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The conserved C terminus of Claspin interacts with Rad9 and promotes rapid activation of Chk1

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Claspin is a key mediator of the ATR-Chk1 checkpoint pathway. In response to DNA damage, Claspin interacts with Rad17 and Chk1 in a phosphorylation-dependent manner, enabling ATR to phosphorylate Chk1 efficiently. Claspin also interacts with Rad9, but how they interact and whether the interaction is functional remains unknown. Unexpectedly, our analysis of two splicing isoforms of Claspin provided an answer to these questions. The Claspin^{1339} isoform contains an evolutionarily conserved C terminus, but the Claspin^{1332} isoform does not. Although the transcripts encoding both Claspin isoforms coexist in HCT116 cells, Claspin^{1339} is the predominant form responsible for Chk1 activation. When expressed in cells depleted of endogenous Claspin, both Claspin^{1339} and Claspin^{1332} are able to mediate Chk1 activation. However, the activation of Chk1 is delayed in Claspin^{1332}-expressing cells compared with Claspin^{1339}-expressing cells. Furthermore, only Claspin^{1339} but not Claspin^{1332} interacts with Rad9 efficiently. Together, these results suggest that the conserved C terminus of Claspin interacts with Rad9 and ensures timely activation of the ATR-Chk1 pathway.

Introduction

The DNA damage checkpoint is a barrier against genomic instability and tumorigenesis.[1,2] In response to DNA damage, the checkpoint is activated to arrest the cell cycle, promote DNA repair or trigger cell death. The ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) kinases are key regulators of the checkpoint.[3,4] Whereas ATM primarily responds to double-stranded DNA breaks (DSBs), ATR is activated by a wide spectrum of DNA damage and DNA replication stress. ATR and its functional partner ATRIP are recruited to RPA-coated single-stranded DNA (ssDNA) at sites of DNA damage or stalled replication forks,[5] and its kinase activity is stimulated by TopBP1.[6] Once activated, ATR phosphorylates and activates the effector kinase Chk1 through a process that depends on the mediator protein Claspin.[7,8] The ATR-Claspin-Chk1 pathway plays a critical role in protecting DNA replication forks against DNA damage.

Claspin is a protein involved in both DNA replication and the response to DNA damage.[8-11] When DNA damage interferes with DNA replication, Claspin interacts with Rad17, a DNA damage sensor required for ATR activation.[12,13] Furthermore, Claspin is phosphorylated by CK1 in an ATR-dependent manner, increasing the affinity of Claspin to Chk1.[14-16] In vitro, Claspin stimulates the phosphorylation of Chk1 by ATR.[17,18] How Claspin regulates Chk1 activation in human cells is not yet fully understood, and it remains an important question for the research of DNA damage response.

The human *CLSPN* gene encoding Claspin is located on chromosome 1p34.2.[7] Several isoforms of the *CLSPN* transcript are included in the GeneBank. Among these, the originally reported *CLSPN* transcript (AF297866.1) encodes a protein of 1332 amino acids (referred to as Claspin^{1332}),[7] whereas the subsequently deposited *CLSPN* transcript 1 (NM_022111.3) encodes a protein of 1339 amino acids (referred to as Claspin^{1339}). Claspin^{1332} and Claspin^{1339} are only distinct in their extreme C termini (see below). The C terminus of Claspin^{1339} but not Claspin^{1332} is evolutionarily conserved. Both Claspin^{1332} and Claspin^{1339} have been widely used in functional analyses by previous studies.[9,10,18-24] However, which of these Claspin isoforms is predominant in cells and whether they are equally functional is not clear.

Here, we show that the *CLSPN* transcripts encoding the two distinct C termini of Claspin coexist in multiple human cancer cell lines. In the colon cancer cell line HCT116, the predominant form of Claspin contains the conserved C terminus. When expressed in cells depleted of endogenous Claspin, both Claspin^{1339} and Claspin^{1332} are able to mediate Chk1 activation in response to UV radiation. However, Chk1 activation in cells expressing Claspin^{1332} is delayed compared with cells expressing Claspin^{1339}. In addition, unlike Claspin^{1339}, Claspin^{1332} is unable to efficiently interact with Rad9, a component of the Rad9-Rad1-Hus1 (9-1-1) complex associated with Rad17. Together, these results suggest that the conserved C terminus of Claspin is a regulatory domain that engages 9-1-1 and promotes the early phase of Chk1 activation.

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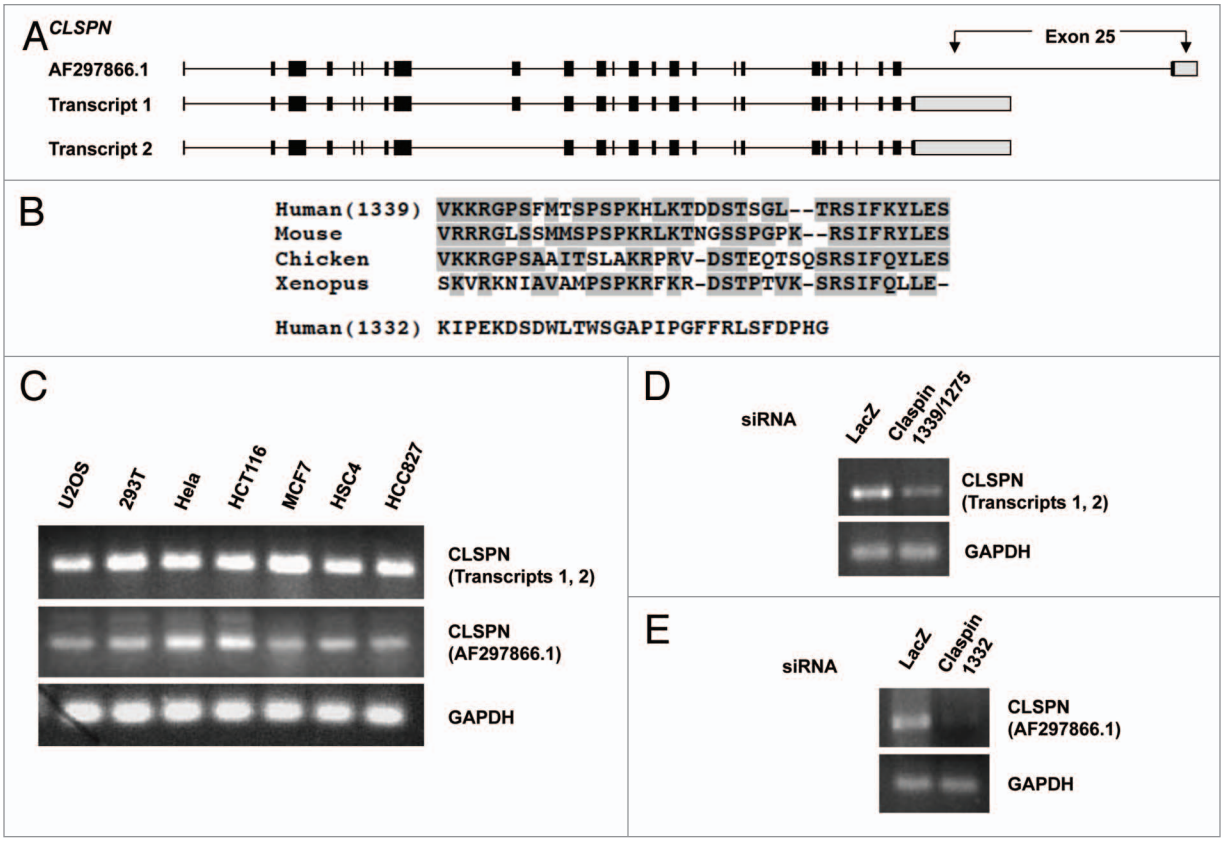

Figure 1. The Claspin transcript isoforms encoding distinct C termini. (A) A schematic representation of the *CLSPN* transcript isoforms. (B) Alignment of the C terminus of human Claspin^{1339} with the corresponding regions in Claspin homologs from other species. The conserved amino acids are shaded. The C terminus of human Claspin^{1332} is also shown. (C) The *CLSPN* transcripts encoding the two distinct C termini were detected in a panel of human cancer cell lines by RT-PCR using specific primers. RT-PRC of GAPDH served as the control for mRNA inputs. (D and E) HCT116 cells were transfected with si1339/1275 and si1332, and mRNA was prepared and analyzed by RT-PCR as in (C).

Results

The *CLSPN* transcripts encoding distinct C termini coexist in human cells. The human *CLSPN* gene was identified based on the homology between its annotated protein product and Xenpous Claspin protein.[7] The originally reported *CLSPN* transcript (AF297866.1) contains 25 exons, and it encodes a protein of 1332 amino acids (Claspin^{1332}). Two additional *CLSPN* transcript isoforms were subsequently deposited to the GeneBank. The *CLSPN* transcript 1 (NM_022111.3) contains 25 exons, whereas the *CLSPN* transcript 2 (NM_001190481.1) contains only 24 exons. The exon 9 of *CLSPN* transcript 1 is absent in the transcript 2 (**Fig. 1A**). The *CLSPN* transcripts 1 and 2 encode two Claspin isoforms of 1339 (Claspin^{1339}) and 1275 amino acids (Claspin^{1275}), respectively. The *CLSPN* transcript AF297866.1 and transcript 1 contain different exons 25 (**Fig. 1A**). As a result, the last 36 amino acids of Claspin^{1339} and the last 29 amino acids of Claspin^{1332} are distinct (**Fig. 1B**). The extreme C terminus of Claspin^{1339} but not Claspin^{1332} is conserved in vertebrates (**Fig. 1B**), suggesting that Claspin^{1332} is either a recently emerged variant or a product in certain cancer cells.

To distinguish the *CLSPN* transcripts encoding the two distinct C termini, we designed two primer sets to specifically amplify the two alternative exons 25. The primer set 1 detects both the *CLSPN* transcripts 1 and 2, whereas the primer set 2 specifically detects the *CLSPN* transcript AF297866.1. RT-PCR was performed using the two primer sets and mRNA derived from a panel of human cancer cell lines (**Fig. 1C**). The *CLSPN* transcripts encoding the two distinct C termini were detected in all cell lines, suggesting that they are present broadly and not mutually exclusive. To confirm the specificity of RT-PCR, we designed two siRNAs (si1339/1275 and si1332) that specifically target the two alternative exons 25 in the *CLSPN* transcripts (**Fig. 1D and E**). The two siRNAs effectively reduced the levels of their respective target mRNAs in HCT116 cells.

Together, these results suggest that the *CLSPN* transcripts encoding the two distinct C termini coexist in human cells. Furthermore, given that the cancer cell lines that we analyzed have different origins, the presence of the *CLSPN* transcript AF297866.1 in all these cell lines suggests that Claspin^{1332} is unlikely associated with a specific oncogenic event. However, we noted that the relative abundance of the *CLSPN* transcripts varied among the cell lines. Whether the ratio of Claspin isoforms is functionally relevant remains to be investigated.

Both Claspin^{1339} and Claspin^{1332} are functional for Chk1 activation. Using the siRNAs that specifically target the Claspin

isoforms with distinct C termini, we next investigated the relative abundance and functional contribution of these proteins. Transfection of HCT116 cells with a pan-isoform Claspin siRNA (siPAN) that targets all three isoforms significantly reduced the levels of Claspin protein (**Fig. 2A**). Consequently, the UV-induced Chk1 phosphorylation was diminished in siPAN-treated cells (**Fig. 2A**). Similar to siPAN, si1339/1275, which targets the conserved C terminus, also significantly reduced the overall levels of Claspin protein and UV-induced Chk1 phosphorylation (**Fig. 2A**). In marked contrast, si1332, which targets the unconserved C terminus, did not reduce the levels of Claspin protein, nor did it reduce the UV-induced Chk1 phosphorylation (**Fig. 2A**). These results suggest that Claspin^{1339} and/or Claspin^{1275}, but not Claspin^{1332}, are predominant in HCT116 cells and primarily responsible for UV-induced Chk1 activation. As shown in **Figure 2B**, the electrophoretic migration of endogenous Claspin was identical to that of Flag-tagged Claspin^{1339}, suggesting that Claspin^{1339} is the predominant isoform.

Since both Claspin^{1332} and Claspin^{1339} have been used in functional analyses in previous studies, we asked if these Claspin isoforms were equally functional. We generated plasmids that express Flag-tagged Claspin^{1332} or Claspin^{1339}. The coding sequences of Claspin in these plasmids were modified to render them resistant to siPAN. When Flag-Claspin^{1332} and Flag-Claspin^{1339} were individually expressed in siPAN-treated cells, they both suppressed the defect in Chk1 phosphorylation 1 h after UV irradiation (**Fig. 2B**). In contrast, Chk1 phosphorylation remained defective in the siPAN-treated cells that were subsequently transfected with empty vector. These results suggest that, although the abundances of Claspin^{1332} and Claspin^{1339} are different in cells, they are both functional in mediating Chk1 activation.

Cells expressing Claspin^{1332} activate Chk1 with a delay. In the experiment where we compared the abilities of Claspin^{1332} and Claspin^{1339} to mediate Chk1 activation (**Fig. 2B**), we noticed that UV-induced Chk1 phosphorylation was slightly more efficient in Claspin^{1339}-expressing cells than in Claspin^{1332}-expressing cells. To compare the functions of these Claspin isoforms more carefully, we performed the same experiment in **Figure 2B** and monitored Chk1 phosphorylation at 0, 0.5, 1, 2, 4 and 8 h after UV irradiation. In Claspin^{1339}-expressing cells, Chk1 became maximally phosphorylated 1 h after UV irradiation (**Fig. 3A**). However, in Claspin^{1332}-expressing cells, Chk1 phosphorylation did not reach its plateau until 4 h after UV irradiation (**Fig. 3B**). Thus, although Claspin^{1332} is capable of mediating Chk1 activation, there is a delay in the timing of Chk1 activation when Claspin^{1332} is the only form of Claspin in cells.

Only Claspin^{1339} but not Claspin^{1332} interacts with Rad9. Given the delay of Chk1 activation in Claspin^{1332}-expressing cells, we postulated that Claspin^{1332} might be defective in its interaction with another checkpoint protein. Claspin is known to interact with Chk1 in a DNA damaged-induced manner.[7] To compare the abilities of Claspin^{1332} and Claspin^{1339} to interact with Chk1, we transiently expressed Flag-Claspin^{1332} or Flag-Claspin^{1339} in HEK293T cells and then irradiated the cells with UV (**Fig. 4A**). Immunoprecipitation of endogenous Chk1 from cell extracts captured both Flag-Claspin^{1332} and Flag-Claspin^{1339} with similar efficiency (**Fig. 4A**). In contrast, neither Claspin isoform was detected in the control immunoprecipitation with IgG. This experiment rules out the possibility that the two Claspin isoforms have different binding affinities to Chk1 after UV damage.

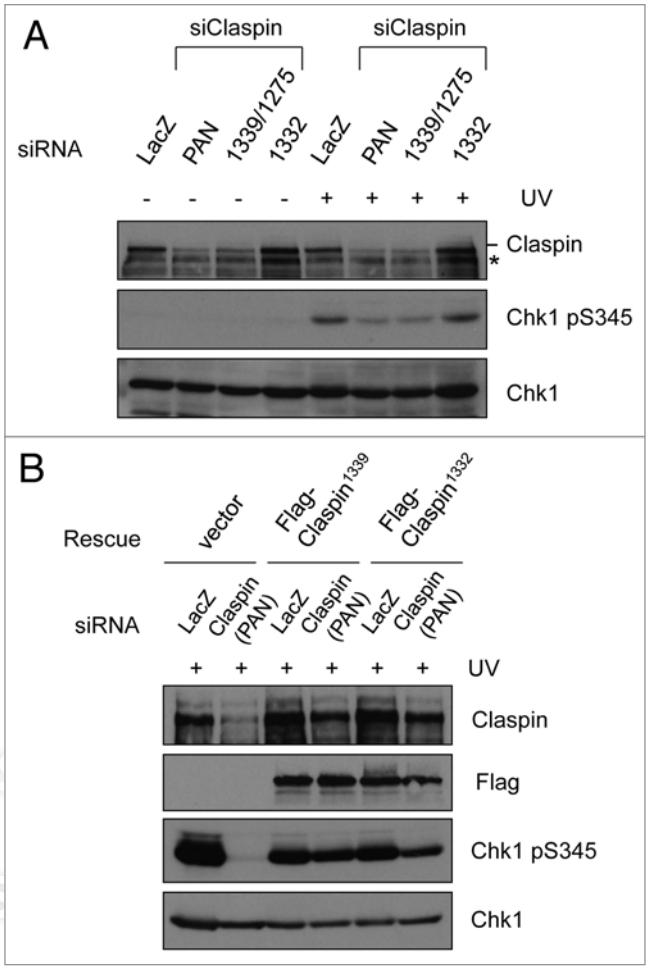

Figure 2. Both Claspin^{1339} and Claspin^{1332} are functional for Chk1 activation. (A) HCT116 cells were transfected with siPAN, si1339/1275 or si1332 and irradiated with 15 J/m^2 UV after 48 h. The phosphorylation of Chk1 and the levels of the indicated proteins were analyzed by western blot 1 h after UV irradiation. *, a protein that cross-reacted with the Claspin antibody. (B) HCT116 cells were first transfected with siLacZ or siPAN, and then transfected again with empty vector, or plasmids expressing Flag-Claspin^{1339} or Flag-Claspin^{1332}. Cells were irradiated with 15 J/m^2 UV, and the levels of phosphorylated Chk1 and the indicated proteins were analyzed by western blot 1 h after UV damage.

In addition to Chk1, Claspin is also known to interact with Rad9,[25] a component of the 9-1-1 complex that is recruited to damaged DNA by the Rad17-RFC complex. To compare the abilities of Claspin^{1132} and Claspin^{1139} to interact with Rad9, we transiently expressed GFP-Rad9 with either Flag-Clapsin^{1339} or Flag-Claspin^{1332} in HEK293T cells, irradiated the cells with UV and then immunoprecipitated Flag-tagged Claspin from cell extracts (**Fig. 4B**). Only Flag-Claspin^{1339} but not Flag-Claspin^{1332} efficiently captured GFP-Rad9 from extracts, suggesting that the conserved C terminus of Claspin is important for the interaction with Rad9. Furthermore, these results suggest that the delay of

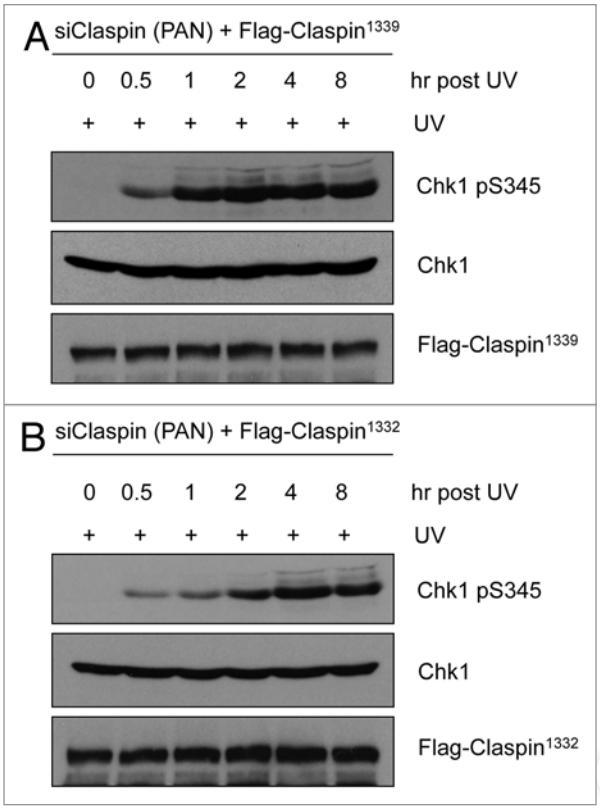

Figure 3. Chk1 phosphorylation is delayed in Claspin^{1332}-expressing cells. (A and B) HCT116 cells were sequentially transfected with siPAN and plasmids expressing Flag-Claspin^{1339} or Flag-Claspin^{1332} vector. Cells were irradiated with 15 J/m^2 UV, and cell extracts were prepared and analyzed by western blot at the indicated time points.

Chk1 activation in Claspin^{1332}-expressing cells may be attributed to the defective Claspin^{1332}-Rad9 interaction.

Discussion

Claspin is a dual functional protein that is involved in both DNA replication and the response to DNA damage. Even in the absence of extrinsic DNA damage, Claspin plays an important role in the progression and stabilization of DNA replication forks, suggesting that Claspin may be a component of the DNA replication machinery.[8,11,26,27] When DNA replication forks encounter DNA damage, increased amounts of ssDNA are generated, leading to recruitment of RPA and the Rad17-RFC complex to stalled replication forks.[12,28,29] The Rad17-RFC complex recognizes the junctions between ssDNA and dsDNA (double-stranded DNA) and loads the 9-1-1 complexes onto damaged DNA.[28,30] Independently of Rad17, RPA coated ssDNA directly recruits the ATR-ATRIP complex and promotes ATR autophosphorylation at Thr 1989.[5,31] Together, the 9-1-1 complex and phosphorylated ATR on damaged DNA bring TopBP1 to the protein complex, which stimulates the kinase activity of ATR-ATRIP and facilitates ATR to recognize its substrates.

How does Claspin contribute to checkpoint activation? Claspin is known to interact with phosphorylated Rad17, a substrate of ATR.[13] This Rad17-Claspin interaction may be established at stalled replication forks after the initial activation of ATR, promoting the subsequent phosphorylation of Claspin by ATR. In an ATR-dependent manner, Claspin is phosphorylated by CK1 at its C terminus, creating a binding site for Chk1.[16] The phosphorylation-mediated interaction between Claspin and Chk1 may transiently recruit Chk1 to stalled replication forks, stimulating the phosphorylation of Chk1 by ATR and Chk1 autophosphorylation.[17,18] In this study, we found that Claspin engages Rad9 through the conserved C terminus. Although this interaction is not absolutely required for Chk1 activation, it is important for efficient Chk1 phosphorylation during the early phase of ATR response. In Xenopus egg extracts, Rad17 and 9-1-1 remain associated after DNA damage.[32] It is possible that the interaction between Claspin and Rad9 enables Claspin to interact with Rad17 more efficiently, thereby accelerating Chk1 recruitment and subsequent activation. Our findings suggest that the protein-protein interactions within the checkpoint-signaling complex control not only the magnitude but also the timing of checkpoint response. To our knowledge, the conserved C terminus of Claspin is the first regulatory domain of a checkpoint protein that has a clear role in the temporal control of DNA damage signaling.

Our results also raise the interesting possibility that alternative splicing of the *CLSPN* transcript may influence the kinetics of ATR response. Given that Claspin^{1332} mediates Chk1 activation with a delay, its presence in replication forks may exert dominant negative effects on certain early events during activation of the ATR-Chk1 pathway. The rapid response of ATR pathway may be important for stabilizing stalled replication forks and promoting specific DNA damage repair or tolerance events. The inability of cells to activate the ATR pathway promptly may compromise genomic stability. The relative abundance of different *CLSPN* transcripts appears to vary among different cancer cell lines (**Fig. 1C**). Due to their difference in 3' UTR, the *CLSPN* transcripts that contain distinct exons 25 may be subjected to differential regulation by miRNAs or other post-transcriptional regulatory mechanisms. It would be interesting to further investigate whether an altered ratio of the Clapsin^{1339} and Claspin^{1332} isoforms is associated with and contributes to tumorigenesis.

Materials and Methods

Cell lines and cell culture. HeLa, HEK293T, U2OS, MCF7, HSC4 and HCC827 cells were maintained in DMEM medium (Invitrogen Corporation) supplemented with 10% fetal bovine serum. HCT116 cells were maintained in McCOY's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum.

siRNAs and plasmids. siRNAs were transfected with Oligofectamine and plasmids were transfected with Lipofectamine following the manufacturer's instructions (Invitrogen). The siRNAs targeting LacZ, Claspin (PAN), Claspin (1339/1275) and Claspin (1332) were synthesized by Invitrogen. The siRNA sequences were as follows: LacZ: AAC GUA CGC GGA AUA CUU CGA; Claspin (PAN): AAG GAA AGA AAG GCA GCC

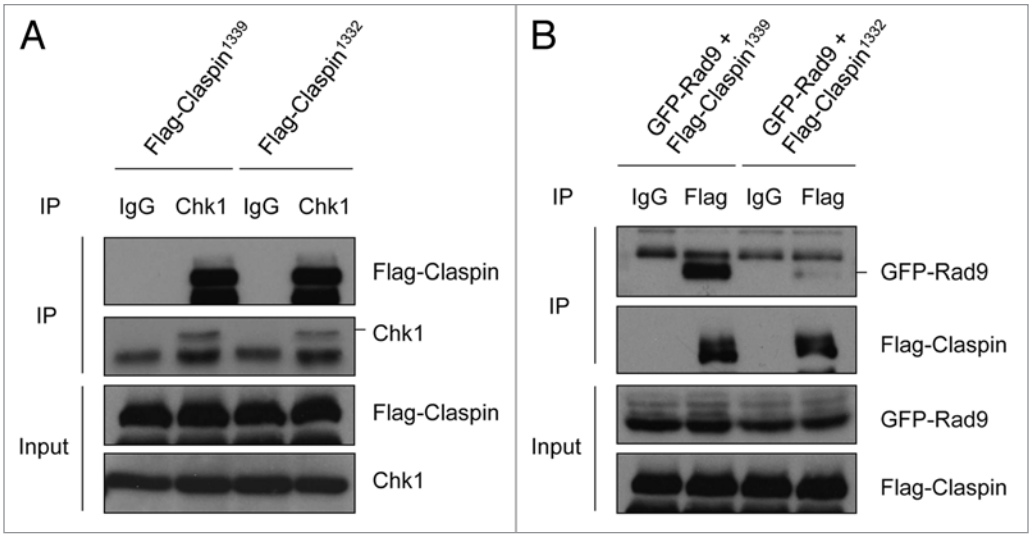

Figure 4. Claspin^{1339} but not Claspin^{1332} interacts with Rad9 efficiently. (A) HEK293T cells transiently expressing Flag-Claspin^{1339} or Flag-Claspin^{1332} were irradiated with 15 J/m^2 UV and lysed after 1 h. Cell extracts were subjected to immunoprecipitation with Chk1 antibodies. The Chk1 and Flag-tagged Claspin in input and precipitates were analyzed by western blot. (B) HEK293T cells co-expressing GFP-Rad9 and Flag-Claspin^{1339} or Flag-Claspin^{1332} were irradiated with UV and analyzed by immunoprecipitation using Flag antibodies. The levels of GFP-Rad9 and Flag-tagged Claspin in input and precipitates were analyzed by western blot.

AGA; Claspin (1339/1275): GGC CTT GGG TTA GGT TTC ACT TCC T; Claspin (1332/1275): CAA AGA GAG GCG GAG AAG GGC TAT T.

To express Flag-tagged Claspin^{1339} in human cells, the Claspin coding sequence was PCR amplified from the I.M.A.G.E. clone 7961377 and cloned into the pFlag-CMV2 vector. Flag-Claspin^{1332} was generated by cloning the Claspin coding sequence from S-Flag-Claspin (a gift of Dr. Junjie Chen) into the pFlag-CMV2 vector.

Antibodies. Chk1 rabbit polyclonal antibody (sc7898), Chk1 mouse monoclonal antibody (G4, sc8408), GFP rabbit polyclonal antibody (sc8334), normal rabbit IgG (sc-2027) and normal mouse IgG (sc-2025) were from Santa Cruz Biotechnology, Inc. Claspin rabbit polyclonal antibody (A300-267) was from Bethyl Laboratories, Inc. Mouse Flag monoclonal antibody (F1804, M2) was from Sigma-aldrich Corporation. Rabbit pChk1s345 monoclonal antibody was from Cell Signaling Technology, Inc. GFP mouse monoclonal antibody (ab1218) was from Abcam PLC.

Immunoprecipitation. HCT116 cells were lysed in NETN buffer [20 mM TRIS-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40] containing 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and a protease inhibitor cocktail (Sigma). Cell extracts were spun at 14,000 rpm for 10 min, and the resulting supernatants were precleared with Protein G/A Sepharose beads. Antibodies to Flag or Chk1 were added to the extracts containing Protein G/A Sepharose beads, and were incubated overnight at 4°C. Subsequently, the Sepharose beads were sedimented and washed four times with NETN buffer. The immunoprecipitates were then subjected to SDS-PAGE and western blot analysis.

RT-PCR. The total mRNA was extracted using the RNeasy Mini kit (Qiagen). After determining the concentration of the mRNA, RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (Qiagen). Primers for the exon 25A are: CTC CTG TCA AGG CTG AGG C and CAA AGC AGT CTC AAT GTA G. Primers for the exon 25B are: CTG ACC ATA ACC CAG TGC T and TTG TTC TGC CCA GAA TAG CC. Primers for GAPDH are: GCC TCA AGA TCA TCA GCA ATG and CCA CGA TAC CAA AGT TGT CAT GG.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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