Published in final edited form as:

Sci Signal. ; 4(174): ra34. doi:10.1126/scisignal.2001684.

p38 α Signaling Induces Anoikis and Lumen Formation During Mammary Morphogenesis

Huei-Chi Wen^{1,2,3}, Alvaro Avivar-Valderas^{1,2}, Maria Soledad Sosa^{1,2}, Nomeda Girnius⁴, Eduardo F. Farias¹, Roger J. Davis⁴, and Julio A. Aguirre-Ghiso^{1,2,*}

¹Department of Medicine, Tisch Cancer Institute at Mount Sinai, Mount Sinai School of Medicine, New York, NY 10029, USA

²Department of Otolaryngology, Tisch Cancer Institute at Mount Sinai, Mount Sinai School of Medicine, New York, NY 10029, USA

³Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Rensselaer, NY 12144, USA

⁴Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA 01605, USA

Abstract

The stress-activated protein kinase (SAPK) p38 can induce apoptosis, and its inhibition facilitates mammary tumorigenesis. We found that during mammary acinar morphogenesis in MCF-10A cells grown in three-dimensional culture, detachment of luminal cells from the basement membrane stimulated mitogen-activated protein kinase (MAPK) kinases 3 and 6 (MKK3/6) and p38 α signaling to promote anoikis. p38 α signaling increased transcription of the death-promoting protein BimEL by phosphorylating the activating transcription factor 2 (ATF-2) and increasing c-Jun protein abundance, leading to cell death by anoikis and acinar lumen formation. Inhibition of p38 α or ATF-2 caused luminal filling reminiscent of that observed in ductal carcinoma in situ (DCIS). The mammary glands of MKK3/6 knockout mice (MKK3^{-/-}/MKK6^{+/-}) showed accelerated branching morphogenesis relative to those of wild-type mice, as well as ductal lumen occlusion due to reduced anoikis. This phenotype was recapitulated by systemic pharmacological inhibition of p38 α and β (p38 α/β) in wild-type mice. Moreover, the development of DCIS-like lesions showing marked ductal occlusion was accelerated in MMTV-Neu transgenic mice treated with inhibitors of p38 α and p38 β . We conclude that p38 α is crucial for the development of hollow ducts during mammary gland development, a function that may be crucial to its ability to suppress breast cancer.

Copyright 2008 by the American Association for the Advancement of Science; all rights reserved.

*To whom correspondence should be addressed. julio.aguirre-ghiso@mssm.edu .

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/4/174/ra34/DC1

Author contributions: H.-C.W. and J.A.A.-G. designed the research, analyzed the data, and wrote the manuscript; H.-C.W. and A.A.-V. performed the experiments and analyzed the data; M.S.S. performed animal experiments and analyzed the data; E.F.F. provided the MMTV-Neu mice, performed the experiments, and analyzed the data; N.G. maintained the MKK3/MKK6 wild-type and KO mice and provided tissue sections under the supervision of R.J.D., who also wrote the manuscript. R.J.D. is an Investigator of the Howard Hughes Medical Institute.

Competing interests: The authors declare that they have no competing interests.

Citation: H.-C. Wen, A. Avivar-Valderas, M. S. Sosa, N. Girnius, E. F. Farias, R. J. Davis, J. A. Aguirre-Ghiso, p38 α signaling induces anoikis and lumen formation during mammary morphogenesis. *Sci. Signal.* **4**, ra34 (2011).

INTRODUCTION

The creation of ducts and alveoli containing luminal spaces surrounded by polarized epithelial cells attached to a basal lamina is a hallmark of postnatal mammary gland development (1). Studies of mammary epithelial cells (MECs) in three-dimensional (3D) cultures and of mammary gland development in vivo have shown that lumen formation results from the removal of cells that have detached from the extracellular matrix (ECM) in the terminal end bud (TEB) and in elongating mammary ducts (2). Lack of cell-ECM adhesion elicits an acute “stress signaling” response that results in caspase-dependent (anoikis) (3) and caspase-independent (4, 5) cell death. Anchorage-independent cell survival and enhanced proliferation are conferred by proteins encoded by oncogenes, such as human epidermal growth factor receptor 2 (known as HER2/neu). Enhanced survival can also be conferred by overexpression of antiapoptotic proteins, such as B cell lymphoma 2 (Bcl-2). Both proteins promote the accumulation of luminal cells characteristic of breast atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS). Clinically, a reduced response to oncogene- and replication-induced stress that results in senescence predicts subsequent tumor formation and worse outcome in DCIS patients (6). However, the precise stress signaling pathways that might be deregulated to promote resistance to anoikis and thereby lumen filling are not fully understood.

The p38 mitogen-activated protein kinase (MAPK) pathway integrates various types of stress signals and mediates anoikis in normal epithelial cells (7-9). Four mammalian isoforms of p38 [*p38α* (*MAPK14*), *p38β* (*MAPK11*), *p38γ* (*MAPK12*), and *p38δ* (*MAPK13*)] have been identified (10). *p38α* promotes growth arrest by inhibiting cyclin D1 gene transcription and protein abundance (11) and by activating the p53 to p21 and p16 to Rb pathways (11-14). *p38α* also regulates the spindle assembly checkpoint (15) and can delay the G₂ to M transition of the cell cycle (16). It also inhibits tumor initiation by sensing oncogene-induced oxidative stress (17). Accordingly, inactivation of *p38α* leads to mouse mammary tumorigenesis in vivo (13, 18), and ~15% of human primary breast carcinomas show amplification of *PPM1D*, the gene that encodes protein phosphatase Mg²⁺/Mn²⁺-dependent 1D, which dephosphorylates *p38α*, thereby inhibiting its activity (12). However, the location and timing of p38 signaling required for mammary tumor suppression remain unknown. The p38 pathway, which inhibits signaling through the ERK1/2 (extracellular signal-regulated kinase 1 and 2) MAPK pathway, is activated by loss of adhesion in MECs (7) and induces anoikis in colonic epithelial cells (8, 9). We thus hypothesized that p38-dependent inhibition of ERK1/2 signaling and thereby induction of anoikis might be central mechanisms to limit the accumulation of luminal cells during mammary morphogenesis.

Here, we used 3D cultures of immortalized nontumorigenic human MCF-10A MECs to show that *p38α*-mediated anoikis acts as an early barrier to prevent the inappropriate survival and growth of luminal cells. Upon cell detachment, MAPK kinase 3 and 6 (MKK3/6)-mediated phosphorylation of *p38α* led to activation of ATF-2 (activating transcription factor 2) and induced c-Jun-dependent transcription of the proapoptotic gene Bim. This led to the induction of caspase-3-dependent anoikis of the detached cells and lumen formation in mammary acini. MECs hypomorphic for *p38α* were incapable of activating ATF-2 and stimulating c-Jun protein increase and the subsequent increase in BimEL (the extra-long form of Bcl-2-interacting mediator of cell death) mRNA and protein, resulting in resistance to anoikis and luminal filling. This phenotype was recapitulated by ATF-2 knockdown. Further, occlusion by excess luminal cells of the mammary gland ducts and TEBs was apparent in MKK3/6 knockout (*MKK3^{-/-}/MKK6^{+/-}*) mice and in mice treated with *p38α/β* inhibitor but not in wild-type controls. Systemic inhibition of p38 had marked effects in transgenic mice in which the *Her2/neu* oncogene is under the control of the mouse mammary tumor virus promoter (MMTV-*Neu*), which developed DCIS-like

lesions after only 2 weeks of treatment. Our data reveal how p38 α signaling shapes normal mammary acinar morphogenesis and inhibits HER2/neu-driven tumorigenesis. Our data also define at what stage of mammary gland development p38 α might act to suppress tumorigenesis and how its inhibition could accelerate disease progression.

RESULTS

Loss of ECM attachment activates MKK6-p38 α signaling and anoikis in MCF-10A cells

Integrin and growth factor signaling become uncoupled in MCF-10A cells grown in suspension, initiating a stress signal that results in cell death (19). Consistent with previous studies (7), immunoblot (IB) analysis indicated that p38 phosphorylation was increased in detached MCF-10A cells relative to that in attached cells (Fig. 1A). We also observed activation of p38 in primary mouse MECs (mMECs) and immortalized mouse embryonic fibroblasts (MEFs) grown in suspension (Fig. 1, A and C). When focusing on MCF-10A cells, we found that increased p38 phosphorylation was accompanied by phosphorylation of its upstream activators MKK3 and 6 (MKK3/6) and of its downstream target, the heat shock protein 27 (HSP27) (Fig. 1A), confirming activation of the p38 signaling pathway. p38 was not activated by centrifugation or trypsinization of cells (fig. S1A). Further, blocking β 1-integrin ligand binding in attached cells with the AIIB2 monoclonal antibody (20) increased p38 phosphorylation to a degree comparable to that induced by growth in suspension (Fig. 1B). The phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²)-specific antibody we used to assess p38 phosphorylation detects all isoforms of activated p38. Using antibodies selective for the different p38 isoforms, we found relatively similar amounts of endogenous p38 α , p38 β , p38 γ , and p38 δ in MCF-10A cells (fig. S1B). We focused mainly on p38 α , and its role in anoikis and mammary morphogenesis.

To identify the upstream MAPK kinase responsible for activating p38 in cells grown in suspension, we assessed p38 phosphorylation in MEFs derived from wild-type, *MKK3*^{-/-}, *MKK6*^{-/-}, or *MKK3*^{-/-}/*6*^{-/-} mice and grown on fibronectin-coated plates or for 24 hours in suspension culture. In adhered conditions, these cells produced both the p38 α and the p38 β isoforms (fig. S1C). MKK6 phosphorylates both isoforms, but predominantly p38 α , whereas MKK3 phosphorylates only p38 α and not p38 β (21, 22). Ablation of MKK3 had no obvious inhibitory effect on p38 phosphorylation in suspended cells, but loss of MKK6 or of both MKK3 and MKK6 markedly inhibited it (Fig. 1C); these findings suggest that both MKK3 and MKK6 contribute, albeit to different degrees, to activation of p38 α in response to cell detachment. In agreement with the MEF data, treatment of detached MCF-10A cells with SB203580 [which inhibits both the p38 α and the p38 β isoforms but not p38 γ or p38 δ (23, 24)] decreased HSP27 phosphorylation (Fig. 2A). Treatment 24 hours before and throughout the assay with SB203580 also significantly inhibited anoikis after 24 and 48 hours in suspension, although this inhibition was no longer apparent at 72 hours (Fig. 1D). Together, these data demonstrate that detachment from ECM can activate the MKK3/6 to p38 α to HSP27 pathway in MCF-10A cells.

Activation of p38 α mediates acinar lumen formation by inducing BimEL and anoikis

Activation of p38 α signaling can induce anoikis or growth arrest (9, 25). We studied anoikis and growth arrest during mammary acinar morphogenesis using MCF-10A 3D cultures (26). As previously reported (27), MCF-10A cells formed acini in 3D culture and, by day 15 of morphogenesis, when luminal cells are cleared through anoikis, MCF-10A acini formed lumens (Fig. 1E). Treatment with SB203580 or small interfering RNA directed against p38 α (siRNAp38 α) blocked luminal clearing, resulting in acini filled with cells (Fig. 1E). Proliferation, as measured by Ki67 staining, was not significantly increased (Fig. 1F), but

acini were slightly larger (~30%) (Fig. 1G). We conclude that the primary function of p38 α during MCF-10A morphogenesis is to promote lumen formation.

BimEL can be transcriptionally activated by p38 after different stress signals (28, 29), and it is required for lumen formation (7, 30). As previously shown (19), cell detachment increased BimEL abundance. We found that increased BimEL abundance correlated with robust phosphorylation of p38 α and HSP27 (Fig. 2A). siRNAp38 α or pharmacological inhibition of p38 α / β with SB203580 or SCIO469 (specific for p38 α) (31, 32) almost completely reversed the increase in *Bim* mRNA and protein in suspended MCF-10A cells (Fig. 2A). Using a *Bim*-luciferase (*Bim*-Luc) reporter construct (33), we determined that RNA interference (RNAi) knockdown of p38 α in MCF-10A cells or deletion of p38 α in MEFs (34) decreased *Bim* promoter activation in cells grown in suspension (Fig. 2B). Expression of a constitutively active mutant form of p38 α (p38 α CA) (35, 36) stimulated the *Bim* promoter to the same extent as did cell detachment (Fig. 2B), indicating that p38 activation is sufficient to activate *Bim* gene transcription. In addition, the increased luciferase activity in response to p38 α CA paralleled increases in endogenous *Bim* mRNA, indicating that the *Bim*-Luc assay is a faithful reporter of *Bim* expression (Fig. 2B). In 3D culture, SB203580-treated acini showed significantly less BimEL than did untreated cells (Fig. 2C). This decrease in BimEL correlated with reduced luminal apoptosis (detected by cleaved caspase-3 staining) in acini formed by SB203580- or siRNAp38 α -treated cells at day 8 and day 10 of morphogenesis, respectively, relative to cells treated with empty vehicle or control siRNA (Fig. 2, D and E, and fig. S1D). Together, these data indicate that p38 α -regulated expression of *Bim* is associated with lumen formation during mammary acinar morphogenesis.

ERK1/2 and p38 α have opposing effects on BimEL abundance

ERK and p38 have opposing effects on apoptosis (37-39) and, whereas p38 activation increases BimEL abundance, ERK1/2 reduced BimEL protein accumulation (28, 29, 40). We hypothesized that a signaling imbalance favoring p38 α over ERK1/2 could increase BimEL abundance in detached luminal cells. Conversely, a high ERK1/2-to-p38 α signaling ratio in ECM-attached cells might decrease BimEL induction.

Either treatment with the MEK1/2 (mitogen-activated or extracellular signal-regulated protein kinase kinase 1 and 2) inhibitor U0126 to decrease ERK signaling (Fig. 3A), or activation of p38 α signaling by expressing either a constitutively active form of MKK6 [Mkk6b(E)] or p38 α CA increased BimEL abundance in adherent MCF-10A cells incubated in full growth media with 5% horse serum (conditions of high basal ERK1/2 activation) (Fig. 3, A and B). SB203580 treatment of adherent cells increased ERK1/2 phosphorylation and prevented induction of BimEL by U0126 so that its abundance did not increase beyond that found under basal conditions (Fig. 3A). Moreover, U0126-mediated induction of cell death was reversed by SB203580 treatment (fig. S1F). These data suggest that, upon inhibition of MEK1/2-ERK signaling, p38 activity is required to induce BimEL production and apoptosis. Furthermore, a p38 α siRNA inhibited Mkk6b(E)-dependent BimEL induction (Fig. 3B). This and the fact that p38 phosphorylation was greatly reduced further support the notion that MKK6 activation of primarily p38 α (the phospho-p38 antibody detects all four isoforms) leads to increased BimEL abundance (Fig. 3B). The increase in BimEL apparent in p38 α CA-transfected MCF-10A cells was enhanced by increasing concentrations of U0126 (Fig. 3C). This additive effect of inhibiting ERK1/2 and activating p38 α suggests that the two pathways converge to regulate BimEL abundance.

We next investigated whether mutual regulation of ERK1/2 and p38 α activities might be required to regulate BimEL production. Consistent with the effects of SB203580 treatment in MCF-10A cells, we found that U0126 did not increase BimEL protein abundance in

p38α^{-/-} MEFs, whereas it did in wild-type cells (Fig. 3D). Loss of BimEL induction in suspended *p38α*^{-/-} MEFs was rescued by transient expression of a *wtp38α* vector (Fig. 3E), indicating that the lack of BimEL induction was not an epiphenomenon of *p38α* deletion. Detachment of wild-type or *p38α*^{-/-} MEFs (Fig. 3F) or of MCF-10A cells transfected with control siRNA or siRNA targeting *p38α* (Fig. 2A) confirmed that cells hypomorphic for *p38α* showed decreased induction of BimEL in response to growth in suspension. SB203580 also inhibited BimEL induction in suspended wild-type MEFs (Fig. 3G), arguing that our pharmacological and genetic approaches specifically target *p38α* or *p38α/β*. Together, these data indicate that *p38α* is essential for the increase in BimEL caused by inhibition of ERK1/2 signaling. We also found sustained ERK1/2 phosphorylation in detached MCF-10A cells treated with SB203580 or transfected with siRNA to MKK6 (Fig. 3H), which suggests that activation of MKK6 and *p38* signaling is in part responsible for inhibition of the ERK1/2 pathway. Furthermore, the activation of ERK1/2 observed upon inhibition of *p38α* in MCF-10A cells (Fig. 3, A and H) was also observed in adhered *p38α*^{-/-} (Fig. 3F) and in *MKK6*^{-/-} MEFs (Fig. 3H) where loss of *p38α* or MKK6 signaling caused enhanced ERK1/2 phosphorylation. However, we did not observe this effect in *p38α*^{-/-} cells in suspension. This may be due to differences between deleting *p38α* and inhibiting its kinase activity or to the involvement of other *p38* isoforms. Nonetheless, *p38α* was required for BimEL induction in all cases (Fig. 3, B to F). Inhibition of ERK1/2 signaling may, by enhancing *Bim* expression (19) and protein stability (40), cooperate with *p38α* to increase BimEL protein abundance in detached cells.

Activation of ERK1/2 and *p38α* in MCF-10A cells grown in 3D culture was observed in separate populations of cells. ERK1/2 phosphorylation was confined to the ECM-bound cells; phosphorylated *p38* (p-*p38*) was detected exclusively in matrix-deprived luminal cells at the center of the acinar structures (Fig. 3I). Low-frequency detection (one to two cells per acinus) of cells with *p38α* phosphorylation was consistently found in the acinar structure throughout days 6 to 10 in culture and lost thereafter (Fig. 3I and fig. S1G). This is most likely due to the transient nature of these phosphorylation events (Fig. 3I and fig. S1G). Thus, whereas ERK1/2 activation is restricted to ECM-attached cells, phosphorylation of *p38* specifically occurs in luminal cells and is apparent at all stages of morphogenesis during which apoptosis occurs. Together, these data indicate that a low ratio of ERK1/2 to *p38α* signaling is required for the increase in BimEL and, consequently, for anoikis of luminal cells and thereby lumen formation during mammary acinar morphogenesis.

ATF-2 and c-Jun mediate *p38α*-dependent expression of *BimEL* during mammary acinar morphogenesis

An increase in BimEL abundance relative to that in attached cells was apparent at 0.5 hours of suspension culture. BimEL abundance remained high for 4 hours and decreased slightly at 8 hours (Fig. 4A). These changes in protein abundance were not accompanied by significant changes in mRNA abundance (Fig. 4B). At 8 hours and up to 16 hours, protein abundance increased steadily, as did mRNA (Fig. 4, A and B). At 24 hours, although *Bim* mRNA abundance was lower than at 16 hours, it was still more than twice that in attached cells, and this was accompanied by increased protein abundance. Changes in the abundance of p-*p38*, p-ERK, and p-HSP27 were apparent for all three proteins at 4 hours and thereafter (Fig. 4, A and B). These data suggest that loss of attachment triggers a transient increase in BimEL stability, possibly due to posttranslational modifications (41, 42), followed by a sustained transcriptional activation initiated by a decrease in the ERK1/2/*p38* activity ratio.

We hypothesized that *p38α*-dependent activation of specific transcription factors (TFs) might stimulate the increase in *Bim* expression apparent 8 hours and beyond in suspension. Neither Forkhead box O3a (FOXO3a) nor C/EBP homologous protein (CHOP), two TFs that stimulate *Bim* expression and apoptosis in response to stress (43, 44), was responsible

for *Bim* mRNA induction in suspension (fig. S1, H and I). Therefore, we focused on ATF-2, a TF that is phosphorylated at Thr⁷¹ and Thr⁶⁹ in response to activation of c-Jun N-terminal kinase (JNK) and p38 α , which in turn increases c-Jun transcription (45-47). Both ATF-2 and c-Jun can also heterodimerize and, along with other components of activating protein 1 (AP-1), they stimulate the transcription of genes that promote cell death (48, 49). Mice lacking an ATF-2 allele develop invasive ductal mammary carcinomas that display a solid tubular structure (50). We found that detached MCF-10A and mMEC cells strongly induced ATF-2 phosphorylation (Fig. 4C). When focusing on MCF-10A cells, we found that suspension-induced ATF-2 phosphorylation also correlated with increases in c-Jun and BimEL abundance (Fig. 4C). ATF-2 phosphorylation and the increase in c-Jun abundance were not observed in detached *p38 α ^{-/-}* MEFs or in siRNA_{p38 α} -transfected MCF-10A cells (Fig. 4, D and E); these findings suggest that p38 α played a critical role in activating ATF-2 and inducing c-Jun production in suspended cells.

Both ATF-2 phosphorylation and c-Jun abundance were increased in cells located at the center of the acini that were not attached to the ECM (Fig. 5A), and SB203580 treatment decreased ATF-2 phosphorylation (Fig. 5B); hence, in suspended MCF-10A cells, ATF-2, c-Jun, or both might mediate luminal cell anoikis. To determine whether c-Jun was responsible for BimEL induction, we briefly incubated suspended MCF-10A cells transfected with control or c-Jun expression vector. We detected c-Jun and BimEL as early as 2 to 4 hours in suspension (Fig. 5C), at a time when transcriptional activity is submaximal (Fig. 4B). Next, we determined whether inhibition of c-Jun or ATF-2 could block the increase in BimEL in detached cells. Transfection of a dominant-negative form of c-Jun, TAM-67 (51), inhibited the increase in BimEL (Fig. 5C) and of its mRNA (fig. S1J) and activation of its promoter (Fig. 5C). Similarly, knockdown of ATF-2 inhibited activation of the *Bim* promoter in suspended cells (Fig. 5D). Thus, ATF-2 and c-Jun appear to mediate increased *Bim* expression in response to p38 α signaling in cells detached from the ECM, and thereby potentially contribute to acinar lumen formation.

To determine their role in lumen formation conclusively, we knocked down ATF-2 during acinar morphogenesis (Fig. 5E) and found that this prevented proper lumen formation in enlarged MCF-10A acinar structures (Fig. 5E). These structures showed significant reduction of BimEL and cleaved caspase-3 staining in cells filling the lumen (Fig. 5E). Our monolayer and 3D studies in MECs show that activation of ERK1/2 and p38 α is temporally and spatially regulated so that in luminal cells, p38 α activation is mutually exclusive with that of ERK1/2. The increase in p38 activity leads to activation of ATF-2 and production of c-Jun, which, combined with the loss of ERK1/2 activity, induce BimEL and anoikis of luminal cells.

p38 α / β inhibition triggers luminal filling in ducts and TEB in normal and MMTV-Neu mammary glands

MMTV-PPM1D mice have been studied in the context of HER2/neu signaling in tumor development studies (52). However, when and how p38 signaling might antagonize the development of HER2/neu-induced tumors was not studied. Furthermore, whether *PPM1D* overexpression affects specific stages of normal mammary gland morphogenesis in vivo in the absence of HER2/neu through inhibition of p38 signaling was not reported. To determine this, we studied mammary ductal branching and lumen formation in a strain of normal inbred FVB female mice treated with SB203580. We also compared mammary gland development in the C57B strain of wild-type female or *MKK3^{-/-}/MKK6^{+/-}* C57B mice, where p38 activation is greatly decreased in many tissues (53, 54).

We injected 4-week-old FVB female mice with vehicle or SB203580 (10 mg/kg) intraperitoneally every 48 hours (12, 13) for 2 or 4 weeks. Analysis of stained whole-mount

mammary glands showed that SB203580 accelerated ductal tree elongation and branching (Fig. 6, A and B). Close examination revealed thickened ducts and elongated solid TEBs in SB203580-treated glands, in contrast to the well-defined hollow ducts and normal TEB structures found in the control group (Fig. 6, C and D). Histological analysis of hematoxylin and eosin (H&E)-stained sections (Fig. 6, E to J) confirmed the whole-mount analysis. In ducts, the myoepithelial and epithelial cell layers showed increased cellularity after p38 inhibition, and occupancy of lumens by epithelial cells was common (Fig. 6, G to J, and fig. S2A). The same pattern of myoepithelial and epithelial cell layer organization was corroborated with immunofluorescence to detect α smooth muscle actin (α -SMA, as a marker of myoepithelium) and cytokeratins 8 and 18 (CK8/18, as a marker of epithelium) (fig. S2A). Detection and quantification of cleaved caspase-3 revealed predominantly apoptotic luminal cells in normal ducts (Fig. 7O). SB203580-treated mice showed a significant reduction in apoptosis of luminal cells (Fig. 7O). All of the above characteristics of SB203580-treated mice were recapitulated in mice carrying homozygous or heterozygous deletion of the *MKK3* and *MKK6* alleles in all tissues (53, 54) (Fig. 6, K to T); mice homozygous for deletion of both *MKK3* and *MKK6* are embryonic lethal (53). *MKK3*^{-/-}/*MKK6*^{+/-} mice (5 weeks old) showed reduced ATF-2 phosphorylation in epithelial and stromal cells, confirming reduction of MKK3/6-p38 signaling in these tissues (fig. S2C). These mice also showed a marked acceleration of ductal tree expansion, so that it almost completely filled the mammary fat pad (Fig. 6, K and L). Additional analysis (Fig. 6, O to T) showed that the ducts and TEBs of *MKK3*^{-/-}/*MKK6*^{+/-} mammary glands, unlike those of wild-type mice, had filled lumens (Fig. 6, R to T). This correlated with reduced apoptosis in *MKK3*^{-/-}/*MKK6*^{+/-} mice as determined by cleaved caspase-3 staining (Fig. 7O). There was a nonsignificant trend toward reduced percentage of small- and medium-sized TEBs in SB203580-treated mice or *MKK3*^{-/-}/*MKK6*^{+/-} mice relative to their respective controls and a significant increase in the percentage of larger-size TEBs (fig. S2D). p38 α / β inhibition had no effect on cell proliferation, as measured by p-Rb (Ser^{807/811}) or p-H3 (Ser¹⁰) staining in situ (fig. S3), in either the pharmacological or the genetic analyses. Thus, using two different strains of mice and two different experimental approaches, we showed that MKK3, MKK6, and p38 signaling regulate mammary branching morphogenesis and restrict growth of epithelial and myoepithelial cells and TEB size. At the time points analyzed, these effects coincided with p38-induced luminal anoikis, but not with inhibition of proliferation.

Next, we analyzed whole-mount sections of mammary glands of uniparous MMTV-Neu (*wtp38a*) mice (20 to 24 weeks old) treated with vehicle control or SB203580. After only 2 weeks of treatment, the number of ducts had increased by 40% ($P = 0.004$, Mann-Whitney test) in the SB203580 group ($n = 5$ per group) (Fig. 7, A, B, and P); this was accompanied by an increased ductal density and an increase in the size and number of side buds and TEBs (Fig. 7, C to F). Histological analysis showed occluded lumens in ductal structures ($48 \pm 0.6\%$ control versus $72 \pm 19\%$ SB203580-treated mice, $P < 0.05$), with a substantial increase in the thickness of the epithelial and myoepithelial cell layers, which was confirmed by α -SMA and CK8/18 immunofluorescence (Fig. 7, G to J, and fig. S2B). MMTV-Neu control mice showed decreased luminal apoptosis relative to control FVB mice (Fig. 7O). The increased number of occluded ducts correlated with a 58% ($P = 0.027$) decrease in the percentage of luminal apoptotic cells (determined by cleaved caspase-3 staining) in SB203580-versus control-treated mice (Fig. 7, K, L, and O). As in normal mammary epithelium, p38 α / β inhibition with SB203580 did not enhance in situ cell proliferation (fig. S3). We also observed hyperplastic accumulations of intermingled epithelial and myoepithelial cells emerging from ductal walls in SB203580-treated mice (Fig. 7, I and J, and fig. S2B). Many of these structures were solid and highly disorganized, resembling dysplastic foci or early neoplastic lesions (Fig. 7, D, F, H, and J). As indicated earlier, when during mammary gland development p38 inhibits HER2/neu tumor formation has been unclear. Here, we conclude that MKK3/6 and p38 α / β are critical regulators of proper

branching morphogenesis and that, at least at the time points we tested, p38 α / β might restrict HER2/neu-induced tumors at the time of hyperplasia development. This occurs by promoting luminal cell apoptosis, but not by decreasing cell proliferation. Our in vivo data thus confirm the results of the 3D morphogenesis assay and show that p38 α limits luminal filling, excessive side branching, and tissue expansion in mammary epithelium expressing the HER2/neu oncogene.

DISCUSSION

Here, we show that p38 α has a previously unrecognized role in promoting anoikis of luminal cells during acinar morphogenesis, thereby preventing the accumulation of luminal cells. We found that either pharmacological or genetic inhibition of the p38 stress-signaling axis resulted in resistance to apoptosis, leading to disruption of the normal acinar architecture. These data also support a role for both MKK3 and MKK6 in controlling lumen formation and maintaining normal architecture of the mammary epithelium. Consistent with previous studies (27, 30), our data show that anoikis is required to prevent lumen occlusion in ducts and TEBs and that lack of apoptosis—without any obvious increase in cell proliferation—has a marked effect on ductal tree expansion. It is possible that inhibition of p38 signaling affected stem and progenitor cells in the mammary tissue, contributing to the enhanced branching morphogenesis. If these and other more differentiated cells were protected from apoptosis by p38 inhibition, then the net effect would be larger than anticipated, because all surviving cells would contribute to the expanding mammary epithelium.

We found that inhibition of ERK1/2 signaling in ECM-detached cells mediated BimEL induction and anoikis. We also found that the balance between ERK1/2 and p38 α signaling, which controls tumor cell entry into quiescence (55), liver development (56), and the expression of genes required for suppressing transformation and tumorigenesis (17, 57), provides an important signal integration point for cells to commit to survival and proliferation or to anoikis. However, whether ERK1/2 inactivation is solely due to lack of growth factor and ECM signaling has been unknown. Our results show that MKK6 and p38 α activation in cells grown in suspension is necessary for inhibition of ERK1/2. We further showed that the combined inhibition of ERK1/2 activity and p38 α activation contribute to the expression of *Bim*. Results by Reginato *et al.* (19) suggest that reduced ERK1/2 activity contributes to *Bim* mRNA induction. ERK1/2 is thought to inhibit BimEL expression at the transcriptional (58) and also at the posttranslational level (41). p38 α phosphorylation of BimEL at Ser⁶⁵ has been proposed as a mechanism for increasing BimEL's apoptotic function, but whether this was only due to enhanced protein stability was not shown (59). In contrast, phosphorylation of BimEL by ERK1/2 at Ser⁶⁵ increases protein degradation (40, 60). The phosphorylation by ERK1/2 depends on the previous phosphorylation of BimEL by p90 ribosomal S6 kinase (p90RSK), an ERK1/2 but not p38 target. This dual phosphorylation by ERK1/2 and p90 RSK targets BimEL for degradation (61). Here, we show that ECM detachment induces a biphasic regulation of BimEL abundance in MCF-10A cells. The first phase is most likely posttranslational and accounts for the increase in BimEL abundance up to 8 hours in suspension. This could be due to the loss of ERK phosphorylation at Ser⁶⁵ (Ser⁶⁹ in human) among other mechanisms. After 8 hours, the much stronger increase in BimEL abundance depends on enhanced transcription. Although posttranslational regulation in suspended cells may still occur at times beyond 8 hours, it seems that both transcriptional and posttranslational mechanisms ensure that the increase in BimEL abundance is sufficient to commit cells to anoikis.

The TFs that control increased *Bim* expression during anoikis have been unknown. We now show that neither FOXO3a nor CHOP appears to increase *Bim* transcription in cells cultured in suspension, whereas ATF-2 and c-Jun act as TFs downstream of ERK and p38 to increase

Bim transcription. Both steady-state measurements of *Bim* mRNA and *Bim*-Luc reporter activity support the notion that p38 α increases the transcription of *Bim* through ATF-2. Mice hypomorphic for ATF-2 develop lumen-occluded invasive ductal mammary carcinomas (50), suggesting a role for this TF in lumen formation. We found that ATF-2 knockdown phenocopied the effects of p38 α abrogation. Our results indicate that ATF-2 phosphorylation by p38 α is required to increase the abundance of c-Jun in luminal cells destined to undergo apoptosis. Arguably, ATF-2 can also be pro-tumorigenic (62), and the role of c-Jun downstream of p38 α depends on the cell tissue context. For example, in squamous carcinoma cells where p38 α/β was inhibited (63), or in livers or MEFs of p38 $\alpha^{-/-}$ mice, c-Jun abundance and phosphorylation are increased (64). However, in mouse lungs in which p38 α is conditionally deleted, c-Jun phosphorylation is enhanced but not its abundance (65). Nevertheless, our data are consistent with previous results (50) indicating a critical role for ATF-2 in regulating acinar lumen formation. Knockdown of ATF-2 caused a more profound effect on acinar enlargement than did loss or inhibition of p38 α , suggesting that multiple stress signals might converge on ATF-2. Moreover, our 3D morphogenesis data correlated with the role of p38 in regulating luminal apoptosis in ducts and TEBs in vivo. Surprisingly, genetic inhibition of MKK3/6 or pharmacologic inhibition of p38 α/β increased the number of epithelial and myoepithelial cells without any apparent increase in proliferation. It is possible that the pro-proliferative effect of p38 α/β inhibition may occur at earlier times during mammary gland development. Although our data analysis focused on the p38 α and β isoforms, we cannot eliminate the possibility that p38 γ and p38 δ , both of which are substrates of MKK3 and MKK6 (66), might also play a role in inducing mammary epithelial apoptosis in vivo.

In cancer, p38 appears to execute various and sometimes opposing programs, which might depend on the state of oncogenic progression (17, 63, 67). Despite these complexities, the tumor-suppressive function of p38 is well established (68). Although p38 does not appear to be mutated in cancer, its tumor-suppressive functions could be abrogated by different means. For example, in breast cancer, amplification of Wip1, a p38 phosphatase, appears to circumvent p38's antitumor effects. Genetic or pharmacologic inhibition of p38 α/β isoforms accelerates mammary tumor progression (13, 52). We explored the possibility that p38-mediated anoikis of luminal cells during mammary acinar development could be a crucial point at which p38 might act to curtail breast cancer development. In MMTV-Neu mammary tissue, SB203580 treatment accelerated epithelial tissue growth and the development of hyperplastic ducts with occluded lumens, supporting the notion that p38 α/β signaling could limit the development of Her2/neu-induced hyperplasia. The massive expansion of the ductal tree in MMTV-Neu mammary tissue upon p38 α/β inhibition indicates that p38 α/β acts to restrain HER2/neu signaling. Thus, upon p38 α/β inhibition, unrestricted expansion occurs, perhaps through the same mechanisms as in the FVB mice treated with SB203580 or in the *MKK3^{-/-}/MKK6^{+/-}* mice.

In summary, our data shed light into the morphogenetic and temporal windows (for example, during branching morphogenesis and lumen formation and maintenance) during which p38 α/β activation might block ductal filling, and the development of hyperplasia. p38 α/β signaling, which mediates apoptosis and growth arrest, increases with aging as do that of tumor-suppressive cell cycle inhibitors that limit unscheduled proliferation (69). Thus, it is tempting to speculate that loss of p38 signaling could facilitate survival and proliferation of immortalized or transformed ductal or alveolar luminal cells. Future studies will determine whether these events indeed precede the development of lesions during early steps of tumorigenesis.

MATERIALS AND METHODS

Reagents and plasmids

p38 inhibitors—SB203580 was purchased from Calbiochem and LC Laboratories. SCIO469 was a gift from A. Verma (Albert Einstein Cancer Center, New York). MEK1 inhibitor U0126 was purchased from Promega.

Transfections—Lipofectamine RNAiMax was purchased from Invitrogen and FuGENE HD from Roche. The Dual-Luciferase Assay kit was purchased from Promega. The expression plasmid MKK6b(E) was described previously (63). The p38 α D176A + F327S construct was a gift from O. Livnah (The Hebrew University of Jerusalem, Givat Ram, Jerusalem). c-Jun and TAM-67 were gifts from M. J. Birrer [Center for Cancer Research, National Cancer Institute (NCI), Bethesda, MD]. The pGL3-BimP-luc reporter plasmid was a gift from L. A. Greene (Columbia University, New York, NY).

RNAi—p38 α MAPK siRNA II was purchased from Cell Signaling Technology, MKK6 from Santa Cruz Biotechnology, ATF-2 from Dharmacon, and CHOP and Silencer negative control from Ambion.

Antibodies

Antibodies used in this study were directed against p38 α , p-p38 (T¹⁸⁰/Y¹⁸²), and ERK1 (BD Biosciences); p-ERK1/2 (T²⁰²/Y²⁰⁴) and CHOP (Santa Cruz Biotechnology); GAPDH (glyceraldehyde phosphate dehydrogenase) and Bim (Calbiochem); HA (hemagglutinin; Roche); Ki67 (Zymed); p-MKK3/6(S^{189/207}), p-p38(T¹⁸⁰/Y¹⁸²), p-HSP27(S⁸²), p-Rb(S^{807/811}), p-histone H3 (S¹⁰), ATF-2, p-ATF-2 (T⁷¹), cleaved caspase-3 (N¹⁷⁵), p38 β , p38 γ , p38 δ , and c-Jun N-terminal-specific (Cell Signaling Technology); and c-Jun DNA binding domain-specific (Santa Cruz Biotechnology). In addition, horseradish peroxidase-conjugated (Vector Laboratories and Chemicon International) and Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and polyclonal antibody to cytokeratins 8 and 18 (Progen Biotechnik) were used. Monoclonal antibody against α -SMA was a gift from A. Bernstein (Mount Sinai School of Medicine, New York, NY).

RT-PCR primers

Forward and reverse primer sequences were as follows: Bim as previously described (21); human p38 α , 5'-TCCAGACCATTTTCAGTCCAT-3' and 5'-AAAAACGTCCAACAGACCAA-3'; mouse p38 α , 5'-CCCCAGAGATCATGCTGAAT-3' and 5'-AGGTCAGGCTCTTCCACTCA-3'; human p38 β , 5'-TACTTGGTGACCACCCTGAT-3' and 5'-GCTGGTAAACCAGGAATTGA-3'; mouse p38 β , 5'-ATTCTACCGGCAAGAGCTGA-3' and 5'-GTCCTCGTTCACCGCTACAT-3'; human p38 γ , 5'-TGATGAGACCCTGGATGACT-3' and 5'-TCGCCTAGCTTCTCATGTTT-3'; mouse p38 γ , 5'-AGGCAGGCAGACAGTGAGAT-3' and 5'-AGGGTGCGGTCTACATCATC-3'; human p38 δ , 5'-ATCCTCAGCTGGATGCACTA-3' and 5'-CCCCTGAACAGAGTTTCC-3'; and mouse p38 δ , 5'-ACAAGACTGCCTGGGAGCTA-3' and 5'-CCCAAAGTCCAGGATCTTCA-3'.

Cells, culture conditions, and 3D morphogenesis assays

MCF-10A and MEF monolayer cell culture was maintained as described (26, 53). MCF-10A cells have been described previously (70). Wild-type and knockout MKK3, MKK6, and MKK3/6 MEFs were previously described (26, 53), and wild-type and knockout p38 α MEFs were a gift from A. Porras (Universidad Complutense de Madrid). For suspension assay, MCF-10A cells were pretreated overnight with or without 10 μ M SB203580 or SCIO469, or

transfected with negative control or specific siRNA for 48 hours in complete growth media. Subsequently, MCF-10A or MEF cells (4×10^5 /ml) were incubated in ultra-low attachment plates (Corning), kept in their respective complete growth medium with or without treatment, and harvested at the indicated time points. To quantify percentage of cell viability, detached cells were washed with phosphate-buffered saline, disaggregated, and collected as single-cell suspensions with cell-strained cap (BD Falcon) to be incubated in 1:2 trypan blue stain (BioWhittaker). The number of total and nonviable cells was determined with a counting chamber. MCF-10A 3D morphogenesis assay was carried out as described (26). For treatments and transfections during morphogenesis, 5 μ M SB203580 or 20 nM p38 α or 60 nM ATF-2 and MKK6 siRNA was supplemented to the assay media every 24 or 48 hours, respectively.

RNAi and complementary DNA transfections and luciferase reporter assay

Transfections of plasmids and siRNA oligonucleotides were done in six-well plates with 2 μ g of DNA mixed with 6 μ l of FuGENE HD or 3.2 nM (p38 α) or 9.6 nM (ATF-2 and MKK6) siRNA oligonucleotides in 8 or 22.5 μ l of Lipofectamine RNAiMax per well, respectively, and incubated for 24 and 48 hours, following the manufacturer's instructions. For reporter assay, parental or transfected MCF-10A cells (described above) and MEF cells were cotransfected with 0.5 μ g of Bim reporter plasmid and 0.1 μ g of *Renilla* vector in 2.5 ml of complete growth media in six-well dishes with FuGENE HD. Twenty-four or 48 hours later, cells were washed, lysed, and harvested with buffer provided in the Promega Luciferase System. Relative luciferase activities were obtained with a TD-20/20 luminometer (Turner Designs) and analyzed by normalizing the luciferase activity to *Renilla* luciferase activity.

Western blotting, immunofluorescence, and image processing

MCF-10A and MEF cells were lysed, and protein was analyzed by immunoblotting as previously described (70). MCF-10A 3D acinar structures were fixed at day 6, 8, 10, or 15 and processed for size and immunofluorescence microscopy analysis as previously described (70). Detection of phosphoproteins was improved by incubating, fixing, and treating acinar structures with phosphatase inhibitors. Confocal analyses were performed with the Leica SP5 DM confocal microscopy system equipped with four lasers: an ultraviolet (UV) diode (405 nm), an argon laser (458, 476, 488, and 514 nm), a 543-nm HeNe laser, and a 633-nm HeNe laser. Pictures of luminal spaces from the equatorial section (the largest diameter from top to bottom) of mammary spheres were taken with 63 $\times$ magnification. Quantitative measurements of optical density in IB were performed with ImageJ image processing program by National Institutes of Health. Numbers under the IB bands show fold change for the indicated protein ratio and are expressed as means $\pm$ SEM. Values for control conditions were set at 1. When indicated by an asterisk (*), the differences were statistically significant at $P < 0.05$. All IB results are representative of at least three independent experiments ($n = 3$). Acinar size was calculated with SPOT software following the equation $[(\text{length} \times \text{width}^2)/2 = \text{acini volume (mm}^3)]$ and plotted with GraphPad Prism.

In vivo experiments, mammary gland whole mounts, and immunohistochemistry

Four-week-old FvB (NCI) female mice were injected intraperitoneally with dimethyl sulfoxide (DMSO) (five mice) or SB203580 (five mice, 10 mg/kg) every 48 hours for 4 weeks. SB203580 compound from two sources (see Reagents and plasmids section) was tested in vivo and in vitro, and no apparent differences in efficacy were observed. The same strategy was followed for 24- to 32-week-old MMTV-Neu (71) female mice. C57 *MKK3*^{+/+}/*MKK6*^{+/+} and *MKK3*^{-/-}/*MKK6*^{+/+} mice were previously described (53, 54). Mammary gland whole-mount staining was performed as previously described (72). Briefly, mammary

glands were excised and fixed in 10% buffered formalin overnight. Next, the samples were incubated in Carnoy's fixative solution for 4 hours followed by serial hydration. Finally, mammary glands were incubated in carmine alum solution overnight, dehydrated, and then left in xylol for 16 hours. Stained whole mounts were preserved in methyl salicylate solution. To measure ductal tree density, four transecting lines (2.4 cm long) were drawn across four areas of the mammary gland proximal and distal to the nipple. The number of ducts intersecting these lines was counted and averaged for each animal and expressed as number of ducts per centimeter (unpaired *t* test). Immunohistochemistry from embedded paraffin sections was performed as previously described (72). The sections were processed with VectaStain ABC Elite Kit (Vector Laboratories), and the signal was detected with DAB Substrate Kit for peroxidase (Vector Laboratories).

Statistics

Statistical analysis was performed with MS Excel or GraphPad Prism 5.0 software. *P* values were calculated with one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post test or the unpaired *t* test with *P* < 0.05 considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Confocal laser scanning microscopy was performed at the Mount Sinai School of Medicine Microscopy Shared Resources Facility, supported with funding from NIH-NCI shared resources (grant 5R24CA095823-04), National Science Foundation Major Research Instrumentation (grant DBI-9724504), and NIH shared instrumentation (grant 1 S10RR0 9145-01).

Funding: This work is supported by grants from the Samuel Waxman Cancer Research Foundation Tumor Dormancy Program, NIH/NCI (CA109182), National Institute of Environmental Health Sciences (ES017146), and New York State Stem Cell Science (NYSTEM) to J.A.A.-G.

REFERENCES AND NOTES

1. Bissell MJ, Rizki A, Mian IS. Tissue architecture: The ultimate regulator of breast epithelial function. *Curr. Opin. Cell Biol.* 2003; 15:753–762. [PubMed: 14644202]
2. Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, Reed JC, Rosen JM. Apoptosis in the terminal endbud of the murine mammary gland: A mechanism of ductal morphogenesis. *Development.* 1996; 122:4013–4022. [PubMed: 9012521]
3. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr. Opin. Cell Biol.* 2001; 13:555–562. [PubMed: 11544023]
4. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, White E. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev.* 2007; 21:1621–1635. [PubMed: 17606641]
5. Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101:3438–3443. [PubMed: 14993595]
6. Gauthier ML, Berman HK, Miller C, Kozakeiwicz K, Chew K, Moore D, Rabban J, Chen YY, Kerlikowske K, Tlsty TD. Abrogated response to cellular stress identifies DCIS associated with subsequent tumor events and defines basal-like breast tumors. *Cancer Cell.* 2007; 12:479–491. [PubMed: 17996651]
7. Mailleux AA, Overholtzer M, Schmelzle T, Bouillet P, Strasser A, Brugge JS. BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. *Dev. Cell.* 2007; 12:221–234. [PubMed: 17276340]

8. Rosen K, Shi W, Calabretta B, Filmus J. Cell detachment triggers p38 mitogen-activated protein kinase-dependent overexpression of Fas ligand. A novel mechanism of anoikis of intestinal epithelial cells. *J. Biol. Chem.* 2002; 277:46123–46130. [PubMed: 12356751]
9. Vachon PH, Harnois C, Grenier A, Dufour G, Bouchard V, Han J, Landry J, Beaulieu JF, Vézina A, Dydensborg AB, Gauthier R, Côté A, Drolet JF, Lareau F. Differentiation state-selective roles of p38 isoforms in human intestinal epithelial cell anoikis. *Gastroenterology.* 2002; 123:1980–1991. [PubMed: 12454855]
10. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* 2005; 15:11–18. [PubMed: 15686620]
11. Sun P, Yoshizuka N, New L, Moser BA, Li Y, Liao R, Xie C, Chen J, Deng Q, Yamout M, Dong MQ, Frangou CG, Yates JR III, Wright PE, Han J. PRAK is essential for ras-induced senescence and tumor suppression. *Cell.* 2007; 128:295–308. [PubMed: 17254968]
12. Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ Jr. Appella E. Amplification of *PPM1D* in human tumors abrogates p53 tumor-suppressor activity. *Nat. Genet.* 2002; 31:210–215. [PubMed: 12021785]
13. Bulavin DV, Phillips C, Nannenga B, Timofeev O, Donehower LA, Anderson CW, Appella E, Fornace AJ Jr. Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16^{Ink4a}-p19^{Arf} pathway. *Nat. Genet.* 2004; 36:343–350. [PubMed: 14991053]
14. Wang W, Chen JX, Liao R, Deng Q, Zhou JJ, Huang S, Sun P. Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. *Mol. Cell. Biol.* 2002; 22:3389–3403. [PubMed: 11971971]
15. Takenaka K, Moriguchi T, Nishida E. Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science.* 1998; 280:599–602. [PubMed: 9554853]
16. Mikhailov A, Patel D, McCance DJ, Rieder CL. The G2 p38-mediated stress-activated checkpoint pathway becomes attenuated in transformed cells. *Curr. Biol.* 2007; 17:2162–2168. [PubMed: 18060783]
17. Dolado I, Swat A, Ajenjo N, De Vita G, Cuadrado A, Nebreda AR. p38 α MAP kinase as a sensor of reactive oxygen species in tumorigenesis. *Cancer Cell.* 2007; 11:191–205. [PubMed: 17292829]
18. Qi X, Pramanik R, Wang J, Schultz RM, Maitra RK, Han J, DeLuca HF, Chen G. The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D₃-induced growth inhibition. *J. Biol. Chem.* 2002; 277:25884–25892. [PubMed: 11983707]
19. Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the proapoptotic protein Bim to prevent anoikis. *Nat. Cell Biol.* 2003; 5:733–740. [PubMed: 12844146]
20. Howlett AR, Bailey N, Damsky C, Petersen OW, Bissell MJ. Cellular growth and survival are mediated by β 1 integrins in normal human breast epithelium but not in breast carcinoma. *J. Cell Sci.* 1995; 108(Pt 5):1945–1957. [PubMed: 7544798]
21. Cuenda A, Cohen P, Buee-Scherrer V, Goedert M. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.* 1997; 16:295–305. [PubMed: 9029150]
22. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 1995; 364:229–233. [PubMed: 7750577]
23. Enslen H, Branch DM, Davis RJ. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. *EMBO J.* 2000; 19:1301–1311. [PubMed: 10716930]
24. Raingeaud J, Whitmarsh AJ, Barrett T, Dérjard B, Davis RJ. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* 1996; 16:1247–1255. [PubMed: 8622669]

25. Kültz D, Madhany S, Burg MB. Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. *J. Biol. Chem.* 1998; 273:13645–13651. [PubMed: 9593703]
26. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods.* 2003; 30:256–268. [PubMed: 12798140]
27. Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell.* 2002; 111:29–40. [PubMed: 12372298]
28. Cai B, Xia Z. p38 MAP kinase mediates arsenite-induced apoptosis through FOXO3a activation and induction of Bim transcription. *Apoptosis.* 2008; 13:803–810. [PubMed: 18465250]
29. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. *FEBS Lett.* 2006; 580:3539–3544. [PubMed: 16730715]
30. Reginato MJ, Mills KR, Becker EBE, Lynch DK, Bonni A, Muthuswamy SK, Brugge JS. Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. *Mol. Cell. Biol.* 2005; 25:4591–4601. [PubMed: 15899862]
31. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 2000; 351:95–105. [PubMed: 10998351]
32. Medicherla S, Reddy M, Ying J, Navas TA, Li L, Nguyen AN, Kerr I, Hanjarappa N, Protter AA, Higgins LS. p38 α -selective MAP kinase inhibitor reduces tumor growth in mouse xenograft models of multiple myeloma. *Anticancer Res.* 2008; 28:3827–3833. [PubMed: 19189670]
33. Biswas SC, Liu DX, Greene LA. Bim is a direct target of a neuronal E2F-dependent apoptotic pathway. *J. Neurosci.* 2005; 25:8349–8358. [PubMed: 16162916]
34. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR. Essential role of p38 α MAP kinase in placental but not embryonic cardiovascular development. *Mol. Cell.* 2000; 6:109–116. [PubMed: 10949032]
35. Diskin R, Askari N, Capone R, Engelberg D, Livnah O. Active mutants of the human p38 α mitogen-activated protein kinase. *J. Biol. Chem.* 2004; 279:47040–47049. [PubMed: 15284239]
36. Diskin R, Lebendiker M, Engelberg D, Livnah O. Structures of p38 α active mutants reveal conformational changes in L16 loop that induce autophosphorylation and activation. *J. Mol. Biol.* 2007; 365:66–76. [PubMed: 17059827]
37. Lee J, Hong F, Kwon S, Kim SS, Kim DO, Kang HS, Lee SJ, Ha J, Kim SS. Activation of p38 MAPK induces cell cycle arrest via inhibition of Raf/ERK pathway during muscle differentiation. *Biochem. Biophys. Res. Commun.* 2002; 298:765–771. [PubMed: 12419320]
38. Li SP, Junttila MR, Han J, Kahäri VM, Westermark J. p38 mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase1,2. *Cancer Res.* 2003; 63:3473–3477. [PubMed: 12839928]
39. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science.* 1995; 270:1326–1331. [PubMed: 7481820]
40. Hübner A, Barrett T, Flavell RA, Davis RJ. Multisite phosphorylation regulates Bim stability and apoptotic activity. *Mol. Cell.* 2008; 30:415–425. [PubMed: 18498746]
41. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein. Bim. *J. Biol. Chem.* 2003; 278:18811–18816.
42. Luciano F, Jacquell A, Colosetti P, Herrant M, Cagnol S, Pages G, Auberger P. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene.* 2003; 22:6785–6793. [PubMed: 14555991]
43. Essafi A, Fernández de Mattos S, Hassen YA, Soeiro I, Mufti GJ, Thomas NS, Medema RH, Lam EW. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene.* 2005; 24:2317–2329. [PubMed: 15688014]
44. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, Bouillet P, Strasser A. ER stress

- triggers apoptosis by activating BH3-only protein Bim. *Cell*. 2007; 129:1337–1349. [PubMed: 17604722]
45. Gupta S, Campbell D, Dériard B, Davis RJ. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*. 1995; 267:389–393. [PubMed: 7824938]
 46. Livingstone C, Patel G, Jones N. ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J*. 1995; 14:1785–1797. [PubMed: 7737129]
 47. van Dam H, Wilhelm D, Herr I, Steffen A, Herrlich P, Angel P. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J*. 1995; 14:1798–1811. [PubMed: 7737130]
 48. Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron*. 1995; 14:927–939. [PubMed: 7748560]
 49. Hayakawa J, Mittal S, Wang Y, Korkmaz KS, Adamson E, English C, Ohmichi M, McClelland M, Mercola D. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. *Mol. Cell*. 2004; 16:521–535. [PubMed: 15546613]
 50. Maekawa T, Shinagawa T, Sano Y, Sakuma T, Nomura S, Nagasaki K, Miki Y, Saito-Ohara F, Inazawa J, Kohno T, Yokota J, Ishii S. Reduced levels of ATF-2 pre-dispose mice to mammary tumors. *Mol. Cell. Biol*. 2007; 27:1730–1744. [PubMed: 17189429]
 51. Brown PH, Alani R, Preis LH, Szabo E, Birrer MJ. Suppression of oncogene-induced transformation by a deletion mutant of c-jun. *Oncogene*. 1993; 8:877–886. [PubMed: 8455942]
 52. Demidov ON, Kek C, Shreeram S, Timofeev O, Fornace AJ, Appella E, Bulavin DV. The role of the MKK6/p38 MAPK pathway in Wip1-dependent regulation of ErbB2-driven mammary gland tumorigenesis. *Oncogene*. 2007; 26:2502–2506. [PubMed: 17016428]
 53. Branchio D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ. Mechanism of p38 MAP kinase activation in vivo. *Genes Dev*. 2003; 17:1969–1978. [PubMed: 12893778]
 54. Greenblatt MB, Shim JH, Zou W, Sitara D, Schweitzer M, Hu D, Lotinun S, Sano Y, Baron R, Park JM, Arthur S, Xie M, Schneider MD, Zhai B, Gygi S, Davis R, Glimcher LH. The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. *J. Clin. Invest*. 2010; 120:2457–2473. [PubMed: 20551513]
 55. Aguirre-Ghiso JA, Liu D, Mignatti A, Kovalski K, Ossowski L. Urokinase receptor and fibronectin regulate the ERK^{MAPK} to p38^{MAPK} activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Mol. Biol. Cell*. 2001; 12:863–879. [PubMed: 11294892]
 56. Carreras MC, Converso DP, Lorenti AS, Barbich M, Levisman DM, Jaitovich A, Antico Arciuch VG, Galli S, Poderoso JJ. Mitochondrial nitric oxide synthase drives redox signals for proliferation and quiescence in rat liver development. *Hepatology*. 2004; 40:157–166. [PubMed: 15239099]
 57. Shields JM, Mehta H, Pruitt K, Der CJ. Opposing roles of the extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades in Ras-mediated downregulation of tropomyosin. *Mol. Cell. Biol*. 2002; 22:2304–2317. [PubMed: 11884615]
 58. Weston CR, Balmanno K, Chalmers C, Hadfield K, Molton SA, Ley R, Wagner EF, Cook SJ. Activation of ERK1/2 by ΔRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways. *Oncogene*. 2003; 22:1281–1293. [PubMed: 12618753]
 59. Cai B, Chang SH, Becker EB, Bonni A, Xia Z. p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. *J. Biol. Chem*. 2006; 281:25215–25222. [PubMed: 16818494]
 60. Ley R, Ewings KE, Hadfield K, Cook SJ. Regulatory phosphorylation of Bim: Sorting out the ERK from the JNK. *Cell Death Differ*. 2005; 12:1008–1014. [PubMed: 15947788]
 61. Dehan E, Bassermann F, Guardavaccaro D, Vasiliver-Shamis G, Cohen M, Lowes KN, Dustin M, Huang DC, Taunton J, Pagano M. βTrCP- and Rsk1/2-mediated degradation of BimEL inhibits apoptosis. *Mol. Cell*. 2009; 33:109–116. [PubMed: 19150432]
 62. Eferl R, Wagner EF. AP-1: A double-edged sword in tumorigenesis. *Nat. Rev. Cancer*. 2003; 3:859–868. [PubMed: 14668816]
 63. Adam AP, George A, Schewe D, Bragado P, Iglesias BV, Ranganathan AC, Kourtidis A, Conklin DS, Aguirre-Ghiso JA. Computational identification of a p38^{SAPK}-regulated transcription factor

- network required for tumor cell quiescence. *Cancer Res.* 2009; 69:5664–5672. [PubMed: 19584293]
64. Hui L, Bakiri L, Mairhorfer A, Schweifer N, Haslinger C, Kenner L, Komnenovic V, Scheuch H, Beug H, Wagner EF. p38 α suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. *Nat. Genet.* 2007; 39:741–749. [PubMed: 17468757]
 65. Ventura JJ, Tenbaum S, Perdiguero E, Huth M, Guerra C, Barbacid M, Pasparakis M, Nebreda AR. p38 α MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. *Nat. Genet.* 2007; 39:750–758. [PubMed: 17468755]
 66. Enslen H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J. Biol. Chem.* 1998; 273:1741–1748. [PubMed: 9430721]
 67. Junttila MR, Ala-Aho R, Jokilehto T, Peltonen J, Kallajoki M, Grenman R, Jaakkola P, Westermarck J, Kähäri VM. p38 α and p38 Δ mitogen-activated protein kinase isoforms regulate invasion and growth of head and neck squamous carcinoma cells. *Oncogene.* 2007; 26:5267–5279. [PubMed: 17334397]
 68. Han J, Sun P. The pathways to tumor suppression via route p38. *Trends Biochem. Sci.* 2007; 32:364–371. [PubMed: 17624785]
 69. Wong ES, Le Guezennec X, Demidov ON, Marshall NT, Wang ST, Krishnamurthy J, Sharpless NE, Dunn NR, Bulavin DV. p38MAPK controls expression of multiple cell cycle inhibitors and islet proliferation with advancing age. *Dev. Cell.* 2009; 17:142–149. [PubMed: 19619499]
 70. Sequeira SJ, Ranganathan AC, Adam AP, Iglesias BV, Farias EF, Aguirre-Ghiso JA. Inhibition of proliferation by PERK regulates mammary acinar morphogenesis and tumor formation. *PloS One.* 2007; 2:e615. [PubMed: 17637831]
 71. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell.* 1988; 54:105–115. [PubMed: 2898299]
 72. Lu Y, Bertran S, Samuels TA, Mira-y-Lopez R, Farias EF. Mechanism of inhibition of MMTV-neu and MMTV-wnt1 induced mammary oncogenesis by RAR α agonist AM580. *Oncogene.* 2010; 29:3665–3676. [PubMed: 20453882]

Fig. 1. p38 α activation in suspension culture and its effects on lumen formation. **(A)** Lysates from attached (Att) or suspended (Susp) cells were probed by immunoblot (IB) for the indicated antigens. Phospho-(p-p38) and total p38 α were also measured in mouse MEC (mMEC) lysates. **(B)** Lysates of attached cells untreated or treated with anti-integrin- β 1 blocking antibodies (AIB2, 10 μ g/ml) or isotype-matched control immunoglobulin G (IgG, 10 μ g/ml) or suspended cells were used to detect p-p38 and p38. **(C)** Lysates of adhered or suspended wild-type (WT), *MKK3*^{-/-}, *MKK6*^{-/-}, or *MKK3/MKK6*^{-/-} MEFs were probed for the indicated antigens. **(D)** Viability (assessed by trypan blue exclusion) of suspended MCF-10A cells treated with DMSO (CTRL) or 10 μ M SB203580. **(E)** Confocal images of MCF-10A acini treated with SB203580 (5 μ M) or vehicle DMSO (CONTROL) from days 4 to 15 of morphogenesis (left panel) or cells transfected with p38 α or control siRNAs from days 4 to 10 of culture (right panel). Blue, DAPI (4',6-diamidino-2-phenylindole) staining. Scale bars, 25 μ m. The bar graph shows the number of equatorial section intraluminal cells per acinus ($n = 45$ acini). p38 α knockdown in 3D culture is shown by IB. **(F)** Ki67 staining was scored and the percentage of proliferating cells per acinus was calculated ($n = 50$ acini). NS, not significant. **(G)** Size of control (DMSO) or 5 μ M SB203580-treated MCF-10A acini or control or p38 α siRNA-transfected acini at day 8; Mann-Whitney test, $n = 50$ acini.

Fig. 2. p38 α increases BimEL abundance during anoikis and lumen formation. (A) Lysates from attached or suspended cells treated with or without the p38 α inhibitors SB203580 or SCIO469 (10 μ M) or DMSO (left panel), or cells transfected with p38 α or control siRNAs, were probed by IB (right panel). Reverse transcription polymerase chain reaction (RT-PCR) (lower right panel) from attached (Att) or suspended (Susp) MCF-10A cells treated with SB203580 (10 μ M) or anisomycin (5 μ M) as indicated. RT-PCR controls were performed without reverse transcriptase (–RT) or complementary DNA (cDNA) (–PCR); $n = 3$ experiments. (B) Bim-Luc activity [see scheme: adapted from (33)] was detected in (i) adhered or detached MCF-10A cells transfected with p38 α or control siRNAs (upper left), (ii) control (pcDNA) or constitutively active p38 α (p38 α D176A + F327S; p38 α CA) vectors or nontransfected (NT) (upper right), (iii) WT or p38 $\alpha^{-/-}$ MEFs (lower left), or (iv) adhered MCF-10A transfected with control (pcDNA) or p38 α CA vectors (lower right). All measurements, $n = 3$ experiments. (C and D) Equatorial section of confocal images of day 8 MCF-10A DMSO (CTRL)- or SB203580 (5 μ M)-treated acini stained for Bim (red, arrow and inset) (C) or cleaved caspase-3 (c-C3) (red) (D). Graphs in (C) and (D) show the quantification of Bim- or c-C3-positive staining ($n = 50$ acini). (E) Equatorial confocal section of day 10 MCF-10A acini stained for c-C3 (red) in cells transfected with control siRNA or p38 α siRNA. Graph shows quantification ($n = 50$ acini). Scale bars, 25 μ m [(C) to (E)]. Mann-Whitney test, $P < 0.0001$.

Fig. 3.

Regulation of BimEL abundance by ERK1/2 and p38 α signaling. (**A** to **C**) Lysates of MCF-10A cells were subjected to IB after being treated with DMSO (–), U0126 (5 μ M), or SB203580 (10 μ M) for 48 hours (**A**); transfected with a pcDNA empty vector (EV), constitutively active MKK6 [MKK6b(E)], or HA-p38 α CA (**B**), or with p38 α or control siRNAs (**B**, right panel); and transfected with pcDNA (CTL) or p38 α CA followed by increasing concentrations of U0126 (**C**). In (**B**), HA-p38 α CA was detected with anti-HA epitope antibodies. (**D** to **G**) Lysates of MEFs were analyzed by IB. WT or p38 $\alpha^{-/-}$ MEFs treated with DMSO or U0126 (5 μ M) for 24 hours (**D**); WT untransfected or p38 $\alpha^{-/-}$ MEFs transfected with wtp38 α in attached (Att) or suspended (Susp) cells; control of HA-tagged wtp38 α expression, in lower panel (**E**); attached or suspended WT or p38 $\alpha^{-/-}$ MEFs for 24 hours (**F**). wtp38 α MEFs treated with or without 10 μ M SB203580 in attached versus suspended cells (**G**). (**H**) Lysates of attached or suspended MCF-10A cells treated with or without 10 μ M SB203580 and in WT or MKK6 $^{-/-}$ MEFs in adhered conditions (left panel) and RNAi control or MKK6-transfected MCF-10A cells (right panel) were probed with IB. (**I**) Confocal images of MCF-10A acini fixed at day 8 showing ECM-attached outer rim cells containing p-ERK (T²⁰²/Y²⁰⁴) (green) and detached intraluminal cells containing p-p38 (T¹⁸⁰/Y¹⁸²) (red). Graph: percentage of p-ERK- and p-p38-positive cells in basal and luminal acinar compartments.

Fig. 4. Activation of ERK1/2 and p38α signaling regulates ATF-2 and increases in c-Jun and BimEL. (**A** and **B**) Lysates of MCF-10A cells cultured under adhered (A) or suspended (S) conditions were collected at the indicated time points for IB for the indicated antigens (left panel). The optical density measurement for each antigen was then plotted as relative change (S over A for each time point) (A), and quantitative PCR analysis for *Bim* transcript, measured and calculated as relative induction (S over A for each time point), is shown in (B). (**C** to **E**) Lysates of the indicated cells were analyzed by IB with the indicated antibodies: attached (Att) and suspended (Susp) MCF-10A cells or mouse MECs (mMECs) (C), WT or p38α^{-/-} MEFs (D), and siRNA control- or p38α siRNA-transfected MCF-10A cells (E).

Fig. 5. ATF-2 activation and c-Jun induction are required for lumen formation. (A) Confocal images showing intraluminal p-ATF-2- and c-Jun-positive cells (red) at day 8 of morphogenesis. Lower left corner numbers: percentage of p-ATF-2- or c-Jun-positive intraluminal cells; means $\pm$ SEM, $n = 45$ acini. (B) Confocal images of DMSO (CTL)- or SB203580-treated acini stained for p-ATF-2 (left panel). Inset: p-ATF-2-positive cell (arrow). Graph: percentage of p-ATF-2-positive cells per acinus ($n = 50$ acini). Scale bars, 25 μ m. (C) IB analysis of (from left to right) detached MCF-10A cells transfected with an empty vector or c-Jun-expressing vectors (left); adhered or detached MCF-10A cells transfected with empty (pcDNA) or dominant-negative c-Jun (TAM-67) (middle); and Bim-Luc reporter activity in attached or suspended MCF-10A cells transfected with TAM-67 or empty vector (pcDNA) (right). (C and D) Bim-Luc reporter activity in control (CTRL) or ATF-2 siRNA. (C) and (D) show triplicate determinations from three independent experiments. t test, $P < 0.0001$. (E) Confocal images of day 10 MCF-10A acini stained for Bim (upper panels) or cleaved caspase-3 (c-C3) (lower panels) in ATF-2- or control siRNA-transfected acini. Inset: enlarged Bim- and c-C3-positive cells shown by arrows. Scale bars, 25 μ m (control) and 50 μ m (siATF-2). IB showing ATF-2 knockdown in 3D cultures (lower middle panel). Graphs: percentage of Bim- or c-C3-positive cells per acinus ($n = 50$ acini) (upper middle panel); mean size of ATF-2 or control siRNA-transfected acini (upper right panel) and number of intraluminal cells per acinus ($n = 50$ acini) (lower right panel).

Fig. 6. MKK3, MKK6, and p38 α/β are required for normal mammary branching morphogenesis. (A to J) Four-week-old FVB female mice treated with DMSO (control) or SB203580 (see Materials and Methods). (A to D) Whole mounts of control and SB203580-treated mammary glands showed $8 \pm 2.1\%$ and $52 \pm 8.7\%$ (mean $\pm$ SEM) of occluded lumens, respectively. $P = 0.008$; $n = 3$. Solid rectangles are enlarged in inset. Dashed rectangles in (A) and (B) are magnified in (C) and (D). Arrows in (A) and (B): extension of the ductal tree from lymph node (LN). (C and D) TEB morphology in control (C) versus SB203580-treated (D) mice. H&E histology of mammary glands from control (E) versus SB203580-treated (F) mice. (E) and (F) illustrate the increase in the number of ducts in control (E) versus SB203580-treated mice (F). (G and H) H&E histology of ducts in control (G) or SB203580-treated mice (H). (I and J) TEBs in control (I) or SB203580-treated mice (J). (K to T) Mammary gland whole mounts [(K) to (N)] or H&E sections [(O) to (T)] of 6-week-old WT C57B ($MKK3^{+/+}/MKK6^{+/+}$) or $MKK3^{-/-}/MKK6^{+/-}$ mice. WT and $MKK3^{-/-}/MKK6^{+/-}$ mice showed $11.7 \pm 2\%$ and $48 \pm 9\%$ (mean $\pm$ SEM) ductal occlusion, respectively. $P = 0.017$; $n = 3$ mice. Solid rectangles in (K) and (L) are magnified in (M) and (N), respectively. H&E histology section of the mammary gland region distal from the fat pad lymph node in control (O) or in $MKK3^{-/-}/MKK6^{+/-}$ mice (P). (Q to T) Ducts in WT mice [(Q), empty] or in $MKK3^{-/-}/MKK6^{+/-}$ mice [(R) and (T), partially occluded, or (S), completely occluded].

Fig. 7. p38 α/β promotes lumen formation in MMTV-Neu mammary glands. (A to J) MMTV-Neu females treated with DMSO (Control) or the p38 α/β inhibitor (SB203580, 10 mg/kg). (A to D) Whole-mount control [(A) and (C)] or SB203580 groups [(B) and (D)]. Arrows in (C) and (D) identify side buds. (E to J) H&E sections of control [(E), (G), and (I)] or SB203580 groups [(F), (H), and (J)]. (I and J) Detail of control [(I), empty lumen] and SB203580-treated mice [(J), hyperplastic piling of cells emerging from the ductal walls]. (K and L) Paraffin sections stained for cleaved caspase-3 in control [(K), luminal apoptosis] or SB203580-treated mice [(L), no apoptosis]. Insets, higher-magnification views of additional ducts. (M and N) Quantification of ductal tree extension from the lymph node to the end of the fat pad in control [(M), DMSO] versus SB203580-treated FVB mice or WT versus knockout (KO) (*MKK3*^{-/-}/*MKK6*^{+/-}) C57B mice. In (N), ductal tree extension in WT animals is zero because the tree did not extend beyond the lymph node. (O) Quantification of c-C3 staining in mammary gland sections from FVB and MMTV-Neu mice treated with DMSO (CTRL) or SB203580 (SB) or in WT versus KO (*MKK3*^{-/-}/*MKK6*^{+/-}) mice. (P) Quantification of the number of ducts per centimeter in the mammary glands from FVB and MMTV-Neu mice treated with DMSO (CTRL) or SB203580 (SB).