

1 **The p38 MAPK promotes cell survival in response to DNA damage but**  
2 **is not required for G<sub>2</sub> DNA damage checkpoint in human cancer cells**

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23 Running Title: p38 promotes cell survival after DNA damage

24 Word Count: Materials and methods: 862, Introduction, Results & Discussion: 4714

25 **Abstract**

26 The p38 MAPK is rapidly activated by stresses and is believed to play an important role  
27 in stress response. While Chk1 is known to mediate the G<sub>2</sub> DNA damage checkpoint  
28 control, p38 is also reported to have an essential function in this checkpoint control. Here,  
29 we have investigated further the roles of p38 and Chk1 in G<sub>2</sub> DNA damage checkpoint in  
30 cancer cells. We find that although p38 activation is strongly induced by DNA damage,  
31 its activity is not required for the G<sub>2</sub> DNA damage checkpoint. By contrast, Chk1 kinase  
32 is responsible for the execution of G<sub>2</sub> DNA damage checkpoint control in p53-deficient  
33 cells. Inhibition of p38 activity has no effect on Chk1 activation and  $\gamma$ H2AX expression.  
34 Global gene expression profiling of cancer cells in response to TNF $\alpha$  revealed that p38  
35 plays a strong pro-survival role through coordinated down-regulation of pro-apoptotic  
36 genes and up-regulation of pro-survival genes. We show that inhibition of p38 activity  
37 during G<sub>2</sub> DNA damage checkpoint arrest triggers apoptosis in a p53-independent  
38 manner with a concurrent decrease in the level of Bcl2 family proteins. Our results  
39 suggest that although p38 MAPK is not required for the G<sub>2</sub> DNA damage checkpoint  
40 function, it plays an important pro-survival role during G<sub>2</sub> DNA damage checkpoint  
41 response through up-regulation of the Bcl2 family proteins.

42

## 43 **Introduction**

44

45 The p38 MAPK was originally identified as a 38 kD protein that undergoes rapid tyrosine  
46 phosphorylation in response to stress (17). Significant progress has been made in the past  
47 decade to understand the p38 signal transduction pathway and the biological processes  
48 regulated by p38 MAPK. The p38 MAPK is activated in response to stress related  
49 stimuli such as UV light(28), heat (40), osmotic shock (34,40), endotoxins (18) and  
50 inflammatory cytokines like TNF- $\alpha$  and IL-1 (27,35). The p38 pathway is implicated in  
51 inflammatory response as p38 activation induces pro-inflammatory cytokines and  
52 enzymes such as Cox-2 which controls connective tissue remodeling, and inflammation  
53 related adhesion proteins such as VCAM-1 (37), thus making p38 MAPK signaling an  
54 attractive therapeutic target for the mitigation of inflammatory diseases (42). This has led  
55 to the creation of biochemical inhibitors targeting p38 kinase (7,20). The latest  
56 generation of these inhibitors is highly potent and selective; raising possibilities that  
57 therapy involving p38 inhibitors may one day be an effective treatment for inflammatory  
58 diseases.

59

60 Recently p38 MAPK activity has been reported to be critical for the G<sub>2</sub> DNA damage  
61 checkpoint control in response to DNA damage by UV irradiation (5,6,32) or by  
62 genotoxic agents (19,26). The primary mechanism of p38 involvement in G<sub>2</sub> DNA  
63 damage checkpoint is thought to be mediated through inhibition of CDC25B/C  
64 phosphatases which are required for the activation of CDK1 to initiate mitosis (5,32).  
65 Structural analysis of the p38 binding site, however, suggests that it is unlikely that p38  
66 could interact directly with CDC25B. Instead, its direct downstream target MAPKAPK2

67 (MK2) is implicated as the mediator of p38-dependent G<sub>2</sub> DNA damage checkpoint  
68 control (32).

69

70 The ability of cancer cells to establish cell cycle arrest in response to genotoxic agents is  
71 one of the reasons for resistance to chemotherapy (8). Cancer cells that undergo a  
72 reversible cell cycle arrest in response to genotoxic agents such as adriamycin  
73 (Doxorubicin HCL) and cisplatin have the ability to survive chemotherapy and continue  
74 proliferation post therapy, leading to poor patient outcomes. The implication that p38  
75 activity is necessary for G<sub>2</sub> DNA damage checkpoint arrest provides an exciting  
76 possibility for p38 inhibitor as chemo-sensitizer to enhance efficacy of chemotherapies  
77 by abrogating the G<sub>2</sub> DNA damage checkpoint to promote cancer cells to enter mitosis  
78 prematurely.

79

80 Both p38 and Chk1 are activated by DNA damage in mammalian cells and both are  
81 believed to directly inactivate CDC25 family of protein phosphatases to prevent mitotic  
82 entry in the presence of DNA damage (25,45,46). Paradoxically, inhibition of either p38  
83 or Chk1 has been shown to be sufficient to abrogate the G<sub>2</sub> DNA damage checkpoint  
84 (5,21,45). The role of the p38 MAPK pathway in the G<sub>2</sub> DNA damage checkpoint of  
85 cancer cells has recently been called into question by the observation that transformed  
86 cells do not delay entry into mitosis upon activation of the p38 stress pathway by  
87 anisomycin (33). Furthermore, it has been shown recently that RNAi-mediated inhibition  
88 of Chk1, but not Chk2 or MK2, in HeLa and H1299 cancer cells abrogates DNA-damage  
89 induced S-phase or G<sub>2</sub>-phase arrest (46). The requirement for p38 in the G<sub>2</sub> DNA damage  
90 checkpoint control may be cell type specific, or may depend on the type of DNA damage.  
91 While p38 is activated by both ionizing and UV radiation, the p38/MK2 pathway is

92 reported to be only essential in G<sub>2</sub> DNA damage checkpoint in the absence of p53 (39). It  
93 should be noted that older generation of small molecule inhibitors of p38 kinase was used  
94 at very high concentrations in many earlier studies, raising the possibility of off-target  
95 effects (1,10). In this study, we revisited the role of p38 activity in the G<sub>2</sub> DNA damage  
96 checkpoint control in response to several types of DNA damage, and investigated the  
97 relationship between Chk1 and p38 kinases in G<sub>2</sub> DNA damage checkpoint control in  
98 tumor cells with or without functional p53. We also used newer generation of small  
99 molecule kinase inhibitors which are more potent and selective at physiologically  
100 relevant concentrations and independently confirmed and corroborated the small  
101 molecule kinase inhibitor activity with siRNA-mediated inhibition.

102

103 We demonstrate that while p38 is rapidly and strongly induced by DNA damage,  
104 inhibition of p38 activity with a potent and selective inhibitor LY479754 or siRNA  
105 knock-down does not compromise the ability of cancer cells to mount an effective,  
106 checkpoint-mediated G<sub>2</sub> arrest in response to adriamycin, MMS or UV-induced DNA  
107 damage. In contrast, the chemical inhibition and siRNA knock-down of Chk1 efficiently  
108 abolishes the G<sub>2</sub> DNA damage checkpoint in cancer cells with deficient p53. Using an  
109 unbiased whole genome transcriptional analysis, we identified a strong link between p38  
110 activation and the suppression of anti-apoptotic signaling in TNF- $\alpha$ -treated cells.

111 Extending these findings to the G<sub>2</sub> DNA damage checkpoint context, we show that  
112 inhibition of p38 results in a dramatic increase in apoptosis of cells arrested in G<sub>2</sub> in  
113 response to DNA damage. Based on our observations, we propose that although not  
114 required for the G<sub>2</sub> DNA damage checkpoint control, p38 plays an important  
115 cytoprotective role through the regulation of apoptotic and survival pathways to allow  
116 cells to recover from DNA damage.

117 **Results**

118

119 **p38 MAPK is activated by DNA damage at different stages of the cell cycle**

120

121 The p38 MAPK is known to be activated in response to DNA damage. We first assessed  
122 if p38 activation is associated with G<sub>2</sub> arrest induced by different modes of DNA damage.  
123 For these experiments, we used different sources of DNA damage that induce a G<sub>2</sub> arrest  
124 in p53-deficient HeLa cells (Fig. 1A). In conjunction with the establishment of G<sub>2</sub> cell  
125 cycle arrest, p38 is strongly activated by increasing doses of UV-B irradiation (Fig. 1B),  
126 0.01% MMS (Fig. 1C) and 160nM adriamycin (data not shown, also see Fig. 1D) with  
127 similar kinetics. To further confirm that activation of p38 is closely correlated with G<sub>2</sub>  
128 arrest, we synchronized HeLa cells at G<sub>1</sub>/S using the double thymidine block/release  
129 protocol before imposing DNA damage by addition of adriamycin and followed the cell  
130 cycle progression by monitoring multiple parameters. Indeed, adriamycin treatment  
131 caused G<sub>2</sub> arrest and a sustained activation of p38 (Fig. 1D).

132

133 To investigate if p38 activation occurs specifically during G<sub>2</sub> DNA damage checkpoint-  
134 mediated arrest, HeLa cells were synchronized in G<sub>1</sub> by serum-starvation (Fig. 1E), in  
135 early S phase by double thymidine block (Fig. 1F) or in G<sub>2</sub> by CDK1 inhibitor (RO-3306)  
136 (Fig. 1G) and then released into fresh growth medium containing 0.01% MMS. Cells  
137 were subsequently monitored for the activation status of Chk1, p38, and MAPKAPK-2  
138 (MK2) using the respective phosphorylation-specific antibodies. As shown in Fig. 1E-G,  
139 p38 and Chk1 are rapidly activated after MMS treatment in HeLa cells synchronized at  
140 different stages of the cell cycle. Activation of p38 occurred earlier than that of Chk1 in

141 G<sub>1</sub> and S-phase cells, whereas p38 and Chk1 activation in G<sub>2</sub>-phase cells followed a  
142 similar kinetics (Fig. 1E-G).

143

#### 144 **Inhibition of p38 does not abrogate G<sub>2</sub> DNA damage checkpoint control**

145 To test whether p38 pathway activity is essential for the G<sub>2</sub> DNA damage checkpoint in  
146 response to DNA damage, we investigated the effect of chemical inhibition of the p38  
147 pathway activity with LY479754 (p38i), a highly potent and selective p38 inhibitor (7,11),  
148 on G<sub>2</sub> DNA damage checkpoint-mediated arrest in both unsynchronized (Fig. 2A) and  
149 synchronized (Fig. 2B) HeLa cells treated with adriamycin. Nocodazole, a microtubule  
150 depolymerizing agent, was added to the medium to trap in mitosis cells that escape the  
151 checkpoint arrest in unsynchronized cells. Despite a strong inhibition of p38 activity,  
152 seen as complete inhibition of p38-mediated phosphorylation of MK2, HeLa cells were  
153 still able to mount an effective G<sub>2</sub> DNA damage checkpoint control in response to  
154 adriamycin treatment. Inhibition of p38 did not lead to any significant increase in the  
155 mitotic marker phospho-histone H3 over a 24 hour period. Similarly, another small  
156 molecule kinase inhibitor, SB203580, at concentrations above that needed for completion  
157 inhibition of p38, also had no effect on the G<sub>2</sub> DNA damage checkpoint, as HeLa cells  
158 remained arrested in G<sub>2</sub> during a synchronized G<sub>2</sub>/M progression (data not shown).  
159 Inhibition of MK2 also showed no effect on checkpoint activity (Fig. 2B). In contrast,  
160 inhibition of Chk1 with a selective Chk1 inhibitor (Fig. 2A&B) or ATM/ATR inhibition  
161 with caffeine (Fig. 2B) in an identical experimental setting led to a dramatic increase in  
162 phospho-histone H3 levels, indicating effective abrogation of G<sub>2</sub> DNA damage  
163 checkpoint. Consistent with checkpoint abrogation, inhibition of Chk1 or ATM/ATR led  
164 to a marked decrease in Cdk1 phosphorylation on Tyr15 (Fig. 2B). On the other hand,  
165 inhibition of p38 had no effect on the level of Cdk1 phosphorylation at Tyr15 which

166 remained high (Fig. 2B). Furthermore, abrogation of the G<sub>2</sub> DNA damage checkpoint  
167 with either a Chk1 inhibitor or caffeine occurred in the presence of high p38 and MK2  
168 activities (Fig. 2B).

169

170 These analyses were followed by confocal immunofluorescence microscopy in HeLa  
171 cells. Cells treated with either adriamycin alone or adriamycin+p38i for 21 hours had  
172 high levels of  $\gamma$ H2AX in the nucleus. These cells were arrested at G<sub>2</sub> as indicated by  
173 cytoplasmic accumulation of cyclinB1 and 4N DNA content (data not shown; also see  
174 Fig. 1A). No mitosis was observed in the p38-inhibitor treated cells under the microscope.  
175 In contrast, HeLa cells that were treated with adriamycin+Chk1 inhibitor underwent  
176 mitosis as evidenced by mitotic spindles, condensed DNA and strong phospho-histone  
177 H3 signal, indicating effective abrogation of G<sub>2</sub> DNA damage checkpoint (data not  
178 shown). Western blot analysis further showed that inhibition of p38 MAPK has no  
179 apparent impact on  $\gamma$ H2AX expression and activation of Chk1 (Fig. 2C). This shows that  
180 despite potent inhibition of p38 MAPK pathway, DNA damage response to adriamycin  
181 and MMS is unimpeded leading to a strong G<sub>2</sub> DNA damage checkpoint-mediated cell  
182 cycle arrest.

183

184 Previous reports first implicating p38 as a critical kinase in G<sub>2</sub> DNA damage checkpoint  
185 function utilized UV irradiation as a source of DNA damage (5). Since p38 activity does  
186 not appear to be necessary for adriamycin or MMS induced G<sub>2</sub> DNA damage checkpoint  
187 arrest, we thus wanted to investigate further a role of p38 activity in response to UV-  
188 induced DNA damage. Both synchronous and asynchronous HeLa cell cultures were  
189 exposed to UV radiation and incubated with either p38 or Chk1 inhibitors immediately  
190 after UV treatment. Nocodazole was added to the cultures to trap in mitosis cells that had



191 escaped from G<sub>2</sub> DNA damage checkpoint-mediated arrest. Cells were harvested for  
192 analysis of various mitotic markers after 24 h. Again, while pharmacological inhibition  
193 of p38 and MK2 did not lead to any significant increase in mitotic index over 24 h,  
194 inhibition of Chk1 led to a dramatic increase in mitotic index and phospho-histone H3  
195 over the same time period (Fig. 2D&E). These results suggest that as in the case of  
196 adriamycin treatment, UV damage-induced G<sub>2</sub> arrest is not dependent on p38 activity.

197  
198 To rule out the possibility of off-target effects by chemical inhibitors used in the  
199 experiments, we performed a series of siRNA knock-down experiments targeting p38 $\alpha$ ,  
200 MK2 and Chk1 in HeLa cells with 2 specific siRNA oligos for each gene. Both siRNA  
201 oligos effectively inhibited their target gene expression as determined by Western blot  
202 analysis (Fig. 3A). Cells were transfected with appropriate siRNA, transferred to fresh  
203 growth medium after 48 hrs and were then treated with adriamycin for additional 24 h.  
204 Consistent with the data obtained using the small molecule kinase inhibitors, knock-down  
205 of Chk1 using siRNA also abrogated the G<sub>2</sub> DNA damage checkpoint in the presence of  
206 high p38 activity, as evidenced by a decrease in the CDK1 Tyr15 phosphorylation and an  
207 increase in the histone H3 phosphorylation and mitotic index (Fig. 3A&B). Similarly,  
208 siRNA-mediated inhibition of Chk1 also abrogated UV induced G<sub>2</sub> DNA damage  
209 checkpoint arrest (Fig. 3B). In contrast, knock-down of p38 $\alpha$  or MK2 did not affect the  
210 G<sub>2</sub> DNA damage checkpoint arrest induced by adriamycin or UV treatment (Fig. 3A&B).

211  
212 Lastly, to show that the lack of any effect by p38 inhibition on the G<sub>2</sub> DNA damage  
213 checkpoint-induced arrest was not a phenomenon specific to HeLa cells, we conducted  
214 similar experiments using A549 (lung Cancer), U2OS (osteosarcoma) and Calu-6 (lung)  
215 cell-lines. Similar to the results obtained with HeLa cell, inhibition of p38 also had no

216 impact on the ability of these cancer cell lines to mount a strong G<sub>2</sub> DNA damage  
217 checkpoint-imposed cell cycle arrest in response to adriamycin treatment (Fig. 3C).  
218 Again, inhibition of Chk1 was able to abrogate the adriamycin induced G<sub>2</sub> arrest in the  
219 p53 deficient Calu-6 cells, but not in p53-proficient A549 and U2OS cells as reported  
220 previously (22,29). In addition, we attempted to reproduce the effect of UV-C irradiation  
221 in U2OS cells exactly as previously reported (32). We found that two independent siRNA  
222 oligonucleotide targeting MK2, of which one was the same siRNA oligos previously  
223 published (36), effectively inhibited MK2 expression (Fig. 3D). Contrary to the previous  
224 report (36), however, inhibition of MK2 by RNAi had no effect on histone-H3  
225 phosphorylation in response to 20J/M<sup>2</sup> UV-C irradiation as monitored by Western  
226 blotting or flow cytometry after 18 hours in a nocodazole mitotic trap assay (Fig. 3D&E).  
227 Consistent with our siRNA results for HeLa cells, these results indicate that MK2  
228 inhibition does not abrogate the G<sub>2</sub> DNA damage checkpoint function. In addition,  
229 RNAi-mediated inhibition of MK2 also had no effect on  $\gamma$ H2AX expression and  
230 activation of p38 MAPK in response to UV-C treatment (Fig. 3D). We also noticed that a  
231 significant fraction of U2OS cells lost viability when exposed to 20J/M<sup>2</sup> UV-C. Taken  
232 together, these results demonstrate that although the p38 pathway is induced robustly in  
233 response to DNA damages, its activity is not required for the execution or maintenance of  
234 G<sub>2</sub> DNA damage checkpoint control.

235

#### 236 **Non-genotoxic activation of p38 does not inhibit mitotic entry**

237 If p38 activity is indeed important for execution of G<sub>2</sub> DNA damage checkpoint, then  
238 DNA-damage independent activation of p38 would be expected to impede progression  
239 into mitosis by untimely engagement of the G<sub>2</sub> DNA damage checkpoint. Therefore, we  
240 investigated the effect of non-genotoxic activation of p38 by anisomycin, a potent

241 antimicrobial agent, on the onset of mitosis. Short term exposure to anisomycin at  
242 2 $\mu$ g/ml is not known to cause DNA damage, but strongly induces the p38 signaling  
243 pathway at our hands (11). HeLa cells were first synchronized at the G<sub>2</sub> boundary with a  
244 CDK1 inhibitor (43) and then released in the presence or absence of anisomycin. Cell  
245 cycle progression from G<sub>2</sub> was then followed up to 6 h after release from CDK1 inhibitor  
246 block. As expected, p38 activation was strongly induced by anisomycin but high p38  
247 activity had no impact on the ability of synchronized HeLa cells to enter mitosis rapidly  
248 (Fig. 4).

249

#### 250 **p38 MAPK plays a pivotal role in immediate early stress response and cell survival**

251 To uncover a new role for p38 activity in DNA damage response outside the context of  
252 G<sub>2</sub> DNA damage checkpoint, we returned to the original context of p38 activation in  
253 stress response. We first demonstrated that the p38i effectively inhibited TNF $\alpha$  induced  
254 activation of p38 signaling (data not shown). We then profiled effects of p38 inhibition on  
255 global gene expression in cancer cells induced by TNF- $\alpha$ . Calu-6 lung cancer cells were  
256 treated with TNF- $\alpha$  and p38 inhibitor (LY479754) across a time-course. Samples were  
257 run on Affymetrix HG-U133plus2 gene chips to enable an unbiased assessment of  
258 transcriptional changes in response to TNF- $\alpha$  and p38 inhibition across time.

259

260 A total of 853 transcripts showed significant expression changes between TNF- $\alpha$  treated  
261 cells and DMSO controls in at least one of the 5 time points analyzed (data not shown).

262 To understand the primary effects of TNF- $\alpha$  on gene expression, we focused on  
263 transcription changes at 1 h time point after TNF $\alpha$  treatment and identified a total of 115  
264 transcripts corresponding to 72 unique genes which were differentially expressed. Based

265 on their expression patterns across the 5 time points revealed by hierarchical clustering,  
266 they fall into 4 distinct groups (Fig. 5A). The first group includes 10 genes; among them  
267 9 are immediate early response genes encoding transcription factors (Supplementary  
268 Table 1). Not surprisingly, this group of genes responded most rapidly and transiently to  
269 TNF- $\alpha$  treatment (Fig. 5A). The second group is the largest, with 31 genes consisting of  
270 cytokine, chemokine, growth factor genes and genes implicated in stress response  
271 (Supplementary Table 1). This group also responded to TNF- $\alpha$  rapidly, peaking from 1  
272 to 2 hours and then declined more slowly than the genes in the first group (Fig. 5A). The  
273 third group includes 22 genes that responded to TNF- $\alpha$  more slowly and in a lower  
274 magnitude than the first 2 groups (Fig. 5A). Most of the genes in this group have  
275 functions related to immune regulation (Supplementary Table 1). The fourth group of 9  
276 genes negatively responded to TNF- $\alpha$  treatment (Fig. 5A and supplementary Table 1).  
277 Taken together, these genes which were differentially regulated by TNF- $\alpha$  are mostly  
278 associated with stress and immune responses, consistent with the expected function of  
279 TNF- $\alpha$  signaling.

280

281 Treatment of Calu-6 cells with a selective p38 kinase inhibitor (p38i), LY479754, alone  
282 caused expression changes only in three genes across time compared to the DMSO  
283 controls, further demonstrating the extraordinary selectivity of this kinase inhibitor (data  
284 not shown). One of the genes that was down regulated is COX2, a known p38 target gene  
285 (15), while FADD, a pro-apoptotic component of the FAS receptor pathway, was up-  
286 regulated in the early time points (Fig. 5B). Among the 853 transcripts regulated by  
287 TNF $\alpha$ , the p38 kinase inhibitor completely blocked the expression changes of 260  
288 transcripts and also significantly inhibited changes in the expression levels of another 185

289 transcripts induced by TNF $\alpha$  (Supplementary Table 2). Together, 445 (52%) of TNF $\alpha$   
290 regulated genes responded to inactivation of p38, providing strong evidence for a  
291 significant role of p38 MAPK in TNF $\alpha$ -induced stress response. Furthermore,  
292 inactivation of p38 abolished > 70% of the expression changes induced by TNF $\alpha$  at the 1  
293 h time point. As shown in Fig. 5A, expression changes in cluster 1 and 2 genes that  
294 responded most rapidly to TNF $\alpha$  were also most strongly inhibited by the p38i, whereas,  
295 genes in cluster 3 that responded more slowly and in a lower magnitude to TNF $\alpha$  were  
296 much less affected by p38 inactivation. The data further demonstrate that p38 MAPK  
297 plays a pivotal role in early cellular responses to TNF $\alpha$ .

298  
299 Among many genes, networks and canonical pathways that were affected by p38  
300 inhibition of TNF- $\alpha$  treatment, we found a significant representation of genes modulating  
301 the anti-apoptosis and cell survival pathway (Fig. 5A; supplementary Table S2). In  
302 response to TNF- $\alpha$ , Calu6 cells immediately activated a potent cell survival response  
303 including up-regulation of pro-survival pathways such as BCL-xl, IL6, Myc and EGR  
304 (13,41,44), and also down-regulation of pro-apoptotic signaling components such as  
305 TRADD and FADD (Fig. 5B). Inhibition of p38 significantly reversed these pro-survival  
306 responses, resulting in a recovery of TRADD and FADD and a significant decrease in  
307 BCL-xl, IL6, EGR and Myc (Fig. 5B).

308  
309 Gene expression changes were confirmed at the protein level by Western blotting.  
310 Inhibition of p38 pathway with LY479754 indeed led to a significant decrease in the  
311 levels of BCL2 and BCL-xl and the reversal of decreased FADD expression in the  
312 TNF $\alpha$ -treated cells, in line with results from the gene expression study (Fig. 5C).

313 Concurrently, inhibition of p38 also led to an early induction of PARP cleavage, a  
314 cellular marker for apoptotic cell death. To further confirm and quantify apoptotic cell  
315 death, we determined the apoptosis index (% of cells expressing cleaved-PARP) of  
316 TNF $\alpha$ -treated cells in the presence of p38i. We found that p38i in combination with  
317 TNF $\alpha$  indeed led to increased apoptosis compared to TNF $\alpha$  alone as early as 3 hours  
318 after treatment (Fig. 5D). Together, these results strongly suggest that p38 signaling plays  
319 an important role in immediate early response and in induction of pro-survival/anti-  
320 apoptotic signaling in response to TNF- $\alpha$  stress.

321

322 **Inhibition of p38 in response to DNA damage leads to cell death via inhibition of**  
323 **BCL2 family proteins**

324 The discovery that p38 inhibition results in a strong dampening of anti-apoptotic gene  
325 expression in response to TNF- $\alpha$  led us to reason that p38 activity may play a role in  
326 modulating apoptotic induction in the context of DNA damage. If so, then inhibition of  
327 p38 should result in induction of apoptosis of cells treated with DNA damaging agents.  
328 To test this hypothesis, both synchronous and asynchronous HeLa (p53-deficient) and  
329 A549 (p53-proficient) cells were treated with adriamycin or MMS in the presence of the  
330 p38i, LY479754, for up to 48 h and assayed for the apoptotic markers, namely, cleavage  
331 of caspase 3 or 7 and PARP. A dose escalation experiment with the p38 inhibitor in  
332 combination with adriamycin showed a corresponding increase in cleaved-caspase-3  
333 levels measured as apoptotic index at 48 hours post treatment (Fig. 6A). Consistent with  
334 this, additional experiments with siRNA targeting p38 $\alpha$  and MK2 in HeLa cells also  
335 showed a marked increase in apoptotic markers in combination with adriamycin but not  
336 in cells treated with adriamycin alone or non-specific siRNA in the presence of

337 adriamycin (Fig. 6B). Inhibition of p38 with LY479754 also led to a dramatic increase in  
338 PARP cleavage in p53-positive A549 cells after DNA damage by adriamycin (Fig. 6C)  
339  
340 Since we observed a strong inhibition of BCL2 family gene expression upon p38  
341 inhibition in TNF- $\alpha$  treated cells, we wanted to test if inhibition of BCL2 family proteins  
342 may provide a mechanistic explanation for a role of p38 in regulation of apoptosis  
343 following DNA damage. We find that p38 inhibition in response to both adriamycin (Fig.  
344 6C) and MMS (Fig. 6D) damage leads to a dramatic decrease in BCL-x1 protein levels,  
345 matched with a concordant increase in the level of PARP cleavage. Finally, using multi-  
346 parametric cytometry, we also find that the inhibition of p38 induced apoptosis of cells  
347 that were largely arrested in the G<sub>2</sub> phase in the presence of DNA damage (Fig. 6E).  
348 Taken together, these observations suggest that p38 activity is an integral part of the pro-  
349 survival signaling network induced in response to DNA damage.  
350

351 **Discussion**

352

353 In this study, we show that p38 activation is strongly induced by DNA damage and is  
354 correlated with G<sub>2</sub> arrest. Contrary to prior reports (5,26,32). However, our data strongly  
355 suggest that p38 pathway activity is not necessary for the G<sub>2</sub> DNA damage checkpoint  
356 function. Furthermore, inhibition of Chk1 or ATM/ATR kinase abrogates the G<sub>2</sub> DNA  
357 damage checkpoint in the presence of high p38 activity. While HeLa cells were the  
358 primary cell model used in this study, we also show that the inhibition of p38 activity was  
359 unable to abrogate G<sub>2</sub> DNA damage checkpoint control in Calu-6, A549 and U2OS cell-  
360 lines. In concordance with previous reports (24,29), we find that pharmacological  
361 inhibition of Chk1 alone with a selective small molecule kinase inhibitor or siRNA  
362 knock-down was not sufficient to abrogate the G<sub>2</sub> DNA damage checkpoint in the p53  
363 proficient cells. Corroboration of pharmacological inhibition using small molecule kinase  
364 inhibitors with siRNA knock-down rules out the possibility that the observations may be  
365 caused by off-target activity of the chemical kinase inhibitors. Conversely, non-genotoxic  
366 activation of p38 by anisomycin in G<sub>2</sub> was not sufficient to activate the G<sub>2</sub> DNA damage  
367 checkpoint. Taken together, our results strongly suggest that neither the suppression of  
368 p38 activity nor its non-genotoxic activation has an impact on G<sub>2</sub> DNA damage  
369 checkpoint activity.

370

371 Inhibition of CDC25B/C phosphatase activity is believed to be the primary mechanism  
372 through which the p38 pathway participates in the G<sub>2</sub> DNA damage checkpoint control  
373 (5,32). This prevents the formation of active CDK1/cyclinB complex, thus blocking  
374 progression into mitosis. We find that effective inhibition of p38 activity had no  
375 discernable impact on the level of CDK1 Tyr15 phosphorylation in response to



376 adriamycin treatment. This lack of effect of p38 inhibition on CDK1 activation through  
377 Tyr15 dephosphorylation by CDC25 provides further biochemical evidence in support of  
378 the proposition that p38 does not play an important role in G<sub>2</sub> DNA damage checkpoint  
379 control. Alternatively, as Chk1 kinase is activated in a very similar manner in response  
380 to DNA damage, potential pathway redundancies may mitigate the effect of p38  
381 inhibition on CDC25B activity. In p53 deficient cells, however, we find that inactivation  
382 of Chk1 alone effectively abrogated G<sub>2</sub> DNA damage checkpoint. Furthermore, the  
383 abrogation of G<sub>2</sub> DNA damage checkpoint by Chk1 inactivation occurs in the presence of  
384 high p38 kinase pathway activities. Therefore, in agreement with many previous  
385 publications (4,9) our data suggest that the Chk1 signaling pathway is primarily  
386 responsible for the inactivation of CDK1 in response to DNA damage to prevent cells'  
387 progression into mitosis.

388

389 As we were interested in the exciting possibility of using potent and selective p38 kinase  
390 inhibitors as chemo-sensitizers to enhance anticancer efficacy of chemotherapies, the  
391 inability of a highly selective and potent p38 kinase inhibitor to abrogate G<sub>2</sub> DNA  
392 damage checkpoint comes as a surprise. A closer examination of earlier publications,  
393 however, reveals a certain degree of discrepancies concerning the role of p38 in G<sub>2</sub> DNA  
394 damage checkpoint control in response to different types of DNA damage and the  
395 function of p53 (5,32,39). In addition, earlier studies used older generation of p38 kinase  
396 inhibitors at very high concentrations (5,39). At such high concentrations, it is likely that  
397 these p38 kinase inhibitors may have off-target activities as shown recently (1,10). Our  
398 data are consistent with a more recent report that demonstrates that using the RNAi  
399 approach, only Chk1 but not Chk2 or MK2 is responsible for G<sub>2</sub> DNA damage  
400 checkpoint control in cancer cells (46). Furthermore, it is also recently shown that the p38

401 pathway response at the G<sub>2</sub> DNA damage checkpoint is strongly attenuated in  
402 transformed cells (33). Earlier studies that implicated p38 activity in G<sub>2</sub> DNA damage  
403 checkpoint control were performed in untransformed human cells and mouse embryonic  
404 fibroblasts (5,36,39). However, untransformed mammalian cells have intact p53 and  
405 Chk1 functions. Thus, it is unconceivable that normal, untransformed mammalian cells  
406 with functional p53 and Chk1 would depend on p38 alone for G<sub>2</sub> DNA damage  
407 checkpoint function but not cancer cells which are frequently deficient in p53 function.  
408 Indeed, similar to the findings in p53 proficient cancer cells, we find that inhibition of  
409 p38 activity by the small molecule inhibitor LY479754 was unable to abrogate the G<sub>2</sub>  
410 DNA damage checkpoint in HUVEC cells in response to adriamycin treatment (data not  
411 shown). Together, our results, therefore, rule out the feasibility of developing a p38  
412 inhibitor as a chemo-sensitizer to enhance efficacy of chemotherapies.

413

414 To identify a new role for p38 activity in DNA damage response outside cell cycle  
415 checkpoint control, we conducted a genome-wide gene expression profiling analysis of  
416 the effect of p38 inhibition in response to TNF- $\alpha$  stress. We find that inhibition of p38  
417 dramatically dampens the immediate early transcriptional response and the ability of  
418 cancer cells to mount an effective anti-apoptotic/pro-survival response to TNF- $\alpha$ .  
419 Moreover, the pro-survival signaling induced immediately after exposure to TNF- $\alpha$   
420 consisted of the down-regulation of pro-apoptotic factors such as FADD and TRADD  
421 and the up-regulation of anti-apoptosis components including anti-apoptosis BCL2  
422 family proteins.

423

424 Testing the hypothesis derived from the analysis of transcriptional data in the context of  
425 DNA damage, we find that inhibition of p38 in combination with adriamycin leads to

426 strong induction of apoptosis. Increased apoptosis was observed in both p53 deficient  
427 HeLa cells as well as in p53 proficient A549 cells, implying that the link between p38  
428 activity and pro-survival signaling does not depend on p53 status. Further mechanistic  
429 studies in the context of DNA damage show that p38 may confer its pro-survival effect in  
430 response to DNA damage through the regulation of anti-apoptotic BCL2 family proteins.  
431 Consistent with this notion, we find that chemical inhibition or siRNA knock-down of  
432 p38 in the presence of adriamycin or MMS treatment leads to a dramatic decrease in  
433 BCL2 and BCL-xl. The data suggests that p38 activity, while not connected directly with  
434 the proper functioning of the G<sub>2</sub> DNA damage checkpoint, plays a pivotal role in  
435 response to DNA damage.

436

437 We note that the link between p38 activity, pro-survival signaling in response to DNA  
438 damage and stress may be unexpected, given the strong association of p38 activation with  
439 FasL and TNF- $\alpha$  induced apoptosis (14). The behavior of DNA damaged cells in which  
440 checkpoint has been abrogated may be of some relevance. We have observed that Chk1  
441 inhibitor or caffeine mediated abrogation of G<sub>2</sub> DNA damage checkpoint occurs with  
442 high p38 activity. This implies that while inhibition of p38 in conjunction with DNA  
443 damage leads to increased apoptosis, high p38 activity alone does not prevent apoptosis.  
444 Thus, in the case of Chk1 inhibition-mediated mitotic catastrophe, other apoptosis  
445 inducing factors may override the cytoprotective effects of p38 activity. Although the  
446 underlying mechanistic rationale for this observation is unclear, these observations  
447 suggest that there may be a more complex, and context-specific relationship between p38  
448 and apoptosis induction. From a teleological perspective, it can be argued that in an early  
449 response to stress, p38 signaling promotes cell survival to facilitate the evaluation of the  
450 extent of damage to the cell. Once the G<sub>2</sub> DNA damage checkpoint is breached, p38

451 mediated pro-survival signaling is no longer required or sufficient, as elimination of cells  
452 undergoing mitotic catastrophe would be in the best interest of multicellular organisms.

453

454 Our assertion that p38 plays a role in cell survival is supported by a number of recent  
455 reports linking this signaling pathway to increased levels of BCL2 and BCL-xl in  
456 response to DNA damage and stress (12,23). Furthermore, chemical inhibition of p38 has  
457 been strongly associated with increased chemosensitivity in cancer cells (16,31). Based  
458 on our study and correlative evidence from other reports, we propose a new role for p38  
459 in the context of response to DNA damage (Figure 7). According to this scheme, while  
460 p38 is activated in response to DNA damage resulting in a G<sub>2</sub> DNA damage checkpoint-  
461 mediated cell cycle arrest, its activity is not required for the activation or maintenance of  
462 the G<sub>2</sub> DNA damage checkpoint. Instead p38 activity in response to DNA damage  
463 induces pro-survival signaling to prevent the onset of pre-mature apoptosis in the  
464 immediate aftermath of stress of DNA damage and allows recovery from DNA damage.  
465 This anti-apoptosis response likely allows cells to ascertain the extent of damage and to  
466 respond accordingly. It appears that the role of p38 in the regulation of apoptosis is  
467 context dependent and may switch from pro-survival to pro-apoptosis depending on both  
468 the timing and physiological context of the stress induction. Clearly, elucidation of the  
469 full mechanism of p38 in the regulation of apoptosis would require further investigations.

470

471

472

473 **Methods and Materials**

474 **Cell Culture and Synchronization**

475 All cancer cell lines were obtained from ATCC. HeLa cells were grown in DMEM media  
476 supplemented with 10% FBS, Calu-6 cells were grown in MEM media supplemented  
477 with 10% FBS, 1% sodium pyruvate and 1% HEPES, A549 cells were grown in  
478 RPMI1640 media, supplemented with 10% FBS and U2OS cells were grown in McCoy  
479 5A media supplemented with 10% FBS. All cell culture media and additives were  
480 purchased from GIBCO (Invitrogen, Carlsbad CA, USA). All cells were grown in a cell  
481 culture incubator at 37°C and 5% CO<sub>2</sub> in T75 or T150 tissue culture flasks (Corning,  
482 Lowell, MA, USA). Cells were synchronized at G1 using double thymidine block/release  
483 or at G2 using a selective CDK1 inhibitor as previously described (11).

484

485 **Antibodies, Western blot Analysis and Immuno-fluorescence Microscopy**

486 Rabbit polyclonal antibody to phospho-histone H3, Ser10 (Cat No. 06-570) was  
487 purchased from Upstate Inc. (Charlottesville, VA). Rabbit polyclonal anti-phospho-p38  
488 (Cat No. 9216), anti-phospho-MAPKAPK2 (MK2) (Thr334) (Cat No. 3041), anti-  
489 phospho-Chk1 (Ser 345) (Cat No. 2341), anti-phospho-HSP27(ser 17) (Cat No. 2404),  
490 anti-cl-Casp3 (Cat No.9661), anti-cl-Casp7, D198 (Cat No. 9491), anti- $\alpha/\beta$  Tubulin (Cat  
491 No.2148), anti-BCL2 (Cat No. 2872), anti-BCL-x1 (Cat No. 2762), anti- $\gamma$ -H2AX (Cat No.  
492 9718), anti-FADD (Cat No. 2782), anti-p38 $\alpha$  (Cat No. 9218), anti- $\beta$  actin (Cat No.  
493 4967), and mouse monoclonal anti-c-PARP, D214 (Cat No. 9546), were all purchased  
494 from Cell-Signaling Technologies Inc (Beverly, MA, USA). Anti-CyclinB1 (Cat No.  
495 610220) was purchased from BD Transduction Laboratories (San Jose, CA, USA). HRP  
496 conjugated secondary antibodies were purchased from Amersham (Piscataway, NJ, USA)

497 and Alexa-Fluor linked secondary antibodies were purchased from Invitrogen (Carlsbad,  
498 CA, USA).

499

500 Protein lysates of cultured cells were prepared in a lysate buffer containing a cocktail of  
501 phosphatase and protease inhibitors and Western blotting was performed as previously  
502 described (11). Luminescent substrate detection was performed using the ECL Advance  
503 or ECL plus chemi-luminescent kit (Amersham, Piscataway NJ, USA). Chemi-  
504 luminescent signal was detected using a high resolution GE Gel-blot imager.

505

506 Cells were plated for confocal microscopy in Lab-Tek 4 Chamber slides (Fisher  
507 Scientific). Cells were fixed with 4% para-formaldehyde in PBS and then permeabilized  
508 with 0.2% Triton-X 100. After blocking for 1 hour in 1% BSA in PBS, the cells were  
509 incubated with anti- $\gamma$ -H2AX (1:200), and anti-cyclin B1 (1:500) antibodies in block  
510 solution for one hour at room temperature. The cells were washed 3X in PBS and  
511 incubated with secondary antibody (1:1000) and DNA stain (Sytox Green, Invitrogen,  
512 1:20000) for one hour at room temperature. The cells were washed 3X with PBS and  
513 imaged. Cell imaging was acquired with a Zeiss LSM510 confocal microscope.

514

515

#### 516 **Use of Chemical Agents and Inhibitors**

517 The use of biochemical inhibitors and chemical genotoxic compounds in this study was  
518 performed as previously described (11,47). Chemical inhibitors used in this study were  
519 synthesized by Lilly chemists. Kinase inhibitors used in this study were: p38 $\alpha$ / $\beta$  inhibitor  
520 LY479754 (7,11), MAPKAPK2 (MK2) inhibitor(38) and Chk1 inhibitor PF-00477736  
521 (2). CDK1 inhibitor RO-3306 was purchased from Calbiochem (San Diego, CA, USA).

522 All other chemical reagents used in this study were purchased from Sigma Aldrich (St  
523 Louis, MI, USA).

524

#### 525 **siRNA Transfection**

526 Transfection of 21-nucleotide siRNA duplexes (Qiagen Sciences, Germantown  
527 MD, USA) for targeting endogenous genes was carried out using Lipofectamine  
528 RNAiMax (Invitrogen, Carlsbad CA, USA) as previously described in low serum (Opti-  
529 mem) medium (11). The following validated commercial siRNAs from Qiagen were used  
530 in this study: si-p38 $\alpha$ : (SI00300769, SI00605157), si-MK2: (SI02223697, SI00288246),  
531 si-Chk1: (SI0266000, SI00299859). In addition, a MK2-specific siRNA oligonucleotide  
532 described by Manke et al. (36) was synthesized by Dharmacon and used.

533

#### 534 **Acumen Explorer High Content Imaging Analysis**

535 HeLa cells were plated in 96 well Beckman Dickinson Biocoat plates at 2000 cells per  
536 well in 100  $\mu$ l medium, and incubated in 5% CO<sub>2</sub> at 37°C for 24 hours before treatment  
537 with compounds diluted in growth media with 10% FBS and 0.25% DMSO. All liquids  
538 were handled with an automated 96-channel pipette (Multimek 96, BECKMAN) to  
539 process the plates. Cells were fixed with the Prefer fixative (Anatech LTD, MI, USA) at  
540 25°C for 30 min, permeabilized with 0.1% Triton X100 in PBS (GIBCO, Carlsbad, CA)  
541 for 15 min, and then treated with RNase A (50 $\mu$ g/ml in PBS) (Sigma, St Louis, MO) at  
542 37°C for 60 min. Immuno-staining of cells and counter staining with propidium iodide  
543 (PI) for high throughput quantitative analysis by Acumen Explorer were similarly done as  
544 described (3).

545

546

547 **UV irradiation and FACS analysis**

548 UV irradiation was performed at 254 nm (UV-C) using a Stratalinker 2400 (Stratagene)  
549 on U2OS cells under the same conditions as described by Manke et al (36). U2OS cells  
550 were prepared for FACS analysis also as described by Manke et al (36).

551

552 In addition to experiments reproducing the Manke et al. UV damage data, additional UV  
553 experiments were performed at 290nm (UV-B) using a Bio-Link BLX (Vilber Lourmat)  
554 computerized UV cross linker. For all UV-B experiments, cells were treated with UV-B,  
555 as indicated in the figure legends, after removal of cell growth media, followed  
556 immediately by re-introduction of growth media with indicated chemical inhibitor  
557 treatments. Western Blot, FACS and Acumen high content imaging experiments were  
558 performed as previously described (3, 11, 47).

559

560 **Gene expression profiling analysis**

561 Microarray analysis was performed as previously described (30). Briefly, total RNA from  
562 Calu6 cells was isolated with RNA STAT-60 (Tel-Test) according to the manufacturer's  
563 protocol. 5 micrograms of total RNA were labeled and hybridized to Affymetrix  
564 U133plus2 arrays according to the Affymetrix protocol. All samples were assessed for  
565 RNA quality such as microarray scaling factors, background levels, percent present calls,  
566  $\beta$ -actin and GAPDH 3'/5' ratios, etc. Signal intensities as gene expression values were  
567 obtained from Microarray Suite version 5.0 (MAS5) using the default settings, except 2%  
568 trimmed mean was set to 1500. To apply statistical analysis, a two-sided t-test was used  
569 to identify genes differentially expressed between two groups. The p values of the t-tests  
570 were adjusted for multiple testing using the false discovery rate. The adjusted P values,  
571 or the false discovery rates (FDR), are designated as Q values, where  $Q = P*n/I$  (n = total



572 number of probe sets on the microarrays;  $i$  = sorted rank of P values). The fold change  
573 was calculated as the ratio of the two group means based on the observed signal values  
574 from MAS5 and the gene expression signal change was calculated as the difference of the  
575 two group means. The criteria to define differential gene expression are  $FDR < 0.05$ , fold  
576 change  $> 1.4$  and absolute change  $> 250$ . Differentially expressed genes were mapped to  
577 Gene Ontology (GO) biological process categories and KEGG pathways. The  
578 significance of GO terms or KEGG pathways over-represented in differentially expressed  
579 genes was tested using the hyper-geometric distribution function adjusted with family-  
580 wise error rates for multiple pairwise tests.  
581  
582

583 **Figure Legends**

584

585 **Figure 1:** DNA damage induces G<sub>2</sub> arrest and activates p38 MAPK and Chk1 pathways.

586 (A) Cell-cycle profiles of control HeLa cells or HeLa cells treated with 160nM

587 adriamycin or 500J/M<sup>2</sup> UV irradiation or 0.01% MMS for 24 hours; (B,C) Western blot

588 analysis of activation of p38 $\alpha$  and phosphorylation of its substrate MAPKAPK-2 (MK2)

589 in unsynchronized HeLa cells treated with increasing doses of UV irradiation with

590 10 $\mu$ g/ml anisomycin treatment as a positive control (B); with 0.01% MMS for various

591 time intervals for up to 10 hours (C); (D) synchronized HeLa cells treated with 160nM

592 adriamycin 5 hours after release from second thymidine block. Cell cycle progression and

593 p38 activation were analyzed by flow cytometry and Western blot at time points

594 indicated after release; (E-G) DNA Damage in synchronized cells: HeLa cells

595 synchronized at G<sub>1</sub> by serum starvation (E), at G<sub>1</sub>/S by double thymidine block (F) and at

596 G<sub>2</sub> with CDK1 inhibitor RO-3306 (G) and then were released into 0.01% MMS-

597 containing fresh medium. Activation of p38 and Chk1 kinases were followed by Western

598 blot at time intervals indicated.

599

600 **Figure 2:** Inhibition of Chk1 but not p38 abrogates G<sub>2</sub> DNA damage checkpoint in HeLa

601 cells. (A) Mitotic index of unsynchronized HeLa cells that were pre-treated for 20 hours

602 with adriamycin prior to the addition of 320nM p38 inhibitor (p38i), or 1.25 $\mu$ M Chk1

603 inhibitor (Chk1i) and 150nM nocodazole. Mitotic index were quantified using the high

604 content/high throughput Acumen Explorer for cells expressing phospho-histone H3 at

605 time points indicated after inhibitor treatment; (B) Western blot analysis of double

606 thymidine synchronized HeLa cells treated with various kinase inhibitors in the presence

607 of 160nM adriamycin and 150 nM nocodazole 20 hours after release from thymidine  
608 block. Lane 1: double thymidine treated cells; lane 2: untreated HeLa cells; Lanes 3-9:  
609 synchronized cells treated with adriamycin; lane 3: adriamycin alone; lane 4: plus 320nM  
610 p38 inhibitor (p38i); lane 5: plus 2 $\mu$ M MK2 inhibitor (MK2i); lane 6: plus 1.25 $\mu$ M Chk1  
611 inhibitor (Chk1i); lane 7: plus 6mM caffeine; lane 8: plus 1.25 $\mu$ M Chk1i+320nM p38i;  
612 lane 9: plus 6mM caffeine +320nM p38i; (C) Western blot analysis of the relationship  
613 between p38 activation/inactivation and DNA damage response. Thymidine synchronized  
614 HeLa cells were released into 0.01% MMS and cells were analyzed at times indicated; (D)  
615 Mitotic index of unsynchronized Hela cells treated with 500J/M<sup>2</sup> UV-B irradiation for 20  
616 hours prior to addition of 320nM p38 inhibitor (p38i) or 1.25 $\mu$ M Chk1 inhibitor (Chk1i)  
617 in the presence of 150nM nocodazole (Noc); (E) Western Blot of HeLa cells treated with  
618 1000J/M<sup>2</sup> UV-B and indicated doses of p38 inhibitor (p38i), MK2 inhibitor (MK2i) or  
619 Chk1 inhibitor (Chk1i) 24 hours after kinase inhibitor treatment.

620

621 **Figure 3:** Inhibition of Chk1 but not p38 by small molecule kinase inhibitors or RNAi  
622 abrogates UV induced G<sub>2</sub> DNA damage checkpoint. (A) Western blot analysis of  
623 response of cells after inactivation of Chk1, p38 or MK2 by 2 specific and validated  
624 siRNA oligonucleotides directed against each gene to adriamycin; (B) Mitotic Index plot  
625 of HeLa cells with p38 or Chk1 knockdown and treated with 160nM adriamycin or  
626 500J/M<sup>2</sup> UV for 24 hours; (C) p53-dependent abrogation of G<sub>2</sub> DNA damage checkpoint  
627 by Chk1 inhibition. A549, U2OS and Calu-6 cancer cells were treated with 160 nM  
628 adriamycin for 24 hours before addition of kinase inhibitors (320nM p38i or 1.25 $\mu$ M  
629 Chk1i). Mitotic index were determined at 3 and 24 hours after addition of kinase  
630 inhibitors; (D-E) MK2 is not required for G<sub>2</sub> DNA damage checkpoint control following

631 UV-C irradiation. U2OS cells treated with GFP or MK2 (MAPKAP kinase-2) siRNA  
 632 were irradiated with 20J/M<sup>2</sup> of UV-C as described previously (32), and then placed in  
 633 50ng/ml nocodazole-containing medium for additional 16 hours. Target protein  
 634 knockdown, DNA content and phospho-histone H3 of cells were analyzed by Western  
 635 blotting (D) and flow cytometry (E).

636

637 **Figure 4:** Non-genotoxic activation of p38 with anisomycin does not impede entry into  
 638 mitosis in HeLa cells. (A) Mitotic Index (phospho-histone H3) of HeLa cells  
 639 synchronized with CDK1 inhibitor RO-3306 in the presence or absence of 2μg/ml  
 640 anisomycin; (B) Western Blot for p38 activation of HeLa cells synchronized with CDK1  
 641 inhibitor RO3306 by 2μg/ml anisomycin.

642

643 **Figure 5:** Inactivation of p38 inhibits immediate early response and anti-apoptotic  
 644 pathways in TNF-α treated Calu-6 cells. (A) Cluster analysis of genes differentially  
 645 expressed 1 hour after TNF-α treatment and effect of 320nM p38i LY479754, on these  
 646 expression changes. Gene expression values are represented by colors with red and green  
 647 color indicate high and low expressions, respectively; (B) Box-plots of TNFα response of  
 648 immediate early response genes and members of apoptosis pathway component genes at  
 649 1 hour time point in the presence or absence of p38 kinase inhibitor. (C) Western Blots of  
 650 p38 activation, apoptosis and cell death/survival pathway protein expression in Calu-6  
 651 cells treated with TNF-α in the presence of 320nM p38i, LY479754. (D) Apoptotic index  
 652 (% of cells expressing cleaved-PARP) of Calu-6 cells treated with 25ng/ml TNF-α and  
 653 various concentrations of p38i using an Acumen Explorer high content imaging assay.  
 654 CTRL = control with no treatment.

655

656 **Figure 6:** Inhibition of p38 MAPK sensitizes cells to adriamycin and induces cell death.

657 (A) Apoptosis Index (% of cells expressing cleaved-caspase 3) of HeLa cells treated with

658 increasing doses of p38i, LY479754, in the presence of 160nM adriamycin for 48 hours;

659 (B) Western blot for apoptosis of HeLa cells treated with siRNA targeting p38 $\alpha$ , MK2 or

660 Chk1 in the presence or absence of 160nM adriamycin for 48 hours; (C) Western Blot

661 analysis of synchronized A549 cells by serum starvation for 48 hours after treatment with

662 1 $\mu$ M p38i, LY479754, in the presence of DNA damage induced by 160nM adriamycin

663 for 48 hours; (D) Western Blot of A549 cells treated with 0.01% MMS and 1 $\mu$ M p38i,

664 LY479754, for 48 hours; (E) apoptosis induced by p38 inhibition in the presence of DNA

665 damage is associated with G2 arrest. Apoptosis Index and cell cycle state were

666 determined in A549 cells treated with 160nM adriamycin and 1 $\mu$ M p38i, LY479754,

667 using the Acumen Explorer.

668

669 **Figure 7:** A new Model for a novel role of p38 MAPK in DNA damage response in

670 cancer cells. DNA damage elicits the activation of cell cycle checkpoint, DNA repair and

671 cell survival signaling pathways. In response to DNA damage, Chk1-mediated G<sub>2</sub> DNA

672 damage checkpoint is activated to prevent entry into mitosis with damaged DNA,

673 whereas p38-mediated cell survival pathway is activated to keep cells alive. Together,

674 these two pathways afford cell the time to repair DNA damage and ultimately to allow

675 recovery from DNA damage.

676

677

678

679

680 **Acknowledgements**

681 The authors would like to thank Li Fan of Ye's lab and Chris Sim Kim Siew, Connie Er  
682 Poh Nee at Systems Biology, Lilly Singapore Center for Drug Discovery Pte Ltd. for  
683 their technical assistance. We would like to thank Dr. Song Qing Na and Dr. Mark  
684 Marshall for providing the MAPKAPK2 (MK2) inhibitor and the Chk1 inhibitor,  
685 respectively. We would like to acknowledge Xi Lin for her assistance in running the  
686 microarrays and Dr. Shuguang Huang for initial gene expression profiling analysis. We  
687 would also like to thank Dr. Gunaretnam (Guna) Rajagopal (NJ Cancer Institute) for his  
688 help in this work.

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Figure 1

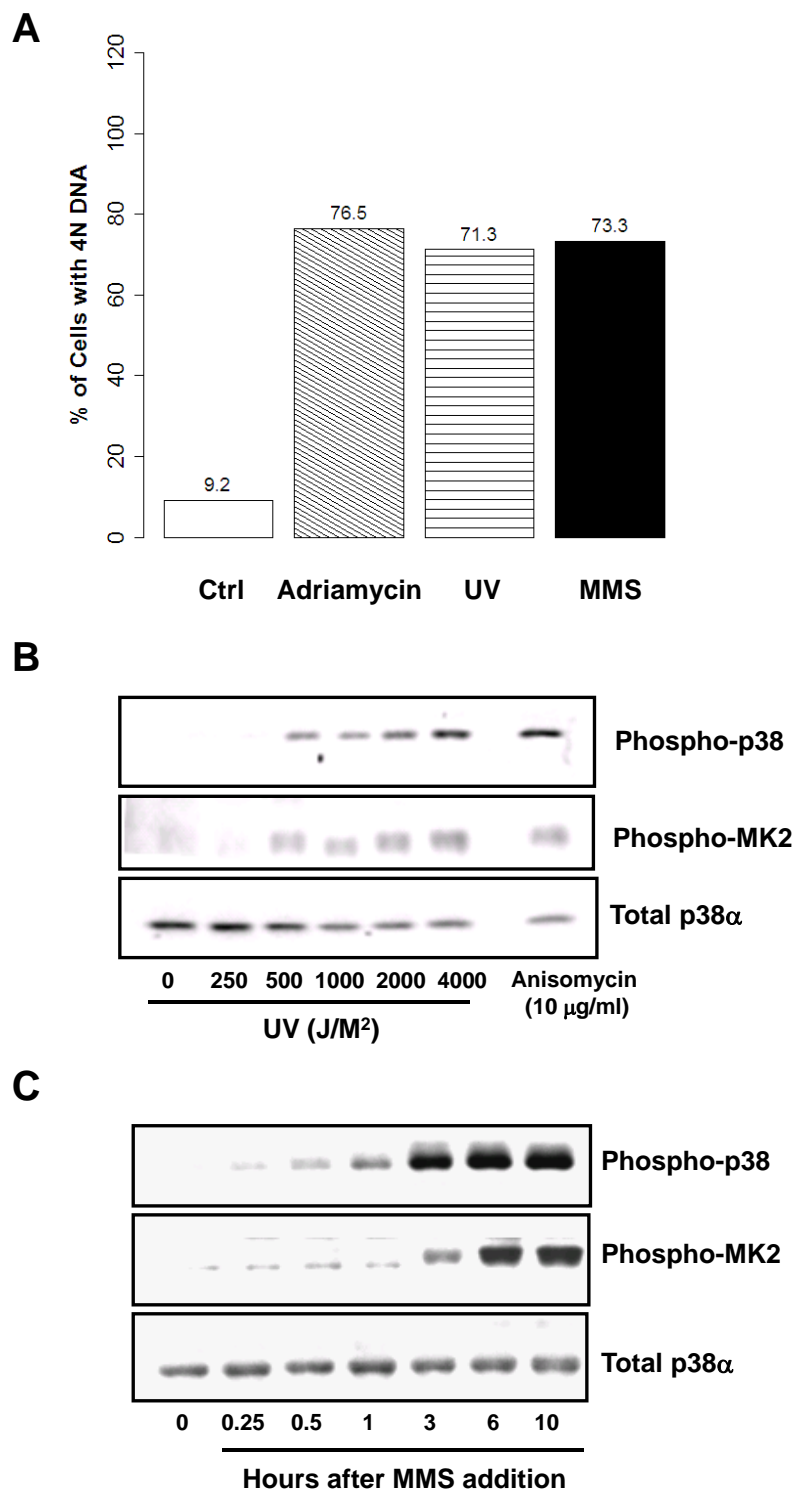




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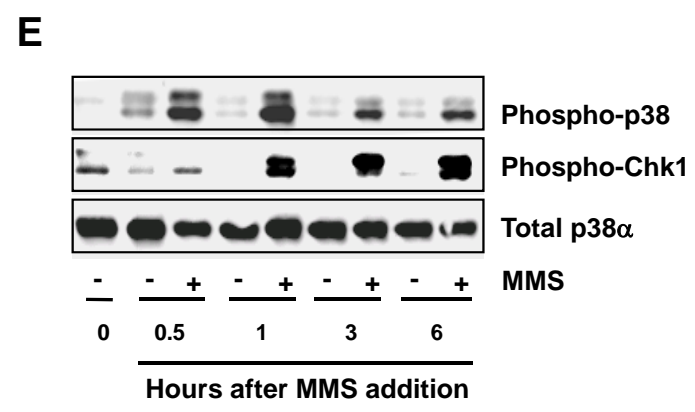
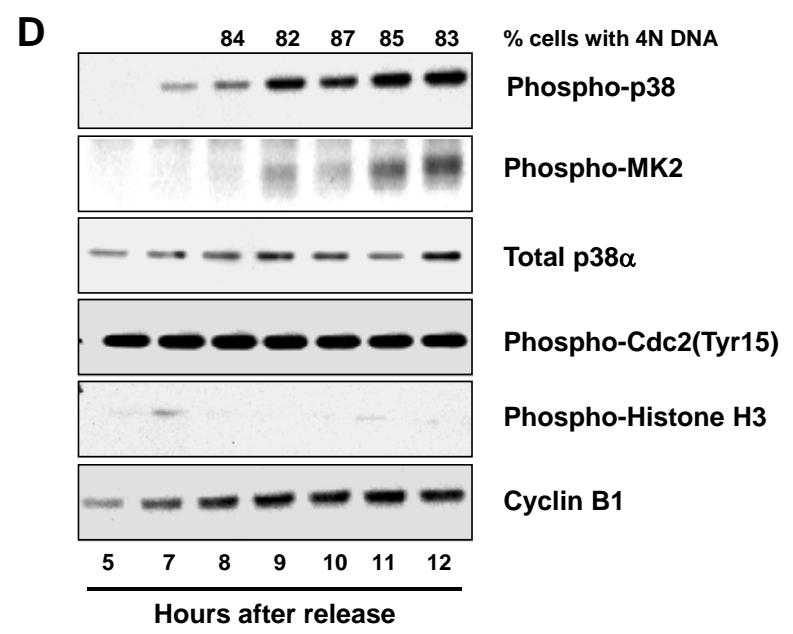
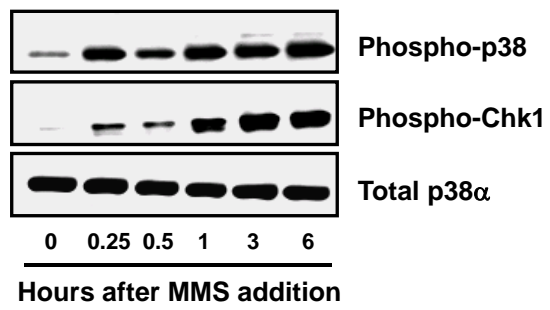


Figure 1, (Continued)

**F**



**G**

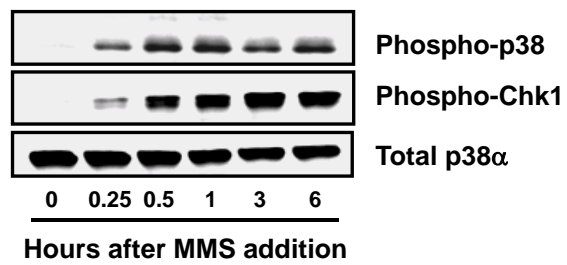
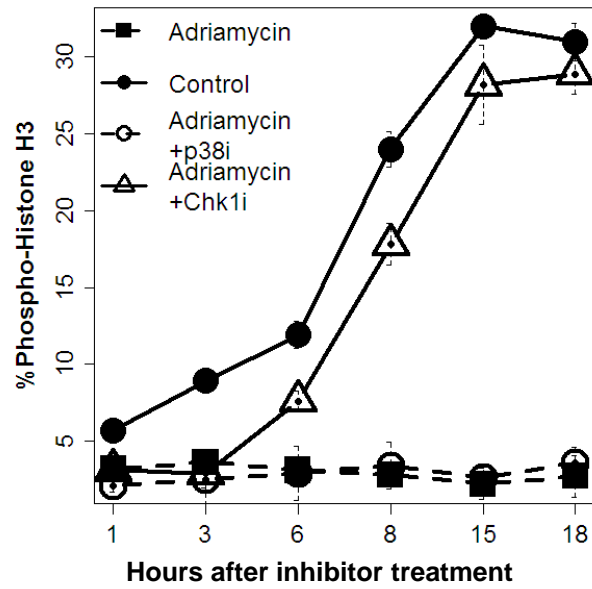


Figure 2

A



B

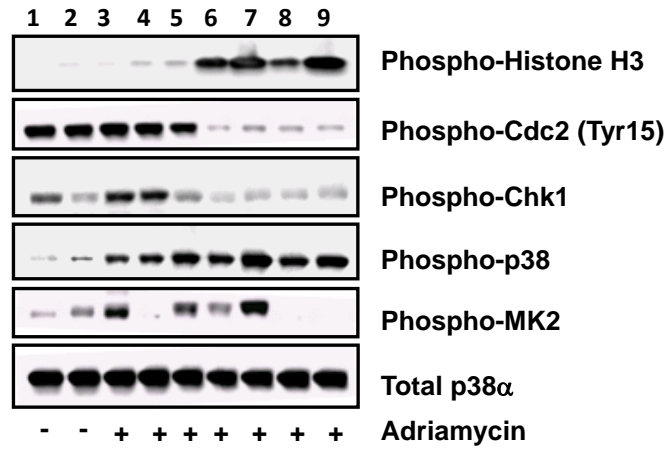


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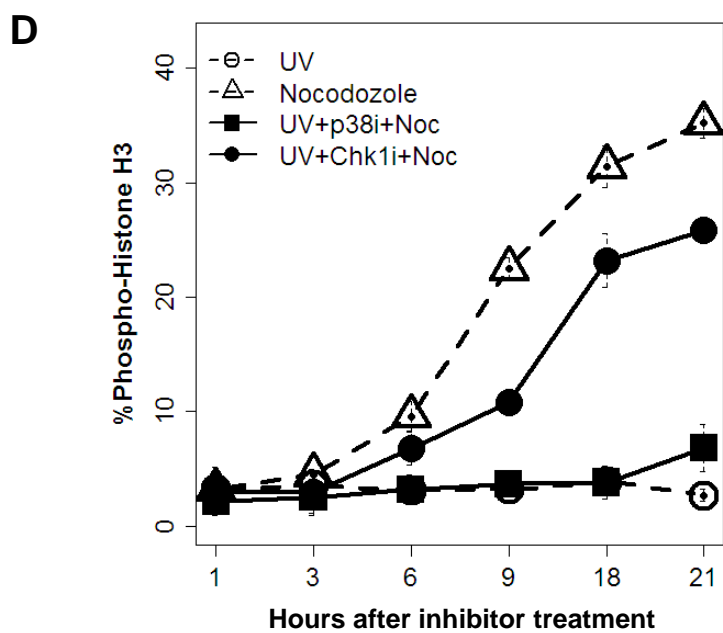
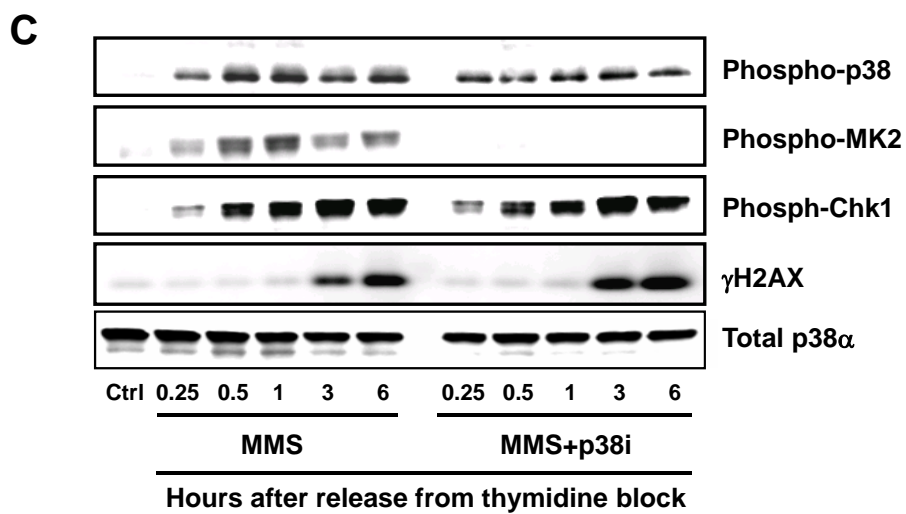


Figure 2, (continued)

**E**

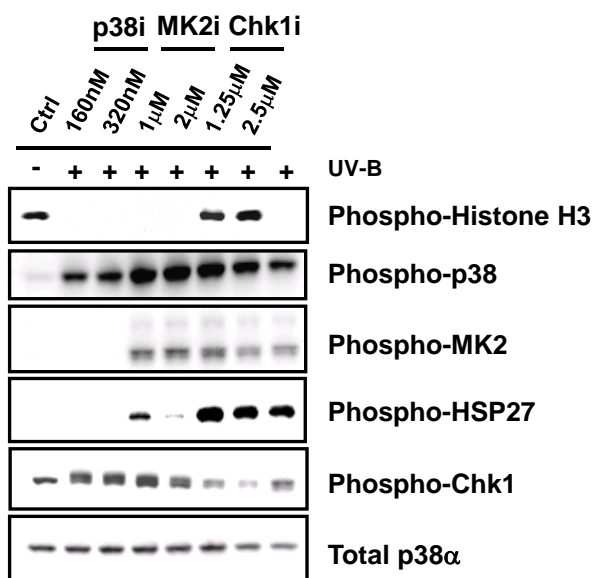


Figure 3

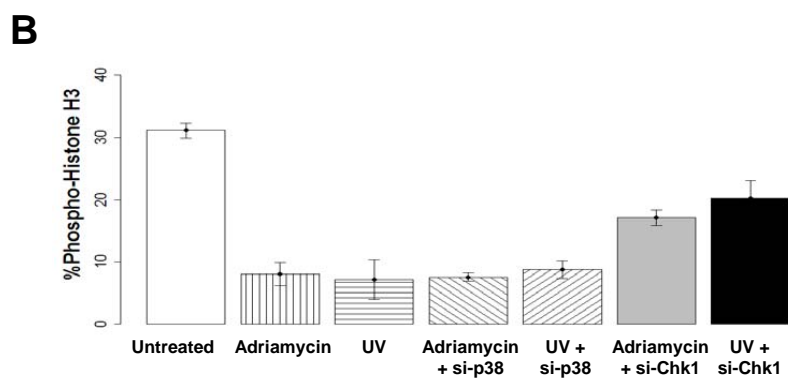
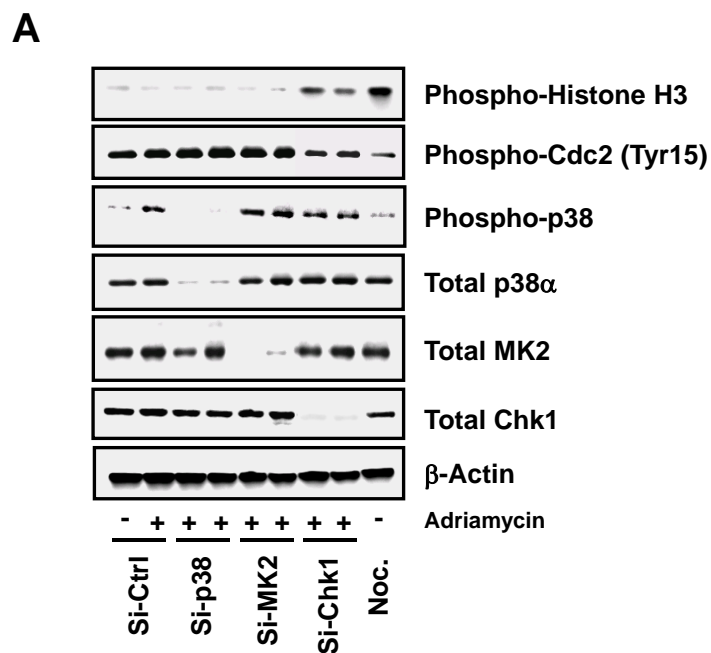


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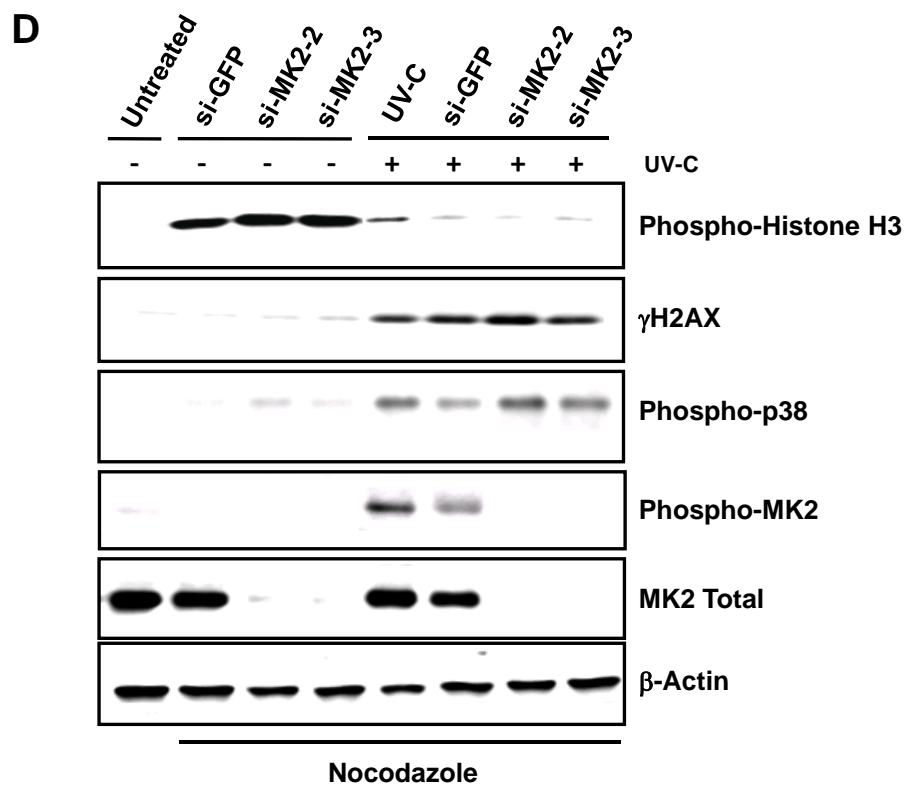
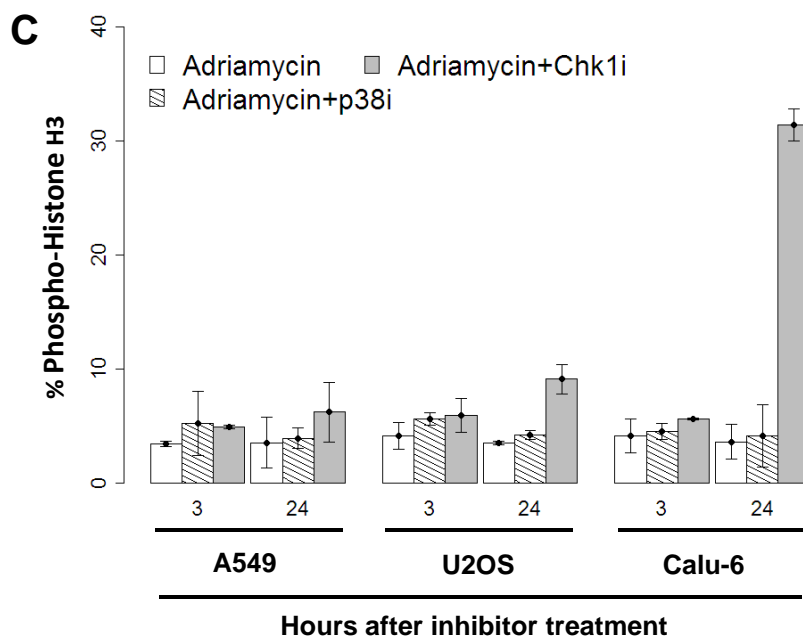


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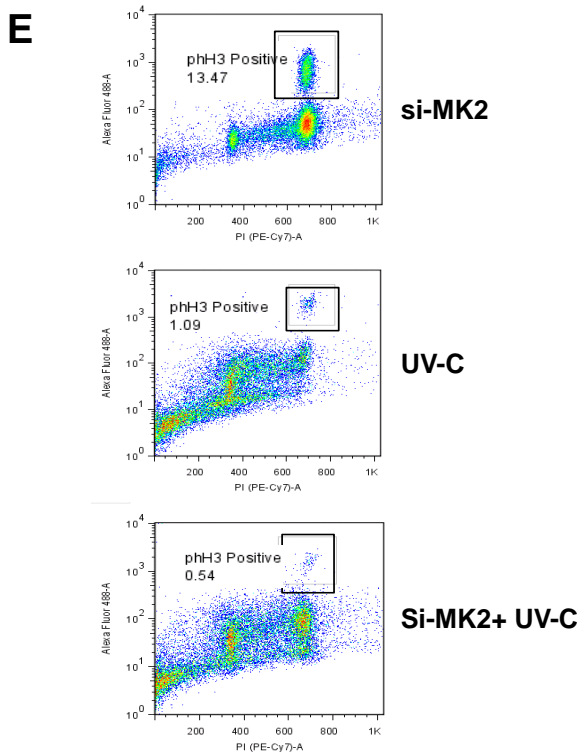




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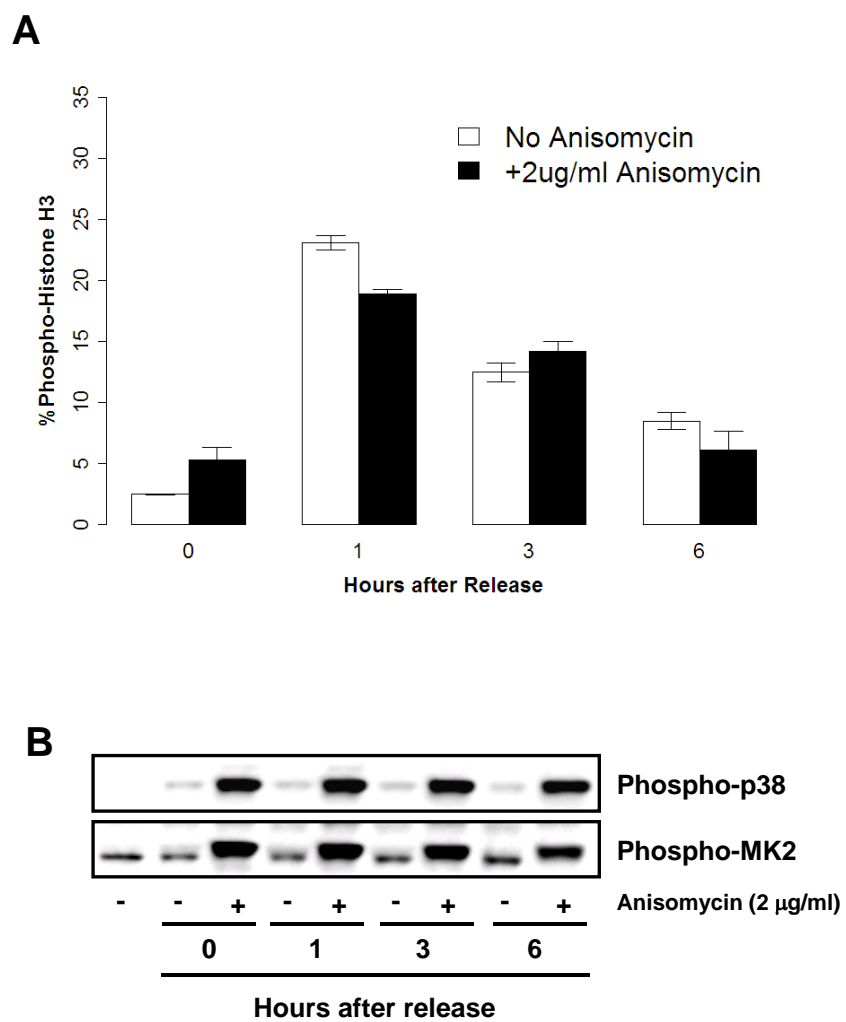


Figure 5

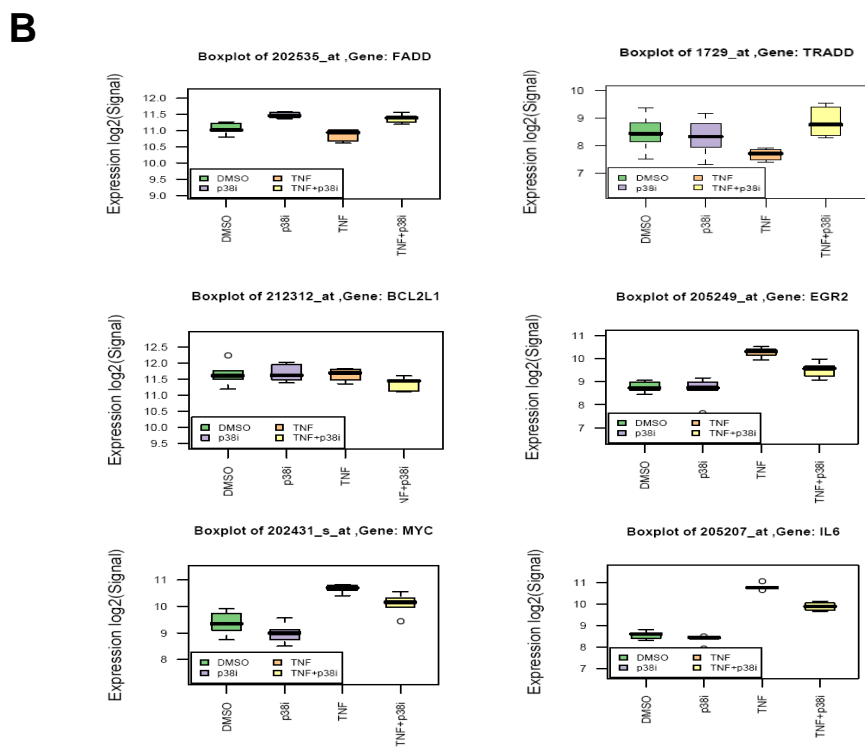
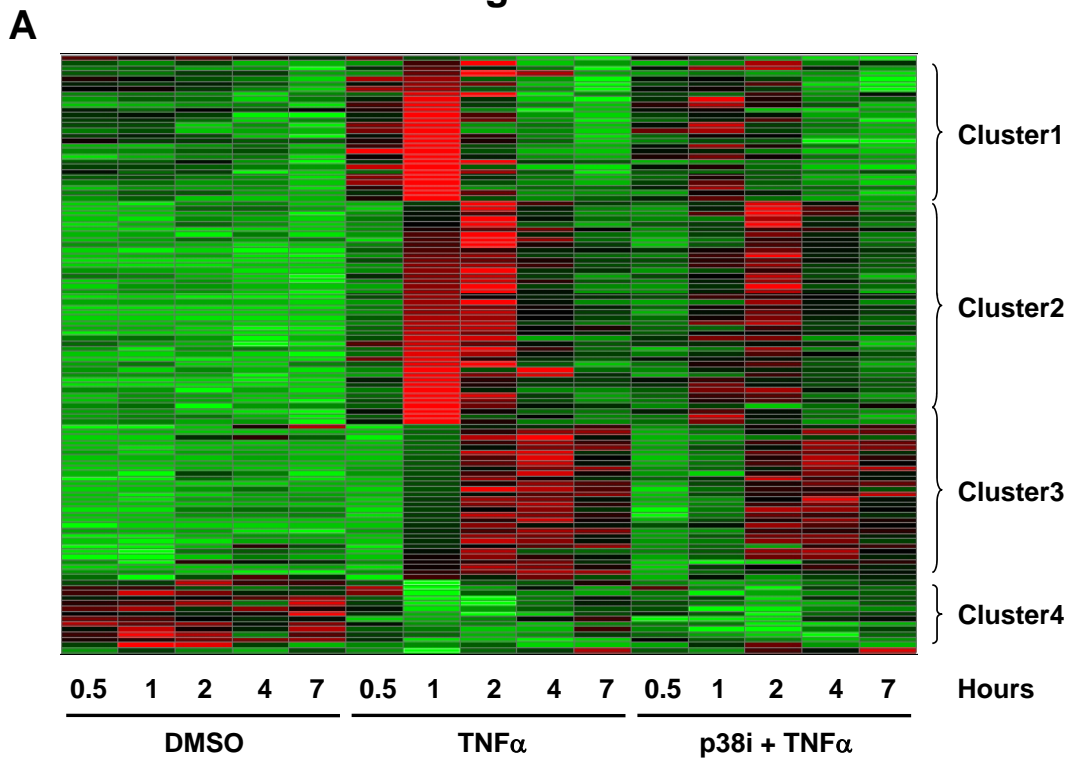
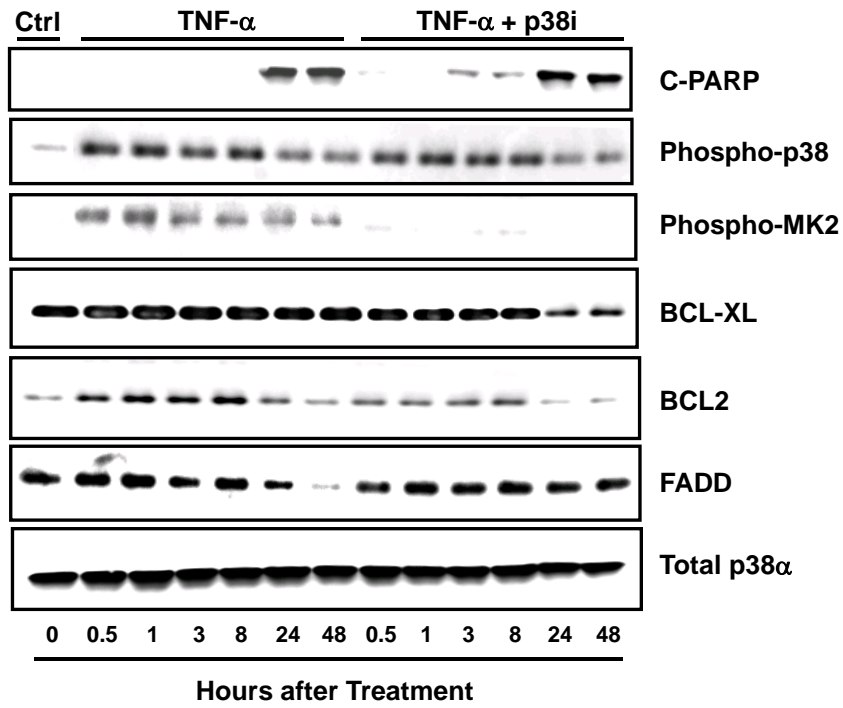


Figure 5, (continued)

C



D

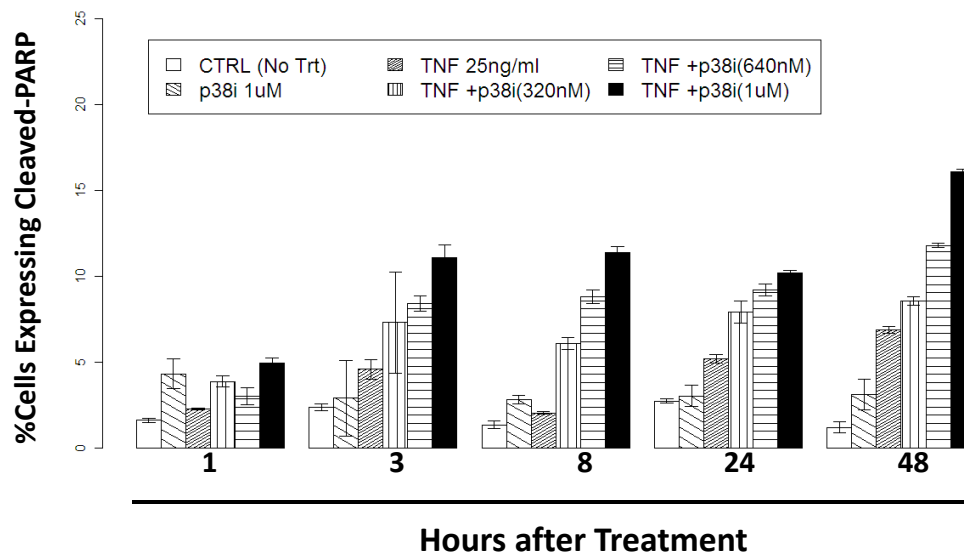


Figure 6

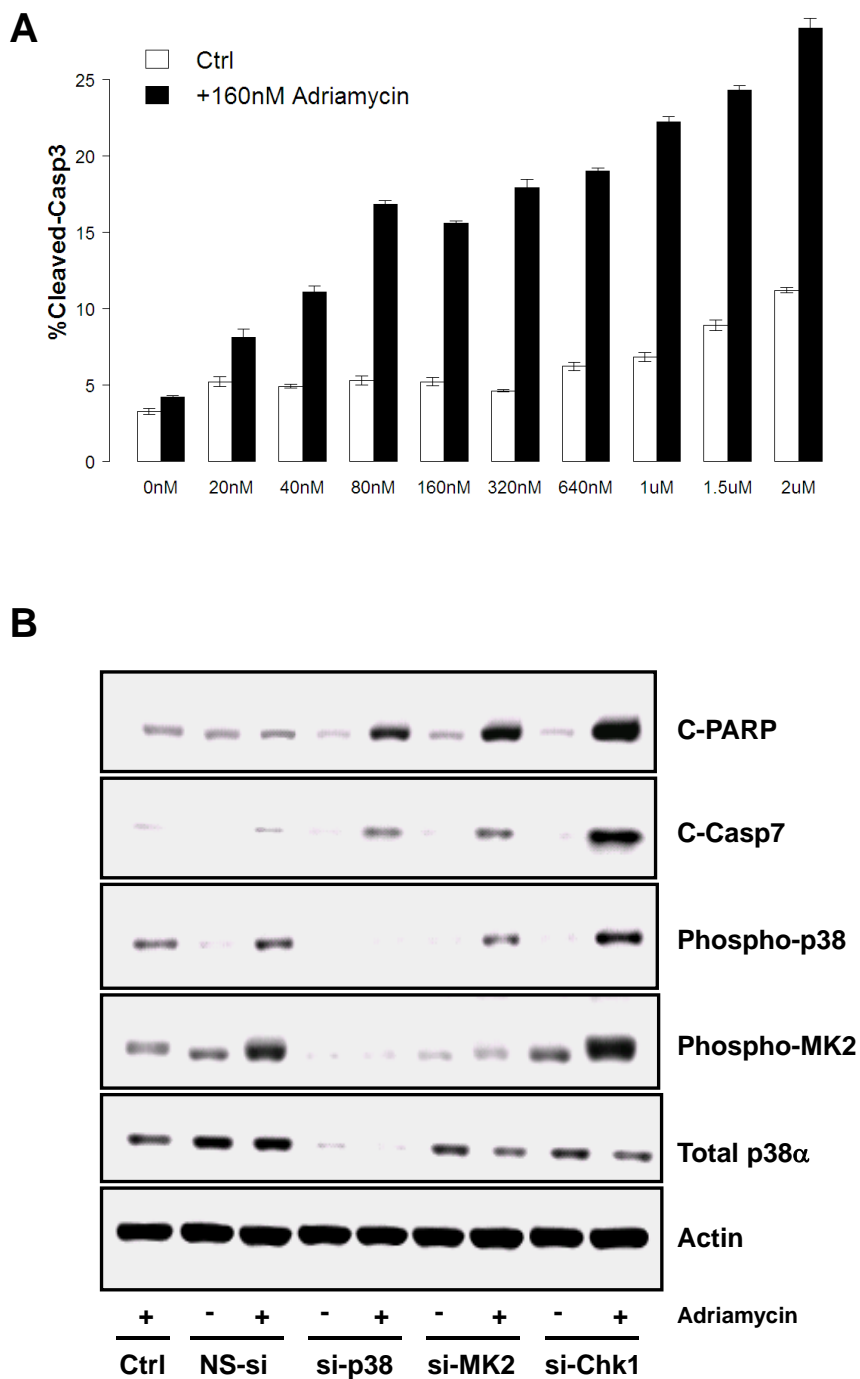
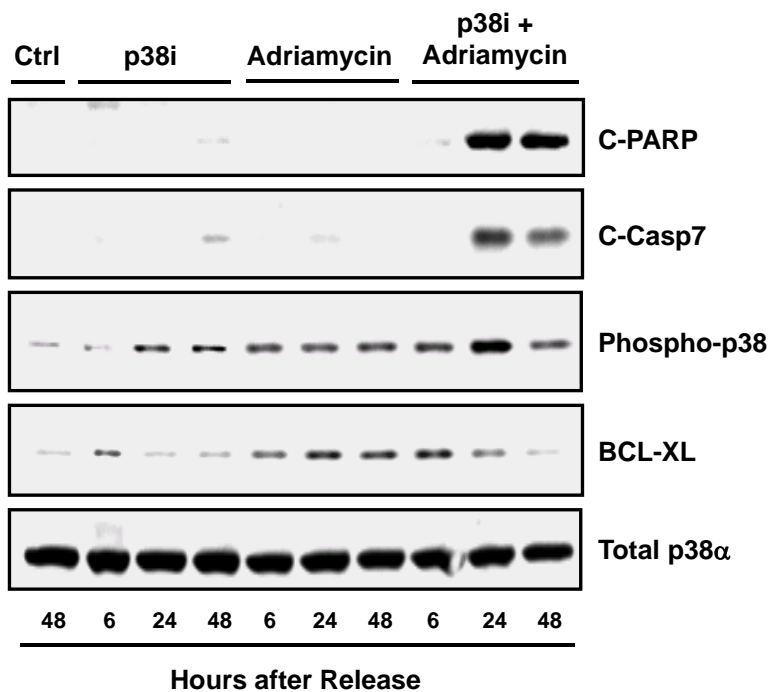


Figure 6, (continued)

C



D

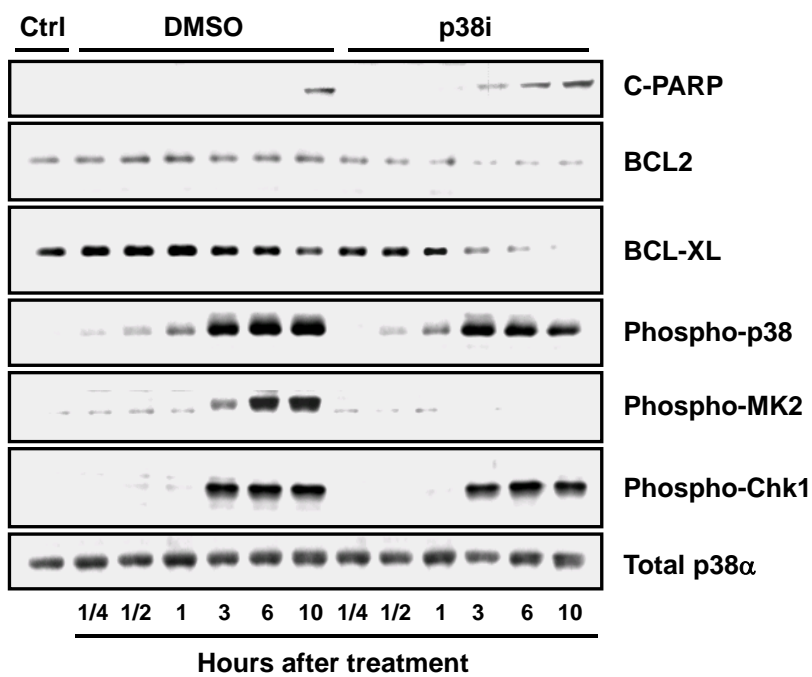


Figure 6, (continued)

**E**

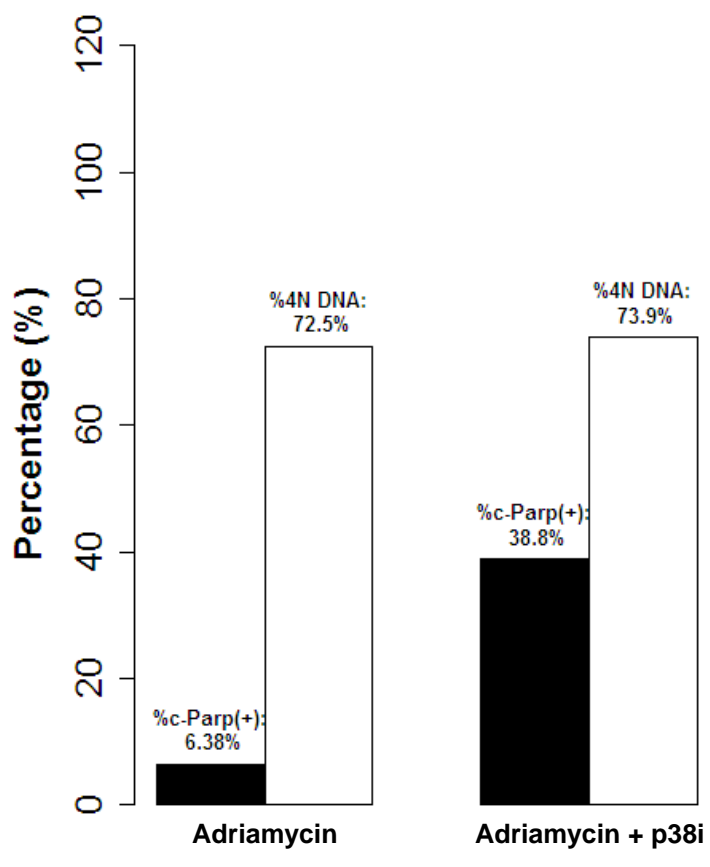


Figure 7

