EGF receptor and PKCδ kinase activate DNA damage–induced pro-survival and pro-apoptotic signaling via biphasic activation of ERK and MSK1 kinases

 Angela M. Ohm[1], Trisiani Affandi[1], and Mary E. Reyland[2]

From the Department of Craniofacial Biology, School of Dental Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045

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DNA damage–mediated activation of extracellular signal–regulated kinase (ERK) can regulate both cell survival and cell death. We show here that ERK activation in this context is biphasic and that early and late activation events are mediated by distinct upstream signals that drive cell survival and apoptosis, respectively. We identified the nuclear kinase mitogen-sensitive kinase 1 (MSK1) as a downstream target of both early and late ERK activation. We also observed that activation of ERK→MSK1 up to 4 h after DNA damage depends on epidermal growth factor receptor (EGFR), as EGFR or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)/ERK inhibitors or short hairpin RNA–mediated MSK1 depletion enhanced cell death. This prosurvival response was partially mediated through enhanced DNA repair, as EGFR or MEK/ERK inhibitors delayed DNA damage resolution. In contrast, the second phase of ERK→MSK1 activation drove apoptosis and required protein kinase Cδ (PKCδ) but not EGFR. Genetic disruption of PKCδ reduced ERK activation in an *in vivo* irradiation model, as did short hairpin RNA–mediated depletion of PKCδ *in vitro*. In both models, PKCδ inhibition preferentially suppressed late activation of ERK. We have shown previously that nuclear localization of PKCδ is necessary and sufficient for apoptosis. Here we identified a nuclear PKCδ→ERK→MSK1 signaling module that regulates apoptosis. We also show that expression of nuclear PKCδ activates ERK and MSK1, that ERK activation is required for MSK1 activation, and that both ERK and MSK1 activation are required for apoptosis. Our findings suggest that location-specific activation by distinct upstream regulators may enable distinct functional outputs from common signaling pathways.

Irradiation (IR)[3] and chemotherapy are widely used for the treatment of cancer; however, adjacent healthy tissues can also be damaged, including the mucosal tissues of the gut and oral cavity. Such collateral tissue damage can result in significant comorbidities and, in some cases, limit the course of therapy (1). As IR and chemotherapies work in part by inducing apoptosis, modulation of apoptotic mediators specifically in nontumor cells could offer protection against collateral tissue damage, resulting in improved quality of life and, in some cases, better tumor eradication (2–4). PKCδ is a ubiquitously expressed serine–threonine kinase that controls a wide variety of cell functions, including proliferation, cell survival, invasion and migration, and cell death (5). PKCδ has been identified as an important mediator of DNA damage–induced apoptosis, and our laboratory and others have shown that inhibition of PKCδ activation can reduce IR and chemotherapy-induced apoptosis *in vitro* and toxicity to nontumor tissues *in vivo* (6–9). Remarkably, inhibition of PKCδ has been shown to preserve salivary gland function in mice exposed to head and neck IR but did not impact treatment of the tumor (8). This supports previous data from our laboratory that suggests that, in contrast to normal cells, in some tumor cells PKCδ does not regulate apoptosis but may instead have a prosurvival role (10–12).

Studies from our laboratory indicate that nuclear PKCδ is required for apoptosis, suggesting that PKCδ function may be dictated in part by its subcellular localization (13–18). Further, most studies suggest that PKCδ does not directly regulate the apoptotic machinery but may instead integrate upstream signals to regulate cell fate decisions in response to cell distress or damage (2, 17, 19, 20). In this regard, PKCδ has been shown to regulate signaling through the mitogen-activated protein kinase (MAPK) pathways (extracellular signal–regulated kinases (ERK), c-Jun N-terminal kinases, and p38 MAPKs), primarily downstream of growth factor receptors (21–23) but also in response to DNA damage (24).

The MEK/ERK pathway has well-established roles in proliferation and survival and regulates cell cycle arrest and apoptosis in response to DNA damage (21, 22, 25–27). In damaged cells, the duration, magnitude, and subcellular localization of ERK1/2 activation may be critical in determining whether the outcome is prosurvival or pro-apoptotic (21, 25). We show that activation of ERK in response to DNA damage agents is bipha-

Figure 1. Biphasic activation of ERK drives survival and apoptosis. *A*, ParC5 cells were treated with 50 μM etoposide for the indicated times (hours). Cells were harvested and immunoblotted for pERK and stripped and reprobed for ERK and β-actin. *B*, quantitation by densitometry of three separate experiments similar to that shown in *A*. Data represent pERK/ERK/actin ratios $\pm$ S.E. Statistics represent one-way ANOVA and *post hoc* Tukey's multiple comparisons. **, $p <$ 0.001; *, $p <$ 0.05. *C*, ParC5 cells were pretreated with the DMSO control (*black columns*) or 10 μM PD98059 (*gray columns*) prior to addition of 50 μM etoposide for the indicated times (hours). *D*, ParC5 cells were pretreated with the DMSO control (*black columns*), 20 μM PD98059 (*dark gray columns*), or 20 μM U0126 (*white columns*) prior to addition of 0, 0.5, 5, or 50 μM etoposide for 18 h. *E*, ParC5 cells were pretreated with the DMSO control (*black columns*), 20 μM or 40 μM PD98059 (*dark gray columns*), or 20 μM or 40 μM U0126 (*light gray* and *white columns*) and then left untreated or treated with 10 Gy of IR. Cells were harvested 48 h after IR. Caspase-3 activity (*C* and *E*) and DNA fragmentation (*D*) were assayed as described under "Experimental procedures." Data shown are representative experiments; statistics represent two-way ANOVA for comparison of time point or treatment with the corresponding control. **, $p <$ 0.001; *, $p <$ 0.05. *Error bars* represent S.E. from triplicate samples.

sic, consisting of an early prosurvival phase and a later pro-apoptotic phase. These phases are mediated by distinct upstream regulators, with EGFR activating ERK in the early phase and PKCδ activating late-phase ERK. Furthermore, we identify a unique ERK→MSK1 signaling module regulated by nuclear PKCδ that is essential for apoptosis. Our study shows that DNA damage induces temporally distinct prosurvival and pro-apoptotic signaling pathways and suggest that the functional output of ERK→MSK1 activation in response to DNA damage is regulated, at least in part, by the upstream activator.

Results

### In response to DNA damage, biphasic activation of ERK drives survival and apoptosis

ParC5 rat parotid acinar cells provide a useful model to study DNA damage–induced cell death, as their response to irradiation is similar to that observed in salivary acinar cells *in vivo* (3). In parC5 cells treated with etoposide, ERK activation is biphasic, with an initial peak at around 2 h and a second peak at 6–8 h (Fig. 1*A*). Similar results were seen when cells were treated with IR (Fig. 2*B*). Quantification of pERK/ERK across multiple time points from three independent experiments is shown in Fig. 1*B*. To determine whether one or both of peaks of ERK activity are required for apoptosis, we assayed activation of caspase-3 in cells pretreated with PD98059, which inhibits

MEK, the upstream regulator of ERK. When apoptosis was assayed 2–4 h after addition of etoposide, pretreatment with PD98059 resulted in an up to 4-fold increase in apoptosis, demonstrating that early activation of ERK conveys a survival signal (Fig. 1*C*). However, at 8 h, PD98059 slightly suppresses apoptosis. Consistent with this, when parC5 cells were pretreated with PD98059 or a second MEK inhibitor, U0126, and then treated with etoposide for 18 h, apoptosis was significantly reduced compared with cells treated with etoposide alone (Fig. 1*D*). Similar results were seen with pretreatment using MEK inhibitors followed by IR (Fig. 1*E*). These data demonstrate that ERK activation in response to DNA damage can drive both survival and apoptosis and that these distinct functional outputs may be dictated by the kinetics of activation.

Activation of EGFR in response to DNA damage promotes cell survival

Activation of EGFR occurs rapidly but transiently in response to DNA damage, with kinetics similar to the early phase of ERK activation (Fig. 2*A*, *left*). Early activation of ERK requires EGFR activity, as it is abolished in cells pretreated with gefitinib (Fig. 2*A*, *right*). Notably, EGFR inhibition only blocked early activation of ERK (up to 4 h), with no effect on late activation of ERK (Fig. 2, *A* and *B*). However, both EGFR inhibitors increased apoptosis up to 8 h after addition of etoposide (Fig.
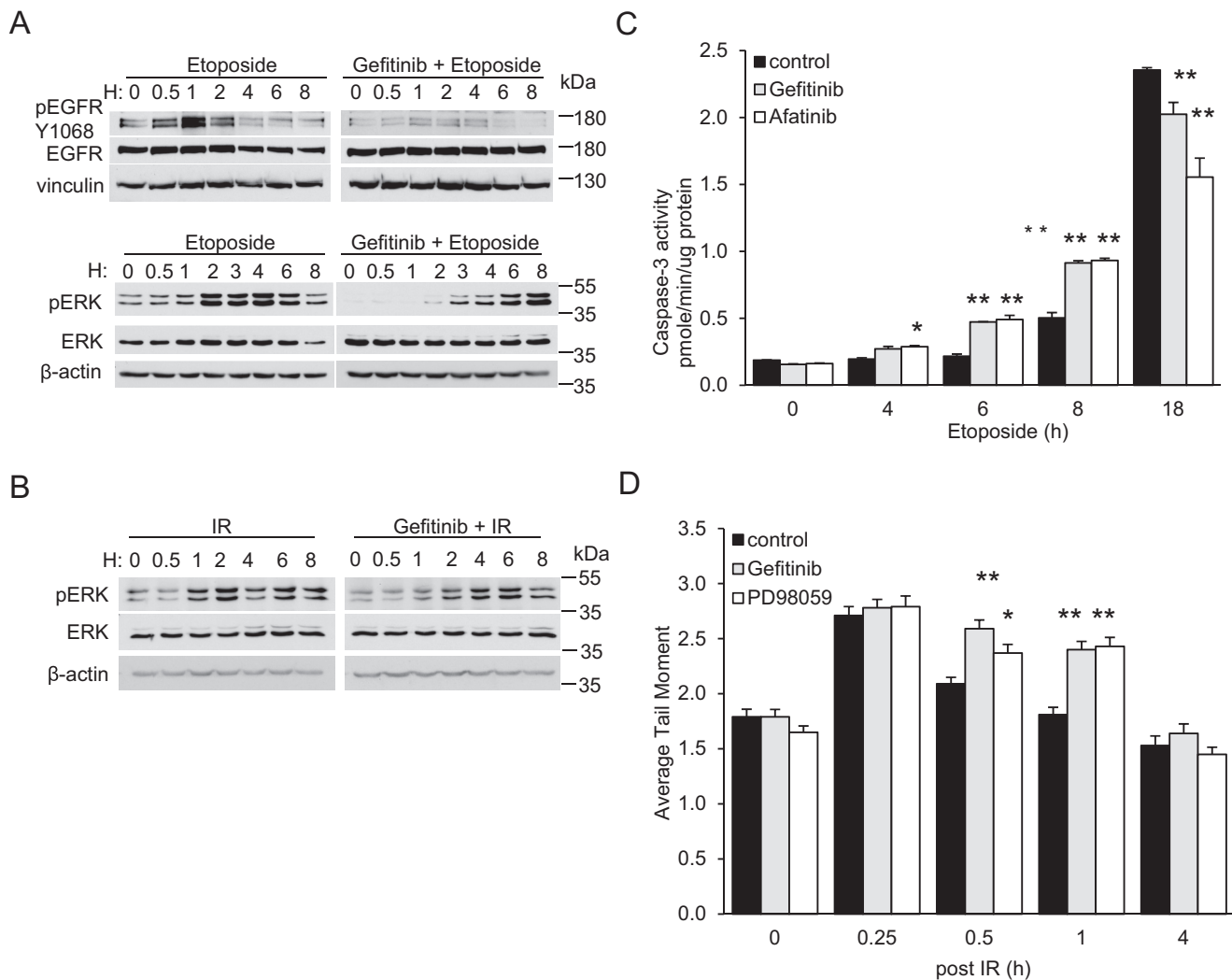
Figure 2. EGFR activation of ERK promotes cell survival. *A*, ParC5 cells were pretreated with the DMSO control or 600 nm gefitinib prior to addition of 50 μM etoposide for the indicated times (hours). Cells were harvested and immunoblotted for the indicated proteins. *B*, ParC5 cells were pretreated with the DMSO control or 300 nm gefitinib prior to treatment with 10 Gy of IR. Cells were harvested at the indicated times (hours) post-IR and immunoblotted for the indicated proteins. *C*, ParC5 cells were pretreated with the DMSO control or afatinib prior to addition of 50 μM etoposide. Cells were harvested for measurement of caspase-3 activity at the indicated times (hours). *D*, ParC5 cells were pretreated with DMSO, 300 nm gefitinib, or 20 μM PD98059 prior to irradiation with 10 Gy of IR. Following IR, cells were harvested at various time points (hours) and prepared using a neutral comet assay according to the Trevigen comet assay protocol. Data shown are representative experiments. Statistics represent two-way ANOVA for comparison of time point or treatment with the corresponding control. **, $p < 0.001$; *, $p < 0.05$.

2*C*), similar to that seen with the MEK inhibitor PD98059 (Fig. 1*C*).

Previous studies have suggested a role for EGFR and ERK in regulation of DNA double-stranded break repair, which could in part explain the prosurvival phenotype we observed (29–33). To test this, we assayed DNA damage using a comet assay in cells treated with IR and pretreated with gefitinib or PD98059. Although untreated cells and inhibitor-treated cells had a similar amount of DNA damage at 15 min, DNA damage was increased in inhibitor-treated cells 30 min and 1 h after IR compared with the control, indicating a delay in DNA repair with inhibition of ERK or EGFR (Fig. 2*D*). Our study shows that prosurvival signaling through EGFR→ERK correlates with enhanced DNA repair in the first few hours after DNA damage. This could occur via direct regulation of DNA repair by EGFR→ERK or through an indirect mechanism, such as prolonged activation of cell cycle checkpoints to allow repair.

PKCδ activation of ERK in response to DNA damage drives apoptosis

PKCδ is a pro-apoptotic kinase that also regulates ERK signaling in some biological contexts (5). We investigated the kinetics and magnitude of ERK activation in parC5 cells depleted of PKCδ using shRNA (Fig. 3*A*) and in salivary gland tissue lysates from PKCδ$^{+/+}$ and PKCδ$^{-/-}$ mice that were treated with IR to the head and neck (Fig. 3*B*). In PKCδ$^{+/+}$ mice, the pattern of ERK activation by IR was similar to that observed in cells treated with etoposide, with two phases of ERK activation at 2 and 6 h (Fig. 3*B*). In PKCδ$^{-/-}$ mice, both phases of ERK activation were reduced; quantitation of pERK/ERK by densitometry shows that the second peak (6 h) was more dramatically reduced than the first peak (2 h) (45% *versus* 80% reduction, respectively) (Fig. 3*B*). This suggests that depletion of PKCδ primarily inhibits the "pro-apoptotic" phase of
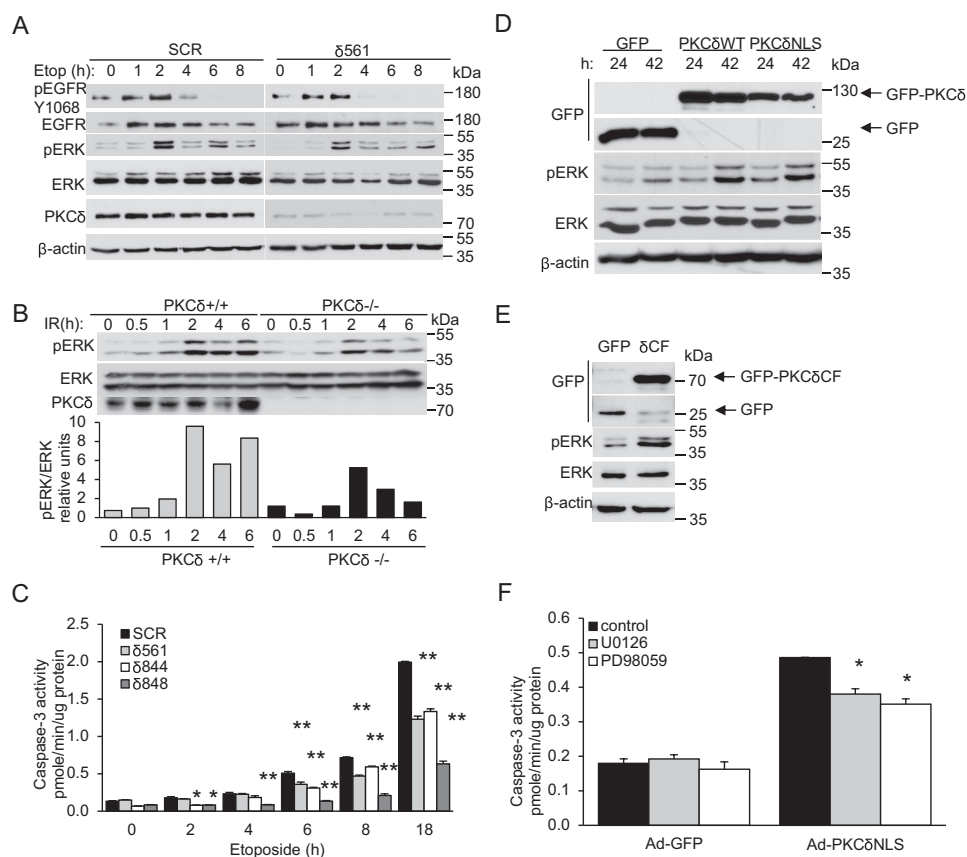
Figure 3. Nuclear PKCδ activates ERK to drive apoptosis. *A*, ParC5 cells stably expressing either PKCδ shRNA (δ561) or a scrambled control were treated with 50 μM etoposide (*Etop*) for the indicated times (hours). Lysates were immunoblotted for the indicated proteins. *B*, 6-week-old female PKCδ$^{+/+}$ and PKCδ$^{-/-}$ mice were irradiated with 5 Gy of IR and harvested at the indicated times (hours) post-IR. Protein lysates were prepared from the parotid gland and immunoblotted for pERK, ERK, and PKCδ. The graph shows relative densitometry units of pERK normalized to total ERK bands, with *gray* and *black columns* indicating PKCδ$^{+/+}$ and PKCδ$^{-/-}$, respectively. *C*, ParC5 cells stably expressing either PKCδ shRNA (δ561) or a scrambled control were treated with 50 μM etoposide for the indicated times (hours). Lysates were assayed for caspase-3 activity. *D*, ParC5 cells were transfected with constructs expressing GFP, PKCδWT, or PKCδNLS for the indicated times (hours). *E*, ParC5 cells were transfected with constructs expressing GFP or GFP-PKCδCF for 24 h. For both *D* and *E*, cells were harvested and immunoblotted for GFP, pERK, ERK, or β-actin. *F*, ParC5 cells were transduced with either Ad-GFP or Ad-GFP-PKCδNLS and plated in the presence of 10 μM U0126 (*gray columns*) or 20 μM PD98059 (*white columns*) or treated with the DMSO control (*black columns*). Cells were harvested and assayed 48 h post-transduction for caspase-3 activity. Data shown are representative experiments. Statistics represent two-way ANOVA for comparison of time point or treatment with the corresponding control. **, $p < 0.001$; *, $p < 0.05$.

ERK activation. To confirm this, we assayed ERK activation in parC5 cells depleted of PKCδ with shRNA (δ561) or a scrambled shRNA (SCR). Similar to PKCδ$^{-/-}$ mice, depletion of PKCδ resulted in a small decrease in the early phase of ERK activation by etoposide at 2 h and a more dramatic effect on later activation of ERK at 6 h (Fig. 3A). Our data suggest that PKCδ primarily regulates the second phase of ERK activation to drive apoptosis, independent of prosurvival signaling by ERK. Depletion of PKCδ also slightly decreased activation of EGFR in cells treated with etoposide (Fig. 3A), consistent with partial inhibition of the early phase of ERK activation (Fig. 3, *A* and *B*). To directly ask whether PKCδ contributes to cell survival in response to DNA damage, we analyzed caspase-3 activation in parC5 cells depleted of PKCδ. Depletion of PKCδ using three independent shRNAs suppressed apoptosis at all time points, indicating that PKCδ drives apoptosis and does not play a role in the early prosurvival effect through EGFR→ERK (Fig. 3C). Our study demonstrates that, in response to DNA damage, PKCδ activation of ERK appears to solely drive cell death signaling.

Nuclear translocation of PKCδ is required for DNA damage–induced apoptosis, and direct targeting of PKCδ to the nucleus induces apoptosis (13, 16). Therefore, we investigated the hypothesis that the pro-apoptotic phase of ERK activation is mediated through nuclear PKCδ. ParC5 cells were transduced with Ad-GFP, Ad-GFP-PKCδWT, or Ad-GFP-PKCδNLS, a construct where we added an SV40 NLS to PKCδ, resulting in direct targeting of PKCδ to the nucleus (15, 16). Remarkably, expression of either Ad-GFP-PKCδWT or Ad-GFP-PKCδNLS resulted in increased ERK activation (Fig. 3D). PKCδCF is a caspase-cleaved form of PKCδ generated in response to DNA damage and is a potent inducer of apoptosis (28). PKCδCF is constitutively nuclear because of loss of constraints imposed by the regulatory domain of the kinase (16). As shown in Fig. 3E, expression of pEGFPN1-PKCδCF robustly induces pERK, further supporting our conclusion that nuclear localization of PKCδ is sufficient to activate ERK. To determine whether ERK activation is required for apoptosis induced by nuclearly targeted PKCδ, parC5 cells transduced with Ad-GFP-PKCδNLS were treated with the MEK inhibitors PD98059 and U0126.
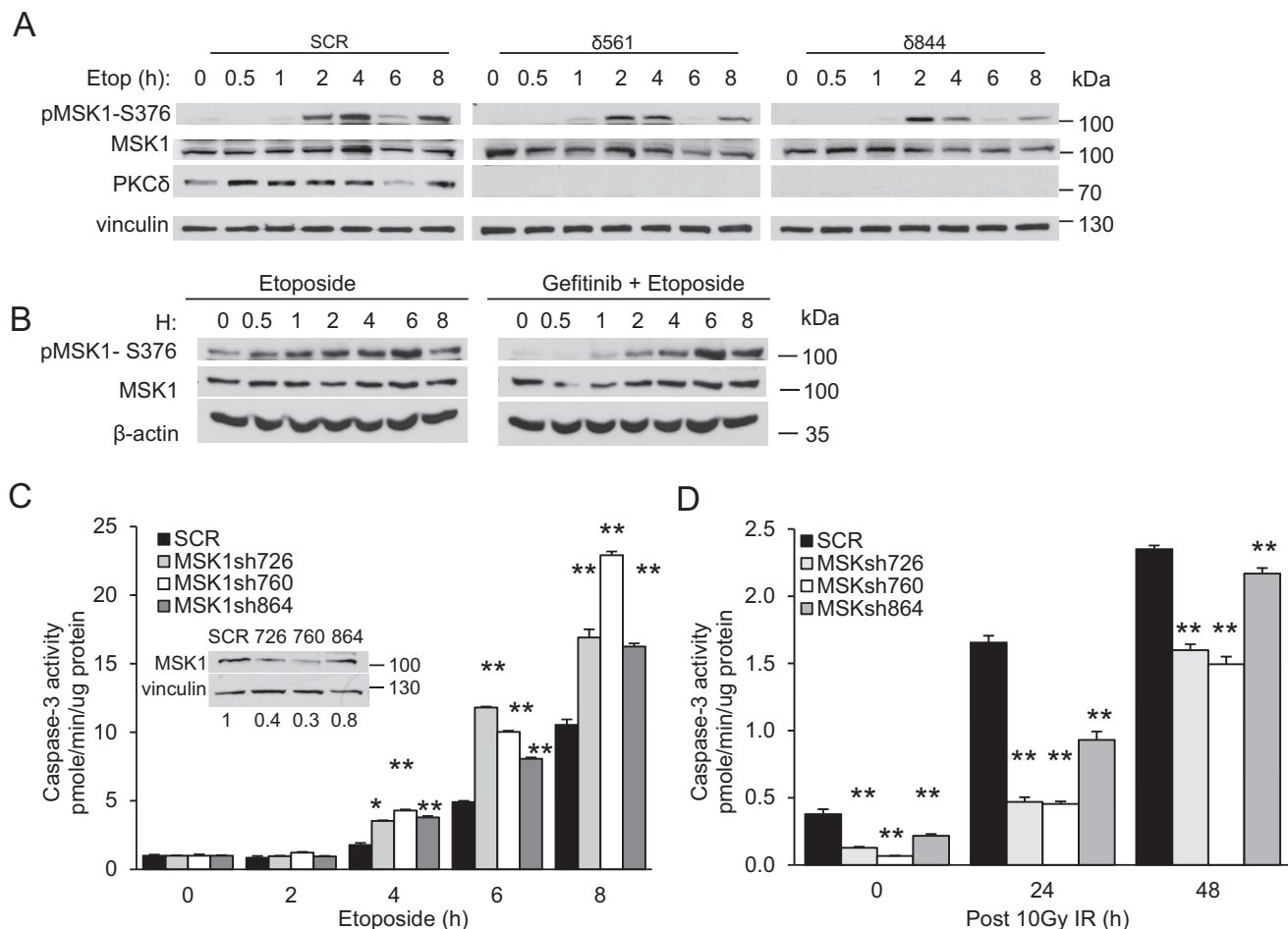
Figure 4. ERK activates MSK1 in response to DNA damage. *A*, ParC5 cells stably expressing either control (SCR) or PKCδ shRNA (δ561 or δ844) were treated with 50 μM etoposide (*Etop*) for the indicated times (hours). Cells were harvested and immunoblotted for the indicated proteins. *B*, ParC5 cells were pretreated with the DMSO control or 600 nM gefitinib prior to addition of 50 μM etoposide for the indicated times (hours). Cells were harvested and immunoblotted for the indicated proteins. *C* and *D*, ParC5 cells stably expressing either SCR control or MSK1 shRNA were treated with 50 μM etoposide (*C*) or irradiated with 10 Gy of IR (*D*). Cells were harvested for caspase-3 activity at the indicated times. The blot in *C* demonstrates the magnitude of MSK1 depletion in the cells used in *C* and *D*. Data shown are representative experiments. Statistics represent two-way ANOVA for comparison of time point or treatment with the corresponding control. **, $p < 0.001$; *, $p < 0.05$.

Expression of Ad-GFP-PKCδNLS induced caspase-3 activation, as shown previously, and this could be suppressed by both MEK inhibitors, indicating a requirement for ERK activation downstream of PKCδ for induction of apoptosis (Fig. 3*F*).

ERK activates MSK1 in response to DNA damage

Our study shows that nuclear PKCδ induces apoptosis via activation of ERK; however, downstream targets of ERK that drive apoptosis are largely unknown. MSK1/2 kinases are activated downstream of ERK and p38 and predominantly localize to the nucleus, where they phosphorylate transcription factors and regulate chromatin remodeling (29–31). In response to DNA damage, MSK1 is activated in a biphasic manner with kinetics similar to what we have shown previously for ERK (Figs. 4*A* and 1*A*). Activation of MSK1 in δ561 and δ844 cells is suppressed compared with SCR cells (Fig. 4*A*). Similar to ERK (Fig. 3, *A* and *B*), loss of PKCδ suppresses late MSK1 activation (6 to 8 h) to a greater extent than early activation (2 to 4 h) (Fig. 4*A*), whereas pretreatment with gefitinib only suppresses early activation of MSK1 (Fig. 4*B*).

Our observation that MSK1 and ERK activation are co-regulated in response to DNA damage suggests that activation

of MSK1 may contribute to prosurvival signaling through EGFR→ERK and pro-apoptotic signaling through PKCδ→ERK. Indeed, depletion of MSK1 using three unique shRNAs results in increased apoptosis up to 8 h after addition of etoposide, indicating that, like ERK, early activation of MSK1 is prosurvival (Fig. 4*C*). In contrast, depletion of MSK1 dramatically decreases cell death 24–48 h after IR (Fig. 4*D*), analogous to what we described previously when MEK/ERK was inhibited (Fig. 1*E*). Our data support a model whereby, in response to DNA damage, EGFR activation of ERK and MSK1 promotes cell survival in the first few hours after DNA damage, whereas late activation of MSK1 downstream of PKCδ→ERK may be required for apoptosis.

PKCδ drives DNA damage–induced apoptosis via ERK→MSK1

As targeting PKCδ to the nucleus activates ERK (Fig. 3*D*), we asked whether nuclearly targeted PKCδ also activates MSK1 as evidenced by phosphorylation on Ser-376. As seen in Fig. 5*A*, transduction of parC5 cells with Ad-GFP-PKCδNLS results in activation of MSK1. MSK1 activation by Ad-GFP-PKCδNLS could be suppressed by pretreatment with the MEK/ERK inhibitor PD98059 but not the p38MAPK inhibitor SB20358 (Fig.
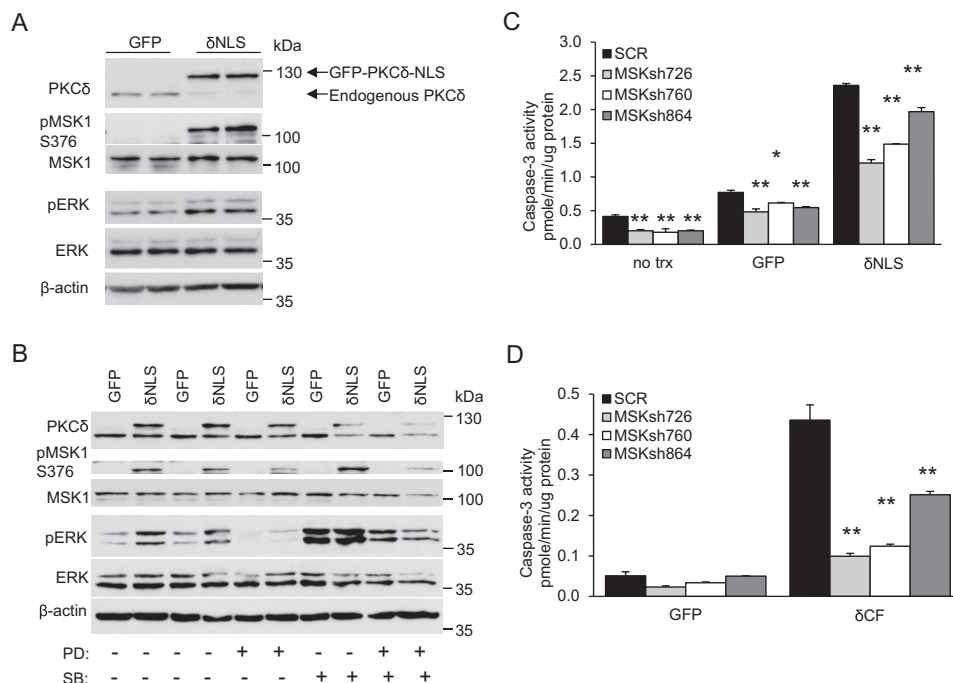
Figure 5. PKCδ drives DNA damage–induced apoptosis via ERK→MSK1. *A*, ParC5 cells were transduced with Ad-GFP or Ad-GFP-PKCδNLS (m.o.i. 200) for 24 h. Cells were harvested at the indicated times post-IR and immunoblotted for the indicated proteins. *B*, ParC5 cells were pretreated with 20 μM PD98059 (*PD*), 5 μM SB203580 (*SB*), or a combination of both concurrently with transduction of either Ad-GFP or Ad-GFP-PKCδNLS adenovirus (m.o.i. 200). Cells were then harvested and immunoblotted for the indicated proteins. *C* and *D*, ParC5 cells stably expressing either SCR control or MSK1 shRNA were either left untransfected or transfected with GFP, GFP-PKCδNLS (*C*), or GFP-PKCδCF (*D*) plasmid for 24 h. Cells were harvested and assayed for caspase-3 activity. Data shown are representative experiments. Statistics represent two-way ANOVA for comparison of time point or treatment with the corresponding control. **, $p < 0.001$; *, $p < 0.05$.

5*B*). Unexpectedly, inhibition of p38MAPK with SB20358 increased pERK in both Ad-GFP and Ad-GFP-PKCδNLS transduced cells; however, pMSK1 was only increased in cells that were also transduced with Ad-PKCδNLS (Fig. 5*B*). Notably, p38MAPK inhibition has no effect on etoposide induced apoptosis (Fig. S1), indicating that this pathway does not contribute to apoptotic signaling through PKCδ.

To verify a pro-apoptotic function for MSK1 downstream of nuclear PKCδ, we analyzed the ability of Ad-GFP-PKCδNLS to induce apoptosis in parC5 cells in which MSK1 was depleted with shRNA. Apoptosis driven by expression of Ad-GFP-PKCδNLS was suppressed in cells depleted of MSK1, and the degree of suppression was correlated with the amount of MSK1 depletion (Figs. 5*C* and 4*C*). Finally, we asked whether MSK1 is required for apoptosis induced by PKCδCF. Expression of pEGFPN1-PKCδCF potently induced apoptosis in control parC5 cells, but apoptosis was severely reduced in cells depleted of MSK1 (Fig. 5*D*).

Our study defines a common ERK→MSK1 pathway that, when activated by EGFR in response to DNA damage, drives cell survival but when activated by nuclear PKCδ drives apoptosis (Fig. 6). Moreover, we define a nuclear specific function for PKCδ in driving apoptosis through ERK→MSK1. Activation of ERK→MSK1 for survival *versus* apoptosis is temporally distinct, which may reflect access to specific upstream regulators.

Discussion

We show that, in the context of DNA damage, activation of ERK can have a dramatically different biological outcome
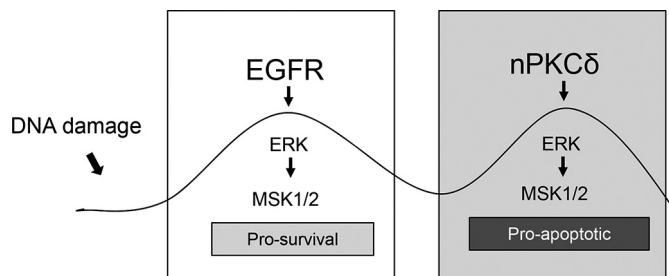
Figure 6. Biphasic activation of ERK and MSK1 in response to DNA damage regulates survival and apoptosis. Our study defines a common ERK→MSK1 pathway that, when activated by EGFR in response to DNA damage, drives cell survival but when activated by nuclear PKCδ drives apoptosis.

depending on when, how, and where in the cell it is activated. We identify temporally distinct prosurvival and pro-apoptotic signaling pathways that are both mediated through ERK→MSK1. Prosurvival signaling through EGFR→ERK enhances DNA repair in the first few hours after DNA damage through a PKCδ-independent pathway. In contrast, pro-apoptotic signaling through ERK occurs 6–8 h after DNA damage and is independent of EGFR but dependent on PKCδ. Furthermore, our study identifies a unique ERK→MSK1 signaling module regulated by nuclear PKCδ that is essential for DNA damage–induced apoptosis. Our study suggests that location-specific activation by distinct upstream regulators may enable common signaling pathways to have unique functional outputs.

The MEK/ERK pathway has well established roles in proliferation and survival and paradoxically regulates cell cycle arrest

and apoptosis, particularly in response to DNA damage (21, 22, 25–27). We show that biphasic activation of ERK in response to DNA-damaging agents sequentially activates prosurvival and pro-apoptotic signaling pathways. EGFR activation is required for the early, prosurvival phase of ERK activation in response to DNA damage. Notably, pretreatment of cells with gefitinib only suppressed the early phase of ERK activation, confirming that early and late ERK activation have distinct upstream regulators. The kinetics of activation of EGFR and early activation of ERK are similar to the kinetics of DNA repair, as assayed by comet, supporting published studies that show a role for EGFR in the regulation of IR-induced DNA double-stranded break repair (32–35). In some cases, EGFR regulation of DNA repair is associated with nuclear translocation of EGFR, and EGFR has been shown to interact with and regulate DNA repair machinery, including DNA-dependent protein kinase (32). EGFR can also regulate chromatin remodeling and DNA repair through tyrosine phosphorylation of histone H4 (36).

The second, pro-apoptotic phase of ERK activation is largely dependent on PKCδ and independent of EGFR. A pro-apoptotic function for PKCδ is supported by extensive studies *in vitro* and *in vivo* that demonstrate a role for PKCδ in apoptosis in response to many types of cell and tissue damage (3, 4, 10, 17, 18, 20). Furthermore, our study confirms that PKCδ does not contribute to cell survival signaling in response to DNA damage, as depletion of PKCδ suppresses the apoptotic response at all time points (Fig. 3C). However, the fact that ERK activation is only partially suppressed in irradiated tissues from a δ knockout mouse supports data suggesting that other pathways, such as Akt and NF-κB, can contribute to ERK activation in response to DNA damage (22).

MSK1/2 kinases, members of the AGC kinase family, are activated by the ERK1/2 and p38 pathways in response to extracellular stimuli and predominantly localize to the nucleus (29–31). Activation of MSK1/2 results in phosphorylation of transcription factors required for immediate-early gene expression and chromatin-associated proteins, including histone H3 (30, 37–39). In our study, MSK1 was activated by DNA damage with similar kinetics as ERK, and early MSK1 activation, like early ERK activation, was EGFR-dependent. We show that early activation of MSK1 through an EGFR→ERK pathway promotes cell survival, consistent with studies that show a role in stress resistance (30) and enhanced apoptosis in an MSK1/2 knockout mouse (40). Given its known role in chromatin modification, it is interesting to speculate that MSK1 could contribute to the increase in EGFR-induced DNA repair we observed in the first few hours after DNA damage.

Notably, prosurvival signaling through EGFR→ERK→MSK1 is transient; however, our study does not address how this signal is turned off. Nyati *et al.* (41) show that ataxia telangiectasia–mutated (ATM) activation can down-regulate ERK through activation of dual specificity phosphatase-1 (DUSP1), also known as MKP-1. Likewise, nuclear localized DUSPs, such as DUSP5, are candidates for regulation of the nuclear level of activated ERK (42). Inhibition of ERK activation through DUSP6 has been shown to enhance the DNA damage response and increase sensitivity to EGFR inhibitors (43). Finally, it is

possible that prosurvival ERK signaling does not need to be "turned off" for PKCδ to induce pro-apoptotic ERK signaling, as sustained ERK activation has been shown to induce apoptosis (21, 25).

As PKCδ is ubiquitously expressed and regulates a wide variety of cell functions in addition to apoptosis, it is important to understand how apoptotic specific signaling by PKCδ is mediated (5). We have shown that nuclear accumulation of PKCδ is necessary for apoptosis in response to DNA-damaging agents and sufficient for apoptosis in many cell types (13–18). This suggests that the subcellular localization of PKCδ in the nucleus may dictate the apoptosis-specific functions of PKCδ. Conversely, it is tempting to speculate that localization of PKCδ in other cellular compartments may dictate other functions, such as proliferation and cell migration. In this study, we use a constitutively nuclear targeted form of PKCδ to address the role of nuclear PKCδ in the regulation of pro-apoptotic signaling through ERK→MSK1. Expression of PKCδNLS potently activates ERK and MSK1, and activation of these kinases is necessary for PKCδNLS to induce apoptosis (Fig. 5). Likewise, expression of δCF also induces ERK and apoptosis through an MSK1-dependent pathway (Figs. 3E and 5D). Although our study shows that nuclear targeting of PKCδ is sufficient to activate ERK→MSK1, it should be noted that PKCδWT can also activate ERK (Fig. 3D). Thus we cannot rule out a role for PKCδ in activating cytoplasmic ERK→MSK1. Indeed, PKCδ is a known effector of growth factor signaling and has been shown to activate ERK in this context (9).

MSK1 is largely associated with cell survival through regulation of transcription of stress-related genes (34). This is consistent with the prosurvival role we demonstrate for early activation of MSK1 (Fig. 4C). Early activation of MSK1, like ERK, occurs independently of PKCδ but depends on EGFR (Fig. 4, A and B). As MSK1 is predominantly a nuclear kinase, further studies are needed to clarify whether EGFR activates a cytoplasmic or nuclear pool of MSK1. Our study defines an additional role for MSK1 in the regulation of cell death and shows that it is a component of the signaling cascade regulated by nuclear PKCδ that activates the apoptotic response. How PKCδ regulates nuclear ERK→MSK1 signaling is not known, although unpublished studies from our laboratory suggest that PKCδ may regulate the abundance of nuclear and cytoplasmic DUSPs. Likewise, additional studies will be needed to decipher how the ERK→MSK1 pathways induce apoptosis. It is tempting to speculate that, as a chromatin-modifying kinase, MSK1 could contribute to DNA repair by regulating the accessibility of damaged DNA to repair complexes.

Our identification of an ERK→MSK1 signaling cascade that can regulate cell survival or apoptosis, depending on how and where it is activated, suggests a common mechanism that can be tuned according to the desired outcome. Conceivably, these pathways could also have shared targets that promote different functional outcomes dictated by the specific biological context. Clarification of prosurvival and pro-apoptotic targets of ERK→MSK1 is essential to maximize the therapeutic potential of targeting these pathways.

Experimental procedures

Cell culture and generation of shRNA stable knockdown cell lines

Culture of the parC5 cell line has been described previously (44). ParC5 cells were stably depleted of target protein by transducing with either a nontargeting lentiviral shRNA (SCR) or lentiviral shRNAs against Protein Kinase C Delta TRCN0000280561 (δ561), TRCN0000022844 (δ844), TRCN0000022848 (δ848) (Sigma-Aldrich, St. Louis, MO), or Rps6ka5 (MSK1) Smartvector rat lentiviral set V3SR11245-14EG314384 (MSK1sh726, MSK1sh760, and MSK1sh864) (Dharmacon, Lafayette, CO). Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium/high-glucose medium (Thermo Scientific, Logan, UT; SH30243.02) supplemented with 10% fetal bovine serum (Sigma-Aldrich, F2442) and transfected using FuGENE 6 (Promega, Madison, WI) to produce a lentivirus containing the shRNA described above. The MEK inhibitors PD98059 and U0126 and the P38 inhibitor SB203580 were obtained from Tocris (Minneapolis, MN). The EGFR inhibitors gefitinib and afatinib were obtained from Selleckchem (Houston, TX). All inhibitors were added 30 min prior to addition of either 50 μM etoposide (Sigma-Aldrich) or exposure to γ-irradiation from a cesium-137 source.

Immunoblot analysis

Immunoblot analysis was performed as described previously (3). Polyvinylidene difluoride membranes were stained with Ponceau S (Sigma-Aldrich, P3504) following transfer to confirm equal transfer and loading. The following antibodies were obtained from Cell Signaling Technology (Danvers, MA): phospho-p44/42 MAPK (ERK1/2) (Thr-202/Tyr-204) (CST4370), p44/42 MAPK (ERK1/2) (CST4696), phospho-EGFR (Tyr-1068) (CST3777), EGFR (CST2232), phospho-MSK1 (Ser-376) (CST9591), p38 (CST9212), PKCδ (CST2058), and vinculin (CST13901). Antibodies to β-actin (ab49900) and GFP (ab290) were obtained from Abcam (Cambridge, MA). Antibodies to phospho-p38 (Thr-180, Tyr-182; NB500-138) and MSK1 (bs-2995R) were obtained from Novus (Centennial, CO) and Bioss Antibodies Inc. (Woburn, MA), respectively. Area density analysis was performed on the indicated blots using VisionWorks software from Analytikjena.

Comet assay

Materials for the comet assay were purchased from Trevigen (Gaithersburg, MD). ParC5 cells were treated with DMSO, 300 nM gefitinib, or 20 μM PD98059 and incubated at 37 °C for 1 h prior to irradiation using a cesium-137 source (10 Gy). Following IR, cells were harvested at the indicated times for measurement of DNA damage using the neutral comet assay according to the Trevigen comet assay protocol. Images were analyzed by Trevigen Comet Analysis Software (version 1.3d). Tail moment was used to quantify DNA damage.

Expression of PKCδ constructs

Overexpression of all PKCδ constructs was achieved using either adenoviral transduction or plasmid transfection, as described previously (13, 16). For adenoviral infection, parC5 cells were transduced with Ad-PKCδWT-GFP, Ad-PKCδNLS-GFP, or Ad-GFP at m.o.i. ranging from 50 to 200 focus-forming units/cell. Infection was allowed to proceed for 24–48 h, as indicated. Transient transfections of plasmid pEGFPN1 and pEGFPN1-PKCδ constructs (δWT, δNLS, and δCF) were performed using Jetprime transfection reagent (Genesee Scientific, El Cajon, CA).

In vivo irradiation

C57Bl/6 PKCδ$^{+/+}$ and PKCδ$^{-/-}$ mice (45) were maintained at the University of Colorado Anschutz Medical Campus in accordance with laboratory animal care guidelines and protocols and with approval of the University of Colorado Denver Institutional Animal Use and Care Committee. Six- to eight-week-old female mice were anesthetized as described, and the head and neck regions were irradiated using a cesium-137 source, whereas the remainder of the body was shielded with lead. Salivary glands were harvested and tissue extracts prepared as described previously (3).

Analysis of apoptosis in vitro

Active caspase-3 was detected with the Caspase-3 Cellular Activity Assay Kit PLUS (Biomol, Farmingdale, NY; BML-ALK7030001), which uses *N*-acetyl-DEVD-p-nitroaniline as a substrate, according to the manufacturer's instructions. DNA fragmentation was assayed using the Cell Death Detection ELISA Plus Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Statistics

Data shown in figures are representative experiments repeated a minimum of three times. *Error bars* indicate standard error. Caspase-3 activity, DNA fragmentation, and comet assays were designed with triplicate biological samples. Statistics were determined using GraphPad Prism 7 software. A two-way ANOVA ($\alpha = 0.05$) with Dunnett's multiple comparisons ($^{**}, p < 0.001; ^{*}, p < 0.05$) was performed within each time point or treatment, comparing each column (*gray* or *white*) with the corresponding control (*black*).

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