JNK Promotes Epithelial Cell Anoikis by Transcriptional and Post-translational Regulation of BH3-Only Proteins

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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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In Brief
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 NH2-terminal kinase (JNK), which increases BIM expression and phosphorylates BMF to engage BAK/BAX-dependent apoptosis.

Data and Software Availability
GSE88856
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 NH₂-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.

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; Piñón et al., 2008; Czabotar et al., 2014). BAK and BAX can release cytochrome c from mitochondria, thereby committing cells to apoptosis (Jürgensmeier 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 NH₂-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; Hübner et al., 2008, 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 Francois, 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 both essential (Frisch et al., 1998) and dispensable (Khawaja 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 showed 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 vitro.
suspension (1 or 48 hr). The number of apoptotic (Annexin V+ 7-aminoactinomycin D [7-AAD]−) cells was measured by flow cytometry. Suspension culture (48 hr) 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 (1 or 24 hr). 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 (JNK1 CA). Immunoblot analysis confirmed that treatment with doxycycline induced the expression of JNK1 CA (Figure 1E). When cultured in suspension (1 or 24 hr), JNK1 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.

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 and activation of the apoptosis effector caspase-3 by cleavage (Figure 1D) confirmed that JNK is required for efficient epithelial cell anoikis.

See also Figure S1.
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 and BAX contribute to JNK-promoted anoikis, we examined BAK/BAX KO epithelial cells transduced with a lentiviral vector that conditionally expresses JNK1 CA. Expression of JNK1 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 JNK1 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 JNKKO epithelial cells (Figure S2B) and therefore cannot account for the resistance of JNKKO 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 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), although
only very low levels of Hrk gene expression were detected (Figure S4A). This analysis indicates that BIM and BMF may mediate the effects 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 (Figures 3A, S3, and 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 (Hübner et al., 2010; Labi et al., 2014). These cells were cultured...
in suspension (1 and 24 hr) 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-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 (JNK1CA). 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 (Figures 4B and 4C). However, this BIM deficiency and BMF deficiency significantly decreased the number of apoptotic (Annexin V− 7-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 Are Required for Epithelial Cell Anoikis In Vivo**

It is established that lumen formation in terminal end buds (TEBs) and ducts in mammary glands is mediated by apoptosis (Humphreys et al., 1996) and that defective anoikis causes TEB/ductal occlusion (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- to 6-week-old) or mature (6-month-old) mice (Figures 5A, 5B, and 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 (Figures 5A and 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 proliferating cell nuclear antigen (PCNA) staining (Figures 5C and 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 keratin 5 and keratin 8 antibodies. 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−/− (JNKKO) epithelial cells (Figure 1) and Bcl2l11−/−/Bmf−/− (BIM/BMFKO) 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; Hübner et al., 2008, 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 Thr112 on BIM and Ser74 on BMF (Hübner et al., 2008, 2010). We also examined a Bcl2l11 mutant that is resistant to ERK-promoted proteasomal degradation due to mutations at the ERK phosphorylation sites Ser55, Ser65, and Ser73 (BIM3SA) (Hübner 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 Thr112 (replacement with Ala) caused no change in anoikis, including studies using epithelial cells on a sensitized genetic background (Bmf−/−) (Figures 7A and 7B). In contrast, mutation of the BMF phosphorylation site Ser74 (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 Ser74, 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 a limited amount of morphogens (e.g., a transforming growth factor β [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; Uhlirva 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 overexpression approaches in Madin Darby canine kidney (MDCK) epithelial cells both supported a required role for JNK in anoikis (Frisch et al., 1996) and refuted a role for JNK in anoikis (Khwaja and Downward 1997). More recently, studies using the drug SP600125, which 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/C0/Mapk9/C0 mammary epithelial cells that demonstrated a requirement of JNK for the luminal clearance of mammary ducts and TEBs 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 and 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 cells, but constitutively activated JNK promotes apoptosis of detached epithelial cells (Figures 1 and 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-apoptotic 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 and 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.
BIM and BMF cooperate to cause anoikis. BIM plays a key role, while the pro-apoptotic functions of BMF are partially redundant with BIM (Figure 3). This cooperation 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 (Figures 5A and 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 cooperative roles of BIM and BMF during cell death (Hübner 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; Hübner et al., 2008, 2010). Studies of mice with germline point mutations in BIM phosphorylation sites (Ser55, Ser65, Ser73, or Thr112) demonstrated that BIM phosphorylation was not required for anoikis (Figure 7). In contrast, studies of mice with a germline point mutation in BMF at the JNK phosphorylation site Ser74 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 (Figures 3A and 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 cooperative roles of BIM and BMF during cell death (Hübner 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).

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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<sup>b−/−</sup> mice (RRID:IMSR_JAX:011024), Bmf<sup>b−/−A53T/441A</sup> mice (RRID:IMSR_JAX:011022), Bcl2<sup>111T124A/111T124A</sup> mice (RRID:IMSR_JAX:011026), Bcl2<sup>111T124A/111T124A</sup> mice (RRID:IMSR_JAX:011025) (Hübner et al., 2008, 2010), and Jnk1<sup>LacZ/Flw</sup> <sup>−/−</sup> RosaCre<sup>ERT</sup> mice (Zas et al., 2007), C57BL/6J mice (stock 000664; RRID:IMSR_JAX:000664) (Das et al., 2007). C57BL/6J mice (RRID:IMSR_JAX:000664) were obtained from The Jackson Laboratory. Virgin females 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.

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 weeks old) digested at 37°C (-2 hr) with 0.1% collagenase, 0.1% trypsin, and 150 mM NaCl in DMEM (Life Technologies). These cells were maintained in DMEM/F12 media containing 10% fetal bovine serum and supplemented with 150 mM NaCl and 150 mM KCl in poly-HEMA-coated plates (Sigma). Murine kidney epithelial cells were suspended (1.2 × 10<sup>5</sup> cells/mL) in serum-free DMEM/F12 media supplemented with 0.5% methylcellulose in poly-HEMA-coated plates (Sigma). Where indicated, cells were treated with 2 μM JNK-in-8 (Millipore) 24 hr prior to the anoikis assay. Cell death was measured using colony-formation assays and Annexin V staining.

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 aprotinin plus leupeptin). Extracts (30 μg) were subjected to immunoblot analysis with antibodies to caspase-3 (Cell Signaling Technology, catalog number 9662 RRID:AB_331439; dilution 1:500), ERK2 (Santa Cruz Biotechnology, catalog sc-1647; RRID:AB_627547; dilution 1:1,000), FLAG (Sigma-Aldrich, catalog number F3165 RRID:AB_259529; dilution 1:5,000), GAPDH (Santa Cruz Biotechnology, catalog number sc-25778; RRID:AB_1016688; dilution 1:1,000), JNK (R&D Systems, catalog number AF1387 RRID:AB_2140743R&D; dilution 1:1,000), p-ERK (Cell Signaling Technology, catalog number 9101 RRID:AB_2315114; dilution 1:1,000), AKT (Cell Signaling Technology, catalog number 9272 RRID:AB_329827; dilution 1:1,000), p-Ser473 AKT RRID:AB_292742; dilution 1:1,000), p-Ser70 S6K1 RRID:AB_200383; dilution 1:1,000), p-ERK (Cell Signaling Technology, catalog number 9101 RRID:AB_2315114; dilution 1:1,000), AKT (Cell Signaling Technology, catalog number 9272 RRID:AB_329827; dilution 1:1,000), p-Ser473 AKT RRID:AB_292742; dilution 1:1,000), Bcl2 (Cell Signaling Technology, catalog number ab32503 RRID:AB_725697), and α-tubulin (Sigma-Aldrich, catalog number T5168; RRID:AB_477579). Immune complexes were detected with IRDye 680LT conjugated-donkey anti-mouse immunoglobulin G (lgG) antibody (LI-COR Biosciences, catalog number 926-68022 RRID:AB_10715072) and IRDye 800CW conjugated-goat anti-rabbit IgG (LI-COR Biosciences, catalog number 926-3221 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 (RNA integrity number [RIN] >9) was verified using a Bioanalyzer 2100 System (Agilent Technologies). Total RNA (10 μg) was used to prepare each RNA-sequencing (RNA-seq) library 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. 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 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.

mRNA expression was also examined by qRT-PCR analysis using a Quantsstudio 12K Flex machine (Life Technologies). TaqMan assays were used to quantify Bad (MM00432042_m1), Bbc3 (MM00519268_m1), Bcl2 (MM00437797_m1), Bcl2 (MM00432073_m1), Bcl2 (MM00056773_m1), Hrk (MM01208086_m1), and Pmaip1 (MM00451763_m1) mRNA (Life Technologies). Relative mRNA expression was normalized by measuring the amount of 18S RNA in each sample using TaqMan assays (catalog number 4398329; Life Technologies).

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

Colony-Formation Assay
Cells were washed with PBS, replated in 24-well plates, and cultured (24 hr) prior to fixation (100% methanol, −20°C) and staining with 0.1% crystal violet dissolved in 20% methanol/80% PBS. 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-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<sup>-</sup> 7-AAD<sup>+</sup>) population. 7-AAD<sup>+</sup> cells were gated using single-stained controls. The Annexin V<sup>-</sup> and
Annexin V+ populations were defined using cells suspended for 1 hr. The data obtained were analyzed using FlowJo version 9.7.6 (Tree Star).

Mammary Gland Analysis

The fourth 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 alun, and imaged using a Zeiss SteREO Discovery.V12 microscope. 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 H&E and imaged using a Zeiss Axioscience 200M. Sections were also treated with antibodies to keratin 5 (BioLegend, catalog number 905501 RRID:AB_2565050; 1:50 dilution) and keratin 8 (Developmental Studies Hybridoma Bank, catalog number TROMA-I RRID:AB_331826; 1:100 dilution), and immune complexes were detected using Alexa-Fluor-546-conjugated-goat anti-rabbit IgG (H+L) antibody (Molecular Probes, catalog number A11035 RRID:AB_134051) and Alexa-488-conjugated-goat anti-rat IgG (H+L) antibody (Molecular Probes, catalog number A11006 RRID:AB_141373) and counterstained with DAPI. Proliferating cells were stained using the endogenous biotin blocking kit (Thermo Fisher Scientific, E21390), biotin-conjugated PCNA antibody (Thermo Fisher Scientific, catalog number 13-3940 RRID:AB_2533; dilution 1:50), and Alexa-Fluor-633-conjugated streptavidin (Thermo Fisher Scientific, catalog number S-21375 RRID:AB_231350). Immunofluorescence was examined using a Leica SP2 confocal microscope.

Plasmids

We have previously described the plasmid pCDNA3-FLAG-MKK7 [2-Jnk1x1a] (Lei et al, 2002). The FLAG-MKK7[2-Jnk1x1a] cDNA fragment was excised by PCR using the primers 5′-AACACGCCGCCGCCGGGTGGAGACTATA AGGACGATGA-3′ and 5′-AAATCTAGATCTGCTGTACCCCTTACAGAAG GAG-3′, restricted using SacII and XbaI, and cloned in the SacII and XbaI sites of the entry vector pEn_Tmric (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-MKK7[2-Jnk1x1a]-Hygro.

Transduction Assays

HEK293T cells (American Type Culture Collection, CRL-3216) were transfected with 7.5 μg each of the packaging plasmids pMD2.G (Addgene, plasmid 12259; Naldini et al, 1996) and psPAX2 (Addgene, plasmid 12260; Naldini et al, 1996) plus 10 μg pSLIK-Hygro or pSLIK-FLAG-MKK7[2-Jnk1x1a]-Hygro using Lipofectamine 2000 (Life Technologies). The culture supernatant was collected at 24 hr post-transfection and filtered (0.45 μm). Primary epithelial cells were transduced (x2) with the lentivirus plus polybrene (8 μg/mL). The transduced epithelial cells were selected at 48 hr post-infection using medium supplemented with 8 μg/mL hygromycin (Life Technologies). Cells were maintained in selection medium with tetracycline-free fetal bovine serum (Clontech Laboratories). To induce expression of the MKK7-JNK1 fusion protein, cells were treated with 1 μg/mL doxycycline (24 hr).

Statistical Analysis

Data are presented as the mean and SE. 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). Pairwise comparisons of data with similar variance were performed using a t test to determine significance (p < 0.05). Pairwise 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%.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE88856. The accession number for the flow cytometry data reported in this paper is FlowRepository: FR-FCM-ZYCR.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.067.

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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