Cdc5L interacts with ATR and is required for the S-phase cell-cycle checkpoint

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Cell division cycle 5-like protein (Cdc5L) is a core component of the putative E3 ubiquitin ligase complex containing Prp19/Pso4, Plrg1 and Spf27. This complex has been shown to have a role in pre-messenger RNA splicing from yeast to humans; however, more recent studies have described a function for this complex in the cellular response to DNA damage. Here, we show that Cdc5L interacts physically with the cell-cycle checkpoint kinase ataxia-telangiectasia and Rad3-related (ATR). Depletion of Cdc5L by RNA-mediated interference methods results in a defective S-phase cell-cycle checkpoint and cellular sensitivity in response to replication-fork blocking agents. Furthermore, we show that Cdc5L is required for the activation of downstream effectors or mediators of ATR checkpoint function such as checkpoint kinase 1 (Chk1), cell cycle checkpoint protein Rad 17 (Rad17) and Fanconi anaemia complementation group D2 protein (FancD2). In addition, we have mapped the ATR-binding region in Cdc5L and show that a deletion mutant that is unable to interact with ATR is defective in the rescue of the checkpoint deficiency in Cdc5L-depleted cells. These findings show a new function for Cdc5L in the regulation of the ATR-mediated cell-cycle checkpoint in response to genotoxic agents.

Keywords: ATR; Cdc5L; checkpoint; DNA damage

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INTRODUCTION

Precursor messenger RNA-processing factor 19 (Prp19)/Pso4 and cell division cycle 5-like protein (Cdc5L) are highly conserved eukaryotic proteins that have a defined role in precursor messenger RNA (pre-mRNA) splicing from yeast to humans (Cheng *et al*, 1993; Tarn *et al*, 1993; Grey *et al*, 1996; McDonald *et al*, 1999; Ajuh *et al*, 2000; Ohi & Gould, 2002). However, recent findings indicate that these proteins also have a distinct and direct role in the cellular response to DNA damage. By using biochemical approaches, we isolated a four-protein complex composed of Prp19/Pso4, Cdc5L, pleiotropic regulator 1 (Plrg1) and spliceosome-associated protein 27

(SPF27; Pso4 complex), which is required for the processing of DNA interstrand cross-links (ICLs) *in vitro* (Zhang *et al*, 2005a). We have also shown that Prp19/Pso4 is modified by ubiquitination in response to DNA damage and that this modification reduces its affinity for other members of the Pso4 complex (Lu & Legerski, 2007). Others have shown that Prp19/Pso4 interacts with the terminal deoxynucleotidyl transferase, and that depletion of Prp19/Pso4 results in the accumulation of double-strand breaks, apoptosis and decreased survival on exposure of cells to ionizing radiation (Mahajan & Mitchell, 2003). Prp19/Pso4 has also been shown to be a DNA-binding protein and is required for the recruitment of the DNA-repair protein Metnase to sites of double-strand breaks (Mahajan & Mitchell, 2003; Beck *et al*, 2008). Prp19/Pso4 is highly conserved in eukaryotes, and in budding yeast *pso4* mutants show broad hypersensitivity to DNA damage-inducing agents, but are particularly sensitive to psoralen and other ICL-inducing drugs (Henriques *et al*, 1989; Grey *et al*, 1996). Taken together, these findings clearly indicate that the Pso4 complex has a role in the DNA-damage response (DDR), although the nature of its function in these pathways remains to be determined.

The only identified catalytic centre in any of the four Pso4 complex members is a U-box domain located in the amino terminus of Prp19. U-box domains have been shown to contain E3 ubiquitin ligase activity (Hatakeyama *et al*, 2001; Zhang *et al*, 2005a); such activity has also been shown for Prp19 *in vitro* (Ohi *et al*, 2003; Loscher *et al*, 2005; Vander Kooi *et al*, 2006) and this function is required for pre-mRNA splicing *in vivo* (Ohi *et al*, 2003; Loscher *et al*, 2005). Two members of the Pso4 complex—Prp19 and Plrg1—contain WD-40 domains, which have been shown to represent scaffolds for protein–protein interactions (Gettemans *et al*, 2003), and often function as substrate adaptors and as components of E3 ubiquitin ligases.

Ataxia-telangiectasia and Rad3-related protein (ATR) is a crucial signalling kinase that mediates pleiotropic responses to DNA damage, including activation of cell-cycle checkpoints, DNA repair pathways, transcription and apoptosis (reviewed in Zhou & Elledge, 2000; Abraham, 2001; Durocher & Jackson, 2001; Shiloh, 2003). ATR is a member of a family of large phosphatidylinositol-3-OH kinase-like kinases that also include the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia-telangiectasia mutated protein (ATM). ATR is activated particularly by agents that induce replication-fork stalling such as ultraviolet irradiation, hydroxyurea and interstrand cross-linking

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drugs. In response to stalled replication forks encountered during S phase of the cell cycle, ATR is recruited to damaged sites by its cofactor, ATR-interacting protein (ATRIP), which binds to replication protein A (RPA)-coated single-stranded DNA (Cortez *et al*, 2001; Zou & Elledge, 2003). Once recruited to the replication fork, it acts in combination with other effectors and mediators such as the 9–1–1 complex, claspin and TopBP1, to activate downstream branched S-phase checkpoint pathways involving Chk1–Cdc25A/C and Nbs1–Smc1 (Abraham, 2001; Bartek *et al*, 2004; Lambert & Carr, 2005; Harper & Elledge, 2007).

RESULTS

Cdc5L and ATR interact

In our previous study on the involvement of the Pso4 complex in ICL repair, we showed that Cdc5L interacted with the WRN (for Werner syndrome) protein (Zhang *et al*, 2005a). This finding prompted us to examine whether other DNA repair or cell-cycle checkpoint factors interact with Cdc5L. As shown in Fig 1A, reciprocal co-immunoprecipitations of Cdc5L and ATR showed that these two proteins associate physically in HeLa cell extracts. Pre-treatment of cells with ultraviolet irradiation did not affect the interaction (data not shown). Furthermore, pull-down assays performed with purified recombinant proteins confirmed this interaction and indicated that it is probably direct (Fig 1B; supplementary Fig S1 online).

Cdc5L is required for the S-phase cell-cycle checkpoint

To study the effects of Cdc5L on cell-cycle regulation, we prepared a clone of HCT-116 cells that contained an inducible transgene expressing a short hairpin RNA (shRNA) targeted to Cdc5L. Induction of the transgene by doxycycline resulted in strong depletion of Cdc5L between 48 and 72 h after addition of the drug (Fig 2A). To determine whether Cdc5L has a role in the S-phase checkpoint mediated by ATR, we depleted Cdc5L by induction of the shRNA transgene and exposed the cells to specific doses of ultraviolet irradiation (Fig 2B). This experiment showed that the increase in S-phase cells typical of an ultraviolet radiation-induced checkpoint was greatly reduced in cells depleted of Cdc5L. Depletion of Cdc5L did not affect the cell-cycle distribution of untreated cells (supplementary Fig S2 online), indicating that the differential effects on the cell cycle were in response to ultraviolet irradiation. To verify the role of Cdc5L in the S-phase checkpoint, we monitored DNA synthesis after exposure to DNA damage (Painter & Young, 1980) and found that the depleted cells were severely deficient in the normal reduction observed in DNA synthesis in response to ultraviolet radiation or mitomycin C (MMC; Fig 2C). In addition, the Cdc5L-depleted cells were hypersensitive to both ultraviolet radiation and hydroxyurea (Fig 2D). Together, these results show that Cdc5L is required for the S-phase checkpoint in response to replication-fork blocking lesions, which is consistent with the physical interaction with ATR described earlier.

The ATR-dependent checkpoint pathway is mediated by several proteins that act downstream of this kinase (Bartek *et al*, 2004; Lambert & Carr, 2005; Harper & Elledge, 2007). We therefore examined several of these effector proteins to determine whether Cdc5L has a role in their activation, as suggested by the cell-cycle checkpoint studies described earlier. As shown in Fig 3A,B, depletion of Cdc5L resulted in decreased phosphorylation

Fig 1 | Cdc5L interacts with ATR. (A) Immunoblot showing that Cdc5L and ATR co-immunoprecipitate from HeLa nuclear extracts. (B) Cdc5L and ATR interact as determined in a pull-down assay with purified recombinant proteins. ATR, ataxia-telangiectasia and Rad3-related protein; Cdc5L, cell division cycle 5-like protein; GST, glutathione-S-transferase; Gus, β -glucuronidase; IP, immunoprecipitation; Preimm., pre-immune serum.

of checkpoint kinase 1 (Chk1) and cell cycle checkpoint protein Rad 17 (Rad17) in response to replication-fork blocking agents. As a control, and for verification of these results, we used transient knockdown of Cdc5L by small interfering RNA (siRNA) targeted to a sequence distinct from the inducible shRNA used in the previous experiments. Compared with a nonspecific control siRNA, the phosphorylation of Chk1 was strongly depressed by transfection of the Cdc5L siRNA (Fig 3C,D). ATR has also been shown to be required for the monoubiquitination of Fanconi anaemia complementation group D2 protein (FancD2) in response to replication-fork blocking agents (Andreassen *et al*, 2004). Interestingly, depletion of Cdc5L caused a marked decrease in FancD2 expression levels (Fig 3E). Nevertheless, it can also be noted that the ratio of monoubiquitinated FancD2 to the unmodified protein is also reduced by Cdc5L depletion. Together, these results indicate that Cdc5L is required for the DNA damage-induced modification of effector proteins downstream of ATR, and further support a role for Cdc5L in ATR-mediated checkpoint activation.

Finally, as described in the Introduction, Cdc5L interacts with the ubiquitin E3 ligase Prp19/Pso4. Therefore, we examined whether Prp19/Pso4 was also required for the activation of ATR as indicated by Chk1 phosphorylation (Fig 3F). Depletion of Prp19/Pso4 did impair the activation of ATR; however, depletion of Prp19/Pso4 also resulted in the destabilization of Cdc5L. Interestingly, depletion of Cdc5L did not seem to affect the stabilization of Prp19/Pso4. Thus, it is unclear from these experiments whether Prp19/Pso4 is required directly for ATR activation or whether the observed result is due to the destabilization of Cdc5L.

Fig 2 | Depletion of Cdc5L results in a defective S-phase checkpoint and hypersensitivity in response to DNA damage. (A) Dox was added to HCT-116 cells expressing the Cdc5L shRNA for the indicated time periods. At the 100-h time point Dox was removed and cells were kept in fresh medium for the indicated time periods. (B) Cell-cycle profiles of HCT-116 cells with or without the depletion of Cdc5L. Cells were incubated for 48 h with or without Dox, treated with the indicated dose of ultraviolet (UV) radiation and, 16 h later, analysed by FACS for cell-cycle distribution. Numbers inside the panels indicate the percentage of S-phase cells as determined by the ModFit modelling programme. (C) Quantification of DNA-synthesis assays of HCT-116 cells with or without the depletion of Cdc5L and after being exposed to the indicated genotoxic agents. (D) HCT-116 cells were treated with Dox for 48 h and then exposed to the indicated genotoxic agent; cell survival was determined by a clonogenic assay. Cdc5L, cell division cycle 5-like protein; Dox, doxycycline; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HU, hydroxyurea; MMC, mitomycin C; shRNA, short hairpin RNA.

Cdc5L and ATR interact

As described earlier, Cdc5L and ATR interact physically. To map the site of this interaction, we prepared various truncation constructs of Cdc5L and examined them for interaction with purified Flag-ATR in pull-down assays. As shown in Fig 4, this deletion analysis indicated that a strong binding site for ATR is present between residues 75–251 (construct h) of human CDC5L. A weaker interaction was observed with construct j, suggesting that residues 180–251 contain a minimal binding site for ATR. The additional removal of residues 180–210 (construct k) abolished the interaction, indicating that this region is important for ATR binding. Interestingly, this region of Cdc5L is completely conserved between the *Xenopus*, chicken, mouse and human homologues, and is also highly conserved with the budding yeast homologue Cef1 (supplementary Fig S3 online).

To confirm that this region mediated an interaction with ATR, we prepared a construct of Cdc5L that was lacking residues 180–210 (GST-Cdc5L^D), and expressed this construct and a wild-type control (GST-Cdc5L^R) in the HCT-116 cell clone expressing the inducible shRNA (Fig 5A). Both of these constructs were modified to render them refractory to the shRNA, as shown by their expression in the presence of doxycycline. Pull-down assays showed that the GST-Cdc5L^D mutant did not interact with ATR (Fig 5B), thus confirming that residues 180–210 are required for ATR binding. Next, we examined DNA synthesis after DNA damage in the HCT-116 clones stably expressing these constructs. As shown in Fig 5C,D, the Cdc5L^R construct, but not the Cdc5L^D

construct, was able to rescue the defect in the S-phase checkpoint after exposure to either ultraviolet radiation or MMC. Finally, we also tested whether this ATR-interacting region affected the interaction between Cdc5L and Prp19/Pso4 and found that it did not (supplementary Fig S4 online), thus indicating that the impaired ability of the Cdc5L^D mutant to activate ATR was not due to disruption of the association between these proteins. Together, these results indicate that interaction between Cdc5L and ATR is required for activation of the S-phase checkpoint in response to replication-fork blocking lesions.

DISCUSSION

As indicated in the Introduction, a growing amount of evidence suggests that Cdc5L and Prp19/Pso4 are involved in DDR in mammalian cells. The results presented here extend these findings and show that Cdc5L interacts with ATR, and that this interaction is required for activation of this kinase with respect to downstream mediators and for implementation of the S-phase cell-cycle checkpoint. These findings are, to our knowledge, the first to suggest that Cdc5L is involved in regulation of the cell cycle in response to genotoxic stress.

The exact biochemical functions of Cdc5L and Prp19/Pso4, and by implication the Pso4 complex, in the DDR remain unclear. One possibility is that as Prp19/Pso4 is a DNA-binding protein, it might have a role in recruiting other repair and/or checkpoint proteins to sites of DNA damage, as shown by its interaction with the repair protein Metnase (Beck *et al*, 2008). Furthermore, Cdc5L

Fig 3 | Cdc5L is required for the activation of ATR-mediated checkpoint pathways. (A,B) Immunoblots showing that Cdc5L is required for the phosphorylation of Chk1 and Rad17 in HCT-116 cells. The ultraviolet (UV) radiation dose and HU concentration were 20 J/m² and 2 μ M, respectively. Cells were fixed for analysis 1 h after ultraviolet radiation treatment and 3 h after the introduction of HU. (C–E) Knockdown of Cdc5L by siRNA in HeLa cells results in defective phosphorylation of Chk1, and monoubiquitylation of FancD2. In (E) 'L' and 'S' refer to the ubiquitinated and unmodified forms of FancD2, respectively. (F) Depletion of Prp19/Pso4 destabilizes Cdc5L and causes impairment of ATR activation. Experiments were carried out as described in (C); Ku70 is shown as a loading control. ATR, ataxia-telangiectasia and Rad3-related protein; Cdc5L, cell division cycle 5-like protein; Chk1, checkpoint kinase 1; Ctrl, control; Dox, doxycycline; FancD2, Fanconi anaemia complementation group D2 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HU, hydroxyurea; MMC, mitomycin C; Prp19, precursor messenger RNA-processing factor 19; Rad17, cell cycle checkpoint protein Rad 17; siRNA, small interfering RNA.

contains two Myb-like domains at its N terminus and might therefore also have DNA-binding activity (Neubauer *et al*, 1998; Ajuh *et al*, 2000). Thus, Cdc5L could have a role in recruiting proteins such as ATR and WRN to sites of DNA damage. However, a more probable, but not mutually exclusive, hypothesis is that the Pso4 complex functions as an E3 ubiquitin ligase in the DDR. The role of ubiquitination in the DDR has been shown by the recent demonstration of requirements for the E3 ligases Cul4, ring-finger protein 8 and CHIP (for carboxyl terminus of Hsc70-interacting protein) in both DNA repair and checkpoint pathways (Groisman *et al*, 2003; Sugawara *et al*, 2005; Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007; Wang & Elledge, 2007; Parsons *et al*, 2008). Both CHIP and Prp19 contain U-box domains that are required for the transfer of ubiquitin from an E2 ligase to the substrate (Hatakeyama *et al*, 2001; Zhang *et al*, 2005b). The U-box domain is a modified RING motif that lacks the full complement of zinc-ion-binding ligands (Aravind & Koonin, 2000) and has clearly been shown to function as an E3 ligase. Although numerous substrates have been identified for CHIP (Boudrez *et al*, 2000; Dickey *et al*, 2007; Xia *et al*, 2007; Parsons *et al*, 2008), few, if any, substrates have been identified for Prp19/Pso4, although it has been shown that this protein can undergo autoubiquitination

in vitro (Ohi *et al*, 2005). Furthermore, we have shown that this modification occurs *in vivo* in response to DNA damage (Lu & Legerski, 2007). The role of Cdc5L in the Pso4 complex is not known, but an obvious possibility is that it might act as an adaptor protein to recruit substrates to the E3 ligase complex. The concept that the Pso4 complex acts as an E3 ubiquitin ligase can readily explain its pleiotropic roles in both pre-mRNA splicing and the DDR.

METHODS

Pull-down assay. For pull-down assays Flag-tagged ATR protein expressed in human embryonic kidney-293 cells was bound on Flag-beads overnight at 4 °C. The bound Flag-ATR was washed with TBS (Tris buffer saline) buffer and incubated with TBS with 1 M NaCl on ice for 2 min to remove non-specifically bound proteins (see supplementary Fig S1 online). The salt was removed by washing with TBS three times. Otherwise, the assay was carried out as described previously (Zhang *et al*, 2005a).

Clonogenic assay. Cdc5L was depleted in HCT-116 cells by adding doxycycline to a final concentration of 2 μ g/ml. At 48 h after induction of the shRNA, cells were treated with ultraviolet radiation and hydroxyurea at various doses. After treatment, the cells were trypsinized and plated with fresh media without

Fig 4 | Mapping of the ATR-binding site in Cdc5L. (A) Schematic showing results of ATR binding to various deletion constructs of Cdc5L. (B,C) Western blots (WB) showing pull-down assays between purified Flag-ATR and deletion constructs of GST-Cdc5L. The uppermost band in each lane represents the full-length recombinant protein, whereas the lower bands represent degradation products. ATR, ataxia-telangiectasia and Rad3-related protein; Cdc5L, cell division cycle 5-like protein; GST, glutathione-S-transferase; Gus, β -glucuronidase.

Fig 5 | Deletion of the ATR-interacting region in Cdc5L results in a failure to rescue the S-phase checkpoint. (A) Expression of Cdc5L alleles in HCT-116 cells containing an inducible Cdc5L shRNA. GST-Cdc5L^R indicates an allele of Cdc5L that is refractory to the shRNA. GST-Cdc5L^D contains a deletion of the ATR-interacting domain located in amino-acid residues 181–210; this allele is also refractory to the shRNA. (B) GST-Cdc5L^D does not interact with ATR as indicated in the pull-down assay. (C,D) The GST-Cdc5L^D allele is unable to rescue the S-phase checkpoint defect in HCT-116 cells expressing the Cdc5L shRNA, as indicated by quantification of DNA synthesis. 'KD' indicates knockdown of endogenous Cdc5L by an inducible shRNA. ATR, ataxia-telangiectasia and Rad3-related protein; Cdc5L, cell division cycle 5-like protein; Dox, doxycycline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione-S-transferase; KD, knockdown; MMC, mitomycin C; shRNA, short hairpin RNA; UV, ultraviolet radiation; WB, western blot.

antibiotics. After 12–15 days, colonies were fixed and stained with crystal violet (0.25% crystal violet, 0.2% paraformaldehyde, 72% methanol and 18% water) and counted.

Evaluation of DNA synthesis. HCT-116 cells were incubated with [¹⁴C]thymidine for 24 h. After replacing the medium, cells were exposed to various doses of ultraviolet radiation. The cells were returned to the incubator for 60 min and pulse-labelled in medium containing [³H]thymidine for an additional 120 min (Painter & Young, 1980). The samples were collected and counted by scintillation. The ratio of incorporated ³H to ¹⁴C was used for quantification to standardize the variation in DNA recovery. For MMC treatment, cells were incubated with the drug for 3 h and pulse-labeled for further 3 h.

Cdc5L depletion by RNA-mediated interference. Double-stranded DNA with the sequence 5'-GGAAGAGAGGAGTTGATTATATCAAGAGATATAATCAACTCCTCTCTCC-3' with corresponding sticky ends was cloned into pEntry-H1-TO (Invitrogen, Carlsbad, CA, USA) and transfected into HCT-116 cells that stably express the tetracycline repressor protein. 48 h after transfection, zeocin was added to the medium, and separated colonies were picked and amplified. Cdc5L knockdown in HeLa cells was achieved by using the siRNA sequence 5'-GGAAGAGAGGA GUUGAUUA-3' (Dharmacon, Lafayette, CO, USA). Further methods are detailed in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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