Star-PAP Control of BIK Expression and Apoptosis Is Regulated by Nuclear PIPKIα and PKCδ Signaling

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SUMMARY

BIK protein is an initiator of mitochondrial apoptosis, and BIK expression is induced by proapoptotic signals, including DNA damage. Here, we demonstrate that 3′ end processing and expression of BIK mRNA are controlled by the nuclear PI4,5P2-regulated poly(A) polymerase Star-PAP downstream of DNA damage. Nuclear PKCδ is a key mediator of apoptosis, and DNA damage stimulates PKCδ association with the Star-PAP complex where PKCδ is required for Star-PAP-dependent BIK expression. PKCδ binds the PI4,5P2-generating enzyme PIPKIα, which is essential for PKCδ interaction with the Star-PAP complex, and PKCδ activity is directly stimulated by PI4,5P2. Features in the BIK 3′ UTR uniquely define Star-PAP specificity and may block canonical PAP activity toward BIK mRNA. This reveals a nuclear phosphoinositide signaling nexus where PIPKIα, PI4,5P2, and PKCδ regulate Star-PAP control of BIK expression and induction of apoptosis. This pathway is distinct from the Star-PAP-mediated oxidative stress pathway indicating signal-specific regulation of mRNA 3′ end processing.

INTRODUCTION

BCL2-interacting killer (BIK) protein activates the mitochondrial apoptotic pathway by inducing Ca2+ release from the endoplasmic reticulum (ER) and the remodeling of the mitochondrial cristae that releases cytochrome c and activates caspase 9 (Chinnadurai et al., 2008; Germain et al., 2002). BIK is a tumor suppressor, a prognostic marker, and a therapeutic target for cancers, and BIK expression is induced by DNA damage, viral infection, and cytokines (Chinnadurai et al., 2008). BIK expression inhibits antiapoptotic effects of oncoproteins and viral infection and induces cell apoptosis (Han et al., 1996). In contrast, loss of BIK per se fails to alter apoptotic profile in hematopoietic and endothelial cells, while combined deficiency of BIK and BIM (another BH3-only protein) exhibits additive effect on apoptosis in germ cells (Coultas et al., 2005), indicating a critical yet redundant role of BIK. The cellular levels of BIK are controlled by both posttranslational and transcriptional mechanisms (Hur et al., 2006; Marshansky et al., 2001). However, these regulatory mechanisms for BIK expression and the signaling pathways mediating these steps are not well defined.

Protein kinase C (PKC) isoforms are key regulators of apoptosis (Brodie and Blumberg, 2003; Reyland, 2007) and PKCs, a novel PKC subfamily member, is required for apoptosis in response to several stimuli (Brodie and Blumberg, 2003), including DNA damage (Yoshida, 2007). Mice deficient in PKCδ are resistant to γ-irradiation-induced apoptosis (Humphries et al., 2006). DNA damage-induced p53 activation and protein levels are similar in primary cells derived from PKCδ−/− and PKCδ+/− mice, indicating that PKCδ functions downstream of the DNA damage response (Humphries et al., 2006). PKCδ regulates the mitochondrial apoptosis pathway (Humphries et al., 2006; Steinberg, 2004) and this requires nuclear targeting of PKCδ (Yoshida, 2007).

PKCδ is targeted to different cellular compartments, depending on its phosphorylation status (Rybin et al., 2004). Phosphorylation of tyrosines 64 and 187 on PKCδ appears important for PKCδ’s role in apoptosis induced by DNA damage (Blass et al., 2002). However, tyrosine phosphorylation only serves to amplify the later stages of the apoptosis signal and is not required for the nuclear targeting of full-length PKCδ (Blass et al., 2002). After initiation of apoptosis, PKCδ is cleaved by caspase or other proteases into regulatory and catalytic domains, and these further activate downstream signaling cascades (DeVries et al., 2002). The cleaved catalytic fragment of PKCδ is important for the progression of apoptosis (Ghayur et al., 1996), and the regulatory fragment is required for the optimal function of PKCδ (Blass et al., 2002).

Activated PKCδ could also induce apoptosis without being proteolytically cleaved by caspase (Tanaka et al., 2003). It was proposed that the catalytic fragment and the full-length PKCδ target different nuclear substrates at different stages of apoptosis (Steinberg, 2004). There are substrates for nuclear PKCδ activity, but it is not clear which of these are critical for induction of apoptosis. PKC isoforms are regulated by...
phosphoinositide signaling through generation of diacylglycerol by cleavage of phosphatidylinositol-4,5-bisphosphate (PI4,5P2), and some PKCs directly interact with and are regulated by PI4,5P2 (Chauhan and Brockerhoff, 1988; Huang and Huang, 1991; Lee and Bell, 1991). The role of phosphoinositides in the modulation of nuclear PKCα is not defined.

In nuclei, there is a distinct phosphatidylinositol cycle that is independent of the cytosolic pathway (Barlow et al., 2010; Cocco et al., 1987). Nuclear diacylglycerol kinase theta (DGKθ), phospholipase C beta 1 (PLCβ1), PI4,5P2, and phosphatidylinositol-4-phosphate 5-kinase I alpha (PIPKIα) all were detected and targeted to nuclear speckles (Gonzales and Anderson, 2006; Tabellini et al., 2003). These findings suggest a mechanism where nuclear signals emanating from PI4,5P2 may regulate nuclear localized PKC isoforms.

Recently gene expression has been shown to be directly regulated by nuclear phosphoinositide signaling through a noncanonical poly(A) polymerase, speckle-targeted PIPKIα-regulated poly(A) polymerase (Star-PAP) (Mellman et al., 2008). Star-PAP controls the expression of a subset of genes by assembling into a distinct 3’ end cleavage and polyadenylation complex on its target mRNAs. Star-PAP associates with regulatory proteins such as PIPKIα and casein kinase I (CKI) isoforms α and ε, which are required for 3’ end processing (Gonzales et al., 2008; Laishram et al., 2011; Mellman et al., 2008). PI4,5P2 directly and specifically controls the activity of Star-PAP (Mellman et al., 2008). Star-PAP has been proposed to function as a signal-regulated effector of mRNA 3’ end processing that may integrate into different pathways (Barlow et al., 2010). In this study, we identified BIK as a direct target of Star-PAP and defined a Star-PAP-specific binding region on the BIK mRNA. Upon DNA damage signaling, Star-PAP, PKCα, and PIPKIα form a functional complex required for BIK mRNA cleavage in response to the apoptotic signal induced by etoposide. These data define Star-PAP as a molecular target for nuclear PKCα that controls the expression of BIK, an important switch in the mitochondrial apoptosis pathway.

RESULTS

Star-PAP and Associated PIPKIα Are Required for BIK Expression

Cellular BIK is increased in response to DNA damage signaling (Fu et al., 2007). Gene microarray analysis of Star-PAP knockdown cells implicated BIK as a Star-PAP target (Mellman et al., 2008). To determine the role of Star-PAP in BIK expression, the mRNA and the protein levels of BIK were analyzed in the absence or presence of etoposide treatment. Etoposide (VP-16) is a DNA-damaging compound that inhibits topoisomerase II and induces apoptosis (Baldwin and Osheroff, 2005). The optimal time used for etoposide treatment was determined by time-course experiments. In the DNA fragmentation analysis, DNA condensation was initiated at 4 hr post etoposide treatment (Figure S1A). Concomitantly, BIK protein levels were substantially increased during this time (Figure S1B). Therefore, this time after etoposide treatment was chosen for most of the experiments, except for the DNA laddering and the caspase 3/7 activity assays as described below.

Star-PAP control of BIK expression was measured by quantitative RT-PCR after Star-PAP knockdown by RNA interference (RNAi). BIK mRNA level decreased 8-fold after Star-PAP knockdown, proportional to the efficiency of Star-PAP knockdown (Figure 1A). The etoposide-potentiated BIK expression was reduced to the same extent (Figure 1A). Similarly, when PIPKIα was knocked down, both the basal and the etoposide-induced BIK expression were decreased (Figures 1A and S1D). Remarkably, while etoposide enhanced the cellular BIK protein content, knockdown of Star-PAP or PIPKIα diminished the BIK protein greater than 15-fold in both etoposide treated and nontreated cells (Figures 1A and S1D). Simultaneous knockdown of both Star-PAP and PIPKIα did not significantly enhance the block of BIK mRNA or protein expression (Figure 1A), indicating that Star-PAP and PIPKIα act in the same pathway for BIK expression.

Since Star-PAP is a poly(A) polymerase, we investigated the 3’ end processing and polyadenylation of BIK pre-mRNA using 3’ RACE assays with cDNA synthesized from total RNA isolated from cells after RNAi knockdown of Star-PAP or PIPKIα and etoposide treatment. Distinct RACE products for BIK were observed with the control cell mRNAs and an increase was noted after stimulation with etoposide (Figures 1B and S1D). There was a loss of RACE products upon Star-PAP or PIPKIα knockdown regardless of etoposide treatment (Figures 1B and S1D). Ectopic re-expression of the wild-type (wt) but not the polymerase dead (pd) Star-PAP or the kinase dead (kd) PIPKIα, with silencing mutations (sm) that are resistant to the RNAi, rescued the loss of BIK in Star-PAP or PIPKIα knockdown cells (Figures 1B and S1D). The changes in BIK protein levels followed the similar pattern (Figures 1B and S1D). The Star-PAP nontarget mRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was not affected by knockdown of Star-PAP/PIPKIα nor by stimulation with etoposide (Figures 1B and S1D). These data demonstrate that the Star-PAP poly(A) polymerase activity and the ability of PIPKIα to generate PIP2 are required for BIK expression.

Consistent with the Star-PAP role in the cleavage of its target HO-1 pre-mRNA (Laishram and Anderson, 2010; Mellman et al., 2008), Star-PAP or PIPKIα knockdown resulted in an accumulation of uncleaved BIK pre-mRNA (Figures 1B and S1D). However, DNA damage stimulation reduced the uncleaved BIK message compared to the unstimulated control (Figures 1B and S1D). Expression of Star-PAPwt/sm and Star-PAPkd/sm rescued the knockdown-induced cleavage defect (Figure 1B), indicating that Star-PAP protein but not its activity is required for 3’ end cleavage. In contrast, re-expression of PIPKIαwt/sm but not PIPKIαkd/sm alleviated the cleavage defect resembling the rescue of the BIK mRNA 3’ end formation in the RACE assays. This indicates a role for the PIPKIα kinase activity, PIP2 production, in the regulation of Star-PAP processivity toward BIK. These data collectively demonstrate that Star-PAP controls both the 3’ end cleavage and polyadenylation of the BIK mRNA downstream of DNA damage.

PKCα Associates with and Directly Phosphorylates Star-PAP

To identify signaling molecules that transmit apoptotic/DNA damage signals to Star-PAP-mediated BIK expression, we
investigated protein kinase activities associated with Star-PAP. When affinity-purified Star-PAP complex was treated with phorbol 12-myristate 13-acetate (PMA), associated protein kinase activity toward Star-PAP was stimulated (Figure 2A). PMA activates a number of kinases, including PKC isoforms (Brose and Rosenmund, 2002). Therefore the association of members of the PKC family with Star-PAP was analyzed by immunoblotting (IB). This identified PKC\(\delta\) but not PKC\(\alpha\) (Figure 2B) or any other PKC isoforms (data not shown) in the affinity purified Star-PAP complex under these conditions. The association of PKC\(\delta\) with Star-PAP was further confirmed by immunoprecipitation (IP) and IB of endogenous Star-PAP and PKC\(\delta\) (Figure 2C). However, the GST-tagged PKC\(\delta\) failed to pull down the His-tagged Star-PAP in vitro, indicating that the association of PKC\(\delta\) with Star-PAP is indirect (data not shown).

Star-PAP and the associated PIPK\(\alpha_2\) are spatially targeted to nuclear speckles in the cells (Mellman et al., 2008). Our data demonstrate that PKC\(\delta\) was also targeted to foci that partially colocalized with Star-PAP (Figure 2D, first four panels) and with the speckle marker SC-35 (Figure 2D, middle and last four panels) as shown by immunofluorescence microscopy (IF).

Since PKC\(\delta\) is a component of the Star-PAP complex and may phosphorylate Star-PAP, the effect of exogenous PKC\(\delta\) kinase activity toward Star-PAP was analyzed. Recombinant PKC\(\delta\) phosphorylated the purified Star-PAP complex in a dose-dependent fashion, and PMA treatment further enhanced PKC\(\delta\)-dependent phosphorylation of Star-PAP (Figure 2E). Consistently, PKC\(\delta\) phosphorylated the recombinant His-tagged Star-PAP (Figure 2F), indicating that Star-PAP is a direct substrate of PKC\(\delta\). In both cases, IP of Star-PAP under denaturing conditions demonstrated that the 120 kDa \(^{32}\)P-phosphorylated band was indeed Star-PAP (Figures 2E and 2F, lower panels).

**PKC\(\delta\) Directly Interacts with PIPK\(\alpha_2\), and PI4,5P\(2\) Stimulated PKC\(\delta\) Kinase Activity toward Star-PAP**

PIP\(\alpha_2\) and PKC\(\delta\) are components of Star-PAP complex. It is reported that some PKC isoforms interact with and are regulated by PI4,5P\(2\) (Chauhan and Brockerhoff, 1988; Huang and Huang, 1991; Lee and Bell, 1991). We hypothesized that PI4,5P\(2\) could be a direct regulator of PKC\(\delta\). As PIP kinases often define PI4,5P\(2\) signaling specificity by interacting with PI4,5P\(2\) effectors (Heck et al., 2007), the interaction between

![Figure 1. BIK Expression Is Controlled by Star-PAP and PIPK\(\alpha_2\)](image-url)
PIPKIα and PKCδ was investigated by in vitro GST pull-down assays. This demonstrated direct binding of PIPKIα to PKCδ (Figure 3A). Moreover, endogenous PKCδ was coimmunoprecipitated with PIPKIα and vice versa (Figure 3B), indicating that PKCδ and PIPKIα associate in vivo. To determine if PKCδ activity is regulated by PI4,5P2, PKCδ kinase activity was assayed using myelin basic protein (MBP) as generic PKCδ substrate in the presence of an increasing amount of PI4,5P2. PI4,5P2 stimulated PKCδ kinase activity toward MBP (Figure 3C). Most importantly, PI4,5P2 stimulated PKCδ activity toward Star-PAP purified from cell lysates (Figure 3D).

**Figure 2. PKCδ Associates with Star-PAP in the Nucleus and Directly Phosphorylates Star-PAP**

(A) Affinity purified FLAG-tagged Star-PAP complex contains PMA-stimulated kinase activities. Coomassie staining of the SDS-PAGE gel showed total protein loading.

(B) PKCδ was detected in the FLAG-Star-PAP complex by IB.

(C) PKCδ was coloPed with endogenous Star-PAP and was confirmed by reverse IP.

(D) PKCδ colocalized with Star-PAP in the nucleus and targeted to nuclear speckles as shown by IF. The boxed areas were magnified and shown as insets. Scale bar = 10 μm.

(E) Purified recombinant PKCδ dose-dependently phosphorylated the Star-PAP complex, and the phosphorylation was further stimulated by PMA.

(F) The recombinant PKCδ directly phosphorylated His-tagged Star-PAP. In addition to protein staining, the Star-PAP protein in the reactions was examined by IP/IB using Star-PAP antibody, and the 32P-phosphorylated Star-PAP was identified by phosphorimaging after IP (E and F bottom panels).

**PIPKIα Is Required for DNA Damage-Stimulated Interaction of PKCδ and Star-PAP**

PKCδ translocates into the nucleus in response to DNA damage, and this nuclear translocation is required for the initiation of apoptosis (DeVries et al., 2002). As Star-PAP controls BIK expression and interacts with PKCδ in the nucleus, the impact of the apoptotic signal on PKCδ association with Star-PAP was assessed by reciprocal IP/IB of Star-PAP and PKCδ after DNA damage. PKCδ protein levels increased and peaked at 4 to 8 hr post etoposide treatment (Figure S1B), and the association of PKCδ with Star-PAP was concurrently enhanced (Figures 4A...
PKCα and PI4,5P₂ Regulate Star-PAP Control of BIK

Figure 3. PKCα Directly Interacts with PIPKIα, and PI4,5P₂ Stimulates PKCα Kinase Activity

(A) GST pull-down assay revealed direct interaction of PKCα and PIPKIα.
(B) Endogenous PKCα coIPed with PIPKIα.
(C) PI4,5P₂ dose-dependently increased PKCα kinase activity toward MBP.
(D) PI4,5P₂ augmented PKCα phosphorylation of the affinity purified Star-PAP. The phosphorimages were quantified. Error bars represent standard deviation of three independent experiments (middle panels of C and D). Total proteins in the gels were stained with Coomassie blue. Star-PAP contents and specific phosphorylation in the reactions were evaluated by IP followed by phosphorimaging and IB (D bottom panels).

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PKCα and S1C). The increased PKCα interaction with Star-PAP was supported by IF staining, which demonstrated that PKCα increased its nuclear speckle colocalization with SC-35 by 1.5 fold and with Star-PAP by 2.3 fold after etoposide treatment (Figure S2A).

PIPKIα and PKCα directly interact and are integrated into the Star-PAP complex, and DNA damage enhances PKCα association with Star-PAP. Thus, it is plausible that PIPKIα and the locally produced PI4,5P₂ may modulate PKCα activity and assembly into the Star-PAP complex. To determine the impact of PIPKIα on the interaction of PKCα with Star-PAP, PIPKIα was knocked down and the cells were treated with or without etoposide. PIPKIα knockdown diminished the association of PKCα with Star-PAP (Figures 4B and 4C), confirming that PIPKIα is required for the interaction of PKCα with the Star-PAP complex. However, DNA damage had no detectable impact on the association of PIPKIα with Star-PAP (Figures S2B and S2C). In addition, PKCα knockdown did not alter the association of Star-PAP with PIPKIα in both etoposide-treated and non-treated cells (Figures S2D and S2E). Star-PAP is incorporated into the transcriptional complex, and the association of RNA Pol II with the Star-PAP complex was also impaired after PIPKIα knockdown (Figures 4B, 4C, S2D, and S2E), indicating that the interaction of the Star-PAP complex with the transcription machinery requires PIPKIα and PKCα.

These data demonstrate that PIPKIα within the Star-PAP complex is required for the recruitment of and serves as a docking site for PKCα in vivo. This suggests that PIPKIα could be a substrate for PKCα or may directly modulate its activity. Recombinant PKCα failed to phosphorylate PIPKIα in vitro (data not shown). To determine if PIPKIα directly regulates PKCα activity, increasing PIPKIα was combined with PKCα, and kinase activity toward MBP or Star-PAP was assayed. Remarkably, PIPKIα inhibited PKCα activity toward Star-PAP (Figure S3A) and MBP (data not shown) in a dose-dependent fashion. The PIPKIα inhibition of PKCα activity was relieved by addition of PI4,5P₂ (Figures 4D and S3B), again indicating that PIPKIα activity and PI4,5P₂ production modulate PKCα activity. Surprisingly, PMA failed to stimulate PKCα activity toward recombinant Star-PAP when PKCα was associated with PIPKIα (Figure S3C).
This supports the idea that PI4,5P2 is the primary lipid messenger for PKCδ activation in this signaling pathway.

**PKCδ Regulates Star-PAP Activity, BIK Expression, and Is Associated with BIK mRNA**

BIK and PKCδ both mediate the mitochondrial apoptosis pathway (Germain et al., 2002; Humphries et al., 2006). As Star-PAP controls BIK expression and the association of PKCδ with Star-PAP is increased in response to DNA damage, the possibility that PKCδ modulates BIK expression was explored. Knockdown of PKCδ dramatically reduced both basal and eto-poside-stimulated BIK mRNA and protein levels (Figure 5A), equivalently to Star-PAP and PIPKIα knockdown (Figures 1B and S1D). This phenotype was rescued by the expression of PKCδwt/sm but not PKCδkd/sm (Figure 5A). Expression of PKCδkd/sm also decreased the RACE product similarly to PKCδ knockdown (Figure 5A). The control GAPDH was not affected by knockdown or ectopic expression of PKCδ or DNA damage (Figure 5A). However, knockdown of PKCδ had no effect on the cleavage of BIK pre-mRNA in control cells, but increased the uncleaved message upon DNA damage (Figure S4A). While PKCδwt/sm overexpression rescued the cleavage defect, PKCδkd/sm failed to restore BIK pre-mRNA cleavage (Figure S4A). These data demonstrate that PKCδ is required for the generation of mature BIK mRNA in vivo. Consistent with this role in BIK mRNA 3’ end processing, PKCδ and PIPKIα associate with the BIK UTR RNA along with Star-PAP, as shown by RNA immunoprecipitation (RIP).

As PKCδ interacts with and phosphorylates Star-PAP, the role of PKCδ in the 3’ end processing of BIK mRNA was investigated by using 3’ RACE assays. Knockdown of PKCδ resulted in a loss of RACE product in control and DNA-damaged cells (Figure 5A), similarly to the knockdown of Star-PAP and PIPKIα (Figures 1B and S1D). This phenotype was rescued by the expression of PKCδwt/sm but not PKCδkd/sm (Figure 5A). Expression of PKCδkd/sm also decreased the RACE product similarly to PKCδ knockdown (Figure 5A). The control GAPDH was not affected by knockdown or ectopic expression of PKCδ or DNA damage (Figure 5A). However, knockdown of PKCδ had no effect on the cleavage of BIK pre-mRNA in control cells, but increased the uncleaved message upon DNA damage (Figure S4A). While PKCδwt/sm overexpression rescued the cleavage defect, PKCδkd/sm failed to restore BIK pre-mRNA cleavage (Figure S4A). These data demonstrate that PKCδ is required for the generation of mature BIK mRNA in vivo. Consistent with this role in BIK mRNA 3’ end processing, PKCδ and PIPKIα associate with the BIK UTR RNA along with Star-PAP, as shown by RNA immunoprecipitation (RIP).
In contrast, PAP\textsubscript{a} was associated with the Star-PAP nontarget GCLC RNA, but not with BIK RNA. PIPKI\textsubscript{a} was also not associated with GCLC RNA, whereas PKC\textsubscript{d} showed weak association (Figure 5B), indicating that BIK mRNA is a selective target for the Star-PAP pre-mRNA processing complex.

To further explore whether PKC\textsubscript{d} directly modulates Star-PAP poly(A) polymerase activity, Star-PAP was affinity-purified from the cells stably expressing FLAG-Star-PAP with or without PKC\textsubscript{d} knockdown or/and etoposide treatment (Figures S4B and S4C). The purified Star-PAP was used in the in vitro poly(A) polymerase assays as previously described (Mellman et al., 2008). Upon DNA damage (etoposide treatment), the poly(A) polymerase activity of Star-PAP was stimulated and the addition of PI4,5P\textsubscript{2} further enhanced this activity (Figure 5C). Star-PAP poly(A) polymerase activity under these conditions was highly processive adding >600 adenine residues after initiation (Figure S5B). Nevertheless, Star-PAP has linear enzyme kinetics in its polyadenylation activity over this time range (data not shown). The PI4,5P\textsubscript{2} stimulation of Star-PAP activity also required priming by etoposide treatment (Figure 5C).

Remarkably, PKC\textsubscript{d} was required for Star-PAP activation, as knockdown of PKC\textsubscript{d} (Figure S4B) blocked both the DNA damage stimulation and the priming required for PI4,5P\textsubscript{2} stimulation of Star-PAP activity (Figure 5C). These results were confirmed by assaying at the varying concentrations of Star-PAP purified from cells that were ± PKC\textsubscript{d} RNAi in the presence or absence of etoposide treatment (Figure S5A). The combined data demonstrate that PKC\textsubscript{d} is required for Star-PAP activity toward BIK downstream of DNA damage stimulation.

**Star-PAP Binds to the BIK 3′ UTR RNA**

Star-PAP directly binds to its target HO-1 pre-mRNA in the UTR upstream of the poly(A) signal and recruits the cleavage and polyadenylation specificity factor (CPSF) 160 and 73 subunits (Laishram and Anderson, 2010). Since Star-PAP associated with the BIK mRNA in vivo, a direct interaction was explored. A fragment of BIK 3′ UTR RNA (~145 to +120 with respect to cleavage site), containing the cis-regulatory elements and the equivalent Star-PAP regulatory region as identified in HO-1, and an RNA fragment from the equivalent
region of the Star-PAP nontarget gene GCLC were transcribed. In vitro RNA electrophoretic mobility shift assays (EMSA) using radiolabeled BIK or GCLC UTR RNA were carried out. A slower migrating binary complex of Star-PAP and BIK RNA was detected with increasing Star-PAP concentration, but there was no detectable interaction with the GCLC RNA (Figure 6A). This BIK RNA fragment was also confirmed as the minimum upstream sequence from the BIK cleavage site that showed optimum binding to Star-PAP by deletion analysis of the BIK UTR (data not shown).

In competition experiments, the Star-PAP-BIK RNA complex was competed with 20-fold molar excess of cold BIK UTR RNA but not by a nonspecific RNA of similar length (Figure 6B). Addition of Star-PAP antibody but not a control antibody resulted in a supershift of the Star-PAP-BIK RNA complex (Figure S6A). PAPα did not bind to the BIK RNA at similar concentrations to that of Star-PAP (data not shown). These results are consistent with the RIP data (Figure 5B) and with previous reports that PAPα has low affinity for RNA (Wahle, 1991), and they demonstrate that Star-PAP...
directly and specifically interacts with the 3' UTR of the BIK pre-mRNA. To map the Star-PAP binding region on the BIK UTR, an RNA footprinting assay was employed using the BIK UTR RNA substrate used in EMSA experiments. A ladder of digested fragments of BIK RNA was generated with different concentrations of RNase S (Figure 6C, lanes 5 and 6). The site of protection by Star-PAP binding was obtained after comparing with that received His-Star-PAP (Figure 6C, lanes 7 and 8). Several positions within the BIK UTR were specifically protected by Star-PAP against RNase S cleavage. The protected region extended from ~120 to ~70 nucleotides upstream of the cleavage site (taken as +1) with a Star-PAP dose for protection (Figure 6C). A similar result was also observed from footprinting with RNase T1 (Figure S6C). The putative Star-PAP binding region on BIK UTR RNA was deleted and reassayed for binding using EMSA. The results showed that Star-PAP did not bind the BIK RNA lacking this region (Figure 6D). These data defined the Star-PAP binding sequence in BIK UTR (Figure 6E).

The BIK UTR Sequence Suggests a Mechanism for Star-PAP Specificity

Comparison of the Star-PAP binding regions in BIK and HO-1 UTR showed similar nucleotide sequences with high GC contents (>65%) (Figure S6B). Using a bioinformatics approach, the UTR sequences of all genes that were downregulated by Star-PAP knockdown in the microarray analysis (Mellman et al., 2008) were analyzed. This demonstrated that there is an enrichment of G and C nucleotides upstream of cleavage site in the putative Star-PAP target mRNAs compared to the UTRs of Star-PAP nonregulated genes (Figure 6F). In addition, we observed enrichment of G-rich 4-mers (CUGG, GGGG, GGGA, GGAG, etc.) around the cleavage site of Star-PAP targets (Figure 6G). This is consistent with the Star-PAP footprint on HO-1 and BIK, and indicates that Star-PAP directly binds GC-rich sequence upstream of the poly(A) signal.

Most interestingly, this analysis showed a deficiency in U-content 3' to cleavage site among the Star-PAP target genes, especially in the region of downstream sequence elements (DSE) (Figure 6F). This is important because CstF complex recognizes the U-rich or GU-rich DSE and cooperates with CPSF-160 binding to form a stable cleavage complex, required for canonical 3' end processing (Murthy and Manley, 1995; Zhao et al., 1999). Our analysis also revealed that U-rich 4-mers (UUUU, UAUU, UUGU, etc.) were highly depleted in this region (Figure 6G) among the Star-PAP targets. Markedly, sequence analysis of the BIK UTR sequence demonstrated that in addition to the high GC-rich Star-PAP binding region upstream of the poly(A) signal, there was no U/GU-rich consensus or optimal DSE for CstF binding 3' to cleavage site (Figure 6E). This indicates that the deficiency of a CstF binding region might render the canonical PAPz-based 3' end processing complex unable to access BIK and potentially other Star-PAP target mRNAs. As Star-PAP functions distinctly by directly binding and recruiting the CPSF 160 and 73 subunits to the RNA (Laishram and Anderson, 2010), this may reduce the requirement for CstF binding.

Star-PAP Is Required for DNA Damage-Induced Apoptosis

PKCδ and BIK play key roles in apoptosis (Chinnadurai et al., 2008; Humphries et al., 2006; Reiland, 2007). As nuclear PIPKδ2 and PKCδ regulate Star-PAP-control of BIK mRNA processing and expression, Star-PAP may regulate the apoptotic response to DNA damage. To test this hypothesis, total genomic DNA was extracted from cells treated with or without Star-PAP knockdown or/and etoposide and assayed for DNA ladder-termination of chromatin DNA fragmentation. In non-etoposide-treated cells, basal DNA fragmentation level was observed and reduced in Star-PAP knockdown cells (Figure 7A). Etoposide induced DNA fragmentation and this was blocked by Star-PAP knockdown (Figure 7A). These results were corroborated by an analysis of caspase 9 and caspase 3/7 activation. DNA damage-induced cellular caspase 9 and caspase 3/7 activities were abolished by loss of Star-PAP (Figure 7B). It was reported that a requirement of BIK for apoptosis is likely cell-type-dependent, and loss of another BH3-only protein BIM could impair the proapoptotic effect (Coultas et al., 2005). To examine the combined effect of the loss of BIK expression via the Star-PAP-controlled pathway and the loss BIM expression, Star-PAP and/or BIM were knocked down followed by etoposide treatment and specific caspase activities were measured. While single knockdown of Star-PAP or BIM could each reduce caspase 9 and caspase 3/7 activities in etoposide-treated cells, double knockdown of Star-PAP and BIM further prevented the apoptotic effect (Figure S7).

PKCδ Regulation of the Star-PAP Specificity and Activity Is Specific for DNA Damage but Not Oxidative Stress

Star-PAP regulates a subset of genes, including HO-1, that have roles in oxidative stress response (Laishram and Anderson, 2010; Mellman et al., 2008). PKCδ is reported to play roles in aspects of the oxidative stress response (Konishi et al., 1997). Yet, there is no evidence for PKCδ regulation of HO-1 expression. As PKCδ regulates Star-PAP in the DNA damage-induced BIK expression pathway, we examined if PKCδ is also required for oxidative stress-mediated Star-PAP regulation of BIK and HO-1 expression. While the oxidative stress agonist tBHQ induced dramatic increase in HO-1 protein level, the cellular BIK level remained unaffected (Figure 7C, lanes 1 and 5). As expected, Star-PAP or PIPKδ2 knockdown attenuated both HO-1 and BIK expression regardless of the presence of tBHQ (Figure 7C, lanes 2, 3, 6, and 7). Yet, knockdown of PKCδ diminished BIK but not HO-1 protein levels in both tBHQ non-treated and treated cells (Figure 7C, lanes 1, 4, 5, and 8). Moreover, an in vitro poly(A) polymerase assay, using affinity-purified FLAG-Star-PAP from cells treated with or without PKCδ knockdown in the presence or absence of tBHQ or etoposide treatment, showed that while PKCδ knockdown blocked the etoposide priming of PI4,5P2-stimulation of Star-PAP activity (Figure 7D, lanes 3, 4, 7, and 8), tBHQ- and PI4,5P2-stimulated Star-PAP poly(A) polymerase activities were not impacted by the loss of PKCδ (Figure 7D, lanes 9, 10, 11, and 12). These data indicate that PKCδ regulation of Star-PAP activity is specific to the DNA damage-signaling pathway where Star-PAP regulates BIK expression.
BIK expression is a key switch for the initiation of the mitochondrial apoptosis pathway. BIK is also recognized as a tumor suppressor (Chinnadurai et al., 2008) whose transcription is regulated in part by p53 (Hur et al., 2006; Mathai et al., 2002) and DNA damage stimuli (Real et al., 2006). Although the mechanisms for BIK transcription have been studied, the role of BIK 3′ end processing in its expression and translation remains uncharacterized. As BIK acts as an early initiator for apoptosis, the processing of the message is a fundamental step in the expression pathway.

Here, we have shown that Star-PAP controls the 3′ end cleavage and polyadenylation of the BIK pre-mRNA, a process essential for stability, export, and translation of the message. Although BIK 3′ UTR has an intact poly(A) signal (AAUAAA), it cannot be processed by the PAPα canonical 3′ end processing complex. PAPα has very low affinity for RNA substrate (Martin and Keller, 1996; Wahle, 1991) and lacks RNA binding specificity (Zhao et al., 1999). Thus, PAPα is recruited to pre-mRNA by the cooperative binding of CPSF to the poly(A) signal and CstF to a U/GU-rich element that is 3′ to the cleavage site (Keller et al., 1991; Murthy and Manley, 1995). There are examples of suboptimal poly(A) sequences, where an intact AAUAAA is not sufficient for the CPSF complex to bind the pre-mRNA and recruit PAPα (Gilmartin et al., 1995). There are also examples of suboptimal CstF U/GU-rich element sites (Hu et al., 2005; Maciolek and McNally, 2008; Tian et al., 2005).

Star-PAP directly binds to the 3′ end of pre-mRNAs upstream of the poly(A) signal (Figure 6). The Star-PAP protection region on both BIK and HO-1 shows a high GC-rich sequence motif. Star-PAP interacts with RNA harboring multiple binding motifs...
PKCδ and PI4,5P₂ Regulate Star-PAP Control of BIK

that have a relatively large footprint on the UTR, Star-PAP binding to the pre-mRNA recruits CPSF160 and CPSF73 by direct protein-protein interactions (Laishram and Anderson, 2010). Unlike canonical PAPs, Star-PAP, CPSF160, and CPSF73 are sufficient to reconstitute specific 3’ end cleavage in vitro, indicating that the CstF complex plays a less important role for Star-PAP targets (Laishram and Anderson, 2010). Thus, for some Star-PAP exclusive pre-mRNA targets a plausible explanation for loss of PAPz usage could be the loss of CPSF-CstF complex assembly onto the target mRNAs, resulting in deficient recruitment of PAPz to the pre-mRNAs. Sequence analysis of Star-PAP-regulated genes, including BIK, has demonstrated that apart from the GC-rich sequence around the cleavage site, there is depletion of U-rich sequence in the region of DSE resulting in a suboptimal CstF recognition site (Figure 6). The deficiency of CstF binding would impede both the stable assembly of CPSF-160 on the poly(A) signal and recruitment of PAPz to the cleavage site of BIK UTR. These changes could render BIK an exclusive Star-PAP target.

PKCδ is a central switch that controls the apoptosis pathway, and this requires the nuclear localization and cleavage of PKCδ by caspase-3 (Reyland, 2007). Yet, considerable evidence indicates that the full-length PKCδ is required for the priming of Star-PAP such that PI4,5P₂ generated by PIPKIα can also be activated by DAG that is generated by PIPKIγ and this interaction blocked PKCδ activity, but PI4,5P₂ - and PKCδ-mediated recruitment of PAP by lipid messengers integrates Star-PAP, PKCδ, and PI4,5P₂ into a signaling pathway essential for BIK expression and initiation of apoptosis, and therefore has wide ramifications for mRNA processing and gene expression.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Treatments

Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (ATCC) and cultured in 1 x DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (50 U/ml) and were grown at 37°C in 5% CO₂. Transfection with the plasmids and the siRNAs (Supplemental Information) was performed as described (Mellman et al., 2008). Whenever required, cells were treated with 100 μM etoposide/tBHQ or the vehicle control, DMSO, for 4 hr or prolonged time as indicated and harvested for analysis.

Quantitative Real-Time RT-PCR

Total RNA was extracted and reverse transcribed. Target mRNA was quantified with the MyiQ single-color real-time PCR detection system (Bio-Rad) as described previously (Mellman et al., 2008). Single-product amplification was confirmed by melting-curve analysis, and primer efficiency was near 100% in all experiments. Target mRNA abundance was normalized to GAPDH expression. Primers used for mRNA expression and 3’ end cleavage are shown in Supplemental Information.

Immunoblot, Immunoprecipitation, and Antibodies

IB and IP were carried out as described (Mellman et al., 2008). The intensity of the bands on images was quantified using ImageJ software. Input = 10% of the total protein used for the IP. The antibodies used are shown in Supplemental Information.

Protein Kinase Assay

The kinase assay was performed as detailed in the Supplemental Information.

Immunofluorescence Microscopy

This was performed as previously described (Boronenkov et al., 1998). The ratios for comparing protein associations were generated by computerized counting of the overlaid yellow spots in 20 cells for each assay condition from a representative experiment.

GST Pull-Down Assay

Human recombinant GST-tagged PKCδ (echelon, #E-K037) and His-tagged Star-PAP or PIPKIγ (affinity purified) were subjected to GST pull-down assay as described previously (Ling et al., 2007). Input = 1% of the total protein used for the pull-down.

RNA Immunoprecipitation

RIP was performed as described (Mellman et al., 2008), using antibodies against endogenous RNA Pol II, Star-PAP, PAPα, PIPKIγ, and PKCδ. The primers used to detect the BIK or GLC3 UTR are shown in Supplemental Information.

3’ RACE and mRNA 3’ End Cleavage Measurement

3’ RACE was performed with the GeneRacer system (Invitrogen, #L1500-02) using BIK or GAPDH gene-specific primers and the 3’ adapter primer, following the manufacturer’s instructions. PCR products were run on agarose gel, and individual DNA bands were excised, purified, and ligated into the pGEM-T Easy Vector (Promega, #A1360) for sequencing. For the 3’ end cleavage, total RNA was isolated and the uncleaved pre-mRNA level was measured by real-time PCR, as described (Mellman et al., 2008). The
noncleaved message was represented as fold over the total mRNA and normalized against the nontreated control sample.

Poly(A) Polymerase Assay
The FLAG-tagged Star-PAP was affinity purified, and the poly(A) polymerase assay was carried out using the 45-mer RNA oligonucleotide (UAGGGA)_{5A15} as an RNA substrate, as described previously (Mellman et al., 2008).

RNA EMSA and RNase Footprinting
Uniformly radiolabeled BIK UTR RNA substrates were prepared by run-off in vitro transcription from the linearized plasmid pTZ-BIK (harbors T7 promoter and BIK 3’ UTR) using the T7 transcription kit (Fermentas). EMSA was carried out in 20 μl EMSA binding buffer, as described (Laishram and Anderson, 2010). The RNA Footprinting was carried out as detailed in the Supplemental Information.

Sequence Analysis of Star-PAP Target Gene UTRs
The microarray data for Star-PAP knockdown in HEK293 cells (Mellman et al., 2008) were analyzed. Cleavage site information was obtained from PolyA_DB (Lee et al., 2007). The Robust Multichip Average (RMA) method was used for data normalization, and the cutoff of p value < 0.05 (t test) and fold change > 1.5 was used to select genes showing significant downregulation, which were considered as Star-PAP targets. Those with one poly(A) signal only as based on the cDNA and EST evidence were used for nucleotide and cis-element analyses. The proportional test and the Fisher’s exact test were used to assess the difference in nucleotide content and the significance of 4-mers in different regions surrounding cleavage sites, respectively.

DNA Laddering Assay
The assay was performed as described (Gong et al., 1994). Equal amounts of cells from each experimental condition were collected 24 hr after etoposide treatment and fixed with ice-cold 70% ethanol. DNA was extracted using phosphate-citrate buffer, treated with RNase and proteinase K, and analyzed by agarose gel electrophoresis.

Detection of Caspase Activities
Caspase activities under the experimental conditions were measured using commercially available assay kits as described in the Supplemental Information.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.11.017.

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Supplemental Information

Star-PAP Control of BIK Expression and Apoptosis

Is Regulated by Nuclear PIPKια and PKCδ Signaling

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Figure S1. Effect of Etoposide on Nuclear DNA, Protein levels, PKCδ and Star-PAP Association, and Requirement of PIPKIα in BIK mRNA Expression

(A) IF staining of nuclear DNA with DAPI in cells treated with etoposide at different time points.

(B) BIK, BAX, PKCδ, and Star-PAP protein levels at different times (h = hour) of etoposide treatment.

(C) IP of Star-PAP from cells treated with etoposide and detection of PKCδ and Star-PAP levels with IB. * = unspecific bands.

(D) PIPKIα kinase activity is required for BIK expression. Knockdown of PIPKIα abolished basal and etoposide-stimulated BIK mRNA and protein generation similar to the impact on BIK mRNA 3'-end cleavage and tail-message production, which were rescued by wild type but not kinase dead PIPKIα (**). The Star-PAP non-target gene GAPDH was used as a negative control in the 3'-RACE assays. See also Figure 1 and Figure 4.
Figure S2. Etoposide Treatment Increased PKCδ and Star-PAP Association within the Cells but Resemble PKCδ Knockdown, Had No Effect on Star-PAP Association with PIPKιαα

(A) IF staining showed enriched nuclear speckle occupancy of PKCδ and its association with Star-PAP after etoposide treatment. The fluorescent colors for PKCδ, SC-35, Star-PAP are indicated. The ratios (derived from image analysis with MetaMorph software) are for comparison of the merged yellow spots within nuclei between etoposide non-treated and treated cells. Scale bar = 10 μm.

(B–E) Star-PAP and PIPKιαα association was neither affected by etoposide (B and C) nor by PKCδ knockdown (D and E) as evaluated by IP and IB. See also Figure 4.
Figure S3. PIPKα Inhibited PKCδ Phosphorylation of Star-PAP which Can Be Restored by PI4,5P2 but Not PMA

(A) The PKCδ kinase activity against Star-PAP was quenched by adding PIPKα.

(B) PIPKα-blocked PKCδ phosphorylation of Flag-Star-PAP was rescued by PI4,5P2. The band indicated as phosphorylated Star-PAP was further confirmed by IP and IB of Star-PAP from the denatured reaction mix.

(C) PMA failed to restore the PIPKα-inhibited PKCδ kinase activity toward Star-PAP. Quantification of the kinase activities was normalized to the substrate-only lane. Total protein used in the kinase reaction was detected by Coomassie staining. See also Figure 4.
Figure S4. PKCδ Kinase Activity Is Required for BIK mRNA 3’-End Cleavage under DNA Damage Conditions

(A) Knockdown of PKCδ impaired the cleavage of BIK mRNA in the etoposide treated but not the non-treated cells. Overexpressing PKCδ<sup>wt/sm</sup> and not PKCδ<sup>kd/sm</sup> rescued the cleavage defect. Error bars represent SEM of 3 independent experiments with triplicate for each experimental condition.

(B) IB showing knockdown of PKCδ in presence or absence of etoposide treatment in Flag-Star-PAP stably expressing HEK 293 cells.

(C) The affinity purified Flag-tagged Star-PAP complexes from cells treated with or without PKCδ knockdown and etoposide (100 μM, 4 h) for in vitro poly(A) polymerase assays shown in Figure 5. * = unspecific bands. See also Figure 5.
Figure S5. PKCδ Is Required for DNA Damage Signal-Mediated Star-PAP Polyadenylation Activities

(A) In vitro poly(A) polymerase assay in the presence of PI4,5P₂ using A₄₅-oligo and increasing concentrations of Flag-Star-PAP complex purified from HEK 293 cells stably expressing Flag-Star-PAP +/- PKCδ knockdown and with or without etoposide treatment. Star-PAP shows PKCδ-dependent polyadenylation of A₄₅-substrate and etoposide stimulates both initiation and processivity of Star-PAP polyadenylation.

(B) In vitro Star-PAP polyadenylation assay with A₄₅-oligos at increasing time points as indicated (1-60 min) in presence of PI4,5P₂. Quantification indicates a linear increase in Star-PAP polyadenylation activity with time (at least up to 60 min) (data not shown). The origin of the gel and markers are indicated. See also Figure 5.
Figure S6. Analysis of Star-PAP Binding to BIK 3'-UTR and Comparison of the Binding Regions in BIK and HO-1 UTR RNAs

(A) EMSA of BIK UTR RNA with 15 nM Star-PAP in presence of increasing Star-PAP antibody (antibody super shift) (left panel) or β-tubulin antibody (right panel). Unbound RNA (F), Star-PAP-BIK RNA binary complex (B) and antibody, Star-PAP and RNA ternary complexes (T) are indicated.

(B) Sequence alignment of the Star-PAP binding regions on the BIK and HO-1 UTR RNAs. * indicates fully conserved residues highlighted in blue.

(C) Footprint of Star-PAP on BIK UTR RNA by RNase T1 probing. The digestion pattern with different RNase T1 concentrations (mU) in absence of Star-PAP (lane 7-8), or in presence of 10 nM or 15 nM of Star-PAP (lane 5-6), and the untreated BIK RNA (lane 9) are indicated. Sequencing ladder is shown in lane 1-4. Numbers refer to the position of nucleotides with respect to cleavage site (CS). PAS = poly(A) signal. See also Figure 6.
Figure S7. Star-PAP and BIM Knockdown Prevented Etoposide-Induced Apoptosis

Caspase 9 and caspase 3/7 activities were measured to evaluate the degree of apoptosis. The efficiency of Star-PAP and BIM knockdown was illustrated by IB at the bottom panels. Error bars represent standard error of the mean (SEM) of 3 independent experiments with triplicate for each experimental condition. See also Figure 7.
Supplemental Experimental Procedures

Plasmids and Constructs
The wild type and the polymerase-dead (D216/218A) Star-PAP as well as the wild type and the kinase dead (D322A) PIPK\(\alpha\) mutant cDNAs were inserted into the pCMV-Tag4a (Stratagene) vector through EcoRI and SalI restriction sites. PKC\(\delta\) wild type and kinase-dead plasmids were purchased from “Addgene (#16386, 16389)”. Silence-resistant mutations were generated in the Star-PAP, PIPK\(\alpha\), and PKC\(\delta\) cDNAs to render it insensitive to the siRNA oligos.

Protein Kinase Assay
The kinase assay was carried out in 1X kinase buffer containing 4 mM MOPS pH 7.2, 5 mM MgCl\(_2\), 3 mM MnCl\(_2\), 1 \(\mu\)M ATP, 0.5 mM DTT, 5 \(\mu\)M sodium orthovanadate, supplemented with 2 \(\mu\)g of MBP or affinity purified Star-PAP, PMA, PI4,5P\(_2\) micelles in NP-40 (0.04\%, final). The assay was initiated by adding \([\gamma-^{32}P]\)ATP to the mixture and further incubated at room temperature (20 – 25 °C) for 30 min. The reaction was stopped by adding sample buffer to the mix and heated at 95 °C for 5 min. The samples were then subjected to SDS-PAGE followed by phosphorimaging with a Storm 840 phosphorimager (Molecular Dynamics) and quantified using the ImageQuant 5.1 software (Molecular Dynamics). Quantification of the kinase activities was normalized to the substrate-only lane.

RNase Footprinting
The RNA Footprinting was carried out by probing the BIK UTR RNA fragment with RNase S or RNase T1 followed by the detection of the cleaved fragments by extension of a primer complementary to the 3’-end of BIK RNA as described (Damgaard et al.,
Around 0.5 nM of RNA was incubated in 20 μl of EMSA buffer in presence or absence of 0 - 15 nM of Star-PAP followed by digestions with indicated amounts of either RNase S or RNase T1 (2 – 4 mU) at RT for 15 min and primer extension after the enzymatic cleavage as described (Laishram and Anderson, 2010). A marker sequence was generated by dideoxy sequencing (Sequenase Quick-Denature sequencing Kit, USB Corps) from the template DNA of BIK UTR RNA with the same primer used for extension.

**Detection of Caspase Activities**

After RNAi knockdown, 10^4 cells/well were reseeded in 96-well plates and let grow for 24 h followed by etoposide treatment. Caspase 9 (after 4 h, Caspase-Glo9 Assay kit, Promega #G8210) and Caspase 3/7 activities (after 24 h, Apo-ONE Homogeneous Assay kit, Promega, #G7792) were measured using BioTek Synergy 2 microplate reader (BellBrook Labs) according to the manufacturers’ instructions. Triplicate wells for each experimental condition were used.

**SiRNAs Used in the Experiments**

scrambled non-targeting: AGGUAGUGUAUUCGCCUUG

Star-PAP: GUGUGUUUGUCAGUGGCUU

PIPKα: GAAGUUGGAGCACUCUUGG

PKCδ: AACCAUGUUAUUCGCCACC

BIM: CAGAGAUAUGGAUCGCCCAAGAGUU

**Primers Used for Gene Expression**

BIK forward: 5’-TCTTGATGGAGACCCTCCTG-3’
BIK reverse: 5’-GTCCTCCATAGGGTCCAGGT-3’

GAPDH forward: 5’-GAAGGTCCGGAGTCAACCGGATT-3’

GAPDH reverse 5’-GAATTTGCCATGGGTGGAAT-3’

**Primers Used for RIP**

*BIK* forward: 5’-GTCACACCCCTGTGTGATATGTGATGC-3’

*BIK* reverse: 5’-GCAGGGAAGGATCTGATTAGGAGCACACAG-3’

*GCLC* forward: 5’-GATGATTAAGAATGCCTGGTT-3’

*GCLC* reverse: 5’-TATGCTTTCTTTCTAGAAACATC-3’

**Primers Used for mRNA 3’-End Cleavage**

*BIK* forward: 5’-GTCACACCCCTGTGTGATATGTGATGC-3’

*BIK* reverse: 5’-GCGAGAGGAAGCCCGTGCTGGTGCTGCC-3’

**Primers Used for mRNA 3’-RACE**

*BIK*: 5’-GGCCTGCTGCTGCTGTATCTTT-3’

*GAPDH*: 5’-GTATGACGACGACATTTGGCTACAGCAAC-3’

**Primer Used for Extension and Sequencing in RNA Footprinting**

5’- TGCCTCCCCTTTTGCAGGGGA -3’.

**Antibodies Used**

Rabbit polyclonal anti-Star-PAP and anti-PIPKια (Anderson’s Lab homemade) (Mellman et al., 2008); rabbit polyclonal anti-PKCδ (Santa Cruz Biotechnology, #sc-937); mouse monoclonal anti-PKCδ (Zymed, #41-0300); mouse monoclonal anti-SC35 (BD-Pharmingen, #556363); mouse monoclonal anti-Flag (Sigma Aldrich, #F1804); goat
polyclonal anti-BIK (Santa Cruz Biotechnology, #sc-1710); rabbit polyclonal anti-BIM (Santa Cruz Biotechnology, #sc-11425); mouse monoclonal anti-RNA Pol II (neoclon, #WP011); rabbit polyclonal anti-BAX (Santa Cruz Biotechnology, #sc-493); mouse monoclonal anti-T7.Tag (Novagen, #69048); mouse monoclonal anti-HA (Covance, #MMS-101P); mouse monoclonal anti-β-tubulin (Upstate Biotechnology, #05-661); mouse monoclonal anti-actin (MP Biomedical, #691002).

**Supplemental References**
