

# Doxorubicin bypasses the cytoprotective effects of eIF2 $\alpha$ phosphorylation and promotes PKR-mediated cell death

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The eukaryotic cell responds to various forms of environmental stress by adjusting the rates of mRNA translation thus facilitating adaptation to the assaulting stress. One of the major pathways that control protein synthesis involves the phosphorylation of the  $\alpha$ -subunit of eukaryotic initiation factor eIF2 at serine 51. Different forms of DNA damage were shown to induce eIF2 $\alpha$  phosphorylation by using PERK, GCN2 or PKR. However, the specificity of the eIF2 $\alpha$  kinases and the biological role of eIF2 $\alpha$  phosphorylation pathway in the DNA damage response (DDR) induced by chemotherapeutics are not known. Herein, we show that PKR is the eIF2 $\alpha$  kinase that responds to DDR induced by doxorubicin. We show that activation of PKR integrates two signaling pathways with opposing biological outcomes. More specifically, induction of eIF2 $\alpha$  phosphorylation has a cytoprotective role, whereas activation of c-jun N-terminal kinase (JNK) by PKR promotes cell death in response to doxorubicin. We further show that the proapoptotic effects of JNK activation prevail over the cytoprotection mediated by eIF2 $\alpha$  phosphorylation. These findings reveal that PKR can be an important inducer of cell death in response to chemotherapies through its ability to act independently of eIF2 $\alpha$  phosphorylation.

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The eukaryotic cell has established intricate mechanisms to cope with environmental stress. It does so by changing protein expression in a manner that promotes adaptation to the assaulting stress. Protein expression within the cell is regulated at the transcriptional, translational and post-translational levels. Translational control is often used under conditions of cellular stress because it allows immediate and selective changes in protein levels.<sup>1</sup> Translation itself is divided into three distinct phases: initiation, elongation and termination. By far the most explored step is that of initiation, which has been shown to be regulated by different extracellular stimuli.<sup>1</sup> One of the major pathways that control translation initiation is that of the phosphorylation of the  $\alpha$ -subunit of translation initiation factor eIF2.<sup>2</sup> Phosphorylation of eIF2 $\alpha$  at serine 51 (S51) leads to the inhibition of global protein synthesis providing the cell with the opportunity to elicit adaptive responses not only by saving energy but also by preventing the accumulation of unwanted proteins that could interfere with cellular functions.<sup>1</sup>

There are four eIF2 $\alpha$  kinases that share a homologous kinase domain (KD) but possess different regulatory domains

that enable them to become activated by distinct stimuli.<sup>2</sup> The eIF2 $\alpha$  kinases are the heme-regulated inhibitor, which is found mainly in erythroid cells and is activated by heme deficiency; the endoplasmic reticulum (ER)-resident kinase (PERK), which is activated by ER stress and inhibits protein synthesis as part of the unfolded protein response; the general amino-acid nonderepressing kinase 2 (GCN2), which responds to amino-acid deprivation and becomes activated by uncharged tRNA and the RNA-dependent protein kinase PKR, an interferon-inducible protein that becomes activated by double-stranded (ds) RNA.<sup>2</sup> Previous work suggested that induction of eIF2 $\alpha$  phosphorylation can be either cytoprotective or proapoptotic. That is, transient induction of eIF2 $\alpha$  phosphorylation functions mostly cytoprotectively through the activation of pathways that promote cell survival such as the phosphatidylinositol-3 kinase (PI3K)<sup>3</sup> and nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathways.<sup>4,5</sup> However, prolonged induction of eIF2 $\alpha$  phosphorylation is mainly proapoptotic.<sup>5,6</sup> Although eIF2 $\alpha$  phosphorylation has a major role in mediating the biological effects of the eIF2 $\alpha$  kinases, their ability to function independently of eIF2 $\alpha$  phosphorylation has been reported.<sup>7,8</sup>

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**Keywords:** PKR; doxorubicin; eIF2 $\alpha$ ; JNK; protein phosphorylation; apoptosis

**Abbreviations:** ASK1, apoptosis signal-regulating kinase 1; ATF4, activating transcription factor 4; ATM, ataxia-telangiectasia mutated; DDR, DNA damage response; DMSO, dimethyl sulphoxide; DNA-PK, DNA-dependent protein kinase; dsRNA, double-stranded RNA; eIF2 $\alpha$ , eukaryotic initiation factor 2 subunit- $\alpha$ ; eIF2B, eukaryotic initiation factor 2B; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; GCN2, general control nonderepressible 2; H2AX, histone 2AX; JNK, c-jun N-terminal kinase; KD, kinase domain; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; NF- $\kappa$ B, nuclear factor of  $\kappa$ -light polypeptide gene enhancer in B cells; PERK, PKR-like endoplasmic reticulum-resident kinase; PI, propidium iodide; PI3K, phosphatidylinositol 3 kinase; PKR, double-stranded RNA-dependent protein kinase

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Previous findings provided evidence that eIF2 $\alpha$  phosphorylation is induced by genotoxic stress. That is, GCN2, PERK and PKR have been implicated in the DNA damage response (DDR) by various stimuli.<sup>9–12</sup> However, the specificity of the eIF2 $\alpha$  kinases and the role of eIF2 $\alpha$  phosphorylation in DDR induced by chemotherapeutics have not been elucidated. Herein, we show that PKR is specifically activated by doxorubicin leading to the induction of eIF2 $\alpha$  phosphorylation and c-jun N-terminal kinase (JNK) activity. We also show that eIF2 $\alpha$  phosphorylation conveys a cytoprotective effect, which is counteracted by JNK activation, the latter being required for PKR-mediated cell death. These data reveal a dual function for PKR in response to DNA damage with potential implications in treatments with chemotherapeutic drugs.

## Results

**PKR promotes cell death in response to doxorubicin treatment.** Previous work from our group showed that PKR mediates the induction of G<sub>1</sub> arrest by enhancing the activation of the tumor suppressor p53 and is involved in p53 phosphorylation at serine 18 in mouse embryonic fibroblasts (MEFs) subjected to DNA damage.<sup>13</sup> We examined the biological role of PKR in response to DNA damage in MEFs that are deficient in p53 due to spontaneous immortalization. For this purpose immortalized MEFs completely deficient in PKR activity (PKR<sup>-/-</sup> MEFs)<sup>14,15</sup> together with their isogenic wild-type counterparts were treated with the chemotherapeutic drug doxorubicin and subjected to analysis of cell death by flow cytometry. We noticed that PKR was required for optimal induction of cell death in response to doxorubicin (Figure 1a). The higher sensitivity of PKR<sup>+/+</sup> MEFs to the cytotoxic effects of doxorubicin was supported by the higher levels of cleaved caspase-3 in these cells compared with PKR<sup>-/-</sup> MEFs for the various periods of treatment (Figure 1b). Moreover doxorubicin treatment did not cause differences in cell-cycle arrest between PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs (Supplementary Figure S1) indicating a specific role of PKR in the induction of cell death.

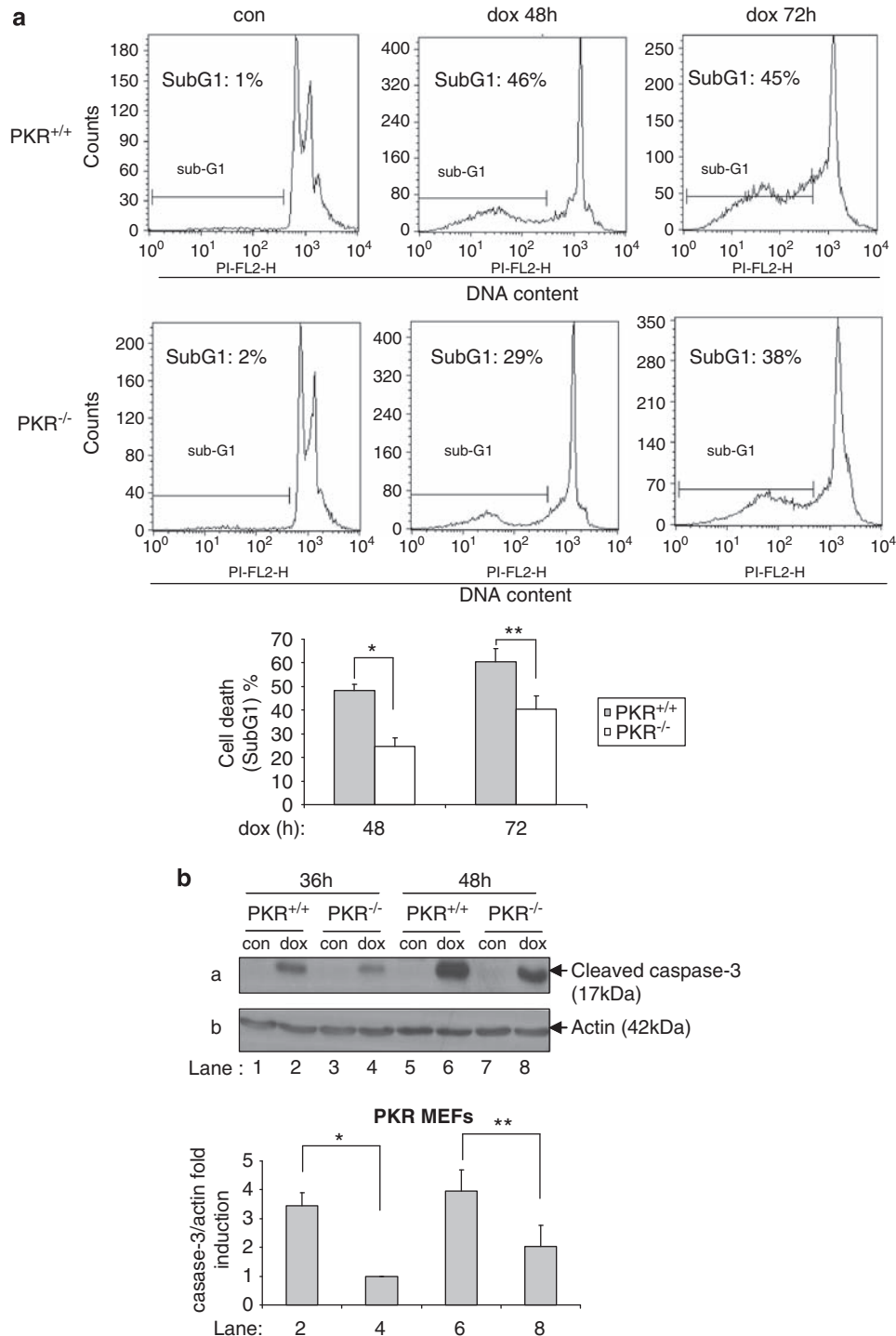
Because both PERK and GCN2 have been shown to respond to DNA damage, we next wanted to examine the specificity of eIF2 $\alpha$  kinases to doxorubicin treatment. To this end, we treated PERK<sup>-/-</sup> and GCN2<sup>-/-</sup> MEFs as well as their isogenic wild-type MEFs with doxorubicin and subjected to fluorescence-activated cell sorting (FACS) analysis. We observed that cell death was equally induced in PERK<sup>+/+</sup> and PERK<sup>-/-</sup> as well as in GCN2<sup>+/+</sup> and GCN2<sup>-/-</sup> MEFs thus excluding a role for either eIF2 $\alpha$  kinase in promoting the apoptotic effects of doxorubicin (Figure 2). Moreover, we did not observe any differences in cell-cycle arrest induced by the drug between PERK<sup>+/+</sup> and PERK<sup>-/-</sup> MEFs, GCN2<sup>+/+</sup> and GCN2<sup>-/-</sup> MEFs (data not shown). Taken together, these data suggested that PKR specifically responds to DNA damage caused by doxorubicin leading to the induction of cell death.

**Phosphorylation of eIF2 $\alpha$  in response to doxorubicin treatment is mediated by PKR and exerts a cytoprotective role.** Next, we determined the role of eIF2 $\alpha$  phosphorylation in doxorubicin-mediated cell death. We first examined the

induction of eIF2 $\alpha$  phosphorylation at S51 in PKR<sup>-/-</sup> MEFs together with their isogenic wild-type counterparts on doxorubicin treatment. We observed an induction of eIF2 $\alpha$  phosphorylation in PKR<sup>+/+</sup> but not in PKR<sup>-/-</sup> MEFs (Figure 3, panel a). Contrary to this, eIF2 $\alpha$  phosphorylation was similarly induced in PERK<sup>-/-</sup> MEFs (panel c) or GCN2<sup>-/-</sup> MEFs (panel e) compared with their corresponding isogenic wild-type MEFs. Induction of eIF2 $\alpha$  phosphorylation by doxorubicin resulted in the upregulation of activating transcription factor 4 (ATF4) in PKR<sup>+/+</sup> but not in PKR<sup>-/-</sup> MEFs (Supplementary Figure S2A), supporting a functional role of eIF2 $\alpha$  phosphorylation in this process. Moreover, induction of eIF2 $\alpha$  phosphorylation by doxorubicin occurred in various human tumor cells (Supplementary Figure S2B) concomitantly with an increased PKR autophosphorylation at T446 (Supplementary Figure S2B, panel c) confirming the activation of PKR by doxorubicin in human cells.

To understand the biological effects of eIF2 $\alpha$  phosphorylation, we used MEFs containing either a wild-type allele of eIF2 $\alpha$  (i.e., eIF2 $\alpha$ <sup>S/S</sup>) or an allele bearing the S51A mutation (i.e., eIF2 $\alpha$ <sup>A/A</sup> MEFs). First, we verified that doxorubicin treatment induced eIF2 $\alpha$  phosphorylation at S51 in eIF2 $\alpha$ <sup>S/S</sup> MEFs (Figure 4a). Next, we evaluated cell death in eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs by flow cytometry. We observed that eIF2 $\alpha$ <sup>A/A</sup> MEFs were more susceptible than eIF2 $\alpha$ <sup>S/S</sup> MEFs to cell death by doxorubicin (Figure 4b). Also, the higher sensitivity of eIF2 $\alpha$ <sup>A/A</sup> MEFs to the proapoptotic effects of doxorubicin was further indicated by the increased expression of cleaved caspase-3 in these cells compared with eIF2 $\alpha$ <sup>S/S</sup> cells after doxorubicin treatment (Figure 4c). These data supported the notion that eIF2 $\alpha$  phosphorylation conveys a cytoprotective effect on doxorubicin-treated MEFs and that PKR-mediated cell death by doxorubicin uses a pathway other than that of eIF2 $\alpha$  phosphorylation.

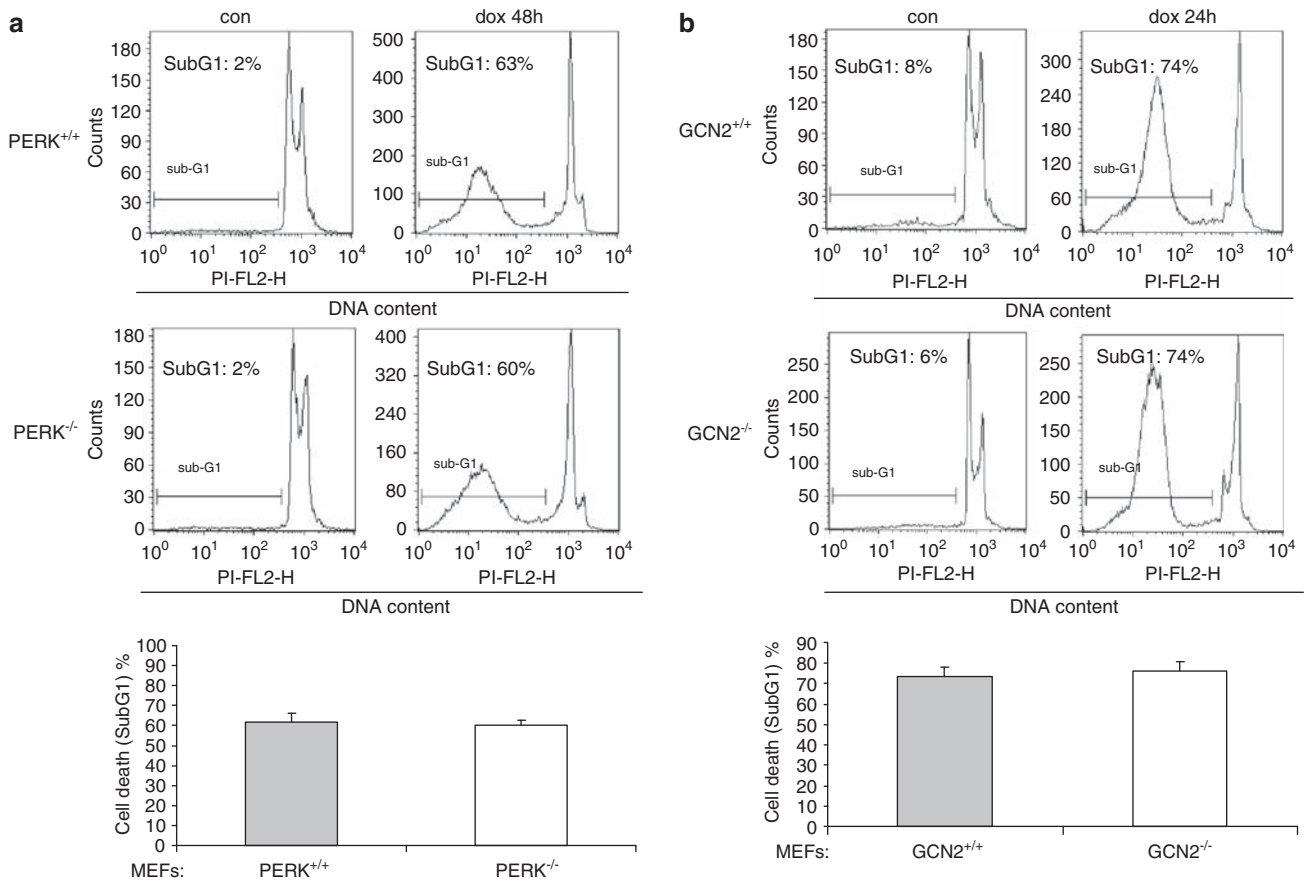
**PKR induces JNK activity in response to doxorubicin treatment.** Previous data established a role of stress-activated mitogen-activated protein kinases (MAPKs; i.e., JNK1/2, p38 and extracellular signal-regulated kinase (ERK) 1/2) in the regulation of cell death by DNA-damaging drugs including doxorubicin.<sup>16</sup> Moreover, PKR was implicated in the activation of the p38 MAPK in response to dsRNA independently of eIF2 $\alpha$  phosphorylation.<sup>17</sup> These findings prompted us to examine a possible role of MAPKs in PKR-mediated cell death by doxorubicin. We observed that doxorubicin treatment resulted in a higher induction of JNK1/2 activity in PKR<sup>+/+</sup> than in PKR<sup>-/-</sup> MEFs (Figure 5a, panel a). PKR specifically mediated the induction of JNK1/2 activity given that neither ERK1/2 nor p38 MAPK phosphorylation was significantly induced in PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs (Figure 5a, panels c and e). Furthermore, JNK1/2 activation in PKR<sup>+/+</sup> MEFs was maintained for long periods of doxorubicin treatment (Figure 5b) and was independent of eIF2 $\alpha$  phosphorylation as JNK1/2 phosphorylation was similarly induced in eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs in response to doxorubicin (Figure 5c). To verify the ability of PKR to mediate JNK activation, we used human HT1080 cells expressing a conditionally active form of PKR, which is activated after treatment of cells with the antibiotic coumestrolin.<sup>6</sup> We found



**Figure 1** PKR promotes doxorubicin-induced cell death. **(a)** PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated time periods. Cells were subjected to FACS analysis after propidium iodide staining. Cell death is represented by the percentage (%) of cells in sub-G<sub>1</sub>. Histograms represent the mean cell death from five independent experiments after subtraction of background cell death (untreated control cells) ( $n=5$ ). Statistical significance of the difference as calculated by Student's *t*-test is with \* $P<0.0003$ , \*\* $P<0.0009$ . **(b)** PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated time periods. Protein extracts (50  $\mu$ g) were subjected to western blot analysis for cleaved caspase-3 (a) and actin (b). Histograms represent the mean value of the ratio of cleaved caspase-3 to actin after normalization to that of lane 4 for the indicated lanes of the western blot from five independent experiments ( $n=5$ ). \* $P<0.005$ , \*\* $P<0.006$

that conditional activation of PKR, which was assessed by the eIF2 $\alpha$  phosphorylation, led to JNK1 activation by phosphorylation as detected by immunoblotting with phospho-specific

antibodies (Supplementary Figure S3). Taken together, these findings showed the ability of PKR to induce JNK activity in mouse and human cells in response to doxorubicin.



**Figure 2** PERK and GCN2 do not contribute to doxorubicin-induced cell death. PERK<sup>+/+</sup> and PERK<sup>-/-</sup> MEFs (a) or GCN2<sup>+/+</sup> and GCN2<sup>-/-</sup> MEFs (b) were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated time periods and subjected to FACS analysis after propidium iodide staining. Cell death is represented by the percentage (%) of cells in sub-G<sub>1</sub>. Histograms represent the mean cell death from three independent experiments after subtraction of background cell death (untreated control cells) ( $n=3$ )

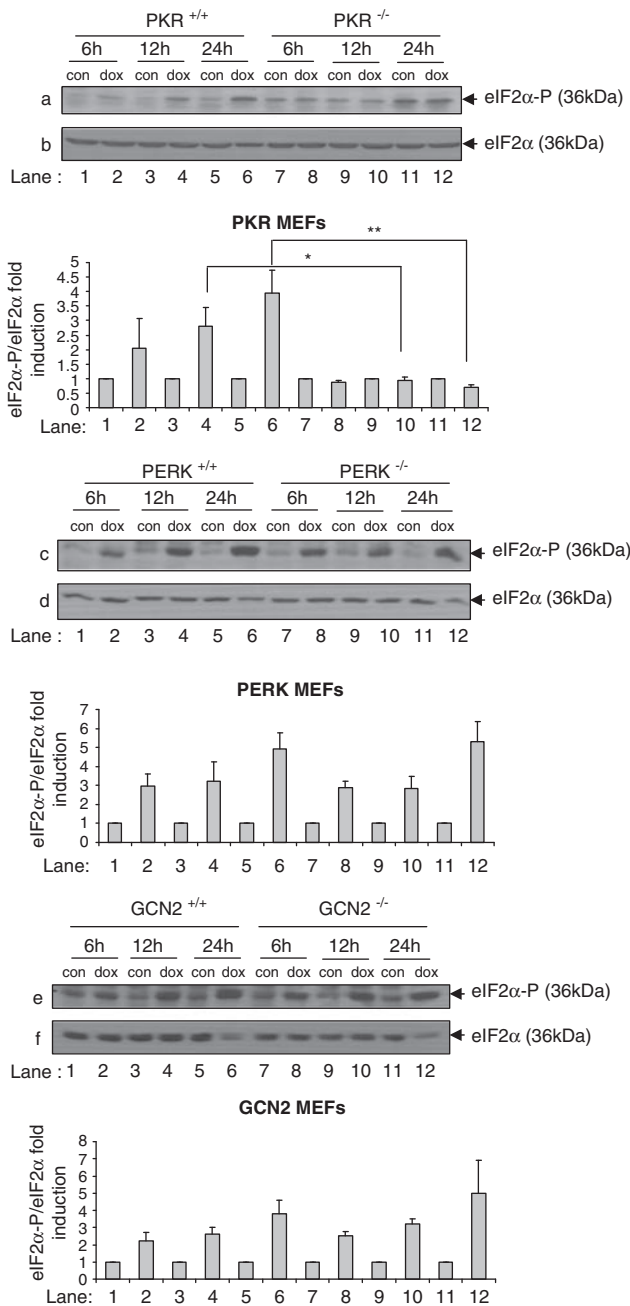
To determine the biological significance of JNK activation, we examined whether pharmacological inhibition of JNK1/2 had any effect on PKR-mediated cell death. To this end, we treated PERK<sup>+/+</sup> and PERK<sup>-/-</sup> MEFs with doxorubicin in the absence or presence of the specific JNK inhibitor SP600125, and assessed cell death by FACS analysis (Figure 6). We observed that SP600125 alone had a minimal cytotoxic effect on both PERK<sup>+/+</sup> and PERK<sup>-/-</sup> MEFs. However in combination with doxorubicin, SP600125 completely blocked the death of PERK<sup>+/+</sup> MEFs and had a modest inhibitory effect on the death of PERK<sup>-/-</sup> MEFs (Figure 6). Collectively, these data suggested that induction of PKR-mediated cell death by doxorubicin requires the activation of JNK1/2.

## Discussion

Our work shows that PKR can function as a mediator of cell survival as well as cell death in response to doxorubicin treatment. The prosurvival properties of PKR which are mediated by the induction of eIF2 $\alpha$  phosphorylation, are counteracted by the proapoptotic effects arbitrated by JNK activation (Figure 7). Doxorubicin is a chemotherapeutic drug that belongs to the family of anthracyclines, which are widely used for the treatment of various forms of cancer. Doxorubicin induces a DDR through its ability to intercalate with DNA,

generates reactive oxygen species and inhibits topoisomerase II activity.<sup>18</sup> Inhibition of topoisomerase II is responsible for the production of ds breaks on DNA, which are recognized by proteins involved in DNA repair as well as proteins that control cell proliferation and cell death.<sup>19</sup>

A major pathway activated by DDR involves the ataxia-telangiectasia mutated (ATM) kinase and the DNA-dependent protein kinase (DNA-PK), both of which are implicated in the induction of the DNA repair.<sup>19</sup> ATM is also important in the induction of the G<sub>1</sub> checkpoint as a result of the activation of the tumor suppressor p53.<sup>20</sup> Previous work from our group showed that PKR is implicated in the phosphorylation and activation of p53 in response to DNA damage. More specifically, using PERK<sup>+/+</sup> and PERK<sup>-/-</sup> MEFs expressing a temperature-sensitive mutant of p53 (tsp53), we showed that PKR promotes G<sub>1</sub> arrest under conditions that tsp53 attains a wild-type conformation.<sup>13</sup> We further showed that PKR phosphorylates p53 at serine 392 *in vitro*<sup>21</sup> and facilitates the phosphorylation of the tsp53 at serine 18 in response to ionizing irradiation or doxorubicin treatment.<sup>13</sup> These data showed that in cells with functional p53, activation of PKR in response to doxorubicin and other types of DNA damage conveys a cytoprotective role by promoting G<sub>1</sub> arrest. Here, we show an alternative function of PKR, which is the induction of cell death in MEFs with deficient p53 caused by



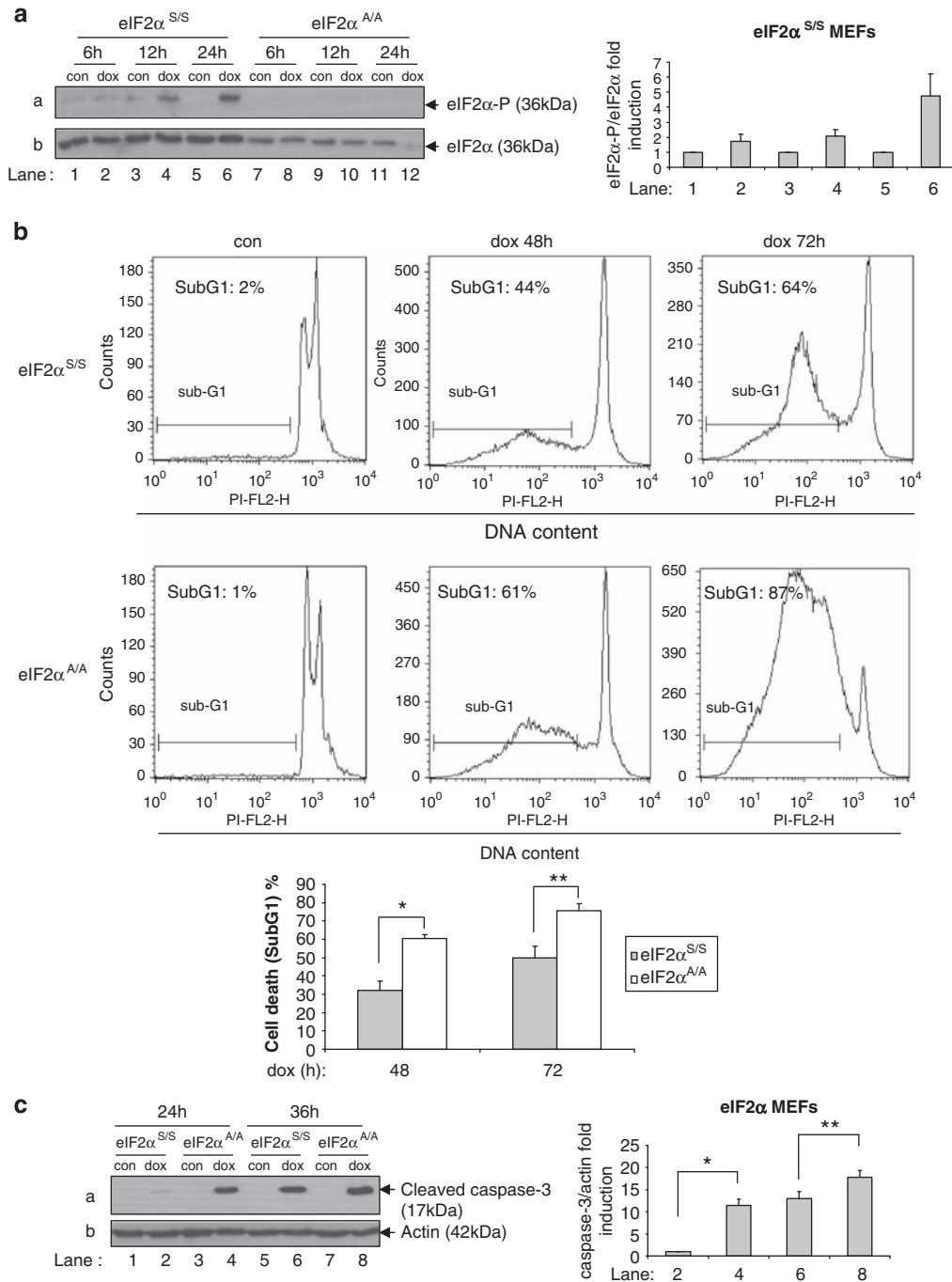
**Figure 3** PKR induces eIF2 $\alpha$  phosphorylation in response to doxorubicin treatment. The indicated types of MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated time periods. Protein extracts (50  $\mu$ g) were subjected to immunoblot analysis for phosphorylated eIF2 $\alpha$  (a, c, e) and total eIF2 $\alpha$  (b, d, f). Histograms represent the mean value of the ratio of eIF2 $\alpha$  phosphorylation levels to total eIF2 $\alpha$  levels for the treated cells normalized to that of their untreated control from six independent experiments for PKR ( $n=6$ ), five independent experiments for PERK ( $n=5$ ) and five independent experiments for GCN2 ( $n=5$ ). Statistical significance of the difference as calculated by Student's  $t$ -test is with \* $P<0.05$ , \*\* $P<0.02$

spontaneous immortalization (data not shown). Our previous findings implied a possible interplay between PKR and ATM/DNA-PK. Nevertheless, PKR is unlikely to act upstream of ATM or DNA-PK given that phosphorylation of histone 2AX

(H2AX) at serine 139 was efficiently induced in both PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs after doxorubicin treatment (Supplementary Figure S4A). Furthermore, pharmacological inhibition of ATM and DNA-PK by wortmannin or ATM alone by KU55933 in combination with doxorubicin increased the index of death similarly in PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs (Supplementary Figure S4B and S4C). Taken together, these results do not support a functional interplay between PKR and ATM and/or DNA-PK in response to doxorubicin.

Previous work by many researchers established a temporal role of eIF2 $\alpha$  phosphorylation in the induction of cell death. That is, short-term induction of eIF2 $\alpha$  phosphorylation leads to cytoprotection, which is mediated by the induction of prosurvival pathways such as the PI3K<sup>3</sup> and NF- $\kappa$ B pathway.<sup>4</sup> However, long-term induction of eIF2 $\alpha$  phosphorylation promotes apoptosis through, at least in part, the activation of ATF4 – CCAAT/enhancer binding homologous protein pathway.<sup>2</sup> However, there has been strong evidence to suggest that the signaling properties of eIF2 $\alpha$  kinases in mammalian cells are not exclusively linked to the induction of eIF2 $\alpha$  phosphorylation. For example, in contrast to DNA damage, activation of PKR or PERK in response to dsRNA or ER stress respectively decreases p53 levels through a mechanism that involves the activation of glycogen synthase kinase 3- $\beta$  and proteasomal degradation of the tumor suppressor protein independently of eIF2 $\alpha$  phosphorylation.<sup>22–24</sup> Also, the antiviral effects of the eIF2 $\alpha$  kinases are not always related to the inhibition of viral protein synthesis due to eIF2 $\alpha$  phosphorylation. Specifically, the ability of PERK and GCN2 to impair vesicular stomatitis virus replication in MEFs does not require eIF2 $\alpha$  phosphorylation.<sup>7</sup> How the eIF2 $\alpha$  kinases mediate their effects independently of eIF2 $\alpha$  phosphorylation is not presently clear. One possibility is that proteins other than eIF2 $\alpha$  can become substrates of the eIF2 $\alpha$  kinases as has been shown for p53<sup>21</sup> and nuclear factors associated with dsRNA proteins.<sup>25</sup>

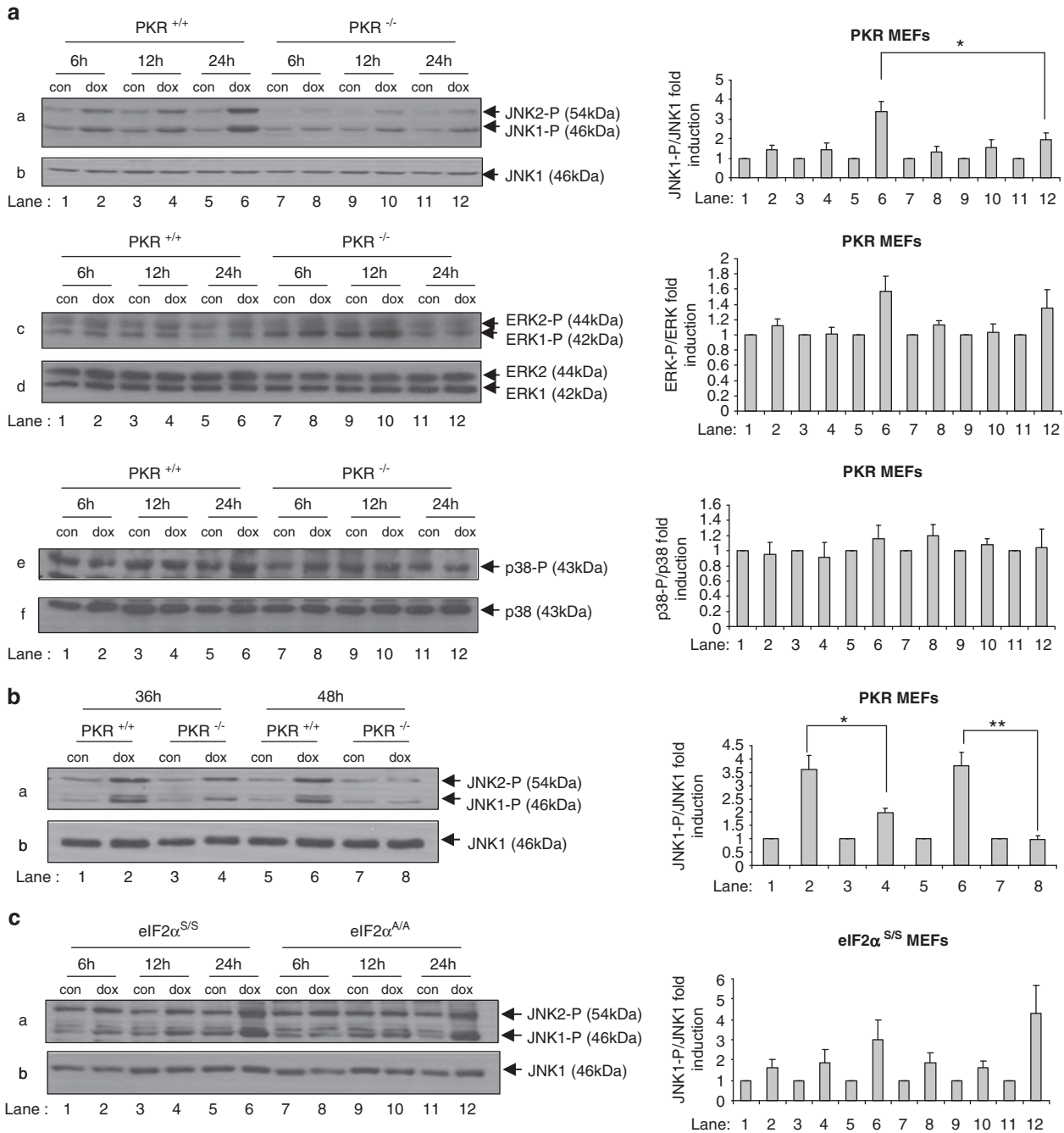
Our work shows that PKR functions upstream of JNK in cells treated with doxorubicin. JNK is a well-established inducer of apoptosis through different mechanisms that involve both nuclear and cytoplasmic events.<sup>26</sup> It has been shown that in response to doxorubicin, JNK activation promotes the nuclear localization of c-Abl, which in turn can induce cell death through the tumor suppressor p73.<sup>27</sup> Conceivably, it is possible that PKR induces the activity of kinases that act upstream of JNK1/2, based on a previous study showing that activation of p38/MAPK by PKR is mediated by MAPK kinase kinase 6 in cells treated with dsRNA.<sup>17</sup> It is also of interest that PKR physically associates with the apoptosis signal-regulating kinase 1 (ASK1) and promotes ASK1-mediated apoptosis induced by serum deprivation.<sup>28</sup> ASK1 functions upstream of JNK,<sup>29</sup> thus providing a tentative link between PKR and JNK activation. It is not presently known how PKR becomes activated by doxorubicin. DNA damage by doxorubicin might promote the interaction of PKR with proteins that function as activators. Another possibility may be that PKR is subjected to post-translational modifications in doxorubicin-treated cells, such as phosphorylation by other kinases and/or dephosphorylation by specific phosphatases, which induce its catalytic activity.



**Figure 4** Phosphorylation of eIF2 $\alpha$  protects cells from doxorubicin-induced death. (a) eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated time periods. Protein extracts (50  $\mu$ g) were subjected to immunoblotting for phosphorylated eIF2 $\alpha$  (a) and total eIF2 $\alpha$  (b). Histograms represent the mean value of the ratio of eIF2 $\alpha$  phosphorylation levels to eIF2 $\alpha$  total levels for the treated cells normalized to that of their untreated control from four independent experiments ( $n = 4$ ) for the eIF2 $\alpha$ <sup>S/S</sup> MEFs. (b) eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated time periods and subjected to FACS analysis after propidium iodide staining. Cell death is represented by the percentage (%) of cells in sub-G<sub>1</sub>. Histograms represent the mean cell death from five independent experiments after subtraction of background cell death (untreated control cells) ( $n = 5$ ). Statistical significance of the difference as calculated by Student's *t*-test is with \* $P < 0.002$ , \*\* $P < 0.02$ . (c) eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated times. Protein extracts (50  $\mu$ g) were subjected to western blot analysis for cleaved caspase-3 (a) and actin (b). Histograms represent the mean value of the ratio of cleaved caspase-3 to actin after normalization to the ratio of lane 2 for the indicated lanes from four independent experiments ( $n = 4$ ). Statistical significance of the difference as calculated by Student's *t*-test is with \* $P < 0.006$ , \*\* $P < 0.04$ .

The ability of PKR to respond to doxorubicin treatment could have potential implications in cancer therapies. For example, it was previously shown that human invasive ductal

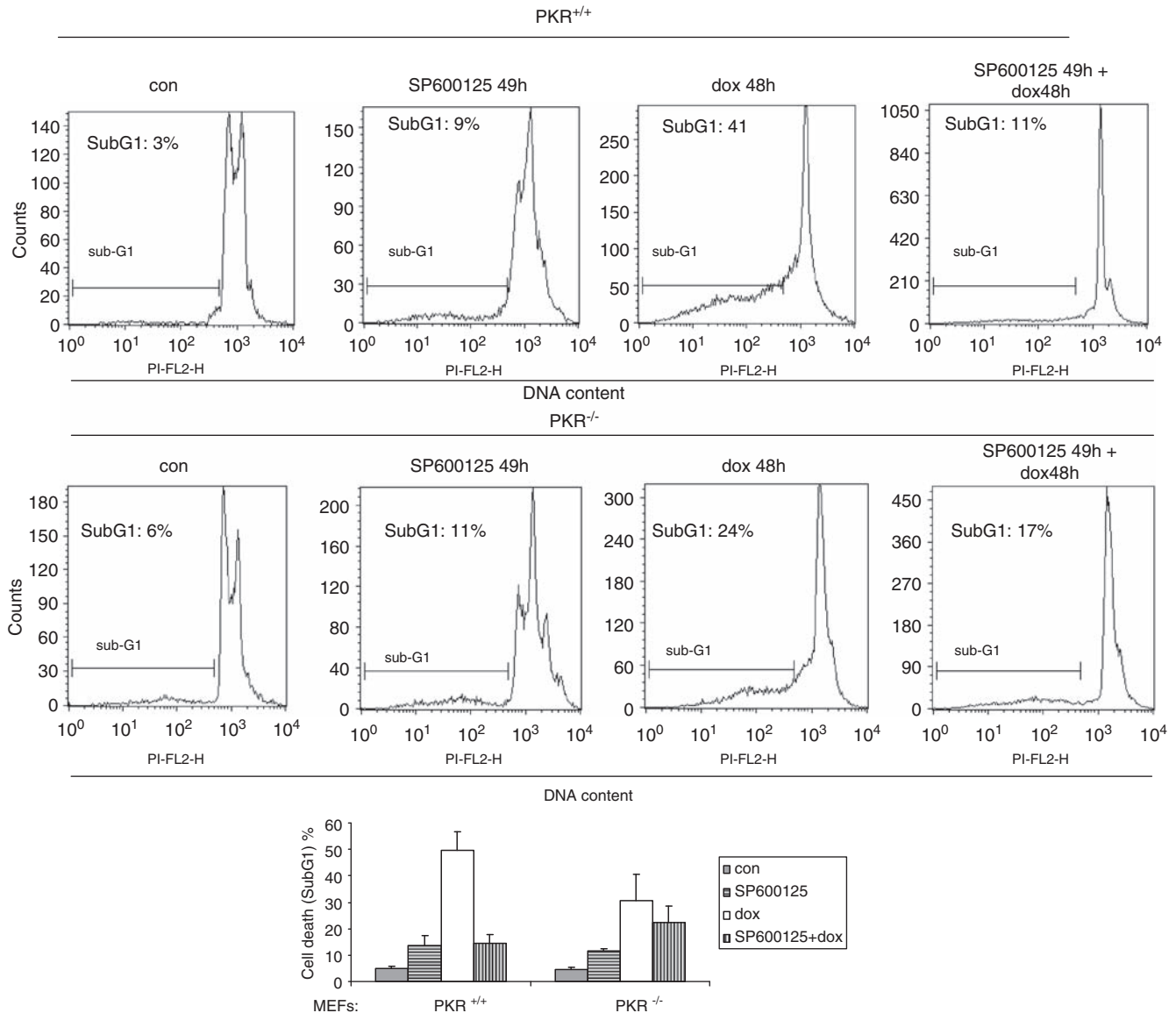
breast carcinomas and several human breast tumor cell lines contain elevated levels of PKR compared with non-malignant breast cells.<sup>30,31</sup> However, the PKR-eIF2 $\alpha$  phosphorylation



**Figure 5** PKR induces JNK phosphorylation in response to doxorubicin. **(a)** PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated times. Protein extracts (50  $\mu$ g) were subjected to immunoblotting for phosphorylated JNK (a), total JNK1 (b), phosphorylated ERK (c), total ERK (d), phosphorylated p38 (e), total p38 (f). Histograms represent the mean value of the ratio of the phosphorylated protein levels to their total levels for the treated cells normalized to that of their untreated control from four independent experiments for JNK ( $n=4$ ), four independent experiments for ERK ( $n=4$ ) and four independent experiments for p38 ( $n=4$ ). Statistical significance of the difference as calculated by Student's  $t$ -test is with  $*P<0.03$ . **(b)** PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated times. Protein extracts (50  $\mu$ g) were subjected to immunoblotting for phosphorylated JNK (a) and total JNK1 (b). Histograms represent the mean value of the ratio of the phosphorylated protein levels to their total levels for the treated cells normalized to that of their untreated control from six independent experiments ( $n=6$ ). Statistical significance of the difference as calculated by Student's  $t$ -test is with  $*P<0.02$ ,  $**P<0.002$ . **(c)** eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs were left untreated (con) or treated with 1  $\mu$ M doxorubicin (dox) for the indicated times. Protein extracts (50  $\mu$ g) were subjected to immunoblotting for phosphorylated JNK (a) and total JNK1 (b). Histograms represent the mean value of the ratio of the phosphorylated protein levels to their total levels for the treated cells normalized to that of their untreated control from six independent experiments ( $n=6$ )

arm was found to be compromised in malignant breast tumor cells due to upregulation of the guanine nucleotide exchange factor eIF2B,<sup>31</sup> which antagonizes the inhibitory effects of

phosphorylated eIF2 $\alpha$  on protein synthesis.<sup>32</sup> Interestingly, increased eIF2B levels were observed in various transformed cells and accounted for the high tolerance of these cells to



**Figure 6** Pharmacological inhibition of JNK blocks PKR-mediated cell death induced by doxorubicin. PKR<sup>+/+</sup> and PKR<sup>-/-</sup> MEFs were treated with 10  $\mu$ M SP600125 (SP600125), 1  $\mu$ M doxorubicin (dox) or both drugs (dox + SP600125) for the indicated time periods and subjected to FACS analysis using propidium iodide staining. Control cells and doxorubicin-treated cells received the equivalent amount of DMSO in which SP600125 was dissolved. DMSO or SP600125 was added an hour before doxorubicin. Cell death is represented by the percentage (%) of cells in sub-G<sub>1</sub>. Histograms represent the mean cell death from three independent experiments ( $n=3$ ). Group statistical significance of the differences as calculated by ANOVA is with  $*P<0.0001$

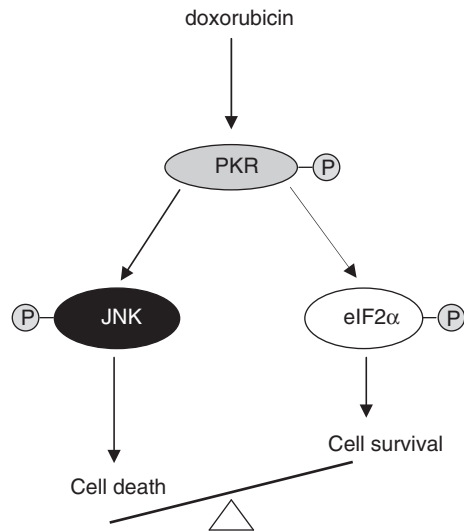
inhibition of protein synthesis by phosphorylated eIF2 $\alpha$ .<sup>33</sup> Based on our findings, the possibility remains that breast tumors and possibly other types of tumors with increased levels of PKR are more susceptible to doxorubicin than tumors with low levels of the kinase. However, such an increased susceptibility could be counteracted by the cytoprotective effects of induced eIF2 $\alpha$  phosphorylation after drug treatment. As such, doxorubicin in combination with compounds designed to impair eIF2 $\alpha$  phosphorylation (e.g., eIF2 $\alpha$  pseudosubstrate peptide-mimetic drugs) might prove to be effective means for the treatment of specific types of cancers with elevated PKR. Nevertheless, the therapeutic potential of PKR may also depend on the type of tumor based on evidence that PKR activation by chemotherapeutic drugs is

proapoptotic for some types of tumors<sup>34</sup> and cytoprotective for others.<sup>35</sup>

#### Materials and Methods

**Cell culture and treatments.** PKR<sup>-/-</sup> MEFs and their isogenic wild-type counterparts<sup>14</sup> were grown in Dulbecco's modified Eagle's medium (DMEM; Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Wisent) and 100 U/ml of penicillin-streptomycin (Wisent). Isogenic wild-type and PERK<sup>-/-</sup> MEFs<sup>36</sup> were grown in DMEM supplemented with 10% heat-inactivated bovine serum (Wisent) and 100 U/ml of penicillin-streptomycin. Isogenic wild-type and GCN2<sup>-/-</sup> MEFs<sup>37</sup> were grown in DMEM and supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin plus 1  $\times$  nonessential amino acids (Invitrogen, Carlsbad, CA, USA). Isogenic eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs<sup>38</sup> were grown in DMEM supplemented with 10% non-heat-inactivated bovine serum (Wisent) and 100 U/ml of penicillin-streptomycin plus 1  $\times$  nonessential amino acids





**Figure 7** A model for PKR function in response to doxorubicin. Activation of PKR by doxorubicin leads to the induction of eIF2 $\alpha$  phosphorylation, which exerts a cytoprotective effect. Induction of PKR activity also leads to the activation of JNK, which promotes cell death. JNK activation proceeds independently of eIF2 $\alpha$  phosphorylation and is sufficient to overcome the cytoprotective effects of eIF2 $\alpha$  phosphorylation in immortalized MEFs

and 1  $\times$  essential amino acids (Invitrogen). HT1080, H1299 and A549 cells were grown in DMEM supplemented with 10% heat-inactivated bovine serum (Wisent) and 100 U/ml of penicillin–streptomycin. Cells were treated with 1  $\mu$ M of doxorubicin hydrochloride (Sigma, Oakville, ON, Canada) dissolved in water as well as 10  $\mu$ M SP600125 (Calbiochem, Burlington, ON, Canada), 30  $\mu$ M wortmannin (Bioshop, Burlington, ON, Canada) or 2  $\mu$ M of KU55933 (Tocris Biosciences, Ellisville, MO, USA) dissolved in dimethyl sulfoxide (DMSO).

**Protein extraction and immunoblotting.** Protein extraction and quantification was performed as described elsewhere.<sup>6</sup> For immunoblotting of caspase-3, we prepared extracts as previously described in Cheong *et al.*<sup>39</sup> Immunoblotting of the rest of proteins was performed as previously described.<sup>6</sup> The primary antibodies used are: rabbit polyclonal phospho-specific against S51 of eIF2 $\alpha$  (Invitrogen), mouse monoclonal to eIF2 $\alpha$  (Cell Signaling, Danvers, MA, USA), rabbit polyclonal against cleaved caspase-3 (Cell signaling), mouse monoclonal to actin (MP Biomedicals, Solon, OH, USA), rabbit polyclonal phospho-specific against T183/Y185 JNK1/2 (Cell Signaling), rabbit polyclonal to JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal phospho-specific against T202/Y204 of ERK1/2 (Cell Signaling), rabbit polyclonal to ERK1/2 (Cell Signaling), rabbit polyclonal phospho-specific against T180/Y182 of p38 (Cell Signaling), rabbit polyclonal to p38 (Cell Signaling), rabbit polyclonal to  $\gamma$ H2AX (Upstate Biotechnology, Temecula, CA, USA), rabbit polyclonal against H2AX (Thermo Scientific, Rockford, IL, USA), rabbit monoclonal phospho-specific to T446 of PKR (Abcam, Cambridge, MA, USA), mouse monoclonal against PKR (F9),<sup>40</sup> rabbit polyclonal against ATF4 (Proteintech Group, Chicago, IL, USA), mouse monoclonal to GyrB (John Innes Enterprises, Norwich, UK). All antibodies were used at a concentration of 0.1–1  $\mu$ g/ml. After incubation with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:1000 dilution; KPL, Gaithersburg, MD, USA), proteins were visualized with the enhanced chemiluminescence detection system according to the manufacturer's protocol (PerkinElmer Life Sciences, Waltham, CA, USA). Quantification of the bands was performed by densitometry using the Scion Image software (Frederick, Maryland, USA).

**Cell staining and flow cytometry analysis.** Cells were subjected to propidium iodide staining and flow cytometry analysis as previously described.<sup>6</sup>

**Statistical analysis.** All quantitative variables are presented as means  $\pm$  S.E. We compared the differences of more than two groups using one-way ANOVA and the differences of two groups using two-tailed Student's *t*-test (GraphPad Prism 5, La Jolla, CA, USA), and  $P < 0.05$  was considered statistically significant.

### Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)