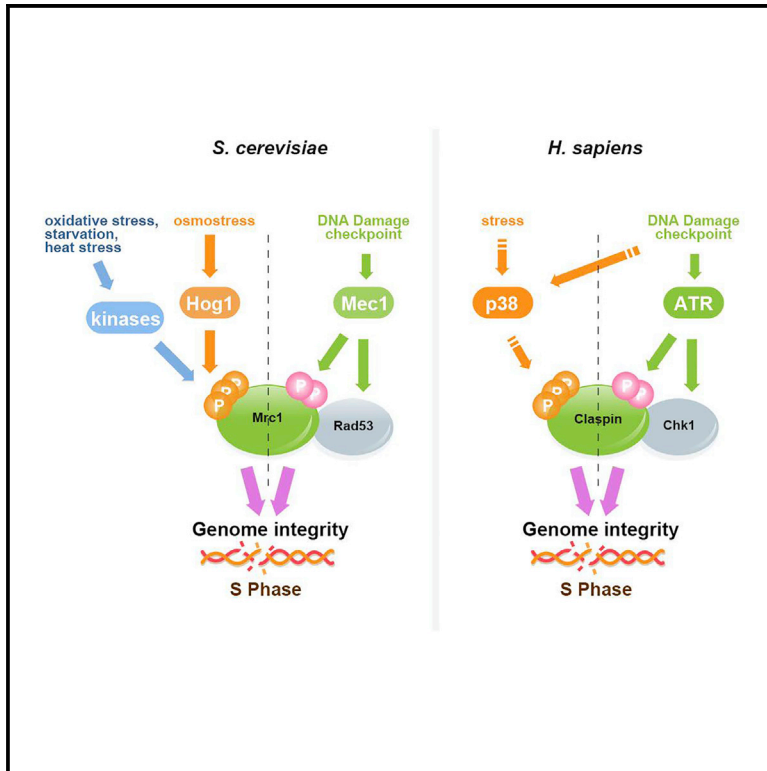


Regulation of Claspin by the p38 stress-activated protein kinase protects cells from DNA damage

Graphical abstract



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In brief

Preventing collisions between the RNA and DNA polymerases during replication is essential to maintain genomic integrity. Here, Ulsamer et al. report that a mechanism involving the p38 SAPK and Claspin, which is conserved from yeast to humans, prevents DNA damage upon stress during S-phase.

Highlights

- p38 and Claspin prevent stress-induced DNA damage in mammals as Hog1 and Mrc1 do in yeast
- p38 and the DNA damage checkpoint drive Claspin phosphorylation on different sites
- p38 SAPK phosphorylation of Claspin protects cells from DNA damage during S-phase
- The p38 sites in Claspin are relevant to promote resistance to cisplatin treatment



Article

Regulation of Claspin by the p38 stress-activated protein kinase protects cells from DNA damage

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SUMMARY

Stress-activated protein kinases (SAPKs) enhance survival in response to environmental changes. In yeast, the Hog1 SAPK and Mrc1, a protein required for DNA replication, define a safeguard mechanism that allows eukaryotic cells to prevent genomic instability upon stress during S-phase. Here we show that, in mammals, the p38 SAPK and Claspin—the functional homolog of Mrc1—protect cells from DNA damage upon osmotic stress during S-phase. We demonstrate that p38 phosphorylates Claspin and either the mutation of the p38-phosphorylation sites in Claspin or p38 inhibition suppresses the protective role of Claspin on DNA damage. In addition, wild-type Claspin but not the p38-unphosphorylatable mutant has a protective effect on cell survival in response to cisplatin treatment. These findings reveal a role of Claspin in response to chemotherapeutic drugs. Thus, this pathway protects S-phase integrity from different insults and it is conserved from yeast to mammals.

INTRODUCTION

Cells are constantly exposed to environmental changes. To ensure viability under the new conditions, they must have the capacity to adapt rapidly. To deal with such challenges, for instance, sudden variations in osmolarity, cells evolved signal transduction pathways that regulate many aspects of cell physiology, ranging from cell-cycle modulation to transcriptional control (Carbonell et al., 2019; de Nadal et al., 2011; Rhind and Russell, 2012; Saito and Posas, 2012; Zhang and Liu, 2002). During DNA replication in S-phase, cells are especially vulnerable to mutations as their chromatin structure is partially unwrapped, which results in unprotected DNA strands. Also, if any environmental challenge occurs during this stage of the cell cycle, adaptation might lead to changes in gene expression to maintain cell fitness. These alterations could potentially result in conflicts between replication and transcription, thus representing a major source of genomic instability (Duch et al., 2012; Garcia-Muse and Aguilera, 2016).

Unprogrammed transcription and DNA replication may occur simultaneously in the same loci, leading to potential transcription-replication conflicts (TRCs), which are often found at promoters and termination regions of highly transcribed genes

and lead to DNA damage. These TRCs may not necessarily be due to a collision between transcription and replication machineries but could also be caused by the effects of transcription on DNA structure and its surrounding chromatin. Accordingly, cells have developed mechanisms to avoid or resolve these conflicts (Duch et al., 2012; Gomez-Gonzalez et al., 2009; Hamperl et al., 2017). Furthermore, in mammalian cells, osmotic stress leads to the rearrangement of the transcription landscape, in which Pol II distribution is reduced genome-wide but enriched at the transcription start sites and gene bodies of osmotic stress-responsive genes. In contrast, transcription termination is impaired, extending mRNA synthesis beyond the transcription end sites, and there is also a marked reorganization of chromatin. These effects have been observed in approximately 1,500 genes (Amat et al., 2019; Ferreiro et al., 2010b; Rosa-Mercado et al., 2021; Vilborg et al., 2015).

In yeast, the stress-activated protein kinase (SAPK) p38 ortholog, Hog1, induces expression of hundreds of genes in response to stress (de Nadal and Posas, 2015; Nadal-Ribelles et al., 2012). When such a general transcription rearrangement is produced during S-phase, Hog1 directly prevents collision between the transcription and DNA replication machineries by phosphorylating the N-terminal region of Mrc1, a basic regulatory



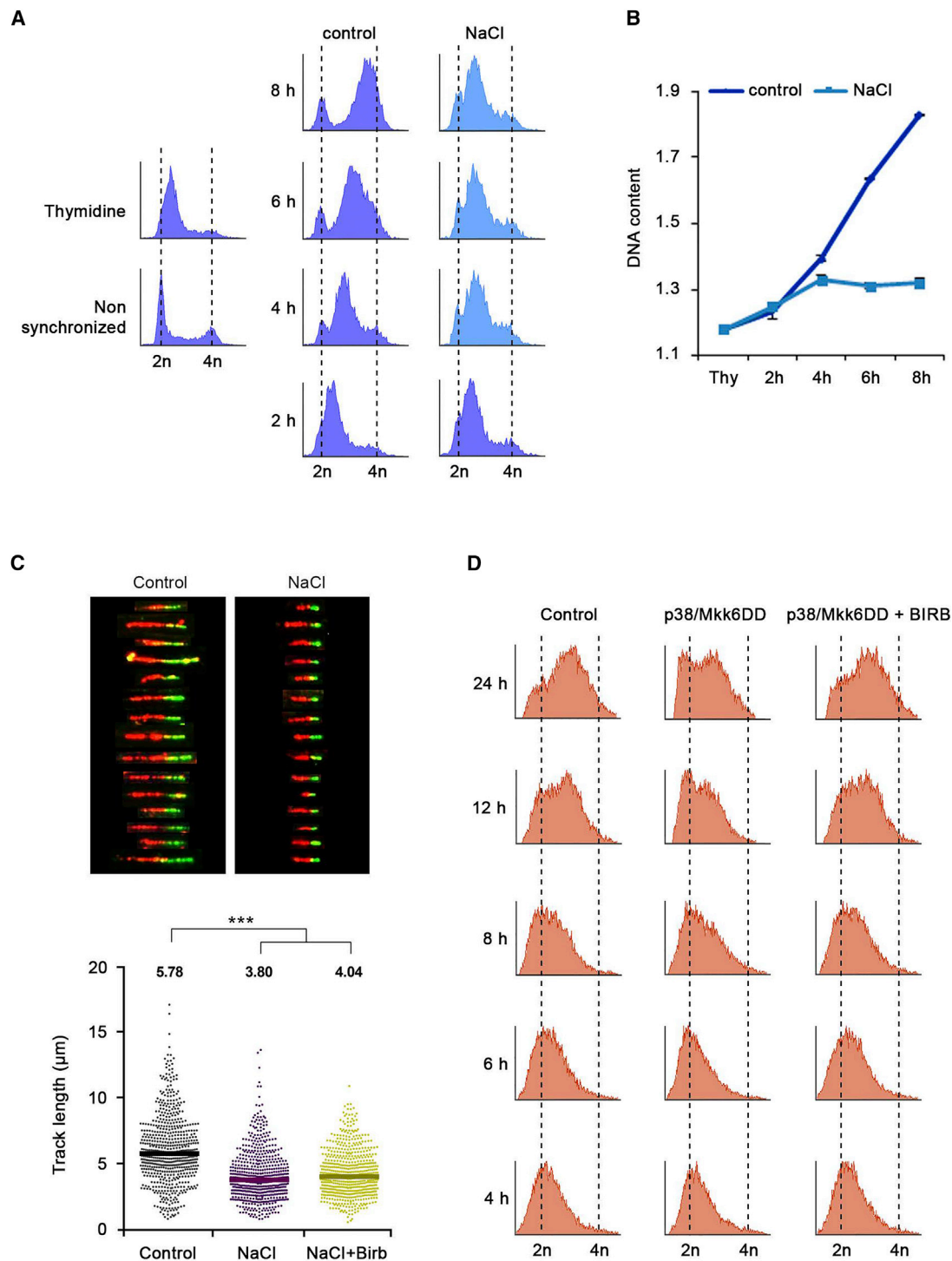


Figure 1. Both osmotic stress and active p38 cause a delay in S-phase progression

(A) U2OS cells were synchronized by thymidine block at the beginning of S-phase and released in the presence or absence of 150 mM NaCl. Total DNA content was assessed by propidium iodide (PI) staining at the indicated times. Osmotic stress-affected time points are labeled in light blue.

(B) Graphic representation of S-phase progression in the presence or absence of 150 mM NaCl. DNA content was assessed by PI staining and the main progression peak was plotted against G1 (= 1). Error bars represent standard deviation of three biological replicates.

(C) DNA fiber length measurement. U2OS cells were treated with or without 150 mM NaCl for 2 h. BIRB796 p38 inhibitor was added 2 h before where indicated. DNA was sequentially labeled with 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU) to track replication. Upper panel: note that, for this image, individual fibers have been cut from their original position in the raw microscopy images and pasted onto a common black background. As for the labeling, only

(legend continued on next page)

component of the replication-pausing complex and also downstream effector of the DNA damage checkpoint pathway (Duch et al., 2013b; Yaakov et al., 2009). This Mrc1 phosphorylation can also be performed by alternative kinases, such as Psk1, Mpk1, or Snf1, in response to oxidative, heat, or nutrient stress, respectively (Duch et al., 2018), and results in cell protection against DNA damage during S-phase. This pathway was named “Mrc1 transcription-replication safeguard mechanism”, and it prevents genomic instability triggered by environmental stress in yeast (Duch et al., 2013a; Hamperl and Cimprich, 2016).

As its yeast counterpart, p38 participates in the regulation of cell-cycle arrest induced by several stimuli at both G1/S and G2/M (Bulavin and Fornace, 2004; Canovas and Nebreda, 2021; Coulthard et al., 2009; Cuadrado and Nebreda, 2010; Martinez-Limon et al., 2020). For instance, at G1-S, p38 phosphorylates the CDK inhibitor p57Kip2, increasing its affinity for the Cdk2 complex and delaying cell-cycle progression (Joaquin et al., 2012). In addition, p38 phosphorylates the tumor suppressor Rb on its N-terminal region to regulate the expression of S-phase genes and prevent S-phase entry (Gubern et al., 2016).

Claspin is the mammalian functional homolog of Mrc1 (Kumagai and Dunphy, 2003; Lee et al., 2003; Smits et al., 2019), sharing roles both in the pausing complex and the DNA damage response (DDR) pathways. There are substantial differences between yeast and mammals in S-phase control. In yeast, replication starts at well-defined sequences called autonomously replicating sequences, while replication origins in higher eukaryotes do not appear to have a specific sequence requirement but rather rely on broader genomic signatures, such as intra-TAD chromatin contact sites (Errico and Costanzo, 2010; Sima et al., 2019). In mammals, p38 is also activated upon DNA damage and is thus directly involved in the DDR pathway (Bulavin et al., 2001; Canovas et al., 2018; Colomer et al., 2019; Reinhardt et al., 2007; Roy et al., 2018). Therefore, in contrast to yeast, it is difficult to discriminate between the intra- and extra-cellular response of the DDR pathway. However, here we sought to explore whether there is an S-phase control mechanism in mammals that acts in a related manner to that of yeast. We found that, indeed, p38 and Claspin also contribute to DNA protection and cell adaptation upon extracellular challenges during S-phase. Also, we describe the involvement of this pathway in promoting cell viability in response to cisplatin treatment.

RESULTS

Osmostress delays S-phase progression

To assess the effect of osmostress on S-phase progression in mammals, U2OS human osteosarcoma cells were synchronized at the beginning of S-phase by thymidine block and then released in the presence or absence of NaCl (150 mM). DNA replication was assessed by DNA staining by flow cytometry at

different time points (Figure 1A). FACS quantification showed significant differences in cell-cycle progression already at 4 h after thymidine release (Figure 1B). Thus, osmostress causes a delay in S-phase progression.

We next examined whether osmostress affects replication fork progression. To this end, non-synchronized U2OS cells were treated with 150 mM NaCl for 2 h and then sequentially labeled with 5-chloro-2'-deoxyuridine and 5-iodo-2'-deoxyuridine, and the rate of fork progression was assessed by stretched DNA fibers. Osmostress caused a delay in fork rate relative to untreated cells, which was not reversed by the addition of the p38 inhibitor BIRB796 (Figure 1C).

The p38 SAPK is activated in response to osmostress leading to control of the progression of different stages of the cell cycle (Canovas and Nebreda, 2021). Hence, we explored the possible role of p38 in delaying S-phase progression in mammalian cells. For that, U2OS cells were synchronized by thymidine block and 24 h later, once completely arrested in S-phase to prevent the effect of p38 in G1, transfected either with mCherry-tagged p38 simultaneously with its upstream activator Mkk6^{DD} (a constitutive allele of the MAP2K MKK6) or with empty mCherry control. At 24 h post-transfection, cells were released from the thymidine block in the presence of Nocodazole (to prevent re-entry into G1), and the DNA content of mCherry-positive cells was quantified by Hoechst 33342 staining. The synchronization procedure affected the timing of replication; however, it allowed us to express proteins specifically at the onset of S-phase and prevented a potential delay in G1. Cells with active p38 showed a significant delay in their progression toward G2 when compared with control cells. The S-phase delay caused by p38 activation was prevented by the addition of the p38 inhibitor BIRB796 (Figure 1D). A similar experiment was performed following the S-phase progression upon p38 activation by EdU staining, with comparable results (Figure S1A). Hence, activation of p38 leads to a delay in S-phase progression.

p38 and Claspin are necessary for cell survival upon osmostress during S-phase

To assess the physiological relevance of p38 for S-phase control and to test whether this response involves Claspin, we examined the effect of knocking down either p38 or Claspin upon osmostress. U2OS cells were transfected with siRNAs against p38, Claspin, or a scrambled DNA control (Figure 2A). Transfected cells were synchronized with thymidine and subjected to stress at the indicated concentration of NaCl. Six hours after release from the synchronization, cell viability (percent of dead cells) was assessed by positive propidium iodide (PI) staining as analyzed by flow cytometry. p38 and Claspin showed a clear protective effect upon osmostress (150 mM NaCl or higher) (Figure 2B). Therefore, these two proteins are required for cell survival upon osmostress during S-phase.

the CldU label (red) is measured in red-green tracks and is plotted in track length (μm). Lower panel: each dot of the plot represents a single measurement of fork rate. A pool of two biological replicates is shown, each one with >300 CldU-IdU tracks identified and measured.

(D) U2OS cells were arrested at the onset of S-phase by thymidine blocking. Once completely arrested, cells were transfected with either mCherry empty plasmid (control) or Mkk6^{DD} and p38 cherry plasmids (p38/Mkk6^{DD}). Twenty-four hours after transfection, cells were released into S-phase in the presence or absence of the BIRB796 (BIRB). Nocodazole was added to avoid cell progression beyond G2/M. The DNA content of cherry-positive cells at the indicated time after release was quantified by Hoechst 33342 staining.

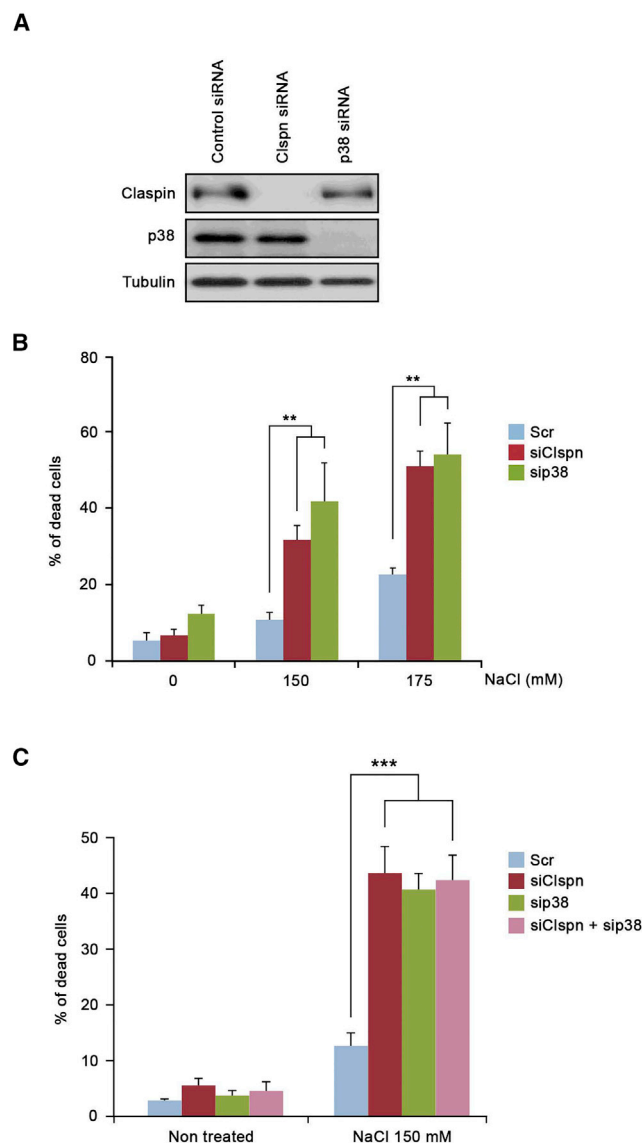


Figure 2. p38 and Claspin are necessary for cell survival upon osmotic stress during S-phase

(A) Representative western blot showing Claspin (Clspn) and p38 siRNA efficiency in U2OS cells.

(B) Cell viability was monitored by PI staining at different NaCl concentrations (6 h) after release into S-phase. siRNA osmotic stress-treated cells were synchronized with thymidine and released in the presence of different NaCl doses. Cell viability was assessed by PI staining. Error bars represent SEM. ** $p < 0.05$ in unpaired, two-tailed t test.

(C) U2OS cells were transfected with siRNA against Claspin, p38, or a combination of both. After 24 h cells were synchronized with thymidine for 24 h, released, and treated with salt (150 mM) for 6 h. Cells were then collected and stained with PI. Error bars represent SEM. *** $p < 0.005$ in unpaired, two-tailed t test.

We hypothesized that p38 and Claspin could act in the same pathway, like their yeast counterparts Hog1 and Mrc1. To test this notion, we studied the effect of simultaneously inhibiting the two proteins combining p38 and Claspin siRNA treatments.

The double knockdown did not cause any significant increase in cell death to the Claspin siRNA or p38 siRNA treatments alone upon osmotic stress. These observations thus indicate that p38 and Claspin act epistatically to increase cell fitness in response to stress during S-phase (Figure 2C).

Next, we examined whether p38 and Claspin per se mediate the cell-cycle delay in S-phase observed upon osmotic stress (Figure 1). To this end, p38 or Claspin were knocked down, and S-phase progression upon osmotic stress was assessed by PI staining and FACS analysis. Neither the downregulation of p38 nor Claspin had any effect on the osmotic stress-induced S-phase delay, as the progression was affected by the addition of salt in a similar manner under control conditions and p38 and Claspin knockdown (Figure S1B). Along the same lines, the addition of p38 inhibitors BIRB796 or SB203580 did not prevent the delay observed upon osmotic stress (Figure S1C). These results, together with those shown in Figure 1C, suggest that, although p38 and Claspin are required for maximal cell survival and p38 activation can delay S-phase progression, additional cell-cycle regulators act to delay cell-cycle progression upon osmotic stress in S-phase.

The pro-survival role of p38 and Claspin correlate with protection against DNA damage

Next, we tested whether the p38/Claspin pro-survival effect could be through protection against DNA damage, as observed in yeast (see Introduction). To explore this notion, we studied DNA damage by two independent approaches, namely using denaturing comet assays, which detect both single- and double-strand breaks, and γ H2AX-staining, which detects double-strand breaks in addition to other forms of DNA damage (Collins et al., 1997; Rogakou et al., 1998). The comet assay was performed on thymidine-synchronized U2OS cells subjected or not to osmotic stress after thymidine release. p38- or Claspin-deficient cells were treated as described before, stressed with different NaCl doses (150 and 175 mM) for 4 h, harvested, embedded in agarose, and visualized using electrophoresis in alkaline conditions. DNA was then stained and damage accumulation was assessed. At both NaCl concentrations, the number of positive comet cells was higher in p38 and Claspin knockdown cells than in control cells (3- and 2.8-fold, respectively, over the control) (Figure 3A). Of note, we observed some DNA damage in the knockout cells in the absence of osmotic stress, which can be attributed to the effect of thymidine synchronization on these cells. To assess DSB more specifically, we performed the comet assays under neutralizing conditions in cells that were synchronized with thymidine and released with or without NaCl (150 mM). Cells with sip38 clearly showed an increase in DNA damage when compared with wild type, providing similar results as in denaturing conditions (Figure S2A).

To avoid the effect of cell synchronization, we monitored γ H2AX. Although it is a less-specific DNA damage marker, it has the advantage that it allows the monitoring of S-phase without the need for thymidine synchronization. Instead, DNA-replicating cells were labeled with EdU (5-ethynyl-2'-deoxyuridine) (Figure S2B) and only EdU-positive cells were analyzed. p38 or Claspin knockdown cells were stressed with different concentrations of NaCl (150 and 175 mM) and

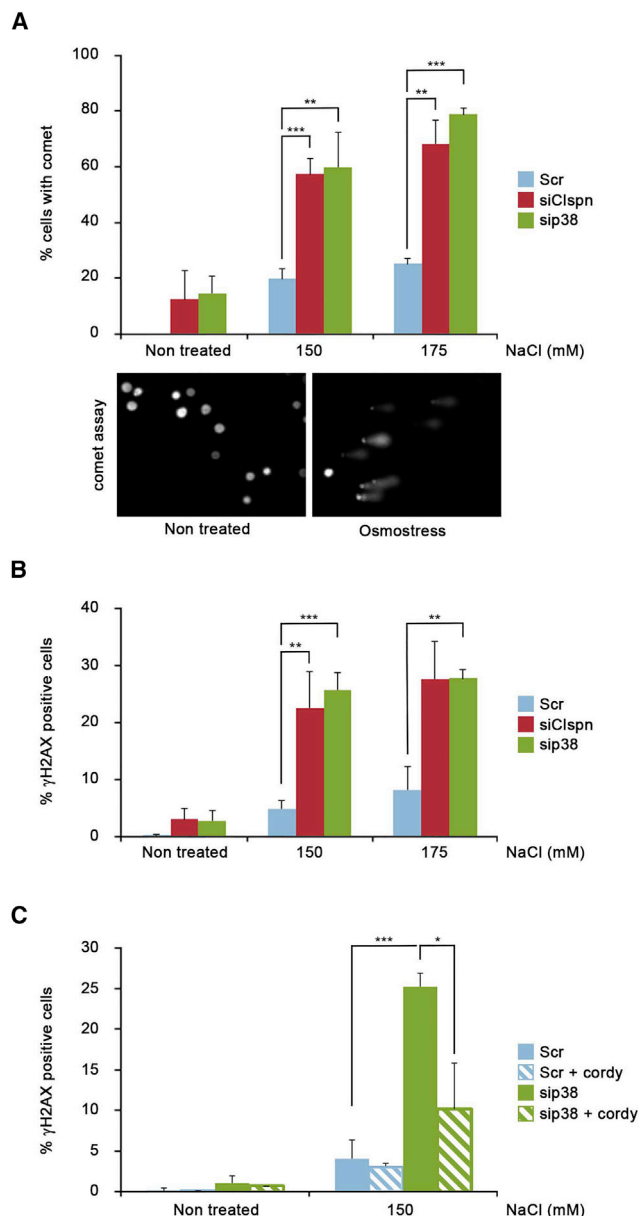


Figure 3. p38 and Claspin knockout reduced viability correlates with increased DNA damage, which is reversed by cordycepin

(A) Claspin- and p38 siRNA-treated cells were synchronized with thymidine and released in the presence of the indicated NaCl doses. Four hours after being released into S-phase, the comet assay under alkaline conditions was performed. Error bars represent SEM. ** $p < 0.05$ and *** $p < 0.005$ in unpaired, two-tailed t test. The lower panel shows a representative image of the comets observed in the comet assay.

(B) Cells were labeled with EdU (5-ethynyl-2'-deoxyuridine) and visualized using click chemistry combined with an antibody against γ H2AX. Only EdU-positive cells are shown in the figure (see Figure S2B). Error bars represent SEM. ** $p < 0.05$ and *** $p < 0.005$ in unpaired, two-tailed t test.

(C) siRNA-treated cells were subjected to cordycepin 2 h before NaCl treatment and labeled with EdU 1 h before collection. Cells were stained for EdU and γ H2AX. Error bars represent SEM. *** $p < 0.01$ and * $p < 0.1$ in unpaired, two-tailed t test.

the presence of γ H2AX foci was then compared with non-treated control cells. p38- and Claspin-deficient cells showed an increase in DNA damage similar to that observed in the comet assay (Figure 3B). Therefore, p38 and Claspin protect cells from DNA damage upon osmotic stress during S-phase. Of note, the double knockout of Claspin and p38 did not add any significant γ H2AX signal (Figure S2C). Therefore, the p38/Claspin pathway protects cells from DNA damage upon osmotic stress during S-phase.

In yeast, it is believed that the induction of stress-activated transcription when cells are replicating leads to conflicts between the two processes (TRCs) and results in DNA damage (Duch et al., 2018; Hamper and Cimprich, 2016). Thus, we examined whether the DNA damage caused by stress in the absence of p38 could be prevented by inhibiting transcription. We followed γ H2AX foci as before in the presence or absence of cordycepin, a derivative of the nucleoside adenosine, which is a transcription inhibitor (Kon-drashov et al., 2012). The appearance of γ H2AX foci was monitored in U2OS cells that were treated with cordycepin for 2 h (50 μ M) before NaCl treatment (150 mM) in control or p38-deficient cells. Cordycepin treatment partially suppressed DNA damage accumulation in p38-deficient cells upon osmotic stress (Figure 3C), suggesting that TRCs are one of the reasons for the accumulation of DNA damage. Interestingly, while cordycepin partially reverts the γ H2AX signal induced by osmotic stress in the absence of p38, it does not reverse the effect of Claspin knock-down. These results point out that there are other functions of Claspin protecting cells from DNA damage, besides those regulated by p38 phosphorylation (Figure S2D).

p38 directly phosphorylates Claspin

Next, we examined whether p38 directly phosphorylates Claspin to maintain genomic integrity. To perform an *in vitro* kinase assay, wild-type Claspin was expressed and purified from yeast by fusing it to GST under the *GAL1* promoter. After GST purification, Claspin was incubated with recombinant forms of p38 and its activator Mkk6^{DD} in the presence of ³²P-labeled ATP. p38 directly phosphorylated Claspin *in vitro* (Figure 4A). p38 is known to phosphorylate SP and TP motifs on its target proteins, and Claspin contains eight SP sites and one TP site. We initially mutated the four SP/TP sites at the C terminus, since this is the part of the protein that has a similar structure to yeast's Mrc1. However, this was not enough to see any effect on cell survival upon osmotic stress, even when some of the sites were clearly phosphorylated by p38. Hence, we mutated all the serines and the threonine on those SP and TP sites to alanine (Claspin^{9A}). Recombinant GST-tagged Claspin^{WT} and Claspin^{9A} were incubated with recombinant p38 and MKK6^{DD} in the presence of ³²P-labeled ATP. p38 directly phosphorylated Claspin^{WT} and the phosphorylation was prevented by the p38 inhibitor SB203580 and abolished in the Claspin^{9A} mutant (Figure 4B). Next, we assessed *in vivo* Claspin phosphorylation upon activation of p38. HEK293T cells were co-transfected with vectors expressing Claspin and p38/Mkk6^{DD} or an empty vector control. Cells were then treated or not with the p38 inhibitor (BIRB796) for 2 h before Claspin was monitored by western blot. We detected a shift in Claspin mobility in the p38/Mkk6^{DD}-transfected cells, which was suppressed by the addition of the p38

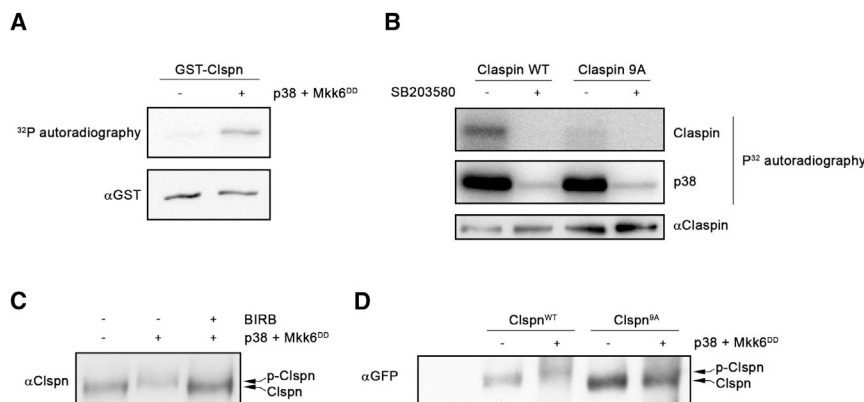


Figure 4. p38 phosphorylates Claspin on its SP/TP sites

(A) *In vitro* phosphorylation of Claspin by p38. GST-tagged Claspin was expressed in yeast and purified by glutathione sepharose. It was then used for an *in vitro* kinase assay with recombinant p38 and Mkk6^{DD} proteins.

(B) *In vitro* phosphorylation of Claspin by p38. Recombinant GST-tagged wild-type (Claspin^{WT}) or a mutant allele containing the mutations on the SP/TP sites to Ala (Claspin^{9A}) Claspin were incubated with recombinant p38 and MKK6^{DD} in the presence of radiolabeled γ³²ATP for 40 min. p38 inhibitor SB203580 was used when indicated.

(C) *In vivo* phosphorylation of Claspin by p38. HA-tagged Claspin was transfected into HEK293T

cells together with either empty or p38/Mkk6^{DD} plasmids. Addition of the p38 inhibitor Birb796 reversed the p38/Mkk6-induced shift. (D) GFP-tagged wild-type (Claspin^{WT}) or 9A (Claspin^{9A}) Claspin were transfected into HEK293T cells with empty or p38/Mkk6^{DD} plasmids.

inhibitor (Figure 4C) as well as by alkaline phosphatase treatment (Figure S3A). We then transfected either Claspin^{WT} or Claspin^{9A} together with p38/Mkk6^{DD} and observed that the p38-induced mobility shift on Claspin was reduced in the Claspin^{9A} mutant (Figure 4D). Therefore, p38 phosphorylates Claspin both *in vitro* and *in vivo*. Osmostress also induced a shift on Claspin, the reversal of which in Claspin^{9A} was much less apparent, but still perceptible (Figure S3B), suggesting that osmostress induces the phosphorylation of additional sites of Claspin in addition to those phosphorylated by p38. Of note, the Claspin^{9A} mutant still contained the Chk1 phosphorylation sites (Clarke and Clarke, 2005), thereby indicating that p38 and Chk1 act on different sites to regulate Claspin activity. To further confirm this functional segregation, we performed an experiment to follow Chk1 S345 phosphorylation as a readout of checkpoint activation in Claspin^{WT} and Claspin^{9A} cells upon knockdown of endogenous Claspin. Both Claspin^{WT} and Claspin^{9A} cells activate checkpoint pathways in response to UV light to a similar extent (Figure S4A). As osmostress also induces DNA damage, we explored whether Chk2 can phosphorylate Claspin. First, in an *in vitro* kinase assay we showed that activated Chk2 can directly phosphorylate Claspin (Figure S4B, left panel). Next, we tested whether the phosphorylation occurred in the same sites to those for p38 and found that the mutation of the SP/TP sites in Claspin^{9A} did not significantly affect the capability of Chk2 to phosphorylate Claspin, in contrast to what is observed by p38 (Figure S4B, right panel). Thus, our data suggest that p38 and Chk2 regulate Claspin acting on different sites.

The Claspin^{9A} mutant is sensitive to osmostress

To assess the physiological relevance of the p38-mediated phosphorylation on Claspin, both siRNA-resistant Claspin^{WT} and Claspin^{9A} were tagged with a GFP protein and stably expressed in a U2OS cell line while knocking down the endogenous gene (Figure 5A). Cells stably expressing each Claspin form were synchronized and challenged with osmostress (6 h, 150 mM NaCl), and cell death was assessed as described previously. While Claspin^{WT} rescued the viability of Claspin-deficient cells (siClaspin), the Claspin^{9A} mutant failed to fully

rescue viability upon osmostress (Figure 5B). Thus, the phosphorylation of Claspin is relevant for cell survival upon osmotic stress. To confirm the previously observed epistatic effect of Claspin and p38 in protecting cell viability, p38 was knocked down by siRNA on either Claspin^{WT} or Claspin^{9A}, and cells were subjected to osmotic stress (6 h, 150 mM NaCl) to assess viability. p38 knockdown induced a similar cell death in Claspin^{WT} as in the Claspin^{9A} mutant. However, it did not add any significant cell death to Claspin^{9A} mutant cells (Figure 5C). Similar results were observed using the p38 inhibitor BIRB796 (Figure S5).

We then assessed the relevance of Claspin phosphorylation on protecting cells from DNA damage upon osmotic stress. Comet assays and γH2AX experiments were performed in cells depleted of endogenous Claspin and expressing either Claspin^{WT} or Claspin^{9A} in the presence or absence of the p38 inhibitor (BIRB796). The Claspin^{9A} mutant showed more DNA damage upon osmotic stress than Claspin^{WT} in both procedures (Figures 5D and 5E). Interestingly, in both cases, p38 inhibition led Claspin^{WT} to display similar levels of DNA damage to the Claspin^{9A} mutant upon osmotic stress (Figures 5D and 5E). Therefore, the phosphorylation of Claspin by p38 is critical to protect cells from DNA damage caused by osmotic stress.

Claspin^{9A} mutant cells display an increase on R-Loop formation upon stress

Our results using the transcription inhibitor cordycepin revealed the induction of TRCs as a possible mechanism causing the DNA damage observed upon osmotic stress. We observed that DNA damage accumulation was partially reversed in U2OS cells overexpressing RNaseH, a well-known enzyme for its activity in R-Loop clearance (Figure 5F). Therefore, we decided to assess the formation of R-Loops as a proxy of TRCs (Costantino and Koshland, 2018; Crossley et al., 2019; Garcia-Muse and Aguilera, 2019; Promonet et al., 2020; Kumar et al., 2021) in Claspin^{WT} and Claspin^{9A} cells by using the S9.6 antibody (which detects R-Loops). There was a significantly higher accumulation of R-Loops in the Claspin^{9A} mutant cells upon osmotic stress when compared with non-stressed cells or wild-type Claspin^{WT} cells (Figures S6A and S6B).

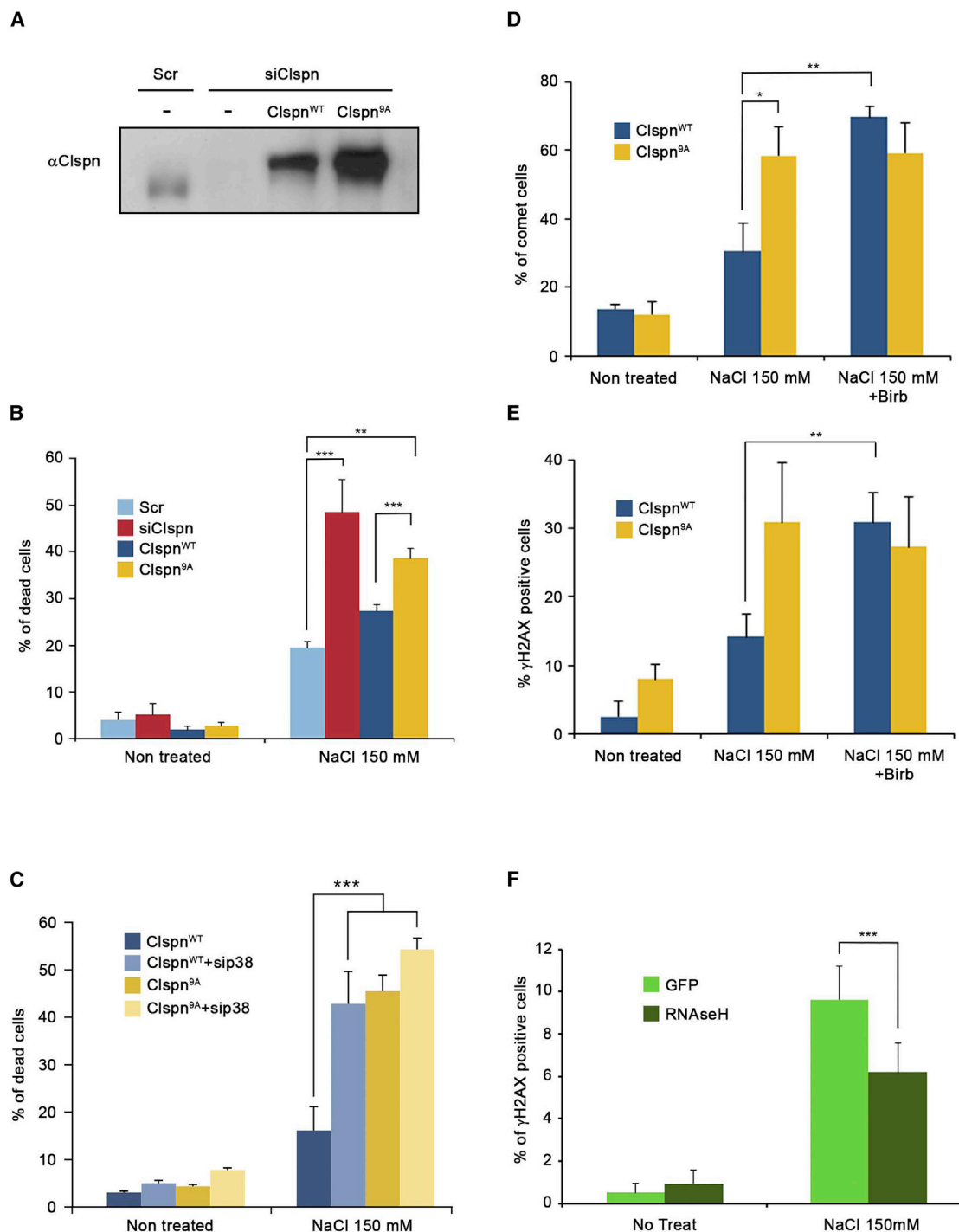


Figure 5. Claspin^{9A} fails to rescue the Claspin^{WT} phenotype and p38 inhibition mimics Claspin^{9A}

(A) Western blot showing stable expression of GFP-Claspin^{WT} and GFP-Claspin^{9A} in U2OS cells and the effect of siRNA addition.

(B) Cell death 6 h after release from thymidine block, in the presence or absence of 150 mM NaCl. Error bars represent SEM. **p < 0.05 and ***p < 0.005 in unpaired, two-tailed t test.

(C) Endogenous Claspin (in all conditions) and p38 (where indicated) were knocked down by siRNA in U2OS cells overexpressing Claspin^{WT} or Claspin^{9A} mutant. Cell death was assessed by PI staining after 6 h release from thymidine with or without 150 mM NaCl. Percentage of PI-positive cells is shown. Error bars represent SEM. ***p < 0.005 in unpaired, two-tailed t test.

(D) Claspin^{WT}- or Claspin^{9A}-expressing cells were synchronized by thymidine blocking. Four hours after release into S-phase with or without 150 mM NaCl treatment, cells were collected for alkaline comet assay. Error bars represent SEM. *p < 0.1 and **p < 0.05 in unpaired, two-tailed t test.

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Phosphorylation of Claspin at the p38 sites increases cell survival upon cisplatin treatment

Since the phosphorylation of Claspin by p38 prevented osmotic stress-induced DNA damage and that p38 is involved in cell survival in response to the administration of several chemotherapeutic drugs during S-phase (Reinhardt et al., 2007), we tested whether cisplatin also led to Claspin phosphorylation. HEK293T cells transfected with either Claspin^{WT} or the Claspin^{9A} mutant fused to GFP were treated with cisplatin (20 μ M, 8 h). Cisplatin induced a clear shift of the Claspin^{WT}, while this shift was prevented in the Claspin^{9A} mutant (Figure 6A). In contrast, 5-FU (an analog of uracil that blocks DNA replication) did not induce a shift in Claspin mobility.

Clonogenic assays are widely used to test the sensitivity of a particular cell type to chemotherapeutic drugs. Therefore, to check the significance of the phosphorylation of Claspin for cell survival upon cisplatin treatment, colony assays were performed to compare Claspin^{WT} and Claspin^{9A} mutant cells. Unsynchronized cells were treated with 2 μ M cisplatin for 24 h. Cisplatin was washed out after the treatment and cells were grown in regular medium for 1 week. The Claspin^{9A} mutant showed smaller and fewer colonies compared with Claspin^{WT} cells (Figures 6B and 6C). Accordingly, the addition of a p38 inhibitor resulted in a reduction in cell viability similar to that observed in Claspin^{9A} mutant cells. Thus, cell survival in response to cisplatin seems to be mediated by the phosphorylation of Claspin.

DISCUSSION

In yeast, regulation of S-phase by Mrc1 in response to stress is critical for cell survival and the maintenance of genomic integrity (Duch et al., 2013b, 2018). Here, we explored the potential conservation in mammalian cells of this S-phase safeguard mechanism. Our results point to a great degree of conservation of the mechanism and physiological effects of this pathway. As in yeast, p38 protects cell viability upon osmotic stress and performs its effects epistatically with Claspin during S-phase, as shown by the lack of additive effects when both Claspin and p38 activity are eliminated. p38 directly phosphorylates Claspin and this phosphorylation is important for cell survival upon osmotic stress, since the Claspin^{9A} mutant is sensitive to osmotic stress. Our observations confirm that mammalian cells have a pathway homologous to that described in yeast. The physiological role of this pathway in protecting cells from DNA damage upon osmotic stress in S-phase is evolutionarily conserved. Data showing that osmotic stress causes DNA damage in mammalian cells were previously described but the role of p38 was not defined (Kultz and Chakraborty, 2001). Our results highlight the importance of a protective mechanism against DNA damage that enhances mammalian adaptation to osmotic stress—and potentially to other kinds of stresses—in S-phase. Moreover, we showed that p38 activation

delays DNA replication, as does Hog1 in yeast. Nevertheless, in mammals, activation of p38 is only partially responsible for the osmotic stress-induced S-phase delay as p38 knockdown per se did not suppress the slow down of osmotic stress-induced replication, thus reflecting greater pathway complexity in mammalian cells in response to osmotic stress, which, according to our preliminary observations, may involve also Chk2 activation.

The coexistence of replication and transcription in mammals is not yet well understood. Replication timing is established during the early G1 phase of the cell cycle (Dileep et al., 2015). Failure of this programming, as occurs when activated oncogenes induce replication origins within highly transcribed genes, results in subsequent TRCs (Macheret and Halazonetis, 2018). Of note, it has been recently described that Claspin overexpression reverts the replication stress induced by oncogene overexpression, independently of ATR signaling (Bianco et al., 2019; Pasero and Tourriere, 2019).

Our results using the transcription-inhibitor cordycepin as well as the RNaseH and R-Loop detection experiments, are consistent with the notion that osmotic stress induces TRCs in mammalian cells, thus contributing to DNA damage, and they are in agreement with our previous studies in yeast, where TAR assays confirmed the transcription dependency of DNA damage upon environmental stress (Duch et al., 2013b, 2018). Reinforcing this notion, in mammalian cells osmotic stress disrupts A/B compartments of chromosome organization, reduces CTCF binding, and impairs TAD domains both at their boundaries and at intra-TAD level, while, in parallel, it induces a massive transcription rearrangement, which includes impaired transcription termination (Amat et al., 2019; Rosa-Mercado et al., 2021; Vilborg et al., 2015). Under these circumstances, unprogrammed replication and deregulated transcriptional activity are described to take place (Sima et al., 2019), creating conditions in which cells would be prone to TRCs (Garcia-Muse and Aguilera, 2016; Hamperl and Cimprich, 2016). Therefore, we concluded that the role of the p38/Claspin pathway in decreasing DNA damage upon stress during S-phase is, at least in part, due to TRC reduction.

Cisplatin induces Claspin phosphorylation at the p38 sites. It has been described that p38 inhibition sensitizes tumor cells to cisplatin-induced apoptosis and that the response to cisplatin treatment is potentiated upon p38 α inhibition (Germani et al., 2014; Pereira et al., 2013). Moreover, p38 is involved in cell survival in response to cisplatin and other drugs through MK2 activation (Reinhardt et al., 2007), which might explain the small but significant additive effect of adding the p38 inhibitor to the cells expressing Claspin^{9A} in the colony assay. Our results show that p38 also mediates cell viability in response to cisplatin through Claspin phosphorylation.

It has been proposed that targeting DNA-repair mechanisms may serve as an anti-cancer strategy (Furgason and Bahassi, 2013; Lecona and Fernandez-Capetillo, 2014). However, although targeting Chk1 has been proposed as a possible

(E) Claspin^{WT}- or Claspin^{9A}-expressing cells were synchronized by thymidine blocking. Four hours after release into the S-phase with or without 150 mM NaCl treatment, cells were fixed and stained for γ H2AX. Error bars represent SEM. **p < 0.05 in unpaired, two-tailed t test.

(F) U2OS cells were transfected with a plasmid encoding GFP-tagged RNaseH1 or a GFP-expressing plasmid as a control. Next day, thymidine was added for 24 h. Then, cells were released from thymidine blocking and treated with or without 150 mM NaCl for 4 h. Next, cells were fixed and stained for γ H2AX. Cells were analyzed by flow cytometry, selecting only the GFP-expressing cells. Graph shows a plotting of the γ H2AX-positive cells from biological triplicates. ***p < 0.005 in unpaired, two-tailed t test.

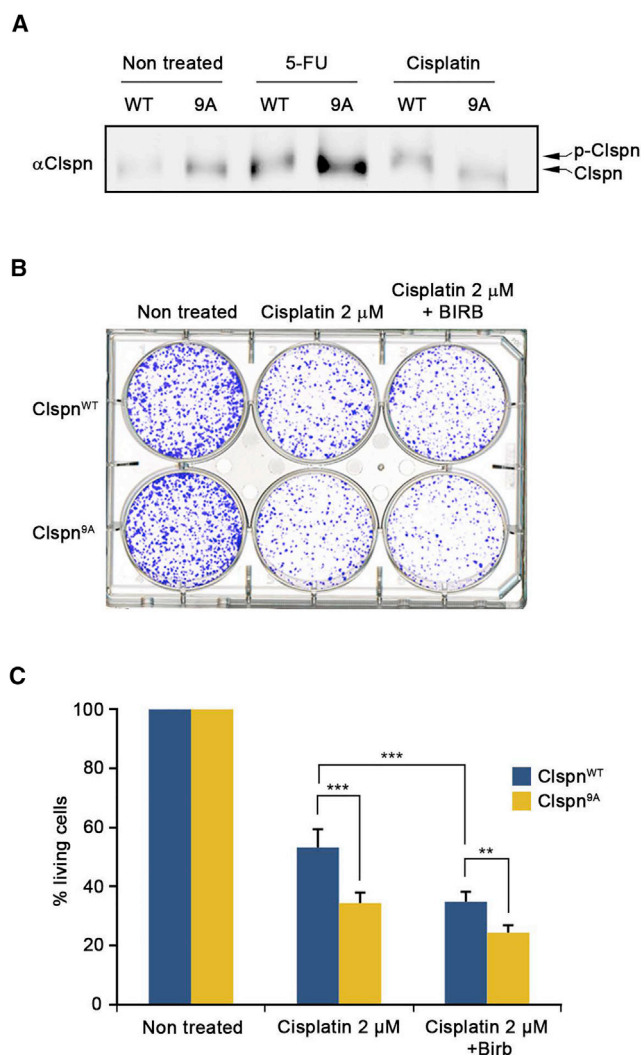


Figure 6. Claspin phosphorylation upon cisplatin treatment is important for cell survival

(A) Cisplatin induces Claspin phosphorylation. Western blot of HEK293T cells stably expressing Claspin^{WT} or Claspin^{9A} upon cisplatin (20 μ M for 8 h) or 5-FU (10 μ M for 8 h) treatments.

(B) U2OS cells stably expressing Claspin^{WT} or Claspin^{9A} were plated in six-well plates (1,000 cells/well). Twenty-four hours later, cells were pre-treated with 0.5 μ M p38 inhibitor (BIRB 796) for 2 h and then 2 μ M cisplatin treatment was applied for 24 h while maintaining the kinase inhibitor. Treatments were washed out and cells were incubated for 1 week before crystal violet staining.

(C) Colony quantification from (B) was carried out using ImageJ and values were normalized to untreated samples.

mechanism of sensitizing cancer cells to apoptosis, it is not clear whether this can sensitize tumoral cells to platinating agents (Wagner and Karnitz, 2009). Here, we showed that Chk1 phosphorylation sites differ from those for p38, and therefore targeting Claspin p38-mediated phosphorylation might provide an approach to render cells more sensitive to cisplatin or other platinating chemotherapeutic drugs.

Since Claspin phosphorylation by p38 protects cells from DNA damage during S-phase and the failure of DNA damage protection

mechanisms might underlie many types of cancer, we reviewed the COSMIC somatic mutation database (Forbes et al., 2017) and found several reported mutations in Claspin SP sites. Of note, most of them are not in the serine but in the proline, altering p38 phosphorylation sites specifically. These observations thus suggest that the p38/Claspin pathway protects cells from carcinogenic DNA mutations, in accordance with the proposed role of Claspin as a tumor suppressor (Azenha et al., 2017; Lee et al., 2020). Our results are in line with those of other studies describing point mutations in Claspin in different types of cancer that are not detected in normal tissues (Wang et al., 2008).

Our results confirm the existence of an evolutionarily conserved p38/Claspin pathway that is activated in S-phase to protect cell integrity. Further research will be necessary to explore the relevance of this pathway in cancer biology and its potential in the design of therapeutic approaches targeting DNA integrity.

Limitations of the study

We identify an evolutionary conserved p38 and Claspin pathway in protecting cells from cell death and DNA damage and provide evidence that the activation of those proteins upon stress prevents TRCs. However, we did not define to what extent TRCs are responsible for induction of DNA damage upon deficient activation of the pathway. Another aspect that is not completely defined is the more relevant SP/TP sites in Claspin phosphorylated by p38 in response to stress. Also, whereas it is clear that p38 phosphorylates Claspin and that this is essential for cell survival, the inhibition of p38 or the Claspin mutant in the p38-phosphorylation sites does not prevent cell-cycle delay and fork progression inhibition, indicating that additional mechanisms may exist that act upon osmotic stress. Regarding the cell model, we have limited the experiments to U2OS and HEK293T cell lines, while it is well known that response to DNA damage can vary across cell lines, for example, in those cell lines deficient for p53 protein.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.111375>.

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AUTHOR CONTRIBUTIONS

A.U. was instrumental in all phases of the study. A.U., S.B., A.M.-L., and S.R.-A. designed and conducted the experiments. A.U., R.F., J.M., E.d.N., and F.P. designed the experiments and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Tubulin	Sigma-Aldrich	RRID: AB_477593
Rabbit monoclonal anti-pp38 SAPK, clone 3D7	Cell Signaling	RRID: AB_331762
Rabbit polyclonal anti-p38 α SAPK	Santa Cruz Biotechnology	RRID: AB_632138
Rabbit polyclonal anti-Claspin (N-Terminal)	(Mamely et al., 2006)	N/A
Mouse monoclonal anti-HA	Made in house from the 12CA5 hybridoma	RRID: AB_514505
Mouse monoclonal S9.6 for R-Loop detection	Kerafast	RRID: AB_2687463
rabbit α -Nucleolin	Abcam	RRID: AB_881762
Mouse monoclonal anti-GFP, JL8	Clontech	RRID: AB_10013427
Mouse monoclonal anti- γ H2AX, clone JBW301	Millipore	RRID: Cat # 05-636; AB_309864
Mouse monoclonal Alexa 647-conjugated γ H2AX antibody	Cell Signaling	RRID: AB_10692910
Anti-mouse Alexa 568	Invitrogen	RRID: AB_2534072
Mouse monoclonal anti-GST	GE Healthcare	RRID: AB_771432
Chemicals, peptides, and recombinant proteins		
BIRB796	Merck-Millipore	Cat # 506172
SB203580	Calbiochem	Cat # 559389
Lipofectamine RNAiMAX	ThermoFisher	Cat # 13778030
Lipofectamine LTX	ThermoFisher	Cat # 15338100
FuGENE 6	Promega Transfection Reagent	Cat # E2691
DMEM	Biological Industries	Cat # 01-055-1A
FBS	Sigma-Aldrich	F2442
Hoechst 33342	Sigma-Aldrich	Cat # 14,533-100MG
DAPI	Sigma-Aldrich	Cat # D9542
ECL detection reagent	Amersham	Cat # NA934V
^{32} P-ATP	PerkinElmer	Cat # NEG002A
Propidium iodide	Sigma-Aldrich	Cat # P4170
Glutathione-Sepharose beads	GE Healthcare	Cat # 17-0756-01
Glass beads (0.4–0.6 mm)	VWR	Cat # 1.04016.0500
GST-MKK6 ^{DD}	(Gubern et al., 2016)	N/A
GST-p38a	(Gubern et al., 2016)	N/A
pcDNA3-2xHA-Claspin (siRNA resistant)	(Semple et al., 2007)	N/A
G418	Gibco	10131035
Thymidine	Sigma-Aldrich	T1895
Thapsigargin	Sigma-Aldrich	T9033
Cisplatin	Sigma-Aldrich	479306
5-FU	Sigma-Aldrich	F6627
Poly-D-Lysine	Sigma-Aldrich	P6407
Nocodazole	Sigma-Aldrich	201954700
BIOMAX XAR films	Kodak	Z358487-50EA
Critical commercial assays		
Click-iT® EdU Alexa Fluor® 488	ThermoFisher	Cat # C10425
Flow Cytometry Assay Kit		
CometAssay® Kit	Trevigen	Cat # 4250-050-K
Experimental models: Cell lines		
U2OS	ATCC	Cat # HTB-96

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
HEK293T	ATCC	Cat # ACS-4500
Experimental models: Organisms/strains		
<i>S. cerevisiae</i> strain BY4741	ATCC	201388
Oligonucleotides		
siRNA8 α Claspin GCACAUACAU GAUAAAGAAAdTdT	Thermoscientific	TMOSLR-001156
Scrambled siRNA GAUAAACACA CGAAGCUAUAdTdT	Thermoscientific	TMOSLR-004650
siRNA α p38 CCAGACCAUUUC AGUCCAUdTdT	Thermoscientific	TMOSLR-001321
Primer to amplify mCherry (for Claspin expression) Fw AGAAGAGGATCCCATGGTGAG CAAGGGCGAGGAG	Sigma-Aldrich (in-house designed)	N/A
Primer to introduce mCherry (for empty control expression) FwGA GAGACTCGAGACCATGGTGAG CAAGGGCGAGGAG	Sigma-Aldrich (in-house designed)	N/A
Primer to amplify mCherry RvTAT ATTGCGGCCGCTACTTGTACA GCTCGTCC	Sigma-Aldrich (in-house designed)	N/A
Primer to amplify GFP FwTTGGTT GGAGATCTACCATGGTGAG CAAGGGCGCCGAGCTGT	Sigma-Aldrich (in-house designed)	N/A
Primer to amplify GFP Rv TTGGTTGGAGATCTCTTGTACA GCTCATCCATGCCGT	Sigma-Aldrich (in-house designed)	N/A
Recombinant DNA		
pcDNA3	Invitrogen	Cat # V79020
pAcGFPN1	Clontech	Cat # 632497
pEFmLink-MKK6 ^{DD}	(Alonso et al., 2000)	N/A
pcDNA3-3HA-p38a	Provided by Dr. Engelberg (The Hebrew University of Israel)	N/A
pcDNA3-flag-p38a	(Enslen et al., 1998)	N/A
p38 α -DSRed	(Ferreiro et al., 2010a)	N/A
pGEX2T1-MKK6 ^{DD}	(Gubern et al., 2016)	N/A
pGEX5x3-p38a	(Gubern et al., 2016)	N/A
pBS34-mCherry	Addgene	83796
p426GAG (PGAL1-GST, URA3, 2 micron)	(Christianson et al., 1992)	N/A
pcDNA3-2xHA-Claspin (siRNA resistant)	(Semple et al., 2007)	N/A
pFRT-TODestGFP_RNAseH1	Addgene	#65784
GST-MKK6 ^{DD}	(Gubern et al., 2016)	N/A
GST-p38a	(Gubern et al., 2016)	N/A
GST-Claspin	This paper	N/A
GFP-Claspin ^{WT} and GFP-Claspin ^{9A} (siRNA resistant)	This paper	N/A
Software and algorithms		
CellQuest	Becton Dickinson	N/A
FACSDiva	NIKON INSTRUMENTS INC	N/A
NIS elements AR software	Nikon	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
FACScalibur flow cytometer	Becton Dickinson	N/A
BD LSRFortessa	Becton Dickinson	N/A
BD FACSaria II-SORP	Becton Dickinson	N/A
Nikon Eclipse Ti microscope	Nikon	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Francesc Posas (francesc.posas@irbbarcelona.org).

Material availability

All unique reagents generated in this study are available from the [lead contact](#) with a completed materials transfer agreement.

Data and code availability

Original data are available from the [lead contact](#) upon request. This paper does not report any original code. Any additional information reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human embryo kidney 293T (HEK 293T) and U2OS cells were maintained in Dulbecco's modified Eagle's medium (Biological Industries) containing 10% fetal calf serum (Sigma), supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin and cultured in a 5% CO₂ humidified incubator at 37°C.

When indicated, cells were incubated with 10 µM SB203580 (Calbiochem) for 30 min or 0.5 µM BIRB796 (Merck) for 2 h, prior to the stress treatment. DNA transfections of HEK 293T cells were performed using FuGENE 6 (Promega) ratio 6 µL reagent:1 µg DNA and transfections of U2OS were performed using Lipofectamine LTX (Invitrogen) ratio of 5 µL LTX:1 µL Plus:1 µg DNA. siRNA transfections were performed using RNAimax (Invitrogen) 0.25 µL reagent:1 nM siRNA. When indicated, thymidine (Sigma) was added at 250 µM for 24 h. Cisplatin (Sigma) was added at the indicated doses and times.

METHOD DETAILS

Stable Claspin expression

To obtain Claspin stable-expressing cells, either siRNA-resistant GFP-Claspin^{WT} or GFP-Claspin^{9A} was transfected into U2OS cells. The next day, treatment started with 1 µg/µL G418 (Gibco). Ten days later, green cells were sorted to discard non-green resistant cells and 1 week later were sorted again (BD FACSaria II-SORP). Claspin knock-down by siRNA was performed on the Claspin-expressing cells two days before each experiment.

Plasmid constructs and siRNAs

p38-mCherry was generated by *Bam*HI-*Not*I substitution of the dsRed in the p38α-DSRed plasmid (Ferreiro et al., 2010a) by mCherry amplification from plasmid pBS34. To generate the empty-mCherry vector, p38-mCherry was removed using *Xho*I-*Not*I and mCherry was reintroduced by PCR. pEFmink-MKK6^{DD} was obtained from Dr. A. R. Nebreda (Alonso et al., 2000). pcDNA3 (Invitrogen) was the empty control when empty mCherry was not used. pFRT-TODestGFP_RNaseH1 was a gift from Thomas Tuschl (Addgene plasmid #65784). pcDNA3-2xHA-Claspin (siRNA8 resistant) was obtained introducing silent mutations with the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) in the original plasmid previously described (Mamely et al., 2006), as was siRNA8: GCACAUACAUGAUAAAGAAAdTdT (Semple et al., 2007). siRNA for p38α was CCAGACCAUUUCAGUCCAAdTdT and the scrambled siRNA control was GAAUAAACACGAAGCUAUAdTdT. For inducible expression in yeast, Claspin was subcloned into the p426GAG (*Bam*HI-*Xho*I) (P_{GAL1}-GST, URA3, 2 micron), which is based on the high copy number yeast vector pRS426 (Christianson et al., 1992). GFP-Claspin was generated by subcloning Claspin *Bam*HI-*Xho*I into an empty pcDNA3 plasmid and adding GFP *Bam*HI/*Bgl*II amplified from pAcGFPN1 (Clontech). Oligonucleotides are indicated in the [Key resources table](#).

Western blot

Transfected cells were washed with ice-cold PBS and scraped into lysis buffer (10 mM Tris-HCl pH 7.5, 1% NP-40, 2 mM EDTA, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM Sodium Vanadate, 1 mM PMSF, 1 mM Benzamide, 5 µg/mL Leupeptine,

5 $\mu\text{g/mL}$ Pepstatine and 2 $\mu\text{g/mL}$ Aprotinin). The lysates were cleared by centrifugation and boiled in Laemmli buffer supplemented with β -Mercaptoethanol. The antibodies used were rabbit polyclonal anti-p38 α (Santa Cruz, sc-535), rabbit monoclonal anti-pp38 (Cell Signaling, clone 3D7), antibody for N-terminal Claspin (Mamely et al., 2006), and mouse monoclonal anti-HA, which was developed in-house from the 12CA5 hybridoma. Anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase and the Enhanced Chemiluminescence kit were from GE Healthcare. To visualize the Claspin mobility shift, proteins were run at 4°C in a 25 cm long gel until Claspin was only 3–5 cm away from the bottom edge.

Claspin expression and purification in yeast

S. cerevisiae strain BY4741 was transformed with the plasmid coding for galactose-inducible GST-Claspin. Colonies were then selected and grown in Ura[−] at 30°C. Before promoter induction, the medium was changed to YP supplemented with 2% raffinose, and 3 h later supplemented with 2% galactose (at an OD600 of 0.8) for 5 h. Cells were collected by centrifugation at 4°C, and pellets were frozen at −80°C. The next day, the pellets were resuspended in HEPES lysis buffer (45 mM Hepes-KOH at pH 7.2, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 1% Triton X-100) containing a cocktail of protease and phosphatase inhibitors (see Western blot methods). An equal volume of glass beads (0.5 mm diameter) was added, and the cells were broken by vortexing at 4°C. The whole extract was clarified by centrifugation for 10 min at 9300 *g* at 4°C. GST-fused proteins were pulled down from the supernatants with 100 μL of 4B glutathione-sepharose beads (GE Healthcare, 50% slurry equilibrated with HEPES Buffer) by mixing them overnight at 4°C. The glutathione-sepharose beads were collected by a brief centrifugation and were washed eight times in HEPES lysis buffer and three times more in Kinase Buffer 1x to proceed to the next section.

In vitro p38 kinase assay

GST-p38 α purified from bacteria was activated *in vitro* in a small volume by mixing with GST-MKK6^{DD} in kinase assay buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2 mM DTT) in the presence of 100 mM cold ATP for 20 min at 30°C. For the control, buffer was added instead of GST-p38 α and GST-MKK6^{DD}. 15 μL of the mix was used for *in vitro* phosphorylation of Sepharose beads containing GST-fused Claspin proteins. The reactions were carried out in kinase assay buffer in the presence of 1 mCi/assay of radiolabeled ³²P- γ -ATP (3000 Ci/mmol from PerkinElmer) in a final volume of 40 μL /assay for 20 min at 30°C. Reactions were stopped by adding SB5X (250 mM Tris-HCl pH 6.8, 0.5 M DTT, 10% SDS, 20% glycerol, 0.5% Bromophenol Blue) and boiling for 10 min. Next, Sepharose beads were discarded by Max speed centrifugation and phosphorylated proteins were subjected to PAGE-SDS, transfer-blotted onto a PVDF membrane, and exposed to BIOMAX XAR films (KODAK).

Cell cycle and cell death measurement

To assess the cell cycle, two procedures were followed. When the cycle experiment was not combined with a fluorochrome, cells were trypsinized and fixed in 70% ice-cold ethanol while vortexing, and left at 4°C overnight. Next, ethanol was washed out and cells were treated with a working solution (0.3 mg/mL RNase A and 3.75 $\mu\text{g/mL}$ Propidium Iodide in PBS) for 24 h at 4°C. The stained cells were acquired on a FACSCalibur cytometer (Becton Dickinson) and analyzed using the CellQuest software. When combined to mCherry fluorochrome expressing cells, Hoechst 33,342 was added to cells at a final concentration of 2 μM 1 h before harvesting, and cells were analyzed without fixation at LSR-FORTESSA (Becton Dickinson) and analyzed using FACSDiva software. When indicated, 1 h after treatment, nocodazole (Sigma) was added to a final concentration of 100 ng/mL to trap the cells in the G2/M phase. Cell death upon osmotic stress was assessed by labeling cells with 1 $\mu\text{g/mL}$ Propidium Iodide in PBS for 10 min on ice, followed by FACS analysis. The stained cells were acquired on an FACSCalibur cytometer (Becton Dickinson) and analyzed using the CellQuest software.

DNA fiber analysis

Cells were pulse-labeled with 50 μM CldU (20 min; #C6891 Sigma) followed by 250 μM IdU (20 min; #I7125 Sigma). Labeled cells were collected and DNA fibers were spread in buffer containing 0.5% SDS, 200 mM Tris pH 7.4, and 50 mM EDTA. For immunodetection of labeled tracks, fibers were incubated with primary antibodies (for CldU, rat anti-BrdU # ab6326 Abcam; for IdU, mouse anti-BrdU #347580 BD Bioscience) for 1 h at RT and developed with the corresponding secondary antibodies (anti-rat IgG AF594, #A-11007; anti-mouse IgG1 AF488, #A-21121; all from Molecular Probes) for 30 min at RT. Mouse anti-ssDNA antibody was used to assess fiber integrity (#MAB3034 Millipore, secondary antibody anti-mouse IgG2a AF647, #A-21241 Molecular Probes). Slides were examined with a Leica DM6000 B microscope, as described (Mouron et al., 2013). The conversion factor used was 1 μm = 2.59 kb (Jackson and Pombo, 1998). In each assay, > 300 tracks were measured to estimate fork rate.

Comet assay and γ H2AX staining

The comet assay was performed using the Trevigen kit and following the manufacturer's instructions. Briefly, cells were harvested after a 4-h treatment, washed in cold PBS, imbedded in melted agarose, and spread in the kit-provided slides until agarose gelified. Lysis was then performed overnight and electrophoresis ran for 30 min at 230 mAmp. Once dried, slides were stained by Sybr Gold and images were captured by a Nikon Eclipse Ti microscope. Comet-positive cells were analyzed by double-blind counting. For the γ H2AX staining, replicating U2OS cells were labeled with EdU (5-ethynyl-2'-deoxyuridine) 1 h before harvesting and visualized using the click chemistry Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen) combined with γ H2AX antibody (Clone

JBW301, Millipore) detected by secondary Alexa 568 anti-mouse antibody (Invitrogen) and 4 μ M Hoechst 33,342. When GFP-positive, synchronized cells were collected and treated with cytofix and cytoperm (Becton Dickinson) and then detected with Alexa 647-conjugated γ H2AX antibody (Cell Signaling).

R-Loop detection

Cells were seeded in 8-well slide coverslips at 15,000 per well. 48 h later cells were treated with 150 mM NaCl for 4 h, washed with PBS and fixed in ice-cold methanol for 10 min, followed by a 1 min ice-cold acetone incubation for permeabilization. Cells were blocked with 2% BSA in PBS for 1 h at room temperature and primary antibodies against R-loops and nucleolin were added, samples were incubated overnight at 4°C. Anti-mouse Alexa 568 and anti-rabbit Alexa 633 were both used at a 1:1000 dilution and incubated for 1 h at room temperature. After PBS wash, samples were incubated with Hoescht 33,342. Quantification was performed selecting the red signal (R-Loops) overlapping with Hoescht (nucleus), then the red signal overlapping with nucleolin signal was subtracted. High-throughput spinning-disk confocal microscope (LIPSI, Nikon) with PrimeBSI sCMOs camera (Teledyne Photometrics) were used to take pictures with a 20x dry lens. At least 500 cells per condition were used for analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Results are expressed as the mean $\pm$ SEM unless stated otherwise. p values were calculated using a Student's two-tailed t test or mean $\pm$ standard error. Statistical details and p values of experiments can be found in the Figure legends.