The stress-activated protein kinases p38α/β and JNK1/2 cooperate with Chk1 to inhibit mitotic entry upon DNA replication arrest

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ABSTRACT

Accurate DNA replication is crucial for the maintenance of genome integrity. To this aim, cells have evolved complex surveillance mechanisms to prevent mitotic entry in the presence of partially replicated DNA. ATR and Chk1 are key elements in the signal transduction pathways of DNA replication checkpoint; however, other kinases also make significant contributions. We show here that the stress kinases p38 and JNK are activated when DNA replication is blocked and that their activity allows S/M, but not G2/M, checkpoint maintenance when Chk1 is inhibited. Activation of both kinases by DNA replication inhibition is not mediated by the caffeine sensitive kinases ATR or ATM. Phosphorylation of MKK3/6 and MKK4, p38 and JNK upstream kinases, was also observed upon DNA replication inhibition. Using a genetic approach, we dissected the p38 pathway and showed that both p38α and p38β isoforms collaborate to inhibit mitotic entry. We further defined MKK3/6 and MK2/3 as the key upstream and downstream elements in the p38 signalling cascade after replication arrest. Accordingly, we found that the stress signalling pathways collaborate with Chk1 to keep Cyclin B1/Cdk1 complexes inactive when DNA replication is inhibited, thereby preventing cell cycle progression when DNA replication is stalled. Our results show a complex response to replication stress, where multiple pathways are activated and fulfill overlapping roles to prevent mitotic entry with unreplicated DNA.

Key words: Chk1, p38, JNK, Hydroxyurea, S/M checkpoint, SAPK

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; Cdc25, cell division cycle 25; Cdk, cyclin-dependent kinase; Chk, checkpoint kinase; Gadd45, growth arrest and DNA damage 45; GAP120, GTPase-activating protein 120; HSP27, heat shock protein 27; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MAP2K, MAPK kinase; MAP3K, MAPK kinase kinase; MAPKAPK, MAPK-activated protein kinase; MK, MAPK-activated protein kinase; MKK, MAPK kinase; Plk1, Polo-like kinase 1; SAPK, stress-activated protein kinase; TAO, thousand-and-one amino acid.

INTRODUCTION

Preventing mitotic entry before completion of DNA replication is critical for the maintenance of genome integrity. For this reason cell surveillance mechanisms have emerged to block the activation of mitosis-promoting factors when replication forks are present. The mechanisms that ensure cell cycle arrest after replication inhibition are part of a wider DNA replication checkpoint. This checkpoint monitors the presence of stalled or ongoing DNA replication forks and elicits signal transduction pathways that lead to the stabilization of arrested forks, the delay of late origin activation, the activation of DNA repair, and also the inhibition of mitotic entry.[1-3] The checkpoint response is essential not only after inhibition of DNA replication caused by the collision of the replication fork with damaged DNA, but also when the progression of the fork is slowed down because of secondary DNA structures or protein barriers, such as those found in natural pausing sites, fragile sites, repetitive sequences, and highly transcribed regions.[4] Checkpoint failure will cause the collapse of replication forks and premature chromosome condensation, thereby increasing chromosomal abnormalities.

In mammalian cells, the central players in this checkpoint are ATR and its downstream effector kinase Chk1. All members of the Cdc25 phosphatase family are phosphorylated by Chk1, in a process that leads to the degradation, inactivation or mislocalization of these phosphatases. Lack of Cdc25 activity prevents Cdk2 and Cdk1 activation, thus inhibiting S-phase progression (intra-S checkpoint response) and mitotic entry (S-M checkpoint response).[5-8] In addition, ATR and Chk1 promote the activation of DNA repair machinery, the stabilization of replication forks and the suppression of late origin activation and homologous recombination.[1,9-11] However, recent studies show that checkpoint response

needs to be locally inactivated in some conditions, since replication resumption relies on neighbour origin activation and homologous recombination mechanisms after DNA damage or long times of DNA synthesis inhibition.[12,13] Coordination of these apparently opposite responses is driven by a not well understood mechanism, although ATR-dependent activation of Plk1 seems to be essential for the local firing of neighbour origins close to stalled forks.[14]

The DNA damage checkpoint shares some common events with the DNA replication checkpoint. Two major signal transduction pathways triggered by DNA damage have been described, the ATM/Chk2 axis, activated after DNA double strand breaks, and the ATR/Chk1 axis, which is mainly induced after lesions that are processed into single strand stretches of DNA. Both pathways elicit p53 signalling and inactivate Cdc25 phosphatases, consequently arresting cell cycle.[15] In parallel to the ATR/Chk1 and ATM/Chk2 axes, the p38 stress-induced mitogen activated protein kinase (p38 MAPK) has been described as the third player in the DNA damage response, contributing to the inhibition of both G1/S and G2/M transitions after DNA damage.[16-20] A crucial element in the p38-dependent DNA damage response is the mitogen-activated protein kinase-activated protein kinase-2 (MK2). MK2 inhibits Cdc25 phosphatases by phosphorylating them on the same sites as those described for Chk1 and Chk2[17,21]and modulates the levels of some proteins critical for the maintenance of checkpoint response, such as Gadd45α, by promoting the stabilization of their mRNAs.[18] Although some studies are contradictory, JNK, another stress-activated protein kinase (SAPK), has been reported to regulate the Cdc25 family of phosphatases. Consequently, this kinase has been implicated in the control of mitotic entry in response to various genotoxic and non-genotoxic stress stimuli.[22-25] Interestingly, Chk1, MK2 and JNK also control the G2/M transition in a normal cell cycle.[26-29]

Using asynchronously growing fibroblasts, we previously showed that p38 MAPK promotes cell cycle arrest when DNA replication is blocked even in cells where Chk1 was inhibited or depleted.[30] Given the relevance of checkpoint functionality in the maintenance of genomic stability,[3] we aimed to better define the stress-induced MAPK signalling cascade responsible for cell cycle arrest upon replication block. Different studies showing a role of SAPKs upon various stimuli which induce DNA damage or replication checkpoint responses have been published. However, to our knowledge, a simultaneous and comprehensive analysis of JNK and p38 pathways in response to replication stress in a non-transformed cell line has not been done.

Our results show that after DNA synthesis inhibition, both p38 α/β and JNK are activated and required to achieve a complete ATR/Chk1–independent arrest in a subset of cells with unreplicated DNA. Accordingly, we demonstrate that stress signalling pathways keep Cyclin B1/Cdk1 complexes inactive in response to DNA replication block when Chk1 is inhibited. We have further defined the upstream and downstream elements needed for p38 and JNK function after DNA synthesis inhibition. Our findings lead to a model where MKK3/6-p38-MK2/MK3 and MKK4/JNK signalling pathways act in parallel and independently to ATR/Chk1 to establish and maintain cell cycle arrest when DNA replication is blocked.

RESULTS

p38 is specifically activated in arrested S-phase cells and maintains cell cycle arrest even when Chk1 is not functional.

We previously showed that Chk1 and p38 collaborate to inhibit entry into mitosis in the presence of HU. To better understand p38 activation under these circumstances, we first analyzed now whether its activation was dependent on DNA replication inhibition. To this end, NIH3T3 fibroblasts were synchronized and HU was added either in G1 or in S-phases. Phosphorylation of p38 was observed only when HU was added in S-phase cells, indicating that the effect was dependent on the presence of arrested DNA replication forks (Fig. 1A and Supplementary Fig. S1A). Interestingly, p38 was only activated at HU concentrations that were completely blocking DNA synthesis, while Chk1 phosphorylation was already observed when DNA replication was slowed down (Fig. 1B and Supplementary Fig. S1B). Other drugs known to inhibit DNA replication as aphidicolin (DNA polymerase α inhibitor), camptothecin (topoisomerase I inhibitor) and etoposide (topoisomerase II inhibitor) also induced p38 phosphorylation when added in S-phase (Fig 1C).

We took advantage of etoposide, which induces DNA damage both in S and G2 phases, to test whether p38 was specifically acting in S-phase. Etoposide was added to a population enriched in S-phase cells, together with Chk1 or p38 inhibitors. As observed in figure 1D, late S and G2 damaged cells relied entirely on Chk1 to arrest cell cycle, while cells with DNA content clearly below 4n remained arrested after Chk1 inhibition. Of note, the observation that a population of cells overrides the cell cycle arrest after UCN01 treatment indicates that the drug is in fact inhibiting Chk1 in our model, and as extensively proved.[31] Single inhibition of p38 after etoposide treatment did not induce mitotic entry under our experimental conditions. In contrast, simultaneous inactivation

of Chk1 and p38 after DNA damage led to the unscheduled mitotic entry of a new subset of cells with DNA content between 2n and 4n, S-phase cells. Similar results were obtained using asynchronously growing MEFs (Fig. 1E). These observations indicate that the activities of both Chk1 and p38 are essential to prevent mitotic entry of S-phase cells with arrested DNA replication. Conversely, p38 activity is dispensable for the cell cycle arrest induction in cells with fully replicated DNA.

Both p38 α and β collaborate to inhibit mitotic entry after DNA replication arrest.

We have previously reported that DNA replication block induces phosphorylation of p38 and its downstream kinase MK2 in p38α knockout (KO) MEFs, suggesting that another isoform, possibly p38β, is also activated by HU.[30] Using p38β KO MEFs, we confirmed here that p38α was phosphorylated after HU treatment (Fig. 2A). Furthermore, phosphorylation of MK2 after DNA replication inhibition was also observed in the p38β KO MEFs, thereby indicating that both isoforms can phosphorylate MK2 in response to HU (Fig. 2A). MK2 phosphorylation was abolished either after chemical inhibition of p38 α/β (SB203580) or in the double p38α and p38β KO MEFs (Fig 2A and Supplementary Fig. S2).

We next analyzed the ability of these cells to arrest cell cycle after a replication stress. We found that the presence of only one of the p38 isoforms (α or β) was sufficient to partially maintain cell cycle arrest after HU and UCN01 treatment, while cells deficient for both isoforms were not able to prevent mitotic entry after Chk1 inactivation (Fig. 2B and Supplementary Fig. S3). Moreover, no further significant mitotic entry was detected after p38 chemical inhibition in these conditions, proving that SB203580 dependent mitotic entry is due to its effect on p38α and p38β. Of note, under our experimental

conditions, UCN01 did not inhibit MK2 activity, measured by HSP27 phosphorylation (Supplementary Fig. S2)

Our findings demonstrate that both p38α and p38β are activated after DNA replication block and cooperate in maintaining cell cycle arrest of cells with non-replicated DNA.

p38 activation after DNA replication block is mediated by MKK3 and MKK6 but is ATR/ATM-independent.

Activation of p38 after DNA damage by doxorubicin and camptothecin is ATR/ATM-dependent, although the mechanism that links ATR/ATM and p38 remains unknown. In contrast, p38 activation induced by UV is not mediated by these kinases.[17,32,33] As is the case with UV, p38 phosphorylation after a replication block is not dependent on ATR/ATM, as it was not inhibited by caffeine addition, either when it was added at the same time or one hour before the HU treatment (Fig. 3A). To further explore the upstream elements needed for p38 activation upon DNA replication block, we analyzed the phosphorylation status of MKK3/6, the main MAPK kinases (MAP2K) reported to act directly upstream of p38. P-MKK3/6 was observed upon HU treatment (Fig 3B), indicating that at least one of them was activated after DNA replication block. We next assessed their role in p38 activation in MKK3 and MKK6 single and double KO MEFs.[34] MKK3/6 double KO MEFs did not activate p38 in response to HU. In contrast, phosphorylation of p38 was detected in these cells when they were exposed to a different stress agent such as anisomycin, confirming that after some stimuli other MAP2K can trigger p38 activation in the absence of MKK3/6 kinases[34] (Fig. 3C). This suggested that p38 phosphorylation upon DNA replication block is mediated by at least one of these kinases. In agreement with these data, MKK3/6 double KO cells were not able to inhibit mitotic entry after DNA synthesis arrest and Chk1 inactivation (Fig. 3D

and Supplementary Fig. S4). We next examined the contribution of each MAP2K to p38 phosphorylation upon DNA replication block. As shown in figure 3C, p38 activation was detected in both MKK3 and MKK6 single depleted cells upon HU treatment. Accordingly, the Chk1-independent induction of cell cycle arrest was partially abrogated in the single KO MEFs (Fig. 3D and Supplementary Fig. S4). These observations demonstrate that both MKK3 and MKK6 participate in the DNA replication checkpoint, working together to activate p38 MAPK and consequently reinforcing the cell cycle arrest induced by DNA replication inhibition.

p38 acts through MK2 and MK3 in response to DNA replication block.

MK2 and MK3 are key p38 effectors in the stress response, with MK2 being the most studied so far.[35,36] It has been shown that in response to DNA damage MK2 is activated by p38, which in turn phosphorylates and inactivates several members of the Cdc25 family, consequently preventing activation of Cdk1 and mitotic entry.[17,21] To test whether the p38 function after DNA replication block was dependent on MK2 activity, we analyzed the checkpoint response in MK2 KO MEFs. The Chk1-independent checkpoint was partially abrogated in MK2 depleted cells, suggesting that MK2 was only partially responsible for the p38-dependent cell cycle arrest (Fig. 4 and Supplementary Fig. S5). As MK3 is able to phosphorylate the same substrates *in vitro* as MK2, and partial functional redundancy between both kinases has been frequently reported,[35-37] we wondered whether MK3 was acting together with MK2 after replication inhibition. To test the role of this kinase in the DNA replication checkpoint, the ability to arrest cell cycle after HU treatment was assayed in MK3 KO cells or double MK2 and MK3 KO MEFs. Cells lacking MK3 also had a partial Chk1-independent response to DNA synthesis arrest. Moreover, when MK2 and MK3 were

absent, a higher proportion of cells entered mitosis by the sole inhibition of Chk1. Only a minor increase in mitotic entry was observed upon additional p38 inhibition in these cells, indicating that the main role of p38 after DNA replication block is driven by its downstream kinases MK2 and MK3 (Fig. 4 and Supplementary Fig. S5). We reasoned that p38 acts through MK2 and MK3 to prevent mitotic entry in response to DNA replication arrest.

Inhibition of DNA replication leads to Cyclin B1 (CycB1) associated kinase inactivation, which correlates with the disappearance of the high-mobility, hypo-phosphorylated band of the Cdk1 associated to Cyclin B1[38] (Figs. 5A and 5B). In concordance with the low percentage of cells entering mitosis after Chk1 inhibition in WT cells (Figs. 2B, 3D and 4), single inactivation of Chk1 did not recover neither the appearance of the high-mobility band of Cdk1 nor the kinase activity of the CycB1/Cdk1 complexes. Electrophoretic mobility of Cdk1 and complete activity of CycB1-associated Cdk1 were only achieved after simultaneous addition of both Chk1 and p38 inhibitors (Fig. 5A), in correlation with the observed increase of mitotic cells (Figs. 2B, 3D and 4). Note that in figure 5A (lower panel), a strong Cdk1 kinase activity was detected after simultaneous Chk1 and p38 inactivation, although less CycB1/Cdk1 complex was immunoprecipitated. Confirming the role of MK2 and MK3 in the S-phase checkpoint, the sole inhibition of Chk1 after replication block in MK2/MK3 double depleted cells led to the appearance of the lower Cdk1 band, and consequently, to the recovery of CycB1/Cdk1 activity (Fig. 5B). Additional inactivation of p38 in these cells did not induce any change neither in Cdk1 electrophoretic mobility nor in its kinase activity. An intermediate scenario was found in single MK2 or MK3 KO MEFs, thereby indicating that not only MK2 but also MK3 regulates CycB1/Cdk1 activity (Fig.

5B). Consequently, we conclude that both, MK2 and MK3, can inhibit CycB1/Cdk1 activation even in the absence of Chk1 activity.

JNK is activated after DNA replication arrest.

We wondered whether other stress pathways were also involved in the DNA replication inhibition response. JNK kinase has been recently reported to phosphorylate and inhibit Cdc25 phosphatases, thereby restraining cell cycle progression and preventing mitotic entry under different stress conditions.[22-25]As shown in figure 6A, HU and aphidicolin treatments led to JNK1/2 phosphorylation. Activation of JNK1/2 after HU addition was confirmed by the detection of c-jun phosphorylation, one of its main substrates (Fig. 6B). As in the case of p38, JNK activation was only observed at HU concentrations that were completely blocking DNA synthesis (Fig. 6C and Supplementary Fig. S1B). We next examined whether DNA replication inhibition was also activating MKK4 and MKK7, the direct upstream JNK kinases. HU treatment triggered MKK4 but not MKK7 phosphorylation (Fig. 6D), suggesting that MKK4 is the MAP2K responsible for JNK activation after DNA replication block.

To further explore the upstream mechanisms involved in MKK4/JNK activation after DNA replication arrest, we assessed the role of ATR and ATM in the induction of JNK phosphorylation. As in case of p38, caffeine treatment did not prevent phosphorylation of JNK (Fig. 6E), indicating that the activation of this SAPK was also independent of ATR and ATM activity.

p38 and JNK stress kinases display similar activation kinetics upon DNA synthesis inhibition.

We sought to analyze the activation profiles of Chk1, p38 and JNK after DNA synthesis arrest. To this end, NIH3T3 were synchronized at S-phase and the phosphorylation of these kinases was examined at different times upon HU addition. As shown in figure 6F, the three kinases were rapidly phosphorylated after DNA replication block. Chk1 maximum activation was achieved one hour after HU treatment. Despite some overlap with Chk1 phosphorylation was detected, p38 and JNK kinase activation displayed slower activation kinetics, reaching their maximum peak 2 hours after the HU addition. Remarkably, both SAPKs had parallel activation profiles. Note that JNK levels remained unchanged during the course of the experiment, indicating that the detected JNK phosphorylation was not due to an increase in its total levels. These findings show that both p38 and JNK kinases are similarly and rapidly activated after DNA replication blockade.

JNK contributes to block mitotic entry after DNA replication arrest.

The similar activation kinetics of p38 and JNK kinases after DNA synthesis block prompted us to analyze the role of JNK in the establishment and maintenance of DNA replication checkpoint. To this end, we evaluated the ability of JNK1/2 depleted MEFs to induce cell cycle arrest upon replication inhibition. Cells lacking JNK1/2 were still able to arrest after HU treatment (Fig. 7A and Supplementary Fig. 6). The sole inhibition of p38 in these cells did not provoke any increase in mitotic entry (Supplementary Fig. 6), indicating that JNK was dispensable in the Chk1-mediated arrest. However, after Chk1 inactivation a percentage of JNK1/2 KO cells entered mitosis, while, confirming our previous results, WT cells were still arrested. Of note, p38 phosphorylation was observed in JNK1/2 KO cells under these conditions (Fig. 7B). Interestingly, simultaneous inhibition of Chk1 and p38 kinases in JNK1/2 KO cells

allowed checkpoint abrogation in a new subset of cells (Fig. 7A and Supplementary Fig. 6). These observations suggest that JNK1/2 participate in parallel to p38 in the Chk1-independent checkpoint response to prevent mitotic entry in the presence of unreplicated DNA.

We next examined the contribution of JNK1/2 to CycB1/Cdk1 inhibition after HU treatment. In contrast to WT cells, the appearance of the high-mobility Cdk1 band and a partial CycB1 associated Cdk1 activation were achieved in HU-treated JNK1/2 DKO MEFs when Chk1 was inhibited (Fig. 7C). Higher activity of Cdk1 co-immunoprecipitated with CycB1 was obtained when p38 was also inactivated, in agreement with the increment of mitotic cells observed in the same condition (Figs. 7A and 7C). Our data demonstrate that JNK1/2 collaborate with p38 and Chk1 to inhibit CycB1/Cdk1 activation after a DNA replication block.

DISCUSSION

Coordination of DNA replication and mitotic entry is essential to ensure genome integrity. It is therefore not surprising to find multiple and redundant pathways that provide a robust mechanism to inhibit progression into M-phase when DNA replication is blocked (S/M checkpoint). We showed here that, when DNA replication is inhibited, the stress kinases p38 and JNK are activated in addition to Chk1, triggering parallel responses that ensure inhibition of mitotic entry even in the absence of Chk1 activity.

The p38 MAPK cascade responds to a wide range of stimuli and drives various cellular outcomes, that will depend on the cell type, the duration and strength of the signal, the crosstalk with other signalling pathways, the different p38 isoforms activated, and the downstream effectors modulated by them.[39] Using genetically modified MEFs, we dissected the essential elements of the p38 MAPK signalling pathway that are involved in the DNA replication checkpoint. After DNA replication block, both p38α and p38β isoforms are activated and participate in promoting cell cycle arrest. Moreover, our results show that p38α/β function is mainly driven by their downstream kinases MK2 and MK3, as double MK2 and MK3 depleted cells do not show an increase in checkpoint abrogation after inhibition of p38 by SB203580.

Recently, in addition to p38 MAPKs, JNK kinases have also been involved in cell cycle arrest induced by either genotoxic or non-genotoxic insults.[22-25] In agreement with these reports, we showed that JNK1/2 depletion leads to an impaired cell cycle arrest in response to replication block when Chk1 is inactivated.

It has been reported that p38α and MK2 are activated and become crucial in the DNA damage checkpoint response of p53-deficient cells.[18,32] In that model, both Chk1 and p38α/MK2 pathways are essential to maintain the G2 arrest, as the sole inhibition of either of them allows unscheduled mitotic entry of cells with unrepaired DNA. Here we

proved that not only p38α and MK2 but also JNK, p38β and MK3 are part of the DNA replication checkpoint response. Single Chk1, p38 or JNK activities are sufficient for the induction and maintenance of the cell cycle arrest after a replication stress in p53-proficient, non-transformed cells. However, after simultaneous inhibition of Chk1 and p38 or JNK kinases some S-phase arrested cells are allowed to escape from cell cycle arrest and enter into mitosis with unreplicated DNA. Moreover, we showed that the response to DNA damage in S-phase synchronized cells mimics the one induced by a replication block, while the DNA damage response of G2 cells strongly relies on Chk1, suggesting the existence of different checkpoint mechanisms to arrest cell cycle in each cell phase.

Correlating with their role in the induction of cell cycle arrest, our results show that JNK and p38α/β-MK2/MK3, collaborate to maintain CycB1/Cdk1 inactive when DNA replication is inhibited in the absence of Chk1 activity. Coordinated dephosphorylation of Cdk1 inhibitory sites by Cdc25 phosphatase family and nuclear translocation of the complex are essential for the correct mitotic entry.[40,41]It is well known that Chk1, MK2 and JNK phosphorylate Cdc25 phosphatases, thereby inducing their cytoplasmic localization, degradation or inactivation, depending on the specific phosphorylation site or the phosphatase isoform targeted.[8,22] We hypothesize that the inactivation of CycB1/Cdk1 complex by MK2, MK3 and JNK1/2 kinases after a DNA replication block occurs through inhibition of Cdc25 activity, although a different mechanism to prevent CycB1/Cdk1 activation cannot be excluded from our data.

The signalling linking the different DNA lesions to SAPK activation is not well understood. Previous reports have shown that p38 activation in response to γ irradiation, camptothecin, cisplatin and doxorubicin is ATM or ATR-dependent, while its activation in response to UV occurs in the absence of these kinases.[20,32] A large family of kinases

with MAPK kinase kinase (MAP3K) activity has been described, and some of them have been reported to simultaneously activate MKK3/6 and MKK4/7, p38 and JNK MAP2Ks respectively.[42] A recent work shows, using a FRET (Förster resonance energy transfer) biosensor to detect MAP3K activity, that activation of these kinases occurs in different cellular compartments depending on the stimulus, MAP3Ks are activated in the cytoplasm by anisomycin and in the nucleus after etoposide treatment.[43] We hypothesize that arrested DNA replication forks may activate a nuclear MAP3K upstream of p38 and JNK. One of the MAP3K that has been described as an important activator after DNA damage is the TAO2 kinase.[33] In that work, the authors demonstrate that ATM is needed to induce TAO2 activation, which in turn activates MKK3/6 kinases after different DNA damage agents or long exposure to HU. Our data indicate that both p38 and JNK activation after replication block are caffeine insensitive, suggesting that a different mechanism of those described is driving the signal from short term stalled forks to stress activated effectors.

We further characterized the MAP2Ks involved in p38 and JNK activation in the S-phase checkpoint. It has been recently shown that MAP2Ks differentially activate each p38 isoform depending on the type of stimulus.[39] Here we show that after DNA replication block both MKK3 and MKK6 are involved in p38α and β activation, since in their absence p38 is not phosphorylated after DNA synthesis inhibition and the p38 replication checkpoint response is not functional. The MAP2Ks reported for JNK activation are MKK4 and MKK7.[42] We propose here that MKK4 is the specific upstream kinase responsible for JNK activation in the DNA replication checkpoint, since only MKK4 but not MKK7 phosphorylation is detected after HU treatment. In contrast to what was reported for UV,[34] after DNA replication block MKK4 cannot compensate the MKK3/6 role in p38 activation, as p38 phosphorylation is not detected

after HU treatment in double depleted MKK3/6 cells. Our data suggest that, after replication block, cells can trigger activation of either MKK3/6-p38-MK2/3 or MKK4-JNK pathways, which collaborate with Chk1 to restrain cell cycle progression.

One possibility is that the role of SAPKs upon DNA replication block is completely redundant with Chk1, therefore allowing a more robust checkpoint response. However, we have found some differences between Chk1 and SAPKs activation. JNK and p38 activation kinetics differ from Chk1 activation profile after HU treatment. The stress-kinases phosphorylation is induced later and it is more sustained than Chk1 phosphorylation. Moreover, p38 and JNK are only activated when DNA replication is completely blocked, while Chk1 phosphorylation is already observed when DNA replication is slowed down. Therefore, p38 and JNK signalling pathways may control additional mechanisms in the replication checkpoint response when cells are exposed to more challenging conditions.

Single cell analysis will help to elucidate whether there are cells that activate one or more SAPKs at the same time. In fact, Chk1 inhibition in JNK-depleted cells abrogates the HU induced S/M checkpoint response in a subset of cells, while another group of cells needs additional inhibition of p38 in order to enter into mitosis with unreplicated DNA. These results could indicate that, whereas all cells activate Chk1 after a replication block, activation of each one of the SAPKs is diverse among the cell population. Their specific activation in a single cell may depend on the S-phase point reached at the moment of DNA replication arrest, the number of stalled replication forks or how long a cell has been exhibiting them. Alternatively, SAPKs could be activated by all cells with stalled DNA replication forks, but different levels of checkpoint kinases activity would be needed to keep CycB1/Cdk1 complexes inactive within the

population, making some cells more sensitive to cell cycle arrest abrogation after partial checkpoint inhibition.

Despite Chk1, p38 and JNK activation are readily detected upon DNA replication block, we noticed that only a subset of S-phase arrested cells abrogates checkpoint and enter mitosis after their simultaneous inactivation. This suggests that additional mechanisms, such as transcriptional repression of mitotic inducers[30] are playing important roles in the checkpoint response. Nevertheless, there is a reproducible, significant increase in mitotic entry of cells with unreplicated DNA when Chk1 and SAPKs are simultaneously inhibited. These mitotic cells with unreplicated or damaged DNA are thought to undergo mitotic catastrophe, a heterogeneous mechanism which has been proposed to be an important oncosupressive process, due to its function in sensing mitotic failures and eventually eliminating aberrant cells. Defects in the execution of this mechanism can ultimately lead to the survival of some damaged, aberrant cells harbouring aneuploidies. Components involved in this process, such as spindle checkpoint or G1 DNA damage response proteins, are commonly mutated in pre-cancerous and cancerous cells.[44] Therefore, we propose that, under replication stress, partial defects in ATR/Chk1 and SAPK signalling pathways could additionally favour genomic instability by increasing the frequence of aberrant mitotic cells.

MATERIALS AND METHODS

Cell culture

Immortalized mouse embryo fibroblasts (MEFs) wild-type (WT), MK2-/-, MK3-/-, MK2/3-/-, MKK3-/-, MKK6-/-, MKK3/6-/- and JNK1/2-/- were established from embryonic day 13.5 mice with the corresponding genotypes as previously described.[34,45-47] MEFs p38α-/-, p38β-/- and double knockouts p38α-/- and p38β-/- were generated from E13.5 mice embryos of the corresponding genotypes by 3T3 spontaneous immortalization protocol. MEFs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS). NIH3T3 cells were grown in DMEM supplemented with 10% donor bovine serum (DBS), and made quiescent by serum starvation.

Drugs

Drugs and their working concentrations were used as follows: 1.5 mM hydroxyurea (HU), 50 μM etoposide, 5μg/ml aphidicolin, 10 μg/ml anisomycin, 10μM bromodeoxyuridine (BrdU), 1μM camptothecin, 5 mM caffeine and 300 nM for UCN01 (all from Sigma, St Louis, MO, USA); 10 μM SB203580 and SB218078 (both from Calbiochem, Merck KGaA, Darmstadt, Germany).

Gel electrophoresis, immunoblotting, immunoprecipitation and Cdk1 kinase assay.

Cell extracts were obtained and run in Laemmli SDS-polyacrylamide gels, and transferred to Nitrocellulose membranes as previously described.[48] Incubation with antibodies was

conducted overnight at 4ºC. Antibodies used: p38 (sc-728; 1:100), Cdk1 (p34(17)sc-54; 1:200), Cyclin B1 (GNS1, sc-245; 1:100), GAP120 (sc-63; 1:100) and Cdk4 (H-303 sc-749; 1:500) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Cyclin B1 (#4138, 1:1000), P-p38 T180/Y182 (#9211; 1:1000), MAPKAPK2 (#3042; 1:1000), P-MAPKAPK2 T334 (#3041; 1:1000), JNK (#9252; 1:1000), P-JNK T183/Y185 (#9211; 1:1000), P-HSP27 S82 (#2401; 1:1000), P-Chk1 S345 (#2341, 1:500), P-MKK3/6 (#9236; 1:1000), P-SEK1/MKK4(Ser257) (#4514; 1:1000), P-MKK7 (Ser271/Thr275) (#4171; 1:1000), SEK1/MKK4 (#9152; 1:1000) and MKK7 (#4172; 1:1000) (all from Cell Signaling; Beverly, MA, USA); P-c-Jun (BS4045; 1:1000) (Bioworld, St. Louis Park, MN, USA). Cyclin B1 was immunoprecipitated with a mouse monoclonal antibody (#05-373, from Millipore). Immunoprecipitation and Cdk1 kinase assays were performed as described in Rodriguez-Bravo et al.[30]

Flow cytometry

Combined analysis of DNA content and phospho-Histone H3 (Ser10) was performed to quantify the percentage of mitotic cells. Cells were trypsinized, washed in ice-cold PBS and fixed with 70% ethanol overnight at -20ºC. Cells were then permeabilized with PBS containing 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) for 10 min at 4ºC, blocked with 2% FCS in PBS, and incubated with anti-phospho-H3 for 45 min at room temperature (Millipore, #06-570; 1:400). After washing, cells were incubated with secondary Alexa488-conjugated antibody (1:500) for 30 min at room temperature, washed, and resuspended in PBS containing 20 μg/ml propidium iodide (Sigma, St. Louis, MO, USA) dissolved in PBS with 0.2 mg/ml RNAse A (Sigma, St. Louis, MO, USA) and 0.1 %(v/v) Triton X-100 prior to analysis by FACS (FACSCalibur).

Double analysis of DNA content and BrdU was performed to determine the progression through the cell cycle of the S-phase cells. Cells were trypsinized, washed in ice-cold PBS and fixed with 70% ethanol overnight at -20ºC. Afterwards, cells were washed and treated with 2N HCl dissolved in PBS containing 0.1 %(v/v) Triton X-100 for 15 min at room temperature. A $Na_2B_4O_7 \cdot 10H_2O$ buffer with a pH of 8.5 was used twice to neutralize the HCl solution. After that, cells were washed and blocked with 3%BSA (Sigma, St. Louis, MO, USA) in PBS- containing 0.05%(v/v) Tween (Sigma, St. Louis, MO, USA) prior the incubation with anti-BrdU (Abcam, ab6326; 1/250) for 1 hour at room temperature. After washing, cells were incubated with secondary Alexa488-conjugated antibody (1:500) for 45 min at room temperature, washed, and resuspended in PBS containing 10 μg/ml propidium iodide dissolved in PBS with 0.1 mg/ml RNAse A, prior to analysis by FACS (FACSCalibur).

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LEGENDS TO FIGURES

Fig. 1. The stress activated protein kinase p38 is activated in response to DNA replication block and prevents mitotic entry of cells with unreplicated DNA. A) Synchronized NIH3T3 were treated with HU during G1 (0h after activation from quiescence) or S-phase (14h after activation from quiescence). p38 and p38 phosphorylation (P-p38) were analyzed by western blot after a 6h treatment. B) S-phase synchronized NIH3T3 cells (14 h after activation from quiescence) were treated with indicated HU concentrations during 2h. p38, p38 phosphorylation (P-p38) and Chk1 S345 phosphorylation (P-Chk1) were analyzed by western blot. GAP120 was used as loading control. C) S-phase NIH3T3 synchronized cells (14 h after activation from quiescence) were treated with aphidicolin (Aph), camptothecin (CPT) or etoposide (Etop) during the indicated time. Cell extracts were analyzed by western blot using antibodies to detect phosphorylated p38 (P-p38) and p38. D) S-phase synchronized NIH3T3 cells were treated with etoposide (Etop) or with etoposide and UCN01 (UCN) or SB203580 (SB) for 3h. DNA content and P-H3 positive cells were analyzed by FACS. E) Asynchronously growing MEFs were treated with etoposide (Etop) for 8h and during the last 3h UCN01 (UCN01) or SB203580 (SB) were added. DNA content and P-H3 positive cells were analyzed by FACS. D) and E) Representative experiments are shown. Total percentages of P-H3 positive cells are shown.

Fig. 2. Both p38α and β cooperate to inhibit mitotic entry in DNA replication checkpoint.

Asynchronously growing MEFs were non-treated (C) or treated with HU for 10h and during the last 5h UCN01 (UCN) or SB203580 (SB) were added. A) Cell extracts were

analyzed by western blot to detect phospho-p38 (P-p38), phospho-MK2 (P-MK2), MK2 and GAP120 (loading control). B) Graphs show the percentage of P-H3 positive cells after each treatment analyzed by FACS. Mean±SEM of at least 3 independent experiments are shown. WT: wild type; p38α KO: p38α knockout; p38β KO: p38β knockout; p38α/β DKO: p38α and β double knockout.

Fig. 3. Both MKK3 and MKK6 collaborate to activate the p38-dependent checkpoint response to HU treatment. A) Asynchronously growing MEFs were left untreated (C), or treated with HU for 5h (HU), caffeine alone for 6h (Caff Pre), or HU and caffeine together for 5h (HU + Caff). Caffeine was also added 1h before the HU treatment (HU + Caff Pre). Cell extracts were analyzed by western blot using antibodies to detect phospho-p38 (P-p38), phospho-Chk1 S345 (P-Chk1) or GAP120 (used as a loading control). B) Asynchronously growing MEFs were treated with HU for the indicated time, 1h with anisomycin (An) or left untreated (C). Cell extracts were analyzed by western blot to detect phospho-MKK3/6 (P-MKK3/6), MKK6 and GAP120 (loading control). C) Asynchronously growing MEFs were left untreated (C), or were treated with anisomycin for 1h (An), or with HU for the indicated times. Cell extracts were analyzed by western blot to detect phospho-p38 (P-p38) or p38. D) Asynchronously growing MEFs were non treated (C) or treated with HU for 10h. UCN01 (UCN) or SB203580 (SB) were added when indicated during the last 5h of the HU treatment. Graphs show the percentage of P-H3 positive cells after each treatment analyzed by FACS. Mean±SEM of at least 3 independent experiments are shown. WT: wild type; MKK3 KO: MKK3 knockout; MKK6 KO: MKK6 knockout; MKK3/6 DKO: MKK3/6 double knockout.

Fig. 4. Both MK2 and MK3 are key p38-downstream elements in the DNA replication checkpoint. Asynchronously growing MEFs were non treated (C) or treated with HU for 10h and during the last 5h SB218078 (2180) or SB203580 (SB) were added. Graphs show the percentage of P-H3 positive cells after each treatment analyzed by FACS. Mean±SEM of at least 3 independent experiments are shown. WT: wild type; MK2 KO: MK2 knockout; MK3 KO: MK3 knockout; MK2/3 DKO: MK2/3 double knockout.

Fig. 5. Inhibition of CycB1 associated kinase activity after replication block is Chk1, p38 and MK2/3-dependent. A) Asynchronously growing MEFs were treated with HU for 10h and during the last 5h SB218078 or SB203580 were added. Cells were lysed and Cyclin B1 immunoprecipitated. Upper panel: Immunoprecipitated Cyclin B1 and associated Cdk1 were analyzed by western blot. Lower panel: after Cyclin B1 immunoprecipitation, Cdk1 activity in each condition was evaluated by performing an H1-kinase assay. In parallel, Cdk1 levels of the same immunoprecipitates were analyzed. (B) MK2 knockout, MK3 knockout and MK2/MK3 double knockout MEFs were treated as in (A). Immunoprecipitated CycB1 and Cdk1-associated to CycB1 were detected by western blot (left panels). In parallel, CycB1-associated Cdk1 activity was assayed (right panels). MK2 KO: MK2 knockout; MK3 KO: MK3 knockout; MK2/3 DKO: MK2/3 double knockout.

Fig 6. **JNK stress kinase is activated after DNA replication block.**

A) Asynchronously growing MEFs were treated with HU or Aphidicolin (Aph) for 10h or with anisomycin (An) for 1h. Cells were harvested and JNK phosphorylation (P-JNK) was analyzed by western blot. B) Asynchronously growing MEFs were treated

with anisomycin (An) for 1h or with HU for 10h and during the last 5h UCN01 (UCN) or SB203580 (SB) were added. Cell extracts were analyzed by western blot using antibodies to detect phosphorylated c-jun (P-c-jun). C) S-phase synchronized NIH3T3 cells (14 h after activation from quiescence) were treated with indicated HU concentrations during 2h. Cell extracts were analyzed by western blot using antibodies to detect phosphorylated JNK (P-JNK). D) Asynchronously growing MEFs were treated with HU for the indicated time or 1h with anisomycin (An). Cell extracts were analyzed by western blot to detect phospho-MKK4 (P-MKK4), phospho-MKK7 (P-MKK7), total MKK4 and total MKK7. E) Asynchronously growing MEFs were treated with HU for 5h, with caffeine for 6h, or with HU plus Caffeine (1h of caffeine pre-incubation prior to HU addition and 5h together with HU). Cell extracts were analyzed by western blot to detect P-JNK. F) S-phase synchronized NIH3T3 were treated with HU for the indicated times. Chk1, p38 and JNK phosphorylation, and total JNK1/2 were determined by western blot using the specific antibodies (P-Chk1, P-p38, P-JNK1/2, and JNK1/2). C: control, non–treated cells. GAP120 was used as loading control.

Fig. 7. JNK collaborates to inhibit entry into mitosis and CycB1-associated kinase activity after HU treatment.

A) Asynchronously growing wild type (WT) and JNK1/2 double knockout (JNK1/2 DKO) MEFs were treated with HU for 10h and during the last 5h UCN01 (UCN) or SB203580 (SB) were added. Graph shows the percentage of P-H3 positive cells (mean ± SEM) after each treatment analyzed by FACS. B) Asynchronously growing JNK1/2 double knockout (JNK KO) MEFs were treated with anisomycin for 1h (An) or with HU for 10h and during the last 5h UCN01 (UCN) or SB203580 (SB) were added. Cell extracts were analyzed by western blot to detect phospho-JNK (P-JNK), phospho-p38

(P-p38), JNK and p38. C) Asynchronously growing wild type (WT) or JNK1/2 double knockout (JNK1/2 DKO) MEFs were treated with HU for 10h, during the last 5h UCN01 (UCN01) or SB203580 (SB) were added. Cells were lysed and Cyclin B1 immunoprecipitated. Immunoprecipitated CycB1 and associated Cdk1 were analyzed by western blot (left panels). In parallel, an H1-kinase assay was performed in CycB1 immunoprecipitated extracts (right panels).

Fig. 1

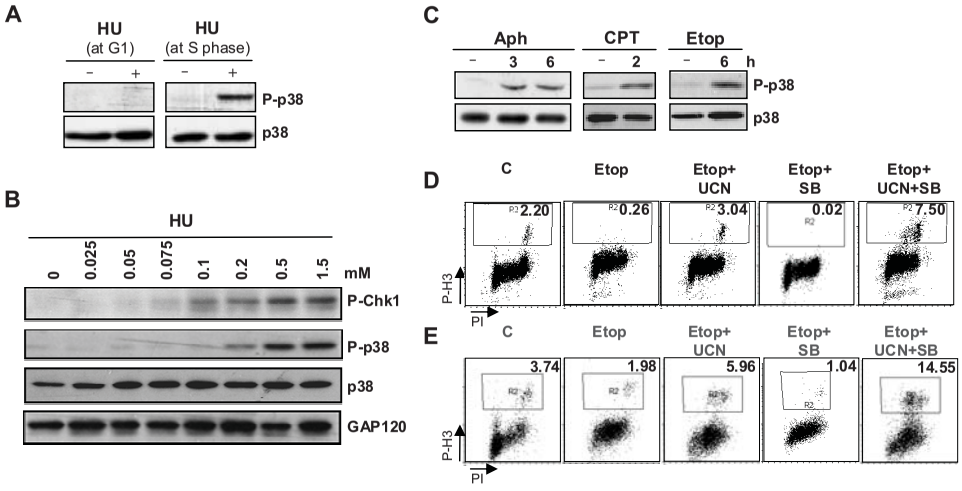
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Fig. 2

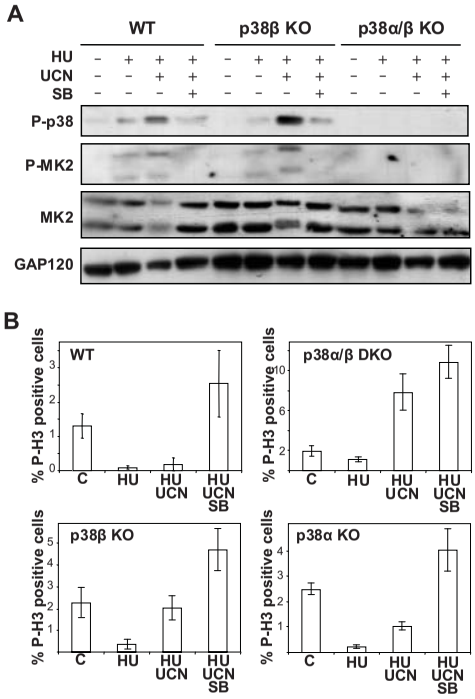
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Fig. 3

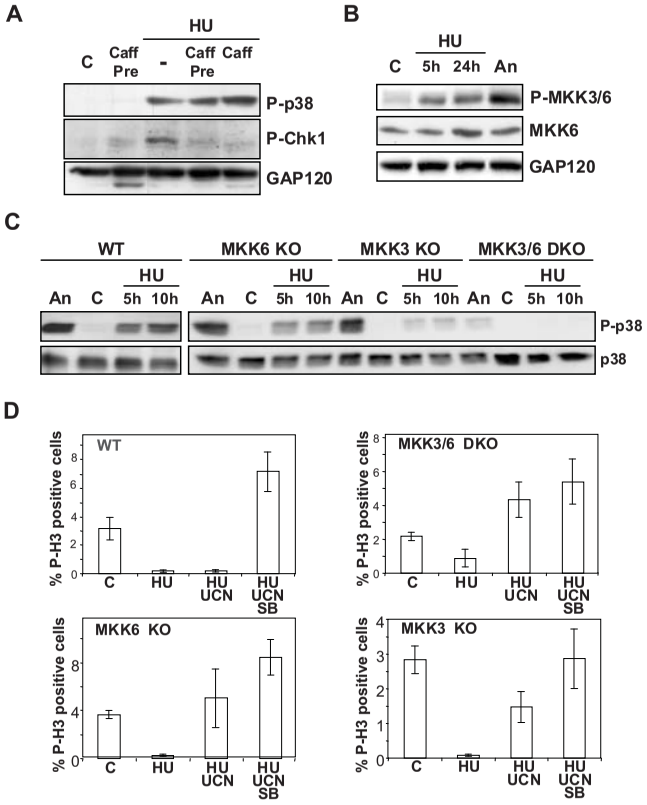
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Fig. 4

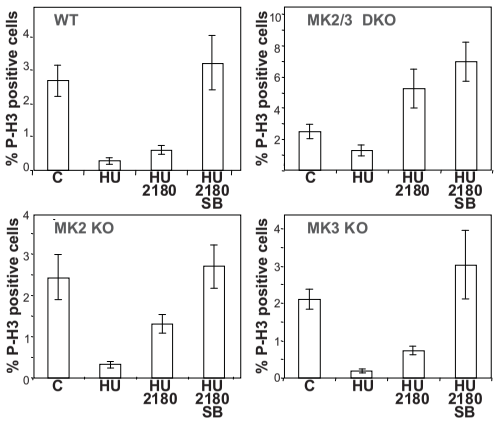
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Fig. 5

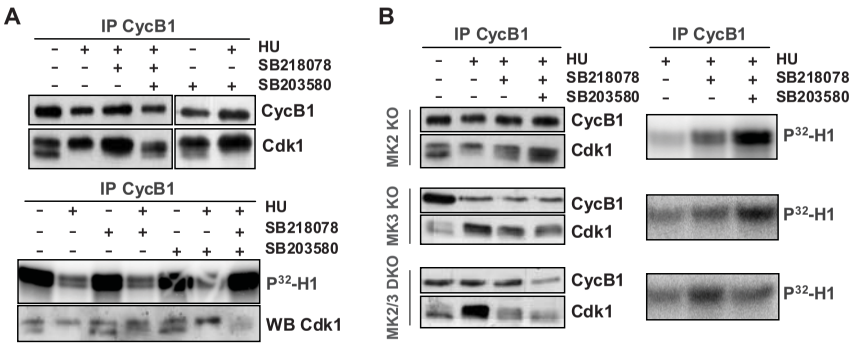
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Fig. 6

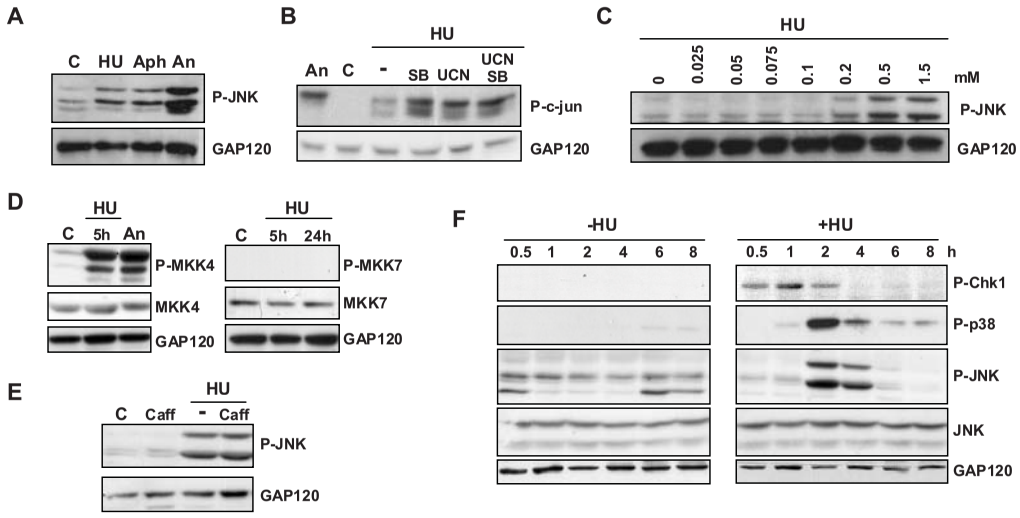
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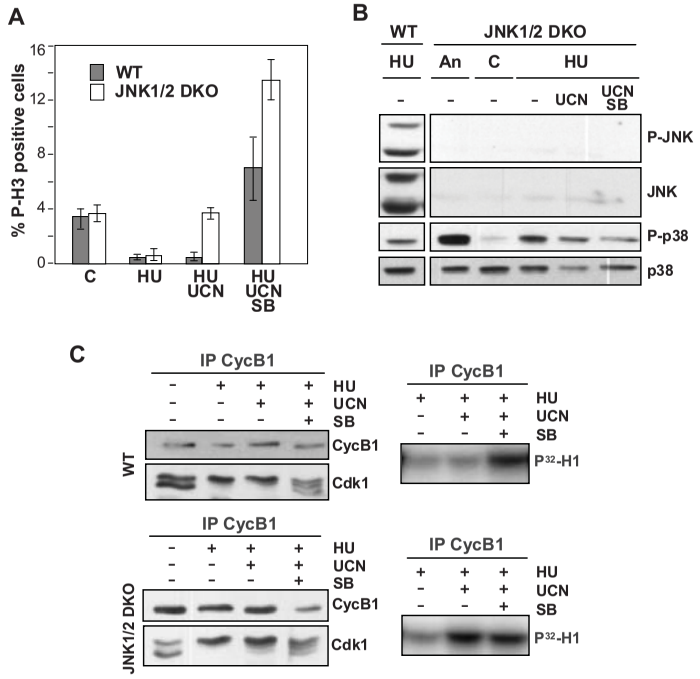
Fig. 7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