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JNK promotes epithelial cell anoikis by transcriptional and post-translational regulation of BH3-only proteins

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Summary

Developmental morphogenesis, tissue injury, and oncogenic transformation can cause the detachment of epithelial cells. These cells are eliminated by a specialized form of apoptosis (anoikis). While the processes that contribute to this form of cell death have been studied, the underlying mechanisms remain unclear. Here we tested the role of the cJUN NH_2-terminal kinase (JNK) signaling pathway using murine models with compound JNK-deficiency in mammary and kidney epithelial cells. These studies demonstrated that JNK is required for efficient anoikis *in vitro* and *in vivo*. Moreover, JNK-promoted anoikis required pro-apoptotic members of the BCL2 family of proteins. We show that JNK acts through a BAK/BAX-dependent apoptotic pathway by increasing BIM expression and phosphorylating BMF leading to death of detached epithelial cells.

eTOC Blurb

Developmental morphogenesis, tissue injury, and oncogenic transformation can cause epithelial cell detachment. These cells are eliminated by a specialized form of apoptosis termed anoikis. Girnius and Davis show that anoikis is mediated by the cJUN NH_2-terminal kinase (JNK), which increases BIM expression and phosphorylates BMF to engage BAK/BAX-dependent apoptosis.

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Author Contributions

N.G. and R.J.D. designed the study. N.G. performed experiments. N.G. and R.J.D. analyzed data and wrote the paper.

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The RNA-seq data were deposited in the Gene Expression Omnibus (GEO) repository with accession number GSE88856. The flow cytometry data were deposited in Flow Repository with accession number FR-FCM-ZYCR.

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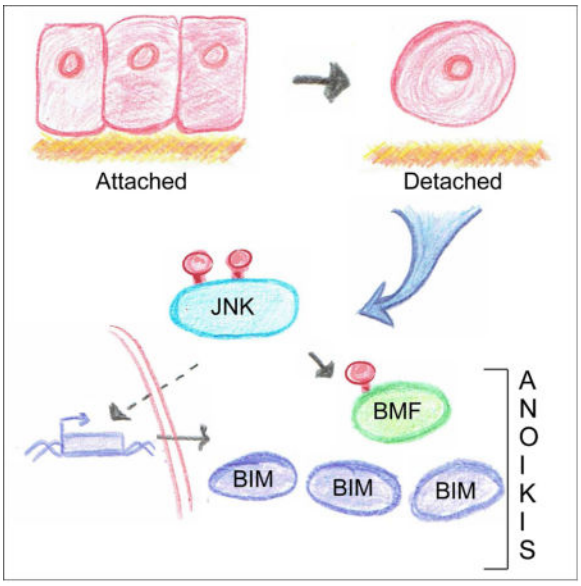

Introduction

Multicellular organisms rely on apoptosis to remove excess cells, mediate cell turnover, and clear damaged cells in order to prevent disease (Fuchs and Steller 2011). Improper regulation of cell death is implicated in pathogenic processes, including cancer (Hanahan and Weinberg 2011). Gaining an understanding of pathways that mediate these forms of cell death is therefore critically important.

Pro-apoptotic BCL2-family proteins, including BAK/BAX-like proteins and BH3-only proteins, can initiate cell death, while anti-apoptotic BCL2-family proteins can suppress cell death (Huang and Strasser 2000; Pinon et al. 2008; Czabotar et al. 2014). BAK and BAX can release cytochrome c from mitochondria, thereby committing cells to apoptosis (Jurgensmeier et al. 1998; Narita et al. 1998). Anti-apoptotic BCL2-like proteins can prevent BAK and BAX activation, while pro-apoptotic BH3-only members of the BCL2 family can initiate BAK/BAX-mediated cell death. Multiple signaling pathways target the BCL2-family proteins, and the balance of these signals determines whether a cell initiates apoptosis (Puthalakath and Strasser 2002). The stress-activated c-JUN NH_2-terminal kinase (JNK) pathway (Davis 2000) is one of these signaling mechanisms (Tournier et al. 2000). Pro-apoptotic targets of JNK signaling include the BH3-only proteins BIM and BMF that can initiate BAK/BAX-dependent apoptotic cell death (Lei et al. 2002; Lei and Davis 2003; Hubner et al. 2008; Hubner et al. 2010).

Anoikis – apoptosis induced by epithelial cell detachment – is implicated in the luminal clearance of developing mammary glands (Humphreys et al. 1996), involution of lactating mammary glands (Boudreau et al. 1995), and cancer metastasis (Douma et al. 2004). The initiation of anoikis is induced by the disruption of epithelial cell interactions with the cell matrix (Frisch and Francis 1994; Frisch and Screaton 2001; Reginato et al. 2003). The role of JNK in anoikis is controversial because it has been reported that JNK is essential (Frisch

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et al. 1996) and also that JNK is dispensable (Khwaja and Downward 1997) for epithelial cell apoptosis in response to detachment. This controversy has yet to be resolved. More recent studies suggest that JNK may promote epithelial cell anoikis *in vitro* (McNally et al. 2011) and *in vivo* (Cellurale et al. 2012).

The purpose of this study was to rigorously test the role of JNK in anoikis using compound ablation of the *Mapk8* and *Mapk9* genes that encode the JNK1 and JNK2 protein kinases (Han et al. 2013) and pharmacological inhibition using a highly specific small molecule (Zhang et al. 2012). These *loss-of-function* studies demonstrated that JNK signaling is required for epithelial cell anoikis. Conversely, *gain-of-function* studies using constitutively activated JNK demonstrated that JNK signaling promotes anoikis. Mechanistic analysis demonstrated that JNK-promoted anoikis requires the pro-apoptotic BCL2-family proteins BAK/BAX and the BH3-only proteins BIM and BMF. We show that JNK-induced BIM expression and JNK-mediated phosphorylation of BMF lead to engagement of the BAK/BAX apoptosis pathway that causes death of detached epithelial cells.

Results

JNK promotes epithelial cell anoikis

To test the role of JNK during epithelial cell anoikis, we examined the effect of JNK inhibition using a small molecule (JNK-IN-8) that selectively and potently blocks JNK activity (Zhang et al. 2012). Normal human mammary epithelial cells were treated with JNK-IN-8 or solvent (DMSO) and then cultured in suspension (1h or 48h). The number of apoptotic (annexin V^+ 7-AAD^-) cells was measured by flow cytometry. Suspension culture (48h) caused a large increase in apoptosis (anoikis) that was strongly suppressed following treatment with the JNK inhibitor (Figure S1).

To obtain genetic evidence for a role of JNK in epithelial cell anoikis, we examined the effect of *Mapk8* (encodes JNK1) and *Mapk9* (encodes JNK2) gene ablation in primary murine kidney epithelial cells. Immunoblot analysis of Control *($Mapk8^{+/+}$ $Mapk9^{+/+}$)* and JNK^{KO} *($Mapk8^{-/-}$ $Mapk9^{-/-}$)* cells confirmed that JNK was expressed in Control, but not JNK^{KO}, epithelial cells (Figure 1A). We examined anoikis of Control and JNK^{KO} epithelial cells caused by suspension culture (1h or 24h). Colony formation assays demonstrated that JNK-deficiency promoted epithelial cell survival (Figure 1B). Quantitation of apoptotic (annexin V^+ 7-AAD^-) cells using flow cytometry (Figure 1C) and activation of the apoptosis effector caspase 3 by cleavage (Figure 1D) confirmed that JNK is required for efficient epithelial cell anoikis.

To test whether JNK promotes anoikis, we examined the effect of conditional expression of constitutively activated JNK using epithelial cells transduced with a doxycycline-inducible lentiviral vector that expresses Flag-Mkk7β2-Jnk1α1 (JNK^{CA}). Immunoblot analysis confirmed that treatment with doxycycline induced the expression of JNK^{CA} (Figure 1E). When cultured in suspension (1h or 24h), JNK^{CA} expression in epithelial cells caused an increase in the number of apoptotic (annexin V^+ 7-AAD^-) cells detected by flow cytometry (Figure 1F). These data demonstrate that JNK functions to promote anoikis.

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JNK-promoted anoikis is mediated by the BAK/BAX pathway

It is established that the pro-apoptotic BCL2 family proteins BAK and BAX play a central role in apoptotic cell death (Lindsten et al. 2000; Wei et al. 2001). To test whether this pathway contributes to anoikis, we examined the effect of suspension culture (1h and 24h) on Control and *Bak1*$^{-/-}$ *Bax*$^{-/-}$ (BAK/BAX^{KO}) epithelial cells. We found that BAK/BAX-deficiency greatly decreased the number of apoptotic (annexin V^{+} 7-AAD^{-}) cells detected by flow cytometry following epithelial cell detachment (Figure 2A). BAK and BAX are therefore key players in anoikis.

To test whether BAK/BAX contribute to JNK-promoted anoikis, we examined BAK/BAX^{KO} epithelial cells transduced with a lentiviral vector that conditionally expresses JNK^{CA}. Expression of JNK^{CA} in doxycycline-treated BAK/BAX^{KO} epithelial cells was confirmed by immunoblot analysis (Figure 2B). Examination of BAK/BAX^{KO} epithelial cell suspension cultures demonstrated that JNK^{CA} expression did not cause increased anoikis (Figure 2C). Together, these data demonstrate that JNK-promoted anoikis is mediated by the BAK/BAX pathway.

BH3-only proteins promote epithelial cell anoikis *in vitro*

The BAK/BAX pathway of cell death can be engaged by BH3-only members of the BCL2 protein family by interacting with pro-survival BCL2-family proteins (Zong et al. 2001; O'Neill et al. 2016). We found that anoikis was not associated with increased BAK or BAX expression (Figure S2A). Consequently, BAK/BAX-mediated cell death may be initiated by either increased pro-apoptotic BH3-only protein function and/or decreased pro-survival BCL2 family protein function.

We examined the potential role of pro-survival members of the BCL2 family. Gene expression studies demonstrated decreased expression of *Bcl2* and *Bcl2l1* during anoikis of primary murine epithelial cells (Figure S2B). Increased expression of pro-survival BCL2 family genes was not detected in JNK^{KO} epithelial cells (Figure S2B) and therefore cannot account for the resistance of JNK^{KO} epithelial cells to anoikis (Figure 1).

Expression of the anti-apoptotic BCL2 family member MCL1 is regulated by ubiquitin-mediated degradation promoted by the AKT-regulated GSK3 signaling pathway (Maurer et al. 2006). We therefore performed immunoblot analysis to examine survival signaling pathways and MCL1 expression during anoikis. We found that suspension culture caused decreased activation of the ERK and AKT signaling pathways and decreased expression of MCL1 protein (Figure S3). However, JNK-deficiency caused no change in MCL1 protein abundance (Figure S3). Together, these data demonstrate that decreased expression of pro-survival BCL2 family member MCL1 protein likely contributes to anoikis, but this mechanism is not targeted by JNK to promote anoikis.

We also examined the role of pro-apoptotic BH3-only members of the BCL2 family in JNK-promoted apoptotic cell death during anoikis. Gene expression analysis demonstrated that the expression of *Bcl2l11* (encoding BIM), *Bmf*, and *Hrk* were increased during epithelial cell anoikis (Figure 3A). However, only very low levels of *Hrk* gene expression were detected (Figure S4A). This analysis indicates that BIM and BMF may mediate the effects

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of JNK on anoikis. However, studies of gene expression by JNK^{KO} epithelial cells demonstrated that only the *Bcl2l11* gene (not the *Bmf* gene) exhibited JNK-dependent expression during anoikis (Figure 3A, S3 & S4B). Thus, JNK promotes BIM expression during anoikis.

To test the role of BIM and BMF in anoikis, we prepared primary epithelial cells from Control mice *(Bcl2l11*$^{+/+}$ *Bmf*$^{+/+}$*)*, BIM^{KO} mice *(Bcl2l11*$^{-/-}$ *Bmf*$^{+/+}$*)*, and BMF^{KO} mice (*Bcl2l11*$^{+/+}$ Bmf $^{-/-}$). We also prepared primary epithelial cells from BIM/BMF^{KO} mice (*Bcl2l11*$^{-/-}$ Bmf$^{-/-}$) because it has previously been established that BIM and BMF have partially redundant functions (Hubner et al. 2010; Labi et al. 2014). These cells were cultured in suspension (1h and 24h) to test the effect of BIM and BMF-deficiency on anoikis. Colony formation assays demonstrated that BIM-deficiency, but not BMF-deficiency, significantly increased survival following suspension culture (Figure 3B). Similarly, BIM-deficiency, but not BMF-deficiency, suppressed cleavage and subsequent activation of the apoptosis effector caspase 3 during anoikis (Figure 3C). However, both BIM-deficiency and BMF-deficiency significantly decreased the number of apoptotic (annexin V^+ $7\text{-}AAD^-$) cells detected by flow cytometry during anoikis (Figure 3D). These data indicate that while BIM and BMF can both contribute to anoikis, BIM (which exhibits JNK-dependent expression) plays a key role during anoikis, while BMF (which is expressed by a JNK-independent mechanism) most likely plays a partially redundant role.

BIM and BMF-deficiency in epithelial cells causes resistance to JNK-promoted anoikis

To confirm that BIM and BMF mediate the effects of JNK to promote anoikis, we examined the effect of compound BIM and BMF-deficiency in epithelial cells that conditionally express constitutively activated JNK (JNK^{CA}). Activated JNK was expressed using a doxycycline-inducible lentiviral vector (Figure 4A). JNK-promoted anoikis in control *(Bcl2l11*$^{+/+}$ *Bmf*$^{+/+}$*)* epithelial cells was detected by flow cytometry of Annexin V staining (Figure 4B, C). However, this JNK-promoted anoikis was suppressed in *Bcl2l11*$^{-/-}$ *Bmf*$^{-/-}$ (BIM/BMF^{KO}) epithelial cells (Figure 4B, C).

The low level of JNK-promoted anoikis detected in *Bim*$^{-/-}$ *Bmf*$^{-/-}$ epithelial cells may be caused by the increased expression of other BH3-only proteins. Indeed, we found that constitutively activated JNK caused increased expression of *Hrk* mRNA by epithelial cells in suspension culture (Figure S5A). Increased *Pmaip1* mRNA (encodes NOXA) expression was also detected (Figure S5B). It is therefore possible that HRK and NOXA may also contribute to anoikis caused by constitutively activated JNK. However, studies of wild-type epithelial cells demonstrated that anoikis was associated with very low levels of *Hrk* mRNA expression (Figure S4A) and only modest changes in *Pmaip1* mRNA expression (Figure 3A). These data suggest that BIM and BMF are the major physiological targets of JNK signaling and that very high levels of activated JNK may also engage the HRK and NOXA pathways.

BIM and BMF are required for epithelial cell anoikis *in vivo*

It is established that lumen formation in terminal end buds (TEBs) and ducts is mediated by apoptosis (Humphreys et al. 1996) and that defective anoikis causes TEB/ductal occlusion

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(Mailleux et al. 2007). Interestingly, JNK-deficiency is associated with TEB/ductal occlusion (Cellurale et al. 2012), suggesting that JNK signaling in the breast epithelium may be required for developmental anoikis. Previous studies have established roles for BIM and BMF in mammary acinar formation (Mailleux et al. 2007; Schmelzle et al. 2007). To elucidate the relative roles of BIM and BMF in this form of anoikis *in vivo*, we examined murine mammary gland development. We found that BMF-deficiency caused no major defects in mammary gland development of young (5–6 wk old) or mature (6 month old) mice (Figure 5A,B & S6A), although BMF-deficiency was found to cause increased duct extension in young mice compared with Control mice (Figure S6B). In contrast, young BIM-deficient mice exhibited a marked defect in duct extension (Figure S6B) and occlusion of TEB and ducts (Figure 5A and S6A) compared with Control mice. Duct occlusion in mature BIM^{KO} mice was not observed (Figure 5B). Interestingly, the developmental defects detected in compound mutant BIM/BMF^{KO} mice were more severe than those detected in either BIM^{KO} mice or BMF^{KO} mice. Compared with BIM^{KO} mice, young BIM/BMF^{KO} mice exhibited a larger duct extension defect and significantly greater occlusion of TEB and ducts (Figure 5A & S6). Moreover, the duct occlusion phenotype persisted in mature BIM/BMF^{KO} mice (Figure 5B) and was not associated with increased proliferation, as monitored by PCNA staining (Figure 5C & S6C). These data demonstrate that BIM plays a key role during mammary gland development, and confirm the conclusion that the anoikis functions of BIM are partially redundant with BMF.

The mammary epithelium is composed of keratin 5^+ myoepithelial cells that form the exterior surface of ducts and keratin 8^+ luminal epithelial cells that form the interior surface of ducts (Deugnier et al. 2002). To determine which of these cell types occluded the ducts of BIM/BMF^{KO} mice, we stained tissue sections with antibodies to keratin 5 and keratin 8. Both myoepithelial and luminal cells contributed to luminal occlusion in young mice (Figure 6A), but we primarily found luminal cells in the occluded lumens of mature mice (Figure 6B). Thus, while myoepithelial and luminal epithelial cells are initially retained within the ducts of the developing mammary glands of BIM/BMF^{KO} mice, it is the luminal epithelial cells that persist in mature mice (Figure 6).

Role of BIM and BMF phosphorylation during anoikis

The anoikis phenotypes of $Mapk8^{-/-}$ $Mapk9^{-/-}$ (JNK^{KO}) epithelial cells (Figure 1) and $Bcl2l11^{-/-}$ $Bmf^{-/-}$ (BIM/BMF^{KO}) epithelial cells (Figure 3) are similar. It is possible that this observation reflects the finding that both BIM and BMF are phosphorylated by JNK (Lei and Davis 2003; Hubner et al. 2008; Hubner et al. 2010). To test whether JNK-mediated phosphorylation of BIM and BMF contributes to JNK-dependent anoikis, we isolated primary epithelial cells from mice harboring point mutations in the *Bcl2l11* and *Bmf* genes at the JNK phosphorylation sites Thr^{112} on BIM and Ser^{74} on BMF (Hubner et al. 2008; Hubner et al. 2010). We also examined a *Bcl2l11* mutant that is resistant to ERK-promoted proteasomal degradation due to mutations at the ERK phosphorylation sites Ser^{55}, Ser^{65}, and Ser^{73} (BIM^{3SA}) (Hubner et al. 2008). We found that mutation of the ERK phosphorylation sites on BIM caused no change in epithelial cell anoikis monitored by flow cytometry analysis of 7-AAD/Annexin V staining (Figure 7A). Similarly, mutation of the JNK phosphorylation site Thr^{112} (replacement with Ala) caused no change in anoikis, including

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studies using epithelial cells on a sensitized genetic background *(Bmf*$^{-/-}$*)* (Figure 7A, B). In contrast, mutation of the BMF phosphorylation site Ser^{74} (replacement with Ala) caused decreased anoikis *in vitro* (Figure 7C), but caused only limited ductal occlusion *in vivo* (Figure S7).

Collectively, these data indicate that BIM and BMF phosphorylation is not essential for anoikis. Indeed, BIM phosphorylation at these sites appears to play no role in anoikis. However, phosphorylation of BMF on Ser^{74}, a site targeted by JNK, partially contributes to anoikis. We conclude that BMF phosphorylation (but not BIM phosphorylation) may contribute to cell death following epithelial cell detachment.

Discussion

Genetic studies of epithelial cell sheet development in *Drosophila* demonstrate a role for extrusion and death of compromised cells. This extrusion mechanism causes the removal of cells from the epithelial cell sheet and JNK activation in the detached epithelial cells subsequently causes apoptosis (Adachi-Yamada et al. 1999; Adachi-Yamada and O'Connor 2002; Gibson and Perrimon 2005; Shen and Dahmann 2005). Similarly, competition between cells in epithelial cell sheets for limiting amounts of morphogen (e.g. a TGF-β ligand) (Moreno et al. 2002) or cell-intrinsic fitness (e.g. cMYC expression) (Moreno and Basler 2004) can cause JNK-dependent elimination of compromised cells. These mechanisms not only ensure normal development of epithelial cell sheets, but also act to suppress tumor formation (Brumby and Richardson 2003; Uhlirova et al. 2005; Igaki 2009). JNK therefore plays a key role in epithelial cell sheet development. Nevertheless, the mechanism of pro-apoptotic signaling by JNK in *Drosophila* is unclear.

Mammalian studies of the role of JNK in epithelial cell detachment and death (anoikis) are controversial because early studies using dominant-negative over-expression approaches in MDCK epithelial cells were interpreted both to support a required role for JNK in anoikis (Frisch et al. 1996) and to refute a role of JNK in anoikis (Khwaja and Downward 1997). More recently, studies using the drug SP600125, that inhibits JNK and many other protein kinases (Bain et al. 2003), indicated that JNK may be required for acinar formation and luminal clearance by human MCF10A mammary epithelial cells (McNally et al. 2011). This observation suggested that JNK may play a role in anoikis. This was later supported by studies of murine mammary gland development using mice with wild-type or compound mutant *Mapk8*$^{-/-}$ *Mapk9*$^{-/-}$ mammary epithelial cells that demonstrated a requirement of JNK for the luminal clearance of mammary ducts and terminal end buds by apoptosis (anoikis) (Cellurale et al. 2012). The present study extends these findings to demonstrate a requirement for JNK in anoikis of human and murine primary epithelial cells (Figures 1 & S1).

This requirement for JNK in anoikis contrasts with the observation that JNK does not contribute to other forms of apoptosis, including cell death mediated by the cell surface receptors FAS and TNFR1 (Tournier et al. 2000; Lamb et al. 2003; Das et al. 2009). These observations indicate that JNK plays a selective role in apoptosis. This is illustrated by the finding that constitutively activated JNK does not cause apoptosis of attached epithelial

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cells, but constitutively activated JNK promotes apoptosis of detached epithelial cells (Figures 1 & 4).

We demonstrate that the mechanism of JNK signaling to cause anoikis requires the pro-apoptotic BCL2 family proteins BAK and BAX (Figure 2) and the pro-apopotic BH3-only proteins BIM and BMF (Figure 3). *Gain-of-function* studies using conditional expression of constitutively activated JNK demonstrated that the JNK-promoted anoikis detected in wild-type epithelial cells was suppressed in BAK/BAX^{KO} epithelial cells (Figure 2) or BIM/BMF^{KO} epithelial cells (Figure 4). The residual cell death detected in BAK/BAX^{KO} and BIM/BMF^{KO} cells may reflect partial compensation by the related pro-apoptotic proteins BOK (Figure S2) and HRK/NOXA (Figures 3 & S5), respectively. Together, these data establish the BH3-only proteins BIM and BMF as mediators of JNK-promoted anoikis caused by activation of the cell intrinsic BAK/BAX mitochondrial apoptosis pathway.

While BIM and BMF co-operate to cause anoikis, BIM plays a key role while the pro-apoptotic functions of BMF are partially redundant with BIM (Figure 3). This co-operation is illustrated by the finding that compound mutant BIM/BMF^{KO} mice exhibited a larger duct extension defect and significantly greater occlusion of TEB and ducts compared with BIM^{KO} mice or BMF^{KO} mice (Figure 5A & S6). Moreover, unlike BIM^{KO} mice or BMF^{KO} mice, the duct occlusion phenotype persisted in mature BIM/BMF^{KO} mice (Figure 5B). These functions of BIM and BMF are consistent with previous observations demonstrating co-operative roles of BIM and BMF during cell death (Hubner et al. 2010; Labi et al. 2014; Sakamoto et al. 2016). Moreover, these roles are consistent with the established functions of BIM and BMF during mammary acinar development (Mailleux et al. 2007; Schmelzle et al. 2007).

Two mechanisms may account for the activation of BIM and BMF by JNK signaling:

1. Anoikis is associated with markedly increased expression of both BIM and BMF (Figure 3A). The increased expression of BIM, but not BMF, was JNK-dependent (Figure 3A). Indeed, it is established that the JNK target cJUN strongly promotes the expression of BIM (Whitfield et al. 2001). This JNK-dependent increase in BIM expression may account for the requirement of both JNK and BIM for anoikis.

2. BIM and BMF are substrates that are phosphorylated by activated JNK (Lei and Davis 2003; Hubner et al. 2008; Hubner et al. 2010). Studies of mice with germ-line point mutations in BIM phosphorylation sites (Ser^{55}, Ser^{65}, Ser^{73} or Thr^{112}) demonstrated that BIM phosphorylation was not required for anoikis (Figure 7). In contrast, studies of mice with a germ-line point mutation in BMF at the JNK phosphorylation site Ser^{74} demonstrated that BMF phosphorylation was required for efficient anoikis (Figure 7).

Collectively, these data establish that JNK promotes anoikis by increasing BIM expression and by phosphorylating BMF. It is possible these roles of BIM and BMF are augmented by effects of JNK on other BH3-only proteins, including NOXA and HRK (Figure 3A). NOXA is expressed by epithelial cells and this expression is modestly increased during anoikis (Figure 3). NOXA may therefore contribute to anoikis under some conditions, particularly

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when a threshold amount of BH3-only protein is required to promote anoikis. HRK is expressed at extremely low levels in primary epithelial cells, but does exhibit increased JNK-dependent expression during anoikis (Figures 3A & S4A). This JNK-dependent HRK expression may reflect the targeting of the *Hrk* gene by the cJUN transcription factor (Ma et al. 2007). Nevertheless, the very low level of HRK expression in epithelial cells indicates that HRK may only contribute to anoikis under specialized circumstances. Interestingly, the expression of HRK and NOXA are increased when epithelial cells expressing constitutively activated JNK are cultured in suspension (Figure S5). It is therefore possible that BIM and BMF mediate the pro-anoikis effects of moderate levels of JNK activation in detached epithelial cells and that very high levels of JNK activity may additionally recruit JNK-dependent expression of HRK and NOXA to promote efficient anoikis.

Genetic analysis of *Drosophila* indicates that JNK plays a role in tumor suppression by promoting the elimination of compromised epithelial cells (Brumby and Richardson 2003; Uhlirova et al. 2005; Igaki 2009; Igaki et al. 2009; Ohsawa et al. 2011). This observation suggests that JNK may play a similar role in mammalian epithelial cells. Thus, loss of JNK function by epithelial cells may lead to survival in luminal spaces and the subsequent acquisition of additional mutations that may cause cancer. Indeed, JNK-deficiency enhances tumor formation in a transplantation model of breast cancer (Cellurale et al. 2012). Moreover, sequencing of human tumors has revealed that two upstream components of the JNK pathway (MAP2K4 and MAP3K1) are frequently mutated in human cancer (Stephens et al. 2012; Nik-Zainal et al. 2016). Whether these human mutations contribute to cancer development is unclear. Studies to test this hypothesis are therefore warranted.

In conclusion, we have demonstrated that JNK is required for efficient anoikis of detached human and mouse epithelial cells. We show that JNK causes increased BIM expression and phosphorylation of BMF following epithelial cell detachment. These BH3-only proteins act as mediators of JNK-promoted anoikis that engage the cell intrinsic BAK/BAX mitochondrial apoptosis pathway.

Experimental Procedures

Animal Care

We have previously described *Bmf*$^{-/-}$ mice (RRID:IMSR_JAX:011024), *Bmf*$^{S74A/S74A}$ mice (RRID:IMSR_JAX:011022), *Bcl2l11*$^{T112A/T112A}$ mice (RRID:IMSR_JAX:011026), *Bcl2l11*$^{S55,65,73A/S55,65,73A}$ (RRID:IMSR_JAX:011025) mice, (Hubner et al. 2008; Hubner et al. 2010), and *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$ *RosaCre*^{ERT} mice (Das et al. 2007). C57BL/6J mice (Stock # 000664; RRID:IMSR_JAX:000664) mice, *B6;*129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT)Nat}$*/J* mice (Stock # 004847; RRID:IMSR_JAX:004847) (Badea et al. 2003), and B6.129S1-*Bcl2l11*$^{1tm1.1Ast}$/J mice (Stock # 004525; RRID:IMSR_JAX:004525) (Bouillet et al. 1999)) were obtained from The Jackson Laboratories. Virgin females (age 6 wks and 6 months) were used for mammary gland studies. Both male and female mice (age 8 wks) were used to establish kidney epithelial cells. The mice were housed in a specific pathogen-free facility accredited by the American Association of Laboratory Animal Care. All animal studies were approved (A-1032) by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

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Cell culture

Tertiary human mammary epithelial cells were purchased and maintained in MammaryLife Media (Lifeline Cell Technology). Primary murine kidney epithelial cells were prepared (Follit et al. 2008) using kidneys from mice (8 wk. old) digested at 37°C (<2 h) with 0.1% collagenase, 0.1% trypsin, and 150 mM NaCl in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies). These cells were maintained in DMEM/F12 media containing 10% fetal bovine serum and supplemented with 150 mM urea plus 150 mM NaCl. Wild-type and *Bak1*$^{-/-}$ *Bax*$^{-/-}$ (BAK/BAX^{KO}) epithelial cells were obtained from Applied Biological Materials (ABM Inc) and maintained in DMEM media containing 10% fetal bovine serum. *Rosa-Cre*^{ERT} kidney epithelial cells were treated with 1 μM 4-hydroxytamoxifen (24 h) to ablate floxed alleles.

Immunoblot Analysis

Cell lysates were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin plus leupeptin). Extracts (30μg) were subjected to immunoblot analysis with antibodies to Caspase 3 (Cell Signaling Technology Cat# 9662 RRID:AB_331439; dilution 1:500), ERK2 (Santa Cruz Biotechnology Cat# sc-1647; RRID:AB_627547; dilution 1:1000), Flag (Sigma-Aldrich Cat# F3165 RRID:AB_259529; dilution 1:5000), GAPDH (Santa Cruz Biotechnology Cat# sc-25778; RRID:AB_10167668; dilution 1:1000), JNK (R & D Systems Cat# AF1387 RRID:AB_2140743R&D; dilution 1:1000), p-ERK (Cell Signaling Technology Cat# 9101 RRID:AB_2315114; dilution 1:1000), AKT (Cell Signaling Technology Cat# 9272 RRID:AB_329827; dilution 1:1000), p-Ser473 AKT (Cell Signaling Technology Cat# 9271 RRID:AB_329825; dilution 1:1000), MCL1 (Rockland Cat# 600-401-394S RRID:AB_11179937; dilution 1:2000), BAX (Abcam Cat# ab32503 RRID:AB_725631; dilution 1:5000), BCLXL (Abcam Cat# ab32370 RRID:AB_725655; dilution 1:2000), BCL2 (Abcam Cat# ab182858 RRID:AB_2715467; dilution 1:2000), BMF (Abcam Cat# ab181148 RRID:AB_2715466; dilution 1:1000), BIM (Abcam Cat# ab32158 RRID:AB_725697), and αTubulin (Sigma-Aldrich Cat# T5168; RRID:AB_477579). Immune complexes were detected with IRDye 680LT conjugated-donkey anti-mouse IgG antibody (LI-COR Biosciences Cat# 926-68022 RRID:AB_10715072) and IRDye 800CW conjugated-goat anti-rabbit IgG (LI-COR Biosciences Cat# 926-32211 RRID:AB_621843), and quantitated using the Odyssey infrared imaging system (LI-COR Biosciences).

Analysis of mRNA

RNA was isolated using the RNeasy kit (Qiagen). RNA quality (RIN > 9) was verified using a Bioanalyzer 2100 System (Agilent Technologies). Total RNA (10μg) was used to prepare each RNA-seq library by following the manufacturer's instructions (Illumina). Three independent libraries were examined for each condition. The cDNA libraries were sequenced by Illumina Hi-Seq with a paired-end 40-bp format. These RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database with accession number GSE88856. Reads from each sample were aligned to the mouse genome (UCSC genome browser mm10 build) using TopHat2 (Kim et al. 2013). The average number of aligned

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reads per library was > 30,000,000. Gene expression was quantitated as fragments per kilobase of exon model per million mapped fragments (FPKM) using Cufflinks (Trapnell et al. 2010). Differentially expressed genes were identified using the Cufflinks tools Cuffmerge and Cuffdiff.

The expression of mRNA was also examined by quantitative RT-PCR analysis using a Quantstudio 12K Flex machine (Life Technologies). TaqMan® assays were used to quantify *Bad* (Mm00432042_m1), *Bbc3* (Mm00519268_m1), *Bcl2l11* (Mm00437797_m1), *Bid* (Mm00432073_m1), *Bik* (Mm00476123_m1), *Bmf* (Mm00506773_m1), *Hrk* (Mm01208086_m1), and *Pmaipl* (Mm00451763_m1) mRNA (Life Technologies). The relative mRNA expression was normalized by measurement of the amount of *18S* RNA in each sample using Taqman© assays (catalog number 4308329; Life Technologies).

Anoikis Assay

Tertiary human mammary epithelial cells were suspended (4×10^5 cells/ml) in MammaryLife media (Lifeline Cell Technology) containing 0.5% methylcellulose (Sigma) in poly-HEMA (Sigma) coated plates. Murine kidney epithelial cells were suspended (1.2×10^5 cells/ml) in serum-free DMEM/F12 media supplemented with 0.5% methylcellulose in poly-HEMA coated plates. Where indicated, cells were treated with 2μM JNK-in-8 (Millipore) at 24 h prior to the anoikis assay. Cell death was measured using colony formation assays and by measurement of annexin V-staining.

Colony Formation Assay

Cells were washed with PBS, replated in 24-well plates, and cultured (24 h) prior to fixation (100% methanol, −20°C) and staining with 0.1% cryst al violet dissolved in 20% methanol/80% PBS. The cells were imaged using a Zeiss SteREO Discovery.V12 microscope and quantitated by extracting the crystal violet dye with 10% acetic acid and measurement of the absorbance at 590 nm (Tecan Instruments).

Flow Cytometry

Cells were washed twice with PBS and stained with phycoerythrin-conjugated annexin V and 7-aminoactinomycin D (7-AAD) using the PE annexin apoptosis detection kit I (BD Pharmingen #559763) and examined by flow cytometry using a FACSCalibur (BD Biosciences) to quantitate the apoptotic (Annexin V^+ 7-AAD^-) population. 7-AAD^+ cells were gated using single-stained controls. The annexin V^+ and annexin V^- populations were defined using cells suspended for 1hr. The data obtained were analyzed using FlowJo version 9.7.6 (Tree Star).

Mammary Gland Analysis

The 4^{th} inguinal mammary glands were harvested from 6-week old and 6-month old virgin female mice. Whole mount preparations were fixed with formalin, stained with carmine alum, and imaged using a Zeiss SteREO Discovery.V12 micoscope. Sections (5 μm) were prepared using tissue fixed in 10% formalin that was dehydrated and embedded in paraffin. A board-certified pathologist examined sections stained with hematoxylin & eosin and imaged using a Zeiss Axiovert 200M. Sections were also stained with antibodies against

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keratin 5 (BioLegend Cat# 905501 RRID:AB_2565050; 1:50 dilution) and keratin 8 (Developmental Studies Hybridoma Bank Cat# TROMA-I RRID:AB_531826; 1:100 dilution), and immune complexes were detected using AlexaFluor 546 conjugated-goat anti-rabbit IgG (H+L) antibody (Molecular Probes Cat# A11035 RRID:AB_143051) and AlexaFluor 488 conjugated-goat anti-rat IgG (H+L) antibody (Molecular Probes Cat# A11006 RRID:AB_141373) and counterstained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI). Proliferating cells were stained using the endogenous biotin blocking kit (Thermo Fisher Scientific E21390), biotin-conjugated PCNA antibody (Thermo Fisher Scientific Cat# 13-3940 RRID:AB_2533; dilution 1:50), and AlexaFluor 633-conjugated streptavidin (Thermo Fisher Scientific Cat# S-21375 RRID:AB_2313500). Immunofluorescence was examined using a Leica SP2 confocal microscope.

Plasmids

We have previously described the plasmid pCDNA3-Flag-MKK7β2-Jnk1α1 (Lei et al. 2002). The Flag-MKK7β2-Jnk1α1 cDNA fragment was excised by PCR using the primers 5´-AAACCGCGGGCCGCCACCATGGACTATAAGGACGATGA-3´ and 5´-AAATCTAGATCACTGCTGCACCTGTGCTAAAGGAG-3´, restricted using *Sac*II and *XbaI*, and cloned in the *Sac*II and *Xba*I sites of the entry vector pEN_Tmirc3 (Addgene plasmid # 25748 (Shin et al. 2006)) before insertion, using Gateway Technology, into the lentiviral vector pSLIK-Hygro (Addgene plasmid # 25737 (Shin et al. 2006)) to create the vector pSLIK-Flag-MKK7β2-Jnk1α1-Hygro.

Transduction assays

HEK293T cells (American Type Culture Collection Cat # CRL-3216) were transfected with 7.5μg each of the packaging plasmids pMD2.G (Addgene plasmid #12259 (Naldini et al. 1996)) and psPAX2 (Addgene plasmid # 12260 (Naldini et al. 1996)) plus 10μg of pSLIK-Hygro or pSLIK-Flag-MKK7β2-Jnk1α1-Hygro using Lipofectamine 2000 (Life Technologies). The culture supernatant was collected at 24 h post-transfection and filtered (0.45 μm). Primary epithelial cells were transduced (×2) with the lentivirus plus polybrene (8 μg/ml). The transduced epithelial cells were selected at 48 h post-infection using medium supplemented with 8 μg/ml hygromicin (Life Technologies). The cells were maintained in selection medium with tetracycline-free fetal bovine serum (Clontech). To induce expression of the MKK7-JNK1 fusion protein, the cells were treated with 1 μg/ml doxycycline (24 h).

Statistical Analysis

Data are presented as the mean and standard error. The n values provided in the figure legends correspond to the number of independent experiments for studies using cultured cells or the number of animals examined. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software). Pair-wise comparisons of data with similar variance were performed using a t-test to determine significance ($p<0.05$). Pair-wise comparisons of data with unequal variance were performed using Welch's unpaired t-test to determine significance ($p<0.05$). When more than two populations were compared ANOVA with Bonferroni's test was used to determine significance with an assumed confidence interval of 95%.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Signaling by JNK is needed for efficient anoikis mediated by the BAX/BAK pathway
- JNK promotes anoikis by increasing BIM expression and BMF phosphorylation
- Clearance of occluded mammary ducts in vivo requires JNK and BIM/BMF

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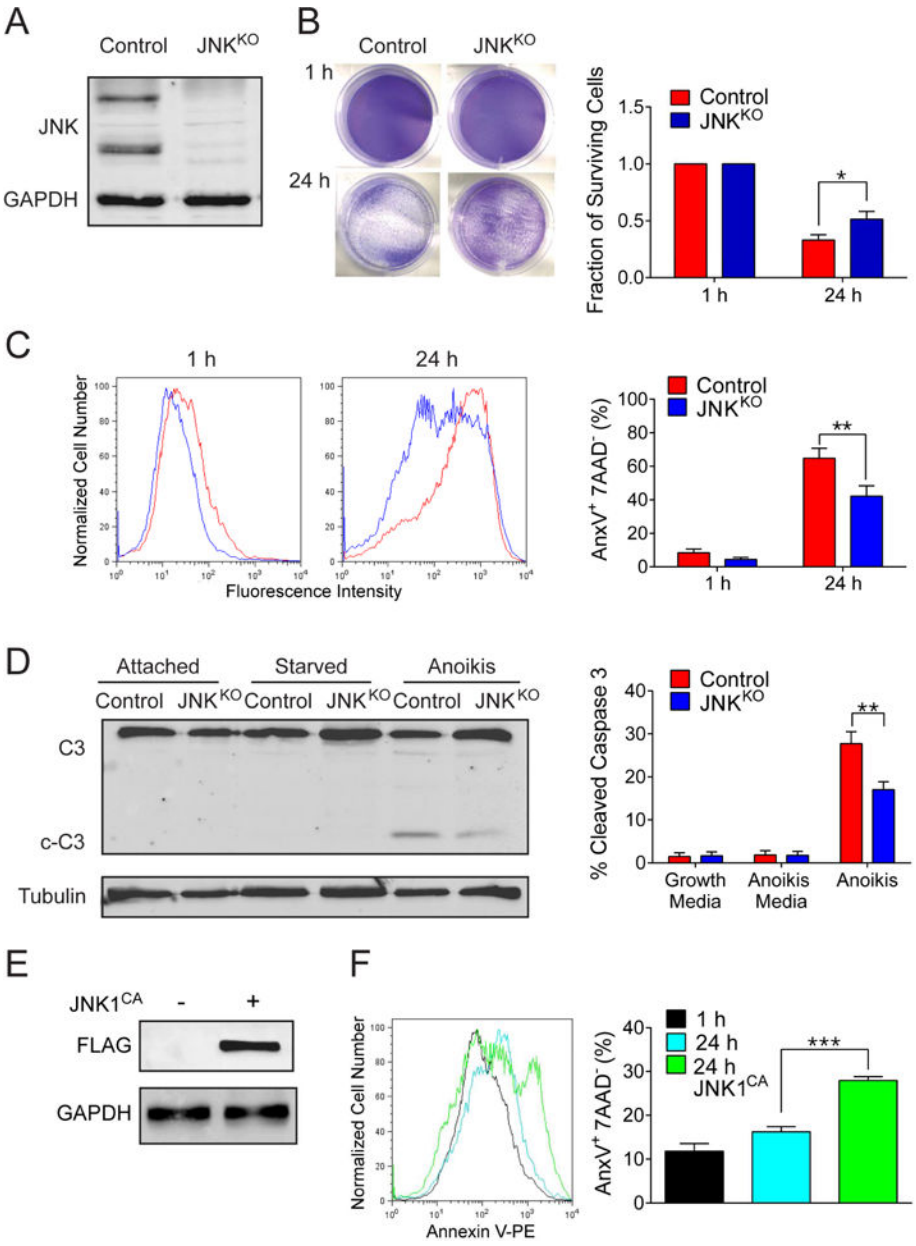

Figure 1. JNK promotes anoikis of murine epithelial cells

A) *Mapk8*$^{LoxP/LoxP}$ *Mapk9*$^{-/-}$ *RosaCre*^{ERT} mouse kidney epithelial cells were treated with 4-hydroxytamoxifen to generate *Mapk8*$^{-/-}$ *Mapk9*$^{-/-}$ cells (JNK^{KO}). JNK expression by Control (*RosaCre*^{ERT}) and JNK^{KO} cells was examined by immunoblot analysis.

B) Control and JNK^{KO} mouse kidney epithelial cells were replated after suspension (1h or 24h) and stained with crystal violet. Representative images of cultures are presented. The fraction of surviving cells was quantitated by staining with crystal violet (mean ± SEM; n=3; * $p<0.05$).

C) Control and JNK^{KO} mouse kidney epithelial cells were cultured in suspension (1h or 24h). Representative flow cytometry data are presented. Apoptotic Control and JNK^{KO} cells

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(annexin V+ ($AnxV^+$) and $7AAD^-$) were quantitated by flow cytometry (mean ± SEM; n=4; ** $p<0.001$).

D) Extracts prepared from Control and JNK^{KO} mouse kidney epithelial cells (attached, attached and starved 24h, and anoikis 24h) were examined by immunoblot analysis of caspase 3 (C3), cleaved caspase 3 (c-C3), and αTubulin. The data were quantitated (mean and SEM; n=3; ** $p<0.01$).

E, F) Control mouse kidney epithelial cells were transduced with an empty vector or a vector that expresses constitutively activated JNK1 (Flag-Mkk7β2-Jnk1α1 ($JNK1^{CA}$)), treated with doxycycline, and examined by immunoblot analysis using antibodies to FLAG and GAPDH (E). The epithelial cells were cultured in suspension (1h or 24 h) and apoptotic cells (annexin V^+ ($AnxV^+$) and $7AAD^-$) were quantitated by flow cytometry (F) (mean ± SEM; n=4; *** $p<0.001$). Representative flow cytometry data are also presented.

See also Figure S1.

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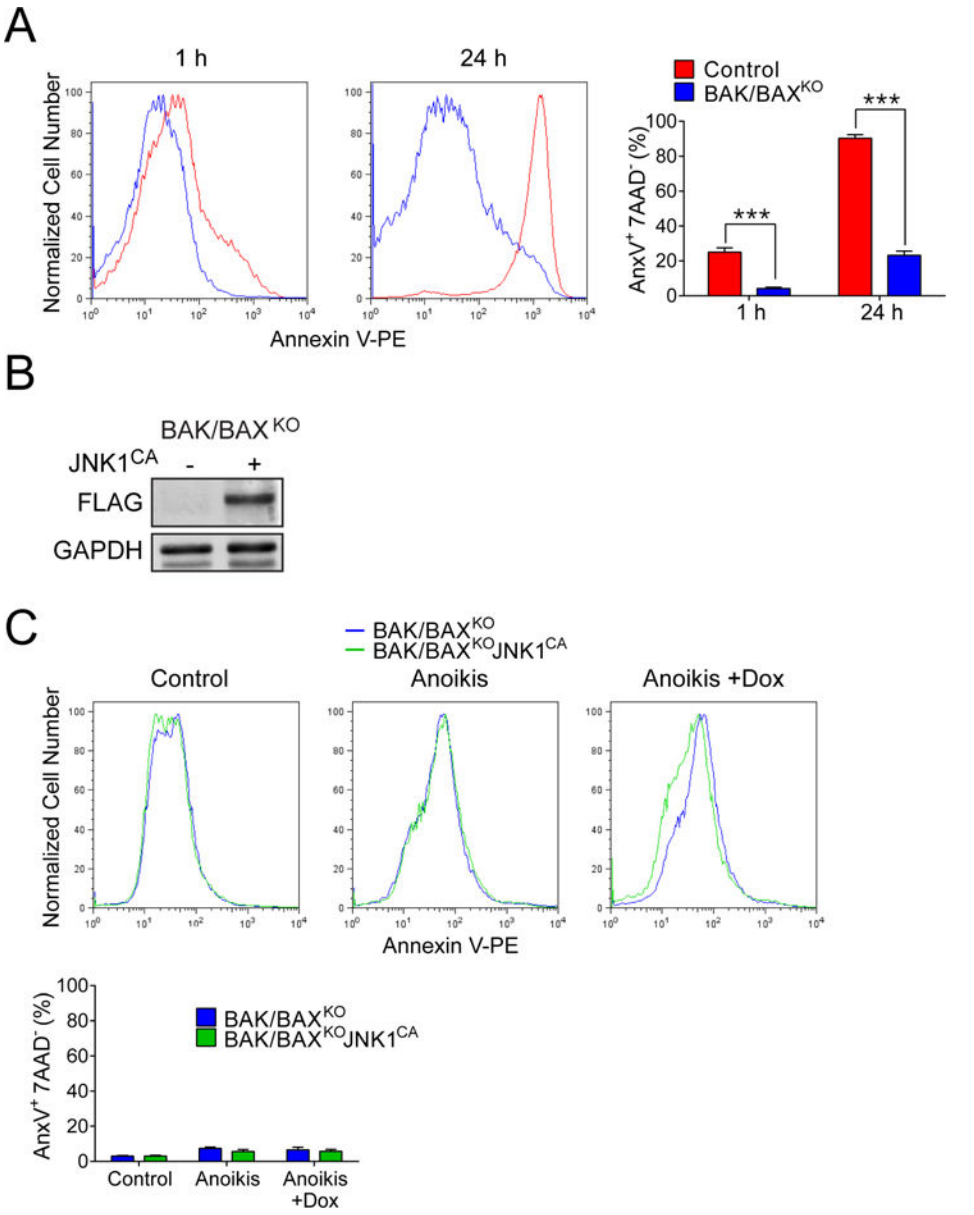

Figure 2. BAK and BAX are required for JNK-promoted anoikis

A) Control and *Bak1*$^{-/-}$ *Bax*$^{-/-}$ (BAK/BAX^{KO}) mouse kidney epithelial cells were cultured in suspension (1h or 24h) and apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were quantitated by flow cytometry (mean ± SEM; n=3; *** p<0.001). Representative flow cytometry data are also presented.

B, C) BAK/BAX^{KO} kidney epithelial cells were transduced with an empty vector or a vector that expresses constitutively activated Flag-tagged JNK1 (JNK1^{CA}), treated with doxycycline, and examined by immunoblot analysis using antibodies to FLAG and GAPDH (B). The cells were cultured in suspension (1h or 24 h) and apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were quantitated by flow cytometry (C). The data shown are the mean ± SEM; n=3). Representative flow cytometry data are also presented.

See also Figure S2.

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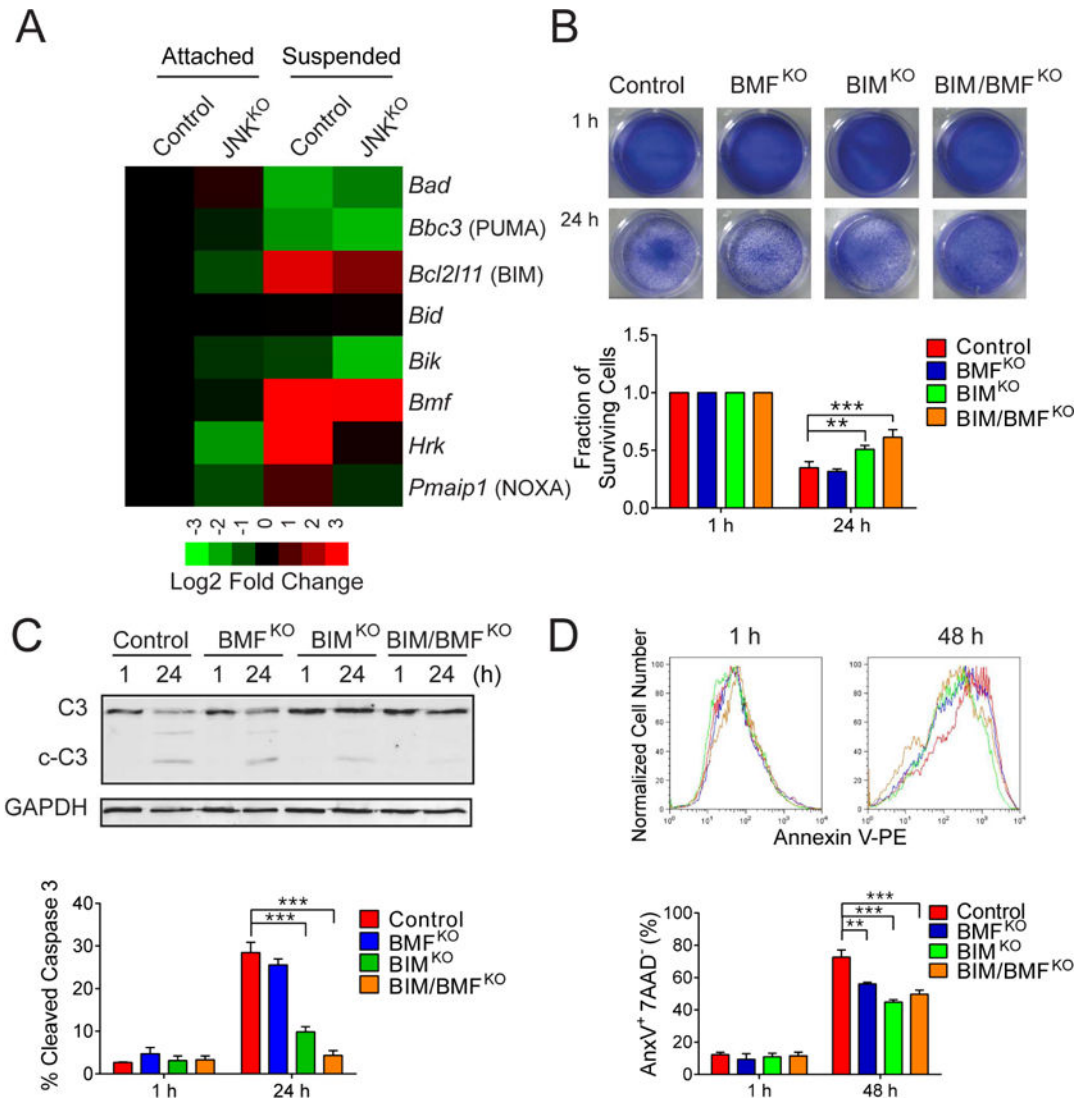

Figure 3. Anoikis causes JNK-dependent increased expression of BH3-only genes

A) Attached and suspended (4h) Control and JNK^{KO} mouse kidney epithelial cells were examined by RNA-seq analysis. The expression of BH3-only genes is presented using a heat map with samples normalized to the attached Control epithelial cells (mean, n=3).

B) Control (n=9), *Bmf*$^{-/-}$ (BMF^{KO}, n=9), *Bcl2l11*$^{-/-}$ (BIM^{KO}, n=12), and compound mutant *Bmf*$^{-/-}$ *Bcl2l11*$^{-/-}$ (BIM/BMF^{KO}, n=10) kidney epithelial cells were cultured in suspension (1h or 24h). Epithelial cell viability was tested by colony formation assays and quantitated by staining with crystal violet (mean ± SEM; ** p<0.01, *** p<0.001). Representative images of cultures are also presented

C) Kidney epithelial cells were cultured in suspension (1h or 24h) and extracts were examined by immunoblot analysis of caspase 3 (C3), cleaved caspase 3 (c-C3), and GAPDH. The data were quantitated (mean and SEM; n=2; *** p<0.001).

D) Kidney epithelial cells were cultured in suspension (1h or 48 h) and apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were quantitated by flow cytometry (mean and SEM;

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Control n=6, BMF^{KO} n=4, BIM^{KO} n=4, BIM/BMF^{KO} n=6; ** $p<0.01$, *** $p<0.001$). Representative flow cytometry data are also presented.
See also Figures S3 & S4.

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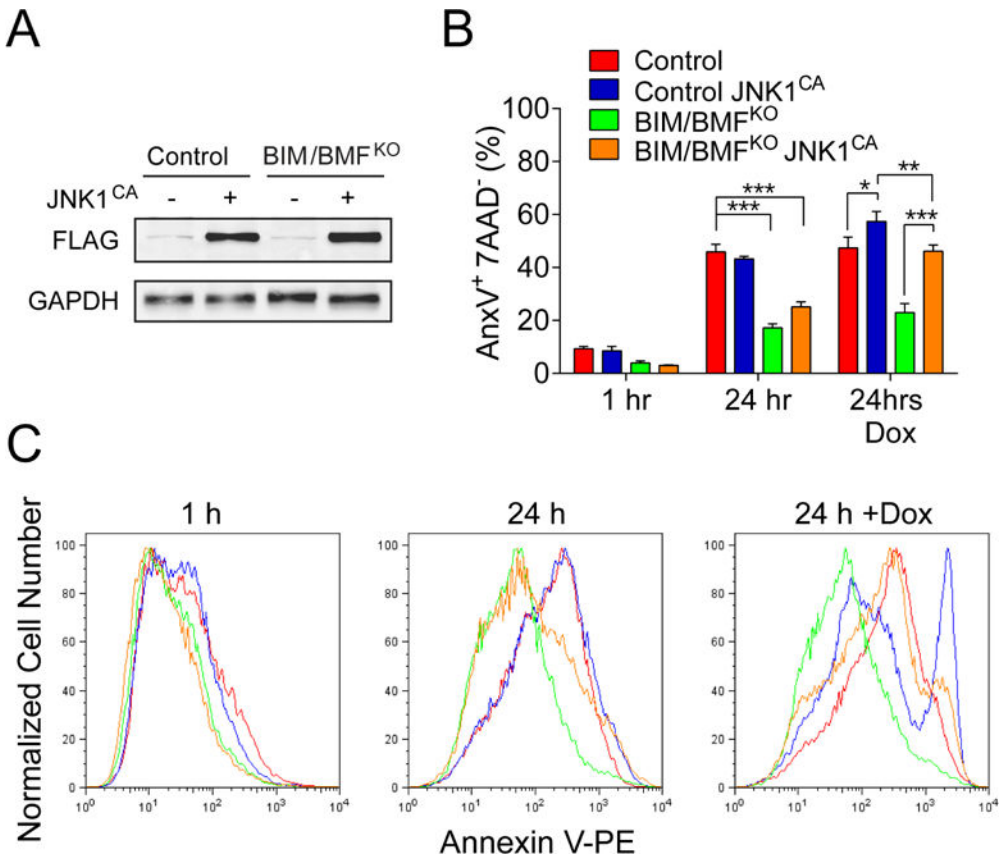

Figure 4. BIM and BMF contribute to JNK-promoted anoikis

A) Control and BIM/BMF^{KO} mouse kidney epithelial cells expressing doxycycline (Dox)-inducible FLAG-tagged constitutively active JNK (JNK1^{CA}) were cultured in suspension (1h or 24h) in medium supplemented without or with doxycycline. The expression of FLAG-JNK1^{CA} and GAPDH was examined by immunoblot analysis.

B, C) The epithelial cells were cultured in suspension (1h or 24 h) and apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were examined by flow cytometry (mean ± SEM; n=6; * $p<0.05$, ** $p<0.01$, *** $p<0.001$) (B). Representative flow cytometry data are presented (C). See also Figure S5.

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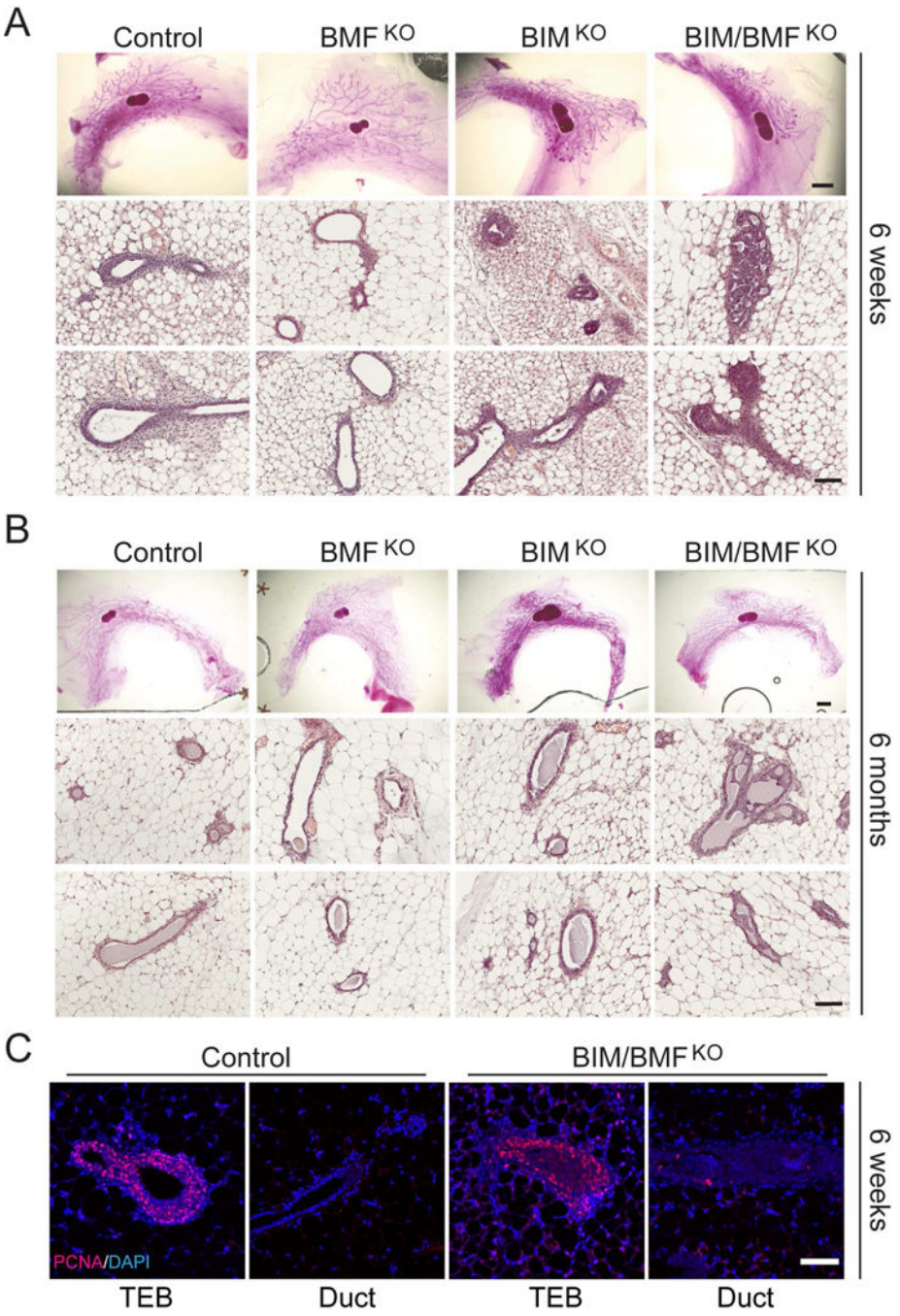

Figure 5. BIM and BMF contribute to normal mammary gland development

A,B) Representative carmine alum-stained whole-mount mammary glands *(upper panels*, scale bar = 2 mm) and hematoxylin and eosin stained sections *(middle and lower panels*, scale bar = 100 μm) from 6 week-old (A) and 6 month-old (B) mice are presented.

C) Representative sections of mammary glands from 6 week-old mice were stained with DAPI (blue) and an antibody to PCNA (red). 5 Control mice and 7 BIM/BMF^{KO} mice were examined. Sections showing ducts and terminal end buds (TEB) are presented. Scale bar = 75μm.

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See also Figure S6.

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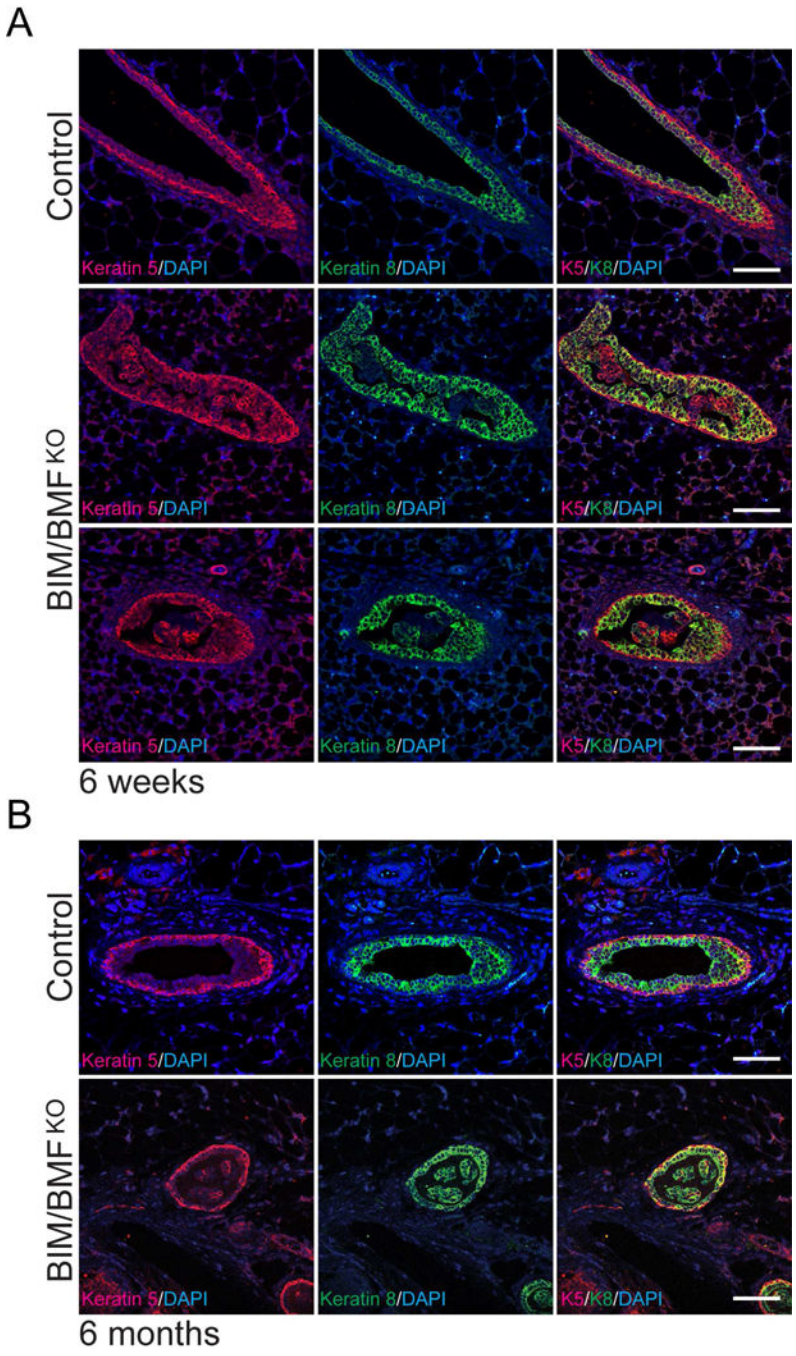

Figure 6. Basal and luminal cells occlude ducts of BIM/BMF^{KO} mammary glands

A, B) Representative sections of mammary glands from Control (upper panels) and BIM/BMF^{KO} (lower panels) mice were stained with antibodies against keratin 5 (red) and keratin 8 (green), and counterstained with DAPI. Scale bars = 75μm. Six week-old (A) and 6 month-old (B) mice are presented.

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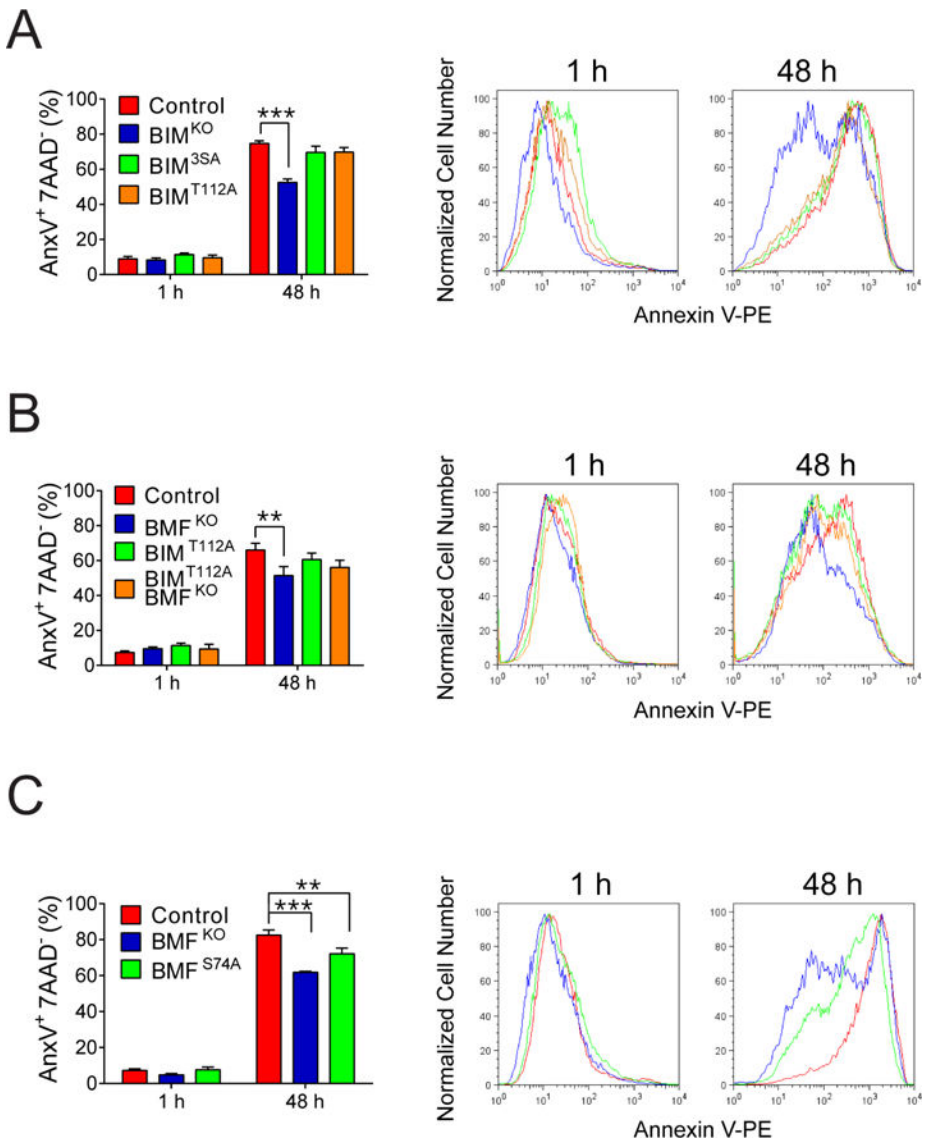

Figure 7. Phosphorylation of BMF, but not BIM, contributes to JNK-promoted anoikis

A)Control, *Bcl2l11*$^{-/-}$ (BIM^{KO}), *Bcl2l11*$^{Ser55,65,73A/Ser55,65,73A}$(BIM^{3SA}), and *Bcl2l11*$^{T112A/T112A}$ (BIM^{112A}) kidney epithelial cells were examined. Apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were quantitated by flow cytometry (mean ± SEM; n=6; ** p<0.01, *** p<0.001). Representative flow cytometry plots are presented.

B) Control, *Bmf*$^{-/-}$ (BMF^{KO}), *Bcl2l11*$^{T112A/T112A}$ (BIM^{T112A}), and *Bmf*$^{-/-}$ *Bcl2l11*$^{T112A/T112A}$ (BMF^{KO} BIM^{T112A}) kidney epithelial cells were examined. Apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were quantitated by flow cytometry (mean ± SEM; n=6; ** p<0.01, *** p<0.001). Representative flow cytometry plots are presented.

C) Control, *Bmf*$^{-/-}$ (BMF^{KO}), and *Bmf*$^{S74A/S74A}$ (BMF^{S74A}) mouse kidney epithelial cells were cultured in suspension (1h or 48 h). Apoptotic cells (annexin V^{+} (AnxV^{+}) and 7AAD^{-}) were quantitated by flow cytometry (mean ± SEM; n=6; ** p<0.01, *** p<0.001). Representative flow cytometry plots are presented.

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See also Figure S7.