MAP4K3 modulates cell death via the post-transcriptional regulation of BH3-only proteins

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Intracellular signal transduction networks involving protein kinases are important modulators of cell survival and cell death in multicellular organisms. Functional compromise of these networks has been linked to aberrant apoptosis in diseases such as cancer. To identify novel kinase regulators of cell death, we conducted an RNAi-based screen to identify modulators of the intrinsic apoptosis pathway. Using this approach, we identified MAP4K3 as a novel apoptosis inducer. Here, we present evidence that this pro-apoptotic kinase orchestrates activation of BAX via the concerted posttranscriptional modulation of PUMA, BAD, and BIM. Additionally, we found decreased levels of this kinase in pancreatic cancer samples, suggesting a tumor suppressor role for MAP4K3 in pancreatic tumorigenesis.

apoptosis | mitochondria | RNAi screen | signal transduction | GLK

Cellular and tissue homeostasis in vertebrates depends on the activities of intracellular signal transduction pathways that modulate cell division and survival, as well as programmed cell death. The ability of tumor cells to increase in number is determined by both the rates of cell proliferation and cellular removal by programmed cell death involving apoptosis. Importantly, acquired resistance to apoptosis is a hallmark of perhaps all types of cancer (reviewed in ref. 1).

Apoptosis can be induced by cell surface receptors (extrinsic pathway) or by various genotoxic agents, metabolic stresses, or transcriptional cues (intrinsic pathway) (reviewed in ref. 2). The intrinsic pathway of apoptosis is initiated by pro-apoptotic BH3-only proteins belonging to the BCL-2 family. BH3-only proteins act as initial sensors of apoptotic signals emanating from multiple cellular processes. BH3-only protein expression can be induced by transcription factors. For example, *PUMA* is induced by the tumor suppressor p53 in response to DNA damage (3). BH3-only proteins can also be activated posttranslationally; for example, BAD is activated upon loss of phosphorylation in response to growth-factor deprivation, and BID is activated through caspase-8-mediated proteolytic processing. Once activated, BH3-only proteins bind and neutralize anti-apoptotic BCL-2 family members, leading to relief of their inhibitory effect toward the pro-apoptotic BCL-2 proteins BAX and BAK. Additionally, it has been reported that the active forms of certain BH3-only proteins (tBID and BIM) interact directly with BAX, leading to its activation (2). When activated, BAX and BAK promote cytochrome *c* release, activation of the caspase-9-containing apoptosome and downstream activation of execution caspases such as caspase-3. Activated execution caspases coordinate apoptotic cell death via the proteolytic processing of several key cellular substrates (4).

Ste20p (sterile 20 protein) is a putative yeast mitogen-activated protein kinase kinase kinase (MAP4K) involved in the mating pathway. MAP4K3 (also known as germinal center-like kinase, GLK) is a member of the Ste20 family of protein kinases and is known to be activated by UV radiation and the pro-inflammatory cytokine TNF- α (5). Here, we report the identification of human MAP4K3, a Ste20/germinal center kinase family MAP4K implicated in Jun N-terminal kinase (JNK) and mammalian Target of Rapamycin (mTOR) signaling (5, 6), as an apoptosis inducer in selective screening for kinase suppressors of the apoptotic intrinsic

pathway. A pathway is elucidated in which MAP4K3 induces caspase-dependent apoptotic cell death. We describe a novel mechanism by which apoptosis induced by MAP4K3 involves the selective induction of BH3-only proteins, leading to BAX activation. MAP4K3 seems to promote an mTORC1-dependent increase in the stability of the BCL-XL neutralizers PUMA and BAD. Additionally, MAP4K3-dependent JNK signaling results in the phosphorylation of BIM, a direct activator of BAX. We have also detected decreased levels of MAP4K3 in pancreatic tumors, which are in line with a recent study suggesting that this kinase might be an important modulator of pancreatic tumorigenesis (7).

Results

Identification of the MAP4K3 Kinase as an Inducer of the Intrinsic Cell Death Pathway Using a Selective RNAi Screen. We conducted a forward genetic screen to identify novel suppressors of the intrinsic cell death pathway using DNA damage as a cell death inducer. To achieve this objective, we used a retrovirus-encoded RNAi library targeting the human kinome (8) according to the approach outlined in Fig. 1A. This screen revealed significant enrichment in the levels of shRNAs targeting the MAP4K3 protein kinase in cells that survived UV-induced cell death (Table S1). Notably, levels of shRNAs targeting *MAP4K3* were also enriched before cell death induction, indicating that suppression of this kinase might also confer a proliferative advantage to cells. We sought to confirm whether suppression of MAP4K3 could enhance resistance to cell death using 3 independent siRNAs targeting *MAP4K3*. Transfection of cells with these siRNAs resulted in a significant decrease in *MAP4K3* mRNA and protein levels (Fig. 1B and C). We next examined the effect of *MAP4K3* knockdown on the survival of cultured cells after treatment with DNA-damaging agents to induce the intrinsic apoptosis pathway. For control purposes, we compared the effects of *MAP4K3* knockdown to those of scrambled siRNAs as well as siRNAs targeting the prosurvival gene *BCL-XL*. These experiments confirmed that suppression of *MAP4K3* results in enhanced resistance to cell death following DNA damage induced by exposure to UV or cisplatin (Fig. 1D–F), suggesting that MAP4K3 acts as a general mediator of DNA damage-induced cell death.

Given that *MAP4K3* shRNAs were present in a substantial proportion of our cell population before UV selection (approximately 6%, see Table S1), we wished to determine whether MAP4K3 suppression resulted in enhanced proliferation. To test this hypothesis, we compared DNA synthesis in *MAP4K3*-silenced cells to that in control cells using a BrdU incorporation ELISA (supporting information (SI) Fig. S1). This analysis showed that

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Fig. 1. Suppression of MAP4K3 results in increased resistance to DNA damage-induced cell death. (A) Diagram illustrating the strategy used for the identification of putative suppressors of UV-induced apoptosis using a viral shRNA library. U2OS cells stably expressing a pool of shRNA constructs were selected following UV irradiation (50 J/m^2) to induce cell death. Surviving cells were expanded and the genomic DNA was isolated from cell pools both before (gray) and after (orange) UV selection. The viral shRNAs were PCR amplified and sequenced. (B) Down-regulation of *MAP4K3* mRNA in U2OS cells. Three independent siRNAs were used to target *MAP4K3* (#1455, #1550, #1640). The fold-change in the *MAP4K3* transcript level in cells transfected with *MAP4K3* siRNAs compared with cells transfected with control siRNA is indicated. Mean values $\pm$ SD are shown; $n = 3$. (C) Down-regulation of MAP4K3 protein by *MAP4K3*-specific siRNA in U2OS cells. Cell lysates were analyzed with a MAP4K3-specific antibody as well as α -tubulin as a loading control. (D) Down-regulation of *MAP4K3* results in enhanced cell survival following UV-mediated DNA damage. Nuclei of surviving cells are stained with Hoechst. (Scale bar, $200 \mu\text{m}$.) (E) Enhanced cell survival following down-regulation of *MAP4K3* in UV-treated cells. Viability is shown relative to nonirradiated control cells for each siRNA transfection. (F) Enhanced cell survival following down-regulation of *MAP4K3* in cisplatin-treated cells ($100 \mu\text{M}$). Viability is shown relative to nontreated control cells for each siRNA transfection. Data are presented as mean values $\pm$ SD; $n = 3$ in each group. Statistically significant values relative to the treated controls are indicated with asterisks (one-way ANOVA with Bonferroni posttest).

suppression of MAP4K3 resulted in enhanced proliferation in asynchronously dividing cells.

MAP4K3 Activates the Mitochondria-Dependent Intrinsic Apoptosis Pathway. Given that suppression of MAP4K3 resulted in significant resistance to DNA damage-induced cell death, we next sought to determine whether its overexpression is detrimental to cell survival. We expressed MAP4K3, as well as several mutant versions of this kinase, in cultured cells (Fig. 2A) to determine the effect of its overexpression on cell death. Expression of the active kinase resulted in cell shrinking and chromatin condensation, which are characteristic of apoptosis. Additionally, we observed that transfection with a kinase-inactive mutant, a deletion mutant lacking the

Fig. 2. MAP4K3 induces mitochondria-dependent apoptotic cell death. (A) Schematic representation of the human MAP4K3 constructs used for overexpression studies. The shaded area corresponds to the N-terminal kinase domain. The arrow indicates the terminal amino acid position of the truncated versions. The white area corresponds to the region of this kinase containing the putative PEST (proline, glutamic acid, serine, and threonine) domains. (B and C) Expression of MAP4K3 results in caspase-dependent cell death. U2OS cells were transiently transfected with control (GFP), the indicated MAP4K3 expression constructs, or a caspase-8-GFP construct, and were left untreated (–) or were treated (+) with the broad-spectrum caspase inhibitor zVADfmk. GFP-positive cells were visualized by fluorescence microscopy. Cells with apoptotic chromatin condensation were scored based on fluorescence. A minimum of 300 GFP-positive cells were analyzed for each transfection. (Scale bar, $10 \mu\text{m}$.) Data are presented as mean values $\pm$ SD; $n = 3$ in each group. Statistically significant values relative to MAP4K3 (FL) are indicated with asterisks (one-way ANOVA with Bonferroni posttest). (D and E) Expression of MAP4K3 results in BAX activation. U2OS cells were transiently transfected with the control vector (GFP) and both kinase-active and kinase-dead MAP4K3 expression constructs in the presence of zVADfmk. BAX activation was assessed in GFP-positive cells by immunofluorescence, using an antibody that detects conformationally active BAX. Between 100 and 300 GFP-positive cells were analyzed in each transfection. (Scale bar, $10 \mu\text{m}$.) Data are presented as mean values $\pm$ SD; $n = 3$ in each group. Asterisks indicate statistically significant values relative to GFP-transfected cells (one-way ANOVA with Bonferroni posttest). (F) Quantitation of active clustered BAX in U2OS cells transfected with control (sc) or *MAP4K3* siRNAs. Analysis was performed using an automated microscope system as previously described (18). RFU; Relative Fluorescence Units. Data are presented as mean values $\pm$ SD; $n = 3$ in each group. Statistically significant values relative to UV-treated cells transfected with control siRNAs are indicated with asterisks (two-way ANOVA with Bonferroni posttest). (G and H) Expression of BCL-XL rescues cell death induced by MAP4K3. U2OS cells were co-transfected with GFP or MAP4K3 and either RFP or BCL-XL fused to RFP. As a control, cells were also transfected with BAX-GFP in the presence of either RFP or BCL-XL-RFP. Apoptotic chromatin condensation was scored by Hoechst staining in cells that were fluorescent in both the red and green channels. A minimum of 300 GFP/RFP-positive cells were analyzed in each transfection. (Scale bar, $10 \mu\text{m}$.) Data are presented as mean values $\pm$ SD; $n = 3$ in each group. Asterisks indicate statistically significant values relative to the cells transfected with MAP4K3 and RFP (one-way ANOVA with Bonferroni posttest).

Fig. 3. MAP4K3 signaling modulates BH3-only proteins at the posttranscriptional level. (A) Expression of MAP4K3 leads to an increase in the levels of the BH3-only proteins PUMA and BAD. Transfected cells (HEK293) were analyzed by Western blotting with the indicated antibodies. (B) MAP4K3-dependent increase in PUMA and BAD is dependent on mTORC1 signaling. HEK293 cells were transfected with the indicated constructs and were either left untreated or treated with rapamycin (1 μ M) or InSolution JNK Inhibitor II (1 μ M) for 24 h. Lysates were subsequently analyzed with the indicated antibodies. (C and D) Stability of PUMA and BAD mRNAs is enhanced upon expression of MAP4K3. Following transfection with GFP (control) or MAP4K3, expression constructs HEK293 cells were incubated in the presence or absence of rapamycin (1 μ M) for 16 h and were then treated with α -amanitin (10 μ g/ml). At the indicated time-points, the levels of PUMA and BAD mRNAs were analyzed by qRT-PCR and presented as mean values $\pm$ SEM. The P-values (two-way ANOVA with Bonferroni posttest) correspond to the significance of the interaction between the 2 variables (transfection conditions and time-point) in the experiment. (E) Inhibition of mTORC1 and JNK signaling suppresses MAP4K3-induced cell death. U2OS cells were transfected with MAP4K3 expression constructs and treated with vehicle, rapamycin (1 μ M) or InSolution JNK Inhibitor II (1 μ M). Cell death was scored using a BAX activation assay. Results are shown relative to GFP-transfected cells. Data are presented as mean values $\pm$ SD; $n = 3$. Statistically significant values relative to cells transfected with MAP4K3 and treated with vehicle are indicated with asterisks (one-way ANOVA with Bonferroni posttest). (F) siRNA-mediated suppression of Raptor partially suppresses MAP4K3-mediated up-regulation of PUMA and BAD. HEK293 cells were cotransfected with MAP4K3 and either control (sc) or Raptor siRNAs. Lysates were subsequently analyzed with the indicated antibodies. (G) siRNA-mediated suppression of Raptor contributes to resistance to MAP4K3-mediated cell death. U2OS cells were cotransfected with MAP4K3 and either control (sc) or Raptor siRNAs. Cell death was scored using a BAX activation assay. Data are presented as mean values $\pm$ SD; $n = 3$. Statistical significance relative to control is indicated with asterisks (two-tailed unpaired t test). (H) MAP4K3 expression results in JNK-dependent BIM phosphorylation. HEK293 cells were transfected with either kinase-inactive (KD) or active MAP4K3 in the presence or absence of InSolution JNK Inhibitor II (1 μ M). Following immunoprecipitation, lysates were analyzed by Western blotting with the indicated antibodies. (I) siRNA-mediated suppression of BAX, PUMA,

kinase domain, and treatment with a broad-spectrum caspase inhibitor (Fig. 2 B and C) suppressed these morphological changes, which suggest that MAP4K3 induces caspase-dependent apoptotic cell death and that its kinase activity is important for this process.

Expression of the MAP4K3 variants was confirmed by Western blotting (Fig. S2A). Interestingly, versions of MAP4K3 containing the amino-terminal kinase domain but lacking the carboxy-terminal predicted PEST domains (5) were more highly expressed compared to the full-length protein. To determine whether the differences in expression levels were linked to proteasomal control, we analyzed the effect of proteasomal inhibitors on MAP4K3 expression. This assay showed that proteasomal inhibition significantly increased the levels of MAP4K3 (Fig. S2B). To confirm that MAP4K3 is a target of the ubiquitin-proteasome system, we immunoprecipitated MAP4K3 from cells cotransfected with an HA-ubiquitin construct. We detected a smear of HA-immunoreactive bands in extracts prepared from cells transfected with MAP4K3, particularly when the carboxy-terminal PEST-containing sequence was present (Fig. S2C). We therefore conclude that MAP4K3 is ubiquitinated and likely is targeted for proteasomal degradation. This finding suggests that cellular levels of this kinase are tightly regulated, perhaps to prevent abnormal induction of apoptosis.

To determine whether MAP4K3 acts upstream of the mitochondrial-dependent cell death pathway, we investigated the effects of MAP4K3 expression on activation of the pro-apoptotic BCL-2 family member BAX. Toward this goal, we transfected cells with MAP4K3 in the presence of a broad-spectrum caspase inhibitor (zVAD-fmk) to block caspase activation downstream of mitochondrial alterations. The presence of active BAX in GFP-positive cells was monitored and quantified by fluorescence microscopy using an antibody that recognizes the conformationally active form of BAX (Fig. 2 D and E). We found that the levels of active BAX increased upon expression of active MAP4K3. In addition, we monitored the levels of active BAX following siRNA-mediated MAP4K3 suppression. This analysis showed that suppression of MAP4K3 significantly reduced the levels of active BAX following UV-mediated induction of cell death (Fig. 2 F and Fig. S3). Taken together, the data indicate that the kinase activity of MAP4K3 results in BAX activation, suggesting that this kinase acts upstream of the mitochondrial intrinsic apoptosis pathway.

To confirm the role of mitochondrial signaling in MAP4K3-induced cell death, we next sought to determine whether inhibition of BAX-dependent apoptosis suppressed MAP4K3-induced cell death. To that end, we coexpressed MAP4K3 together with the prosurvival BCL-2 family member BCL-XL and monitored apoptotic chromatin condensation in cells expressing both proteins by fluorescence microscopy. We also performed control experiments designed to confirm the ability of BCL-XL to suppress BAX-induced killing in our experimental settings. In these experiments, BCL-XL expression suppressed MAP4K3-induced cell death, further confirming that this kinase acts upstream of the mitochondrial intrinsic cell death pathway (Fig. 2 G and H).

MAP4K3 Modulates BH3-Only Proteins at the Posttranscriptional Level.

Activation of BAX is triggered by the BH3-only class of pro-apoptotic Bcl-2 family members (reviewed in ref. 2). We therefore decided to address whether MAP4K3 plays a role in BAX activation through modulation of BH3-only proteins. Given that BCL-XL suppresses MAP4K3-induced cell death, we focused our analysis on PUMA, BAD, and BIM, which comprise a subset of BH3-only

BAD, and BIM partially suppress MAP4K3-induced cell death. U2OS cells were cotransfected with MAP4K3 and either control (sc) or the indicated siRNAs. Cells with apoptotic chromatin condensation were scored based on fluorescence. Between 100 and 300 GFP-positive cells were analyzed for each transfection. Data are presented as mean values $\pm$ SD; $n > 3$. Statistical significance relative to control is indicated with asterisks (one-tailed unpaired t test).

components of the intrinsic apoptotic pathway with high affinity for BCL-XL (2). We compared the levels of these proteins in cells expressing MAP4K3 to cells transfected with a control plasmid by Western blotting (Fig. 3A). MAP4K3 expression resulted in an increase in the levels of the BH3-only proteins PUMA and BAD. Furthermore, the kinase activity of MAP4K3 seems to be required for this up-regulation, since expression of a kinase-inactive version did not result in up-regulation of these proteins (Fig. 3A, lane 4). Given that p53 has been previously implicated in the transcriptional up-regulation of pro-apoptotic Bcl-2 family members, including PUMA (3) and BAX (9), we monitored the levels of p53 and BAX in these experimental settings. We failed to detect any significant changes of either p53 or the p53 target BAX upon MAP4K3 expression suggesting that this transcription factor is unlikely to be involved in MAP4K3-induced apoptosis.

Previous studies have suggested that MAP4K3 contributes to the activation of both the JNK and mTOR signaling pathways (5, 6). To assess whether MAP4K3-dependent activation of either the JNK or mTOR pathways contributed to up-regulation of BH3-only proteins, we assessed the levels of PUMA, BAD, and BIM in cells expressing MAP4K3 and treated with an mTORC1 inhibitor (rapamycin) or JNK inhibitor (InSolution JNK Inhibitor II). We detected enhanced phosphorylation of the mTORC1 target protein S6 kinase and increased phosphorylation of the JNK target c-Jun upon expression of MAP4K3, confirming that this kinase activates both of these signaling cascades. Inhibition of JNK signaling, achieved by treatment with a broad-spectrum JNK inhibitor, failed to suppress the up-regulation of PUMA or BAD in MAP4K3-expressing cells. However, suppression of mTORC1 activity by rapamycin had a significant effect on the regulation of PUMA, as well as a more modest effect on the regulation of BAD, (Fig. 3B) indicating that the mTORC1 pathway acts downstream of MAP4K3 to promote up-regulation of PUMA and BAD.

Additionally, to determine whether MAP4K3 contributes to the up-regulation of PUMA or BAD at the transcriptional level, we analyzed the PUMA and BAD transcript levels by qRT-PCR in cells expressing MAP4K3 and compared these to the appropriate controls. This analysis failed to show any significant differences in the mRNA levels of either PUMA or BAD in the presence of enhanced expression of MAP4K3 (Fig. S4), suggesting that the up-regulation of PUMA and BAD in this context is likely to be posttranscriptional.

mTORC1 modulates the activity of eIF4B and eIF4E, therefore affecting the activity of the CAP-binding translation complex eIF4F. It has recently been proposed that changes in the functional status of eIF4F modulate mRNA stability *in vivo* (10). To test whether MAP4K3 induces PUMA or BAD expression by enhancing mRNA stabilization, we analyzed the stability of these 2 transcripts. Analysis of mRNA decay using the polymerase II inhibitor α -amanitin revealed that expression of MAP4K3 conferred enhanced stability to both PUMA and BAD transcripts and that this effect was reversed upon mTORC1 inhibition with rapamycin (Fig. 3C and D), suggesting that MAP4K3 acts at least in part to stabilize PUMA and BAD mRNA.

MAP4K3 Mediates Apoptosis Through the mTORC1 and JNK Pathways.

Next, to determine the contribution of mTORC1 and JNK signaling to cell death promoted by MAP4K3 activity, we analyzed the levels of active BAX in cells transfected with MAP4K3 in the absence and presence of the mTORC1 inhibitor rapamycin or the JNK inhibitor. Inhibition of either mTORC1 signaling or JNK signaling downstream of MAP4K3 resulted in protection from cell death (Fig. 3E). To confirm the role of mTORC1 in the pro-apoptotic activity of MAP4K3, we performed epistatic analysis by selectively suppressing the mTORC1 component Raptor by siRNA. We used a pool of siRNAs to knock-down Raptor expression in U2OS cells (Fig. S5). Analysis of cells transfected with MAP4K3 together with Raptor siRNAs showed that suppression of Raptor partially suppressed the induction of PUMA and BAD (Fig. 3F) and resulted in a rescue of

MAP4K3-induced cell death (Fig. 3G), further suggesting a role for mTORC1 downstream of MAP4K3 in the apoptotic signaling cascade.

We were intrigued by the observation that inhibition of JNK downstream of MAP4K3 resulted in significant protection from cell death (Fig. 3E), but failed to suppress the up-regulation of PUMA and BAD (Fig. 3B). Notably, JNK phosphorylation of BIM at serine 69 (serine 65 in mouse and rat) has been proposed to lead to its activation through release from dynein and myosin V motor complexes (11, 12). To test whether MAP4K3 signaling although JNK leads to BIM phosphorylation, we immunoprecipitated BIM from cells transfected with wild-type or kinase-inactive (KD) MAP4K3 in the presence or absence of a JNK inhibitor and assessed BIM phosphorylation status in the purified complexes. This revealed that expression of active MAP4K3 results in a significant increase in the level of phospho-BIM. Notably, this increase is partially suppressed by JNK inhibition, suggesting that JNK signaling through MAP4K3 leads to the activation of BIM (Fig. 3H). Finally to assess the epistatic contribution of BAX, PUMA, BAD, and BIM for MAP4K3-induced apoptosis, we performed siRNA-mediated knock-down of these pro-apoptotic BCL-2 family members. After confirming successful knock-down in our experimental settings (Fig. S5), we analyzed apoptotic chromatin condensation in cells transfected with MAP4K3 together with either BAX, PUMA, BAD, or BIM siRNAs. This showed that suppression of either BAX, PUMA, BAD, or BIM partially rescued MAP4K3-induced cell death (Fig. 3I), further supporting a role for these BCL-2 family members downstream of MAP4K3 in this apoptotic signaling cascade.

Reduction of MAP4K3 Expression in Pancreatic Cancer. The results of a study designed to dissect the core signaling pathways altered in pancreatic cancer suggest that MAP4K3 could be an important modulator of pancreatic cancer (7). This work identified a somatic mutation in MAP4K3 (E351K) predicted to contribute to tumorigenesis. We performed a sequence alignment of human MAP4K3 with its mouse, rat, and fly orthologues and noted that the amino acid mutated in pancreatic cancer maps to the PEST domain of this kinase (Fig. 4A), suggesting that stability of this kinase might be compromised in pancreatic cancer cells. We therefore considered whether a decrease in the level of MAP4K3 could contribute to tumorigenesis by making cancer cells more resistant to apoptotic cell death.

Given the recently proposed role for this kinase in pancreatic cancer progression (7), we examined whether MAP4K3 expression is altered in this type of cancer. MAP4K3 expression was detected in normal pancreas and pancreatic cancer tissues by immunohistochemistry using a MAP4K3-specific antibody (Fig. 4B). This analysis revealed significantly lower levels of MAP4K3 in the pancreatic tumor samples (Fig. 4C), suggesting that posttranscriptional regulation of this kinase might be an important contributing factor in pancreatic tumorigenesis.

Discussion

MAP4K3 is a member of the Ste20 family of protein kinases, which are known to be activated by UV radiation and the pro-inflammatory cytokine TNF- α (5). Our observations support an apical role for this kinase in intracellular signal transduction cascades, given that it can induce the concerted activation of both the JNK and mTORC1 signaling pathways.

Ste20 group kinases have various intracellular regulatory effects, including regulation of apoptosis and cytoskeleton remodeling (reviewed in ref. 13). Here, we show for the first time that this Ste20 kinase family member is a modulator of apoptosis. It is noteworthy that RNAi-mediated suppression of MAP4K3 was also linked to enhancement of cellular proliferation, as shown by an increase in BrdU incorporation. Although the focus of this study is the characterization of MAP4K3 in the modulation of cell death, we

Fig. 4. Alterations in the protein levels of MAP4K3 in pancreatic cancer. (A) Alignment of MAP4K3 homologues in different species. Numbers correspond to amino acid positions in the human protein. The shaded domain corresponds to the PEST sequence, and the boxed area indicates the amino acid found mutated in the human *MAP4K3* gene in pancreatic cancer. (B) MAP4K3 staining in normal pancreatic samples and pancreatic tumors. Preimmune staining is shown in the upper panels. Sections were counterstained with haematoxylin. Grades 1, 2 or 3 (G1, G2 and G3) are indicated. (Scale Bar, 100 μ m.) (C) Box-plot analysis of MAP4K3 levels in normal pancreatic and tumor samples. N corresponds to the number of individual cases analyzed for each diagnosis. Statistical significance is indicated by asterisks (one-way ANOVA with Bonferroni posttest).

cannot rule out a role for this kinase in modulating cellular proliferation independent of its pro-apoptotic function. In flies, RNAi-mediated suppression of HPO, a *Drosophila* Ste20 kinase, results in an increase in the level of cyclin E (which drives cell proliferation), as well as resistance to apoptosis (14). As HPO is the orthologue of the pro-apoptotic kinases MST1 and MST2, it is tempting to speculate that MAP4K3 might be involved in the control of cell proliferation as well as apoptosis in a manner similar to HPO in flies.

Our data suggest that MAP4K3 signaling through mTORC1 results in increased expression of PUMA and BAD through a posttranscriptional mechanism. The BH3-only protein PUMA is known to be induced at the transcriptional level by the tumor suppressor p53 in response to DNA damage (3), and by the class O

forkhead transcription factor-3A (FOXO3A) in response to cytokine/growth factor withdrawal (15). It is noteworthy that we found no role for p53 in the MAP4K3 dependent activation of PUMA in our studies. Activation of BAD at the posttranslational level has previously been linked to loss of phosphorylation in response to growth factor deprivation (16). We therefore propose that the MAP4K3-dependent translational control of PUMA and BAD via the mTOR pathway represents a novel mechanism for regulation of this subset of BH3-only proteins.

BH3-only proteins such as PUMA and BAD bind to anti-apoptotic BCL-2 family members such as BCL-XL, thereby preventing them from neutralizing BAX. The parallel activation of BIM through the JNK branch of MAP4K3 signaling leads to direct BAX activation, resulting in the induction of apoptosis (Fig. 5). We propose that the concomitant activation of multiple different signaling pathways by MAP4K3 is an elegant example of the selective complementary usage of various signal transduction pathways to achieve a defined biological outcome.

mTORC1 modulates the activity of eIF4B and eIF4E, a modulator and a direct component of the CAP-binding translation initiation complex eIF4F, respectively. It has recently been proposed that changes in the functional status of eIF4F modulate mRNA stability *in vivo* (10). It is thus possible that effectors of mTORC1, in particular eIF4A and eIF4E, could act downstream of MAP4K3 to modulate polysome disassembly and mRNA decay, particularly in the case of *PUMA* and *BAD* transcripts. Additionally, considering that the mTORC1 inhibitor rapamycin only partially rescued the enhanced expression of PUMA and BAD triggered by MAP4K3 (Fig. 3B), we cannot rule out a role for MAP4K3 in the mTORC1-independent enhancement of *PUMA* or *BAD* translation. It is known that mTOR, when complexed with the rapamycin-insensitive companion of mTOR (RICTOR) as part of mTORC2, can promote the activation of AKT through direct phosphorylation. Activation of this pathway is reported to lead to the phosphorylation and inactivation of pro-apoptotic factors like BAD and FOXO3a (reviewed in ref. 17). We have not excluded the possibility that MAP4K3 can also lead to the activation of mTORC2; however, such activation would be hard to reconcile with the proposed pro-apoptotic role of this kinase.

Given that MAP4K3 is herein reported to be a potent inducer of apoptosis, it is not surprising that its intracellular levels are tightly regulated so as to prevent abnormal cell death. Our data indicate that the carboxy-terminal PEST domain might play a role in the modulation of MAP4K3 stability through regulation by the ubiquitin-proteasome system.

A MAP4K3 mutation resulting in a single amino acid substitution in the PEST domain of this kinase has been recently reported to be associated with pancreatic cancer (7). This suggests that abnormal regulation of MAP4K3 protein levels might be an important contributing factor for this disease. We detected a significant reduction in the levels of MAP4K3 in pancreatic tumor

Fig. 5. Proposed mechanism for MAP4K3 cell death signaling. Activation of mTORC1 by MAP4K3 modulates the activity of eIF4B and eIF4E. Of these, eIF4E is a component of the CAP-binding complex eIF4F whereas eIF4B promotes the RNA helicase activity of eIF4A (reviewed in ref. 19). eIF4F is a modulator of eukaryotic mRNA stability (10). MAP4K3 signaling leads to up-regulation of the BH3-only proteins PUMA and BAD in an mTORC1-dependent manner. PUMA and BAD, in turn, are capable of neutralizing anti-apoptotic BCL-2 family members, leading to BAX activation and caspase-dependent cell death. In parallel, MAP4K3 signaling through JNK induces BIM phosphorylation. The direct activation of BAX by BIM, together with the neutralization of BCL-XL by PUMA or BAD, results in activation of the intrinsic apoptosis pathway.