The stress kinase MKK7 couples oncogenic stress to p53 stability and tumor suppression

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Most preneoplastic lesions are quiescent and do not progress to form overt tumors. It has been proposed that oncogenic stress activates the DNA damage response and the key tumor suppressor p53, which prohibits tumor growth. However, the molecular pathways by which cells sense a premalignant state in vivo are largely unknown. Here we report that tissue-specific inactivation of the stress signaling kinase MKK7 in KRasG12D-driven lung carcinomas and NeuT-driven mammary tumors markedly accelerates tumor onset and reduces overall survival. Mechanistically, MKK7 acts through the kinases JNK1 and JNK2, and this signaling pathway directly couples oncogenic and genotoxic stress to the stability of p53, which is required for cell cycle arrest and suppression of epithelial cancers. These results show that MKK7 functions as a major tumor suppressor in lung and mammary cancer in mouse and identify MKK7 as a vital molecular sensor to set a cellular anti-cancer barrier.

JNK kinases (JNK1, 2 and 3) are required for cells to respond to their extracellular environment and regulate many physiological processes such as proliferation, apoptosis, differentiation and inflammation1. The JNK kinase pathway has also been implicated in tumorigenesis2. However, both tumor-promoting and tumor-suppressive functions have been reported1–8. Many of the inconsistencies in these data might be due to the fact that the studies used whole-body knockout mice or knock-ins of inactive JNK substrates3–5. In such studies, non-cell autonomous functions of JNK, such as inflammatory responses, angiogenesis, stroma interaction or tumor immune surveillance, might contribute to the tumor phenotypes1,2,5,6. Furthermore, previous studies have shown that there are functional redundancies and compensation among isoforms of JNK2. The kinases MKK4 and MKK7, which are encoded by Map2k4 and Map2k7, respectively, are required for optimal activation of JNKs in a synergistic fashion9. However, it has been reported that MKK4 can also affect the second stress kinase pathway p38 (refs. 1, 9). Therefore, to target JNK signaling in a tissue-specific and conditional fashion in vivo, we focused on MKK7, a specific and essential upstream regulator of the JNK signaling cascade.

RESULTS
Conditional deletion of Map2k7
To investigate the role of MKK7 and MKK7-regulated activation of JNK in tumorigenesis, we generated a new mouse line that carries a conditional Map2k7 (also known as Mkk7) allele (Map2k7fl/fl; Supplementary Fig. 1a and Online Methods). We confirmed germline transmission by DNA blotting. We removed the neo cassette using a Flpe deleter line and confirmed the presence of the remaining LoxP sites by sequencing. Map2k7fl/fl mice were viable and fertile and showed no detectable phenotypes. Deletion of Map2k7 in the epidermis (K5-Cre Map2k7fl/fl mice) resulted in an ‘eye-open at birth’ (EOB) phenotype (Supplementary Fig. 1b,c) similar to Mapk8/−/−; Mapk9/−/− (also known as Jnk1−/−; Jnk2−/−) compound mutant mice10 and mice with epidermal deletion of cJun11, a key downstream target of the JNK signaling pathway. Thus, we have generated a Map2k7fl/fl allele that allows tissue-specific inactivation of the MKK7-JNK signaling pathway.

Prolonged survival in MKK7 deficient KRasG12D lung tumors
To test the role of MKK7 in lung cancer driven by the inducible oncogenic KRasG12D allele, we crossed Map2k7fl/fl mice with a Lox-Stop-Lox-KRasG12D strain (termed KRas; Map2k7fl/fl hereafter). Lox-Stop-Lox-KRasG12D mice rapidly develop non–small-cell lung carcinomas (NSCLCs) after induction of the KRasG12D allele12. We achieved expression of KRasG12D and deletion of exons 3–10 of Map2k7 by adenoviral delivery of Cre recombinase through inhalation (AdCre; Supplementary Fig. 1d,e). Notably, deletion of Map2k7 in the KRasG12D-driven model of lung cancer resulted in markedly shortened survival, whereas the mean survival time of littermate controls was 185 days post AdCre infection, the mean survival of KRas; Map2k7fl/fl mice was only 102 days post AdCre infection (Fig. 1a).

Lung tumors from KRas; Map2k7fl/fl mice showed efficient deletion of Map2k7 and impaired activation of the JNK-cJun pathway,
MKK7 controls tumor onset of KRasG12D-driven lung cancer

We next monitored tumor onset at early stages after AdCre infection. Whereas at 4 weeks after infection only 20% of control Kras; Map2k7fl/fl mice (n = 10) developed hyperplasia, all Kras; Map2k7fl/fl mice (n = 9) harbored multiple hyperplastic lesions and even solid adenomas at this point (Fig. 1b and Supplementary Fig. 2a). Six weeks after AdCre infection, we again observed markedly increased numbers of solid adenomas as well as the formation of adenocarcinomas in the lungs of Kras; Map2k7fl/fl mice (Fig. 1c and Supplementary Fig. 2a). Because of the large size of lung adenocarcinomas at 9 weeks after tumor initiation, we could not count individual tumors in Kras; Map2k7fl/fl mice at this time point (Supplementary Fig. 2a,b). Quantification of overall tumor burden confirmed a significant increase at all time points in Kras; Map2k7fl/fl mice compared to Kras; Map2k7fl/fl littermates (Fig. 1d). These results show that deletion of Map2k7 facilitates KrasG12D-driven lung cancer.

We next classified the progression of lung tumors 6 weeks after tumor initiation as previously described13 (Supplementary Fig. 3a). Kras; Map2k7fl/fl mice contained significantly more adenocarcinomas than did littermate controls (Fig. 1e). Moreover, staining for the proliferation marker Ki67 showed that lung tumors in Kras; Map2k7fl/fl mice underwent increased proliferation in the hyperplastic regions (4 weeks after AdCre infection) as well as in solid adenomas (6 weeks after AdCre infection; Supplementary Fig. 3b). We did not detect apoptotic cells in KrasG12D-induced lung lesions from littermate control or Kras; Map2k7fl/fl mice (Supplementary Fig. 3c); this finding is in line with previous studies and is probably due to the fact that oncogenic KrasG12D is a potent inhibitor of apoptosis14. Thus, these data show that MKK7 not only controls tumor initiation but also suppresses malignant progression.

Mapk8−/−; Mapk9−/− mice exhibit enhanced lung tumor growth

MKK7, with MKK4, is essential for phosphorylating and activating JNKS. In the lung, JNK1 and JNK2, but not JNK3, are expressed1. We therefore investigated whether the identified tumor-suppressive functions are mediated by JNK1, JNK2 or both and generated KrasG12D mice that carried mutations in Mapk8 (also known as Jnk1) or Mapk9 (also known as Jnk2). Loss of Mapk8 alone or inactivation of Mapk9 did not affect tumor burden or overall survival during Koncogenic KrasG12D-mediated lung tumorigenesis (Fig. 2a and Supplementary Fig. 4a,b). Mapk8; Mapk9 double-knockout mice die as embryos10, however, compound Mapk8−/−; Mapk9−/− (Jnk1−/−; Jnk2−/−) mice have been used to unmask phenotypes such as eyelid closure defects10. We therefore tested KrasG12D-induced lung tumorigenesis in such compound mutant animals. Both Mapk8−/−; Mapk9−/− and Mapk8+/−; Mapk9−/− compound mutants were born at Mendelian ratios and survived into adulthood. We found no obvious phenotype in adult Mapk8−/−; Mapk9−/− compound mutant mice except the previously reported eyelid closure defect in Mapk8−/−; Mapk9−/− mice, which was absent from Mapk8−/−; Mapk9−/− mutants10. During KrasG12D-induced lung tumorigenesis Mapk8−/−; Mapk9−/−, but not Mapk8−/−; Mapk9−/− compound mutants showed accelerated tumor growth and progression compared to littermate controls (Fig. 2a,b). Protein blotting of total lung lysates showed that Mapk8−/−; Mapk9−/− mice expressed near-normal levels of total JNK (Supplementary Fig. 4c). By contrast, JNK expression was severely compromised in lungs...
from Mapk8+/−; Mapk9−/− compound mutant mice (Fig. 2c), indicating that JNK2 is the major isoform in the lung or that JNK2 can compensate for the loss of JNK in lung tumors. These data show that the MKK7−JNK1/2 signaling axis acts as a tumor suppressor pathway in KRasG12D-induced lung cancer.

**MKK7 deficiency affects the DNA damage response**

How does the MKK7−JNK1/2 pathway suppress epithelial tumorigenesis? We used gene expression profiling to define the molecular mechanisms of MKK7-mediated tumor suppression. As MKK7 affects the early stages in tumor development, we decided to use primary pneumocytes from KRas; Mapk8+/−; and KRas; Mapk8+/−; Mapk9+/− littermates rather than transformed cells from established tumors that may harbor additional, secondary mutations. Immunohistochemical analysis confirmed the presence of SP-C-expressing type II pneumocytes and low numbers of CC10-expressing Clara cells in the primary pneumocyte culture, mirroring the prevalent cell types in tumors that arose in the KRasG12D-driven tumor model (Supplementary Fig. 5a,b), confirming previous data12. We cultured pneumocytes for 3 days and infected them with Ad-Cre-GFP to induce expression of the KRasG12D oncogene and the concomitant deletion of Map2k7 (Supplementary Fig. 7).

**Fig. 2** Mapk8+/−; Mapk9−/− compound mutant mice phenocopy the effect of MKK7 deletion. Genetic dissection of JNK isoform functions in KRasG12D-driven lung tumors. (a) Analysis of tumor burden in Mapk8+/−; Jnk1−/−, Mapk9−/− (Jnk2−/−) and Mapk8−/−G12D compound mutant mice reveals increased tumor-to-lung area in Mapk8+/−; Mapk9−/− but not in Mapk8−/− single, Mapk9−/− single or Mapk8−/−; Mapk9−/− mice. We analyzed the lungs 9 weeks after AdCre infection. Changes are given as fold increases compared to control littermates (control). Data are shown as means ± s.e.m. **P < 0.01; ***P < 0.001 (Student’s t-test). (b) Representative sections (stained with hematoxylin and eosin) of KRas; Mapk8+/−; and KRas; Mapk9−/− littermates 9 weeks after AdCre infection. Insets show an adenoma in a KRas; Mapk8−/−; Mapk9−/− mouse (arrow) and an adenocarcinoma in a KRas; Mapk8+/−; Mapk9−/− mouse (arrowhead). Magnifications are ×40. (c) Expression of JNK and p53 in Mapk8+/−; Mapk9−/− compound mutant KRasG12D-induced tumors. β-actin is shown as loading control. An overexposed blot for total JNK is presented to show residual total JNK expression in lung tumors of Mapk8−/−; Mapk9−/− compound mutant mice. Scale bars, 100 µm for insets and 2 mm for whole-lung pictures.

**Fig. 3** The MKK7−JNK pathway controls p53 expression in lung cancer. (a) Gene profiling of primary pneumocytes from KRas; Map2k7+/− and KRas; Map2k7+/− mice treated with AdCre in vitro. We used gene set enrichment analysis to determine whether a defined set of genes or pathways showed statistically significant enrichment. All datasets will be published online at NCBI. (b) Immunohistochemical analysis of γH2AX, pCHK2 and p53 in premalignant lesions 4 weeks after AdCre infection. Magnifications are ×400. Staining for γH2AX and pCHK2 in hyperplastic lesions from KRas; Map2k7+/− and KRas; Map2k7−/− mice was comparable. Whereas 80% of pneumocytes in adenomas of KRas; Map2k7+/− mice showed intense nuclear p53 immunostaining, only 20% of pneumocytes in hyperplastic regions of KRas; Map2k7+/− and KRas; Map2k7−/− mice showed nuclear p53 staining. Furthermore, p53 staining was more intense in hyperplastic lung lesions from control KRas; Map2k7+/− (arrowhead) than from KRas; Map2k7+/− mice (thin arrow). The bar graphs denote percentage of cells positive for γH2AX, pCHK2 and p53. Data are shown as means ± s.e.m. **P < 0.01 (Student’s t-test). (c,d) Protein blot analysis of p53, MKK7 and total JNK in Map2k7−/−deficient (c) and Mapk8−/−; Mapk9−/− compound mutant (d) KRasG12D-driven lung tumors 6 weeks after AdCre infection. Data from individual tumors isolated from different mice are shown. Scale bars, 50 µm.
There were no alterations in oncocytic stress-induced activation of γH2AX and CHK2 or formation of 53BP1 foci and γH2AX foci in Mkk7 mutant lung hyperplasias (Fig. 3b) or in solid adenomas (Supplementary Fig. 7a,b). There was a significant reduction in p53 protein in Mkk7-deficient lung hyperplasias 4 weeks after induction of KrasG12D (Fig. 3b). Not only was the number of p53-positive cells markedly reduced in Mkk7-deficient lung lesions compared to hyperplastic regions in littermate controls, but also the staining intensity was decreased in the remaining few p53-positive cells in lungs of Kras; Map2k7fl/fl mice.

Protein blotting and immunohistochemistry confirmed that p53 was also markedly decreased in all Mkk7-deficient adenocarcinomas analyzed 6 and 8 weeks after induction of KrasG12D (Fig. 3c and Supplementary Fig. 7c). The residual Mkk7 band in lungs of Kras; Map2k7fl/fl mice might represent residual Mkk7 in the stroma (fibroblasts, vascular cells and so on) of the isolated tumors or incomplete deletion. Moreover, in Mapk8t−/−; Mapk9t−/− mice that develop enhanced lung cancer, but not in Mapk8t−/−; Mapk9f−/− compound mutant mice which had no altered tumorigenesis (Fig. 2a), p53 was also decreased (Fig. 3d). We did not observe any differences in p53 mRNA expression among Mkk7−expressing and Mkk7−deficient KrasG12D-driven lung tumors (Supplementary Fig. 7d).

As Ras promotes oncogene-induced senescence (OIS) by activating CHK2 in vitro and in vivo19–22, we further investigated senescence in our Mkk7−deficient KrasG12D-driven lung tumors. We found markedly decreased staining of the senescence-associated markers p16INK4a and HP1γ, indicating that senescence was decreased in Mkk7−deficient tumors (Fig. 4a). There was also a concomitant increase in proliferation as determined by pronuclear cell nuclear antigen (PCNA) staining (Fig. 4a). These data indicate that the loss of Mkk7 has no apparent effect on transcription of the Tp53 gene but rather regulates p53 protein stability in vivo, which influences oncogene-induced cellular senescence.

Figure 4 Mkk7 regulates senescence and p53 stability. (a) Immunohistochemical analysis of PCNA, p16INK4a and HP1γ in adenomas 6 weeks after AdCre infection. The bar graphs show the percentage of cells positive for PCNA, p16INK4a and HP1γ. Data are shown as means ± s.e.m. *P < 0.05. **P < 0.01 (Student’s t-test). Magnifications are ×400. (b) p53 expression in the human lung tumor cell line A549 after short interfering RNA (siRNA)-mediated knockdown of Mkk7 and treatment with doxorubicin (Dox; 1 µM). Mkk7 and β-actin levels are indicated. C indicates scrambled control cells treated with scrambled siRNA and T denotes Mkk7-specific siRNA. (c) Protein blot analysis of p53 and various phosphorylated (P) forms of p53 after knockdown of Mkk7 and treatment with doxorubicin (Dox; 1 µM). We used the proteasome inhibitor MG132 (30 µM) to ensure equal p53 levels. β-actin is shown as loading control. (d) Protein blot analysis of p53 in A549 cells after siRNA-mediated knockdown of Mkk7. Cells were exposed to 5 Gy of γ-irradiation. (e) Effect of stable Mkk7 knockdown using shRNA in A549 lung tumor cells to form xenograft tumors. We injected 5 × 10⁶ A549 cells transfected with pSIREN shScramble or shMkk7 into nude mice and monitored tumor formation over 26 days. Data are shown as means ± s.e.m. *P < 0.05 (Student’s t-test). Scale bars, 50 µm.

Mkk7 deficiency affects p53 protein stability

We next wanted to test whether endogenous KrasG12D directly induced DNA damage and influenced p53 expression through Mkk7. We therefore isolated primary pneumocytes from Lox-Stop-Lox-KrasG12D mice, infected these cells with Ad-GFP or Ad-Cre-GFP to activate KrasG12D and followed the appearance of 53BP1 foci and induction of γH2AX. Notably, we found that endogenous activation of oncogenic KrasG12D did not itself significantly increase the DDR above baseline activity normally found in cultured cells, nor did it significantly increase p53 levels (Supplementary Fig. 8a,b). This finding is consistent with evidence that endogenous KrasG12V is potent enough not to transform cells directly nor to cause DDR.21,22 However, we did observe a trend toward increased formation of 53BP1 foci (P = 0.097) as well as slightly increased activation of Erk signaling and proliferation in cells harboring activated KrasG12D compared to control cells treated only with Ad-GFP (Supplementary Fig. 8b,c). Therefore, the DDR seen in primary tumors is probably largely due to a second hit in KrasG12D-driven tumors, as previously reported.23,24

We decided to model the DDR using genotoxic stress in human A549 cells, which are derived from a non–small-cell lung cancer, express wild-type p53 and carry a mutation in the KRas oncogene in the same amino acid as in our mouse lung cancer model.25 We treated the cells with doxorubicin, a commonly used chemotherapeutic drug, to trigger double-end breaks and thereby trigger the DDR and induce p53 (ref. 26). Doxorubicin induced fast and sustained INK activation in A549 cells dependent on Mkk7 expression (Supplementary Fig. 9a). Control A549 cells showed strong upregulation of p53 expression after doxorubicin treatment, whereas Mkk7 knockdown cells showed delayed and less sustained p53 expression (Fig. 4b). Consequently, Mkk7 knockdown cells failed to arrest at the G2/M cell cycle checkpoint after DNA damage (Supplementary Fig. 9b). Mechanistically, knockdown of Mkk7 did not affect p53 stability.
Figure 5 Loss of MKK7 can be rescued by overexpression of p53. (a) Genetic inactivation of Map2k7 on a Tp53−/− background does not further increase lung tumor burden. Average tumor-to-lung area is shown 6 weeks after AdCre infection. At least three planes from each lung were stained with hematoxylin and eosin and analyzed in a blinded fashion. At least eight mice per genotype were analyzed. Data are shown as means ± s.e.m. (b–d) Rescue of MKK7 tumor suppressive function by overexpression of p53. (b) Representative histology of littermate control and KRas; Map2k7−/− mice harboring an extra copy of p53 analyzed 9 weeks after AdCre infection. Insets show adenocarcinomas (arrowheads) in KRas; Map2k7−/− and KRas; Map2k7+/− mice and adenomas (arrows) in p53T−; KRas; Map2k7−/− and p53T+; KRas; Map2k7−/− mice. Magnification is ×40. (c) Tumor burden 9 weeks after AdCre infection is reduced to control levels in p53T+; KRas; Map2k7+/− mice. We analyzed at least eight mice per genotype. Data are shown as means ± s.e.m. (d) Protein blot analysis shows increased p53 levels in tumors of p53T+; KRas; Map2k7+/− mice. *P < 0.05 (Student’s t test). Scale bars, 100 μm for insets and 2 mm for whole-lung pictures.

mRNA expression (Supplementary Fig. 9c); rather, in the presence of the proteasome inhibitor MG132, we observed comparable induction and expression of p53 in both Mkk7 knockdown and control A549 cells (Fig. 4e). To test whether this also holds true for genotoxic injuries other than doxorubicin, we tested γ-irradiation. Again, expression of p53 was delayed and less sustained after γ-irradiation in A549 cells after Mkk7 knockdown than in control cells (Fig. 4d).

MKK7 deficiency affects p53 phosphorylation

The JNK pathway has been implicated as both a positive and a negative regulator of p53. For instance, it has been proposed that JNK targets p53 for degradation in non-stressed cells27, MEFs lacking JNK1/2 transcriptionally upregulate p53 mRNA28 and cJun functions as a potent transcriptional suppressor of p53 (ref. 29). Moreover, deletion of c-Jun in adult mouse liver leads to a decrease in the induction of tumors by carcinogens that results from upregulation of p53 (ref. 30). Thus, one would expect that inactivation of the JNK pathway would result in upregulation of p53. By contrast, in vitro JNK can phosphorylate p53 on key residues (Ser6, Ser33 and Thr81) in its N terminus and thereby lead to increased stability31–33. Whether this is functionally relevant in vivo has not been determined. This prompted us to analyze the phosphorylation status of p53 after genotoxic stress. Knockdown of Mkk7 in A549 cells led to reduced phosphorylation of p53 on Ser6, Ser33 and, less strongly, Thr81 after treatment with doxorubicin and in the presence of the proteasome inhibitor MG132 to ensure equal p53 levels (Fig. 4c). Phosphorylation of p53 on Ser15 or Ser392, sites that are regulated by other kinases such as ATM, ATR, DNA-PK, CDK5 and CDK9, was unaffected17 (Fig. 4c). These data show that the Mkk7-JNK pathway couples genotoxic stress to phosphorylation and stability of p53.

Effect of Mkk7 knockdown on human lung cancer cells

To test the effect of Mkk7 knockdown in tumor formation by A549 cells, we established stable pools of pSIREN-mediated Mkk7 knockdown cells and vector control cells (Supplementary Fig. 9d). Mkk7 knockdown cells showed a significant growth advantage over vector control A549 cells after xenografting into nude mice (Fig. 4e). Thus, knockdown of Mkk7 in human A549 lung tumor cells affects p53 protein stability and results in enhanced tumor formation.

To test whether MKK7-JNK signaling is also perturbed in human primary lung tumors, we analyzed MKK7 in samples from human samples. Phosphorylation of MKK7 was upregulated in NSCLCs compared to surrounding healthy tissue. Furthermore, tumors in which p53 was mutated (confirmed by sequencing) showed even higher phosphorylation of MKK7 than did tumors harboring wild-type p53 (Supplementary Fig. 9e). Thus, MKK7 is activated in primary human lung tumors and such hyperactivation seems to depend on p53 status.

p53 overexpression reverts lung tumorigenesis

To provide direct evidence that p53 deregulation is indeed functionally relevant for MKK7-mediated tumor suppression in vivo, we crossed KRas; Map2k7−/−/− mice to Tp53−/− mice. As previously reported, Tp53 heterozygosity did not impinge on lung tumor growth34. Tp53 knockout mice showed accelerated lung cancer progression similar to KRas; Map2k7−/−/− mice (Fig. 5a and Supplementary Fig. 10a). These data are in line with previous reports showing that deletion of Tp53 in KRasG12D-driven lung tumors results in increased multiplicity and accelerated malignant progression14,34,35. Notably, loss of p53 in KRas; Map2k7−/−/− mice did not further accelerate tumor growth, which is consistent with an epistatic effect between MKK7 and p53 (Fig. 5a and Supplementary Fig. 10a). However, there were more adenocarcinomas in Tp53−/−/−; KRas; Map2k7−/−/− mice (Supplementary Fig. 10b), indicating that loss of p53 does not affect tumor burden on a MKK7-deficient background but results in increased numbers of adenocarcinomas.

To test the hypothesis that p53 levels are crucial for the tumor suppressor activity of MKK7, we crossed KRas; Map2k7−/−/− animals with ‘Super p53’ BAC transgenic mice, which harbor an extra copy of p53. We speculated that increasing the p53 gene dose might rescue MKK7 deficiency. Indeed, the p53 transgene (p53T+) markedly reduced the tumor burden in KRas; Map2k7−/−/− mice to levels found in control mice (Fig. 5b,c). In the lung tumors of Super 53 transgenic mice,
p53 levels were markedly increased, although still lower in MKK7-deficient tumors (Fig. 5d). These data provide direct evidence that MKK7 exerts its tumor suppressive function through p53.

MKK7 suppresses NeuT-driven mammary cancer

We next wanted to test whether this finding holds true for another epithelial tissue. To assess the function of stress kinase signaling in epithelial tumorigenesis, we deleted Map2k7 in mammary epithelial cells using a MMTV-Cre deleter mouse. MMTV-Cre Map2k7floxedΔ mice (termed Map2k7fl/fl mice hereafter) were viable and healthy and showed normal mammary gland formation throughout puberty and lactation (Supplementary Fig. 11a and data not shown). MKK7 is highly expressed in ductal epithelial cells of the mammary gland (Supplementary Fig. 11b). Ductal epithelial cells in situ (Supplementary Fig. 11b), as well as isolated primary mammary epithelial cells (MECs), showed almost complete deletion of MKK7 (Supplementary Fig. 11c,d). Moreover, primary MECs isolated from Map2k7fl/fl mice showed impaired activation of JNK and cJun in response to the cytotoxic drug ansominycin or the proinflammatory cytokine TNFα (Supplementary Fig. 11e).

To test whether inactivation of MKK7 had an effect on mammary tumorigenesis, we crossed Map2k7fl/fl mice with MMTV-NeuT transgenic mice, a genetic model of breast cancer, to generate MMTV-NeuT; Map2k7fl/fl mice (termed NeuT; Map2k7fl/fl mice hereafter). In the KRasG12D lung tumor model, we observed a much earlier onset of mammary tumors in NeuT; Map2k7fl/fl mice than in MMTV-NeuT mice (Fig. 6a). Moreover, the overall survival of NeuT; Map2k7fl/fl female mice was markedly reduced compared to that of control littersmates (Fig. 6b). Histopathological analysis revealed that these late-stage tumors in both NeuT; Map2k7fl/fl and control mice were typical adenocarcinomas (Supplementary Fig. 12a). Whole-mount analysis of mammary glands of 10-week-old mice (before the appearance of any palpable tumors) showed that NeuT; Map2k7fl/fl mice had numerous hyperplastic regions (Fig. 6c). At the same time point, we found no such hyperplastic lesions in control littersmates (Fig. 6c). At 16 weeks of age, the entire mammary fat pad contained hundreds of adenocarcinomas in NeuT; Map2k7fl/fl mice, whereas the mammary fat pads of control mice contained only a few lesions (Fig. 6d). These results show that deletion of Map2k7 in MECs facilitates NeuT-driven cancer formation; that is, MKK7 functions as a potent tumor suppressor in the mammary gland.

MKK7 controls p53 protein stability in MECs

We next isolated primary MECs from Map2k7fl/fl mice. We infected these cells with Ad-GFP or Ad-Cre-GFP to delete Map2k7 and treated them with doxorubicin. Doxorubicin induced fast and sustained MKK7-dependent activation of JNK in primary MECs (Supplementary Fig. 13a). As expected, control MECs showed strong activation of the DDR checkpoint kinases CHK1 and CHK2 as well as upregulation of p53 expression after doxorubicin treatment (Fig. 6e). However, although MKK7-deficient cells showed apparently normal activation of CHK1 and CHK2, these MECs showed delayed and less sustained p53 protein expression (Fig. 6e). Consequently, MKK7-deficient MECs failed to arrest at the G2/M cell cycle checkpoint after DNA damage (Supplementary Fig. 13b). Moreover, MKK7-deficient cells failed to induce p53-responsive genes such as p21, Puma, Mdm2 and Gadd45γ (Supplementary Fig. 13c).

Mechanistically, loss of MKK7 did not affect p53 mRNA expression (Supplementary Fig. 13d); rather, in the presence of the prosesome inhibitor MG132, we observed comparable induction and expression of p53 in both MKK7-deficient and MKK7-expressing MECs (Supplementary Fig. 13e). Similar to our lung cancer model, p53 protein levels, but not mRNA levels, were markedly reduced in all primary MKK7-deficient NeuT-driven mammary tumors analyzed, whereas expression and phosphorylation of p38 did not seem to be affected in breast cancer epithelium (Fig. 6f).
and Supplementary Fig. 13f,g). Thus, loss of MKK7 in mammary epithelial cells does not affect transcription of the Tp53 gene but directly affects the stability of p53 protein.

**DISCUSSION**

Most pre-cancerous lesions, such as hyperplasia of the lung and the breast, are quiescent and do not progress to form overt tumors36,37. The mechanism of how cells sense their premalignant state and undergo cell cycle arrest and/or cell death is unclear. In vitro, oncogenic stimulation of DNA replication can lead to double-strand breaks, stalled DNA forks, allelic imbalance at ‘fragile sites’, telomere dysfunction or production of reactive oxygen species, all of which will trigger the DDR and p53-dependent cell cycle arrest, senescence and cell death15–17,19. In line with this concept, immunohistochemical studies have shown that early human lesions of the lung, breast, skin, colon and bladder show activation of DDR and upregulation of p53 (refs. 15,16). Moreover, activated JNK signaling and the importance of the JNK upstream activator Rac1 in have been described in KRasG12D-driven tumors38,39, but the functional importance of activated JNK signaling has not been addressed. We now confirm that the DDR acts as an anti-tumor barrier in an inducible lung cancer model in vivo and identify MKK7-JNK signaling as a crucial component that senses oncogenic stress and thereby links the DDR to p53 stability and tumor suppression (Supplementary Fig. 14). However, we cannot rule out the possibility that the oncogene itself or stromal factors such as cytokines participate to activate MKK7-JNK signaling. Further studies are required to elucidate how oncogenic stress is sensed by MKK7 and how ARE14,17,35,40, ATM22 and ATR17 might be involved in MKK7-mediated stabilization of p53.

Activating mutations in KRAS (10–30%) and loss-of-function point mutations in TP53 (50–70%) are frequently found in human non–small-cell lung cancer, whereas 30% of human breast tumors overexpress Her2 (Neu) and about 40% harbor p53 mutations36,41. Our genetic in vivo data now clearly show that tissue-specific inactivation of MKK7 resulted in impaired p53 protein expression and early onset tumor formation in K-RasG12D-driven lung carcinomas and NeuT-driven breast cancer. Moreover, we also observed enhanced skin tumors in K5-Cre Map2k7f/f mice following challenge with the chemical carcinogen DMBA/TPA (data not shown). These in vivo data show that the stress kinase MKK7 functions as a tumor suppressor for epithelial cancers of the lung, mammary gland and skin downstream of various oncogenes. Our data might also provide a molecular mechanism by which common genotoxic therapies such as doxorubicin or γ-irradiation, which are used in everyday clinical practice, function as anti-cancer therapies. They also provide support for a re-evaluation of JNK inhibitors as therapeutic strategies for inflammatory and fibrotic diseases, as inhibition of the MKK7-JNK pathway in vivo might lead to deregulation of the key tumor suppressor p53.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

**Accession numbers.** Transcriptional profiling of control and MKK7-deficient primary mouse pneumocytes after oncogenic KRasG12 induction was done in biological triplicates. Microarray data and description of experimental design are deposited under GEO number GSE26763.

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**AUTHOR CONTRIBUTIONS**

D.S. designed and performed most experiments. A.K. and V.G.G. performed the DNA damage and p53 immunohistochemistry and analysis. A.M. performed all RT-PCR analyses. T.W. generated the MKK7f/f/f mice. U.E. and V.S. helped with immunohistochemistry. R.H.Z. analyzed the tumor section as the expert pathologist. J.A.P. and G.G.N. helped in microarray and gene set enrichment analysis. G.F. and M.S. contributed to the characterization of the ErbB-2 and Super p53 transgenic mice, respectively. J.M.P. coordinated the project and wrote the manuscript with D.S.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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7. Nateri, A.S., Spencer-Dene, B. & Behrens, A. Interaction of phosphorylated c-Jun N-terminal phosphorylation with p53 transgenic mice, respectively. J.M.P. coordinated the project and wrote the manuscript with D.S.


ONLINE METHODS

Generation of mice carrying a Map2k7floxed allele. A targeting vector was constructed by PCR amplification of a BAC clone carrying the Map2k7 locus. A 3.5-kb-long arm encompassing exon 2 was isolated with HindIII (H) and cloned downstream of the DTA cassette followed by a HindIII-BamHI (B)-digested middle arm spanning exons 3–10. The short arm (exons 12 and 13) was PCR-amplified and placed downstream of the Neo cassette. The construct was linearized and electroporated into A9 embryonic stem cells derived from a 129×C57Bl mixed strain. After germine line transmission, mice were crossed to Flp transgenic mice to delete the flt-flanked Neo cassette. To generate mice carrying a null allele of Map2k7 (Map2k7α allele), Map2k7floxed mice were crossed to β-actin-Cre ubiquitous deleterious mice. Mice carrying the Map2k7floxed or Map2k7α alleles as well as the KrasG12D knockin mice were backcrossed at least ten times onto a C57Bl background before generating the Kras; Map2k7αfloxed mice. MMTV-NeuT mice as well as MMTV-Cre mice have been previously described. Super p53 mice were kindly provided by M. Serrano, JNK1 and JNK2 knockout mice were provided by E. Wagner and have been described previously.45,46 MMTV-Cre mice (stock #003553 and Tprp53m1217) homozygous (p53−/−) knockout mice were obtained from the Jackson Laboratory. Mouse genotypes were determined by PCR and DNA blot analysis. In all experiments described in this paper, only littermate mice from the same breeding were used. All mice were bred and maintained according to an ethical animal license protocol complying with the current Austrian law.

Induction of lung tumorigenesis in Lox-Stop-Lox KrasG12D mice. Infection of lungs with AdCre was performed as previously described12. Briefly, mice were anesthetized with Ketalar/Xylasol and placed on a heated pad. An AdCre-CaCl2 precipitate was produced by mixing 60 µl MEM, 2.5 µl AdCre (1010 pfu/ml; University of Iowa, Gene Transfer Vector Core Iowa, USA) and 0.6 µl CaCl2 (1 M) for each mouse and incubated for 20 min at room temperature. Histology, whole-mount analysis and immunohistochemistry. For histo

Histology, whole-mount analysis and immunohistochemistry. For histological analysis of lung tumors, 4-µm sections from at least three different central planes of the lungs were cut, stained with hematoxylin and eosin and scanned with a Mirax slide scanner. Tumor volume was automatically scored by an algorithm programmed and executed using the definiens software suite program and visually controlled by a pathologist in a blinded fashion. Whole-mount staining of mammary glands was performed as described5. For immunoperoxidase staining, paraffin-embedded sections were dehydrated and antigenic epitopes exposed using a 10-mM citrate buffer and microwaving. Sections were incubated with rabbit polyclonal anti-Ki67 (Novacraft), anti-active caspase 3 (Cell Signaling #9661) or anti-MKK7 (Cell Signaling #4172). Primary antibody staining was visualized using peroxidase-conjugated anti-rabbit IgG. Immunohistochemistry for DDR markers was performed as described13 using the UltraVision LP Detection System (#TL-060-HD, Thermo) according to the manufacturer’s instructions. Primary antibodies were reactive to mouse MKK7 (#1472), MKK4 (#3346), phosphorylated (P) JNK (JNK25), JNK25; detecting JNK1 and JNK2), phosphorylated (P) c-Jun (#9164), c-Jun (#9162), phosphorylated (P) Akt (#3787), Akt (#9272), phosphorylated (P) Erk1/2 (#9101), Erk1/2 (#9102), p38-MAPK (#2122), p38 (#2524), phosphorylated (P) CHK1 (#2348), CHK1 (#2345), phosphorylated (P) CHK2 (#2197), CHK1 (#2662), phosphorylated (P) Ser(392) p53 (#9281), phosphorylated (P) Ser(15) p53 (#9284), phosphorylated (P) Ser(6) p53 (#9285), phosphorylated (P) Ser(33) p53 (#2526), phosphorylated (P) Thr(81) p53 (#2676) (all from Cell Signaling), p38-MAPK (AF689; R&D), γH2AX (Ser139 #07-164 Millipore) and β-actin (Sigma). Blots were washed three times in TBST for 30 min, incubated with HRP-conjugated secondary antibodies (1:2,000; Promega) for 1 h at room temperature, washed 3 times in TBST for 30 min and visualized using enhanced chemiluminescence (ECL). The JNK kinase assay (#9810) and phospho (P) c-Jun ELISA Kit (#7145) were from Cell Signaling.

qRT-PCR. Total RNA of tumors was prepared using the RNasey Mini Kit (QIAGEN) according to the manufacturer’s instructions. Total RNA (2 µg) was transcribed into complementary DNA (Amersham Kit) and subjected to quantitative RT-PCR analysis. The primer sequences are given in Supplementary Table 1.

siRNA- and short hairpin (shRNA)-mediated gene knockdown of MKK7. For knockdown experiments ON-TARGETplus SMARTpool for MAP2K7 (MKK7) from Dharmacon was used (#L-004016-00). Scrambled siRNA was used as a control. For stable knockdown, hairpin primers used for cloning into pSIREN following the manufacturer’s guidelines are given in Supplementary Table 2.

DNA damage responses. For measurement of cell cycle arrest and apoptosis primary mouse mammary epithelial cells and A549 human lung cancer cells were seeded at a cell density of 100,000 cells per well in a 6-well plate and allowed to grow for 24 h at 3% O2. Cells were then treated with doxorubicin or γ-irradiation. Cell cycle distribution was determined by fluorescence-activated cell sorting (FACS) using ethidium bromide (EtBr) staining.

Statistics. All values in the paper are given as means ± s.e.m. Comparisons between groups were made by Student’s t-test. For the Kaplan Meier
analysis of tumor onset, a log rank test was performed. *P* < 0.05 was accepted as statistically significant.


