IKK and NF- κ B-mediated regulation of Claspin impacts on ATR checkpoint function

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In response to replication stress, Claspin mediates the phosphorylation and activation of Chk1 by ATR. Claspin is not only necessary for the propagation of the DNA-damage signal, but its destruction by the ubiquitin-proteasome pathway is required to allow the cell to continue the cell cycle allowing checkpoint recovery. Here, we demonstrate that both the NF- κ B family of transcription factors and their upstream kinase IKK can regulate Claspin levels by controlling its mRNA expression. Furthermore, we show that c-Rel directly controls Claspin gene transcription. Disruption of IKK and specific NF- κ B members impairs ATR-mediated checkpoint function following DNA damage. Importantly, hyperactivation of IKK results in a failure to inactivate Chk1 and impairs the recovery from the DNA checkpoint. These results uncover a novel function for IKK and NF- κ B modulating the DNA-damage checkpoint response, allowing the cell to integrate different signalling pathways with the DNA-damage response.

The EMBO Journal (2010) 29, 2966–2978. doi:10.1038/emboj.2010.171; Published online 23 July 2010

Subject Categories: signal transduction; cell cycle

Keywords: Chk1; Claspin; IKK; NF- κ B

Introduction

In response to cellular insults, which can result in DNA damage or replication stalling, cells can activate genome surveillance pathways that co-operate to preserve genomic integrity, by preventing inappropriate progression of the cell cycle. In this pathway ATR, a protein kinase, is activated and phosphorylates a number of downstream targets that can co-ordinate cell-cycle progression, with repair of the damaged DNA (Kastan and Bartek, 2004). One of such targets is the Chk1 protein kinase, when phosphorylated by ATR at two critical serine residues at positions 317 and 345 propagates the damage response (Zhao and Piwnicka-Worms, 2001). Once activated, Chk1 phosphorylates a number of downstream targets, such as Cdc25, which ultimately results in cyclin-dependent kinase inhibition and cell-cycle delay, allowing the cell time to use the necessary repair mechanisms (Harper and

Elledge, 2007). Such a rapid, but reversible, block of cell-cycle progression is essential for the repair of potentially catastrophic perturbations in their genome.

Claspin was originally identified in *Xenopus* in a screen to identify Chk1-interacting proteins (Kumagai and Dunphy, 2000). It serves as an adaptor protein, binding to both Chk1 and ATR, and is necessary for ATR-dependent phosphorylation of Chk1 in both *Xenopus* and human systems (Chini and Chen, 2003; Kumagai and Dunphy, 2003; Clarke and Clarke, 2005). Claspin protein levels fluctuate during the cell cycle being low during early G1, accumulating to be high in S-phase and eventually degraded in a manner controlled by the protein kinase, Polo-like kinase-1 (Plk1), at the onset of mitosis (Yoo *et al*, 2004; Peschiaroli *et al*, 2006). This cyclical regulation of Claspin has been shown to be involved in the normal progression of the cell cycle, and following checkpoint activation (Peschiaroli *et al*, 2006). Indeed, inappropriate reduction of Claspin levels by siRNA slows replication fork rates (Petermann *et al*, 2008) and, following replication stress, promotes premature entry into mitosis before completing DNA replication (Chini and Chen, 2003); conversely, overexpression of a non-degradable form of the protein results in a delay in the inactivation of Chk1 and recovery from the DNA replication checkpoint (Mailand *et al*, 2006; Mamely *et al*, 2006; Peschiaroli *et al*, 2006). Any process involved in the regulation of Claspin levels would therefore be important for maintaining faithful replication of the DNA, and also proper regulation of the DNA replication checkpoint activated by ATR.

NF- κ B is the collective name for a family of transcription factors. This family is comprised of five genes, encoding seven proteins: RelA, RelB, c-Rel, p105/p50 (NF- κ B1) and p100/p52 (NF- κ B2). NF- κ B activation pathways can be classified as canonical, non-canonical and atypical regarding the mode and involvement of the IKK complex (Perkins, 2007; Chariot, 2009). Genotoxic agents such as chemotherapeutic drugs and radiotherapy can activate NF- κ B through atypical pathways (Perkins, 2007). As such, ATM has been shown to bind and phosphorylate IKK γ /Nemo (NF- κ B essential modulator), which results in activation of the IKK complex and hence NF- κ B (Wu *et al*, 2006). NF- κ B can have both anti- and pro-apoptotic functions and this is dependent on many factors including genetic context, binding partners and posttranslational modifications of the subunits themselves (Perkins and Gilmore, 2006). Despite the existence of many reports concerning the mode of activation and response of NF- κ B following DNA damage, no study thus far as assessed if NF- κ B has a function in any of the cellular checkpoints.

In this study, we demonstrate that the IKK complex can regulate Claspin levels by controlling its mRNA expression. This regulation is dependent on the activity of the IKK β , but independent of the IKK α subunit. Furthermore, we have identified c-Rel as the transcription factor downstream of IKK β directly controlling Claspin gene transcription. We

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Received: 10 February 2010; accepted: 1 July 2010; published online: 23 July 2010

show that disruption of IKK and c-Rel significantly impairs ATR-mediated checkpoint function following DNA damage. Reduction of IKK or c-Rel results in increased H2Ax foci, in a manner similar to that of Claspin depletion. Importantly, hyperactivation of IKK results in delayed inactivation of Chk1 and recovery from the DNA replication checkpoint, with inappropriate activation of the checkpoint in mitosis.

Results

Depletion of IKK β results in a severe reduction of Claspin levels

Previous studies have shown that NF- κ B is activated following DNA damage and that the IKK complex subunit Nemo interacts with ATM (Wu *et al*, 2006). In addition, a recent study linked perturbations of IKK β to genetic instability (Irelan *et al*, 2007). We noticed that the phospho-degron present in the amino terminus of Claspin is highly similar to that of the IKK substrate I κ B- α (Supplementary Figure S1A). As such, we investigated if the IKK complex regulates Claspin. The IKK complex is composed of two catalytic subunits IKK α and IKK β , and a regulatory subunit IKK γ (Perkins, 2007; Chariot, 2009). Although highly homologous IKK α and IKK β have largely distinct functions, because of their different substrate specificities and modes of regulation (Hacker and Karin, 2006). Although we could not detect IKK-mediated phosphorylation of a bacterially expressed fragment of Claspin containing the phospho-degron motif, *in vitro* (Supplementary Figure S1B), we investigated if endogenous IKK could regulate Claspin in cells. RNA interference (RNAi) was used to reduce the expression of the catalytic subunits IKK α or IKK β . Relative to non-targeting controls, IKK α and IKK β protein and mRNA levels were specifically decreased in U2OS cells by the respective RNAi (Figure 1A; Supplementary Figure S2A). Although specific knockdown of IKK α had no effect on Claspin levels, reduction

of IKK β unexpectedly correlated with a visible reduction of Claspin protein levels (Figure 1A). Additional siRNA oligonucleotides targeting IKK β , produced a similar reduction of Claspin protein levels (Supplementary Figure S2B). Furthermore, Claspin protein levels, in cells depleted of endogenous IKK β , could be partially rescued by the expression of an exogenous IKK β construct (Supplementary Figure S2C). IKK β depletion also reduced Claspin levels in MDA-MB-231 and HEK293 cells (Supplementary Figure S2D), demonstrating that this effect is not limited to U2OS cells.

The IKK kinase complex requires the regulatory subunit IKK γ for its activity (Perkins, 2007; Chariot, 2009); we therefore tested if Claspin levels were sensitive to IKK γ levels. RNAi specifically reduced IKK γ levels, but did not alter levels of IKK α or IKK β (Figure 1B). Selective reduction of IKK γ significantly diminished levels of Claspin, relative to control-transfected cells (Figure 1B).

The RNAi results indicate that the presence of IKK β and IKK γ are required for maintaining Claspin protein levels. Given that the IKK complex has kinase activity, we determined if inhibiting this would lower Claspin levels. Inhibition of the IKK complex using an IKK inhibitor (Bay 11-7082), confirmed by a reduction in the basal level of phospho-I κ B α (Figure 1C), resulted in a reduction of Claspin levels by 6 h and almost complete loss of Claspin after 24 h of treatment (Figure 1C). IKK β and IKK γ depletion in unstimulated cells also results in lower NF- κ B activity, assessed using an NF- κ B luciferase reporter assay (Supplementary Figure S2E), further indicating that IKK and NF- κ B have basal activity. In addition, IKK inhibition (Supplementary Figure S3A) or siRNA-mediated depletion of IKK β and IKK γ (Supplementary Figure S3B and C), in mouse embryo fibroblasts also resulted in reduced Claspin levels, indicating that IKK regulates Claspin in mouse cells. Taken together, these results demonstrate that Claspin levels are sensitive to both IKK levels and activity.

Figure 1 IKK β is required for maintaining Claspin protein levels. (A) U2OS cells were depleted of IKK α or IKK β using siRNA. Whole-cell lysates (WCLs) were subjected to immunoblot analysis for the levels of the indicated proteins. (B) U2OS cells were depleted of IKK γ using siRNA. WCLs were subjected to immunoblot to analyse the levels of the indicated proteins. (C) U2OS cells were treated with the IKK inhibitor, Bay 11-7082, for the indicated times. WCLs were prepared and subjected to immunoblot analysis using the indicated antibodies. (D) U2OS were depleted of IKK β using siRNA as in (A), but MG132 was added 3 h prior to WCL preparation, and immunoblot analysis was performed using the indicated antibodies.

Figure 2 Claspin mRNA is regulated by IKK β and the NF- κ B family of transcription factors. **(A)** Semi-quantitative and quantitative RT-PCR analysis of Claspin mRNA prepared from U2OS cells depleted of IKK α , IKK β or Claspin by siRNA. **(B)** U2OS cells were depleted of the individual NF- κ B family members; RelA, RelB, c-Rel, p105/p50 or p100/p52, using siRNA and analysed by immunoblot using the indicated antibodies. **(C)** Semi-quantitative RT-PCR analysis of Claspin, RelA, RelB, c-Rel, p105, p100 and actin mRNA from cells treated as in **(A)**. **(D)** Quantitative RT-PCR analysis of Claspin mRNA in cells depleted of the individual NF- κ B subunits as in **(A)**. ANOVA *t*-tests were performed on the means, and the *P*-values were calculated. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

Previous studies demonstrated that Claspin levels are controlled in the cell by changing protein stability, through a mechanism dependent on phosphorylation and degradation by the proteasome (Mailand *et al*, 2006; Mamely *et al*, 2006; Peschiaroli *et al*, 2006). We therefore tested if depletion of IKK β was altering Claspin protein stability. In cells transfected with non-targeting siRNA, there is a partial elevation of Claspin levels in cells treated with the proteasome inhibitor MG132 (Figure 1D), consistent with previously published observations (Bennett and Clarke, 2006). However, in cells transfected with IKK β siRNA, no recovery of Claspin levels in the presence of proteasome inhibition could be observed (Figure 1D). Immunoblotting using an anti-ubiquitin antibody revealed that accumulation of poly-ubiquitinated proteins was similar in cells treated with either non-targeting RNAi or IKK β RNAi, demonstrating that failure to recover Claspin levels in IKK β -depleted cells, was not due to ineffective proteasome inhibition (Figure 1D, lower panel). Furthermore, MG132 treatment increased the levels of p53 and Cyclin D1 (proteins that are regulated by the proteasome) regardless of IKK β levels. These results indicate that IKK β is not altering Claspin levels by regulating its stability.

IKK β controls the levels of Claspin mRNA through modulation of NF- κ B

As IKK inhibition does not alter Claspin levels by affecting its protein stability, we investigated if depletion of IKK could alter levels of Claspin mRNA. We performed quantitative RT-PCR (qRT-PCR) using RNA from cells depleted of IKK α , IKK β or Claspin. Reducing IKK α levels had no significant effect on Claspin mRNA levels, consistent with the observa-

tions of the Claspin protein (Figure 2A). Specific knockdown of IKK β results in a dramatic reduction of Claspin mRNA levels, to ~20% of the levels observed in the cells transfected with the non-targeting control RNA (Figure 2A). Similar results were also observed using alternative siRNAs targeting IKK β , and in other cells such as MDA-MB-231 (Supplementary Figure S4).

As the best-known function of IKK β is to activate the NF- κ B family of transcription factors (Perkins, 2007), we next investigated if any of the NF- κ B transcription factors could regulate the expression of Claspin mRNA. Using previously validated siRNA sequences (Anderson and Perkins, 2003; Schumm *et al*, 2006; van Uden *et al*, 2008), we reduced the levels of RelA, RelB, c-Rel, p100/p52 and p105/p50 at both the protein and mRNA levels, as determined by western blot and RT-PCR, respectively (Figure 2B-D; Supplementary Figure S5). NF- κ B subunits control each other expression by the presence of κ B sites in their promoters, as such depletion of a single subunit will alter the expression levels of additional ones (Figure 2B). As activation of IKK β usually results in the induction of RelA/p50 heterodimer activity, it was surprising to observe that siRNA oligonucleotides directed against RelA or p105/p50, although significantly reducing the levels of their respective targets, did not alter either the levels of the Claspin mRNA or protein (Figure 2B-D). However, reduction of RelB, c-Rel and p100/p52 resulted in a specific decrease in the levels of the Claspin protein (Figure 2B) and mRNA (Figure 2C and D). Taken together, these results demonstrate for the first time that IKK β and specific NF- κ B subunits regulate Claspin levels, through modulation of its mRNA.

Figure 3 The Claspin gene promoter is directly regulated by c-Rel. **(A)** Schematic of the human Claspin gene promoter with putative NF- κ B sites represented as bars, and ChIP PCR primer sets represented as lines. **(B)** ChIP assays using Control IgG, RelB, c-Rel or p52 antibodies were performed on cell extracts from U2OS cells. Immunoprecipitated DNA was PCR amplified using the indicated primer pairs alongside 2% input genomic DNA. **(C)** ChIP assays using Control IgG and c-Rel antibodies were performed on cell extract from control or cells depleted of c-Rel. Immunoprecipitated DNA was amplified by PCR using the indicated primer pairs. **(D)** As in **(C)** but ChIP was performed using antibodies directed against RNA polymerase II. **(E)** As in **(C)** but ChIP was performed using antibodies directed against acetylated histone H3. Levels of RNA polymerase II recruitment to the κ B sites of the Claspin gene were quantified, normalized to inputs, and numbers indicate the difference between control and c-Rel-depleted samples. **(F)** ChIP assays using Control IgG and c-Rel antibodies were performed on cell extract from control or cells depleted of p52 or RelB. Immunoprecipitated DNA was amplified by PCR using the indicated primer pairs. **(G)** U2OS cells were co-transfected with a non-targeting siRNA or a siRNA to deplete IKK β , along with either a vector control or a p52 or RelB expression constructs, as indicated. WCLs were prepared and analysed by western blot using the indicated antibodies.

c-Rel binds directly to the Claspin promoter

Given the results obtained when analysing the levels of Claspin mRNA, we next determined the function of specific NF- κ B subunits in the control of the Claspin gene. Analysis of the Claspin promoter, revealed the presence of putative NF- κ B-binding site sequences both upstream and downstream of the transcription start site (<http://www.genomatix.de/products/MatInspector>) (Figure 3A). As depletion of RelB, c-Rel or p52 altered Claspin levels at both the protein and mRNA levels (Figure 2), we sought to investigate if this effect was direct. To determine if RelB, c-Rel or p52 are directly bound to the promoter of the Claspin gene *in vivo*, we

performed chromatin immunoprecipitation (ChIP) assays in U2OS cells, using specific antibodies against the respective proteins. Primer sets were designed to encompass the NF- κ B-binding site upstream of the transcription start site (κ B1), at the transcription start site (κ B2), downstream of the transcription start site (κ B3), and a control region 2000 bp upstream (Figure 3A). Promoter occupancy was assayed by PCR using these sets of primer pairs. We detected enrichment of c-Rel using the κ B1 primer pair, indicating binding upstream of the transcription start site (Figure 3B). The specificity of this binding was evident by the lack of signal from the c-Rel ChIP in either the control region or the putative κ B consensus

sites downstream or at the start site of transcription (Figure 3B). However, little or no binding of RelB or p52 was observed on any of the putative-binding sites (Figure 3B). These results indicate that c-Rel can directly bind to the Claspin promoter.

To determine if changes in c-Rel levels directly altered the levels of polymerase recruitment or any hallmark of active transcription at the Claspin promoter, we depleted cells of c-Rel using siRNA and performed ChIPs. When compared with control cells, c-Rel depletion resulted, as expected, in less c-Rel present at the κ B1 site of the Claspin promoter (Figure 3C), demonstrating the specificity of the signal obtained in Figure 3B. Importantly, depletion of c-Rel reduced polymerase occupancy at the start site and within the coding region of the Claspin promoter (Figure 3D). Furthermore, levels of acetylated histone H3 were also reduced but only at the promoter region, suggesting that c-Rel is necessary for maintaining high levels of acetylated histones surrounding the Claspin promoter (Figure 3E).

Given that we have found that p52 and RelB also control Claspin levels, we determined if depletion of these subunits would alter c-Rel binding to the Claspin promoter. We found that c-Rel binding to the Claspin promoter remained unaltered in the absence of either p52 or RelB (Figure 3F). Given these results, we sought to determine the contribution of these NF- κ B subunits in the regulation of Claspin levels. For this purpose, we overexpressed either p52 or RelB in the absence of IKK β and analysed Claspin protein levels (Figure 3G). Our results demonstrate that these subunits cannot rescue IKK β depletion and thus suggest p52 and RelB have indirect functions in the control of Claspin.

IKK β controls the recruitment of c-Rel to the Claspin promoter and is required for active transcription of the Claspin gene

As the IKK complex is immediately upstream of the NF- κ B family of transcription factors, including c-Rel, we next sought to determine if Claspin promoter occupancy by c-Rel was dependent on IKK β . For this purpose, we created U2OS stable cell lines in which IKK β was selectively depleted using shRNA, but other members of the IKK complex remained unchanged (Supplementary Figure S6). Claspin protein levels were reduced in this cell line, consistent with the observed results with the transient transfections (Supplementary Figure S6). Using control cells carrying a non-targeting shRNA and IKK β shRNA cells, ChIP assays were performed for the Claspin promoter. c-Rel binding to the upstream κ B site could be observed in the control cells (Figure 4A). In contrast, when IKK β is not present, c-Rel promoter occupancy was significantly decreased (Figure 4A). We also investigated the function of IKK α and IKK γ , as well IKK β , in the recruitment of c-Rel to the Claspin promoter (Figure 4B). As expected and in accordance with our mRNA and protein analysis, depletion of IKK α did not alter c-Rel levels present at the Claspin promoter (Figure 4B). However, depletion of either IKK β or IKK γ visibly reduced c-Rel recruitment to this promoter (Figure 4B). These results indicate that IKK β and IKK γ control c-Rel recruitment to the Claspin promoter.

As IKK β depletion resulted in lower Claspin mRNA (Figures 1 and 2A), we investigated if this was also evident in RNA polymerase recruitment. When IKK β was depleted, we observed a reduction in RNA polymerase II recruitment to the start site of transcription and a reduction of polymerase loading within the coding region, consistent with these cells

Figure 4 The Claspin gene promoter is directly regulated by c-Rel in an IKK β -dependent manner. (A) ChIP assays using Control IgG or c-Rel antibodies were performed on cell extracts from cells stably expressing a control shRNA plasmid and cells expressing shRNA against IKK β . Immunoprecipitated DNA was PCR amplified using the indicated primer pairs alongside 2% input genomic DNA. (B) ChIP assays using Control IgG and c-Rel antibodies were performed on cell extracts from control or cells depleted of IKK α (α), IKK β (β -sequence B) or IKK γ (γ). Immunoprecipitated DNA was amplified by PCR using the indicated primer pairs. (C) As in (A) but ChIP was performed using antibodies directed against RNA polymerase II. (D) As in (A) but ChIP was performed using antibodies directed against acetylated histone H3. (E) U2OS cells were co-transfected with a non-targeting siRNA or a siRNA to deplete c-Rel, along with either a vector control or a Flag-tagged version of IKK β , as indicated. WCLs were prepared and analysed by western blot using the indicated antibodies.

having lower Claspin expression (Figure 4C). We also compared localized levels of acetylated histone H3, a mark of active transcription, surrounding the Claspin promoter. Acetylated H3 levels at the Claspin promoter, surrounding the c-Rel-binding site, were reduced by IKK β depletion, but remained unaltered at the transcription start site and within the gene in the presence or absence of IKK β (Figure 4D). To elucidate whether c-Rel and IKK β exert their effects on Claspin through alternative pathways, we examined if expression of exogenous IKK β , could rescue Claspin levels in cells depleted of c-Rel. U2OS cells transfected with an siRNA against c-Rel have lower levels of Claspin than those transfected with a control non-targeting siRNA. Overexpression of IKK β was unable to rescue the Claspin defect in c-Rel knockdown cells, consistent with IKK being upstream of c-Rel in this signalling pathway (Figure 4E). These results indicate that IKK β and c-Rel regulate polymerase recruitment and activation of transcription of the Claspin gene (Figures 3 and 4).

Regulation of Claspin mRNA is cell-cycle independent but it is responsive to changes in IKK-NF- κ B activity

It is well established that the Claspin protein is regulated through the cell cycle, but no study has assessed if Claspin mRNA is also regulated in this manner. Claspin protein levels are low during the G1 phase of the cell cycle. As such, we determined if the reduction of Claspin mRNA we had observed in the absence of IKK β was merely a consequence of a cell-cycle block. U2OS cells were arrested in the G1 phase of the cell cycle by depletion of the G1-specific cyclin, Cyclin D1 (Figure 5A and B; Supplementary Figure S7A). Cells depleted of IKK β , Claspin and Cyclin D1 show reduced levels of Claspin protein, compared with the cells depleted of IKK α , or treated with the non-targeting control RNAi (Figure 5A). However, knockdown of Cyclin D1 was not sufficient to reduce the levels of the Claspin mRNA, providing a correlative indication that regulation of Claspin mRNA, unlike the protein, is independent of the cell cycle (Figure 5B; Supplementary Figure S7A).

To test directly if the Claspin transcript is regulated through the cell cycle, U2OS cells were synchronized, either at the G1-S boundary using a double-thymidine block, or in mitosis using nocodazole, then released and followed through the cell cycle by FACS (Figure 5C and D). Using both synchronization approaches, it was possible to observe that Claspin protein levels were maintained through S-phase of the cell cycle before being rapidly degraded during mitosis, consistent with published observations (Figure 5C and D) (Yoo *et al*, 2004; Peschiaroli *et al*, 2006). Our results show that the levels of Claspin mRNA do not change significantly throughout the cell cycle (Figure 5C and D). We did see slight fluctuations in the levels of Claspin mRNA in cells which were still subject to chemical block, but these were not observed in samples corresponding to similar stages of the cell cycle in unperturbed conditions, and can therefore be attributed to the chemical intervention (Figure 5C and D and unpublished data). Consistent with Claspin mRNA not fluctuating during the cell cycle, we do not observe any changes in RelB, c-Rel or p100/p52 levels, or IKK levels/activity during the cell cycle using both synchronization approaches (Supplementary Figure S7B and C). These results indicate that Claspin mRNA is not regulated in a cell-cycle manner.

To further separate the cell-cycle-dependent regulation of Claspin by the ubiquitin/proteasome pathway and the IKK-dependent regulation of Claspin, we measured Claspin mRNA levels in the absence of β -TrCP, the ubiquitin ligase required for its degradation. We reduced β -TrCP levels in U2OS cells using previously validated siRNA sequences (Supplementary Figure S8; Mailand *et al*, 2006; Mamely *et al*, 2006; Peschiaroli *et al*, 2006). Depletion of β -TRCP did not change the levels of phosphorylated and total IKK or I κ B- α in unstimulated cells (Supplementary Figure S8B). In addition, the levels of nuclear c-Rel were also unaffected by depletion of β -TRCP (Supplementary Figure S8C). Importantly, in the absence of β -TrCP, there is no significant change in Claspin mRNA levels, compared with cells treated with a non-targeting control, indicating that the regulation of Claspin mRNA is independent of the protein degradation pathway (Figure 5E).

We next investigated if Claspin mRNA could be regulated by modulation of IKK-NF- κ B activity. IKK responds to a variety of different stimuli such as cytokines, stress signals (Perkins, 2007; Chariot, 2009). We initially tested if Claspin was responsive to TNF- α , but did not detect any significant changes (data not shown); however, when we tested another known NF- κ B inducer, PMA/Ionomycin, we observed a modest, but significant induction of Claspin (Figure 5F; Supplementary Figure S9A). Interestingly, UV treatment did not result in higher levels of Claspin. UV is known to activate RelA/p50 (Campbell *et al*, 2004), but UV-mediated activation of c-Rel has not been reported. Importantly, PMA/Ionomycin-dependent induction of Claspin was lost in cells depleted of IKK β , demonstrating that Claspin induction is through an IKK-dependent signalling cascade (Supplementary Figure S9B). These results indicate that Claspin is responsive to the levels of IKK-NF- κ B activity.

Loss of IKK β disrupts Chk1 phosphorylation by ATR following DNA damage

One of the major functions of Claspin is to act as an adaptor between ATR and Chk1. As such, Claspin is critical for the ATR-dependent phosphorylation of Chk1 (Lee *et al*, 2005). Given our discovery that IKK β can control Claspin levels through regulation of its mRNA, we wanted to determine what were the functional consequences of IKK β depletion on the DNA-damage checkpoint and ultimately on cellular fate. To investigate if loss of IKK function could compromise Chk1 phosphorylation in response to DNA damage, we depleted IKK α , IKK β or IKK γ and then analysed the levels of Chk1 phosphorylation after exposure to UV light (a known stimulus of the ATR-Chk1 pathway). As expected, cells transfected with a non-targeting siRNA displayed a robust level of Chk1 Ser317 phosphorylation when exposed to UV (Figure 6A). Significantly, reduction of IKK β , but not IKK α , by siRNA attenuated the phosphorylation of Chk1 on the Ser317 residue, but had no great effect on Chk1 protein levels (Figure 6A). Indeed, knockdown of IKK β produced a similar reduction on Chk1 Ser317 phosphorylation as depletion of Claspin itself (Figure 6A). Importantly, this result is not dependent on method of checkpoint induction or the cell line as similar defects in Chk1 phosphorylation are seen in response to Hydroxyurea in IKK β -depleted U2OS cells (Supplementary Figure S10A), and UV-induced damage in IKK β -depleted MDA-MB-231 cells (Supplementary

Figure 5 Claspin mRNA is regulated independently of the cell cycle but Claspin is responsive to NF- κ B activation. (A) U2OS cells were depleted of IKK α , IKK β , Claspin or Cyclin D1 using siRNA. WCLs were subjected to western blot analysis for the levels of the indicated proteins. (B) Quantitative RT-PCR analysis of Claspin mRNA prepared from U2OS cells depleted of IKK α , IKK β , Claspin or Cyclin D1 by siRNA. ANOVA *t*-tests were performed on the means, and the *P*-values were calculated. ***P* ≤ 0.01 and ****P* ≤ 0.001. (C) U2OS cells were synchronized at G1/S using a double-thymidine block. Thymidine was subsequently washed and cells were released for the indicated times. Cells were analysed by flow cytometry and percentage of cells in each stage of the cell cycle is indicated. WCLs and RNA were prepared from these cells and analysed by western blot and quantitative RT-PCR, respectively. Specific antibodies and primer sets are indicated. (D) U2OS cells were synchronized at prometaphase using nocodazole. Nocodazole was subsequently washed and cells were analysed by flow cytometry and percentage of cells in each stage of the cell cycle is indicated. WCLs and RNA were prepared and analysed by western blot and quantitative RT-PCR, respectively. Specific antibodies and primer sets are indicated. (E) Quantitative RT-PCR analysis of Claspin mRNA prepared from U2OS cells depleted of β-TrCP by siRNA. (F) U2OS cells were treated with PMA (100 ng/ml)/Ionomycin (0.5 μM) for 4 h. One hour prior to harvesting, cells were treated with UV (40 J/m²) as indicated. WCLs were prepared and Claspin levels were analysed by western blot.

Figure 6 Loss or inhibition of IKK β disrupts Chk1 phosphorylation in response to UV. (A) U2OS cells were depleted of IKK α , IKK β , IKK γ or Claspin with siRNA. Cells were treated with UV (40 J/m²) as indicated, and harvested 4 h later. WCLs were prepared and analysed by western blot using the indicated antibodies. (B) U2OS cell were pretreated with the IKK inhibitor, Bay 11-7082, for 24 h, as indicated. Cells were treated with UV (40 J/m²) as indicated, and harvested 4 h later. WCLs were prepared and analysed by western blot using the indicated antibodies. (C) U2OS cells were depleted of the NF- κ B transcription factors RelA, RelB, c-Rel, p50 or p52 with siRNA. Cells were treated with UV as in (A) and WCLs prepared analysed by western blot using the indicated antibodies. (D) U2OS cells were transfected with control or constitutive active IKK β (left panel) or treated with PMA (100 ng/ml)/Ionomycin (0.5 μ M) for 4 h (right panel) prior to treatment with UV (40 J/m²) as indicated. WCLs were prepared and analysed by western blot using the indicated antibodies. (E) U2OS cells were transfected with a non-targeting siRNA or a siRNA to deplete IKK β . c-Rel was then re-introduced into these cells by transient transfection, and cells were treated with UV (40 J/m²) as indicated, and left to culture for a further 4 h. WCLs were prepared and analysed by western blot using the indicated antibodies. (F) U2OS cells were co-transfected with a non-targeting siRNA or a siRNA to deplete IKK β , along with a DNA construct to re-introduce Claspin. Cells were treated with UV (40 J/m²) as indicated, and left to culture for a further 4 h. WCLs were prepared and analysed by western blot using the indicated antibodies.

Figure S10B). We had previously shown that IKK γ is required for maintaining Claspin levels (Figure 1B). As predicted from our results, U2OS cells depleted of IKK γ displayed lower levels of Chk1 Ser317 phosphorylation in response to UV-induced DNA damage (Figure 6A). In addition, the IKK

inhibitor Bay 11-8072 almost completely abolished UV-induced Chk1 Ser317 phosphorylation when compared with control cells (Figure 6B).

Checkpoint activation by DNA damaging agent such as UV light initiates the action of several signal pathways, which act

in concert to repair the damage. To determine how specific the effect of IKK β depletion on the checkpoint response was, we examined phosphorylation of SMC1 following UV damage, an ATM dependent but Chk1 independent, target of the human S-phase checkpoint (Yazdi *et al*, 2002). Depletion of IKK β had no effect on SMC1 Ser966 phosphorylation in response to UV (Supplementary Figure S10C). These results suggest that not all checkpoint activities are lost in the absence of IKK β , indicating once again a specific effect on Claspin.

We also evaluated the effects of depleting specific NF- κ B subunits on the levels of Chk1 phosphorylation following UV (Figure 6C). As predicted from the effects on Claspin mRNA and protein (Figure 2), depletion of RelA and p105/p50 had no significant effect on UV-induced Chk1 Ser317 phosphorylation. However, depletion of RelB, p100/p52 and c-Rel, visibly reduced this phosphorylation event (Figure 6C). Importantly, modulation of endogenous Claspin levels by changing IKK-NF- κ B activity results in changes in the levels of checkpoint activation as assessed by the levels of phosphorylated Chk1 (Figure 6D). Similar results were also observed in HEK 293 cells (Supplementary Figure S10D).

Expression of exogenous c-Rel in IKK β -depleted cells restores checkpoint activation

We have shown that c-Rel can directly regulate the Claspin promoter and this is dependent on IKK β (Figure 3). In addition, we have found that lack of IKK β compromises ATR-mediated phosphorylation of Chk1 (Figure 6A and B). To firmly establish the function of c-Rel in this ATR-mediated event, we determined if expression of exogenous c-Rel could rescue Chk1 activation in cells lacking IKK β . For this purpose, we transiently expressed c-Rel in IKK β knockdown cells and activated the checkpoint by treating cells with UV. A significant increase in c-Rel levels could be seen in cells transfected with c-Rel expression constructs (Figure 6E). Expression of exogenous c-Rel elevated levels of Claspin in IKK β -depleted cells to approximately the levels of the control cells (Figure 6E). Importantly, expression of c-Rel almost completely overcame the defect in Chk1 Ser317 phosphorylation in response to UV-induced damage in IKK β -depleted cells (Figure 6E, compare lane 2 with lane 8). These results demonstrate the importance of IKK β and c-Rel in the UV-induced ATR/Claspin-mediated Chk1 activation.

Re-introduction of Claspin into IKK β -depleted cells restores checkpoint activation

We have demonstrated the dependence of Claspin mRNA on IKK signalling, and that depleting IKK β , results in impaired Chk1 phosphorylation in response to a UV insult. To determine conclusively that the checkpoint impairment in IKK β cells was due to loss of Claspin, we sought to restore the checkpoint in IKK β -depleted cells through transfection of a Claspin construct. Expression of exogenous Claspin elevated levels of Claspin in IKK β depleted to a level higher than that seen in the control cells (Figure 6F). Importantly, expression of Claspin almost completely overcame the defect in Chk1 Ser317 phosphorylation in response to UV-induced damage in IKK β -depleted cells (Figure 6F, compare lane 2 with lane 4 and lane 6 with lane 8). These results demonstrate that expression of Claspin is sufficient to, at least in part, rescue ATR/Claspin-mediated Chk1 activation in IKK β compromised cells.

IKK β -deficient cells are hypersensitive to UV-induced DNA damage

Cell-cycle checkpoints are vital control mechanisms that ensure the fidelity of cell division in response to cellular insults. As we have discovered that depletion of IKK β specifically compromised Chk1 activation through downregulation of Claspin mRNA, we sought to examine if knockdown of IKK β renders cells sensitive to cellular insults. Using the U2OS cells line stably depleted of IKK β , we measured the proportion of cells with a sub-G1 DNA content following UV treatment. As shown in Figure 7A, following UV treatment, control cells showed an increased proportion of cells containing a sub-G1 DNA content of 3% after 24 h treatment raising to 9% after 48 h. Importantly, cells with reduction in IKK β displayed significantly higher levels of cells with a sub-G1 DNA content, 4.3 and 24%, after 24 and 48 h respectively. This result suggests that IKK β impaired cells have an increased sensitivity to cell death following UV treatment. To rule out any effects of the stable depletion of IKK β , we repeated this analysis using transient siRNA transfection. Furthermore, we also investigated the function of c-Rel in this response (Figure 7B). As observed with the stable IKK β -depleted cells, transient reduction of IKK β resulted in enhanced sensitivity to UV treatment. Consistent with our previous results, c-Rel depletion also sensitized cells to UV-induced death (Figure 7B).

Modulation of IKK activity regulates Claspin functions in the cell

Claspin is required for normal replication forks progression (Chini and Chen, 2003; Petermann *et al*, 2008) and controls genomic stability (Freire *et al*, 2006; Focarelli *et al*, 2009). It has been shown that Claspin depletion results in H2Ax foci in cells (Liu *et al*, 2006). As IKK and NF- κ B regulate Claspin levels, we assessed if knockdown of IKK or c-Rel also resulted in the increase of H2Ax phosphorylation and foci. We depleted cells of control, c-Rel, IKK β and Claspin and analysed by western blot the levels of phosphorylated H2Ax (Figure 7C). Our analysis confirmed the previously published results that Claspin depletion results in high levels of phosphorylated H2Ax. Reduction of c-Rel and IKK β also resulted in increased levels of this marker when compared with control cells. We confirmed these results using immunofluorescence microscopy (Figure 7D; Supplementary Figure S11A).

Claspin needs to be degraded to allow for checkpoint recovery (Mailand *et al*, 2006; Mamely *et al*, 2006; Peschiaroli *et al*, 2006). Indeed, when degradation of Claspin is inhibited following DNA damage, activation of Chk1 persists and cells fail to recover from the checkpoint. We thus investigated if hyperactivation of the NF- κ B signalling pathway resulted in a similar phenotype. For this, we overexpressed a constitutively active form of IKK β in cells. We synchronized them in mitosis using nocodazole and then treated them with UV for 2 h prior to lysis. Cells with a normal control of Claspin should have no Chk1 phosphorylation present in mitosis. This is evident in control-transfected cells, where no Chk1 phosphorylation could be detected (Figure 7E). However, in the presence of active IKK β , phosphorylation of Chk1 was still present (Figure 7E), indicating that cells have failed to inactivate the checkpoint in mitosis. Expression levels of Cyclin B1 indicate that cells were synchronized in mitosis.

Figure 7 Modulation of IKK β and c-Rel activity regulates Claspin cellular functions. **(A)** U2OS cells stably depleted of IKK β were treated with UV (40 J/m²) for 24 and 48 h prior to fixation and flow cytometry analysis. Results are depicted as histograms (left panels) and quantification of sub-G1 content of cells. ANOVA *t*-tests were performed on the means, and the *P*-values were calculated. **P* < 0.05 and ***P* < 0.01. **(B)** U2OS cells were transiently depleted of c-Rel and IKK β prior to treatment with UV (40 J/m²) for 48 h. Cells were fixed and analysed by FACS. Graph depicts the mean plus s.d. of the smaller than G1-DNA content of a minimum of three independent experiments. ANOVA *t*-tests were performed on the means, and the *P*-values were calculated. **P* < 0.05 and ***P* < 0.01. **(C)** U2OS cells were transiently depleted of c-Rel, IKK β and Claspin for 48 h prior to lysis. WCLs were analysed for the levels of phosphorylated H2Ax by western blot. **(D)** U2OS were treated as in **(A)** but cells were fixed and stained for immunofluorescence using the indicated antibodies. Images were acquired using a DeltaVision microscope, deconvolved and processed using OMERO client (opensource software). **(E)** U2OS cells were transfected with control or constitutive active IKK β prior to synchronization with nocodazole for 12 h. Cells were treated with UV (40 J/m²) for 2 h prior to lysis. WCLs were analysed by western blot using the indicated antibodies.

Claspin levels correlate with c-Rel in a number of cancer cell lines

Our analysis demonstrates that IKK and c-Rel control the levels of Claspin in the cells by an alternative mechanism to

cell cycle. Given the importance of Claspin for genomic stability and checkpoint responses, we analysed if in cancer cells, there was a correlation between Claspin and c-Rel levels (Figure 8). For this purpose, we obtained cell lysates from 12

Figure 8 Claspin and c-Rel levels correlate in a variety of different cancer cell lines. WCLs of different cancer cell lines obtained from the NCI cell line collection were analysed for the levels of Claspin and c-Rel by immunoblot. Actin and ponceau staining were used to demonstrate equal loading.

different cell lines from the National Cancer Institute (NCI) collection. Our analysis revealed a good correlation between Claspin levels and c-Rel in the majority of the cell lines analysed with two exceptions, an ovarian cancer cell line IGR-OV1 and a breast cancer cell line HS-578T. However, in the rest of the cell lines tested, Claspin levels were high when c-Rel levels were also elevated.

Our results demonstrate for the first time that interference with IKK β and c-Rel has an impact on the DNA-damage checkpoints through modulation of the Claspin gene.

Discussion

The DNA-damage checkpoint is crucial for the maintenance of cellular integrity and proper function of the cell. Here, we demonstrate a novel function of IKK β as being necessary for the proper activation of this checkpoint, *in vivo*, through regulation of Claspin.

This regulation is dependent on the activity of the IKK complex, and is sensitive to IKK activity modulation with inhibition and activation controlling Claspin function (Figures 6 and 7). Claspin levels are sensitive to disruption of IKK β or IKK γ . RNAi depletion of IKK β , or the regulatory subunit IKK γ , but not IKK α , resulted in reduced levels of Claspin protein (Figures 1 and 2). Claspin levels were also reduced when a small molecule inhibitor of the IKK complex was used, indicating that IKK catalytic activity is required to regulate Claspin.

Previous studies have focused on the regulation of Claspin at the level of protein stability (Yoo *et al*, 2004; Peschiaroli *et al*, 2006). These studies have shown that Claspin protein levels fluctuate through the cell cycle and closely match the activity profile of Plk1 (Peschiaroli *et al*, 2006). Plk1 phosphorylates Claspin in its phospho-degron, which results in the recruitment of the SCF- β -TRCP complex. This targets

Claspin protein for degradation by the proteasome. Here, we have demonstrated that IKK regulation of Claspin is independent of the proteasome (Figures 1D and 5E; Supplementary Figure S8), but instead depletion of IKK β results in reduced levels of Claspin mRNA (Figure 2A). Analysis of the regulation of Claspin mRNA levels through the cell cycle also demonstrated that these do not change significantly in our cells (Figure 5). This revealed that Claspin regulation can be achieved by two independent mechanisms, one mediated by protein stability changes and cell-cycle dependent and one mediated by changes in the transcription of the gene itself, which is cell-cycle independent. Changes of Claspin gene expression have not been studied extensively before. However, one research group has suggested that both Chk1 and Claspin are E2F1 targets (Verlinden *et al*, 2007). These results were based on luciferase assays, but a direct regulation of the Claspin promoter by E2F1 using ChIP has not been demonstrated so far. Furthermore, we did not observe any changes in Claspin mRNA following E2F1 siRNA treatment or either of the synchronization approaches we have used, indicating that in our model systems Claspin mRNA is stable throughout the cell cycle (Figure 5; Supplementary Figure S12).

IKK is the upstream kinase responsible for the activation of the NF- κ B family of transcription factors and our data suggest that depletion of RelB, c-Rel or p52 results in reduction of Claspin both at the protein and RNA level (Figure 2). Taken together with the results obtained with the IKK depletion, this indicates that IKK is acting upstream of NF- κ B to control Claspin mRNA. Indeed, we could see the NF- κ B family member c-Rel bound to the Claspin promoter in an IKK β -dependent manner (Figures 3 and 4). We could not detect either RelB or p52 binding at the κ B sites we have identified in the Claspin promoter (Figure 3). This may be due to RelB/p52 binding at distal sites altering Claspin expression or

because the epitopes recognized by these antibodies are occluded in the context of this promoter. However, RelB/p52 may alter Claspin levels indirectly, by altering an unknown factor that controls Claspin. The exact mechanism by which c-Rel transactivates the Claspin gene is not known, but our results indicate that when c-Rel is lost from a region upstream of the transcription start site there is also a localized drop in the levels of histone H3 acetylation (Figure 3E). This suggests that c-Rel may recruit histone acetyl transferases (HATs) to the Claspin promoter. Although we have not investigated the nature of the HAT activity involved in transactivating the Claspin gene, it is interesting to note that c-Rel can act synergistically with p300 to activate target gene promoters in HEK293 cells (Sun *et al*, 2004). Furthermore, loss of c-Rel also correlated with reduced levels of RNA Polymerase II at the transcription start sites and coding region of the Claspin gene (Figure 3D), indicating an active function for c-Rel on this gene. Claspin protein has been reported to be elevated in cancer cells (Tsimaroutou *et al*, 2007; Verlinden *et al*, 2007) and an additional study has reported that Claspin mRNA is high breast cancer (Verlinden *et al*, 2007). Of note, c-Rel (Belguise and Sonenshein, 2007), RelB (Mineva *et al*, 2009) and p100/p52 (Cogswell *et al*, 2000) have all been reported to be abnormally active in breast cancer. Given our results, it would be interesting to determine if c-Rel, RelB and p100/p52 were responsible for the high levels of Claspin observed. Our own analysis revealed a good correlation between Claspin and c-Rel levels in a variety of different cancer cell lines (Figure 8).

Functionally, depleting IKK β results in reduced phosphorylation of Chk1 in response to UV and Hydroxyurea (Figure 6; Supplementary Figure S10). Importantly, c-Rel overexpression can rescue Claspin levels and the impairment in Chk1 phosphorylation when IKK β is depleted (Figure 6E), further confirming the importance of c-Rel in the control of Claspin. Significantly, expression of exogenous Claspin can functionally rescue the checkpoint defect in IKK β -depleted cells, demonstrating the function of Claspin in this pathway (Figure 6F). The consequences of the reduction in the checkpoint is evident with the differences between IKK β -depleted and control cells in response to long-term stresses. IKK β -depleted cells do not arrest efficiently at the G2/M boundary in response to UV, instead these cells undergo cell death (Figure 7A). Importantly, c-Rel reduction also sensitizes cells to UV-induced cell death (Figure 7B). Depletion of Claspin or Chk1 has been shown to result in similar phenotypes (Chini and Chen, 2003), with cells going through premature mitosis before efficient DNA replication resulting in mitotic catastrophe.

The implications of our results are numerous. They demonstrate that cells depleted of IKK β are more sensitive to UV damage. This makes IKK β an attractive anti-cancer agent in combination with DNA damaging agents inhibition of IKK would not only inhibit Chk1 activation but would also prevent other pro-survival genes from being activated by NF- κ B. Similarly, Chk1 inhibitors are currently being assessed for their properties as anti-cancer agents in phase I/II trials (Bucher and Britten, 2008). A reverse side to both IKK and Chk1 inhibition would be the increased genetic instability. Our own results indicate that inhibition of IKK and c-Rel leads to increased H2Ax foci, a marker of DNA damage (Figure 7C and D; Supplementary Figure S11A). However, as the major-

ity of the cells in the human body are quiescent, inhibition of Chk1 activity (either by IKK or Chk1 inhibition directly) might only affect cycling cells such as cancer cells.

Conversely, hyperactivation of IKK results in a failure to recover from the DNA-damage checkpoint, with Chk1 phosphorylation persisting in mitosis (Figure 7E). This is exactly how cells behave in the presence of a non-degradable form of Claspin (Mailand *et al*, 2006). These results suggest that increased activation of IKK might lead to cell-cycle delays because of failure to switch off the checkpoint.

The findings of this study point to the important function of IKK β and c-Rel in the DNA-damage response pathway. Instead of a single level of control over Claspin through the activity of Plk1, we have uncovered a second and parallel regulatory mechanism. As such, IKK β and c-Rel are required for proper checkpoint function and recovery and thus important for signalling following DNA-damage checkpoint activation. Furthermore, by controlling the IKK-NF- κ B axis and hence Claspin, the cell can integrate signals from various cellular pathways with the DNA-damage response, allowing for different levels of checkpoint activation and hence diverse cellular outcomes.

Materials and methods

Cells

U2OS osteosarcoma, MDA-MB-231 breast carcinoma, mouse embryonic fibroblasts and HEK 293 cell lines were grown in Dulbecco's modified eagle medium (Lonza) supplemented with 10% fetal bovine serum (Gibco), 50 units/ml penicillin (Lonza) and 50 μ g/ml streptomycin (Lonza) for no more than 30 passages. U2OS cells containing stably transfected IKK β or non-targeting shRNAs were created using the pSilencer vectors as described previously (Schumm *et al*, 2006). shRNA sequences can be found in Supplementary data. Cell lysates presented in Figure 8 were a kind gift from Dr G Sapkota and D Bruce (Dundee) and obtained from the NCI collection.

DNA constructs

Expression plasmid for c-Rel was a kind gift from Professor Neil Perkins (Newcastle, UK). Flag-IKK β was a kind gift from Professor Ron Hay (Dundee, UK). pcDNA3-IKK β SS-EE was obtained by site-directed mutagenesis and constructed by the College of Life Sciences University of Dundee cloning service. Claspin expression construct was a kind gift from Professor Paul Clarke (Dundee, UK). Exogenous c-Rel and IKK β expression plasmids were transfected into cells using GeneJuice (Invitrogen) as per the manufacturer's instructions.

Chemicals inhibitors

MG132 (Merck Biosciences) was dissolved in DMSO and used at the final concentration of 50 μ M for 3 h prior to harvest. Bay 11-7082 (Merck Biosciences) was dissolved in DMSO and used at the final concentration of 20 μ M. PMA (Sigma) and Ionomycin (Merck Biosciences) were dissolved in DMSO.

siRNA transfection

siRNA duplex oligonucleotides were synthesized by MWG and transfected using Interferin (Polyplus) per the manufacturer's instructions. siRNA sequences can be found in Supplementary data.

siRNA and DNA co-transfection

siRNA duplex oligonucleotides and DNA constructs were co-transfected using JetPrime (Polyplus) as per the manufacturer's instructions.

RT-PCR, Antibodies, ChIP, Microscopy and other methods can be found in Supplementary data.

Statistical analysis

ANOVA and Student's *t*-tests were performed on the means, and *P*-values were calculated. **P* ≤ 0.050, ***P* ≤ 0.010 and ****P* ≤ 0.001.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

The authors thank Professor Neil Perkins, Professor Julian Blow, Professor Robert White, Professor Paul Clarke, Professor Ron Hay

and Dr John Rouse for helpfully comments, suggestions and reagents. The authors are grateful to Adel Ibrahim from the cloning service for providing the constitutively active IKK β construct and Dr G Sapkota and D Bruce for providing the cancer cell line lysates. NK is funded by an AICR project grant, SR is funded by an RCUK fellowship and the University of Dundee and SM is funded by MRC NIRC.

Conflict of interest

The authors declare that they have no conflict of interest.

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