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Cell cycle-dependent formation of Cdc45–Claspin complexes in human cells is compromized by UV-mediated DNA damage

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Cdc45; claspin; DNA damage response; DNA replication; intra-S-phase checkpoint

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The replication factor Cdc45 has essential functions in the initiation and elongation steps of eukaryotic DNA replication and plays an important role in the intra-S-phase checkpoint. Its interactions with other replication proteins during the cell cycle and after intra-S-phase checkpoint activation are only partially characterized. In the present study, we show that the C terminal part of Cdc45 may mediate its interactions with Claspin. The interactions of human Cdc45 with the three replication factors Claspin, replication protein A and DNA polymerase δ are maximal during the S phase. Following UVC-induced DNA damage, Cdc45–Claspin complex formation is reduced, whereas the binding of Cdc45 to replication protein A is not affected. We also show that treatment of cells with UCN-01 and phosphatidylinositol 3-kinase-like kinase inhibitors does not rescue the UV-induced destabilization of Cdc45–Claspin interactions, suggesting that the loss of the interaction between Cdc45 and Claspin occurs upstream of ataxia telangiectasia and Rad 3-related activation in the intra-S-phase checkpoint.

Structured digital abstract

- Clapsin physically interacts with Cdc45 by anti bait coimmunoprecipitation (View interaction)
- Cdc45 physically interacts with RPA32, Clapsin and p125 Pol delta by pull down (View interaction)
- Cdc45 physically interacts with RPA32 by pull down (View interaction)
- Cdc45 physically interacts with Clapsin by pull down (View interaction)
- Cdc45 physically interacts with Clapsin and RPA32 by pull down (View interaction)
- RPA32 physically interacts with Cdc45 by anti bait coimmunoprecipitation (View interaction)

Introduction

In eukaryotic cells, the replication of the genome is strictly controlled and occurs only once per cell cycle [1,2]. Errors in replication of chromosomal DNA lead to mutations in the genome and create the possibility of transformation of the cell to a malignant state [2,3]. Eukaryotic DNA replication begins with the binding of the origin recognition complex to origins of replication in the early G1 phase of the cell cycle [4,5]. This allows Cdc6 (cell division cycle protein 6) and Cdt1 (Cdc10-dependent target one) to associate with origin recognition complex on chromatin, which in turn is the base for the recruitment of other factors such as the Mcm2–7 (mini-chromosome maintenance 2 to 7) complex, forming the pre-replicative complex.

Abbreviations
aa, amino acids; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad 3-related; BP, basic patch; Chk1, checkpoint kinase 1; CMG complex, Cdc45-Mcm2–7-GINS; DNA-PK, DNA-dependent protein kinase; GINS, go-ichi-ni-san (five-one-two-three); FACS, fluorescence-associated cell sorting; Mcm2–7, mini-chromosome maintenance 2 to 7; NLS, nuclear localization sequence; PIKK, phosphoinositide-3-kinase-related protein kinase; Pol-prim, DNA polymerase α-primase; Pol, DNA polymerase; RPA, replication protein A; RPC, replisome progression complex; ssDNA, single-stranded DNA; TGN buffer, Tris-HCl-β-glycerophosphate–NaCl buffer.

Subsequently Cdc45 and the go-ichi-ni-san (five-one-two-three) (GINS) complex bind to the Mcm2–7 proteins to form the Cdc45-Mcm2–7-GINS (CMG) complex, which is the replicative helicase in eukaryotes [5–11]. Early in the S phase, the CMG helicase unwinds DNA at origins creating bi-directional replication forks and single-stranded DNA (ssDNA), which is subsequently bound by replication protein A (RPA). Following this, DNA polymerase α-primase (Pol-prim) synthesizes the RNA primer at the origin of replication [4,5,11–13]. The binding of these proteins and additional factors outlined below allow the formation of the replisome progression complex (RPC) [14], which facilitates continuous DNA synthesis on the leading strand and discontinuous DNA synthesis on the lagging strand [4,5,7]. In addition, Claspin, T cell immunoglobin domain and mucin domain protein 1 and Tipin comprise the so-called 'fork protection complex' and form a complex with the Pol-prim-binding/chromatin-loading factor acidic nucleoplasmic DNA-binding protein [15,16]. The protein complex binds to RPCs and controls replication rates, checkpoint responses and fork-stabilizing [15,16].

The role of Cdc45 in DNA replication as a part of the CMG complex is regulated by the intra-S-phase DNA damage checkpoint and its expression is tightly regulating in quiescent cells and also when the latter re-enter the cell cycle [5,12,17,18]. In human cells, Cdc45 has been shown to interact only in S phase cells with Mcm5, Mcm7, members of the GINS complex, and replicative DNA polymerase (Pol) δ and ε [6,19]. Cdc45 has also been determined to interact with DNA unwinding element binding protein in pre-initiation complex formation, and with topoisomerase II β-binding protein 1 at the G1/S-transition in human cells [20,21]. Studies in yeast have revealed that Cdc45 interacts with Mcm2, Mcm5, Mcm7, Mcm10, Pol ε, RPA, synthetically lethal with Dpb11-1 and mediator of replication checkpoint protein 1 [2,22–27]. The latter is the yeast homologue for human Claspin. Cdc45 has also been shown to interact with Claspin in *Xenopus* egg extracts [28] and in human cells [29]. Recent studies in human and yeast have determined that Cdc45 has ssDNA binding affinity, binding to 50–60-mer ssDNA [30,31]. Mutant yeast Cdc45, which lacks this ssDNA binding activity, showed an accumulation of ssDNA and helicase-DNA polymerase uncoupling upon induction of replication stress, suggesting that Cdc45 may modulate replication fork stalling [31].

Claspin is a mediator of the ataxia telangiectasia and Rad 3-related (ATR)-dependent intra-S-phase checkpoint in human cells and also promotes DNA replication fork progression and stability [32]. When the RPC encounters DNA lesions or reductions of dNTP levels, such as after cell treatment with hydroxyurea, the Mcm2–7 helicase continues to unwind the DNA, whereas the replicative DNA polymerases are stalled by the encounter with abnormal DNA structures or by the lack of free dNTPs [33,34]. This causes an excess formation of RPA-bound ssDNA, which leads to the recruitment of ATR by ATR-interacting protein [35,36]. The radiation-induced mutation 17– replication factor C 2–5 complex loads the 9-1-1 checkpoint clamp (Rad9-Rad1-hydroxy urea-sensitive 1) at stalled replication forks. The phosphorylation of Rad9 creates a binding site for topoisomerase II β-binding protein 1, an activating cofactor for ATR, which stimulates ATR phosphorylation and leads to subsequent activation of checkpoint kinase 1 (Chk1), which then transduces the checkpoint signal further throughout the cell [1,36]. In this checkpoint control system, the fork protection complex, consisting of Claspin, T cell immunoglobin domain and mucin domain protein 1 and Tipin, acts as a mediator of Chk1 phosphorylation [36].

Claspin has been shown to be a ring-shaped protein that binds to replication fork structures [37] with *Xenopus* Claspin binding to chromatin in a pre-replicative complex- and Cdc45-dependent manner [28]. Currently, it is hypothesized that Claspin mediates this checkpoint response to replication stress by facilitating the phosphorylation of Chk1 by ATR [38]. Human Claspin constitutively associates with ATR, and phosphorylation of Claspin facilitates its interaction with Chk1 [39]. The latter is required for the phosphorylation of Chk1 and the kinase has been shown to stabilize Claspin in HeLa cells [28,40]. The replication fork interaction domain of Claspin in *Xenopus* contains two basic patches (BP1 and BP2). Deletion of either BP1 or BP2 compromises the optimal binding of Claspin to chromatin and the removal of BP2 caused a reduction in Claspin-mediated Chk1-activation [28]. *Xenopus* Claspin contains a small Chk1-activating domain that does not bind stably to chromatin but is fully effective at high concentrations for mediating activation of Chk1 [28]. In addition to its role in mediating the intra-S-phase checkpoint, Claspin also functions in controlling the rates of DNA replication during the normal cell cycle [38]. A recent study showed that Claspin is required for normal rates of global replication fork progression because Claspin-depleted HeLa and HCT116 cells had replication fork progression rates that were slower than wild-type cells and were similar to those observed in Chk1-depleted cells [38].

Cdc45 is part of the RPC and interacts with numerous proteins during eukaryotic DNA replication,

although the functional significance and timing in the cell cycle of its interactions are only poorly understood. In the present study, we show that Cdc45 reciprocally co-immunoprecipitates with both Claspin and RPA in human cells. We found that the C-terminus of Cdc45 is important for its interaction with Claspin. We show that these Cdc45-Claspin interactions are maximal during the S phase. Following UVC-induced DNA damage, Cdc45–Claspin interactions decrease, whereas the binding of Cdc45 to RPA is not reduced. We also show that treatment of cells with UCN-01, caffeine or Wortmannin does not rescue the UVC-mediated reduction of Cdc45–Claspin interactions, suggesting that this process is regulated upstream of ATR activation in the intra-S-phase checkpoint.

Results

Claspin and RPA32 co-immunoprecipitate with ectopically expressed and endogenous Cdc45

Human Cdc45 protein with a single FLAG tag at its C-terminus, later called FLAG-Cdc45, was transiently expressed in HeLa S3 cells to a level three-fold higher than endogenous Cdc45 as determined by quantitative western blotting (data not shown). The affinity-purified recombinant FLAG-Cdc45 co-immunoprecipitated Claspin and RPA32 (Figs 1A,C). The reciprocal immunoprecipitation for each interactor was carried out from HeLa S3 cell extracts using antibodies that recognize endogenous Claspin or RPA32. Here, endogenous Cdc45 co-immunoprecipitated with Claspin and RPA32 (Fig. 1B,D, respectively). These results show that human Cdc45 forms complexes with the replication factors Claspin and RPA *in vivo*.

Cdc45–claspin interaction *in vivo* is deficient upon deletion of Cdc45 C-terminus

To determine the regions of Cdc45 important for the interaction of proteins with FLAG-Cdc45, deletion mutants of FLAG-Cdc45 were generated (Fig. 2A). Plasmid constructs that express mutant Cdc45 lacking amino acids (aa)1–100 (ΔNT), 101–190 Δ(aa101–190), 191–290 Δ(aa191–290), 291–390 Δ(aa291–390), 391–488 Δ(aa391–488) and 489–566 (ΔCT) were generated. These deletion mutants were transiently expressed in HeLa S3 cells to similar levels (data not shown) and localized in the nucleus with the exemption of Cdc45-Δ(aa101–190) (Fig. S1). To direct the deletion mutant Cdc45-Δ(aa101–190) into the nucleus, the SV40 nuclear localization sequence (NLS) was added to this polypeptide and the fusion protein was expressed and found in the nucleus [Δ(aa101–190 + NLS)] (Fig. S1). Interestingly, the monoclonal C45-3G10 antibody used for these experiments did not recognize the Δ(aa101–190 + NLS) deletion mutant, showing that this epitope is necessary for binding of the antibody.

The association of these deletion mutants with Claspin, RPA, Mcm7, Pol δ and Pol ε was tested (Fig. 2B and supplementary figures S2B,C). Deletion of the C-terminal part of Cdc45 (ΔCT mutant) resulted in a reduced association of Claspin with Cdc45 and the detection of Claspin was scarcely above background (Fig. 2B, upper panel; see also summary in Fig. S2A). In contrast, none of the Cdc45 deletion mutants tested showed a strongly decreased physical interaction with RPA (Fig. 2B, second panel). The amount of Claspin co-immunoprecipitating with either FLAG-Cdc45 mutants or full-length Cdc45 was also analyzed by densitometry (Fig. 2B, top and third panel; see also summary in Fig. S2A). The comparison showed that the Cdc45-ΔCT mutant bound approximately 20-fold less efficiently to Claspin than the full-length Cdc45 protein (0.04 and 1.0 AU, respectively), and also had a reduced affinity to Claspin compared to the other Cdc45 deletion mutants (Fig. S2A). These findings suggest that the 78 most C-terminal amino acids of Cdc45 may mediate or at least control Cdc45 binding to Claspin. The overall reduced binding of Cdc45 deletion mutants Δ(aa191–290), Δ(aa291–390) and Δ(aa391–488) to Claspin suggests that several parts of Cdc45 may contribute to the complex formation of Cdc45 with Claspin but that the C terminus of Cdc45 is the most important reagion for this interaction. The similar affinity of all Cdc45 deletion mutants to RPA, Mcm7, Pol δ and Pol ε suggests that the C-terminal deletion mutant Cdc45-ΔCT is specifically defective in its interaction with Claspin. These findings suggest that the deletions of parts of the Cdc45 protein most likely does not interfere with the folding of the protein or with the Cdc45's interactions with the other replication proteins tested (Figs 2B and S2B,C).

Claspin and RPA interact with Cdc45 maximally at the S phase

To analyze the interactions of Cdc45 with Claspin and RPA during the cell cycle, K562 cells transiently expressing FLAG-Cdc45 were fractionated according to their size by centrifugal elutriation; this techniques comprises a stress-free method for obtaining cell populations enriched in various cell cycle stages and does not need any drug treatment or pre-treatment of cells [41–43] Therefore, the data obtained with this method

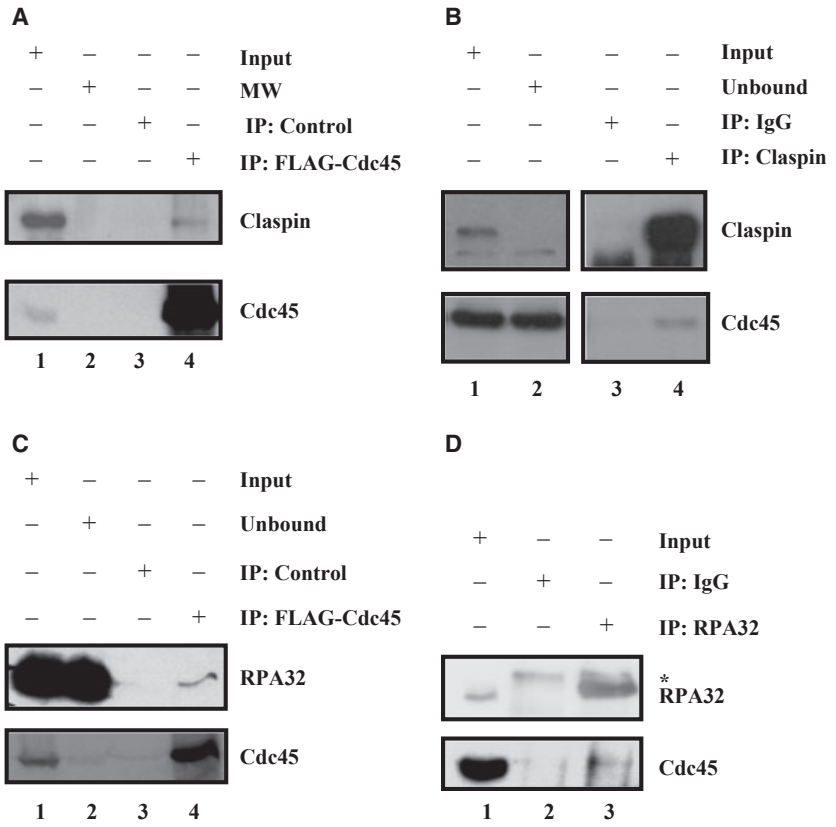

Fig. 1. Claspin and RPA32 interact with Cdc45 in human cells. HeLa S3 cells transiently expressing FLAG-Cdc45 (IP: FLAG-Cdc45) or mock-transfected control cells (IP: Control) were lysed, normalized for protein content and subjected to IP using anti-FLAG serum resin (A, C). Untreated Hela S3 cells were lysed, normalized for protein content and subjected to immunoprecipitation using antibody resin specific for Claspin (B) (IP: Claspin) or RPA (D) (IP: RPA32). The co-immunoprecipitation of Claspin and RPA with FLAG-Cdc45 was determined by western blotting using the indicated antibodies. (A, C) FLAG-bound proteins were eluted with FLAG peptide-containing buffer at 4 °C. Equal amounts of eluate from FLAG resin performed with cells expressing FLAG-Cdc45 (IP: FLAG-Cdc45) and mock-transfected control cells (IP: Control) were loaded to assay Claspin and RPA, respectively, interacting with Cdc45. Lysate from FLAG-Cdc45 expressing cells indicates the position of key proteins by western blotting (Input). (B, D) Proteins bound to nonspecific IgG (IP: IgG) or antibody specific to Claspin or RPA (IP: Claspin or IP: RPA32) were eluted with gel electrophoresis loading buffer and analyzed by western blotting as indicated. MW, molecular weight marker; the asterisk (*) in (D) indicates the light chain of the detected antibody RBF-4E4. Input is lysate from untreated HeLa S3 cells (Input).

are likely to represent the *in vivo* interactions of replication factors throughout the cell cycle [41–43]. The elutriated cells were enriched at various cell cycle stages as confirmed by flow cytometry (Fig. 3A; see also summary in Fig. S3A). Subsequently, the fractionated cells were lysed, protein concentrations were determined and equal amounts of protein were subjected to SDS/PAGE and analyzed by western blotting (Fig. 3B and data not shown). Cdc45, Pol δ and RPA32 were expressed at similar levels throughout the cell cycle, whereas Claspin showed a strongly reduced protein level in G1 cells. In agreement with previous elutriation data [42,43], this G1-enriched cell fraction contained approximately 30% S phase cells (Fig. S3B), which could at least explain in part the residual expression level of Claspin in this fraction (Fig. 3B). In parallel, similar amounts of FLAG-Cdc45 were immunoprecipitated from extracts of these elutriated cells (Fig. 3C). From these elutriated cells, FLAG-Cdc45 bound a maximal amount of p125 of Pol δ from extracts of cells enriched in the S phase (Fig. 3C), which is consistent with previously described findings studying thymidine-arrested and released cells [19]. These results supported the functional activity of the FLAG-Cdc45. The western blots of the FLAG-affinity pull-downs showed that FLAG-Cdc45 co-immunoprecipitated Claspin and RPA32 maximally from cells enriched in the S phase of the cell cycle (Fig. 3C). Densitometry analyses of western blots of immunoprecipitation experiments from two independently

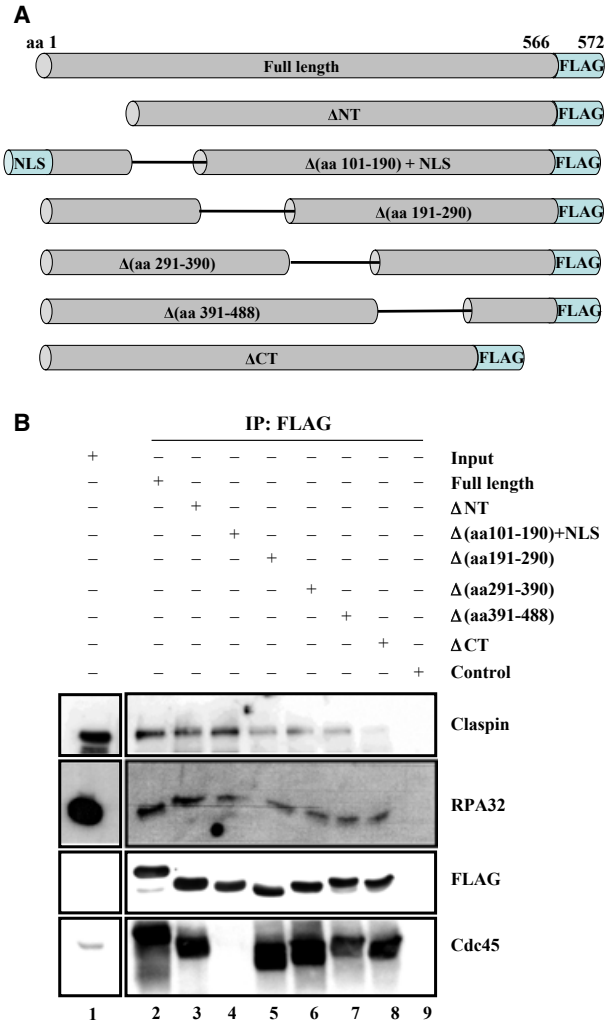

Fig. 2. Interaction of replication proteins with regions of Cdc45. (A) Schematic diagram of full-length FLAG-Cdc45 fusion protein and deletion mutants. Theoretical fusion protein products of plasmids coding for full-length FLAG-Cdc45 (Full Length) and deletion mutants lacking aa1–100 (ΔNT), aa101–190 ([Δ(aa101–190)] but with the addition of the SV40 large T antigen NLS (PKKKRKVG) to its N-terminus [Δ(aa101–190) + NLS]), aa191–290 [Δ(aa191–290)], aa291–390 [Δ(aa291–390)], aa391–488 [Δ(aa391–488)] and aa489–566 (ΔCT) are depicted (A). (B) HeLa S3 cells transiently expressing FLAG-Cdc45, ΔNT (lacking aa1–100), Δ(aa101–190) + NLS, Δ(aa191–290), Δ(aa291–390), Δ(aa391–488), ΔCT (lacking aa489–566) or mock-transfected control cells (IP: Control) were lysed and subjected to FLAG immunoprecipitation. Eluates from each immunoprecipitation were subjected to SDS/PAGE and western blotting using antibodies specific to Claspin, RPA32, FLAG and Cdc45. In the lowest panel, the monoclonal antibody C45-3G10 produced against full-length human Cdc45 does not recognize the Cdc45 mutant Δ(aa101–190 + NLS), suggesting that the monoclonal antibody recognize amino acids in the region aa101–190. Lysate from mock-transfected control cells indicates the position of key proteins by western blotting (Lysate).

elutriated cell populations showed maximal association between Cdc45 and Claspin in the S phase (Fig. S3B). The presence of S phase cells in the G1-enriched cell fraction could explain the residual level of Claspin found in these cells (Fig. 3B) and Cdc45-Claspin complexes determined in cells of this fraction (Fig. 3C). The fractions enriched in S/G2 and G2/M cells also contained late S phase cells (Fig. S3B), which could explain the presence of Claspin and Cdc45-Claspin complex. The amounts of Claspin and the Claspin-containing complexes decreased in these cell fractions, and they closely followed the reduced levels of S phase cells (Fig. 3B,C).

Physical Cdc45–Claspin interactions are diminished after UV treatment

The maximal Cdc45-Claspin and Cdc45-RPA interactions observed in the S phase raised the question whether the binding of Cdc45 to Claspin may be modulated by DNA damage and activation of the intra-S-phase checkpoint. To test the modulation of these interactions after DNA damage, HeLa S3 cells transiently expressing FLAG-Cdc45 were exposed to doses of 5 and 30 $J \cdot m^{-2}$ of UVC and the physical interactions of both endogenous Claspin and RPA32 with Cdc45 were studied 2 h post-treatment by co-immunoprecipitations. Protein levels in these cell extracts were analyzed by SDS/PAGE and western blotting (Fig. 4A). Treatment with UV did not influence the protein levels of Cdc45, Claspin and Chk1. To verify the induction of DNA damage checkpoint pathways in these UV-treated cells, the phosphorylation of checkpoint kinase Chk1 was monitored (Fig. 4A). Both UV treatments resulted in the phosphorylation of Chk1.

In parallel, FLAG-Cdc45 was immunoprecipitated from these cell extracts and Cdc45-associated proteins were determined. Claspin showed a reduction in its co-immunoprecipitation with Cdc45 after 30 $J \cdot m^{-2}$ of UVC but not significantly at the dose of 5 $J \cdot m^{-2}$ of UVC 2 h after UVC treatment compared to the untreated control immunoprecipitation of Cdc45 [Fig. 4B, compare lane 3 (UT; untreated) with lanes 4 and 7 (5 and 30 $J \cdot m^{-2}$, respectively)]. The decrease in association between Claspin and Cdc45 after UVC treatment was also measured by densitometry of western blots of these co-immunoprecipitation experiments (Fig. S4). By contrast to Claspin, no treatment of cells with UVC decreased the co-immunoprecipitation of RPA32 with FLAG-Cdc45 compared to the immunoprecipitation from untreated cells, which served as a positive control (Fig. 4C).

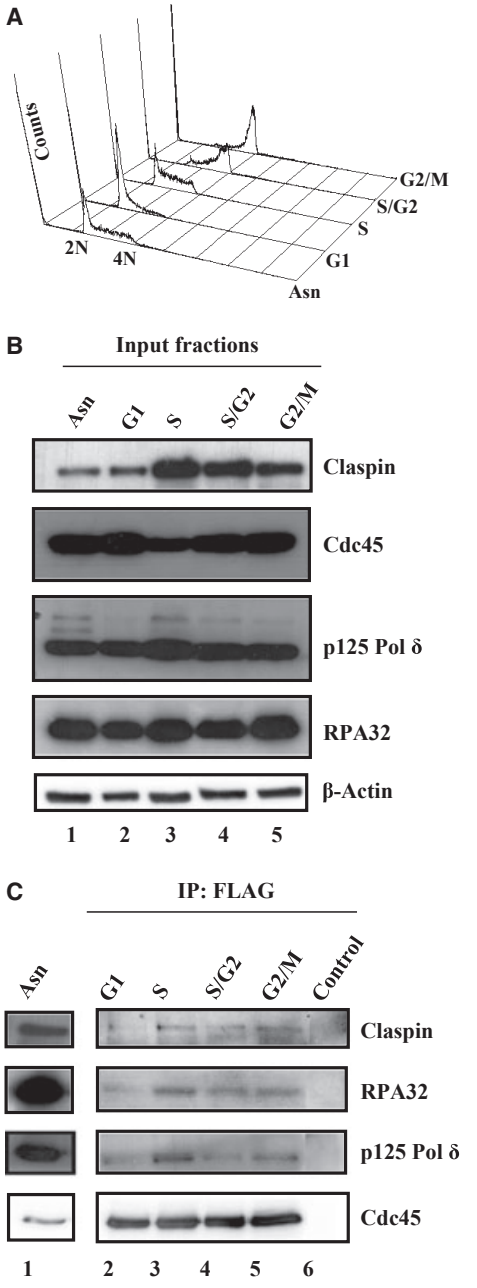

Fig. 3. Claspin and RPA32 interact with Cdc45 maximally in the S phase. (A) 1.5×10^8 K562 cells transiently expressing FLAG-Cdc45 were elutriated, collected and fractions were analyzed by FACS for cell cycle-enriched cells (for additional analysis, see Fig. S3A). (B) Asynchronous control cells (Asn) and cells enriched in G1 phase, S phase, late S/G2 phase and G2/M phase were lysed, normalized for protein content, subjected to SDS/PAGE and western blotting using antibodies raised against Cdc45, Claspin, RPA32 and the p125 subunit of Pol δ. Detection of β-actin serves as a loading control (B). (C) Co-immunoprecipitation of FLAG Cdc45 with Claspin, RPA32 and p125 subunit of Pol δ through the cell cycle was assayed. Extracts of fractions of the elutriation experiment and of asynchronous control cells (IP: Control) were subjected to FLAG-immunoprecipitation. Bound proteins were eluted with FLAG peptide, separated by SDS/PAGE and determined by western blotting using antibodies recognizing the indicated proteins (C). Proteins of lysate from asynchronous control cells were separated by SDS/PAGE and the position of key proteins was determined by western blotting (Asn).

These findings were verified by reciprocal immunoprecipitation of endogenous Claspin. Treating cells with 30 $J \cdot m^{-2}$ UVC did not influence expression levels of Claspin and Cdc45 (Fig. 4D, two top panels), whereas DNA damage signal transduction was activated in these cells as determined by the phosphorylation of Chk1 (Fig. 4D, third panel from the top). Using an antibody specific to Claspin (Fig. 4E), Claspin co-immunoprecipitation with Cdc45 was reduced after UVC treatment of 30 $J \cdot m^{-2}$ (Fig. 4E, lanes 2 and 3).

The reduction in binding of Claspin to FLAG-Cdc45 is insensitive to UCN-01 and phosphoinositide-3-kinase-related protein kinase (PIKK) inhibitor treatment

To investigate whether loss of co-immunoprecipitation between FLAG-Cdc45 and Claspin depends on replication checkpoint signal transduction, small molecule inhibitors UCN01, known to inhibit Chk1 kinase [44], and the PIKK inhibitors caffeine and Wortmannin, inhibiting ataxia telangiectasia mutated (ATM), ATR and DNA-dependent protein kinase (DNA-PK) were used [45,46]. In addition, the ATM-specific inhibitor KU-55933 was employed [47].

After treatment of HeLa S3 cells expressing FLAG-Cdc45 with 30 $J \cdot m^{-2}$ UVC in the presence or absence of 100 nM UCN-01, extracts were prepared and input fractions were subjected to SDS/PAGE and western blotting (Fig. 5A). In parallel, proteins associating with Cdc45 were tested. After treatment of cells with 30 $J \cdot m^{-2}$ UVC, FLAG-Cdc45 co-immunoprecipitated reduced amounts of Claspin compared to control cells but treatment of cells with UV in the presence of UCN-01 did not abolish the decrease in the interaction of Cdc45 with Claspin after DNA damage (Fig. 5B). Treatment of cells with UCN-01 inhibitor alone, however, slightly increased the co-immunoprecipitation of Claspin with FLAG-Cdc45 (Fig. S5). In addition, validation of activity of the UCN-01 drug used by analysing its ability to abrogate Cdk1 phosphorylation in ionizing radiation-treated HeLa S3 cells arrested at mitosis by nocodazole is in agreement with previously published validation experiments [48] (Fig. S6).

To test whether the loss of Claspin–Cdc45 association regulated by PIKK activity, cells were treated

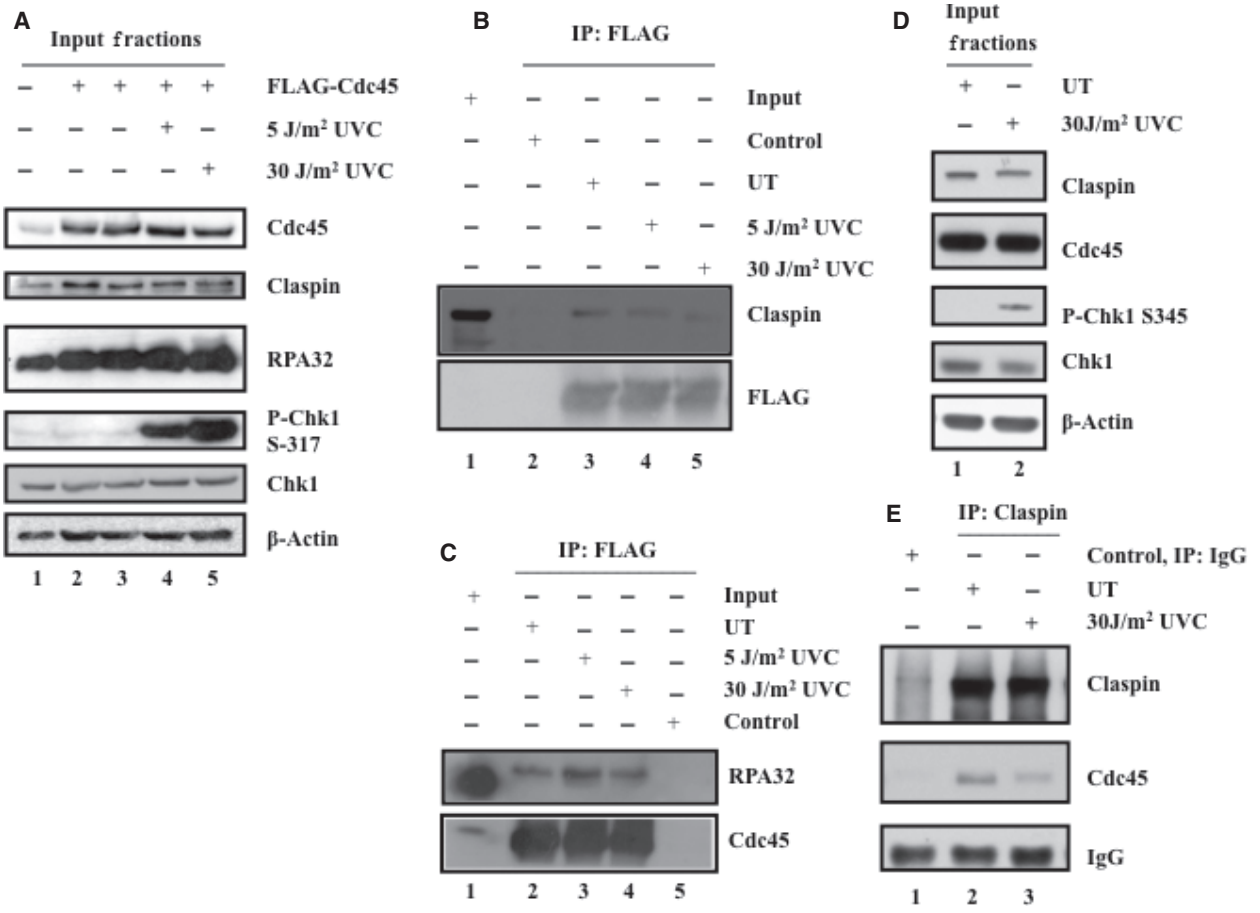

Fig. 4. Effects of UVC treatment on the interactions of Claspin and RPA32 with Cdc45. HeLa S3 cells transiently expressing FLAG-Cdc45 or mock-transfected control cells were treated with doses of 5 and 30 J·m^{-2} of UVC and were harvested 2 h post-treatment. (A) Cells were lysed and subjected to SDS/PAGE and western blotting using antibodies raised against Cdc45, Claspin, RPA32, P-Chk1 S-317 and Chk1. Lanes were equally loaded as detected using β-actin as a loading control (A). (B, C) Lysate from mock-transfected control cells (Lysate) and eluates of FLAG immunoprecipitated from lysates of cells expressing FLAG-Cdc45 (IP: UT), which were exposed to 5 J·m^{-2} UVC (IP: 5 J·m^{-2}), 30 J·m^{-2} UVC (IP: 30 J·m^{-2}) or mock-transfected control cells (IP: Control), were analyzed by SDS/PAGE and western blotting. Antibodies raised against Cdc45, Claspin and RPA were employed to detect FLAG or Cdc45 and associated Claspin (B) and RPA (C). (D) Asynchronous HeLa S3 cells (UT) or cells treated with 30 J·m^{-2} of UVC (30 J·m^{-2}) were harvested 2 h post-treatment and lysed. Input lysates were normalized for protein content and subjected to SDS/PAGE and western blotting, using antibodies raised against Claspin, Cdc45, P-Chk1 S-345 and Chk1. Equal loading of proteins in all lanes was verified by detecting β-actin in parallel, which served as a loading control. (E) Claspin was immunoprecipitated as indicated in lanes 2–4 with binding to a control IgG serving as negative control (lane 1; IP: IgG). Eluates of these immunoprecipitates were subjected to SDS/PAGE and western blotting using the indicated antibodies specific to Claspin, Cdc45 or IgG.

with PIKK inhibitors, caffeine and Wortmannin, and were exposed to 30 J·m^{-2} UVC in the presence or absence of 5 mM caffeine or 100 μM Wortmannin. These inhibitor concentrations abolish the kinase activity of ATM and ATR, or ATM, ATR plus DNA-PK, respectively [44,45]. Neither caffeine, nor Wortmannin treatment affected the amounts of Cdc45, Claspin or RPA32 present in input fractions (Fig. 6A,C). Treatment with caffeine or Wortmannin also did not affect the co-immunoprecipitation of Claspin with FLAG-Cdc45 or the reduction in their co-immunoprecipitation after UVC treatment (Fig. 6B,D). Intriguingly, we observed a slight increase in the co-immunoprecipitation of Claspin with FLAG-Cdc45 only in the presence of Wortmannin (Fig. 6D, top panel, lane 5). The efficacy of the caffeine used was demonstrated by the ability of the drug to abrogate the G2/M checkpoint as determined by pS10-Histone H3 fluorescence-associated cell sorting (FACS) analysis (Fig. S7), whereas the efficacy of the Wortmannin was

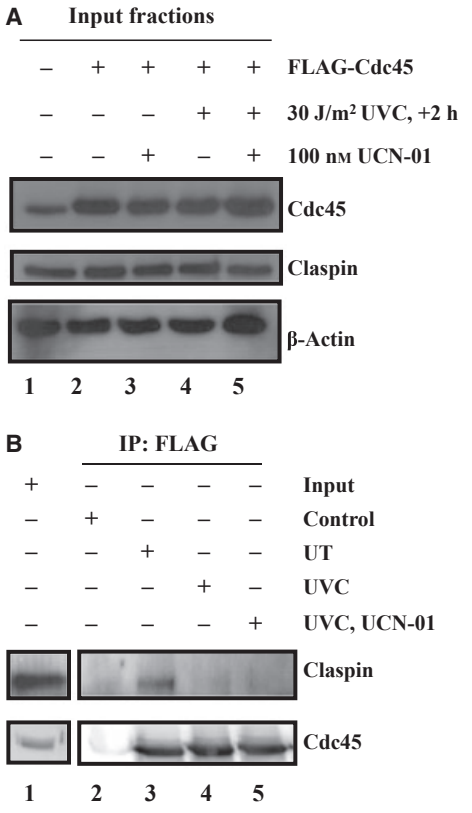

Fig. 5. Inhibition of Chk1 by UCN-01 does not recover the Cdc45–Claspin interaction after UV damage. HeLa S3 cells transiently expressing FLAG-Cdc45 or mock-transfected control cells were treated with 30 $J \cdot m^{-2}$ of UVC with or without a 1 h of pre-treatment with 100 nM UCN-01, a Chk1 inhibitor. Cells were harvested 2 h post UVC treatment in the presence or absence of UCN-01, and lysed. (A) Proteins were subjected to SDS/PAGE and western blotting using antibodies specific to Cdc45 and Claspin. Equal loading of proteins is shown by the detection of β-actin, which acts as a loading control (A). (B) The influence of UCN-01 on the interaction of Cdc45 and Claspin after UV treatment was tested by co-immunoprecipitation using FLAG resin as indicated in (B). The precipitation of proteins from lysates of mock-transfected cells served as a negative control (Control, lane 2). Detection of bound proteins occurred after SDS/PAGE and western blotting of peptide eluates using antibodies specific to Claspin and Cdc45. Lysate from mock-transfected control cells indicates position of key proteins by western blotting (Lysate).

determined by the loss of RPA32 S4/S8 phosphorylation after UVC-treatment in the presence of the drug (Fig. 6C). The specific ATM-inhibitor KU-55933 was also tested for its ability to rescue the Cdc45–Claspin interaction after UVC-treatment (Fig. S8). No rescue was observed after KU-55933 treatment, whereas the efficacy of the inhibitor was demonstrated by reduced Chk2 phosphorylation observed in cells treated with UVC and KU-55933 (Fig. S8).

Discussion

To investigate the interaction of Cdc45 with Claspin and RPA32, C-terminally FLAG-tagged Cdc45 was transiently expressed in human HeLa S3 and K562 cells. FLAG-Cdc45 co-immunoprecipitated Claspin and RPA32. In addition, interactions with previously characterized interaction partners of Cdc45, namely Mcm7, Pols δ and ε, were observed. The detection of these previously identified interactors demonstrates the functionality of the FLAG-Cdc45 fusion protein. The association of Claspin and RPA with endogenous Cdc45 was also confirmed using antibodies specific for Claspin and RPA32. These results agree with recent studies reporting that Claspin co-immunoprecipitates with Cdc45 [29]. These findings also show that Cdc45 and RPA associate with each other in human cells, supporting recent reports about their interaction *in vitro* [49].

To determine the regions of Cdc45 that mediate its interactions with Claspin, RPA32 and other interacting proteins, deletion mutants of Cdc45 were generated and their nuclear localization and association with other proteins were analyzed (Fig. 2, S1 and S2). The Cdc45 deletion mutants tested expressed at similar levels to each other. This allowed the determination of the efficiency of interactions of proteins with each mutant (Fig. 2B and S2). The addition of the SV40 large T antigen nuclear localization sequence (PKKKRKVG) to the Δ(aa101–190) mutant stabilized its expression in HeLa S3 cells (Fig. S2C) and mediated its localization to the nucleus (Fig. S1). All other Cdc45 deletion mutants had nuclear localization, as shown by immunofluorescence microscopy (Fig. S1). In the case of RPA, all Cdc45 deletion mutants showed a similar level of association with this protein, which suggests that these Cdc45 mutants are functional in physical protein–protein interactions. The interaction of RPA32 with Cdc45 is consistent with the recent findings using recombinant Cdc45 and RPA32 [50]. The co-immunoprecipitation of p125 of Pol δ, p261 of Pol ε and Mcm7 with Cdc45 deletion mutants was also investigated, although no deletion mutants deficient for association with these proteins were identified (Fig. S2B,C). These findings suggest that either multiple regions of Cdc45 interact with these proteins, or that these interactions are mediated by other proteins whose binding to Cdc45 is not disturbed by these mutations.

Interestingly, the co-immunoprecipitation of Claspin with the Cdc45 ΔCT deletion mutant was strongly reduced, which suggests that the C-terminal region of Cdc45 mediates the *in vivo* interaction of Cdc45 with Claspin or at least controls the physical association of

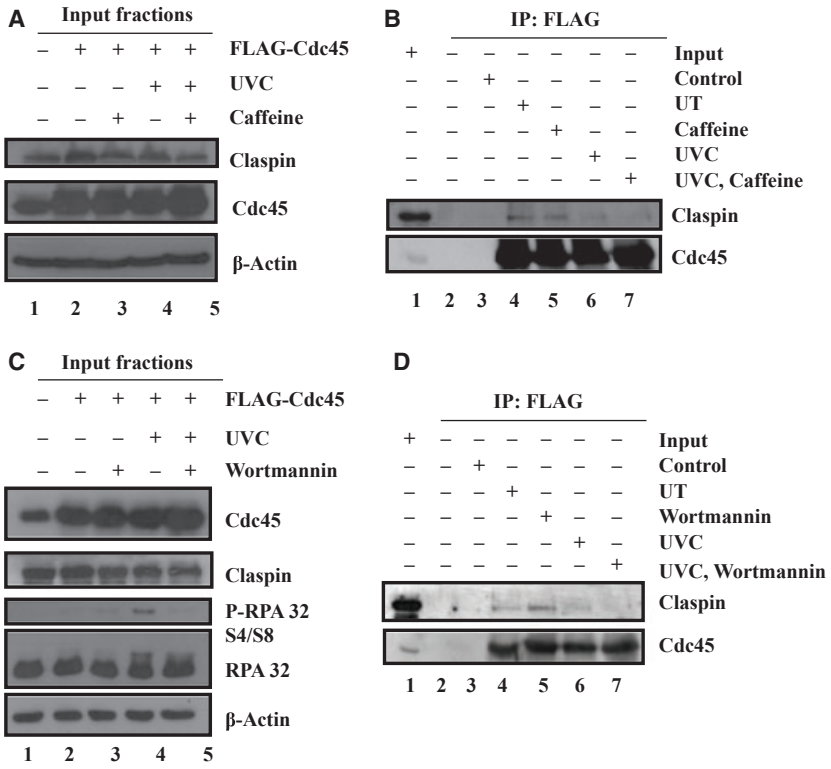

Fig. 6. Inhibition of DNA damage signalling by caffeine and Wortmannin does not recover the Cdc45–Claspin interaction after UV damage. HeLa S3 cells transiently expressing FLAG-Cdc45, or mock-transfected control cells were treated with 30 $J \cdot m^{-2}$ of UVC with or without 1 h of pre-treatment with 5 mM caffeine or 100 μM Wortmannin and were harvested 2 h post-UVC treatment in the presence or absence of inhibitor as indicated. (A) Cells were lysed and subjected to SDS/PAGE using antibodies specific to Cdc45 or Claspin. Detection of β-actin acts as a loading control and shows the equal loading of proteins in all lanes (A). (B) To determine the influence of caffeine, FLAG-IP was performed with lysates of untreated HeLa S3 cells expressing FLAG-Cdc45 (IP: UT), with HeLa S3 cells expressing FLAG-Cdc45 pre-treated for 1 h with 5 mM caffeine (IP: Caffeine), expressing FLAG-Cdc45 exposed to 30 $J \cdot m^{-2}$ of UVC in the absence of caffeine (IP: UVC) and in the presence of 5 mM caffeine plus caffeine pre-treatment (IP: UVC, Caffeine) as described above. Both UVC-exposed cell populations were harvested 2 h post-UVC treatment, whereas caffeine-only incubated cells were collected 3 h after the addition of caffeine. Eluted proteins were separated by SDS/PAGE and analyzed by western blotting using antibodies specific to Claspin and Cdc45 as shown in (B). (C) In addition, HeLa S3 cells transiently expressing FLAG-Cdc45, expressing FLAG-Cdc45 pre-treated for 1 h with 100 μM Wortmannin or mock-transfected control cells were treated with 30 $J \cdot m^{-2}$ of UVC with or without 1 h of pre-treatment with 100 μM Wortmannin and were harvested 2 h post-UVC treatment in the presence or absence of Wortmannin. Cells were lysed and subjected to SDS/PAGE using antibodies specific to Cdc45, Claspin, RPA32 and P-RPA32 S4/S8. Equal loading of proteins was verified by the detection of β-actin, which served as a loading control (C). (B, D) FLAG-immunoprecipitation performed with lysates of HeLa S3 cells after SDS/PAGE and western blotting. Lysate from mock-transfected control cells indicates the position of key proteins by western blotting (Lysate).

these two proteins (Fig. 2B). Because the Cdc45 ΔCT deletion mutant is capable of binding to the other replication proteins tested, these findings strongly suggest that the reduced or lack of interaction of these mutants with Claspin is most likely not caused by lack of proper folding of the mutant protein.

Recent data obtained using small angle X-ray scattering has generated a putative structure for Cdc45 [30]. Here, the predicted structure of Cdc45 resembles the TthRecJ core structure, with the N- and C-termini of the protein arranged on one site of the molecule spatially close to each other. The regions of Claspin that interact with Cdc45 *in vitro* in *Xenopus* and human cells have been characterized, with *Xenopus* Claspin interacting via aa265–605 with *Xenopus* Cdc45 [28] and human Cdc45 to interacting aa1–851 of Claspin [32]. Our data support a model whereby the C-terminus of Cdc45 might mediate its interaction with the N-terminus of Claspin *in vivo*.

To study the regulation of these interactions through the cell cycle, the co-immunoprecipitation of FLAG-Cdc45 with Claspin, RPA32 and p125 of Pol δ was analyzed in elutriated human K562 cells. Claspin and RPA32 interact maximally with FLAG-Cdc45 in

the S phase of the cell cycle. Pol δ served as a positive control in this experiment. In agreement with a previous study showing that endogenous Cdc45 co-immunoprecipitated with Pol δ in S phase cells released from a double thymidine block [19], FLAG-Cdc45 maximally interacted with Pol δ in elutriated, S phase-enriched cells. Maximal co-immunoprecipitations of Claspin, RPA32 and p125 with Cdc45 during the S phase suggests that these interactions of Cdc45 are part of the DNA replication machinery. Previous characterizations of the interaction partners of Cdc45 throughout the cell cycle have used either drug-based methods to synchronize cells, such as a double thymidine block [19], serum depletion [18] or contact inhibition [6]. These synchronization methods may induce replication stress or other stresses [51,52], whereas elutriation centrifugation is a stress-free physical method for obtaining cells enriched in various cell cycle stages and does not need any drug treatment or pre-treatment of cells. Therefore, our data likely represent the *in vivo* interactions of replication factors throughout the cell cycle [41–43].

Previous studies showed that Claspin expression is proliferation-controlled, with a similar patter seen for Mcm proteins and Pol-prim [18,39]. Importantly, the elutriation experiments reported in the present study revealed that Claspin appears to be expressed in a cell cycle-dependent manner, with its levels being low in G1-enriched cells and maximal in S phase cells. By contrast, Cdc45 and RPA32 are constantly expressed throughout the cell cycle at similar levels, which is comparable to the findings previously observed for Pol-prim and RPA in elutriated human cells [42,53] and for Cdc45 in cells released from a double thymidine block [18]. The p125 subunit of Pol δ shows a similar expression pattern with a slight upregulation in S phase cells, which is consistent with previously reported elutriation analyses [54]. In the normal cell cycle, the levels of these replication proteins do not oscillate, whereas Claspin is clearly variable throughout the cell cycle, with an expression pattern reminiscent of Cyclin A [42]. This expression pattern of Claspin may explain its maximal association with Cdc45 in the S phase because the expression levels of in the cell correlate well with Claspin–Cdc45 complex formation.

The treatment of HeLa S3 cells with UVC did not abrogate the Cdc45–RPA interactions at any dose tested, whereas the binding of Cdc45 to Claspin is diminished by UVC in a dose-dependent manner. By contrast to treatment with 5 $J \cdot m^{-2}$ UVC or mock treatment, HeLa S3 cells exposed to 30 $J \cdot m^{-2}$ of UVC showed a reduction in this co-immunoprecipitation. The decreased Cdc45–Claspin interaction with 30 $J \cdot m^{-2}$ UVC of UVC suggests a mechanism whereby low doses of UVC result in a small number of replication stalled forks, whereas higher doses induce a higher number of stalled forks, possibly contributing to the reduction of the observed physical interactions.

To better understand the mechanism and the control of the Cdc45–Claspin interactions, we investigated whether the decreased complex formation of Cdc45 and Claspin after UVC treatment was mediated by the activation or downstream signalling of the intra-S-phase checkpoint. Therefore, co-immunoprecipitations of Claspin with FLAG-Cdc45 were analyzed in the presence or absence of drugs that inhibit different facets of this checkpoint. Neither the compound UCN01, which is known to inhibit Chk1, nor caffeine or Wortmannin, which inhibit the upstream PIKKs, nor even the specifc ATM inhibitor KU-55933, restored the complex formation of Cdc45 and Claspin at 30 $J \cdot m^{-2}$ UVC. The use of caffeine, Wortmannin and KU-55933 also makes it less likely that other branches of the DNA damage response modulate the interactions between Cdc45 and Claspin after UVC damage, such as ATM and DNA-PK. In all of the experiments performed, the concentrations of caffeine and Wortmannin were sufficient to effectively inhibit ATM, DNA-PK and ATR kinases [45,46]. Taken together, these results suggest that the reduction in the interaction between Cdc45 and Claspin may occur upstream of ATR-recruitment and Chk1 activation in the intra-S-phase checkpoint or are independent ways for regulating DNA replication. This regulatory pathway may depend on a change in the conformation in the RPC when the replication machinery becomes stalled, which is upstream of ATR activation in the intra-S-phase checkpoint. Conversely, a reduction in interaction may be mediated by another factor, which is insensitive to KU-55933, caffeine, Wortmannin and UCN-01. Recent findings in yeast suggest that the C terminus of yeast Cdc45 is involved in the recognition of ssDNA and regulates the stalling of helicase after replication stress because the CMG complex is disrupted by long stretches of ssDNA [31]. It is tempting to speculate that the disruption of the Cdc45–Claspin interaction may be involved in the signal transduction of replication stress.

Materials and methods

Cell culture

HeLa S3 cells were cultured in DMEM (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma), 100 units·mL^{-1} penicillin and streptomycin (both Lonza, Basel, Switzerland). K562 cells were cultured in

RPMI media (Sigma) supplemented with 5% fetal bovine serum (Sigma), 100 units·mL^{-1} penicillin and streptomycin (both Lonza).

Antibodies

The following antibodies were used recognizing Cdc45 (C45-3G10) [19], p125 of Pol δ (PDG-5G1) [18] and RPA32 (RBF-4E4) [55,56]. The antibody raised against p261 of Pol ε was obtained from BD Biosciences (Franklin Lakes, NJ, USA) (611238). RPA32 pS4/S8 was purchased from Bethyl Laboratories (Montgomery, TX, USA) (800-338-9579). Mcm7 antibody was obtained from Neomarkers (Fremont, CA, USA) (47DC141). Antibody raised against β-Actin (A5441) and FLAG (F1804) were supplied by Sigma. The Cdk1-specific antibody (sc54) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody recognizing Chk1 pS-317 (#2344) and P-Chk1 pS-345 (#2341) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody raised against Chk1 (DCS-300) was supplied by Neomarkers. Polyclonal rabbit anti-Claspin serum was generated in collaboration with Pocono Rabbit Farm and Laboratory [57].

Generation of plasmid constructs for FLAG-Cdc45 and FLAG-Cdc45 deletion mutants

CDC45L ORF and deletion mutants were amplified by PCR or fusion PCR using KOD DNA polymerase and PCR kit (Novagen, Madison, WI, USA) in accordance with the manufacturer's instructions. PCR products were cloned into the Gateway entry vector pENTR3C (Invitrogen, Carlsbad, CA, USA) in frame between the *Bam*H1 and *Eco*R1 restriction sites and recombined into the Gateway destination vector pT-Rex-DEST30 (Invitrogen) using the LR-Clonase II enzyme mix (Invitrogen) in accordance with the manufacturer's instructions.

Cell treatment

Cells were treated with UVC by removing media from cells, washing once in NaCl/P_i at 37 °C, removing excess NaCl/P_i and exposing cells to UVC for precise amounts of time to control dose using a UVC lamp (Konrad Benda, Wiesloch, Germany) at room temperature [56]. Wortmannin, caffeine, UCN-01 (all from Sigma) and ATM kinase inhibitor KU-55933 (KuDOS Pharmaceuticals Ltd, Cambridge, UK) were dissolved in dimethylsulfoxide as stock solutions of 1 mM (UCN-01 and KU-55933), 20 mM (Wortmannin) or 200 mM (caffeine) and added to cells at the indicated final concentrations. For experiments with these inhibitors and UVC treatment, cells were pre-incubated for 1 h in the presence of 5 mM caffeine, 100 μM Wortmannin, 100 nM UCN-01, 10 μM KU-55933 or dimethylsulfoxide and then treated with UVC. After the UVC treatment, which required the removal of the media during UV exposure of the cells, the UV-treated cells were further incubated and the drug-containing media were put back onto cells until their harvest for further experiments at later time-points. Cells treated in parallel with dimethylsulfoxide under conditions identical to those used for inhibitor-treated cells served as negative controls.

Cell lysis and immunoblotting

Lysates were prepared in TGN buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 50 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 1% Tween-20, 0.2% NP-40) supplemented with phosphatase inhibitor cocktail II (Sigma) and ETDA-free protease inhibitor cocktail (Roche Applied Sciences, Basel, Switzerland). Briefly, cells were lysed for 20 min on ice and centrifuged for 10 min at 13 000 ***g*** at 4 °C. Supernatant fractions were collected and used as input for the immunoprecipitation experiments.

Quantification of western blots

Images of western blots were acquired using a LAS3000 imaging system (Fuji, Tokyo, Japan). Images were analyzed quantitatively using densitometry analysis software (MULTI GAUGE, version 2.2; Fuji) to determine the relative signal intensities of distinct bands.

Immunoprecipitation

FLAG immunoprecipitation experiments were carried out using a FLAG M2 resin and purification kit (Sigma) in accordance with the manufacturer's recommendations. Briefly, 2×10^7 HeLa S3 cells were transfected with 20 μg of plasmid coding for FLAG-Cdc45 fusion protein using Fugene HD or X-treme GENE HP transfection reagent (Roche Applied Sciences), harvested 24 h post-transfection and lysed in TGN buffer. Protein G-Sepharose resin (GE Healthcare, Little Chalfont, UK) was washed three times in 500 μL of TGN buffer, yielding 20 μL of packed resin, and 5 mg of lysate was incubated with this resin for 30 min at 4 °C to pre-clear. In total, 20 μL of packed FLAG resin was washed three times in TGN buffer and incubated with 5 mg of pre-cleared lysate from cells transfected with FLAG-Cdc45 plasmid or from mock-transfected control cells. Lysates were incubated with the resin for 2 h at 4 °C and washed four times with 1 mL of TGN buffer. Bound proteins were eluted by incubation of the FLAG beads in 40 μL of TGN buffer supplemented with 400 μg·mL^{-1} 3 × FLAG peptide (Sigma).

For immunoprecipitation experiments using antibodies raised against Claspin and RPA32, 5 mg of lysate from cells lysed in TGN buffer was incubated with 20 μL of pro-

tein A/G Agarose (Calbiochem, San Diego, CA, USA), which had been washed three times in 500 μL of TGN buffer for 30 min to pre-clear. Either 2 or 5 μg of IgG specific to Claspin or RPA 32, respectively, or an equal amount of nonspecific control IgG was incubated for 2 h at 4 °C with 20 μL of A/G Agarose (Calbiochem), which had been washed three times in 500 μL of TGN buffer to couple the IgG to the beads. IgG-coupled beads were washed three times in 500 μL of TGN buffer and incubated with 5 mg of pre-cleared lysate for 2 h at 4 °C. These beads were then washed four times in 1 mL of TGN buffer before bound proteins were solubilized by boiling the beads in 40 μL of 2 × Laemmli buffer.

Electroporation

Electroporation of K562 cells was carried out using a procedure adapted from a previous study [58]. Briefly, 1×10^7 K562 cells were resuspended in 500 μL of serum- and antibiotic-free RPMI media in a 0.4-mm diameter Gene Pulser® cuvette (Bio-Rad, Hercules, CA, USA). In total, 30 μg of plasmid coding for FLAG-Cdc45 was added to the cuvette and the cells were incubated for 15 min at room temperature. Electroporation was carried out using a Gene Pulser® II electroporation unit and capacitance extender at a voltage of 875 $V \cdot cm^{-2}$ and set to high capacitance. Cells were then reususpended in 10 mL of RPMI media containing fetal bovine serum and antibiotic, placed back in the incubator and harvested for experiments 24 h post-electroporation.

Elutriation

1.5×10^8 K562 cells collected 24 h post-electroporation were elutriated using a JE-5.0 elutriation system (Beckman Coulter, Fullerton, CA, USA) in an Avanti J-26 XP high-performance centrifuge (Beckman Coulter), as described previously [42,43]. Briefly, the elutriation system consisting of the JE-5 rotor and the small chamber was rotated at a constant speed of 1200 r.p.m. at 8 °C. RPMI media supplemented with 5% fetal bovine serum was injected into the elutriation system using a peristaltic pump (Masterflex® L/S; Cole Parmer Instrument Company, Vernon Hills, IL, USA) at a constant initial flow rate. 1.5×10^8 K562 cells were re-suspended in 10 mL of RPMI medium supplemented with 5% fetal bovine serum and a single cell-suspension state was ensured by pipetting cells through a syringe tip (Microlance 3 syringe tip; BD Biosciences). These cells were then introduced into the system and loaded into the elutriation chamber using a constant flow rate. The flow rate was gradually increased and 100-mL fractions at each different flow rate were collected, yielding fractions enriched in G1-, S-, late S/G2- and G2/M-phase cells. Cell synchrony was assayed by FACS analysis for each experiment and appropriately synchronized fractions were used for subsequent immunoprecipitation experiments.

Flow cytometry analysis

1×10^6 HeLa S3 cells were trypsinized or 1×10^6 K562 suspension cells were utilized, washed in $NaCl/P_i$ at 4 °C and re-suspended in 1 mL of $NaCl/P_i$. Ice-cold ethanol was added to a final concentration of 75% to fix samples for flow cytometry as described previously [59]. For propidium iodide staining, samples were then centrifuged at 1500 ***g***, the supernatant was removed, and the cell pellet was re-suspended in 1 mL of propidium iodide with RNAse solution (BD Biosciences) and incubated overnight at 4 °C on an overhead rocker. Cells positive for propidium iodide staining were acquired on a FACS Canto flow cytometer (BD Biosciences) with data analyzed using WINMDI (http://facs.scripps.edu/software.html).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Doc. S1. Drug validation.

Fig. S1. Localization of FLAG-Cdc45 and FLAG-Cdc45 mutants.

Fig. S2. Co-immunoprecipitation of FLAG-Cdc45 deletion mutants with replication proteins.

Fig. S3. Densitometry analysis of the Cdc45–Claspin interaction in the cell cycle.

Fig. S4. Densitometry analysis of the Cdc45–Claspin interaction after UVC treatment.

Fig. S5. FLAG immunoprecipitation in the presence of UCN-01.

Fig. S6. Validation of UCN-01 efficacy.

Fig. S7. Validation of caffeine efficacy.

Fig. S8. KU-55933 treatment does not rescue the Cdc45–Claspin interaction after UVC treatment.