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Drosophila Chk2 is required for DNA damage-mediated cell cycle arrest and apoptosis

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Abstract Chk2 is a major target of ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR). Germline mutations in Chk2 have been identified in a subset of patients with Li–Fraumeni syndrome, suggesting that Chk2 is a tumor suppressor gene. To investigate the role of Chk2 in multicellular organisms, a *Drosophila chk2* (*Dmchk2*) mutant was generated. *Dmchk2* mutants are viable but show defects in maintaining genome stability and are highly sensitive to ionizing radiation. Interestingly, mutating *Dmchk2* completely blocks DNA damage-induced apoptosis and partially blocks DNA damage-induced cell cycle arrest. These results indicate that Chk2 protein plays a crucial role in the DNA damage response pathway mediating cell cycle arrest and apoptosis, and that the ATM-Chk2 pathway is likely conserved in *Drosophila*.

Key words: Apoptosis; Cell cycle checkpoint; Genome stability; *Drosophila* Chk2

1. Introduction

Upon DNA damage or replication block, eukaryotic cells activate checkpoint pathways that delay cell cycle progression. Defects in such controls lead to genomic instability and a predisposition to cancer [1,2]. In mammals, the main transducers of the cell cycle checkpoint pathways are ataxia telangiectasia-mutated (ATM), ATM- and Rad3-related (ATR), Chk1, and Chk2 (Cds1). ATM controls G1 and G2 arrest through Chk2. Chk2 is phosphorylated on Thr68 by ATM kinase when exposed to ionizing radiation [3,4]. In turn Chk2 phosphorylates Cdc25C on Ser216, which interferes with Cdc25C's ability to activate Cdc2 and cause G2 arrest [5]. Chk2 also phosphorylates the transcription factor p53 on Ser20 and stabilizes p53, and consequently causes G1 arrest [6]. Chk1 plays an essential role in the mammalian DNA damage checkpoint. ES cells lacking Chk1 have a defective G2/M DNA damage checkpoint in response to γ-irradiation. Functions of Chk1 are ATR dependent [7].

The cell cycle checkpoint pathway appears to be conserved in *Drosophila*. *Drosophila* homologs of the ATM/ATR (mei-41) and Chk1 (grapes) kinases have been identified [8,9]. *mei-41* cells are extremely sensitive to ionizing radiation, display high levels of mitotic chromosome instability, and fail to show radiation-induced cell cycle arrest [8]. grapes (grp) functions in regulating the DNA replication/damage checkpoint during the late syncytial divisions. Embryos derived from *grp* mutant females enter mitosis with damaged or incompletely replicated DNA [9]. A *Drosophila* p53 homolog has also been identified, which is required for the apoptotic response to DNA damage. Interestingly, inhibition of p53 activity by the expression of dominant negative forms of *Drosophila* p53 blocks radiation-induced apoptosis but does not affect X-ray-induced cell cycle arrest [10–12].

Drosophila chk2 protein (also called Dmnk protein) was previously identified [5,13,14]. It is highly expressed in ovaries and germ cell nuclei during early embryogenesis, suggesting a possible function in meiosis or germline establishment [15]. However, it is not clear if *Drosophila chk2* functions in the cell cycle checkpoint pathway in vivo. Recently studies of *Caenorhabditis elegans chk2* using RNAi showed that *Cechk2* mutants are defective in meiosis but retain a DNA damage checkpoint in response to replication inhibition and ionizing radiation [14,16,17]. Those studies of the *C. elegans chk2* raise the question about the role of Chk2 in the cell cycle checkpoint pathway and during normal development in multicellular organisms. To address this question, we generated and characterized *Drosophila chk2* mutant.

2. Materials and methods

2.1. Molecular analysis of Dmchk2

Dmchk2 cDNA was synthesized using an enhanced avian reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Sigma). Single strand cDNA was synthesized from total RNA of w1118 by RT-PCR with a primer 5′-CGGATTTCCTAGCGTCGCGATCGCTTGGT-3′. RNA was extracted using Trizol (Gibco). For Southern and Northern blots, *Dmchk2* cDNA was labeled with [^{32}P]dCTP using random primer labeling method. To determine the exact sequence that was deleted in our *Dmchk2* mutant, the genomic sequence containing the *chk2* and *barren* genes was PCR amplified and was sequenced using the rhodamine sequencing kit (Perkin-Elmer, Norwalk, CT, USA).

2.2. Fly crosses, imprecise excision, and generation of barren and Dmchk2 rescue flies

All flies were maintained at 25°C unless stated otherwise. The original P element insertion between *Dmchk2* and *barren* (*P1117*), grp^{1}, $mei\text{-}41^{RT1}$ and mwh^{1} were obtained from the Bloomington stock center. *P1117* was excised by crossing with a fly stock carrying transposase Δ2-3. 164 P element excision lines (loss of the red eye color) were established and analyzed for deletions of the *Dmchk2* genomic region.

Since the deletion lines showed deletions of both *barren* and *Dmchk2* sequences, to generate a clean *Dmchk2* mutant that has normal *barren* function (a gene located next to *Dmchk2*), a *barren* rescue construct was generated as described below: *barren* genomic DNA was amplified by PCR using Herculase enhanced polymerase (Strata-

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gene, La Jolla, CA, USA) with the following primers: forward 5′-CCGCTCGAGGTAGCCCCGTAATCTCGTATTCCTGCTATT-3′, reverse 5′-CCGCTCGAGCAGAACAATGCTTCGTTTGTG-3′. The PCR product was cloned into pCaSpeR4, and flies bearing the *barren* rescue construct were generated by P element-mediated transformation.

The *Dmchk2* rescue construct was generated by cloning the *Dmchk2* cDNA into the pCaSpeR-hs vector followed by P element-mediated transformation. To rescue *Dmchk2* mutant phenotypes, third instar larvae of the genotype *Dmchk2; P[W, barren]/P[W, HsDmchk2]* were either mock heat shocked or heat shocked at 37°C for 2 h before irradiation. The mock heat shocked control shows the same phenotypes as the *Dmchk2* mutant.

2.3. Cell death assay

To analyze radiation-induced cell death, late third instar larvae were mock treated or irradiated in an X-ray machine (RS 2000) at 4000 rad. After 4 h at room temperature, wing discs were dissected and stained with acridine orange (1 μg/ml in PBS), a dye that stains apoptotic cells. The stained discs were visualized with a fluorescence microscope using the FITC channel.

2.4. Staining for mitotic cells

To analyze radiation-induced cell cycle arrest, late third instar larvae were mock treated or irradiated with X-ray at 4000 rad. After 1 h at room temperature, wing discs were dissected and fixed in 4% paraformaldehyde in PBS for 30 min. Discs were incubated with an antibody against phosphorylated histone H3, an antibody that specifically stains mitotic cells. Immunohistochemical staining was carried out as described previously [18].

2.5. Radiation sensitivity and genome stability assay

Early third instar larvae of the following genotypes: *Dmchk2; mwh/P[W,barren]*, and *Dmchk2/+; mwh/P[W,barren]* were either mock treated or treated with 3 Gy of radiation. Wings were mounted in Gary's magic mounting medium [19]. Intervein wing hairs of eight wings were examined for each genotype. For radiation sensitivity assays, wandering third instar larvae of *Dmchk2* mutant or heterozygotes were treated with the indicated doses of radiation (5–40 Gy). The survival rate to adulthood of irradiated larvae were scored.

3. Results and discussion

3.1. Generation of Dmchk2 mutant

Imprecise excision of the P element that inserted near *Dmchk2* was carried out. A total of 164 excision lines were established, among which 93 lines appear to be precise excision by Southern analysis (data not shown). These lines were viable and showed no deletions of the *Dmchk2* coding sequences (data not shown). All the remaining lines were homozygous lethal. Four lines were identified that appear to have deletion of the *Dmchk2* coding sequence by Southern blot analysis. As shown in Fig. 1, insertion of a P element between *Dmchk2* and *barren* leads to the appearance of a 3.6 kb fragment. In the E51 line, this 3.6 kb fragment was shifted to 2.0 kb, suggesting the deletion of sequences near *Dmchk2* took place. Sequencing analysis revealed the deletion in line E51 removes the start of the open reading frame as well as all of the promoter sequences of both *Dmchk2* and *barren* genes. To generate flies with mutation in *Dmchk2* but not *barren*, we generated a *barren* genomic rescue construct. Introduction of the barren rescue construct completely rescued the lethality of our E51 deletion line, indicating that the lethality of the E51 line we observed is due to deletion of *barren* function. Consistent with the idea that the rescued E51 line is a mutant for *Dmchk2*, no expression of *Dmchk2* was detected in rescued female while the *Dmchk2* message was abundant in wild-type control (Fig. 1C). In summary, these results show that the *barren*-rescued E51 line is an allele of *Dmchk2* mutant.

3.2. Dmchk2 mutant is sensitive to irradiation and failed to prevent genomic instability

Studies of *Cechk2* suggested that *Cechk2* is essential for meiosis but dispensable for DNA damage checkpoint control [14,16,17]. To determine if Dmchk2 in *Drosophila* may have a function in DNA damage checkpoint control, radiation sensitivity of *Dmchk2* mutant larvae was compared to *Dmchk2* heterozygotes. As shown in Fig. 2A, treatment of *Dmchk2* mutant larvae leads to a significantly higher number of larvae or pupae lethality compared to *Dmchk2* heterozygote. This result suggests that *Dmchk2* mutants are more sensitive to ionizing radiation. In addition, we tested if *Dmchk2* mutants show increased genome instability using the loss of heterozygosity (LOH) assay [20]. As expected, mutation of *Dmchk2* leads to a much higher LOH rate. Furthermore, the rate of LOH is increased upon low dose (3 Gy) IR challenge (Fig. 2B). These phenotypes of *Dmchk2* mutants suggest that *Drosophila* Chk2 has an important role in maintaining genome stability and in DNA damage checkpoint response.

3.3. Dmchk2 mutation blocks irradiation-induced apoptosis

During *Drosophila* development, DNA damage leads to increased cell death and cell cycle arrest in wing discs. To further characterize the role of Dmchk2 in the response to DNA damage, we tested the effect of the *Dmchk2* mutation on ap-

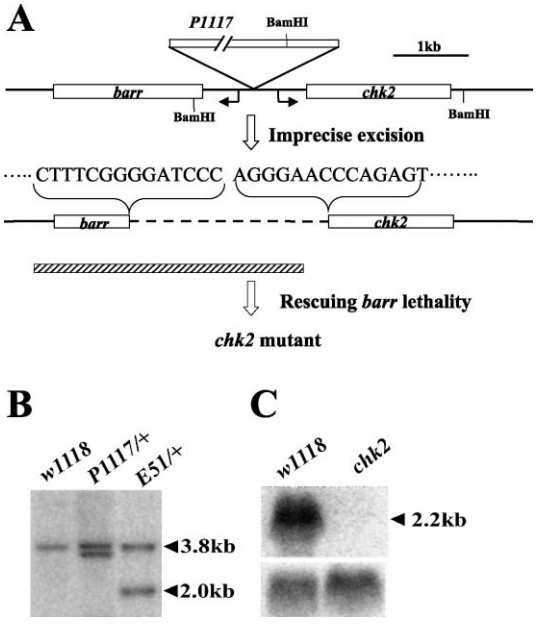

Fig. 1. Generation of *Dmchk2* mutant. A: A diagram of the *Dmchk2* genomic locus and the strategy used to generate the *Dmchk2* mutant. A P element (*P1117*) is inserted between the *Dmchk2* and *barren* (*barr*) gene. Open bars indicate coding sequence; arrows indicate the start of transcription, and a stripped bar represents genomic DNA used to rescue the *barr* mutation. B: A southern blot analysis using *Dmchk2* cDNA as probe. As predicted from genomic sequences diagramed in A, a 3.8 kb genomic fragment was found to hybridize with *Dmchk2* cDNA in wild-type flies. Insertion of the original P element (*P1117*) results in the appearance of a slightly smaller fragment, which is further reduced in an excision line (E51) that shows deletion of the *Dmchk2* coding sequences. C: A Northern blot that shows the 2.2 kb *Dmchk2* transcript was detected from wild-type female RNA but not from *Dmchk2* mutant female RNA. The same blot was reprobed with RBF cDNA as loading control.

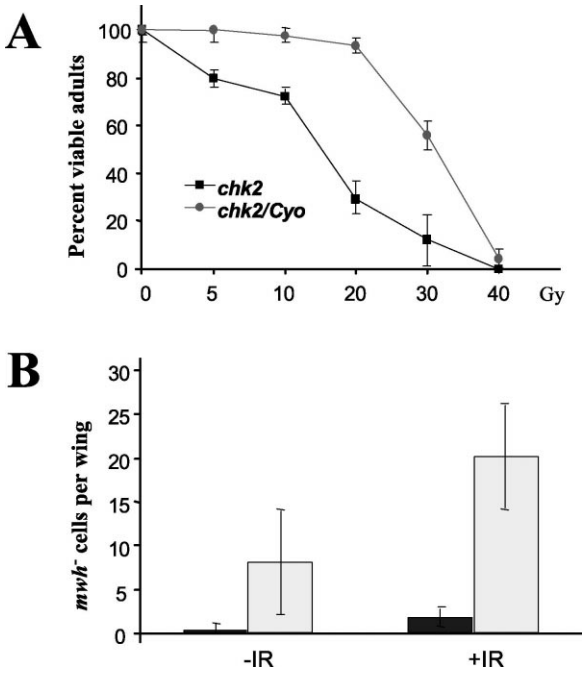

Fig. 2. *Dmchk2* mutants show increased radiation sensitivity and genomic instability. A: Lethality of *Dmchk2* mutants is increased after irradiation. Percent viable adults developed from *Dmchk2* mutant (■) or *Dmchk2* heterozygotes (●) were shown after the third instar larvae of each genotype were treated with the indicated amount radiation. B: *Dmchk2* mutants show increased genomic instability. The average number of mwh$^-$ cells in wings from the *Dmchk2* mutant (dotted bar) or from the *Dmchk2* heterozygotes (filled bar) were shown. With or without IR treatment, *Dmchk2* mutants show an increased number of mwh$^-$ cells.

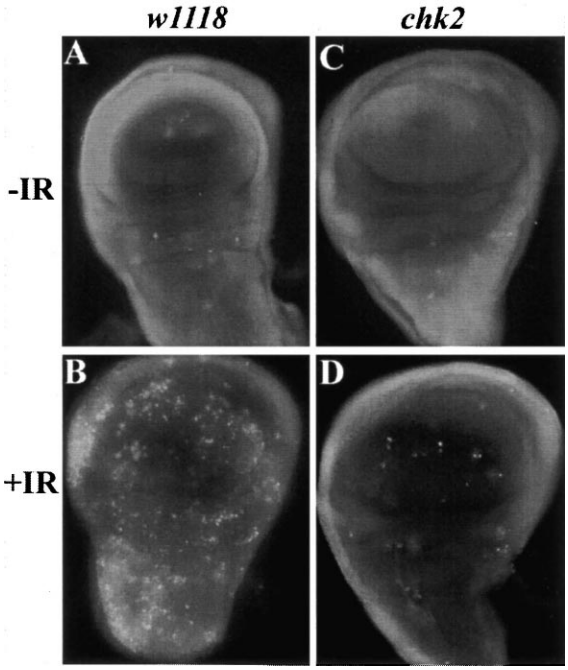

Fig. 3. *Dmchk2* mutants block radiation-induced apoptosis. Acridine orange staining was used to visualize cells that are undergoing apoptosis. In wild-type wing discs, there are a very few apoptotic cells without treatment (A), a large number of apoptotic cells are observed after treatment with X-ray irradiation (B). In contrast, wing discs from *Dmchk2* mutants show little increase in the number of apoptotic cells (C, D).

optosis and cell cycle arrest. Apoptotic cells can be visualized by the dye acridine orange (AO). In wild-type wing discs, DNA damage leads to significant increase in the amount of apoptosis (Fig. 3A, B). In contrast, no significant increase in apoptosis was observed in *Dmchk2* mutants upon irradiation (Fig. 3C, D). To demonstrate that the observed block of increased apoptosis is due to lack of *Drosophila chk2* function, we expressed Dmchk2 protein in the *Dmchk2* mutant background upon irradiation. As shown in Fig. 5, expression of Dmchk2 rescues the apoptosis block in *Dmchk2* mutants after irradiation (Fig. 5C). These results indicate that the Dmchk2 protein is required for radiation-induced apoptosis in the fly. It has been shown previously that expression of *Drosophila* dominant negative p53 in the wing discs also blocks radiation-induced apoptosis [10,11]. Thus our observations are sugges-

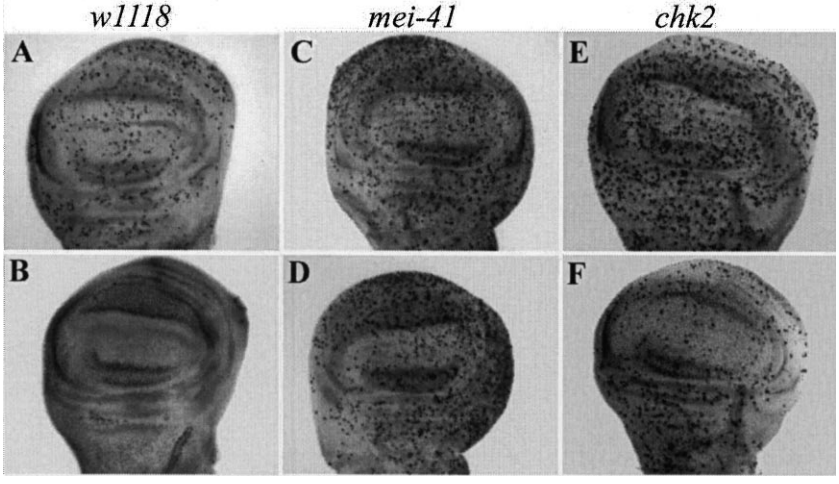

Fig. 4. *Dmchk2* mutants show partial loss of radiation-induced cell cycle checkpoint. Third instar larvae were either mock treated (A, C, E) or treated with X-ray irradiation (B, D, F). Wing disc cells in mitosis were visualized by staining with the anti-phospho H3 antibody. Without IR treatment, all the wing discs have large number of cells in mitosis (A, C, E). After IR treatment, no mitotic cells are found in wild-type wing discs (B); while the number of mitotic cells in *mei-41* mutant wing discs are similar to the unirradiated wing discs (D). There is a significant number of mitotic cells in the *Dmchk2* mutant wing discs, although the number of mitotic cells is reduced compared to the mitotic cells in unirradiated wing discs (F).

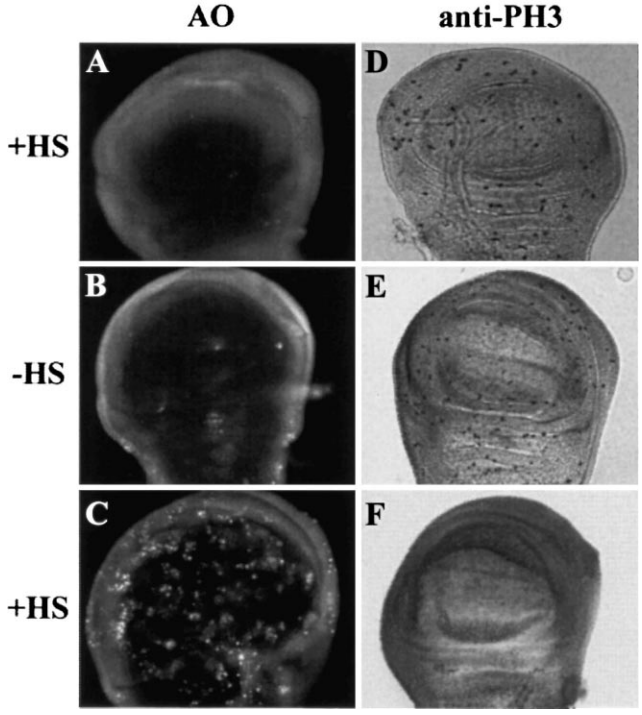

Fig. 5. Expression of wild-type Dmchk2 rescues the *Dmchk2* mutant phenotypes. Expression of wild-type Dmchk2 protein by heat shock restores the radiation-induced cell death in the *Dmchk2* mutant wing discs (C) as well as cell cycle arrest (F). As control, the *Dmchk2* mutants carrying the heat shock *Dmchk2* transgene without heat shock (B, E) or the *Dmchk2* mutants with heat shock (A, D) show the same phenotype as normal *Dmchk2* mutants (see Figs. 3 and 4).

tive that *Drosophila* Chk2 functions in the same pathway as p53 in radiation-induced apoptosis. These results, in conjunction with the observations in mammalian systems that *chk2* null ES cells exhibit resistance to apoptosis in response to DNA damage due to loss of p53 activation [21], suggest the Chk2-p53 pathway is likely conserved between *Drosophila* and mammals in response to DNA damage.

3.4. *Dmchk2 mutation partially blocks irradiation-induced cell cycle arrest*

To examine the effects of *Dmchk2* mutation on radiation-induced cell cycle arrest, we analyzed the ability of cells to enter mitosis after irradiation. As shown previously, wild-type wing disc cells are arrested after irradiation (Fig. 4A and B). In contrast, wing discs from *mei-41* mutant larvae do not show cell cycle block, as there is a similar number of cells in M phase as in non-irradiated wing discs (Fig. 4C and D). Interestingly, while there are significant numbers of cells that enter mitosis upon irradiation of *Dmchk2* mutant wing discs, the numbers are much fewer than those observed in *mei-41* discs or in unirradiated *Dmchk2* discs (Fig. 4E and F). These results indicate that Dmchk2 protein is required for complete cell cycle arrest following DNA damage. To demonstrate the observed defect in cell cycle arrest is due to lack of Dmchk2 function, rescue experiments were carried out to test if expression of Dmchk2 can restore the cell cycle arrest upon irradiation. As shown in Fig. 5, expression of Dmchk2 in the *Dmchk2* mutant background rescues the bypass of radiation-induced cell cycle arrest in *Dmchk2* mutants (Fig. 5F).

A null mutation of the *Drosophila chk1* homolog *grp* also has a partial checkpoint defect [20], similar to the partial DNA damage-induced cell cycle arrest found in *Dmchk2* mutants. In contrast, *mei-41* mutants show a complete defect in cell cycle check arrest after DNA damage. These observations are consistent with the idea that *Dmchk2* and *grp* both function downstream of *mei-41* to bring about the cell cycle arrest. In contrast to the effect of *Dmchk2* in radiation-induced apoptosis, we found that the *grp* mutant has no effect in IR-induced cell death (unpublished data), suggesting that Dmchk2 is the only target of mei-41 that mediates radiation-induced apoptosis, while both *Dmchk2* and *grp* function in the DNA damage-induced cell cycle arrest pathway.

The cell cycle arrest function of *Drosophila* Chk2 is not likely to be mediated through Dmp53 since it was shown previously that overexpression of dominant negative Dmp53 doesn't block IR-induced cell cycle arrest [10]. In yeast, both Chk1 and Cds1 can phosphorylate Cdc25 to inhibit the activation of Cdc2 kinase activity [22–25]. In mammals, it seems that hChk1 and hCds1(Chk2) have redundant functions in regulating DNA damage-induced cell cycle arrest by phosphorylating both p53 and Cdc25 [5,6,26]. Considering the conservation of the checkpoint pathway, a likely target of *Drosophila* Chk2 for the DNA damage-induced cell cycle arrest is the *Drosophila* Cdc25 homolog string.

Our results demonstrate that *Drosophila* Chk2 plays a crucial role in IR-induced apoptosis and cell cycle arrest. Loss of this DNA damage checkpoint leads to genomic instability and high radiation sensitivity. Since the ATM-Chk2 pathway is well conserved in *Drosophila*, it provides the possibility to use this model in cancer research.

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