



Research Repository UCD

Title	Cell cycle-dependent formation of Cdc45-Claspin complexes in human cells is compromised by UV-mediated DNA damage
Authors(s)	Broderick, Ronan, Rainey, Michael D., Santocanale, Corrado, et al.
Publication date	2013-08-27
Publication information	Broderick, Ronan, Michael D. Rainey, Corrado Santocanale, and et al. "Cell Cycle-Dependent Formation of Cdc45-Claspin Complexes in Human Cells Is Compromized by UV-Mediated DNA Damage." Wiley Blackwell (Blackwell Publishing), August 27, 2013. https://doi.org/10.1111/febs.12465 .
Publisher	Wiley Blackwell (Blackwell Publishing)
Item record/more information	http://hdl.handle.net/10197/5053
Publisher's statement	This is the author's version of the following article: Ronan Broderick, Michael D. Rainey, Corrado Santocanale, & Heinz P. Nasheuer (2013) "Cell cycle-dependent formation of Cdc45-Claspin complexes in human cells is compromised by UV-mediated DNA damage" FEBS Journal, 280 : 4888-4902 which has been published in final form at http://dx.doi.org/10.1111/febs.12465 .
Publisher's version (DOI)	10.1111/febs.12465

Downloaded 2025-12-04 22:43:51

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)



© Some rights reserved. For more information

Cell cycle-dependent formation of Cdc45-Claspin complexes in human cells are compromised by UV-mediated DNA damage.

Ronan Broderick*, Michael D. Rainey, Corrado Santocanale & Heinz Peter Nasheuer§.

Centre for Chromosome Biology, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland.

Keywords: DNA replication, DNA damage response, intra-S-phase checkpoint, Cdc45, Claspin, Replication Protein A.

Running title: Formation of Cdc45-Claspin complexes.

Corresponding Author

§ Dr. Heinz Peter Nasheuer,
Centre for Chromosome Biology,
School of Natural Sciences, Biochemistry
National University of Ireland Galway,
University Road, Galway, Ireland.

Tel.: 00353-91-492430

Email: h.nasheuer@nuigalway.ie

* Current address: DNA Replication and Repair Laboratory, The Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DS, UK

Conflict of interest

The authors hereby declare there has been no conflict of interest in the production of this manuscript.

Abbreviations:

9-1-1: Rad9-Rad1-Hus1

And1: Acidic nucleoplasmic DNA-binding protein

ATM: Ataxia telangiectasia mutated

ATR: Ataxia telangiectasia and Rad 3–related

ATRIP: ATR-interacting protein

BP1: basic patch 1

Cdc6: cell division cycle protein 6

Cdt1: Cdc10-dependent target 1

Chk1: checkpoint kinase 1

CMG complex: Cdc45-Mcm2-7-GINS

DUE-B: DNA unwinding element binding protein

GINS: go-ichi-ni-san (five-one-two-three)

FACS: fluorescence-associated cell sorting

FLAG-Cdc45: Cdc45 protein with a C terminal FLAG tag

Hus1: hydroxy urea-sensitive 1

IP: immunoprecipitation

Mcm2–7: mini-chromosome maintenance 2 to 7

Mrc1: mediator of replication checkpoint protein 1

ORC: origin recognition complex

Pol-prim: DNA polymerase α -primase

Pol δ : DNA polymerase δ

Pol ϵ : DNA polymerase ϵ

preRC: pre-replicative complex

Rad17: radiation-induced mutation 17

RFC: replication factor C

RPA: replication protein A

RPC: replisome progression complex

Sld3: synthetically lethal with Dpb11-1

SDS PAGE: sodium dodecyl sulfat polyacrylamide gel electrophoresis

ssDNA: single-stranded DNA

Tim1: T Cell Immunoglobulin Domain and Mucin Domain Protein 1

TGN buffer: Tris-HCl - β -glycerophosphate – NaCl buffer

Abstract

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. Here, we present 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 (RPA) and DNA polymerase δ are maximal during S phase. Following UVC-induced DNA damage, Cdc45-Claspin complex formation is reduced whereas the binding of Cdc45 to RPA 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 destabilisation of Cdc45-Claspin interactions, suggesting that the loss of interaction between Cdc45 and Claspin occurs upstream of ATR activation in the intra-S-phase checkpoint.

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 (ORC) to origins of replication in 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 ORC 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 (preRC). Subsequently Cdc45 and the GINS [go-ichi-ni-san (five-one-two-three)] complex bind to the Mcm2-7 proteins to form the CMG complex (Cdc45-Mcm2-7-GINS), which is the replicative helicase in eukaryotes [5-11]. Early in 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, Tim1 and Tipin comprise the so-called “fork protection complex” and form a complex with the Pol-prim-binding/chromatin-loading factor And1 [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 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 the replicative Pols δ and ϵ [6, 19]. Cdc45 has also been determined to interact with the DUE-B protein in pre-initiation complex formation, and with TopBP1 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, Sld3 and Mrc1 protein [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 determined that Cdc45 has ssDNA binding affinity, binding to 50 to 60mer ssDNA [30, 31]. Mutant yeast Cdc45, which lacks this ssDNA binding activity, showed 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 ATR- (Ataxia telangiectasia and Rad 3-related) 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 ATRIP (ATR-interacting protein) [35, 36]. The Rad17-RFC2-5 complex loads the 9-1-1 checkpoint clamp (Rad9-Rad1-Hus1) at stalled replication forks. The phosphorylation of Rad9 creates a binding site for TopBP1, an activating cofactor for ATR, which stimulates ATR phosphorylation and leads to subsequent activation of Chk1 kinase, which then transduces the checkpoint signal further throughout the cell [1, 36]. In this checkpoint control system, the fork protection complex, consisting of Claspin, Tim1 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 preRC- 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 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 since Claspin-depleted HeLa and HCT116 cells had replication fork progression rates which 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 but the functional significance and timing in the cell cycle of its interactions are only poorly understood. In this study, we show that Cdc45 reciprocally co-immunoprecipitates with both, Claspin and RPA, in human cells. Here, we found that the C-terminus of Cdc45 is important for its interaction with Claspin. We showed that these Cdc45-Claspin interactions are maximal during 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 on average 3-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 (Figure 1A and 1C). 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 (Figure 1B and 1D, 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 (Figure 2A). Plasmid constructs which express mutant Cdc45 lacking amino acids 1-100 (Δ NT), 101-190 (Δ (aa101-190)), 191-290 (Δ (aa191-290)), 291-390 (Δ (aa291-390)), 391-488 (Δ (aa 391-488)) and 489-566 (Δ CT) 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) (Supplementary Figure 1). To direct the deletion mutant Cdc45- Δ (aa101-190) into the nucleus the SV40 NLS was added to this polypeptide and the fusion protein was expressed and found in the nucleus (Δ (aa101-190+NLS) (Supplementary Figure 1). 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 the binding of the antibody.

The association of these deletion mutants with Claspin, RPA, Mcm7, Pol δ and Pol ϵ was tested (Figure 2B and Supplementary Figures). Deletion of the C-terminal part of Cdc45 (Δ CT mutant) resulted in a reduced association of Claspin with Cdc45 and the detection of Claspin was hardly above background (Figure 2B upper panel, and summarised in Supplementary Figure 2A). In contrast, none of the Cdc45 deletion mutants tested showed a strongly decreased physical interaction with RPA (Figure 2B,

second panel). The amount of Claspin co-immunoprecipitating with either FLAG-Cdc45 mutants or full length Cdc45 was also analysed by densitometry (Figure 2B, top and third panel; summarized in Supplementary Figure 2A). The comparison showed that the Cdc45- Δ CT mutant bound about 20 times less efficiently to Claspin than the full length Cdc45 protein (0.04 AU and 1.0 AU, respectively), and also had a reduced affinity to Claspin in comparison to the other Cdc45 deletion mutants (Supplementary Figure 2A). These findings suggest that the 78 most C-terminal aa 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 region 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- Δ CT 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 (Figure 2B and Supplementary Figures 2B and 2C).

Claspin and RPA interact with Cdc45 maximally at S phase

To analyse 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 technique is a stress-free method to obtain cell populations enriched in various cell cycle stages and does not need any drug treatment or pre-treatment of cells [41-43]. Therefore, data produced with this method 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 (Figure 3A) (summarized in Supplementary Figure 3A). Subsequently the fractionated cells were lysed, protein concentrations were determined and equal amounts of protein were subjected to SDS PAGE and analysed by western blotting (Figure 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 (Supplementary Figure 3B), which could at

least explain in part the residual expression level of Claspin in this fraction (Figure 3B). In parallel, similar amounts of FLAG-Cdc45 were immunoprecipitated from extracts of these elutriated cells (Figure 3C). From these elutriated cells, FLAG-Cdc45 bound a maximal amount of p125 of Pol δ from extracts of cells enriched in S phase (Figure 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 S phase of the cell cycle (Figure 3C). Densitometry analyses of western blots of immunoprecipitation experiments from two independently elutriated cell populations showed maximal association between Cdc45 and Claspin in S phase (Supplementary Figure 3B). The presence of S phase cells in G1-enriched cell fraction could explain the residual level of Claspin found in these cells (Figure 3B) and Cdc45-Claspin complexes determined in cells of this fraction (Figure 3C). The fractions enriched in S/G2 and G2/M cells also contained late S phase cells (Supplementary Figure 3B), 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 (Figure 3B and 3C).

Physical Cdc45-Claspin interactions are diminished following UV treatment.

The maximal Cdc45-Claspin and Cdc45-RPA interactions observed in 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 J/m² and 30 J/m² 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 analysed by SDS-PAGE and western blotting (Figure 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 (Figure 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 following 30 J/m² of UVC but not significantly at the dose of 5 J/m² of UVC two hours after UVC treatment compared to the untreated control immunoprecipitation of Cdc45 (Figure 4B, compare lane 3 (UT: untreated) with lanes 4 and 7, 5 J/m² and 30 J/m², respectively). The decrease in association between Claspin and Cdc45 following UVC treatment was also measured by densitometry of western blots of these co-immunoprecipitation experiments (Supplementary Figure 4). In 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 (Figure 4C).

These findings were verified by reciprocal immunoprecipitation of endogenous Claspin. Treating cells with 30 J/m² UVC did not influence expression levels of Claspin and Cdc45 (Figure 4D, the two top panels) whereas the DNA damage signal transduction was activated in these cells as determined by the phosphorylation of Chk1 (Figure 4D, third panel from the top). Using an antibody specific to Claspin (Figure 4E), Claspin co-immunoprecipitation with Cdc45 was reduced following a UVC treatment of 30 J/m² (Figure 4E, compare lanes 2 and 3).

The reduction in binding of Claspin to FLAG-Cdc45 is insensitive to UCN-01, and PIKK inhibitor treatment.

In order 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 ATM, ATR and 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/m² 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 (Figure 5A). In parallel, proteins associating with Cdc45 were tested. After treatment of cells with 30 J/m² 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 interaction of Cdc45 with Claspin after DNA damage (Figure 5B). Treatment of cells with UCN-01 inhibitor alone, however, slightly increased the co-immunoprecipitation of

Claspin with FLAG-Cdc45 (Supplementary Figure 5). In addition, validation of activity of the UCN-01 drug used by analysing its ability to abrogate Cdk1 phosphorylation in IR-treated HeLa S3 cells arrested at mitosis by nocodazole, agrees with previously published validation experiments [48] (Supplementary Figure 6).

To test whether the loss of Claspin-Cdc45 association regulated by PIKK activity cells were treated with PIKK inhibitors, Caffeine and Wortmannin, and were exposed to 30 J/m² UVC in the presence or absence of 5 mM Caffeine or 100 μ M 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 (Figure 6A and 6C). Treatment with Caffeine or Wortmannin also did not affect the co-immunoprecipitation of Claspin with FLAG-Cdc45 or the reduction in their co-immunoprecipitation following UVC treatment (Figure 6B and 6D). Intriguingly we observed a slight increase in the co-immunoprecipitation of Claspin with FLAG-Cdc45 only in the presence of Wortmannin (Figure 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 FACS analysis (Supplementary Figure 7) whereas the efficacy of the Wortmannin was determined by the loss of RPA32 S4/S8 phosphorylation following UVC-treatment in the presence of the drug (Figure 6C). The specific ATM-inhibitor KU-55933 was also tested for its ability to rescue Cdc45-Claspin interaction following UVC-treatment (Supplementary figure 8). No rescue was observed following KU-55933 treatment, whereas the efficacy of the inhibitor was demonstrated by the reduced Chk2 phosphorylation observed in cells treated with UVC and KU-55933 (Supplementary figure 8).

Discussion

In order 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 reports 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 localisation and association with other proteins were analysed (Figure 2, Supplementary Figures 1 and 2). The Cdc45 deletion mutants tested expressed at comparable levels to each other. This allowed the determination of the efficiency of interactions of proteins with each mutant (Figure 2B and Supplementary Figure 2). The addition of the SV40 large T antigen nuclear localisation sequence (NLS: PKKKRKVG) to the Δ (aa101-190) mutant stabilized its expression in HeLa S3 cells (Supplementary Figure 2C) and mediated its localisation to the nucleus (Supplementary Figure 1). All other Cdc45 deletion mutants had nuclear localisation as shown by immunofluorescence microscopy (Supplementary Figure 1). 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 were also investigated but no deletion mutants was deficient for association with these proteins were identified (Supplementary Figure 2B and 2C). 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 Δ CT 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 these two proteins (Figure 2B). Since the Cdc45 Δ CT deletion mutant is capable to bind 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, which interact with Cdc45 *in vitro* in *Xenopus* and human cells have been characterized, with *Xenopus* Claspin interacting via aa 265-605 with *Xenopus* Cdc45 [28] and human Cdc45 shown to interact with aa 1-851 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*.

In order 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 analysed in elutriated human K562 cells. Claspin and RPA32 interact maximally with FLAG-Cdc45 in S phase of the cell cycle. Pol δ served as a positive control in this experiment. In agreement with a previous report, which showed 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 S phase suggests that these interactions of Cdc45 are part of the DNA replication machinery. Previous characterisations of the interaction partners of Cdc45 throughout the cell cycle have used either drug-based methods to synchronize cells, such as double thymidine block [19], serum depletion [18] or contact inhibition [6]. These synchronisation methods may induce replication stress or other stresses [51, 52], whereas elutriation centrifugation is a stress-free physical method to obtain cells enriched in various cell cycle stages and does not need any drug treatment or pre-treatment of cells. Therefore, our data are likely to represent the *in vivo* interactions of replication factors throughout the cell cycle [41-43].

Previous results showed that Claspin expression is proliferation-controlled, with a similar pattern seen for Mcm proteins and Pol-prim [18, 39]. Importantly, the elutriation experiments presented here revealed that Claspin appears to be expressed in a cell cycle-dependent manner with its levels being low in G1-enriched cells and maximally in S phase cells. In 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 double thymidine block [18]. The p125 subunit of Pol δ shows a similar expression pattern with a slight up-regulation in S phase cells which is consistent with previously published 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, having an expression pattern reminiscent of Cyclin A [42]. This expression pattern of Claspin may explain its maximal association with Cdc45 in S phase since the expression levels of in the cell correlate well with the 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. In contrast to treatment with 5 J/m² UVC or mock treatment, HeLa S3 cells exposed to 30 J/m² of UVC showed a reduction in this co-immunoprecipitation. The decreased Cdc45-Claspin interaction with 30 J/m² UVC of UVC suggests a mechanism whereby low doses of UVC causing small numbers of replication fork stalling 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 it was investigated if the decreased complex formation of Cdc45 and Claspin following 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 analysed in the presence or absence of drugs, which 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 the specific ATM inhibitor KU-55933 restored the complex formation of Cdc45 and Claspin at 30 J/m² 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 following UVC damage, such as ATM and DNA-PK. In all 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 up-stream of ATR-recruitment and Chk1 activation in the intra-S-phase checkpoint or are independent ways to regulate DNA replication. This regulatory pathway may depend on a change in conformation in the RPC when the replication machinery becomes stalled which is upstream of ATR activation in the intra-S-phase checkpoint. Conversely, the 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 since 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 the replication stress.

Materials and Methods

Cell Culture

HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal calf serum (Sigma), 100 units/ml penicillin and streptomycin (both Lonza). K562 cells were cultured in RPMI media (Sigma) supplemented with 5% foetal calf serum (Sigma), 100 units/ml penicillin and streptomycin (both Lonza).

Antibodies

Antibodies 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 (611238). RPA32 pS4/S8 was purchased from Bethyl Laboratories (800-338-9579). Mcm7 antibody was obtained from Neomarkers (47DC141). Antibody raised against β -Actin (A5441) and FLAG (F1804) were supplied by Sigma. The Cdk1-specific antibody (sc54) was purchased from Santa Cruz. Antibody recognizing Chk1 pS-317 (#2344) and P-Chk1 pS-345 (#2341) were obtained from Cell Signalling Tech. Antibody raised against Chk1 (DCS-300) was supplied by Neomarkers. A rabbit polyclonal anti-Claspin antibody 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) according to the manufacturer's recommendations. PCR products were cloned into the Gateway entry vector pENTR3C (Invitrogen) in frame between the BamH1 and EcoR1 restriction sites and recombined into the Gateway destination vector pT-Rex-DEST30 (Invitrogen) using the LR-Clonase II enzyme mix (Invitrogen) according to the manufacturers recommendations.

Cell treatment

Cells were treated with UVC by removing media from cells, washing once in PBS at 37°C, removing excess PBS and exposing cells to UVC for precise amounts of time to

control dose using a UVC lamp (Benda, Germany) at room temperature [56]. Wortmannin, Caffeine, UCN-01 (all three Sigma) and ATM kinase inhibitor KU-55933 (provided by KuDOS Pharmaceuticals Ltd, Cambridge, UK, now AstraZeneca plc) were dissolved in DMSO 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 μ M Wortmannin, 100 nM UCN-01, 10 μ M KU-55933, or DMSO 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 DMSO under identical conditions as 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). Briefly, cells were lysed for 20 min on ice and centrifuged for 10 min at 13,000 x g at 4°C. Supernatant fractions were collected and used as input for immunoprecipitation experiments.

Quantification of western blots

Images of western blots were acquired using a LAS3000 imaging system (Fuji). Images were analysed quantitatively using densitometry analysis software (Multi Gauge V2.2 (Fuji)) to determine the relative signal intensities of distinct bands.

Immunoprecipitation

FLAG immunoprecipitation experiments were carried out using FLAG M2 resin and purification kit (Sigma) in line with the manufacturer's recommendations. Briefly, 2×10^7 HeLa S3 cells were transfected with 20 μ g 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) was washed 3 times in 500 μ l TGN buffer, yielding 20 μ l packed resin and 5 mg of lysate was incubated with this resin for 30 min at 4°C to pre-clear. 20 μ l packed FLAG resin was washed 3 times in TGN buffer and incubated with 5 mg 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 4 times with 1 ml of TGN buffer. Bound proteins were eluted by incubation of the FLAG beads in 40 μ l TGN buffer supplemented with 400 μ g/ml 3x FLAG peptide (Sigma).

For IP experiments using antibodies raised against Claspin and RPA32, 5 mg of lysate from cells lysed in TGN buffer was incubated with 20 μ l protein A/G Agarose (Calbiochem) which had been washed 3 times in 500 μ l TGN buffer for 30 min to pre-clear. Either 2 or 5 μ g of IgG specific to Claspin or RPA 32 respectively, or an equal amount of non-specific control IgG was incubated for 2 h at 4°C with 20 μ l A/G Agarose (Calbiochem) which had been washed 3x in 500 μ l TGN buffer to couple the IgG to the beads. IgG-coupled beads were washed 3x in 500 μ l TGN buffer and incubated with 5 mg of pre-cleared lysate for 2 h at 4°C. These beads were then washed 4 times in 1 ml TGN buffer before bound proteins were solubilized by boiling the beads in 40 μ l of 2x Laemmli buffer.

Electroporation

Electroporation of K562 cells was carried out using a procedure adapted from [58]. Briefly, 1×10^7 K562 cells were resuspended in 500 μ l serum- and antibiotic-free RPMI media in a 0.4mm diameter Gene Pulser® cuvette (BioRad). 30 μ g 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/cm² and set to high capacitance. Cells were then resuspended in 10 ml RPMI media containing FCS and antibiotic, placed back in the incubator and were 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) in an Avanti J-26 XP high-performance centrifuge

(Beckman Coulter) as previously described [42, 43]. Briefly, the elutriation system was rotated at a constant speed of 1,200 rpm at 8°C. RPMI media supplemented with 5% FCS was injected into the elutriation system using a peristaltic pump (Masterflex ® L/S, Cole Parmer Instrument Company) at a constant initial flow-rate. 1.5×10^8 K562 cells were re-suspended in 10 ml RPMI medium supplemented with 5% FCS and a single cell-suspension state was ensured by pipetting cells through a syringe tip (Beckton Dickinson, Microlance 3 syringe tip). 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 utilised, washed in PBS at 4°C and re-suspended in 1 ml PBS. Ice-cold ethanol was added to a final concentration of 75% to fix samples for flow cytometry as previously described [59]. For propidium iodide staining, samples were then centrifuged at $1,500 \times g$, the supernatant was removed, and the cell pellet was re-suspended in 1 ml propidium iodide with RNase solution (BD Biosciences) and incubated overnight at 4°C on an overhead rocker. Cells positive for PI staining were acquired on a FACS Canto flow cytometer (BD Biosciences) with data analysed using WinMDi software.

Acknowledgements:

We thank Dr. E. Kremmer (Helmholtz Zentrum, Munich) for providing the Cdc45 and RPA antibodies, and Dr. M. Carty for providing the ATM inhibitor KU-55933. This work was supported by Science Foundation Ireland (SFI) grants 07/RFP/GENF472 and Systems Biology Ireland support to HPN, and by the Thomas Crawford Hayes Fund and Irish Research Council for Science, Engineering and Technology (IRCSET) to RB. MDR was supported by SFI grant 08/IN.1/B2064 to CS.

References

1. Nasheuer HP, Smith R, Bauerschmidt C, Grosse F & Weisshart K (2002) Initiation of eukaryotic DNA replication: regulation and mechanisms. *Prog Nucleic Acid Res Mol Biol* **72**, 41-94.
2. Zegerman P & Diffley JF (2009) DNA replication as a target of the DNA damage checkpoint. *DNA Repair (Amst)* **8**, 1077-1088.
3. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366-374.
4. Blow JJ & Dutta A (2005) Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol* **6**, 476-486.
5. Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N & Oda M (2010) Eukaryotic chromosome DNA replication: where, when, and how? *Annu Rev Biochem* **79**, 89-130.
6. Aparicio T, Guillou E, Coloma J, Montoya G & Mendez J (2009) The human GINS complex associates with Cdc45 and MCM and is essential for DNA replication. *Nucleic Acids Res* **37**, 2087-2095.
7. Nasheuer HP, Pospiech H & Syväoja J (2006) Progress towards the anatomy of the eukaryotic DNA replication fork. In *Genome Integrity: Facets and Perspectives*, (Lankenau DH, ed.), pp. 27 - 68. Springer, (ed.) [DOI: 10.1007/7050_012] *Genome Dynamics & Stability*, Vol. 1, Berlin-Heidelberg-NewYork.
8. Moyer SE, Lewis PW & Botchan MR (2006) Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc Natl Acad Sci USA* **103**, 10236-10241.
9. Pospiech H, Grosse F & Pisani FM (2010) The initiation step of eukaryotic DNA replication. *Subcell Biochem* **50**, 79-104.
10. Tsakraklides V & Bell SP (2010) Dynamics of pre-replicative complex assembly. *J Biol Chem* **285**, 9437-9443.
11. Broderick R & Nasheuer HP (2009) Regulation of Cdc45 in the cell cycle and after DNA damage. *Biochem Soc Trans* **37**, 926-930.
12. Ilves I, Petojevic T, Pesavento JJ & Botchan MR (2010) Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. *Mol Cell* **37**, 247-258.
13. Zhu W, Ukomadu C, Jha S, Senga T, Dhar SK, Wohlschlegel JA, Nutt LK, Kornbluth S & Dutta A (2007) Mcm10 and And-1/CTF4 recruit DNA polymerase alpha to chromatin for initiation of DNA replication. *Genes Dev* **21**, 2288-2299.
14. Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD & Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat Cell Biol* **8**, 358-366.
15. Szyjka SJ, Viggiani CJ & Aparicio OM (2005) Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae*. *Mol Cell* **19**, 691-697.
16. Errico A, Cosentino C, Rivera T, Losada A, Schwob E, Hunt T & Costanzo V (2009) Tipin/Tim1/And1 protein complex promotes Pol alpha chromatin binding and sister chromatid cohesion. *EMBO J* **28**, 3681-3692.
17. Liu P, Barkley LR, Day T, Bi X, Slater DM, Alexandrow MG, Nasheuer HP & Vaziri C (2006) The Chk1-mediated S-phase checkpoint targets initiation factor Cdc45 via a Cdc25A/Cdk2-independent mechanism. *J Biol Chem* **281**, 30631-30644.
18. Pollok S, Bauerschmidt C, Sanger J, Nasheuer HP & Grosse F (2007) Human Cdc45 is a proliferation-associated antigen. *Febs J* **274**, 3669-3684.
19. Bauerschmidt C, Pollok S, Kremmer E, Nasheuer HP & Grosse F (2007) Interactions of human Cdc45 with the Mcm2-7 complex, the GINS complex, and DNA polymerases delta and epsilon during S phase. *Genes Cells* **12**, 745-758.
20. Chowdhury A, Liu G, Kemp M, Chen X, Katrangi N, Myers S, Ghosh M, Yao J, Gao Y, Bubulya P & Leffak M The DNA unwinding element binding protein DUE-B interacts with Cdc45 in preinitiation complex formation. *Mol Cell Biol* **30**, 1495-1507.

21. Schmidt U, Wollmann Y, Franke C, Grosse F, Saluz HP & Hanel F (2008) Characterization of the interaction between the human DNA topoisomerase II β -binding protein 1 (TopBP1) and the cell division cycle 45 (Cdc45) protein. *Biochem J* **409**, 169-177.
22. Araki Y, Kawasaki Y, Sasanuma H, Tye BK & Sugino A (2003) Budding yeast mcm10/dna43 mutant requires a novel repair pathway for viability. *Genes Cells* **8**, 465-480.
23. Dalton S & Hopwood B (1997) Characterization of Cdc47p-minichromosome maintenance complexes in *Saccharomyces cerevisiae*: identification of Cdc45p as a subunit. *Mol Cell Biol* **17**, 5867-5875.
24. Hopwood B & Dalton S (1996) Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. *Proc Natl Acad Sci USA* **93**, 12309-12314.
25. Kamimura Y, Tak YS, Sugino A & Araki H (2001) Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. *Embo J* **20**, 2097-2107.
26. Zhao H & Russell P (2004) DNA binding domain in the replication checkpoint protein Mrc1 of *Schizosaccharomyces pombe*. *J Biol Chem* **279**, 53023-53027.
27. Zou L & Stillman B (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol Cell Biol* **20**, 3086-3096.
28. Lee J, Gold DA, Shevchenko A & Dunphy WG (2005) Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response. *Mol Biol Cell* **16**, 5269-5282.
29. Uno S & Masai H (2011) Efficient expression and purification of human replication fork-stabilizing factor, Claspin, from mammalian cells: DNA-binding activity and novel protein interactions. *Genes Cells* **16**, 842-856.
30. Krastanova I, Sannino V, Amenitsch H, Gileadi O, Pisani FM & Onesti S (2011) Structural and functional insights into the DNA replication factor Cdc45 reveal an evolutionary relationship to the DHH family of phosphoesterases. *J Biol Chem*. **287**, 4121-4128
31. Bruck I & Kaplan DL (2013) Cdc45 Protein-Single-stranded DNA Interaction Is Important for Stalling the Helicase during Replication Stress. *J Biol Chem* **288**, 7550-7563.
32. Sercin O & Kemp MG (2011) Characterization of functional domains in human Claspin. *Cell Cycle* **10**, 1599-1606.
33. Smith KD, Fu MA & Brown EJ (2009) Tim-Tipin dysfunction creates an indispensable reliance on the ATR-Chk1 pathway for continued DNA synthesis. *J Cell Biol* **187**, 15-23.
34. Van C, Yan S, Michael WM, Waga S & Cimprich KA (2010) Continued primer synthesis at stalled replication forks contributes to checkpoint activation. *J Cell Biol* **189**, 233-246.
35. Broderick S, Rehmet K, Concannon C & Nasheuer HP (2010) Eukaryotic single-stranded DNA binding proteins: central factors in genome stability. *Subcell Biochem* **50**, 143-163.
36. Kaufmann WK (2009) The human intra-S checkpoint response to UVC-induced DNA damage. *Carcinogenesis* **31**, 751-765.
37. Sar F, Lindsey-Boltz LA, Subramanian D, Croteau DL, Hutsell SQ, Griffith JD & Sancar A (2004) Human claspin is a ring-shaped DNA-binding protein with high affinity to branched DNA structures. *J Biol Chem* **279**, 39289-39295.
38. Petermann E, Helleday T & Caldecott KW (2008) Claspin promotes normal replication fork rates in human cells. *Mol Biol Cell* **19**, 2373-2378.
39. Chini CC & Chen J (2003) Human claspin is required for replication checkpoint control. *J Biol Chem* **278**, 30057-30062.

40. Chini CC & Chen J (2006) Repeated phosphopeptide motifs in human Claspin are phosphorylated by Chk1 and mediate Claspin function. *J Biol Chem* **281**, 33276-33282.
41. Zhang S, Roche K, Nasheuer HP & Lowndes NF (2011) Modification of histones by sugar beta-N-acetylglucosamine (GlcNAc) occurs on multiple residues, including histone H3 serine 10, and is cell cycle-regulated. *J Biol Chem* **286**, 37483-37495.
42. Dehde S, Rohaly G, Schub O, Nasheuer HP, Bohn W, Chemnitz J, Deppert W & Dornreiter I (2001) Two immunologically distinct human DNA polymerase alpha-primase subpopulations are involved in cellular DNA replication. *Mol Cell Biol* **21**, 2581-2593.
43. Nasheuer HP, Moore A, Wahl AF & Wang TS (1991) Cell cycle-dependent phosphorylation of human DNA polymerase α . *J Biol Chem* **266**, 7893-7903.
44. Zhao B, Bower MJ, McDevitt PJ, Zhao H, Davis ST, Johanson KO, Green SM, Concha NO & Zhou BB (2002) Structural basis for Chk1 inhibition by UCN-01. *J Biol Chem* **277**, 46609-46615.
45. Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE & Abraham RT (1998) Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Res* **58**, 4375-4382.
46. Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM & Abraham RT (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* **59**, 4375-4382.
47. Li Y & Yang DQ (2011) The ATM inhibitor KU-55933 suppresses cell proliferation and induces apoptosis by blocking Akt in cancer cells with overactivated Akt. *Mol Cancer Ther* **9**, 113-125.
48. Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA & O'Connor PM (1996) UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst* **88**, 956-965.
49. Nakaya R, Takaya J, Onuki T, Moritani M, Nozaki N & Ishimi Y (2010) Identification of proteins that may directly interact with human RPA. *J Biochem* **148**, 539-547.
50. Takaya J, Kusunoki S & Ishimi Y (2013) Protein interaction and cellular localization of human CDC45. *J Biochem* **153**, 381-388.
51. Darzynkiewicz Z, Halicka HD, Zhao H & Podhorecka M (2011) Cell synchronization by inhibitors of DNA replication induces replication stress and DNA damage response: analysis by flow cytometry. *Methods Mol Biol* **761**, 85-96.
52. Gaballah M, Slisz M & Hutter-Lobo D (2012) Role of JNK-1 regulation in the protection of contact-inhibited fibroblasts from oxidative stress. *Mol Cell Biochem* **359**, 105-113.
53. Din S, Brill SJ, Fairman MP & Stillman B (1990) Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. *Genes Dev* **4**, 968-977.
54. Zeng XR, Hao H, Jiang Y & Lee MY (1994) Regulation of human DNA polymerase delta during the cell cycle. *J Biol Chem* **269**, 24027-24033.
55. Pestryakov PE, Weisschart K, Schlott B, Khodyreva SN, Kremmer E, Grosse F, Lavrik OI & Nasheuer HP (2003) Human replication protein A: The C-terminal RPA70 and the central RPA32 domains are involved in the interactions with the 3'-end of a primer-template DNA. *J Biol Chem* **278**, 17515-17524.
56. Stephan H, Concannon C, Kremmer E, Carty MP & Nasheuer HP (2009) Ionizing radiation-dependent and independent phosphorylation of the 32-kDa subunit of replication protein A during mitosis. *Nucleic Acids Res* **37**, 6028-6041.
57. Rainey MD, Harhen B, Wang GN, Murphy PV & Santocanale C (2013) Cdc7-dependent and -independent phosphorylation of Claspin in the induction of the DNA replication checkpoint. *Cell Cycle* **12**, 1560-1568.
58. Delgado-Canedo A, Santos DG, Chies JA, Kvitko K & Nardi NB (2006) Optimization of an electroporation protocol using the K562 cell line as a model: role of cell cycle phase and cytoplasmic DNases. *Cytotechnology* **51**, 141-148.

59. Broderick R, Ramadurai S, Toth K, Togashi DM, Ryder AG, Langowski J & Nasheuer HP (2012) Cell Cycle-Dependent Mobility of Cdc45 Determined in vivo by Fluorescence Correlation Spectroscopy. *PLoS One* 7, e35537.

Supporting information

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

Supporting information: Drug validation

Supporting information Figure S1: Localisation of FLAG-Cdc45 and FLAG-Cdc45 mutants.

Supporting information Figure S2: Co-immunoprecipitation of FLAG-Cdc45 deletion mutants with replication proteins.

Supporting information Figure S3: Densitometry analysis of Cdc45-Claspin interaction in the cell cycle.

Supporting information Figure S4: Densitometry analysis of Cdc45-Claspin interaction after UVC treatment.

Supporting information Figure S5: FLAG immunoprecipitation in the presence of UCN-01.

Supporting information Figure S7: Validation of Caffeine efficacy.

Supporting information Figure S8: KU-55933 treatment does not rescue Cdc45-Claspin interaction following UVC treatment.

Figure legends

Figure 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 antibody resin (panels A and C). Untreated HeLa S3 cells were lysed, normalised for protein content and subjected to IP using antibody resin specific for Claspin (panel B; (IP: Claspin)) or RPA (panel D; (IP: RPA32)). The co-immunoprecipitation of Claspin and RPA with FLAG-Cdc45 was determined by western blotting using the indicated antibodies. Panels A and 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. Here lysate from FLAG-Cdc45 expressing cells indicates the position of key proteins by western blotting (Input). In panels B and D, proteins bound to non-specific IgG (IP: IgG) or an antibody specific to Claspin or RPA (IP: Claspin or IP: RPA32) were eluted with gel electrophoresis loading buffer and analysed by western blotting as indicated. MW: molecular weight marker; the asterisk * in panel D marks the light chain of the antibody RBF-4E4 detected here. Here input is lysate from untreated HeLa S3 cells (Input).

Figure 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 amino acids (aa) 1-100 (Δ NT), aa 101-190 [Δ (aa101-190)] but with the addition of the SV40 large T antigen NLS (PKKKRKVG) to its N-terminus (Δ (aa101-190)+NLS], aa 191-290 (Δ (aa191-290)), aa 291-390 (Δ (aa291-390)), aa 391-488 (Δ (aa391-488)) and aa 489-566 (Δ CT) are depicted (panel A). **B.** HeLa S3 cells transiently expressing FLAG-Cdc45, Δ NT (lacking aa 1-100), Δ (aa101-190)+NLS, Δ (aa191-290), Δ (aa291-390), Δ (aa391-488), Δ CT (lacking aa 489-566) 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 $\Delta(\text{aa}101-190+\text{NLS})$ suggesting that the monoclonal antibody recognize aa in the region aa101-190. Lysate from mock transfected control cells indicates the position of key proteins by western blotting (Lysate).

Figure 3: Claspin and RPA32 interact with Cdc45 maximally in S phase.

A. 1.5×10^8 K562 cells transiently expressing FLAG-Cdc45 were elutriated, collected and fractions were analysed by FACS for cell cycle-enriched cells (see Supplementary Figure 3A for additional analysis). **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 (panel 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 recognising the indicated proteins (panel 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).

Figure 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 J/m^2 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 (panel A). In panels B and 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), exposed to 30 J/m^2 UVC (IP: 30 J/m^2) or mock transfected control cells (IP: Control) were analysed by SDS-PAGE and western blotting. Antibodies raised against Cdc45, Claspin and RPA were employed to detect

FLAG or Cdc45 and associated Claspin (panel B) and RPA (panel C). In panel D, asynchronous HeLa S3 cells (UT) or cells treated with 30 J/m² of UVC (30 J/m²) 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. In panel E, Claspin was immunoprecipitated as indicated in lanes 2 to 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.

Figure 5: Inhibition of Chk1 by UCN-01 does not recover Cdc45-Claspin interaction after UV damage

HeLa S3 cells transiently expressing FLAG-Cdc45, or mock transfected control cells were treated with 30 J/m² of UVC with or without a 1 h 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 (panel 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 panel 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).

Figure 6: Inhibition of DNA damage signalling by Caffeine and Wortmannin does not recover Cdc45-Claspin interaction after UV damage

HeLa S3 cells transiently expressing FLAG-Cdc45, or mock transfected control cells were treated with 30 J/m² of UVC with or without a 1 h pre-treatment with 5 mM Caffeine or 100 μ M 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 (panel 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/m² 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 after 3 h of the addition of Caffeine. Eluted proteins were separated by SDS-PAGE and analysed by western blotting using antibodies specific to Claspin and Cdc45 as shown in panel B. **C.** In addition, HeLa S3 cells transiently expressing FLAG-Cdc45, expressing FLAG-Cdc45 pre-treated for 1 h with 100 μ M Wortmannin or mock transfected control cells were treated with 30 J/m² of UVC with or without a 1 h pre-treatment with 100 μ M 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 P-RPA32 S4/S8. Equal loading of proteins was verified by the detection of β -Actin, which served as a loading control (panel C). Panels B and D present the FLAG-immunoprecipitation performed with lysates HeLa S3 cells after SDS-PAGE and western blotting. Here, lysate from mock transfected control cells indicates the position of key proteins by western blotting (Lysate).

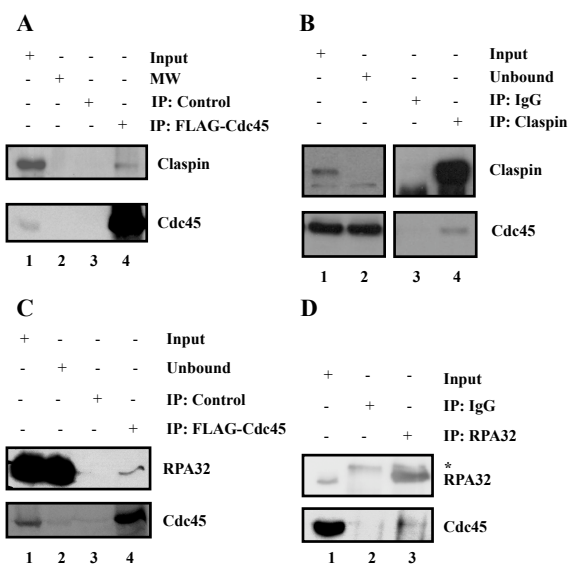
Figure 1

Figure 2

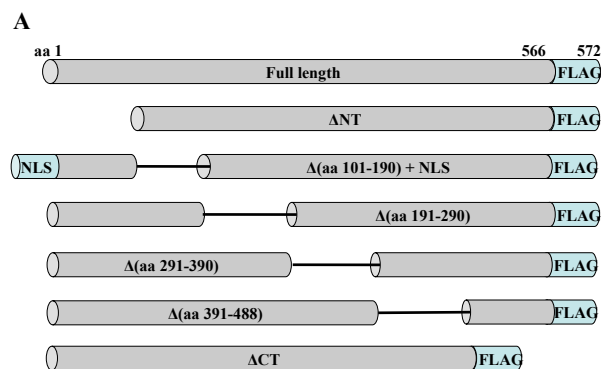


Figure 2

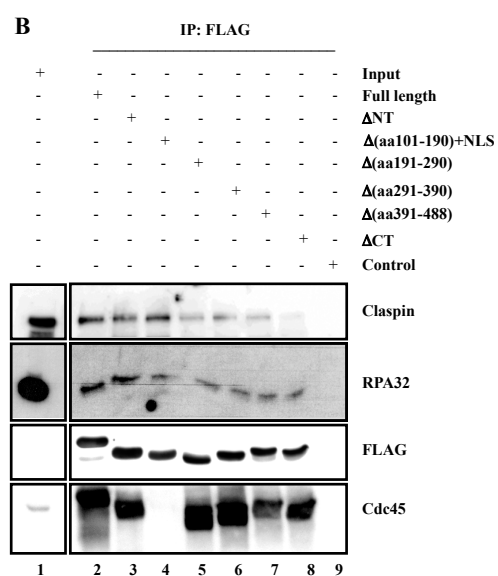


Figure 3

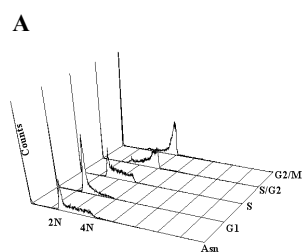


Figure 3

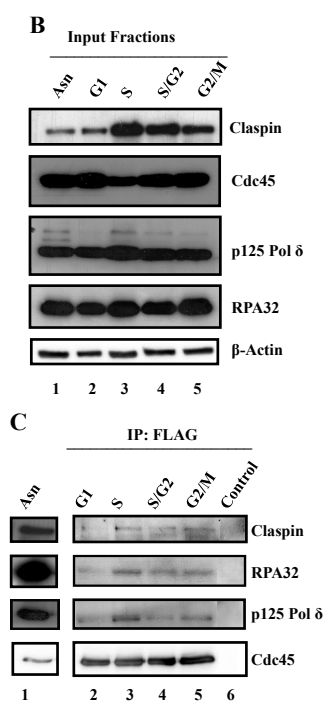


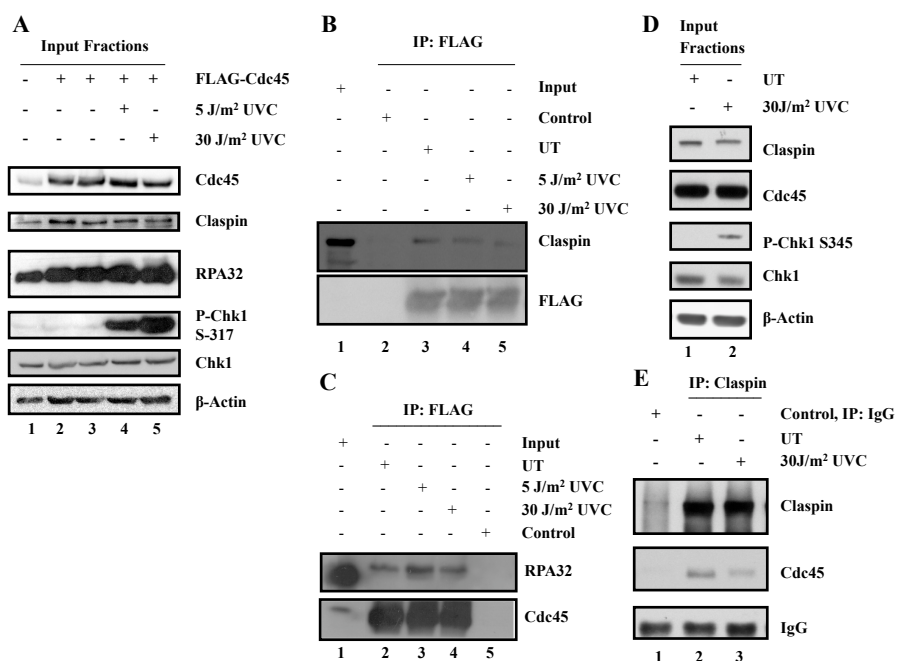
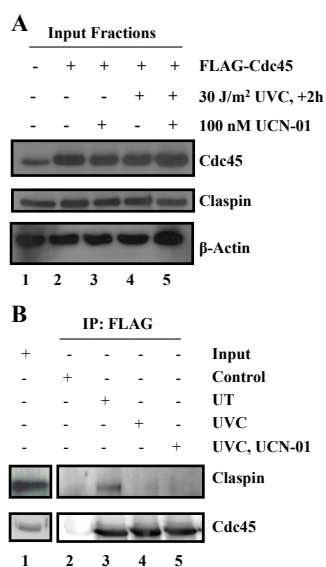
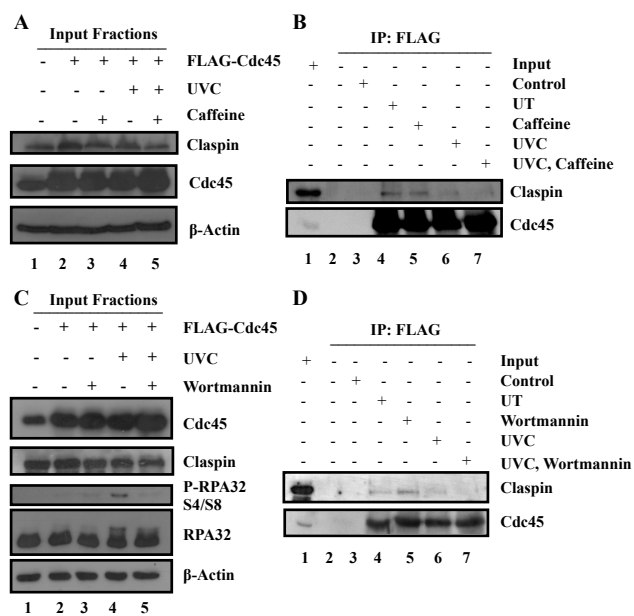
Figure 4**Figure 5**

Figure 6

Cell cycle-dependent formation of Cdc45-Claspin complexes in human cells are compromised by UV-mediated DNA damage.

Ronan Broderick, Michael D. Rainey, Corrado Santocanale & Heinz Peter Nasheuer§.

Centre for Chromosome Biology, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland.

Supplementary information:

Drug validation

Validation of Caffeine activity was carried out as previously described [1]. Briefly HeLa S3 cells were pre-treated with 5 mM Caffeine for 1 h before irradiation with 2 Gy I.R. and cells were harvested 1 h post-irradiation. Cells were treated with ionizing radiation (IR) in the presence of serum-containing medium, using a gamma irradiator (Mainance Engineering Ltd, UK) at room temperature.

Cells positive for p-S10 on histone H3 were determined by FACS analysis. For analysis of pS10-Histone H3 staining, 1×10^6 cells were fixed as normal, centrifuged at $1,500 \times g$ for 5 min at 4°C and re-suspended in 50 µl of PBS supplemented with 1% BSA, 0.5% Triton X-100 and pS10-Histone H3 antibody at a dilution of 1:50, with cells incubated with primary antibody for 2 h at room temperature. Cells were then washed twice with 1 ml PBS supplemented with 1% BSA before re-suspension in 50 µl of PBS supplemented with 1% BSA, 0.5% Triton X100 and anti-rabbit-FITC antibody at a dilution of 1:50, incubating for 1 h with secondary antibody at room temperature in the dark. Cells were then centrifuged and washed twice in 1 ml PBS supplemented with 1% BSA before re-suspended in 1 ml propidium iodide with RNase solution (BD Biosciences) for 1 h at room temperature in the dark on an overhead rocker. Cells positive for PI staining and/or for pS10-Histone H3 staining were acquired on a FACS canto flow cytometer (BD Biosciences) with data analysed using WinMDi software. Antibody recognising pS10-Histone H3 (06-570) was obtained from Upstate. Goat-anti-rabbit-FITC (111-096-042) was obtained from Jackson Immunoresearch.

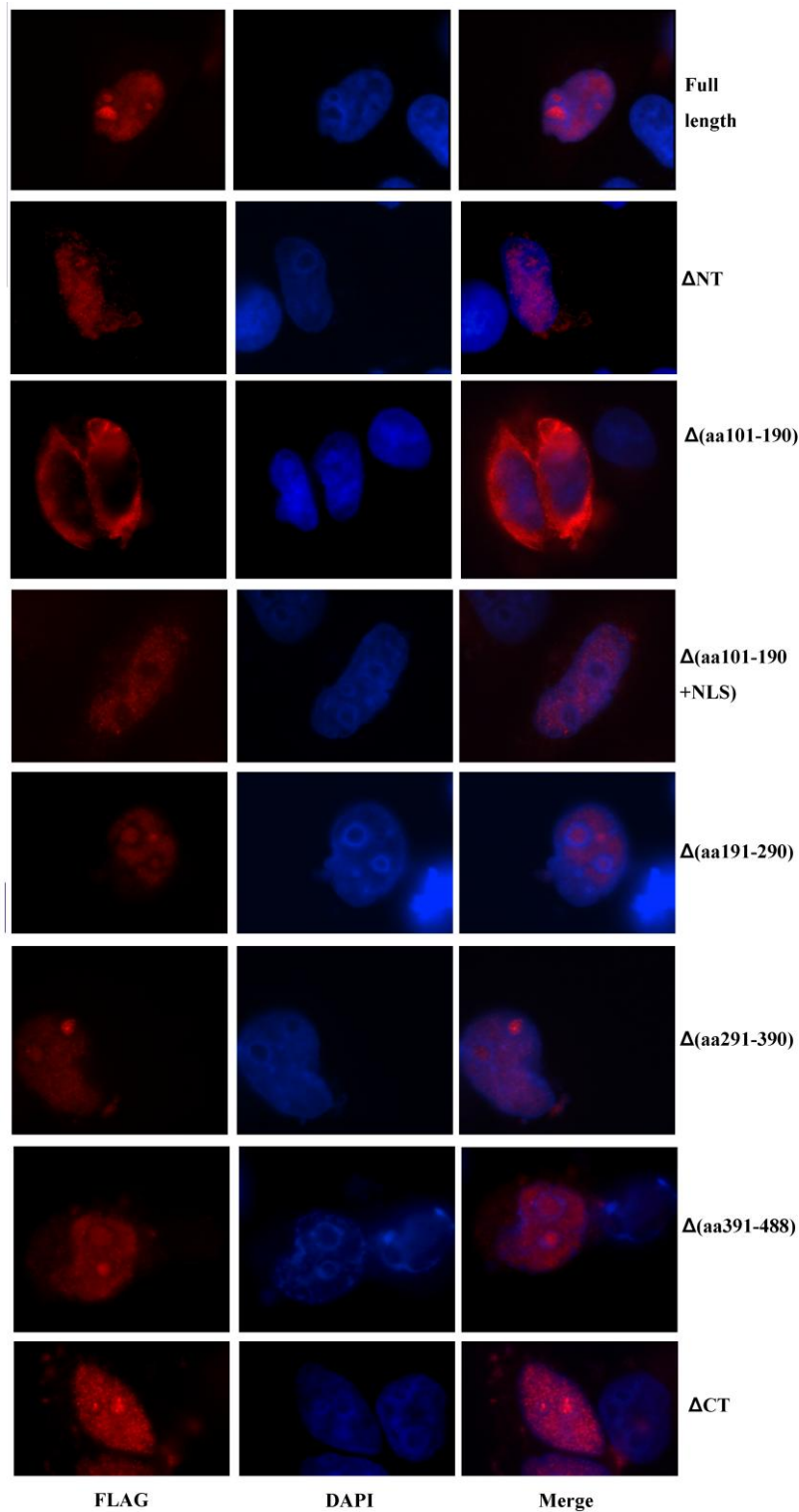
Validation of UCN-01 activity was carried out as previously described [2]. Briefly, HeLa S3 cells were irradiated with 6.3 Gy IR and nocodazole (Sigma) was added to cells at a concentration of 400 ng/ml for 17 h to synchronise cells in mitosis. 6 h before the

end of the nocodazole block, UCN-01 was added at a concentration of 100 nM to cells in media containing nocodazole. Cells were harvested for SDS-PAGE and western blotting with Cdk1 phosphorylation assessed using an antibody against total Cdk1. Total cell lysates were prepared in RIPA buffer (1% Triton X-100, 0.5% deoxycholate, 1% Sodium Dodecyl Sulphate (SDS) in PBS, pH 7.4) supplemented with phosphatase inhibitor cocktail II (Sigma) and EDTA free protease inhibitor cocktail (Roche Applied Sciences). Briefly, cells were lysed for 20 min at room temperature and centrifuged at 18,000 x g for 10 min at 4°C. Supernatants were collected and used for subsequent experiments. Then validation of Wortmannin activity was carried out by analysing RPA32 S4/S8 phosphorylation by SDS-PAGE and western blotting as previously described [3].

Immunofluorescence microscopy was carried out as follows. Cells were grown on glass coverslips (Alpha-Hartenstein, Germany) in culture, were washed twice in PBS and fixed using 4% paraformaldehyde (PFA) for 10 min at 37°C and rinsed 3 times in PBS. Cells were permeabilised with 0.2% Triton-X100 in PBS for 10 mins at 37°C and rinsed 3 times in PBS and transferred to a humidified chamber. Fixed cells were blocked using blocking solution (5% BSA, 10% Goat Serum in PBS-T (0.025% Tween)). Primary antibodies were diluted in blocking solution and blocked cells were incubated for 1h at 37°C with primary antibody. Coverslips were then washed 3 times for 5 min in PBS-T (0.025% Tween) before incubation with secondary antibodies. Secondary antibodies were appropriately diluted in blocking solution and coverslips were incubated with secondary antibody for 1h at 37°C. Cover slips were washed 3 times for 5 min with PBS-T (0.025% Tween) before mounting on glass slides (Alpha-Hartenstein, Germany) using Vectashield mounting medium with DAPI (Vector Laboratories). Images were acquired using an Olympus IX51 Brightfield microscope and Cell R software.

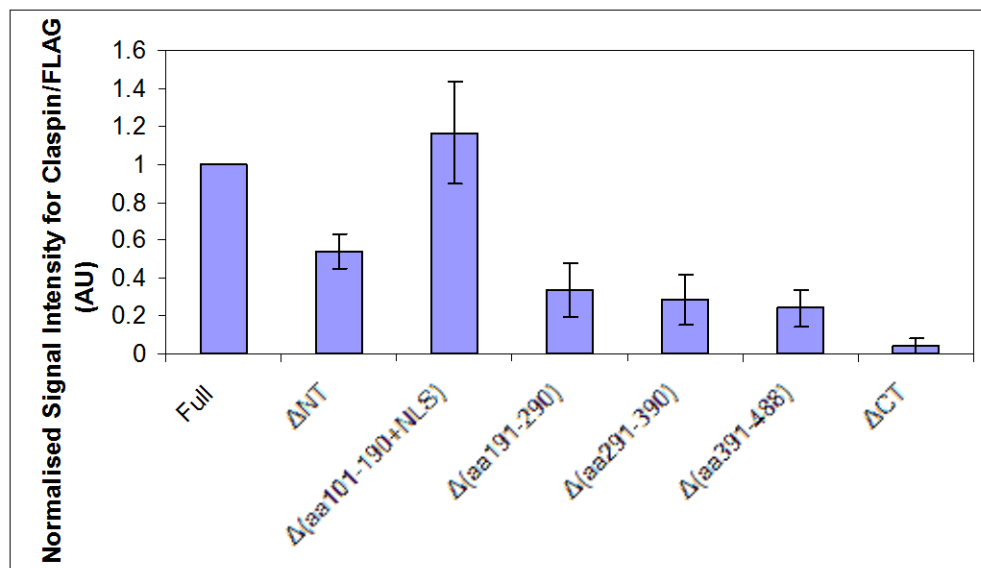
ATM kinase inhibitor KU-55933 (provided by KuDOS Pharmaceuticals Ltd, Cambridge, UK, now AstraZeneca plc) was dissolved as stock solution of 1 mM in DMSO and added at indicated concentrations to the cells with DMSO serving as a negative control.

Antibody raised against phospho-Chk2 (Thr 68) and Chk2 was obtained from Cell signalling (#2661 and #2662, respectively).

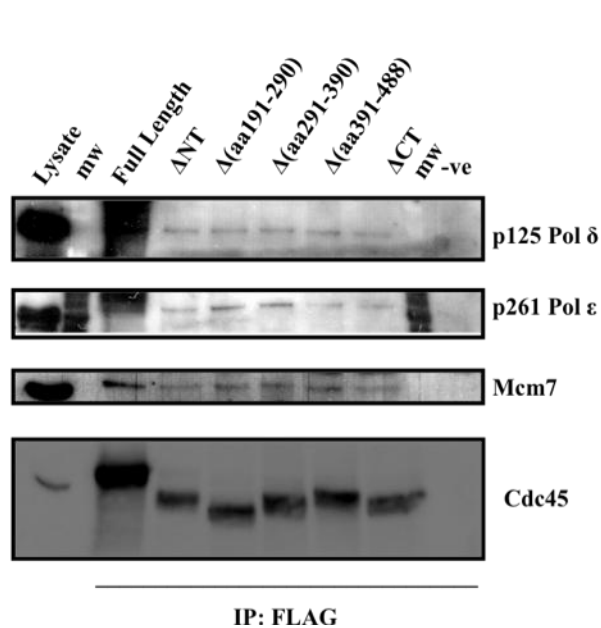


Supporting information Figure S1: Localisation of FLAG-Cdc45 and FLAG-Cdc45 mutants. HeLa S3 cells were transfected with plasmid encoding C-terminally FLAG-tagged Cdc45 (Full length) or with plasmids encoding C-terminally FLAG-tagged deletion mutants (Δ NT (lacking amino acids (aa) 1-100), Δ (aa101-190), Δ (aa101-190+NLS), Δ (aa191-290), Δ (aa291-390), Δ (aa391-488) and Δ CT (lacking aa 489-566)). Cells were harvested, fixed and subjected to immunofluorescence microscopy using an antibody specific for FLAG and Cy3-conjugated secondary antibodies for detection. DAPI staining acts as a marker for the nucleus.

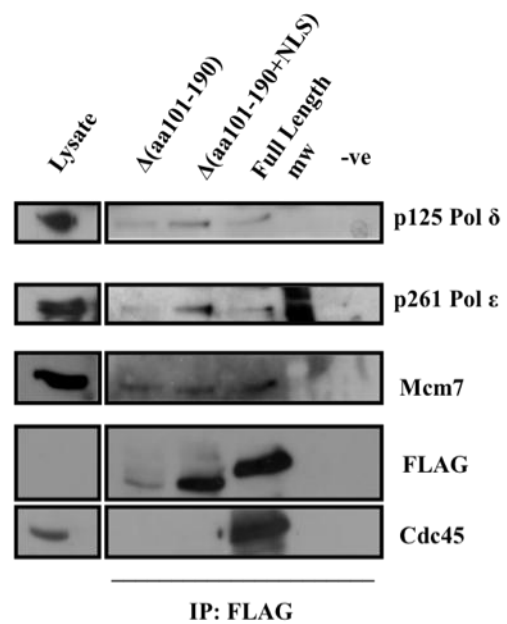
A



B

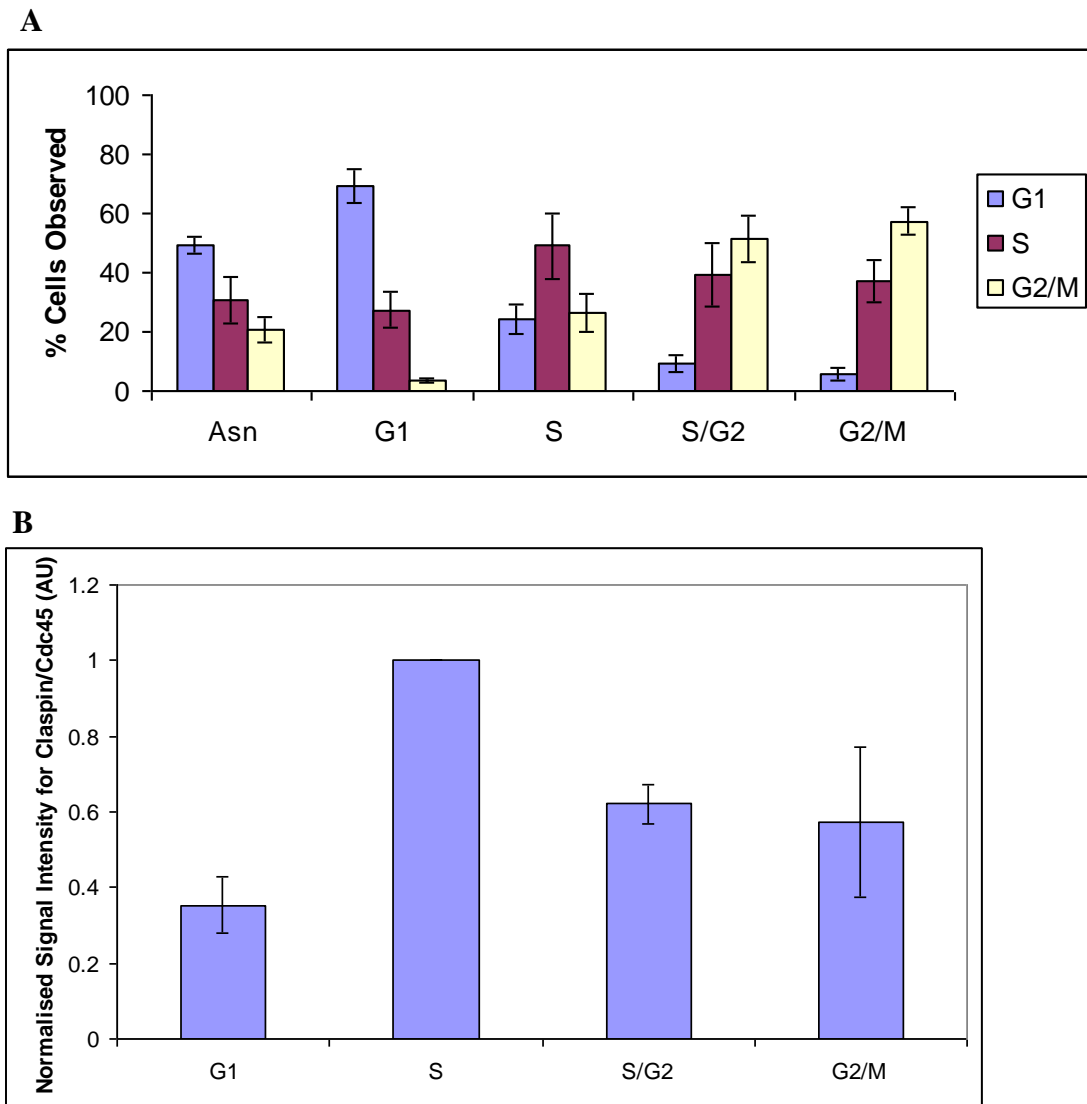


C

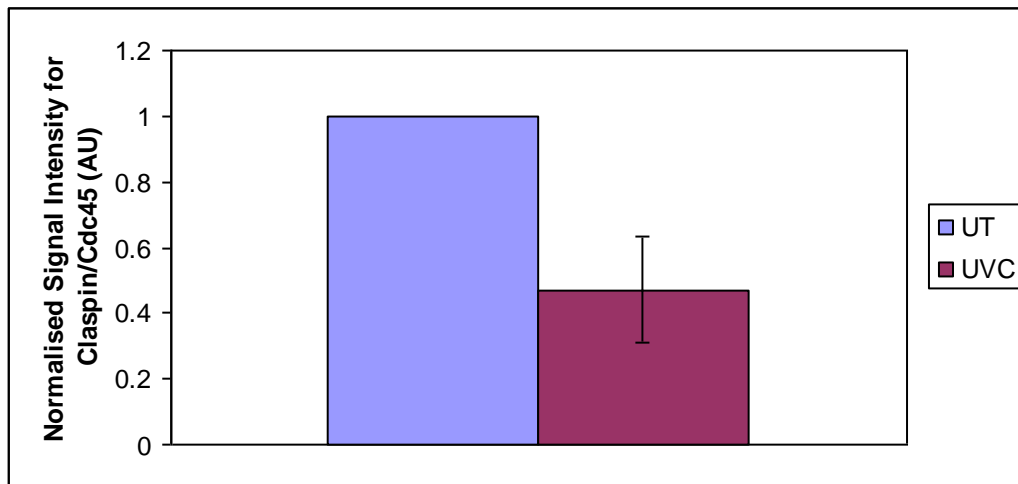


Supporting information Figure S2:

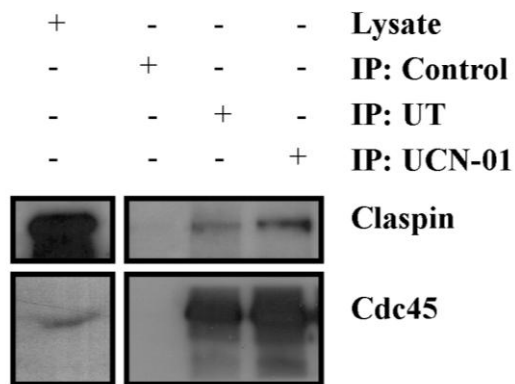
Supporting information Figure S2: Co-immunoprecipitation of FLAG-Cdc45 deletion mutants with replication proteins. A. The ratios of signal intensities for Claspin (minus background)/FLAG (minus background) from three immunoprecipitation experiments of FLAG-Cdc45 and mutant FLAG-Cdc45 expressed in HeLa S3 cells were determined by densitometry. Signal intensities minus background for Claspin in each experiment were obtained in the same way. The ratio of Claspin signal to immunoprecipitated full length FLAG-Cdc45 (Full) was arbitrarily set to 1. Ratio of signal for Claspin/FLAG for full length FLAG-Cdc45 (Full), Δ NT (lacking aa 1-100), Δ (aa101-190+NLS), Δ (aa191-290), Δ (aa291-390), Δ (aa391-488) and Δ CT (lacking aa 489-566) are depicted with standard deviation from the mean indicated by error bars. AU = Arbitrary Units. **B.** HeLa S3 cells transiently expressing full length FLAG-Cdc45 (Full Length), Δ NT, Δ (aa191-290), Δ (aa291-390), Δ (aa391-488), Δ CT or mock transfected control cells (-ve) (panel A) or FLAG-Cdc45 (full length), Δ (aa101-190), Δ (aa101-190+NLS) or mock transfected control cells (-ve) (panel B) were lysed and subjected to FLAG IP. Eluates from each IP were analysed by SDS-PAGE and western blotting using antibodies specific to p125 of Pol δ , p261 of Pol ϵ , Mcm7 and Cdc45 to determine co-IP for each interactor with FLAG-Cdc45. Lysate from mock-transfected control cells acts as a marker for positions of key proteins by western blotting (Lysate).



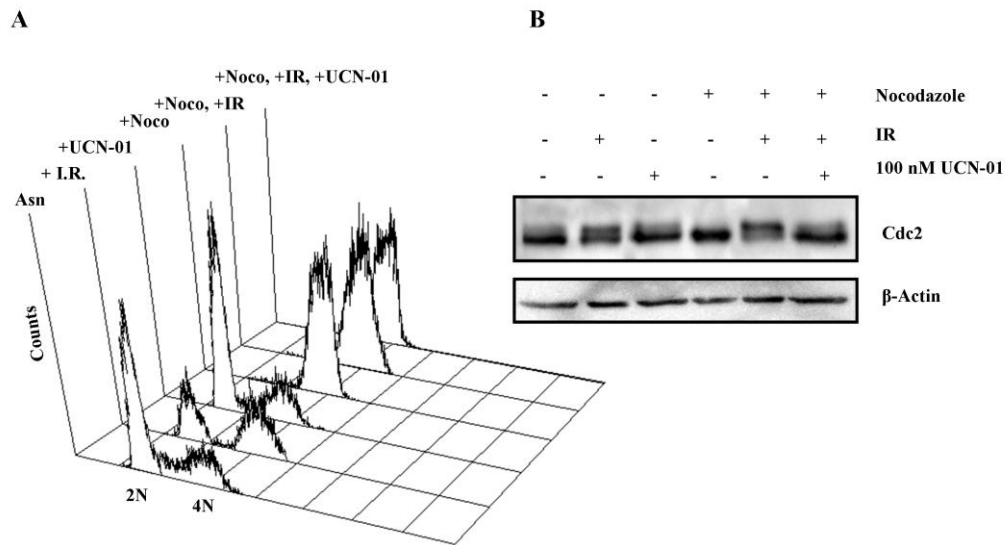
Supporting information Figure S3: Densitometry analysis of Cdc45-Claspins interaction in the cell cycle. **A.** Human exponentially growing K562 cells transiently expressing FLAG-Cdc45 were separated by elutriation into four fractions, which were named G1, S, S/G2 and G2/M following the main population of cells in these fractions. The numbers presented are the average and standard deviation of two independent elutriation experiments from an asynchronous control cells (Asn) or from cells enriched at the G1, S, S/G2 or G2/M stages of the cell cycle were measured by flow cytometry after propidium iodide staining. **B.** The ratios of signal intensities for Claspins (minus background) and Cdc45 of two independent Co-IP experiments of FLAG-Cdc45 from elutriated K562 cells were determined by densitometry. Signal intensities minus background for Cdc45 and Claspins in each experiment were determined by densitometry (G1 signal for Cdc45 and S phase Claspins signal were normalised to 1). Ratio of signal for Claspins/Cdc45 for G1, S, S/G2 and G2/M cells are depicted with standard deviation from the mean indicated by error bars. AU = Arbitrary Units



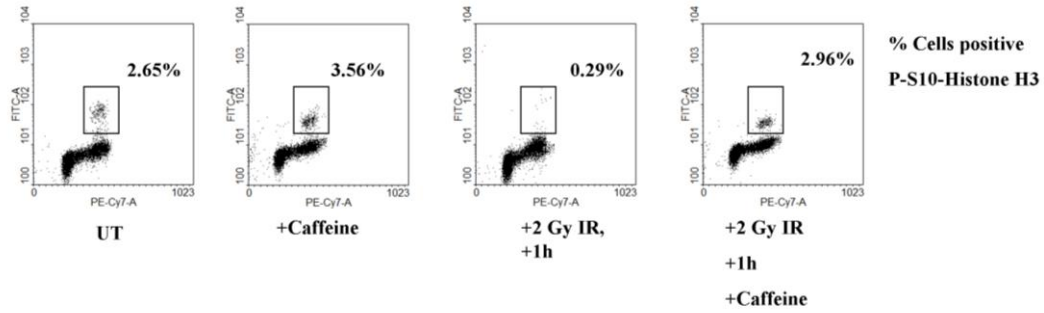
Supporting information Figure S4: Densitometry analysis of Cdc45-Claspin interaction after UVC treatment. The ratios of signal intensities for Claspin (minus background)/Cdc45 (minus background) from 4 different immunoprecipitation experiments of FLAG-Cdc45 from HeLa S3 cells were determined by densitometry. The difference in ratio between untreated (UT, arbitrarily normalised to 1) and UVC-treated (UVC) cells with standard deviation from the mean indicated by error bars are depicted. AU = Arbitrary Units



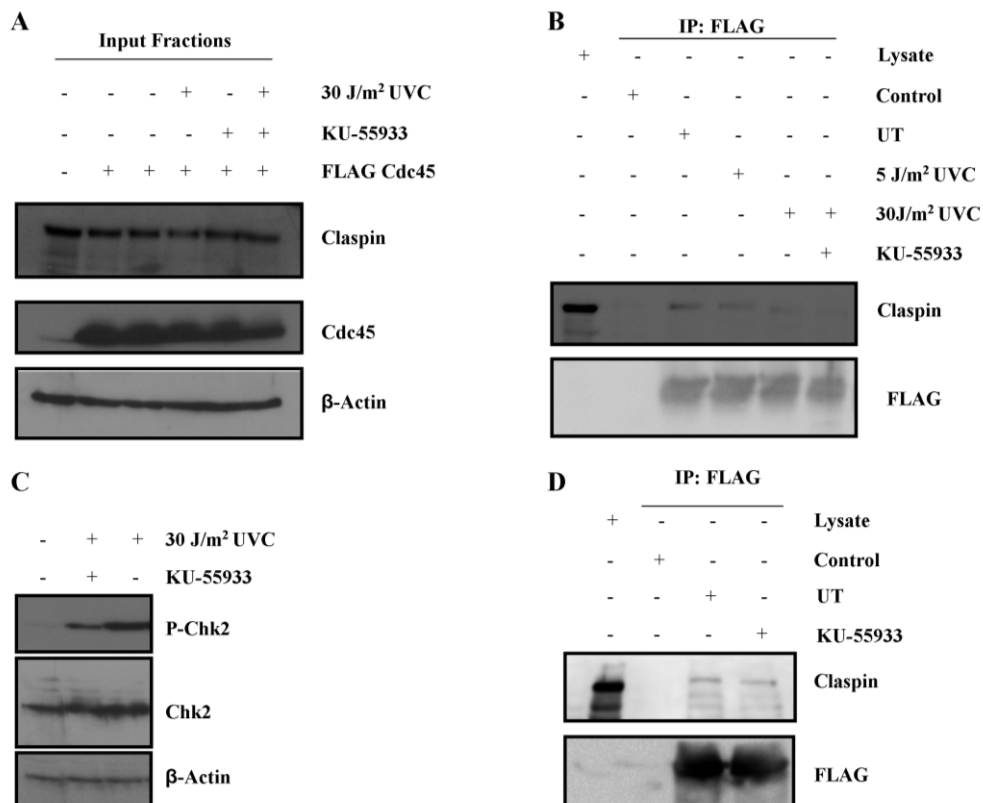
Supporting information Figure S5: FLAG immunoprecipitation in the presence of UCN-01. HeLa S3 cells transiently expressing FLAG-Cdc45 or mock-transfected control cells were left untreated or treated for 1 h in the presence of 100 nM UCN-01. Cell lysates were normalised for protein content and subjected to FLAG immunoprecipitation. HeLa S3 lysate from mock treated control cells (Lysate), and eluates from immunoprecipitations of mock transfected control cells (IP: Control), cells expressing FLAG-Cdc45 (IP: UT) and cells expressing FLAG-Cdc45 in the presence of UCN-01 (IP: UCN-01) were separated by SDS-PAGE and the Co-IP of Claspín with FLAG-Cdc45 was assayed using antibodies raised against Cdc45 and Claspín.



Supporting information Figure S6: Validation of UCN-01 efficacy. HeLa S3 cells were irradiated with 6.3 Gy IR and then incubated in the presence or absence of 400 ng/ml nocodazole for 17 h. 11h post-irradiation, UCN-01 was added at a concentration of 100 nM as indicated. Asynchronous (Asn), irradiated (+IR), UCN-01 treated (+UCN-01), nocodazole treated (+Noc) cells, and cells given a combination of treatments (+Noc, +IR) and (+Noc, +IR, +UCN-01) were fixed and subjected to propidium iodide staining and FACS analysis (panel A). **B.** Cells were TGN-lysed, normalised for protein content and subjected to SDS-PAGE and western blotting using antibodies specific to Cdk1 kinase and β -Actin. The former validates UCN-01 efficacy and the latter acts as a loading control, respectively.



Supporting information Figure S7: Validation of Caffeine efficacy. Asynchronous HeLa S3 cells (UT), cells incubated with 5 mM Caffeine for 2 h (+Caffeine), cells exposed to 2 Gy ionizing radiation (IR) and harvested 1 h post-treatment (+2 Gy IR, + 1h) or cells pre-treated for 1h with Caffeine, exposed to 2 Gy IR and harvested after 1h in the presence of Caffeine (+2 Gy IR, +1 h, +Caffeine) were fixed and harvested for FACS analysis. pS-10-Histone H3 staining was employed and used to determine the % of cells positive for staining.



Supporting information Figure S8: KU-55933 treatment does not rescue Cdc45-Claspin interaction following UVC treatment.

HeLa S3 cells transiently expressing FLAG-Cdc45, or mock-transfected control cells were treated with 30 J/m² of UVC with or without a 1 h pre-treatment of 10 μM KU-55933 and were harvested 2 h post-UVC treatment in the presence or absence of drug as indicated. **A.** Cells were lysed and subjected to SDS-PAGE and western blotting using antibodies specific to Cdc45, Claspin. Detection of β-Actin acts as a loading control (panel A). **B.** To determine the influence of the ATM kinase inhibitor KU-55933, FLAG-IP was performed with lysates of untreated HeLa S3 cells expressing FLAG-Cdc45 (IP: UT), with HeLa S3 cells expressing FLAG-Cdc45 exposed to a 1 h pre-treatment with 10 μM KU-55933, expressing FLAG-Cdc45 exposed to 30 J/m² of UVC in the presence of DMSO only but no KU-55933 and in the presence of KU-55933 as described above. Both UVC-exposed cell populations were harvested 2 h post-UV treatment whereas Caffeine-only incubated cells were collected after 3 h of the addition of Caffeine. Eluted proteins were separated by SDS-PAGE and analysed by western blotting using antibodies specific to Claspin and FLAG as shown in panel B. Lysate from mock-transfected control cells indicates position of key proteins by western blotting (Lysate). **C.** To determine the efficacy of KU-55933 HeLa S3 cells treated with 30 J/m² of UVC with or without a 1 h pre-treatment with 10 μM KU-55933 were harvested 2 h post-UVC treatment in the presence or absence of drug as indicated. Cells were lysed and subjected to SDS-PAGE and western blotting using antibodies specific to phospho-Chk2 (T68), Chk2 and β-Actin, which serves as a loading control. **D.** To determine if KU-55933 treatment alone affects Cdc45-Claspin interaction, FLAG-IP was performed with lysates of untreated HeLa S3 cells expressing FLAG-Cdc45 (IP: UT), or with HeLa S3 cells expressing FLAG-Cdc45 exposed to a 3 h treatment with 10 μM KU-55933. Control: FLAG-IP of mock-transfected cells. Lysate from mock-transfected control cells marks the position of key proteins by western blotting (Lysate).

References

1. Rainey MD, Charlton ME, Stanton RV & Kastan MB (2008) Transient inhibition of ATM kinase is sufficient to enhance cellular sensitivity to ionizing radiation. *Cancer Res* **68**, 7466-7474.
2. Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA & O'Connor PM (1996) UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst* **88**, 956-965.
3. Stephan H, Concannon C, Kremmer E, Carty MP & Nasheuer HP (2009) Ionizing radiation-dependent and independent phosphorylation of the 32-kDa subunit of replication protein A during mitosis. *Nucleic acids research* **37**, 6028-6041.