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Grp/DChk1 is required for G₂-M checkpoint activation in *Drosophila* S2 cells, whereas Dmnk/DChk2 is dispensable

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Summary

Cell-cycle checkpoints are signal-transduction pathways required to maintain genomic stability in dividing cells. Previously, it was reported that two kinases essential for checkpoint signalling, Chk1 and Chk2 are structurally conserved. In contrast to yeast, *Xenopus* and mammals, the Chk1- and Chk2-dependent pathways in *Drosophila* are not understood in detail. Here, we report the function of these checkpoint kinases, referred to as Grp/DChk1 and Dmnk/DChk2 in *Drosophila* Schneider's cells, and identify an upstream regulator as well as downstream targets of Grp/DChk1. First, we demonstrate that S2 cells are a suitable model for G₂/M checkpoint studies. S2 cells display Grp/DChk1-dependent and Dmnk/DChk2-independent cell-cycle-checkpoint activation in response to hydroxyurea and ionizing radiation. S2 cells depleted for Grp/DChk1 using RNA interference enter mitosis in the presence of impaired DNA integrity, resulting in prolonged mitosis and mitotic catastrophe. Grp/DChk1 is phosphorylated in a Mei-41/DATR-dependent manner in response to hydroxyurea and ionizing radiation, indicating that Mei-

41/ATR is an upstream component in the Grp/DChk1 DNA replication and DNA-damage-response pathways. The level of Cdc25^{Stg} and phosphorylation status of Cdc2 are modulated in a Grp/DChk1-dependent manner in response to hydroxyurea and irradiation, indicating that these cell-cycle regulators are downstream targets of the Grp/DChk1-dependent DNA replication and DNA-damage responses. By contrast, depletion of Dmnk/DChk2 by RNA interference had little effect on checkpoint responses to hydroxyurea and irradiation. We conclude that Grp/DChk1, and not Dmnk/DChk2, is the main effector kinase involved in G₂/M checkpoint control in *Drosophila* cells.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/118/9/1833/DC1>

Key words: Chk1, Chk2, Hydroxyurea, Ionizing radiation, RNA interference

Introduction

Cell-cycle checkpoints are regulatory pathways that monitor the integrity and replication status of the genome before cells commit to either replicate or segregate their DNA (Hartwell and Weinert, 1989). One of the evolutionarily conserved genes involved in checkpoint control is *Checkpoint kinase 1* (*Chk1*). *Chk1* was originally identified in fission yeast (*Schizosaccharomyces pombe*) as an essential component of the DNA-damage checkpoint (Al-Khodairy et al., 1994; Walworth et al., 1993). Homologues of Chk1 have also been found in other species, such as budding yeast (*Saccharomyces cerevisiae*) (Sanchez et al., 1999), *Caenorhabditis elegans* (Brauchle et al., 2003), *Drosophila melanogaster* (Fogarty et al., 1997; Sibon et al., 1997), *Xenopus* (Kumagai et al., 1998; Nakajo et al., 1999), mice (Flaggs et al., 1997) and human cells (Sanchez et al., 1997). Although Chk1 is highly conserved throughout evolution, signals that Chk1 respond to have diverged in eukaryotes.

In fission yeast, the DNA-damage pathway requires Chk1

(Al-Khodairy et al., 1994; Walworth et al., 1993) and Chk1 is phosphorylated in response to DNA damage (Walworth and Bernards, 1996). In budding yeast, Chk1 is not required for checkpoint control in response to DNA damage or incompletely replicated DNA when cells are grown under normal conditions (Sanchez et al., 1999). In contrast to the function of Chk1 in budding yeast, Chk1 plays a role in both the DNA-damage and the DNA-replication checkpoints in *Xenopus* and mammals (Guo et al., 2000; Kumagai et al., 1998; Liu et al., 2000; Melo and Toczyski, 2002; Sanchez et al., 1997; Takai et al., 2000).

Upon DNA damage, fission-yeast Chk1 is activated in a manner dependent on the function of several Rad gene products including Rad3 (Walworth and Bernards, 1996). In budding yeast, phosphorylation of Chk1 requires Mec1 (Sanchez et al., 1999), whereas, in *Xenopus* and mammals, Chk1 is mainly regulated by the ATR (ATM- and Rad3-related) signalling pathway (Guo et al., 2000; Heffernan et al., 2002; Hekmat-Nejad et al., 2000; Liu et al., 2000; Zhao and Piwnicka-Worms,

2001). In fission yeast, *Xenopus* and mammals, cell-cycle arrest is mediated by phosphorylation of the dual-specific protein phosphatase Cdc25 by Chk1 (Furnari et al., 1997; Kumagai et al., 1998; Peng et al., 1997; Sanchez et al., 1997; Zeng et al., 1998). Phosphorylated Cdc25 is inactive and unable to dephosphorylate and activate Cdc2, resulting in cell-cycle arrest (Donzelli et al., 2002; Smits and Medema, 2001).

The *Drosophila* Chk1 homologue *grp/Dchk1* was identified as a maternal effect gene required for normal cell-cycle progression during nuclear divisions that precede cellularization in the embryo. In addition, Grp/DChk1 is required for the maternal to zygotic transition (MZT) and the onset of zygotic transcription, an event analogous to the mid-blastula transition (MBT) of vertebrate embryos (Fogarty et al., 1997; Sibon et al., 1997). During larval development, *grp/Dchk1* homozygous mutants are more sensitive to hydroxyurea (HU) (Sibon et al., 1997; Sibon et al., 1999) and methyl methanesulfonate (MMS) (Sibon et al., 1999) than wild-type larvae, although exactly how Grp/DChk1 is required to survive these genotoxins remains to be elucidated and it is currently unknown whether Grp/DChk1 is activated and modified in response to HU or MMS. Mutations in the *Drosophila* ATR homologue *mei-41* (Mei-41/DATR) (Laurencon et al., 2003) show genetic interaction with *grp/Dchk1* mutations with regard to MMS and HU sensitivity, suggesting (but not proving) that the two genes function in the same checkpoint pathway (Sibon et al., 1999).

Another evolutionarily conserved gene involved in checkpoint control is *checkpoint kinase 2* (*chk2*). The Chk2 homologue in budding yeast (Rad53) is phosphorylated and activated in a Mec1-dependent manner in response to DNA-damage and -replication defects induced by HU (Allen et al., 1994; Sanchez et al., 1996; Sun et al., 1996). In fission yeast, Chk1 is the main effector kinase of the DNA-damage-response pathway, whereas the Chk2 homologue, Cds1, is the main effector of the replication-checkpoint pathway (Boddy and Russell, 1999; Murakami and Okayama, 1995). This indicates checkpoint signal specificity of *chk1* and *cds1/chk2* in fission yeast. Other data, however, show that the functions of Chk1 and Cds1/Chk2 partly overlap because, in the absence of Cds1/Chk2, Chk1 is phosphorylated and mediates a checkpoint arrest in response to incomplete DNA replication (Boddy et al., 1998; Lindsay et al., 1998; Murakami and Okayama, 1995). In mammalian cells, Cds1 is phosphorylated by ATM in response to ionizing radiation (IR) (Brown et al., 1999; Chaturvedi et al., 1999; Matsuoka et al., 1998; Tominaga et al., 1999) and is required for IR-induced stabilization of p53 (Hirao et al., 2000). ATM-independent phosphorylation of Cds1 occurs in response to ultraviolet, HU (Chaturvedi et al., 1999; Matsuoka et al., 1998) and MMS (Tominaga et al., 1999). It is currently unknown whether, in mammalian cells, the function of Chk1 and Chk2 homologues partially overlap, like the function of Chk1 and Cds1 in fission yeast.

In *Drosophila*, the maternal nuclear kinase (Dmnk) protein is the homologue of mammalian Chk2 (referred to as Dmnk/DChk2) (Oishi et al., 1998) and is required for IR-induced modification of p53 (Brodsky et al., 2004; Peters et al., 2002). *Dmnk/Dchk2* mutant larvae are more sensitive to IR (but not to HU or MMS) than wild-type larvae (Masrouha et al., 2003; Xu et al., 2001). During *Drosophila* embryogenesis, Dmnk/DChk2 is involved in a DNA-damage checkpoint

induced by IR (Masrouha et al., 2003). Other than p53, no upstream components or downstream targets of Dmnk/DChk2 are known in *Drosophila*.

Taken together, in contrast to yeast, *Xenopus* and mammals, the Chk1- and Chk2-dependent pathways in *Drosophila* are not understood in detail. A relatively straightforward cellular system to investigate this is currently lacking and will be valuable for improving our understanding of cell-cycle regulation. Here, we first tested whether *Drosophila* S2 cells are a valuable model to study G₂/M checkpoint regulation. After establishing this, we examined the role of Grp/DChk1, Dmnk/DChk2 and Mei-41/DATR in checkpoint regulation. Finally, we investigated the cellular consequences of defective checkpoint function in vivo, demonstrating that progression through mitosis in the presence of impaired DNA integrity leads to severe division errors.

Materials and Methods

Fly stocks

Fly stocks were maintained on standard cornmeal-yeast agar medium at 22°C. The *grapes* stock *grp^{fs(A)4}* used in this study is a functional null allele and has been described previously (Fogarty et al., 1997; Sibon et al., 1997). The Oregon-R stock was used as the wild type.

Cell culture and treatment of cells with HU or IR

S2 cells were cultured at 22°C in Schneider's *Drosophila* culture medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, St Louis, MO, USA), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. For experimental use, cells were grown in 35 mm dishes and treated with 10 mM HU (Sigma, St Louis, MO, USA) for the indicated time. Treatment of S2 cells with 150 Gy IR was performed at 0°C using a Philips MGC-41 X-ray machine operating at 200 kV and 10 mA at a dose rate of 27 Gy minute⁻¹. After irradiation, cells were placed at 22°C to recover from irradiation-induced DNA damage for the indicated periods.

Anti-Grp/DChk1 antibody generation

A *grp/Dchk1* cDNA cloned into pGEM was obtained from a published source (Fogarty et al., 1997). The *grp/Dchk1* open reading frame from *MscI* to *EcoRI* sites (corresponding to a sequence from amino acid 261 to beyond the stop codon) was subcloned into pRSETB expression vector cut with *EcoRI* and *PvuII*. *Escherichia coli* BL-21 bacteria were transformed with the resulting plasmid. Grp/DChk1 polypeptide was induced with isopropyl-βD-thiogalactopyranoside (IPTG) and showed the predicted molecular weight. Approximately 0.5 mg polypeptide was used to immunize rabbits commercially (Cocalico Biologicals, Reamstown, PA, USA). Antisera were affinity purified against recombinant Grp/Dchk1 antigen immobilized on nitrocellulose. The nitrocellulose strip carrying ~1 mg antigen was diced into 2 mm squares, blocked with 1% bovine serum albumin (BSA), 0.5% Tween-20 in PBS for 30 minutes, washed with wash buffer (0.1% BSA, 0.05% Tween-20 in PBS) and finally incubated with 1.0 ml crude antisera with 0.5 ml wash buffer for at least 3 hours. The antisera were then removed and the nitrocellulose squares washed quickly three times with wash buffer. The antibody was eluted with 300 µl low-pH elution buffer (5 mM glycine, pH 2.3, 0.5 M NaCl) for 1 minute. The elution was repeated again with 300 µl low-pH elution buffer for 1 minute and 900 µl low-pH elution buffer for 3 minutes. All eluted samples were pooled and the antibody was concentrated and the buffer replaced with PBS using a centrifugal filtration device (Millipore biomax 10K, Billerica, MA, USA).

Western blotting

For SDS-PAGE western-blot analysis of Grp/DChk1, Dmnk, γ -tubulin and CP190, total protein of all samples was loaded to 10% polyacrylamide gels (Minutesi-Protean II, BioRad, CA, USA) supplemented with 1 mM Na_3VO_4 . Western-blot analysis of Cdc25^{Stg} and Cdc2 was performed as previously described (Edgar et al., 1994) using 12.5% polyacrylamide gels (Protean II, BioRad, CA, USA). Cdc25^{Stg} and Cdc2 antibodies were a generous gift from B. A. Edgar. All proteins were transferred to nitrocellulose membranes and probed for the different proteins of interest. S2 cells were lysed in RIPA buffer [1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in PBS with 0.2 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4 and 40 μl proteinase inhibitor cocktail stock (catalogue number 1697498, Roche, Mannheim, Germany)] and sonicated. Lysates were equally diluted in 2 \times sample buffer (0.1 M Trizma base, pH 6.8, 2% SDS, 0.05% bromophenol blue, 4% glycerol, 20% β -mercaptoethanol) and boiled for 5 minutes. Ovaries were dissected in 0.3% Tween-20 in PBS, taken up in 1 \times sample buffer, sonicated and boiled for 5 minutes. Embryos were treated as previously described (Edgar et al., 1994). Grp/DChk1 protein was detected using the rabbit anti-Grapes polyclonal antibody (1:1000 in 3% BSA, 0.1% Tween-20 in PBS, pH 8.0). Dmnk protein was detected using polyclonal rabbit anti-Dmnk-L antibody [generous gift from I. Oishi, Kobe-Shi, Japan (Oishi et al., 1998)] 1:500 in 0.05% Tween-20 in PBS. As loading controls, protein levels of γ -tubulin (T6557, Sigma, St Louis, MO, USA) diluted 1:2000 in 3% BSA, 0.05% Tween-20 in PBS or of the centrosomal protein CP190 (Kellogg et al., 1995) (gift from J. W. Raff, Cambridge, UK) diluted 1:3000 in 5% skimmed milk powder, 0.05% Tween-20 in PBS were detected. Binding of anti-rabbit and anti-mouse secondary antibodies was detected by chemiluminescence (Amersham Biosciences, Little Chalfont, UK). All standard chemicals were provided by Sigma (St Louis, MO, USA).

Immunofluorescence

Cells were plated on 35 mm dishes on cover slips. Cells were washed twice with PBS, fixed for 15 minutes with 3.7% (v/v) formaldehyde (Merck, Germany) in PBS, washed sequentially three times for 5 minutes each with PBS and permeabilized for 15 minutes with 0.2% Triton X-100 in PBS followed by 10 minutes incubation in 100 mM glycine in PBS. Cells were blocked for 30 minutes in 3% BSA in PBS and incubated overnight at 4°C with mouse anti-phosphorylated-histone-H3 (anti-PH3) monoclonal antibody (mAb) (Cell Signaling Technology, Beverly, MA, USA) 1:5000 in 5% BSA, 0.1% Tween-20 in PBS. Cells were washed three times for 10 minutes each with 0.1% Tween-20 in PBS and incubated for 1 hour with CY3-conjugated anti-mouse antibody (Amersham Biosciences, Little Chalfont, UK) diluted 1:500 in 5% BSA, 0.1% Tween-20 in PBS. After three washes with 0.1% Tween-20 in PBS, the DNA was stained for 10 minutes with 0.2 $\mu\text{g ml}^{-1}$ DAPI (Sigma, St Louis, MO, USA). Cover slips were washed three times with PBS and mounted with Citifluor AF1 (Agar Scientific, Stansted, UK). Images were made with a confocal laser-scanning microscope (Leica TCS SP2, Leica microsystems, Heidelberg, Germany).

Flow cytometry

To determine the proportion of mitotic S2 cells by flow cytometry, cells were harvested, washed once with PBS, fixed in 3.7% (v/v) formaldehyde in PBS for 15 minutes, washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 15 minutes. After permeabilization, cells were washed once with PBS followed by overnight incubation with mouse anti-PH3 mAb diluted 1:250 in 5% BSA, 0.1% Tween-20 in PBS at 4°C. Mitotic chromosomes were visualized with secondary goat anti-mouse FITC (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:50 in 5% BSA, 0.1% Tween-20 in PBS. For analysis of cell-cycle distribution, S2

cells were harvested, counted for viability, washed twice with PBS containing 5 mM MgCl_2 and fixed in 2 ml 80% ethanol/acetone (1:1) overnight at 4°C. Cells were washed in 5 ml PBS containing 5 mM MgCl_2 and the pellet was resuspended in 20 μl DNase-free RNase A (Sigma, St Louis, MO, USA) and incubated at 37°C for 30 minutes. Next, 400 μl propidium iodide (PI; Sigma, St Louis, MO, USA) was added (10 $\mu\text{g ml}^{-1}$ in PBS containing 5 mM MgCl_2) and the cells were incubated at 4°C overnight. The DNA content was determined with a FACScalibur (Becton Dickinson, San Jose, CA). Samples were analysed on a Coulter Epics-Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA) with Elite version 4.01 acquisition software; 20,000 events were analysed for each sample. Data analysis was done with WinList (version 5.0; Verity Software House, Topsham, ME, USA).

Conditions for RNA interference in S2 cells

Individual DNA fragments ~700 bp long containing coding sequences for the proteins to be knocked down were amplified as described previously (Clemens et al., 2000). Primer sequences used to generate specific double-stranded (dsRNAs) were obtained as follows (numbers indicate base numbers in the database sequence specified): Grp/DChk1, GenBank accession number AF057041, sense primer 493-509 and antisense primer 1185-1200; Mei-41/DATR, GenBank accession number NM_078645, sense primer 5455-5473 and antisense primer 6152-6170; MNK/DChk2, GenBank accession number NM_165318, sense primer 666-683 and antisense primer 1395-1413. S2 cells were diluted in a final concentration of 1×10^6 cells ml^{-1} in *Drosophila* SFM (Gibco, Paisley, UK) supplemented with L-glutamine (Gibco, Paisley, UK). Cells (1×10^6) were plated in 35 mm dishes and dsRNAs were added directly to the medium to a final concentration of 15 μg per dish. Cells were incubated for 60 minutes at 22°C followed by addition of 2 ml complete Schneider's medium. Cells were incubated for 96 hours to allow for turnover of the proteins of interest.

Time-lapse imaging

Cells were pretreated with dsRNA encoding Grp/DChk1 for 96 hours or not treated. Cells (2.5×10^6) were grown overnight on round cover slips in 2 ml normal growth medium. On the following day, a CaPO_4 transfection procedure was used to transfect S2 cells transiently with pMT-H2B-GFP for 8 hours. The construct pMT-H2B-GFP was created by insertion of a *KpnI-NorI* fragment from pBOS-H2B-GFP (BD Pharmingen, San Diego, CA, USA) into the pMT/V5-HisA vector (Invitrogen, San Diego, CA, USA). After transfection, cells were placed on normal culture medium supplemented with 0.7 M CuSO_4 to allow induction of promoter activity. To keep cells alive during the experiments, cells were cultured at 22°C in 2 ml normal growth medium. Cells were imaged on a 1 minute cycle using a confocal laser-scanning microscope (Leica TCS SP2, Leica microsystems, Heidelberg, Germany).

Results

Drosophila Schneider's S2 cells are a valid model to study G₂-M checkpoint regulation

In order to investigate the function of Grp/DChk1, Dmnk/DChk2 and Mei-41/DATR, we first tested whether *Drosophila* Schneider's S2 (S2) cells could serve as an appropriate model to study G₂-M checkpoint regulation. To this end, S2 cells were treated with the DNA synthesis inhibitor HU or with IR. Mitotic chromosomes were stained with an antibody against the mitosis-specific form of PH3 and the proportion of cells in mitosis was determined by flow

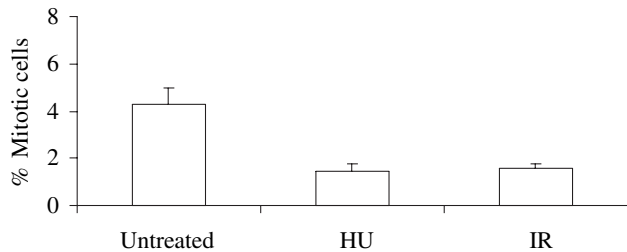


Fig. 1. S2 cells display cell-cycle arrest in response to HU or IR. S2 cells were treated with HU for 15 hours or treated with 150 Gy IR followed by 2 hours of recovery. Cells positively stained for PH3 were counted using flow cytometry. S2 cells showed a decrease in the proportion of mitotic cells when treated with HU (1.5%) or IR (1.6%) compared with untreated cells (4.3%). Error bars represent s.e.m. ($n=13$).

cytometry. HU or IR treatment resulted in a significant reduction in the proportion of mitotic S2 cells compared with untreated S2 cells (Fig. 1). Examination of DNA content using fluorescence-activated cell sorting (FACS) analysis after PI staining of DNA demonstrated that, in response to HU, S2 cells do not progress through S phase and an accumulation of cells in G₁ and a decrease of cells in G₂ was observed (see Fig. S1A in supplementary material). These FACS data are consistent with the decrease in mitotic cells seen in Fig. 1. After irradiation, FACS profiles of DNA content show that cells do accumulate in G₂ (see Fig. S1B in supplementary material), indicating that S2 cells possess a functional G₂-M checkpoint that delays entry of cells into mitosis when DNA is damaged.

Decreased protein levels of Cdc25^{Stg} and inhibitory phosphorylation of Cdc2 indicates checkpoint activation in S2 cells

In fission yeast and mammalian cells, mitotic delay occurs when Cdc25 is inactivated by phosphorylation. Inactive phosphorylated Cdc25 is unable to activate Cdc2, resulting in the accumulation of inhibitory phosphorylated isoforms of Cdc2 and inhibition of cell-cycle progression. Therefore, we examined whether the delay in cell-cycle progression of S2 cells that occurred in response to replication defects or damaged DNA accompanied changes in String (*Drosophila* homologue of Cdc25, referred to as Cdc25^{Stg}) and Cdc2. In *Drosophila* embryos, four phosphoisoforms of Cdc2 are present (Edgar et al., 1994) (Fig. 2). In Fig. 2, '1' and '2' represent, respectively, the active and the unphosphorylated isoforms of Cdc2 that are present during rapid division cycles that precedes the MZT, at which time Cdc25^{Stg} is present (Edgar et al., 1994) (Fig. 2, lane 1). Passage through MZT is accompanied by an increase in cell cycle length, a decrease in protein levels of Cdc25^{Stg} (Fig. 2, lane 2) and an accumulation of phosphorylated, inactive isoforms ('3' and '4') of Cdc2 (Fig. 2, lane 2) (Edgar et al., 1994).

S2 cells produce Cdc25^{Stg} and this form of Cdc25^{Stg} migrates with the same mobility as Cdc25^{Stg} from early *Drosophila* embryos (Fig. 2, lane 3). In S2 cells, only three isoforms of Cdc2 were detected – isoforms 3 and 4, which represent inhibitory phosphorylated Cdc2, and isoform 2, which represents the unphosphorylated isoform of Cdc2 (Fig.

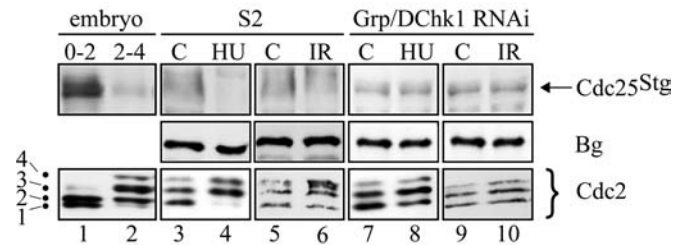


Fig. 2. HU- and IR-induced modifications of Cdc25^{Stg} and Cdc2 in control S2 cells but not in Grp/DChk1-depleted S2 cells. Wild-type embryos at 0–2 hours of age (lane 1) expressed the active phosphorylated isoform (form 1) and the unphosphorylated neutral isoform (form 2) of Cdc2. Wild-type embryos at 2–4 hours of age (lane 2) showed accumulation of inhibitory phosphorylated isoforms (forms 3 and 4) of Cdc2. In early embryos (lane 1) Cdc25^{Stg} was expressed, whereas Cdc25^{Stg} protein was almost absent from in older embryos (lane 2). In S2 cells (lanes 3 and 5), Cdc2 isoforms 2–4 were detected and Cdc25^{Stg} was present. In S2 cells treated with 10 mM HU during 15 hours (lane 4) the Cdc2 isoforms 3 and 4 accumulated, Cdc2 isoform 2 was almost absent and Cdc25^{Stg} levels decreased. S2 cells treated with 150 Gy IR followed by 2 hours of recovery (lane 6) showed accumulation of Cdc2 isoform 4 and presence of Cdc2 isoforms 2 and 3. Cdc25^{Stg} levels decreased moderately in response to IR. S2 cells depleted for Grp/DChk1 by RNAi (lanes 7 and 9) expressed Cdc25^{Stg} and all Cdc2 isoforms (forms 2–4). Grp/DChk1-depleted cells treated with 10 mM HU (lane 8) or treated with 150 Gy IR followed by 2 hours recovery (lane 10) did not show modifications of Cdc25^{Stg} and Cdc2. Equal numbers ($n=20$) of *Drosophila* embryos were loaded in lanes 1 and 2. For lanes 3–10, a background band (Bg) recognized by the Cdc25^{Stg} antibody was used as a loading control.

2, lane 3). Treatment of S2 cells with HU (Fig. 2, lane 4) resulted in a reduction in Cdc25^{Stg} levels, the accumulation of inhibitory phosphorylated Cdc2 (isoforms 3 and 4) and in a reduction of Cdc2 isoform 2. Treatment of S2 cells with IR (Fig. 2, lane 6) resulted in a moderate decrease in Cdc25^{Stg} levels. In contrast to HU-treated cells, only accumulation of the inhibitory phosphorylated Cdc2 isoform 4 was observed, whereas Cdc2 isoform 2 remained present in response to IR (Fig. 2, lane 6). These results demonstrate that Cdc25^{Stg} and Cdc2 are both involved in checkpoint-induced cell-cycle delay in S2 cells and validate the use of S2 cells as a model to study these checkpoint mechanism(s).

Grp/DChk1 is phosphorylated in response to HU or IR

Next, we investigated whether the conserved checkpoint effector kinase Grp/DChk1 is modulated in response to DNA-replication inhibition or damaged DNA. First, we determined whether Grp/DChk1 is expressed in exponentially and asynchronously growing S2 cells. Grp/DChk1 was detected in extracts from wild-type embryos and ovaries, and in S2 cells as a 56 kDa protein (see Fig. S2, lanes 1,3,5, in supplementary material); however, as expected, Grp/DChk1 was not detected in embryos and ovaries from homozygous *grp/Dchk1* females (see Fig. S2, lanes 2,4, in supplementary material). Treatment of S2 cells with HU (Fig. 3A, lane 2) or IR (Fig. 3B, lane 2) resulted in a slower migrating form of Grp/DChk1, and this effect was more prominent in response to HU. The change in mobility of Grp/DChk1 could be reverted with alkaline

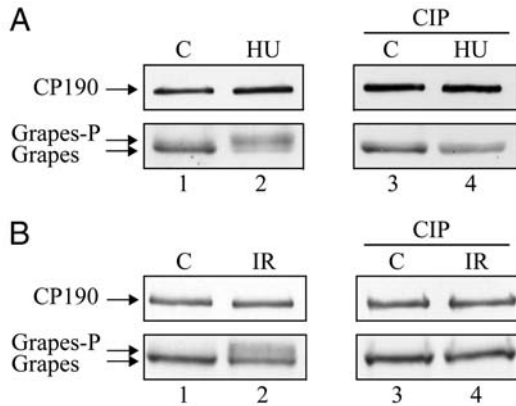


Fig. 3. Grp/DChk1 is phosphorylated in S2 cells in response to HU or IR. Western-blot analysis of Grp/DChk1 of S2 cells treated with 10 mM HU during 15 hours or with 150 Gy IR followed by 2 hours recovery. Grp/DChk1 was detected as a slower migrating protein in S2 cells treated with HU (A, lane 2) or treated with IR (B, lane 2) compared with untreated S2 cells (A,B, lanes 1,3). A shift in mobility of Grp/DChk1 was not detected in S2 cells treated with HU or IR and calf-intestine alkaline phosphatase (CIP; A,B, lane 4). In both A and B, CP190 protein levels were detected as a loading control.

phosphatase (CIP) treatment (Fig. 3A, lane 4 and Fig. 3B, lane 4), implying that the mobility shift was caused by phosphorylation. In conclusion, these data show that, in S2 cells, Grp/DChk1 is phosphorylated in response to incompletely replicated or damaged DNA.

Cdc25^{Stg} and *Cdc2* mediate cell-cycle arrest in response to HU or IR in a Grp/DChk1-dependent manner

To obtain further insight into the mechanism that regulates Grp/DChk1-dependent checkpoint control, Grp/DChk1-deficient S2 cells were generated using RNA interference (RNAi). Treatment of S2 cells with *grp/Dchk1* dsRNA for 96 hours reduced Grp/DChk1 levels below detection (Fig. 4A, lane 2; further indicated as Grp/DChk1-depleted cells). Time-course analysis of cells depleted for Grp/DChk1 showed reduced Grp/DChk1 levels at 24 hours after addition of dsRNA and the effect of dsRNA treatment peaked after 96 hours of dsRNA incubation (data not shown). Silencing of Grp/DChk1 protein production had no effect on the proportion of cells in mitosis (compare Fig. 1, untreated, with Fig. 4B, untreated) and cell-cycle progression (data not shown), indicating that Grp/DChk1 is not essential for S2 cell proliferation.

Next, we assayed whether depletion of Grp/DChk1 affected cell-cycle regulation in response to incompletely replicated or damaged DNA. Control and Grp/DChk1-depleted cells were treated with HU or IR and the proportion of cells in mitosis was determined by counting PH3-positive cells by flow cytometry. HU and IR treatment of Grp/DChk1-depleted cells did not result in a change in the proportion of mitotic cells (5.2% and 4.4% respectively, Fig. 4B) compared with untreated Grp/DChk1-depleted cells. These results demonstrate that cell-cycle arrest in response to HU or IR is compromised in Grp/DChk1-depleted cells and are in agreement with previous studies showing that Grp/DChk1 is required to prevent

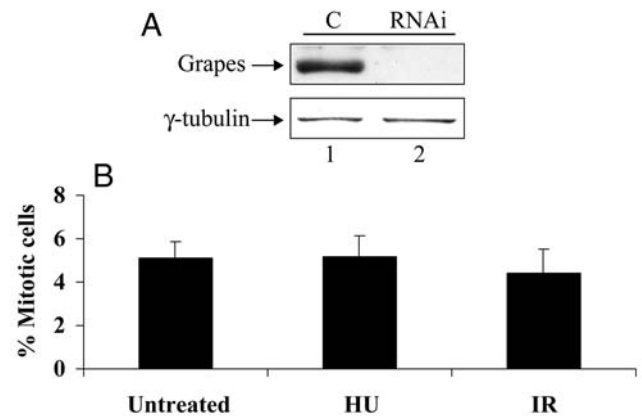


Fig. 4. (A) Downregulation of Grp/DChk1 protein levels in S2 cells using RNAi. Grp/DChk1 expression in S2 cells (Fig. 4A, lane 1) and S2 cells treated with dsRNA of Grp/DChk1 (Fig. 4A, lane 2). Incubation of S2 cells with dsRNA of Grp/DChk1 for 96 hours reduced Grp/DChk1 expression to below detection levels. As a loading control, protein levels of γ -tubulin were detected. (B) Grp/DChk1-depleted cells are defective in accomplishing cell-cycle arrest in response to HU or IR. Grp/DChk1 levels were downregulated by RNAi before cells were treated with 10 mM HU for 15 hours or treated with 150 Gy ionizing radiation followed by 2 hours of recovery. Grp/DChk1-depleted cells positively stained for PH3 were counted using flow cytometry. In Grp/DChk1-depleted cells left untreated 5.1% of the cells was in mitosis. In Grp/DChk1-depleted cells treated with HU also 5.1% of the cells was in mitosis and, in IR-treated Grp/DChk1-depleted cells, 4.4% of the cells were in mitosis. Error bars represent s.e.m. ($n=13$).

progression into mitosis in larval imaginal discs in response to HU and IR (Brodsky et al., 2000; Jaklevic and Su, 2004).

To determine whether *Cdc25^{Stg}* and *Cdc2* are downstream targets of Grp/DChk1-dependent cell-cycle delay in S2 cells, we examined whether *Cdc25^{Stg}* and *Cdc2* were modulated in control S2 cells and in Grp/DChk1-depleted cells treated with HU or IR (Fig. 2). Grp/DChk1-depleted cells showed normal *Cdc25^{Stg}* levels and the three *Cdc2* isoforms (2, 3 and 4; Fig. 2, lanes 7,9) that were also observed in control S2 cells (Fig. 2, lanes 3,5). However, in Grp/DChk1-depleted cells, no decrease in *Cdc25^{Stg}* levels and no changes in *Cdc2* mobility were observed in response to HU or IR (Fig. 2, lanes 8,10). These results demonstrate that *Cdc25^{Stg}* and *Cdc2* are downstream targets of Grp/DChk1 when DNA is incompletely replicated or damaged.

Grp/DChk1 is a downstream target of Mei-41/DATR

In *Xenopus* and humans, Chk1 is directly activated and phosphorylated by ATR (Guo et al., 2000; Zhao and Piwnicka-Worms, 2001). We analysed whether *Drosophila* Grp/DChk1 is a downstream target of *Drosophila* ATR (Mei-41/DATR) (Laurencon et al., 2003). Mei-41/DATR expression was downregulated using RNAi, and downregulation of Mei-41/DATR was confirmed by the observation that Mei-41/DATR-depleted S2 cells failed to inhibit progression into mitosis in response to HU or IR (data not shown). In Mei-41/DATR-depleted cells, no electrophoretic-mobility shift of Grp/DChk1 was observed when cells were treated with HU

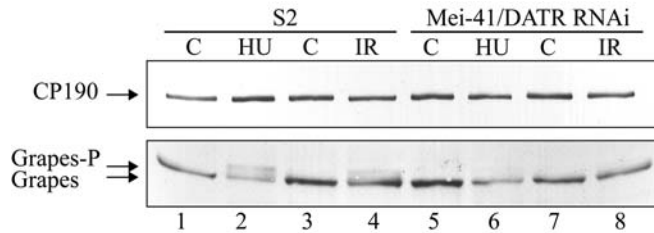


Fig. 5. Phosphorylation of Grp/DChk1 in response to HU or IR is dependent on the expression of Mei-41/DATR. Western-blot analysis of Grp/DChk1 in control S2 cells and in Mei-41/DATR-depleted S2 cells treated with 10 mM HU during 15 hours or treated with 150 Gy IR followed by 2 hours of recovery. Compared with untreated cells (lanes 1,3,5,7), phosphorylated Grp/DChk1 was detected as a slower-migrating protein in control S2 cells treated with HU (lane 2) or IR (lane 4) and in Mei-41/DATR-depleted S2 cells treated with HU (lane 6) or IR (lane 8), no phosphorylation of Grp/DChk1 was observed. As a loading control, CP190 protein levels were detected.

(Fig. 5, lane 6) or IR (Fig. 5, lane 8). From these results, we concluded that Mei-41/DATR is required to phosphorylate Grp/DChk1 in response to incompletely replicated and damaged DNA.

Grp/DChk1 is essential to prevent mitotic catastrophe in response to incompletely replicated or damaged DNA

To determine the consequences of ongoing cell-cycle progression when Grp/DChk1 is depleted and DNA integrity is impaired, we examined mitoses in Grp/DChk1-depleted cells after HU or IR treatment. Under normal conditions, S2 cells (data not shown) and Grp/DChk1-depleted cells both displayed normal mitotic figures (Fig. 6A-C). Using flow cytometry, we observed cell-cycle arrest in control S2 cells in response to incompletely replicated and damaged DNA (Fig. 1). However, HU- or IR-treated Grp/DChk1-depleted cells showed an increase in the proportion of PH3-stained cells compared with control cells treated with HU or IR (Fig. 1, Fig. 4B), and these PH3-positive cells displayed chromosome fragmentation in response to HU (Fig. 6D) or IR (Fig. 6E).

To study the dynamics of chromosome fragmentation and to examine the fate of cells that progress through mitosis in the presence of impaired DNA integrity, Grp/DChk1-depleted S2 cells were transiently transfected with a construct encoding a fusion between histone H2B and green fluorescent protein (GFP), treated with HU or IR and analysed using time-lapse confocal microscopy. In control S2 cells, condensed chromosomes aligned correctly at the metaphase plate before separation and segregation towards the spindle poles during anaphase. When cytokinesis was completed, two distinct cells were formed in which decondensed chromosomes indicated entry into G₁ (Fig. 7A; see Movie 1 in supplementary material). The start of mitosis was defined as the moment at which chromosome condensation became apparent, and mitotic exit was defined as the moment when two daughter cells were formed. Using these criteria, the length of mitosis was on average 40 minutes ($n=36$) in control cells. The fact that we can readily observe control cells progressing through mitosis with no obvious chromosome damage suggests that the intensity of the laser beam did not cause significant cell-cycle

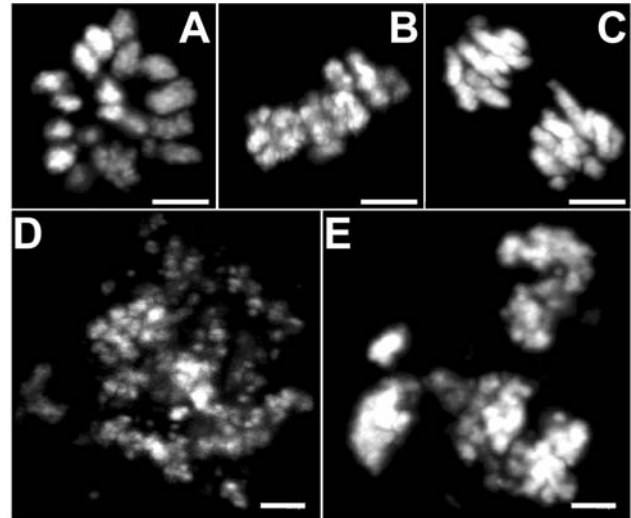


Fig. 6. Analysis of mitotic chromosomes from wild-type S2 cells and Grp/DChk1-depleted S2 cells in response to HU or IR. S2 cells were grown on cover slips and mitotic chromosomes were labelled with the anti-PH3 antibody. Images were made by confocal microscopy. (A-C) Mitotic chromosomes of Grp/DChk1-depleted S2 cells under control conditions in prophase (A), metaphase (B) and anaphase (C). (D,E) Fragmentation of mitotic chromosomes in Grp/DChk1-depleted cells treated with 10 mM HU (15 hours) (D) or IR (150 Gy, 2 hours recovery) (E). Scale bar, 1 μ m.

arrest and allowed mitosis to occur normally. Mitoses were not observed in cells treated with HU or IR owing to cell-cycle arrest (data not shown). Grp/DChk1-depleted cells showed no obvious differences in progression through mitoses compared with control S2 cells (data not shown). However, all recorded Grp/DChk1-depleted cells treated with HU ($n=5$; Fig. 7B; see Movie 2 in supplementary material) or IR ($n=3$; Fig. 7C; see Movie 3 in supplementary material) entered mitosis with abnormal chromosomal condensation and individual chromosome arms could not be distinguished. During mitosis, chromosomes gradually fragmented. Alignment of chromosomes in the metaphase plate was never observed in Grp/DChk1-depleted cells treated with HU and IR. Exit from mitosis was severely prolonged (duration of mitosis varied between 50 minutes and 3 hours) and characterized by unequal chromosome segregation followed by the formation of micronuclei. These data show that Grp/DChk1 is required to prevent mitosis when DNA integrity is impaired and demonstrate that when G₂-M checkpoint fails, mitosis is prolonged, chromosomal organization is abnormal and mitotic catastrophe occurs.

Dmnk/DChk2-depleted cells show normal cell cycle arrest in response to HU or IR and normal phosphorylation of Grp/DChk1

Another *Drosophila* cell-cycle-checkpoint kinase is Dmnk [the *Drosophila* homologue of mammalian Chk2 (Oishi et al., 1998), budding-yeast Rad53 (Allen et al., 1994) and fission-yeast Cds1 (Murakami and Okayama, 1995)]. We tested whether, in *Drosophila*, modifications of Dmnk/DChk2 could be detected in response to HU or IR (Fig. 8A). In S2 cells, no

mobility shift of Dmnk/DChk2 was observed when cells were treated with HU (Fig. 8A, lane 2) or IR (Fig. 8A, lane 3). To investigate further a possible function of Dmnk/DChk2 in checkpoint regulation, Dmnk/DChk2 protein levels were downregulated using RNAi (Fig. 8A, lane 4). Dmnk/DChk2-depleted cells show normal proliferation (data not shown), indicating that Dmnk/DChk2 is not required for cell-cycle progression under normal conditions. Dmnk/DChk2-depleted cells showed normal cell-cycle arrest in response to HU or IR (data not shown). Next, we tested whether, in *Drosophila*, S2 cell modifications of Dmnk/DChk2 could be observed in the absence of Grp/DChk1. Therefore, Grp/DChk1-depleted cells were treated with HU or IR and Dmnk/DChk2 was analysed by western blotting (Fig. 8A). Changes in mobility of Dmnk/DChk2 were not observed in HU-treated (Fig. 8A, lane 6) or IR-treated (Fig. 8A, lane 8) Grp/DChk1-depleted cells. In addition, Grp/DChk1 phosphorylation in response to HU or IR was unaltered in Dmnk/DChk2-depleted cells (Fig. 8B, lanes 6,8). Altogether, Dmnk/DChk2 is not involved in inhibiting progression into mitosis in response to incompletely replicated or damage DNA in S2 cells, changes in mobility of Dmnk/DChk2 were not observed in response to HU or IR, and phosphorylation of Grp/DChk1 in response to HU or IR is not affected by the absence of Dmnk/DChk2. These data suggest that the function of Dmnk/DChk2 and Grp/DChk1 do not overlap in S2 cells and Dmnk/DChk2 plays a minor role in responses to HU and IR in S2 cells.

Discussion

In this report, we demonstrate that *Drosophila* S2 cells are a valuable tool with which to study checkpoint regulation. We used this model system to demonstrate that HU- and IR-induced cell cycle delay depends on the activation and phosphorylation of Grp/DChk1 in a Mei-41/DATR-dependent manner, and depends on modulation of the Grp/DChk1 downstream targets Cdc25^{Stg} and Cdc2. Dmnk/DChk2, by contrast, is not essential for cell-cycle-checkpoint activation in response to HU or IR, nor can Dmnk/DChk2 compensate for checkpoint activation in the absence of Grp/DChk1. We further show that,

when checkpoint regulation fails, mitotic abnormalities and mitotic catastrophe occur in the presence of impaired DNA integrity. In *Xenopus* and mammals, two essential transducers

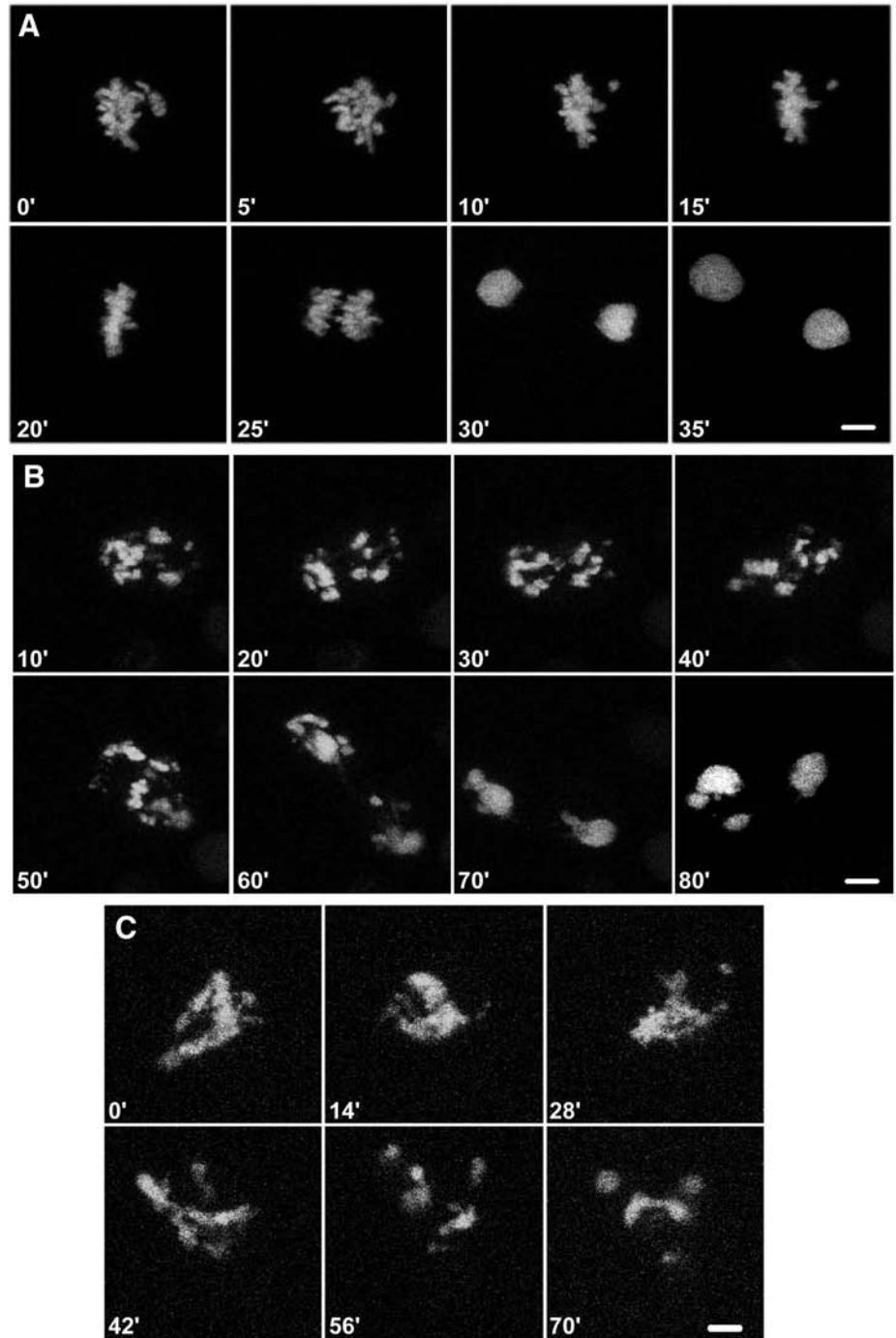


Fig. 7. Grp/DChk1-depleted cells display mitotic catastrophe in the presence of incompletely replicated and damaged DNA. (A) Time-lapse confocal analysis allowed normal cell-cycle progression of S2 cells transiently expressing histone H2B-GFP under control conditions. (B,C) Time-lapse confocal analysis of Grp/DChk1-depleted cells transiently expressing histone H2B-GFP and treated with 10 mM HU (B) for 15 hours or with 150 Gy (C) IR followed by 2 hours of recovery. Mitotic chromosomes were irregularly condensed and did not align in the metaphase plate. After prolonged mitosis, chromosomes separated disproportionately and micronuclei were formed. Times are indicated in minutes. Scale bars, 1 μm .

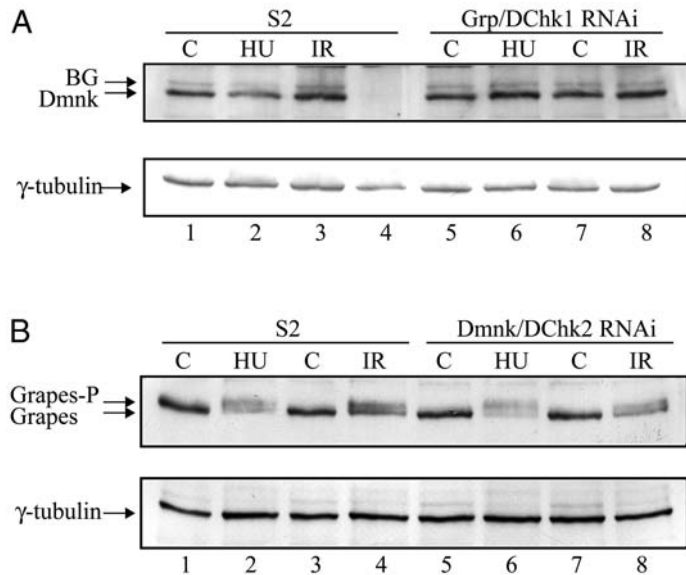


Fig. 8. (A) No mobility shift is observed for Dmnk/DChk2 in response to HU or IR, irrespective of the presence of Grp/DChk1. Western-blot analysis of Dmnk/DChk2 protein in S2 cells and Grp/DChk1-depleted S2 cells treated with 10 mM HU for 15 hours or treated with 150 Gy IR followed by 2 hours of recovery. In S2 cells, Dmnk/DChk2 protein was detected (lane 1) and, in response to HU (lane 2) or IR (lane 3), no shift in mobility of Dmnk/DChk2 was observed. (lane 4) Downregulation of Dmnk/DChk2 protein levels using RNAi. Depletion of Grp/DChk1 using RNAi did not affect the mobility of Dmnk/DChk2 protein in untreated (lanes 5,7), HU-treated (lane 6) or IR-treated (lane 8) S2 cells. As a loading control, γ -tubulin protein levels were detected. (B) Phosphorylation of Grp/DChk1 in response to HU or IR is unaffected in Dmnk/DChk2-depleted cells. Western-blot analysis of Grp/DChk1 in S2 cells and Dmnk/DChk2-depleted cells treated with HU (10 mM, 15 hours) or IR (150 Gy, 2 hours recovery). In S2 cells and in Dmnk/DChk2-depleted cells, a slower-migrating form of Grp/DChk1 was detected in response to HU (lanes 2,6) or IR (lanes 4,8). As a loading control, γ -tubulin protein levels were detected.

of signals from DNA damage and DNA replication defects are known: ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related). Generally, the ATM/Chk2 pathway mainly responds to DNA double-strand breaks induced by IR, whereas the ATR/Chk1 pathway primarily responds to IR-independent forms of DNA damage (such as ultraviolet light) and DNA replication defects (Rhind and Russell, 2000; Zhou and Elledge, 2000). In *Drosophila*, Mei-41/DATR is structurally and functionally related to ATR (Laurencon et al., 2003), and the *Drosophila* Genome Project recently identified a gene (CG6535) that is structurally more related to ATM. These data suggest that there might be an ATM/Chk2-specific pathway and an ATR/Chk1-specific pathway in *Drosophila* as well. Our results demonstrate that Mei-41/DATR is required for the phosphorylation of Grp/DChk1 in response to HU as well as in response to IR, although it is unknown whether Grp/DChk1 is also a direct substrate of Mei-41/DATR.

In contrast to yeast and mammals, Dmnk/DChk2 is not involved in a *Drosophila* DNA-replication-response pathway and the function of Dmnk/DChk2 in a *Drosophila* DNA-damage-response pathway varies between developmental stages of *Drosophila*. Imaginal discs isolated from *dmnk/dchk2* mutant larvae show a mild defect in IR-induced cell-cycle arrest (Brodsky et al., 2004; Xu et al., 2001) and Dmnk/DChk2 is required for IR-induced activation of the embryonic DNA-damage checkpoint during cell cycle 14 (Masrouha et al., 2003). By contrast, we show that normal cell-cycle arrest occurred in IR-treated Dmnk/DChk2-depleted cells. We further show that, in the absence of Grp/DChk1, Dmnk/DChk2 is unable to compensate for cell-cycle-checkpoint activation in response to HU or IR. This demonstrates that, unlike their redundancy in yeast (Boddy et al., 1998; Lindsay et al., 1998; Murakami and Okayama, 1995), Chk1 and Chk2 might not have overlapping functions in *Drosophila* S2 cells.

Similar to Chk1-dependent cell-cycle arrest in yeast and mammals, Cdc25^{Sig} and Cdc2 appear to act downstream of Grp/DChk1 in S2 cells. In fission yeast, *Xenopus* and human, Chk1 has been shown to phosphorylate Cdc25 (Furnari et al., 1997; Kumagai et al., 1998; Peng et al., 1997; Sanchez et al., 1997; Zeng et al., 1998). In human and *Xenopus*, Chk1

phosphorylates Cdc25 to promote its ubiquitin-mediated proteolysis (Shimuta et al., 2002). In S2 cells levels of Cdc25^{Sig} decrease in response to HU and IR but we were not able to detect differently phosphorylated isoforms of Cdc25^{Sig} in the presence of impaired DNA integrity. It is possible that, in *Drosophila*, Cdc25^{Sig} is rapidly degraded upon phosphorylation, that phosphorylation does not cause a detectable mobility change of Cdc25^{Sig}, or that Cdc25^{Sig} is downregulated via an alternative mechanism(s) in response to HU or IR. In response to IR, a moderate decrease in Cdc25^{Sig} levels was observed in S2 cells and only accumulation of Cdc2 isoform 4 was observed, whereas Cdc2 isoform 2 levels were not affected (Fig. 2). Compared with early wild-type embryos, the fastest-migrating Cdc2 isoform (form 1) was undetectable in S2 cells. It is unclear whether isoform 1 of Cdc2 is absent, whether the expression of it is below detection level or whether the actively phosphorylated isoform 1 migrates with the unphosphorylated neutral isoform of Cdc2 in S2 cells.

As shown in Fig. 3A,B, phosphorylation of Grp/DChk1 in response to HU was more evident than phosphorylation of Grp/DChk1 in response to IR. This might be due to technical differences in treatment between HU (15 hours) and IR (150 Gy, 27 Gy minute⁻¹, followed by 2 hours recovery), or to the fact that HU or IR affect cells at different cell-cycle stages. Irrespective of these differences, Cdc25^{Sig} and Cdc2 are downstream targets of the effector kinase Grp/DChk1 in response to HU and IR in S2 cells.

Time-lapse confocal analysis of Grp/DChk1-depleted cells treated with HU or IR revealed the consequences of impaired checkpoint function. Cells undergo a mitotic catastrophe characterized by a succession of four main features: (1) entry into mitosis in the presence of impaired DNA integrity; (2) abnormal condensation of mitotic chromosomes that fail to align in the metaphase plate and individual chromosome arms that could not be detected; (3) severely prolonged mitosis; and (4) disproportional segregation of chromosomes and formation of micronuclei. The above-mentioned features were also observed in HU or irradiated S2 cells that sporadically escaped G₂-M checkpoint control (data not shown; see Table S1 in supplementary material). Therefore, mitotic delay and mitotic

catastrophe as observed in Grp/DChk1-depleted cells treated with HU and IR are Grp/DChk1-independent consequences of entry into mitosis when DNA is incompletely replicated or damaged. Mammalian cells show comparable responses when G₂-M checkpoint fails and cells enter mitosis with incompletely replicated DNA (Hut et al., 2003) indicating that the mitotic response to impaired DNA integrity is a conserved mechanism. In summary, our data show the presence of a Mei-41/DATR and Grp/DChk1 dependent (but Dmnk/DChk2 independent) checkpoint pathway in S2 cells and we demonstrate that, when this checkpoint fails, a mitotic checkpoint is activated, resulting in severe mitotic delay, chromosomal abnormalities and mitotic catastrophe. With the current availability of *Drosophila* RNAi libraries (Echard et al., 2004), the complete sequence of the *Drosophila* genome and with the ease of (simultaneously) downregulating genes in S2 cells (Goshima and Vale, 2003), it would be of interest to use this cellular system in combination with RNAi screens to identify novel genes involved in cell-cycle-checkpoint pathways and genes involved in mitotic responses to impaired DNA integrity.

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