An integrated PKD1-dependent signaling network amplifies IRE1 prosurvival signaling

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Following the accumulation of improperly folded proteins in the endoplasmic reticulum (ER), a condition known as ER stress in this compartment triggers an adaptive signaling pathway referred to as the unfolded protein response (UPR). The UPR aims at restoring ER homeostasis; if the ER stress cannot be resolved, apoptosis is triggered. However, the mechanisms responsible for regulating the balance between cell life and death decisions that occur after exposure to ER stress remain unclear. Protein kinase D1 (PKD1) has been reported to initiate protective signaling against oxidative stress or ischemia, two conditions that impinge on the induction of ER stress. In addition, the high levels of expression of PKD1, observed in highly proliferative cancers and tumors with poor prognosis, contribute to enhanced resistance to chemotherapy. In this study, we show that the ER stress inducers tunicamycin and thapsigargin lead to the activation of PKD1 in human prostate cancer PC-3 cells and in hepatoma HepG2 cells through a PKCδ-dependent mechanism. Moreover, our data indicate that PKD1 is required for the stabilization of inositol-requiring enzyme 1 (IRE1) and the subsequent regulation of its activity. PKD1 activation contributes to the phosphorylation of mitogen-activated protein kinase phosphatase 1, resulting in decreased IRE1-mediated c-Jun N-terminal kinase activation. This study unveils the existence of a novel PKD1-dependent prosurvival mechanism that is activated upon ER stress and selectively enhances IRE1 prosurvival signaling.

The endoplasmic reticulum (ER)4 is responsible for the synthesis, posttranslational modification, and trafficking of secretory and transmembrane proteins and is also a site for calcium storage and lipid synthesis (1–3). There is a strict quality control system evolved for newly synthesized proteins in the ER, which allows for the delivery of properly folded and modified proteins to the Golgi complex. When improperly folded proteins accumulate in the ER lumen, ER functions are altered, thus prompting a cellular condition known as ER stress (4, 5). Subsequently, the unfolded protein response (UPR) is initiated, which is characterized by increased ER folding and clearance capacities as well as attenuated protein synthesis (5). The UPR is mediated by three different ER stress sensors, including inositol-requiring enzyme 1 (IRE1), protein kinase R–like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (4). The UPR is initially aimed at relieving ER stress to restore the compartment’s homeostasis. When the UPR fails to bring cells back to normal, such as that observed under severe or sustained ER stress, apoptosis is triggered (5). As such, UPR signaling is a double-edged signaling pathway, which is extensively involved in inflammatory and metabolic diseases, diabetes, neurodegenerative diseases, and cancer (6).

As the most conserved pathway for the UPR, IRE1 signaling is deeply involved in the regulation of both prosurvival and pro-death processes (7, 8). IRE1 is a type 1 transmembrane protein with both endonuclease (RNase) and kinase catalytic activities located in the cytosol. In response to ER stress, IRE1 forms dimers or oligomers, followed by trans-autophosphorylation and activation of the RNase domain (9, 10). IRE1 has three principal effects, namely the removal of a 26-nucleotide intron from the mRNA encoding XBP1, degradation of a subset of mRNAs via a process called regulated IRE1-dependent decay, and activation of the c-Jun N-terminal kinase (JNK) cascade through the tumor necrosis factor receptor–associated factor 2 (TRAF2)/apoptosis signal-regulating kinase 1 (ASK1) signaling axis (7). The cleavage of the XBP1 mRNA by the active IRE1 RNase, followed by a ligation process mediated by a tRNA ligase, leads to the change of XBP1 ORF, resulting in the expression of a spliced form of XBP1 referred to as XBP1s (11, 12). XBP1s is a transcription factor that has been implicated in the transcription of genes responsible for protein folding, endoplasmic reticulum-associated degradation (ERAD), and protein secretion and appears to be heavily involved in re-establishing ER quality control and redox homeostasis (7). Aside from its RNase activity, IRE1 also activates the JNK signaling cascade through the activation of its kinase domain and the recruitment of the adaptor protein TRAF2 (13). The IRE1/JNK pathway plays a role in cell fate decision, because sustained and intense

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4 The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; PKD, protein kinase D; IRE1, inositol-requiring enzyme 1; PERK, protein kinase R–like endoplasmic reticulum kinase; TRAF2, tumor necrosis factor receptor–associated factor 2; ATF6, activating transcription factor 6; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; TSC, TSC1, terminal cell lysates; kb, PKD inhibitor kb-NB 142-70; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nPKC, novel PKC isoform; MPK1, mitogen-activated protein kinase phosphatase 1; TM, tunicamycin; TM, thapsigargin; PLC, phospholipase C; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; CHX, cycloheximide; PARP, poly(ADP-ribose) polymerase.
PKD1 enhances IRE1 prosurvival signaling

Protein kinase D (PKD) is a serine/threonine kinase family that consists of three members, PKD1, -2, and -3, and belongs to the Ca\(^{2+}\)/calmodulin-dependent protein kinase superfamily (15). In most cases, PKD is a substrate of protein kinase C (PKC), especially the novel PKC isoforms (nPKCs, such as PKCδ) (16). Specific PKCs have been reported to directly bind and phosphorylate PKD1 at two evolutionarily conserved serine residues, namely Ser\(^{738}\)/Ser\(^{742}\) in the catalytic domain, leading to the activation of PKD1 (17, 18). The different PKD family members have unique and nonredundant roles. PKD1 is expressed in a wide range of cells and tissues and participates in a number of intracellular signaling pathways to regulate several essential processes, such as cell survival, inflammation, and vesicle trafficking (19). As reported previously, PKD1 activation following \(\text{H}_2\text{O}_2\) treatment promotes autophagy and cell survival (20, 21) and is required for the regulation of \(\text{H}_2\text{O}_2\)-induced JNK signaling (21, 22), and this may be mediated by a direct complex formation with JNK (23). Considering the key role of JNK signaling in determining cell fate, we explored the possibility of PKD1 implication in UPR signaling.

Herein, we demonstrate that following ER stress, PKD1 is activated in a PKC-dependent fashion, which contributes to an enhanced resistance of the cell to ER stress–induced apoptosis. Activation of the PKC/PKD1 axis, which functions as a noncanonical signaling pathway in the ER stress response, in turn modulates the canonical IRE1 signaling pathway in the UPR. PKD1 is found to be required for IRE1 stability and downstream XBP1 signaling. PKD1 activation is also responsible for regulating JNK signaling by modulating mitogen-activated protein kinase phosphatase 1 (MKP1) phosphorylation, which turns off the pro-apoptotic effect of JNK signaling in the UPR. This study, therefore, provides evidence for the role of PKD1 in the control of selective IRE1 prosurvival signaling.

Results

ER stress induces the activation of PKD1

Considering its ability to modulate the kinetics of JNK signaling, PKD may also function in balancing UPR signaling, thereby determining cell fate. To this end, we first examined whether PKD1 is activated following the induction of ER stress. To achieve this, two well-characterized ER stress inducers were used, namely tunicamycin (TM; an inhibitor of protein \(N\)-linked glycosylation) and thapsigargin (TG; a Ca\(^{2+}\)-ATPase inhibitor that can disturb intracellular calcium homeostasis). In PC-3 and HepG2 cells, both ER stress inducers were able to initiate the canonical UPR signaling pathway, as characterized by TM-induced increases in IRE1 phosphorylation at Ser\(^{724}\) as well as increased PERK phosphorylation (as shown by its decreased electrophoretic mobility) (Fig. S1). The process by which PKD1 is activated involves the transphosphorylation of two conserved serine residues, namely Ser\(^{738}\) and Ser\(^{742}\), by nPKC, and subsequent autophosphorylation at multiple sites, including Ser\(^{910}\), which then conferred full activation (15, 24, 25).

To examine the kinetics of PKD1 activation, PC-3 or HepG2 cells were treated with either TM or TG for different periods of time up to 2 h, and extracts from the treated cells were subjected to Western blotting using antibodies against phospho-Ser\(^{738}\)/Ser\(^{742}\)-PKD1, phospho-Ser\(^{910}\)-PKD1, and PKD1. This kinetic analysis of PKD1 activation revealed that TM induced the phosphorylation of PKD1, which peaked after 30 min of treatment and then decreased (Fig. 1, A and B). TG induced PKD1 phosphorylation in a faster and more transient manner, peaking at 10 min and then gradually returning to basal levels (Fig. 1, C and D). We also examined the phosphorylation levels of PKD1 following treatment with various concentrations of TM or TG. Compared with TG, TM induced PKD1 activation in a more concentration-dependent manner (Fig. S2, A and B).

To further confirm that ER stress mediates the activation of PKD1, we also evaluated the phosphorylation status of putative PKD substrates using phospho-(Ser/Thr) PKD substrate antibodies. Treatment with TM led to the enhanced phosphorylation of PKD substrates (Fig. 1E), an effect that was dampened upon pretreatment with the PKD inhibitor kb-NB 142-70 (referred to as kb hereafter) (Fig. 1E). Taken together, our data support the notion that PKD1 is activated in response to ER stress.

ER stress–mediated activation of PKD1 depends on PKCδ

Accumulating evidence suggests that PKD1 is a point of convergence and integration for multiple stimuli, such as neurotransmitters, growth factors, and oxidative stress (19). In most cases, the activation of PKD1 is mediated by PKCs. With the aim of understanding whether ER stress–mediated PKD1 activation is PKC-dependent, we pretreated PC-3 and HepG2 cells with the pan-PKC inhibitor Gö6983 for 1 h and then treated cells with TM for 30 min. In both cell lines, pretreatment with Gö6983 potently blocked the PKD1 activation induced by TM (Fig. 2A and Fig. S3A). Similarly, TG-induced PKD1 phosphorylation was also abrogated by prior treatment with Gö6983 (Fig. 2B and Fig. S3B), suggesting that the ER stress–mediated PKD1 activation is mediated through PKC. PKCδ, one nPKC isoform, has been reported to play a crucial role in ER stress–induced apoptosis (26, 27) and is able to directly phosphorylate PKD1 in HeLa cells subjected to oxidative stress (18), which suggests the possibility that there is a PKCδ-dependent PKD1 activation in response to ER stress. To test this hypothesis, a dominant negative mutant of PKCδ (PKCδ.DN) was transfected into PC-3 and HepG2 cells, and the phosphorylation of PKD1 was examined following TM or TG treatment. Overexpression of PKCδ.DN inhibited ER stress–mediated PKD1 activation (Fig. 2, C and D and Fig. S3C). As has been reported, both conventional and novel PKCs are activated through the same signal transduction pathway mediated by phospholipase C (PLC) (28). Accordingly, we pretreated cells with the PLC inhibitor U73122, which also blocked the effect of ER stress on PKD1 activation (Fig. 2E). Our data, therefore, indicate that ER stress promotes PKD1 activation through a typical PLC/PKC (most likely PKCδ) pathway.
PKD1 is required for IRE1 stability and function

PERK, IRE1, and ATF6 are regarded as canonical UPR regulators (29). However, additional reports have also demonstrated that other stress-signaling pathways might be induced upon ER stress, thus extending the scope of the canonical UPR (30–33). Based on our initial observations, we next sought to investigate whether the PKC/PKD1 axis could regulate canonical UPR signaling. To this end, PKD1 siRNAs were introduced into PC-3 and HepG2 cells to down-regulate the expression of PKD1. Knockdown of PKD1 led to a decrease in TM-mediated IRE1 activation, as shown by the reduced IRE1 phosphorylation levels (Fig. 3A and Fig. S4A). In contrast, TM-mediated PERK activation was not impacted, as shown by the similar phosphorylation levels of PERK and eIF2α that were seen under all conditions (Fig. 3A and Fig. S4A). To our surprise, we observed a decrease in total IRE1 protein levels in cells transfected with PKD1 siRNAs (Fig. 3A and Fig. S4A). This observation was similar to the effect seen following exposure to TG (Fig. 3B and

Figure 1. ER stress induces the activation of PKD1. A, PC-3 cells were starved for 6 h, followed by treatment with 20 μg/ml TM for the indicated time. Total cell lysates (TCL) were analyzed by immunoblotting using antibodies against phospho-PKD (Ser738/Ser742), phospho-PKD (Ser910), PKD, and α-tubulin. α-Tubulin represents the internal loading control of each sample. Right, immunoblot quantification of the phosphorylation level of PKD. B, HepG2 cells were treated as described in A, and the indicated proteins were detected by Western blotting, with quantification shown on the right. C and D, PC-3 and HepG2 cells were starved for 6 h, followed by treatment with 1 μM TG for the indicated time. TCL were subjected to immunoblotting using the indicated antibodies, with quantification shown on the right. E, after 6-h starvation, PC-3 cells were untreated or pretreated with 10 μM kb for 1 h, followed by TM treatment alone or together with kb for another 30 min, and the phosphorylation of PKD and its substrates was detected by Western blot analysis. Relative amounts of phospho-PKD on Ser738/Ser742 and Ser910 were determined by scanning densitometry of Western blots from three independent experiments with ImageJ software. Data are represented as mean ± S.D. (error bars) of three independent experiments.

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Figure 2. PKD1 is activated downstream of PKCδ upon ER stress. A and B, after 6-h starvation, PC-3 cells were untreated or pretreated with 2.5 μM G66893 for 1 h, followed by 20 μg/ml TM treatment alone or together with G66893 for 30 min (A) or 1 μM TG treatment alone or together with G66893 for 15 min (B). TCL were subjected to immunoblotting using the indicated antibodies, with quantification of phospho-PKD shown on the right. C and D, PC-3 cells were transfected with pcDNA3.1 or PKCδ.DN for 24 h and subjected to TM for 30 min (C) or 1 μM TG for 15 min (D). TCL were subjected to immunoblotting using the indicated antibodies, with quantification of phospho-PKD shown on the right. Relative amounts of phospho-PKD were determined from three independent experiments. Data are represented as mean ± S.D. (error bars) of three independent experiments (Student’s t test; *, p < 0.05; **, p < 0.01; *** or ###, p < 0.001; **** or ####, p < 0.0001; *, compared with control; #, compared with TM or TG treatment alone or mock subjected to ER stress).

Fig. S4B). Consistent with these data, overexpression of WT PKD1 in PC-3 cells clearly increased the levels of IRE1 (Fig. 3C). A double phosphorylation site–defective form of PKD1, referred to as PKD.2A (S738A/S742A) has been reported to be inactive (18). Based on PKD.2A, we constructed PKD.3A (S738A/S742A/S910A), a new phosphorylation-defective form of PKD1. Overexpression of PKD.3A had no impact on the protein levels of IRE1 (Fig. 3C), thereby supporting the notion that PKD1 activity is involved in modulating IRE1 protein levels. We further assessed the impact of the PKD inhibitor kb on IRE1 protein expression under ER stress conditions. Consistent with other reports (34), TM treatment alone led to an increase in IRE1 protein expression levels (Fig. 3D). In contrast, treatment with kb reduced the IRE1 protein levels in all cases (Fig. 3D). Interestingly, treatment with PKD1 siRNAs (Fig. 3E and Fig. S4C) or inhibitor (Fig. 3F) did not affect IRE1 mRNA expression levels, suggesting that the effects of PKD1 on IRE1 protein expression might occur at the translational or post-translational levels. To further explore this, a time-course experiment revealed that incubation of the cells with kb resulted in a gradual decrease in total IRE1 protein levels (Fig. 3G). Next, PC-3 cells were treated with cycloheximide (CHX), a protein synthesis inhibitor, followed by kb challenge. CHX treatment alone led to a time-dependent depletion of the IRE1 protein, whereas combination treatment with both kb and CHX accelerated the loss of the IRE1 protein (Fig. 3G), thus ruling out the possibility that kb may act through the inhibition of IRE1 translation. Our data, therefore, suggest that PKD1 might be implicated in the regulation of IRE1 protein stability. As expected, combination treatment with MG132, a commonly used proteasome inhibitor, resulted in the inhibition of the kb-mediated decrease in IRE1 protein levels (Fig. 3H). Our data indicate that PKD1 plays an important role in maintaining IRE1 protein stability.

Because PKD1 was implicated in the regulation of IRE1 stability, we further assessed the effect of PKD1 inactivation on the IRE1/XBP1 pathway. To address this, PC-3 cells were treated with both TM and kb, and the effect on XBP1s mRNA levels was assessed. As a result, the TM-induced increase in the levels of the XBP1s mRNA was blocked by kb treatment (Fig. 4A), accounting for the decreased mRNA levels of XBP1s target genes, including BiP, P58IPK, and ERdj4 (Fig. 4A). The total protein levels of XBP1s were examined following kb treatment in the presence of ER stress. Consistent with the changes observed in mRNA level, XBP1s protein expression levels were reduced following combination treatment with kb and TM (Fig. 4B). The siRNA-mediated knockdown of PKD1 also impacted the ER stress–induced expression of XBP1s protein, as was seen with the PKD1 inhibitor kb (Fig. 4C). In contrast, the overexpression of PKD1 up-regulated the TM-enhanced expression levels of XBP1s, whereas the mutant PKD.3A failed to modulate
the effect of TM on XBP1s protein levels (Fig. 4D). Taken together, these data suggest that the activation of PKD1 induced by ER stress regulates XBP1s and its target genes by modulating IRE1 stability and its subsequent activity.

Given that PKC appears to mediate the activation of PKD1 under ER stress conditions, we also assessed the effects on IRE1 protein stability and activity in PC-3 cells treated with the PKC inhibitor Gö6983 or expressing PKCδ.DN. Gö6983 treatment alone had no effect on the expression levels of the IRE1 protein (Fig. S5, A and B). The phosphorylation levels of IRE1 and the levels of both the IRE1 and XBP1s proteins in cells treated with a combination of Gö6983 and TM or TG were no different than in TM- or TG-treated cells (Fig. S5, A and B). Moreover, the overexpression of PKCδ.DN also failed to modulate the stability and activity of IRE1 upon ER stress, as was seen in the unaltered expression levels of the IRE1 and XBP1s proteins (Fig. S5, C and D).
PKD1 enhances IRE1 prosurvival signaling

As mentioned previously, IRE1 signals through both TRAF2 and ASK1 to activate the JNK pathway, which is closely connected with ER stress-induced cell apoptosis. Numerous reports have demonstrated that PKD1 can negatively regulate JNK activation to promote survival in cells subjected to various stressors (35). Our data suggest that PKD1 is required for IRE1 signaling under conditions of ER stress. The question of whether PKD1 affects activation of the JNK cascade in response to ER stress also needed to be addressed. We pretreated PC-3 cells with kb and then treated them with TM. Treatment with kb switched the ER stress–induced activation mode of JNK from a slight and transient increase to an intense and prolonged increase (Fig. 5A). Silencing of PKD1 led to increased basal phosphorylation level of JNK and enhanced TM-mediated JNK phosphorylation (Fig. 5B). As shown above, knockdown of PKD1 leads to the degradation of the IRE1 protein, suggesting that the enhanced JNK activation seen upon PKD1 silencing does not occur directly through the IRE1 pathway. We thus hypothesized that PKD1 knockdown may also bypass the PKD1-mediated repression of JNK signaling. To test this hypothesis, we reasoned that the enhanced ER stress-induced JNK activation (at an early stage) seen following pretreatment with kb was an outcome of a regulation imbalance between unimpaired IRE1-mediated JNK activation and impaired PKD1-mediated dephosphorylation of JNK. Consistent with this, the overexpression of either PKD1 WT or PKD.3A had no effect on ER stress-induced JNK activation (Fig. 5C), a convergent outcome from both IRE1/JNK and PKD1/JNK signaling. The duration and intensity of JNK activation that occurs upon exposure of cells to ER stress is considered to be a key determinant in life–death decisions. These data, therefore, reveal that activation of PKD1 prevents the pro-apoptotic effect of intense and sustained JNK activation downstream of the IRE1 pathway.

We next sought to characterize how PKD1 modulates the kinetics of JNK activation following exposure to ER stress. MKP1, a dual-specificity protein phosphatase, has been heavily implicated in the down-regulation of extracellular signal–regulated kinase 1/2, JNK, and p38 activities (36). MKP1 is known to be involved in ER stress signaling by regulating the activation kinetics of JNK to improve cell survival and resistance to ER stressors (14). We pretreated PC-3 cells with the MKP1 inhibitor triptolide (TP), which led to an enhanced JNK activation following exposure of cells to ER stress (Fig. S6A). Based on these data, it appeared that MKP1 may represent a good candidate for mediating the effect of PKD1 on JNK signaling during ER stress. As expected, pretreatment with kb abrogated TM-induced MKP1 phosphorylation (Fig. 5D), which suggests that the MKP1 phosphorylation induced by ER stress is PKD1-dependent. Consistent with this, the down-regulation of PKD1 led to a similar effect on the phosphorylation levels of
MKP1 in cells subjected to ER stress (Fig. 5E). In contrast, TM-enhanced MKP1 phosphorylation was further up-regulated by the overexpression of WT PKD1, but not by the overexpression of PKD.3A (Fig. 5F). Moreover, pretreatment with the PKC inhibitor Gö6983 led to reduced MKP1 phosphorylation and enhanced JNK activation following exposure to ER stress (Fig. 5G), mimicking the effect of the PKD inhibitor on MKP1/JNK signaling. MKP1 was also essential for cell survival following exposure to ER stress, because the MKP1 inhibitor enhanced ER stress-induced caspase-3 and PARP cleavage (Fig. S6B). Pretreatment of cells with the MKP1 inhibitor further reduced viability and promoted ER stress–induced apoptosis (Fig. S6, C and D). These data demonstrate that ER stress–mediated PKD1 activation down-regulates JNK signaling by promoting MKP1 phosphorylation.

**PKD1 activation improves cell resistance to ER stress–induced apoptosis**

It has been demonstrated that PKD1 plays an important role in promoting cell survival through inhibiting JNK activation and beyond (37). Our study here has shown that PKD1 activation following exposure to ER stress regulates IRE1 signaling, selectively increasing the prosurvival IRE1/XBP1 pathway and attenuating the pro-apoptotic IRE1/JNK through different mechanisms. We assumed that activation of PKD1 plays a pivotal role in regulating ER stress–mediated cell death. To test this hypothesis, we treated cells with kb and then assessed the cleavage of both caspase-3 and its substrate PARP following exposure to ER stress using Western blotting. The levels of both cleaved caspase-3 and cleaved PARP were increased upon the combination treatment with kb and TM (Fig. 6A). A TUNEL assay also revealed that inhibition of PKD1 led to an increase in the number of apoptotic cells seen following exposure to ER stress (Fig. 6B). To confirm the role of PKD1 in the modulation of UPR, we investigated the effect of PKD1 knockdown on the survival of cells subjected to ER stress. Silencing of PKD1 by siRNAs enhanced the sensitivity of PC-3 cells toward ER stress, as shown by the increased levels of cleaved caspase-3 and cleaved PARP (Fig. 6C, left). This observation was similar to the
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**Discussion**

ER stress is a complex biological process that occurs in all eukaryotic cells. Increasing evidence has associated ER stress with various cellular dysfunctions or diseases. IRE1 signaling, as the most conserved UPR signaling branch, has been found to play an essential role in determining cell fate (38, 39). The IRE1/XBP1 signaling pathway is able to promote cell resistance to ER stress and control the survival of immune cells (40, 41). The other signaling pathway mediated by IRE1 is involved in the activation of JNK signaling. Accumulating evidence has demonstrated that the kinetics of JNK activation play a critical role in determining cell fate under diverse stress conditions (14, 42).

Because of this dual function of IRE1 that is mediated by the two different output pathways, a tuning mechanism must exist to balance the life and death decisions that arise from signaling through the IRE1 node. In the present study, we monitored PKD1 activation following ER stress and examined its function in the UPR. Our results demonstrate for the first time that PKD1 enhances IRE1 prosurvival signaling.
PKD1 functions as a fine tuner of IRE1 signaling to amplify the prosurvival impact of IRE1 signaling.

**A noncanonical UPR pathway mediating PKD1 activation protects cells from ER stress–induced apoptosis**

It has been reported that the activation of PKD1, as a convergent point, can be triggered by a variety of stimuli, including oxidative stress (19). Generally, PKD1 activation is mediated through the canonical PKC signaling cascade (16). PKCs, especially nPKCs, directly bind to and phosphorylate PKD1 (17, 18, 43). Our present work proves that the ER stress inducers, TM and TG, are able to trigger PKD1 activation. PKD1 activation that occurs following exposure to ER stress was completely abrogated by a PKC inhibitor. In keeping with this, PKCδ.DN also abrogated the increased PKD1 phosphorylation levels induced by ER stress, suggesting that ER stress–induced PKD1 activation is mediated through PKCs, particularly PKCδ. The data here suggest that conventional ER stressor triggers activation of the PKC/PKD signaling, which constitutes a part of the noncanonical UPR.

PKD1 can mediate multiple cellular processes by signaling through diverse pathways (19, 44). Our data show that either silencing or inactivation of PKD1 in cancer cells reduces their resistance to ER stress, as evidenced by the increased cleavage of both caspase-3 and PARP and the number of apoptotic cells that were observed when these cells were exposed to ER stress. Taken together, the present study reveals that PKD1 activation via a noncanonical UPR pathway is critical for the survival of cells exposed to ER stress.

**PKD1 enhances IRE1/XBP1 signaling by modulating the stability of the IRE1 protein**

As has been reported, a noncanonical UPR extensively increases UPR transcriptional output and can drive a diverse number of physiological changes (31, 32), usually by regulating canonical UPR signaling (45). Here, we show that the PKC/PKD signaling axis, a new noncanonical UPR pathway, is required for IRE1 stability. Suppression of PKD1 either through the use of an inhibitor or by silencing PKD1 expression using siRNA results in significant depletion of the IRE1 protein, with no effect on the expression of IRE1 mRNA. The protein synthesis inhibitor CHX enhances the effect of the PKD inhibitor on IRE1 protein expression, indicating that PKD1 plays a role in maintaining the IRE1 protein stability.

HSP90, as an IRE1 chaperone, can increase IRE1α stability by directly binding to the cytosolic domain of IRE1. Phosphorylation of HSP90 at Thr2/Thr7 facilitates its nuclear import, which might result in the increased degradation of a wide number of proteins (46–48). Interestingly, our recent data reveal that PKD1 inactivation caused a clear increase in HSP90 phosphorylation at Thr2/Thr7 (data not shown), which might account for the increased instability of the IRE1 protein. Qi and colleagues (34) have demonstrated that IRE1 is a substrate of ERAD and that ER stress attenuates ERAD-mediated IRE1 degradation. Under conditions of ER stress, the PKC/PKD axis may negatively regulate this degradation process, increasing IRE1 stabilization.

One traditional pathway that is known to be mediated by IRE1 is XBP1 signaling, which is related to cell survival. Our study demonstrates that as a result of the instability of the IRE1 protein that occurs upon PKD1 inhibition or knockdown, the expression levels of both XBP1s mRNA and protein are down-regulated in response to ER stress, which then results in a decrease in the expression levels of XBP1 target genes, including BiP. Taken together, these data suggest that the PKD1 activation that occurs as a result of ER stress in turn increases IRE1 stability and increases the strength of signaling along the IRE1/XBP1s prosurvival axis.

**PKD1 rewires IRE1-mediated pro-apoptotic JNK signaling toward prosurvival signal through MKP1**

Acting through diverse pathways, PKD1 is able to regulate multiple normal and abnormal biological processes, including cell survival, migration, and tumorigenesis (35). One such PKD1–regulated pathway is the JNK pathway. Previous studies, including ours, have demonstrated that the role of JNK in cell apoptosis is highly dependent on its activation kinetics. If there is an intense prolonged JNK activation, the cells undergo apoptosis (14, 42, 49). In contrast, JNK signaling is also known to be critical for cell survival (50). For example, in compound mutant jnk1−/−/jnk2−/− embryos, increased apoptosis is observed in the developing forebrain and hindbrain regions (51, 52). Furthermore, the transient activation of JNK is necessary for cell survival in CGNs subjected to ER stress (14). This study reveals that following exposure to ER stress, the resulting activation of PKD1 changes the activation kinetics of JNK, from a pro-apoptotic signal (i.e. the sustained and intense activation mode) to a prosurvival signal (transient activation mode).

The mechanism by which PKD1 regulates JNK signaling is cell context–dependent. In Rat-1 fibroblasts, for example, platelet-derived growth factor–mediated PKD1 activation suppresses EGF-induced JNK activation by phosphorylating the EGFR (53). In contrast, H2O2–induced ASK-JNK signaling depends on the autophosphorylation of PKD1 at its 14-3-3-binding sites (22). PKD1 has even been found to form a complex with JNK (23). Our early research has proven that different stimuli that cause the induction of ER stress can lead to opposite effects on JNK signaling and cell fate by modulating the phosphorylation of MKP1 at Ser359 (14). In this study, we found that PKD1 activation increases the degree of phosphorylation of MKP1 at Ser359. Inhibition of MKP1 also causes a switch in the kinetics of JNK activation similar to that seen with PKD1 inhibition or knockdown, which accounts for the increased cell apoptosis. The present study clearly demonstrates the existence of a novel mechanism by which PKD1 activation, in response to exposure to ER stress, modulates JNK signaling by enhancing the phosphorylation of MKP1. This leads to a more transient activation of JNK and a switch to the prosurvival mode. The exact mechanisms by which PKD1 is able to increase MKP1 phosphorylation still remain to be defined.

In summary, our data clearly show that ER stress triggers the activation of PKD1 via PKCδ and that activated PKD1 protects the cell from ER stress–induced apoptosis by promoting prosurvival IRE1 signaling while attenuating IRE1-dependent JNK activity (Fig. 6F). There are two principal mechanisms that are
PKD1 enhances IRE1 prosurvival signaling

responsible for this effect. First, PKD1 increases the stability of the IRE1 protein and its activity, which leads to an increase in the expression of XBP1s and its target genes. This positive regulatory loop from PKD1 enhances the contribution of IRE1 signaling to the release of ER stress and the increase of cell resistance to ER stress. Second, PKD1 activation switches the kinetics of JNK activation from a sustained to a transient mode through MKP1, which reverses the pro-apoptotic effect of the IRE1/JNK signaling axis. These two diverse ways by which PKD1 regulates IRE1 signaling result in a common prosurvival outcome. PKD1 may, therefore, function as an important component of a fine-tuning mechanism to control UPR outputs.

Experimental procedures

Antibodies and reagents

TM was obtained from Calbiochem. TG was obtained from Sigma. MG132, CHX, U73122, and triptolide (TP) were purchased from MedChem Express. The selective PKD inhibitor kb-NB 142-70 was from Selleck (Houston, TX). Antibodies against phospho-PKD (Ser738/742), phospho-PKD (Ser916), PKD, phospho-JNK (Thr183/Tyr185), JNK, phospho-MKP1 (Ser359), PARP, caspase-3, IRE1α, phospho-eIF2α (Ser51), eIF2α, phospho-(Ser/Thr) PKD substrate, and horseradish peroxidase–conjugated secondary antibodies against mouse and rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA). The antibody against α-tubulin was from Sungene Biotech (Tianjin Province, China), and those against XBP1 (M-186) and MKP1 (V-15) were from Santa Cruz Biotechnology (sc-37007 and sc-36245); siPKD1-2, siIRE1-1, and siIRE1-2 were purchased from GenePharm (Suzhou, Jiangsu, China). The siRNA sequences used are shown in Table 1. After 72 h, the transfected cells were left untreated or were treated with TM/TG for the indicated periods of time.

Immunoblot analysis

A total of 2.5 × 10⁵ cells were seeded into 35-mm plates and grown in complete medium for 1 day at 37 °C in an atmosphere of 5% CO₂. Cells were then serum-starved for 6 h by incubation in serum-free medium before being treated with the various compounds as described. At the end of each treatment, samples were obtained and detected as described previously (14).

RT-PCR and quantitative PCR

Total cellular RNA was isolated using TRIzol as described by the manufacturer (Invitrogen) and reverse-transcribed into cDNA using the Superscript first-strand synthesis system (Invitrogen), as described previously (14). 1 μl of the reverse transcription product was mixed with TaqDNA mix (New England Biolabs, Ipswich, MA) and 20 pmol of each of the sense and antisense primers in a volume of 25 μl. The primers used for quantitative PCR are listed in Table 2. The Roche FastStart Universal SYBR Green master (RoX) was used according to the manufacturer’s instructions.

Cell viability

Cell viability was determined using the MTT assay. After treatment, the cells were incubated with MTT (0.5 mg/ml) for 2 h at 37 °C. After medium removal, the formazan blue formed was extracted with DMSO and quantified by measuring its absorbance at 490 nm using a spectrophotometer.

TUNEL assay

Cell apoptosis was measured using a commercially available TUNEL Kit, as described by the manufacturer (Promega, MI). Briefly, cells were seeded onto coverslips for 24 h. After treatment, the coverslips were incubated one by one in the assay solution, as described. Finally, 4',6-diamidino-2-phenylindole was used to stain the nucleus. After washing two times with PBS, the coverslips were visualized using a fluorescent microscope. Cells showing a green fluorescent signal were regarded as undergoing apoptosis, and blue fluorescent signal provided an estimate of the total number of cells.

Table 1

| Name        | Sequence                                                                 |
|-------------|--------------------------------------------------------------------------|
| siPKD1-1, 2 | is a siRNA pool including                                                |
|             | 1, 5′-CGCAGAAUUAUGCUGUAAAUUAUU-3′                                      |
|             | 2, 5′-GAACCAAAUGUCACAGAGAUAUU-3′                                       |
|             | 3, 5′-GGUCUGAAUUAACCAAAGAUAUUAU-3′                                     |
|             | 4, 5′-GGCAUAUACGCACGAGCAAUUU-3′                                        |
|             | 5′-GCCUUAAUUCAGGCAACUUTTGTT-3′                                         |

References:
[54] A total of 2.5 × 10⁵ cells were seeded into 35-mm plates and grown in complete medium for 1 day at 37 °C in an atmosphere of 5% CO₂. Cells were then serum-starved for 6 h by incubation in serum-free medium before being treated with the various compounds as described. At the end of each treatment, samples were obtained and detected as described previously (14).
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Statistical analysis

Immunoblot data were quantitated using ImageJ software, and the optical density of each band was normalized. Statistical analyses were performed using GraphPad Prism version 7 software. Data from at least three independent experiments are presented as the mean ± S.D. Statistical comparisons between groups were performed with Student’s t test. Data obtained from dose–response experiments were analyzed using nonlinear curve fitting. Significance levels are indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Table 2

| Primers used for qPCR analysis and site-directed mutation | Name | Sequence |
|----------------------------------------------------------|------|----------|
| XBP1s                                                    | Forward | 5'-CTGAGTCGGGAGCGCTCGA-3' |
|                                                         | Reverse  | 5'-CTCAGCGCTGAGAGACTCG-3' |
| PSBP                                                     | Forward | 5'-GCCAGCTGCTCAGAAGAC-3' |
|                                                         | Reverse  | 5'-AGTACAGCTGCTGAAAT-3' |
| EBD1                                                    | Forward | 5'-TGCGGTTTACAGTACGAC-3' |
|                                                         | Reverse  | 5'-TCACCCAGAGCTCG-3' |
| IRE1α                                                   | Forward | 5'-CCACCTGAGCGCTCTCGA-3' |
|                                                         | Reverse  | 5'-GCCGCTGCTGAGAGACTCG-3' |
| CHOP                                                    | Forward | 5'-AGGAAAGGAGGATGAC-3' |
|                                                         | Reverse  | 5'-CTGCTGAAACCCGCTTCTC-3' |
| BiP                                                     | Forward | 5'-ATTCGGCGCTGCTGCTCTC-3' |
|                                                         | Reverse  | 5'-GCTTGGAGCTGCTGCTCTC-3' |
| GAPDH                                                   | Forward | 5'-AAACGGCGATGACGCTCAA-3' |
|                                                         | Reverse  | 5'-CTGCTGGAATCAGAGCTCTC-3' |

Primers for site-directed mutation (PKD.3A)

| Name | Sequence |
|------|----------|
| Forward | 5'-CTCGGTTTACAGTACGAC-3' |
| Reverse  | 5'-CCGCTCAGGAGATGACGACACCGCTCA-3' |

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