitivity of *mei-41* heterozygotes. The ratio of heterozygous to wild-type females recovered at different MMS concentrations is shown for three alleles. Homozygous mutants for all three alleles show complete lethality at all MMS doses shown here except for 0% MMS (data not shown).

We find that the number of mitotic cells in the dorsal ectoderm was reduced at 20 min after irradiation in wild type and in homozygous *mei-41^{29D}* embryos from heterozygous mothers, but not in homozygous *mei-41^{D12}*



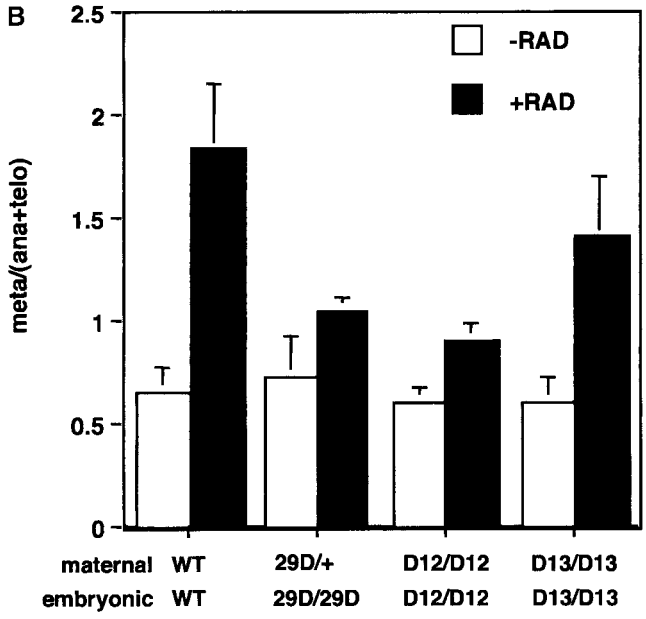


FIGURE 4.—Embryonic mitotic checkpoints that respond to irradiation require *mei-41*. (A) Mitotic entry checkpoint in the embryo. Embryos (325–335 min old) in which the dorsal ectodermal cells are in interphase of cycle 16 (FOE *et al.* 1993) were irradiated with 0 (−RAD) or 5.7 Gy of X rays (+RAD). Embryos were fixed 20 min later, when dorsal ectodermal cells should be in mitosis of cycle 16, and stained with an antibody to phosphorylated histone H3 to visualize mitotic cells (red). Embryos were also stained to visualize DNA (blue). Mitotic cells are counted in the dorsal ectoderm region, enclosed by brackets. Irradiation reduced the number of mitotic cells in wild type (WT; from 111 ± 14 to 19 ± 11) and $mei41^{29D}$ homozygous embryos from heterozygous mothers (29D; from 103 ± 9 to 16 ± 5), indicating a delay in entry into mitosis. Irradiated $mei41^{D12}$ homozygous embryos (D12) from homozygous mothers showed only twofold reduction in the number of mitotic cells compared to unirradiated controls of the same genotype (63 ± 7 to 29 ± 4), indicating that the ability to regulate the entry into mitosis after irradiation has been compromised in these mutants. D12 mutant embryos in these experiments are at the same developmental stage as WT, 355 ± 5 min, but present a significantly lower number of mitotic cells when untreated. We do not address this issue. (B) Metaphase/anaphase checkpoint in *mei-41* mutants. The ratio of the number of metaphase cells to the sum of anaphase and telophase cells in mitosis 16 in the dorsal ectoderm was quantified from unirradiated (−RAD) embryos or embryos that had been irradiated 40 min before fixing (+RAD). Cells in wild-type embryos delay metaphase-anaphase transition after irradiation, as indicated by a threefold increase in meta/(ana$^+$telo) ratio. This increase is diminished in *mei-41* mutants. Data are compiled from at least 3600 mitotic cells from at least 30 embryos for each genotype in two or three experiments. Metaphase/(aNa$^+$telo) ratio was also measured at 20 min after irradiation in $mei41^{D12}$ mutants with similar results (not shown). $mei\text{-}41^{29D}$ homozygous embryos from heterozygous mothers were identified by the lack of β-galactosidase staining from the *FM7, ftz:lacZ* chromosome. $mei\text{-}41^{D12}$ and $mei\text{-}41^{D13}$ embryos are from homozygous mutant mothers.

embryos from homozygous $mei\text{-}41^{D12}$ mothers (Figure 4A; see figure legend for mitotic indices). There are several possible explanations for these observations. First, homozygous $mei\text{-}41^{29D}$ embryos would have inherited wild-type *mei-41* gene products from their heterozygous mothers, and the maternal product may persist long enough to enforce the mitotic entry checkpoint; D12 embryos from homozygous D12 mothers, in contrast, would not have inherited any wild-type *mei-41* gene products. It is also possible that *mei-41* is dispensable for the regulation of mitotic entry after X-ray damage in embryos or plays a redundant role with a second ATM/ATR homolog that exists in Drosophila.

More important for this work, at longer times (40 min) after irradiation when cells have recovered and entered mitosis, the ratio of metaphase to {anaphase + telophase} increased in wild type; this increase was severely diminished in $mei\text{-}41^{29D}$ and $mei\text{-}41^{D12}$ embryos and partially diminished in $mei\text{-}41^{D13}$ embryos (Figure 4B). Thus, all three *mei-41* alleles tested are defective in the metaphase/anaphase checkpoint, implicating this gene in the regulation of mitotic progression in response to X-ray damage.

$mei\text{-}41^{29D}$ embryos have an intact mitotic entry checkpoint but not an intact metaphase/anaphase checkpoint. This could be either because the latter is more

TABLE 1

The phenotypes of *mei-41* alleles studied

	MMS sensitivity	% fertility	% loss of G_2/M checkpoint	Mutation	
				Nucleotide	Amino acids
mei-41^{29D}	++++	3	100	Deletion at 2401	Stop after 39 aa
mei-41^{D3}	++++	1	98	$T^{3768}A$	$A^{384}V$
mei-41^{D5}	+++	50	74	$C^{8924}T$	$P^{2159}L$
mei-41^{D9}	++	77	70	$C^{4257}T$	$S^{647}F$
mei-41^{D12}	+	97	63	$C^{8053}T$	$H^{1869}Y$
mei-41^{D13}	+	98	35	$T^{2585}A$	$W^{90}T$
mei-41^{D14}	++	55	15	$G^{4339}T$	$L^{655}F$
mei-41^{D15}	++	84	6	$A^{4328}C$	$T^{652}P$

MMS sensitivity, female fertility, and loss of G_2/M DNA damage checkpoint are shown for each allele, along with unique nucleotide and amino acid changes. Data for the null allele, *mei-41*^{29D}, are given as a reference. For MMS sensitivity, ++++, +++, ++, and + refer to complete lethality at 0.01%, 0.025%, 0.05%, and 0.08%, respectively. Female fertility is expressed as percentage of eggs from homozygous mutant females that hatch into larvae. Loss of G_2/M checkpoint represents the average number of mitotic cells per eye disc after exposure of male hemizygous mutant larvae to 500 R of X rays expressed as percentage of the same number before irradiation. WT values range from 5 to 15%.

sensitive to the level of maternal *mei-41* gene products that persists or because maternal *mei-41* products are able to substitute for zygotic *mei-41* products in the first checkpoint but not in the second. Thus, the data presented here implicate *mei-41* in the regulation of metaphase/anaphase transition after DNA damage but do not conclusively address the role of *mei-41* in the embryonic mitotic entry checkpoint. Nonetheless, metaphase/anaphase regulation is defective in *mei-41* mutants that are either able (29D) or unable (D12) to regulate the entry into mitosis. This result excludes the possibility that metaphase/anaphase defects are due to variations in the time of entry into mitosis between wild type and mutants.

The phenotype of partial loss-of-function mutants: ATR homologs act as signal transducers in DNA replication and damage checkpoint pathways. The presence of DNA defects activates ATR homologs, which are kinases, which in turn carry out their function by phosphorylation of downstream substrates. Drosophila ATM/ATR, MEI-41, when mutated, leads to a number of phenotypes as described above, ranging from female sterility to MMS sensitivity. If the same checkpoint pathway and components are at work in processes whose failure generates these phenotypes, we might expect that an allele that affects one phenotype will affect others to a similar extent. If, on the other hand, the function of *mei-41* is being executed via different partners/substrates in different processes, we might expect that an allele that affects one phenotype may not necessarily affect another.

To address these possibilities, we compared the phenotypes of 7 previously isolated alleles to each other and to the null allele we generated. Five (D9, D12, D13, D14, and D15) were chosen out of 33 tested alleles (named in MATERIALS AND METHODS) because they behave as wild type in two standard assays for meiotic functions, namely X chromosome nondisjunction frequency and meiotic recombination levels (MASON *et al.* 1981, 1989). Thus, we can rule out the contribution of meiotic defects to female sterility. The sixth, D5, was selected because it affected partially all *mei-41* phenotypes described herein (Table 1); mutant females are meioticaly impaired, showing 5% of X nondisjunction. Finally, D3 was selected because it behaved as a null and we thus expected to find a mutation abolishing all *mei-41* functions. Additionally, because all 7 alleles were generated during a single EMS mutagenesis, we reasoned that they will share a common background and therefore sequence analysis would reveal mutations affecting *mei-41* functions. Results of phenotypic comparison are presented in Figure 4B for metaphase/anaphase delay (in 3 alleles) and summarized in Table 1 for other phenotypes.

A comparison of *mei-41* alleles leads us to two conclusions. First, not all phenotypes are affected equally by each allele. For instance, D12 and D13 alleles present wild-type levels of female fertility but defective metaphase/anaphase checkpoints in the embryo (Figure 4) and defective mitotic checkpoints in larval discs (Table 1). Conversely, the larval mitotic checkpoint is as robust in D15 as in wild type, even though D15 mutants are MMS sensitive and partially female sterile. Second, an allele that is more defective than another with regard to one phenotype is not necessarily so with regard to another phenotype. For instance, the D14 allele is more severe than D9 in female fertility but is less severe than D9 in the mitotic checkpoint in larval discs. Likewise, D12 shows less severe female fertility and MMS sensitivity than D15 does, but is more severe than D15 in larval

TABLE 2

Neutral mutations

	mei-41 alleles						
Nucleotide	D3	D5	D9	D12	D13	D14	D15
2824 G	G	T	T	T	T	G	T
3364 C	C	T	T	T	T	C	T
3424 C	C	T	T	T	T	C	T
3529 G	G	T	T	T	T	G	T
3821 C	C	A	A	A	A	C	A
3916 G	G	A	A	A	A	G	A
4513 A	A	G	G	G	G	A	G
4541 T	T	C	C	C	C	T	C
4549 T	T	C	C	C	C	T	C
4626 G	T	G	G	G	G	T	G
5133 T	T	G	G	G	G	T	G
5397 C	A	C	C	C	C	A	C
6078 G	A	G	G	G	G	A	G
6090 G	A	G	G	G	G	A	G
6195 T	C	T	T	T	T	C	T
6210 A	C	A	A	A	A	C	A
6255 T	C	T	T	T	T	C	T
6927 T	T	C	C	C	C	T	C
7033 T	G	T	T	T	T	G	T
7230 T	C	T	T	T	T	C	T
7701 T	C	T	T	T	T	C	C
7848 C	T	C	C	C	C	T	T
7938 T	C	T	T	T	T	C	T
8043 T	C	T	T	T	T	C	T

(*continued*)

TABLE 2

(Continued)

	mei-41 alleles						
Nucleotide	D3	D5	D9	D12	D13	D14	D15
2413 T	T	T	C	C	C	T	C
3445 C	C	C	T	C	C	C	T
3622 C	T	C	T	C	C	T	T
4678 C	C	G	C	G	G	C	C
4681 T	T	T	T	G	T	T	T
4857 G	G	A	G	A	A	G	G
5052 A	A	A	A	A	A	G	A
5103 G	G	G	G	G	G	T	G
5136 G	G	G	A	G	G	G	A
5316 A	A	A	T	A	A	A	T
5400 G	G	G	A	G	G	G	A
5577 C	C	T	C	T	T	C	C
5625 T	T	C	T	C	C	T	T
5721 A	A	G	A	G	G	A	A
5904 C	T	C	T	C	C	T	T
7044 G	G	T	T	T	T	T	T
7164 A	G	A	G	A	A	G	G
7440 G	T	G	G	G	G	G	T
7599 G	G	G	G	A	G	G	G
7602 T	G	T	T	T	T	T	G
7881 G	A	G	G	G	G	G	G
7896 C	G	C	C	C	C	G	G
8199 C	C	T	C	T	T	C	C
8256 C	C	G	C	C	C	G	C
8310 T	T	C	T	C	C	T	T
8376 T	C	C	T	C	C	C	T
8445 G	A	A	G	A	A	A	G
8946 C	C	T	C	T	T	C	C
9006 G	T	T	G	T	T	T	G
9066 C	T	T	C	T	T	T	C
9162 C	T	T	C	T	T	T	C
9381 A	G	G	A	G	G	G	A
9420 T	A	A	T	T	T	A	T
9517 T	T	C	T	C	C	T	T

Neutral mutations in *mei-41* alleles fall into two groups: those in the top half of the table show nucleotide variations common to D3 and D14 alleles but not shared by D5, D9, D12, D13, and D15 alleles. All alleles show the following mutations compared to the GenBank accession no. U34925 genomic sequence: 2857, T → C; 3934, A → T; 4069, G → A; 4261, C → T; 4974, A → G; 5157, G → T; 5817, A → G; 6735, A → C; 6750, A → G; 7002, A → G; 7008, T → A; and 7011, A → C.

mitotic checkpoint regulation. These observations raise the possibility that some aspects of the *mei-41* phenotype may reflect the specific mutational ablation of domains critical for a specific function. To address this, we determined the sequence of the mutant alleles.

DNA sequence changes in partial loss-of-function alleles: Sequence analysis revealed several differences between mutant alleles and the published *mei-41* sequence. We found 70 silent mutations, 12 of which are shared by all seven mutant alleles (Table 2), and 10 mutations leading to amino-acid changes, all of which are shared by two or more alleles (Table 3). These deviations could be due to variability originally present in the mutagenized population (as indicated by common mutations within each of two different groups: D3 and D14 in one group and D5, D9, D12, D13, and D15 in the other; Table 2) and/or accumulation of mutations in *mei-41* mutants. In addition to these common mutations, a single unique mutation was found for each allele and may account for the phenotype. For $mei\text{-}41^{D3}$, a T → A change at nucleotide 3768 is predicted to convert a nonpolar A to a nonpolar V. We postulate additional mutations in the noncoding region for $mei\text{-}41^{D3}$ because we detect very little protein in these mutants (Sibon *et al.* 1999; Figure 5). The $mei\text{-}41^{D5}$ mutation changes proline^{2159} in the kinase domain to a leucine. This proline is conserved among all members of the PI3K-L family except for UVSB (which has a T) and Ce-atl-1 (which has an A). $mei\text{-}41^{D5}$ shows a partial defect in all assayed phenotypes, suggesting that kinase activity of MEI-41 is important for all its functions. Likewise, mutations in the kinase domain affect all functions known for RAD3 and ATR (Bentley *et al.* 1996; Cliby *et al.* 1998; Martinho *et al.* 1998).

Interestingly, unique mutations in the other five alleles analyzed here fall outside of the kinase domain. Among PI3K-l family members, there is little sequence

TABLE 3

Mutations that are predicted to cause amino acid changes

	mei-41 alleles						
Nucleotide	D3	D5	D9	D12	D13	D14	D15
3096		S → F	S → F	S → F	S → F		S → F
3210		Q → R	Q → R	Q → R	Q → R		Q → R
3332		H → Y	H → Y	H → Y	H → Y		H → Y
3493	E → D					E → D	
7126	L → V					L → V	
2345		L → I		L → I	L → I		
2580		A → V		A → V	A → V		
2585					W → T		
3452		N → H	N → H	N → H	N → H	N → H	N → H
3768	A → V						
4257			S → F				
4328							T → P
4339						L → F	
7606	A → P						A → P
8053				H → Y			
8188	L → G	L → G		L → G	L → G	L → G	
8924		P → L					

Predicted amino acid changes in D3, D5, D9, D12, D13, D14, and D15 alleles are shown. Entries in the top part show variations differentiating D3 and D14 from the other alleles. Single amino acid changes that are unique to each allele are underlined. Mutations common to seven alleles are: 2840, L → M; 2861, Y → H; 3002, M → L; 3530, G → S; 3965, A → T; and 4166, L → V. Standard one-letter code for amino acids is used.

similarity outside of the kinase domain. The exception is a computationally defined region of ~500 amino acids, called the FAT domain that is shared with the TRRAP family of proteins (Bosotti *et al.* 2000). This domain is a part of the rad3 domain, which is conserved only in a subgroup that includes Mec1p, Rad3, ATR, and MEI-41, but not ATM. We find that the *mei-41^{D12}* mutation affects a conserved histidine within the rad3/FAT domain (Figure 1E). *mei-41^{D13}* is a mutation in the N terminus where an algorithm designed to detect DNA-binding domains detects a putative helix-turn-helix, I^{82}MWSAVAKWLDMGCMTRQELKR (Network Protein Sequence Analysis; Dodd and Egan 1990). We find that *mei-41^{D9}*, *mei-41^{D14}*, and *mei-41^{D15}* affect a predicted α-helix in the N terminus (Figure 1D). The *mei-41^{D14}* and *mei-41^{D15}* mutations change, respectively, a leucine to a phenylalanine and a threonine to a proline and are likely to disorganize the putative α-helix (Table 1). *mei-41^{D9}* mutants show a more severe defect in larval mitotic checkpoint than do *mei-41^{D14}* and *mei-41^{D15}* mutants; this suggests that the predicted change of a reactive serine^{647} to a hydrophobic phenylalanine in *mei-41^{D9}* may not only destabilize the putative helix but also specifically disrupt interaction(s) in which this helix participates. In sum, the sequence analysis of partial loss-of-function alleles suggests the importance of N-terminal sequences and identifies mutations that presumably affect one phenotype more severely than another.

DISCUSSION

A large body of work indicates that ATM/ATR homologs play a central role in DNA checkpoints in eukaryotes. Loss of ATM/ATR function leads to pleiotropic defects, which in humans range from sterility and mental retardation to increased killing by ionizing radiation. The diversity of these phenotypes is recapitulated in model organisms such as Drosophila, which are amenable to genetic manipulation and provide an opportunity to understand the function of these key molecules. A question of interest is whether the pleiotropic nature of mutations in ATM/ATR homologs is due to the participation of these molecules in different molecular pathways or their participation in a single signal transduction pathway that is important for several cellular processes. Our identification of several alleles of *mei-41* that affect some phenotypes more severely than others is consistent with the function of *mei-41* in several molecular contexts.

ATR homologs act to stall mitosis in response to two types of DNA defects, namely, incompletely replicated DNA and damaged DNA. In Drosophila, *mei-41* is required to stall mitosis when DNA replication is blocked experimentally during embryonic cleavage divisions (Sibon *et al.* 1999), which occur in a syncytium and are driven by maternally supplied gene products. Embryos from homozygous mothers of strong *mei-41* alleles such as D3 and 29D do not progress beyond syncytial cycles

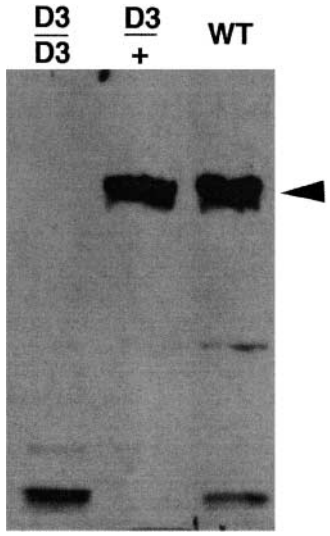


FIGURE 5.—Western blot analysis of *mei-41*^{D3} mutants. Extracts from 1- to 5-hr-old embryos from homozygous *mei-41*^{D3} mutant mothers (D3/D3) and heterozygous *mei-41*^{D3}/FM7 mutant mothers (D3/+) or 0- to 2-hr-old wild-type Sevelen (WT) embryos were analyzed by Western blotting using an affinity-purified antibody against MEI-41. Equal loading among lanes was ensured by controlling for the number of embryo-equivalent material loaded (100 per lane). On the basis of the location of size markers (MATERIALS AND METHODS), the band that is present in WT and *mei-41*^{D3} heterozygotes and absent in *mei-41*^{D3} homozygotes is calculated to have a MW of 242 kD (arrowhead) and is presumed to represent the MEI-41 protein (predicted MW of 258 kD).

(SIBON *et al.* 1999). Therefore, in the absence of meiotic defects, female sterility may be attributed to the failure of syncytial divisions. Syncytial division defects have been proposed to occur due to a failure to delay mitosis in the presence of ongoing DNA replication during these rapid division cycles (SIBON *et al.* 1999).

According to the above discussion, *mei-41*^{D12} and *mei-41*^{D13} mutants, which present a wild-type level of syncytial division function (because they are female fertile) but are unable to regulate metaphase/anaphase in cellularized embryos or G_2/M transition in embryos and larvae following DNA damage, may be responsive to incompletely replicated DNA replication but not to damaged DNA. This could be because syncytial divisions simply require less MEI-41 activity than larval and embryonic checkpoints do. Indeed, 29D heterozygotes, which presumably have wild-type MEI-41 but at reduced levels, have normal female fertility but an ~50% loss of larval G_2/M checkpoint (Figure 3 legend). If so, however, we would expect *mei-41*^{D14} mutants that show a more severe fertility phenotype to be more defective than *mei-41*^{D12} or *mei-41*^{D13} mutants for larval mitotic checkpoint. This is not the case. Therefore, we propose, instead, that *mei-41*^{D12} and *mei-41*^{D13} represent separation-of-function alleles that retained normal activity for syncytial divisions but not for DNA damage checkpoints. Conversely, *mei-41*^{D14} allele is compromised for syncytial cycle function while retaining nearly wild-type activity for larval mitotic checkpoints. Such alleles should be potentially useful for identifying genes that interact with *mei-41* in one context but not another. Additionally, all five alleles (*i.e.*, all except D3 and D5) show a wild-type level of meiotic function as previously described and yet show defects in mitotic cycles during embryogenesis and larval development. Thus, all represent separation-of-function alleles that have normal meiotic function but are defective for regulation of mitotic proliferation.

Interestingly, *mei-41*^{D15} mutants that have a wild-type level of mitotic checkpoint are more MMS sensitive than *mei-41*^{D12} mutants that have a severely defective mitotic checkpoint. Likewise, *mei-41*^{D14} and *mei-41*^{D15} mutants show significantly different levels of female fertility (thus, possibly different DNA replication checkpoint activity) and yet have similar MMS sensitivity. We propose that defects in cell cycle regulation cannot fully explain the MMS sensitivity of *mei-41* mutants. It is likely that the role of *mei-41* in DNA repair, cell death, and other yet-to-be-characterized processes contributes to MMS sensitivity.

One key question concerning checkpoint proteins is whether they are essential for viability in unperturbed cell cycles or they are essential only in the presence of genetic aberrations. Some PI3K-l proteins are essential for cellular viability, while others are not. *mec1* deletion mutants of budding yeast are inviable and *ATR* mutant mice die after the blastocyst stage (KATO and OGAWA 1994; BROWN and BALTIMORE 2000). However, the *S. pombe rad3*Δ strain, in which *rad3* is disrupted, is viable and the *ATM* mutations observed to date in mice are fully viable (JIMENEZ *et al.* 1992; SEATON *et al.* 1992; BARLOW *et al.* 1996; ELSON *et al.* 1996; XU and BALTIMORE 1996). We find that null mutants of *mei-41* are viable as homozygous mutant flies are produced from heterozygous parents. It is possible, however, that homozygous *mei-41* mutant progeny survive due to a supply of wild-type MEI-41 deposited into the eggs by heterozygous mothers. Because embryos from homozygous mutant females fail to progress beyond cleavage divisions (SIBON *et al.* 1999), *mei-41* does have an essential role in early embryogenesis.

Interestingly, heterozygotes for the null allele of *mei-41* show checkpoint defects and MMS sensitivity. Heterozygous phenotypes have been described for *ATM*-deficient cells of human and mouse, including increased sensitivity to killing by mutagens, defective cell cycle checkpoints, and chromosome aberrations, among others (NAEIM *et al.* 1994; SCOTT *et al.* 1994; TCHIRKOV *et al.* 1997; DJUZENOVA *et al.* 1999). In mice, heterozygous *ATR*ES cells did not display increased sensitivity to DNA-damaging agents although other phenotypes such as cell cycle regulation remain to be assayed (DE KLEIN *et al.* 2000). Haplo-insufficiency of ATM/ATR homologs for checkpoint regulation may explain why heterozygous mutant mice display increased tumor incidence (BROWN and BALTIMORE 2000). This notion remains

controversial, however, because recent work suggests that the ATM heterozygotes in question may harbor a mutant allele that acts in a dominant negative manner to inhibit the remaining wild-type allele (Spring *et al.* 2002). Our null allele, *mei-41*^{29D}, is predicted to encode only 39 aa and is therefore unlikely to produce a dominant negative MEI-41. As such, MEI-41 may be truly haplo-insufficient for optimal checkpoint regulation.

We document here a novel role for *mei-41* in the regulation of metazoan mitotic progress. DNA damage blocks mitosis but the exact mitotic step blocked can differ from cell type to cell type (Elledge 1996). In fission yeast, the entry into mitosis is blocked whereas in budding yeast, chromosome segregation and metaphase/anaphase transition are blocked. Drosophila and human cells, on the other hand, appear capable of blocking both the entry into mitosis and the metaphase/anaphase transition (Smits *et al.* 2000; Su *et al.* 2000; Su and Jaklevic 2001). The role of *mei-41* in blocking the entry into mitosis after DNA damage has been documented before, but this report documents for the first time that an ATM/ATR homolog is needed to block mitotic progression in metazoa. Moreover, our results rule out the possibility that damaged chromosomes present a physical barrier to their separation and consequently delay anaphase. Rather, the delay of anaphase is more likely to be an active response since it requires a checkpoint gene.

We describe here several mutations that fall outside of the kinase domain but appear to affect *mei-41* function profoundly. Although unique mutations described here are most likely culprits for the phenotype of each allele, we cannot rule out the contribution of other mutations present within or without the *mei-41* coding region. Site-directed mutagenesis to obliterate domains implicated by our data would be valuable in addressing this issue unequivocally. Nonetheless, in fission yeast, N terminus of RAD3 and, in particular, a putative leucine zipper and a putative protein-protein interaction domain can confer dominant functions when overexpressed (Chapman *et al.* 1999). In humans, N-terminal 247 aa of ATM are required for interaction with p53 *in vitro* (Khanna *et al.* 1998). Thus, N-terminal sequences may contribute to the function of ATM/ATR family members via protein-protein interaction. Interestingly, in two-hybrid assays, a MEI-41 N-terminal fragment containing this helix interacts with MUS304, a protein needed for larval DNA damage checkpoint and a homolog of mammalian ATRIP proteins (Brodsky *et al.* 2000; M. Brodsky, personal communication).

In conclusion, this study demonstrates that *mei-41* null mutants are viable and show dose-sensitive defects in cell cycle checkpoints and MMS sensitivity. Sequence analysis reveals the importance of the kinase domain in all aspects of MEI-41 function and identifies a putative α-helix in the N terminus, which may be important for *mei-41* function in DNA-damage-induced G_2/M checkpoint and syncytial divisions. Two other mutations located in a putative helix-loop-helix in the N terminus and a conserved amino acid in the rad3/FAT domain may be causing defective DNA-damage checkpoint while sparing syncytial division functions. These possibly represent separation-of-function alleles of *mei-41*, which may be useful in screens for interacting genes. The fact that not all phenotypes are affected equally by the five alleles we studied is consistent with the idea that *mei-41* operates in many different molecular contexts to carry out its many functions. There is precedent for this idea because *grp*, a Drosophila *chk1* homolog, that is thought to function downstream of *mei-41*, appears to do so in regulation of mitosis but not of meiosis (Sibon *et al.* 1999; McKim *et al.* 2000). Our data suggest that even in mitotic cycles, signaling networks in which *mei-41* participates may not be rigid, but change at different stages in development (syncytial *vs.* larval) or in response to different kinds of DNA defects (DNA damage *vs.* incomplete replication).

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