p38δ Mitogen-Activated Protein Kinase Is Essential for Skin Tumor Development in Mice

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Abstract

Activating Ras mutations occur in a large portion of human tumors. Yet, the signaling pathways involved in Ras-induced tumor formation remain incompletely understood. The mitogen-activated protein kinase pathways are among the best studied Ras effector pathways. The p38 mitogen-activated protein kinase isoforms are important regulators of key biological processes including cell proliferation, differentiation, survival, inflammation, senescence, and tumorigenesis. However, the specific *in vivo* contribution of individual p38 isoforms to skin tumor development has not been elucidated. Recent studies have shown that p38δ, a p38 family member, functions as an important regulator of epidermal keratinocyte differentiation and survival. In the present study, we have assessed the effect of p38δ deficiency on skin tumor development *in vivo* by subjecting p38δ knockout mice to a two-stage 7,12-dimethylbenz(*a*)anthracene/12-*O*-tetradecanoylphorbol-13-acetate chemical skin carcinogenesis protocol. We report that mice lacking *p38δ* gene exhibited a marked resistance to development of 7,12-dimethylbenz(*a*)anthracene/12-*O*-tetradecanoylphorbol-13-acetate-induced skin papillomas, with increased latency and greatly reduced incidence, multiplicity, and size of tumors compared with wild-type mice. Our data suggest that the underlying mechanism for reduced susceptibility to skin carcinogenesis in p38δ-null mice involves a defect in proliferative response associated with aberrant signaling through the two major transformation-promoting pathways: extracellular signal-regulated kinase 1/2-activator protein 1 and signal transducer and activator of transcription 3. These findings strongly suggest an *in vivo* role for p38δ in promoting cell proliferation and tumor development in epidermis and may have therapeutic implication for skin cancer. [Cancer Res 2009;69(11):4648–55]

Introduction

Cutaneous malignancies are widespread and increasing in incidence (1). Thus, elucidation of the molecular mechanisms that contribute to pathogenesis of skin cancer is of great importance. Epidermis is a multilayered self-renewing tissue protecting the host from environmental insults and providing a barrier against the loss of water and nutrients. Normal epidermal morphogenesis and homeostasis are ensured by a tightly regulated balance of epidermal keratinocyte growth and differentiation (2–5). Malfunctions in control of this balance can lead to development of diseases, including cancer.

Activating Ras mutations contribute to malignant transformation and occur in a large portion of human tumors. Therefore, signaling pathways involved in Ras-driven tumor formation are of considerable interest. Mitogen-activated protein kinase (MAPK) pathways are among the best characterized Ras effector pathways. MAPKs, including extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), and p38s, are serine/threonine kinases that transmit extracellular stimuli to intracellular responses to regulate vital biological processes (6–8). The p38 subgroup includes p38α, p38β, p38γ, and p38δ isoforms, which differ in their pattern of expression, substrate specificity, and sensitivity to chemical inhibitors (8–10). p38 isoforms are activated by stress such as UV radiation, osmotic shock, and cytokines, and less often by mitogenic stimuli. p38 MAPKs regulate diverse cellular responses including proliferation, differentiation, transformation, and programmed cell death. Physiologic outcome of p38 activation is cell type- and stimulus-dependent, and the specific functions of individual p38 isoforms are not well defined (8, 10). In addition, the roles of p38 isoform-specific signaling in various pathologic conditions remain to be elucidated (8, 10).

Normal epidermal keratinocytes express p38α, p38β, and p38δ (11). *p38β* and *p38γ* transcripts are increased in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated mouse epidermis (12), whereas *p38α* and *p38δ* levels are increased in human squamous cell carcinomas (SCC; ref. 13). Previous studies implicate p38δ isoform as an important regulator of keratinocyte function (11, 14–20). p38δ is involved in regulation of involucrin gene expression (11, 18, 20). p38δ is activated by a host of differentiation stimuli, including TPA, calcium, okadaic acid, and green tea polyphenol (16–18). However, the induction of involucrin expression by cholesterol-depleting agents involves p38*a*, suggesting that p38δ is not a sole p38 isoform involved in regulating keratinocyte differentiation (8, 21). p38δ has a role in keratinocyte apoptosis (19, 22). Although these findings suggest a function for p38δ in regulating keratinocyte differentiation and apoptosis in cultured cells, the *in vivo* role of p38δ in controlling skin homeostasis and tumorigenesis has not been examined. In the present study, we investigated the effect of p38δ deficiency on skin tumor development using mouse model of multistage skin carcinogenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

E.M. Schindler and A. Hindes contributed equally to this work.

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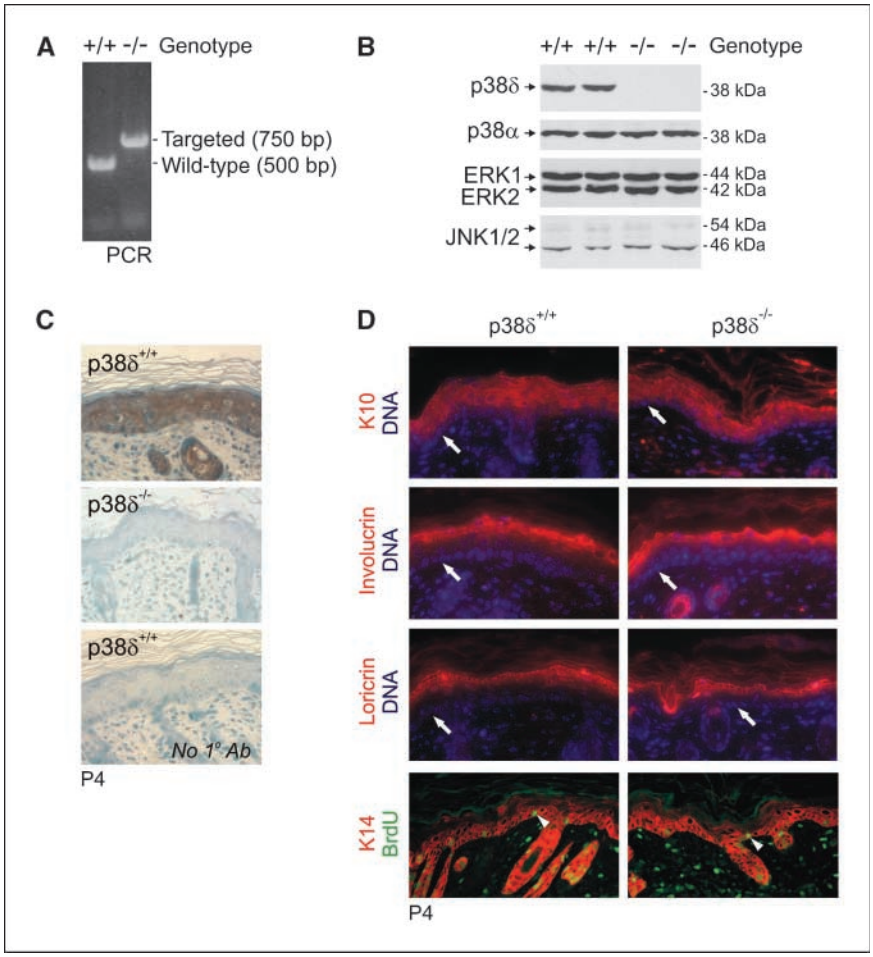

Figure 1. p38δ-null mice exhibit normal skin phenotype. *A,* genotyping PCR using genomic DNA from wild-type (+/+) and p38δ-null (-/-) mice. *B,* immunoblot analysis of p38, ERK, and JNK expression in epidermal extracts of wild-type (+/+) and p38δ-null (-/-) skin. *C,* immunohistochemical analysis of p38δ expression in wild-type ($p38\delta^{+/+}$) and p38δ-null ($p38\delta^{-/-}$) skin using postnatal day 4 (*P4*) mouse skin sections. Positive staining for p38δ is shown by brown staining. Secondary antibody alone was used as a negative control (*bottom*). Magnification, ×20. *D,* immunofluorescence analysis of keratinocyte differentiation and proliferation markers in wild-type ($p38\delta^{+/+}$) and p38δ-null ($p38\delta^{-/-}$) epidermis. Postnatal day 4 skin sections were stained with antibodies against keratin 10 (*K10*), involucrin, loricrin, keratin 14 (*K14*), and BrdUrd. Nuclei were counterstained with Hoechst. *Arrows,* dermal-epidermal junction; *arrowheads,* representative BrdUrd (*BrdU*)-positive nuclei (*green*) in the basal epidermal layer. Representative tissue fields. Magnification, ×40.

Our data show that skin tumor development is inhibited in p38δ-null background, suggesting that p38δ is of functional importance for skin tumor formation.

Materials and Methods

Reagents and antibodies. 7,12-Dimethylbenz(*a*)anthracene (DMBA), TPA, bromodeoxyuridine (BrdUrd), Hoechst 33342, ERK1/2, p38α, and β-actin antibodies were purchased from Sigma. p38δ, Fra-1, JunB, and JunD antibodies were from Santa Cruz Biotechnology. JNK1/2, phospho-ERK1/2, phosho-p38, and phospho-signal transducer and activator of transcription 3 (Stat3; Tyr^{705}) antibodies were from Cell Signaling Technology. BrdUrd antibody was from Chemicon. p38δ antibody, raised in sheep and affinity purified as described (23), was obtained from the Division of Signal Transduction Therapy, University of Dundee, Dundee, United Kingdom. Antibodies against keratin 10, keratin 14, involucrin, and loricrin were from Covance Research Products.

p38δ knockout mice. Generation of p38δ-null mice was described previously (24). These mice are viable and fertile and exhibit no obvious health problems. Genotyping was done as detailed (24).

Skin chemical carcinogenesis. Mice were maintained in animal facility in compliance with Washington University animal care regulations. Experimental groups consisted of animals generated from separate intercrosses of wild-type ($p38\delta^{+/+}$) and p38δ knockout ($p38\delta^{-/-}$) mice, backcrossed to C57BL/6 genetic background for six generations. Studies were conducted using both male and female $p38\delta^{+/+}$ and $p38\delta^{-/-}$ mice. Mice in experiment 1 ($p38\delta^{+/+}$, $n = 9$; $p38\delta^{-/-}$, $n = 6$; 8-14 weeks old) were initiated with a single topical application of 10 μg DMBA in 200 μL acetone to shaved dorsal skin. Starting 1 week after DMBA treatment, mice were treated topically twice weekly for 21 weeks with 10 μg TPA in 200 μL acetone. Mice in experiment 2 ($p38\delta^{+/+}$, $n = 35$; $p38\delta^{-/-}$, $n = 34$; 8 weeks old) were subjected to a single topical application of 100 μg DMBA in 200 μL acetone. Starting 1 week after DMBA treatment, mice were treated twice weekly for 25 weeks with 12.5 μg TPA in 200 μL acetone. The onset of tumor formation and the number and size of tumors were recorded weekly.

Histology, immunofluorescence, and immunohistochemistry. Dorsal skin samples from newborn and adult mice were fixed in 4% paraformaldehyde in PBS, paraffin-embedded, and sectioned at 5 μm. For immunofluorescence detection, sections were subjected to microwave antigen retrieval in 10 mmol/L citrate buffer (pH 6.0), blocking, and incubation with primary antibody at 4°C overnight followed by incubation with the appropriate fluorochrome-conjugated secondary antibody for 2 h at room temperature. Sections were visualized under a Zeiss fluorescence microscope (Carl Zeiss MicroImaging). For immunohistochemical detection, sections were incubated with primary antibodies at 4°C overnight followed by incubation with biotinylated IgG at room temperature for 30 min. Detection of the signal was carried out using Vectastain ABC kit (Vector Laboratories) and diaminobenzidine substrate kit (Pierce Biotechnology) following the manufacturer's instructions. The sections were counterstained with hematoxylin.

Analysis of proliferation and apoptosis after acute TPA treatment of adult mouse skin. Mice were treated with a single TPA (5 μg in 200 μL acetone) or acetone vehicle and sacrificed at the indicated times after treatment. Mice were injected intraperitoneally with BrdUrd (100 μg/g body weight) in PBS 1 h before sacrifice. Tissue sections were immunostained with anti-BrdUrd antibody. Epidermal tissue from the five acetone-treated and nine TPA-treated mice was analyzed, scoring the number of BrdUrd-positive cells per a minimum of 250 basal cells for each sample. Epidermal proliferation rate at postnatal day 4 was measured in skin tissue from two wild-type and two knockout mice, scoring the number of BrdUrd-positive

cells per a minimum of 450 basal cells for each sample. Apoptosis was evaluated using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay kit (Promega) according to the manufacturer's protocol.

Tissue protein isolation and immunoblot analysis. Epidermal samples were collected as described (25). Briefly, dorsal mouse skin was flash-frozen with dry ice, and epidermis was removed by scraping it off with a cold scalpel. Epidermal lysates were prepared in T-PER lysis buffer supplemented with protease inhibitor cocktail (Pierce Biotechnology). The protein content of the samples was determined using Bradford protein assay reagent (Bio-Rad). Immunoblot analysis was done as described (26).

Statistical methods. Time to first tumor (tumor latency) was compared between $p38\delta^{+/+}$ and $p38\delta^{-/-}$ groups using the log-rank statistic. Proportions of animals having tumors at each week were compared using Fisher's exact test. Numbers of tumors per animal observed each week were compared using repeated-measures ANOVA. Numbers of tumors per tumor-bearing animal each week, volume of individual tumors at week 25, and total volume of tumors at week 25 per tumor-bearing animal were compared between $p38\delta^{+/+}$ and $p38\delta^{-/-}$ groups using Mann-Whitney tests. All P values are one-sided.

Results

p38δ knockout mice exhibit normal skin phenotype. To confirm genotype of the p38δ knockouts, we examined genomic DNA samples isolated from $p38\delta^{+/+}$ and $p38\delta^{-/-}$ mice by PCR. *p38δ* DNA was not detected in homozygous $p38\delta^{-/-}$ mice (Fig. 1*A*). Targeted disruption of *p38δ* gene led to a total lack of p38δ protein in $p38\delta^{-/-}$ mice, whereas p38α, ERK1/2, and JNK1/2 proteins were expressed at similar levels in the epidermis of $p38\delta^{+/+}$ and $p38\delta^{-/-}$ mice, as assessed by immunoblot analysis (Fig. 1*B*). Immunohistologic staining of newborn mouse skin sections showed p38δ expression throughout the epidermis in both undifferentiated and differentiated layers in wild-type but not in p38δ-null mice (Fig. 1*C*). Consistent with the absence of overt skin abnormalities in $p38\delta^{-/-}$ mice, normal epidermal tissue architecture is maintained, and expression of differentiation markers keratin 10, involucrin, and loricrin is normal in p38δ-null epidermis (Fig. 1*D*). Epidermal proliferation measured by BrdUrd incorporation is also normal in p38δ knockout mice, as no significant difference is observed in the percentage of BrdUrd-positive basal keratinocytes in wild-type and knockout epidermal tissue (Fig. 1*D, bottom*): 14.87 ± 0.10 (wild-type) and 12.38 ± 0.42 (knockout), $P = 0.074$ (mean ± SE). These data indicate that p38δ is not essential for epidermal homeostasis.

p38δ is required for skin tumor development in mouse model of chemical epithelial carcinogenesis. To begin evaluating the role of p38δ as a contributor to carcinogenesis, we used a two-stage chemical skin tumor induction protocol (27–29).

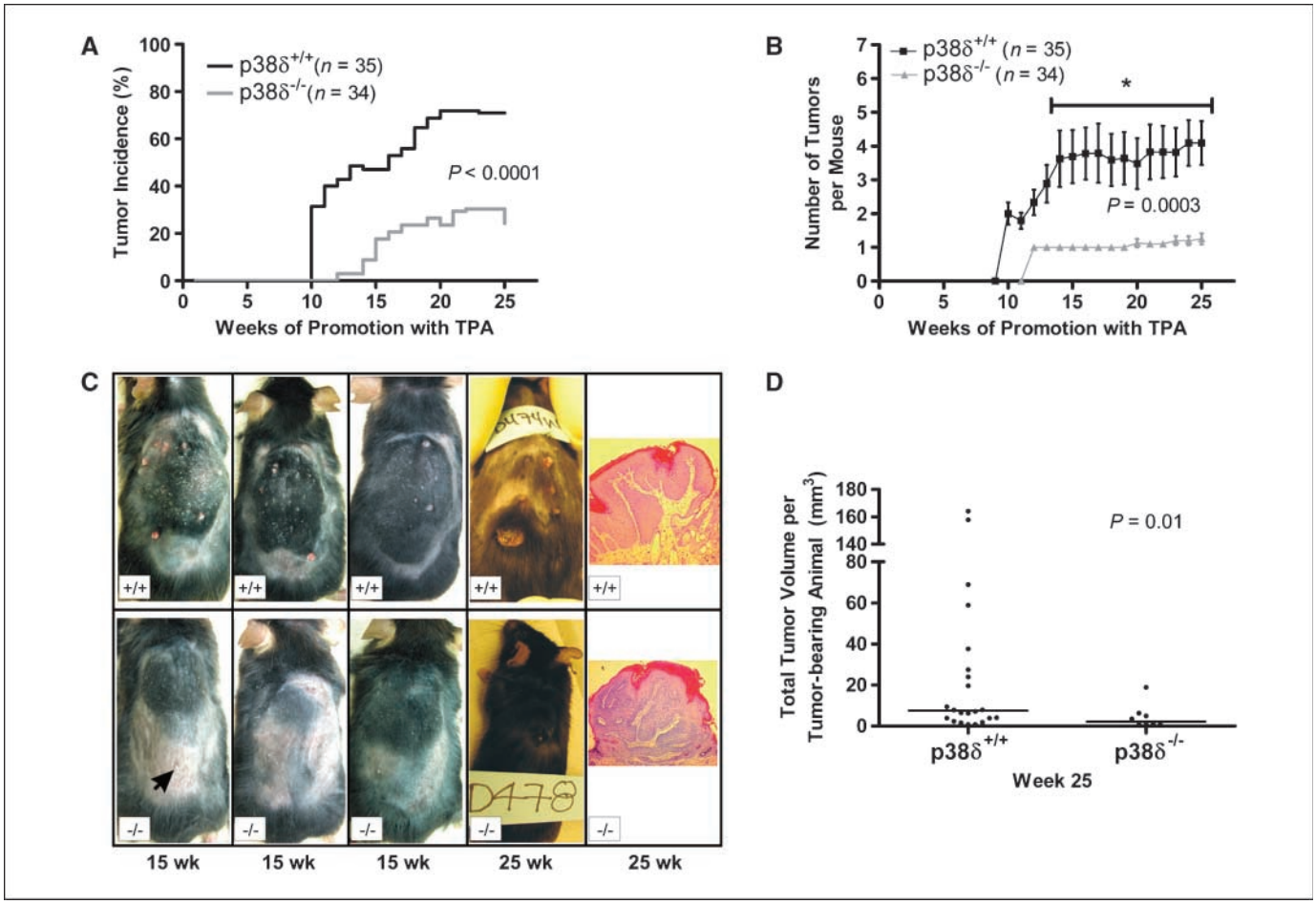

Figure 2. p38δ deficiency inhibits skin tumor development. $p38\delta^{+/+}$ and $p38\delta^{-/-}$ mice were treated as described in Materials and Methods. *A,* tumor incidence. *B,* average number of tumors per mouse in tumor-bearing mice. *, $P < 0.05$. *C,* general appearance of skin tumors in $p38\delta^{+/+}$ (+/+) and $p38\delta^{-/-}$ (-/-) skin following 15 or 25 weeks of twice weekly TPA promotion. *Top* and *bottom right,* H&E staining of a segment of a typical $p38\delta^{+/+}$ papilloma (+/+) and a typical $p38\delta^{-/-}$ (-/-) papilloma. Magnification, ×40. *D,* total tumor volume per mouse in tumor-bearing mice. Individual tumor volumes were calculated based on the formula: V (mm^3) = $p(\text{radius})^2\text{height}$. Total tumor volume was obtained by adding the individual tumor volumes per animal. *Lines,* geometric means.

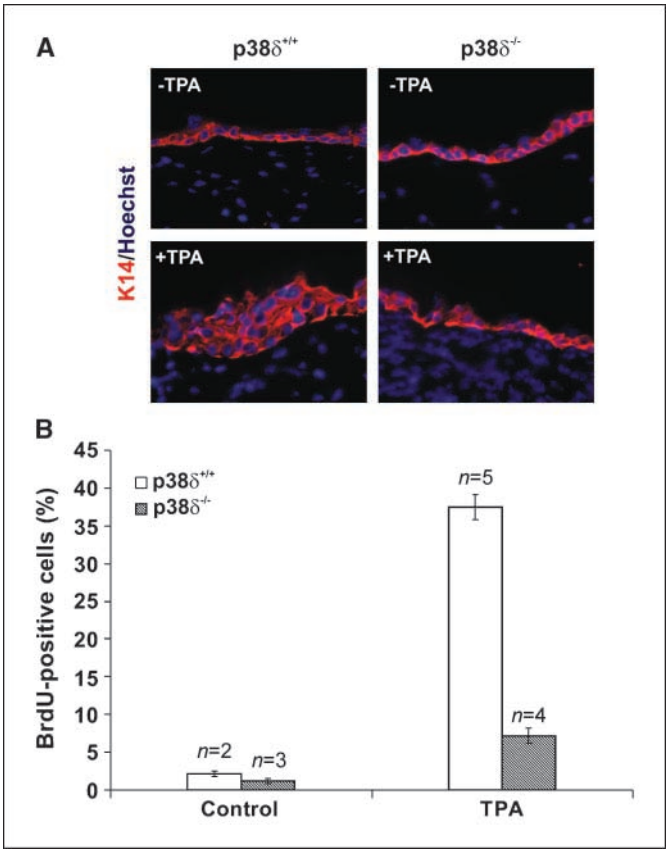

Figure 3. Reduced proliferation in p38δ-null epidermis in response to TPA. The dorsal skin of wild-type and p38δ-null mice was treated topically with acetone vehicle (-TPA) or a single dose of TPA (5 μg in 200 μL acetone), and the mice were sacrificed 24 h later. BrdUrd was injected 1 h before sacrifice. *A,* reduced TPA-stimulated hyperplasia in p38δ-null epidermis. Tissue sections were stained with anti-keratin 14 and Hoechst. Magnification, ×40. *B,* epidermal tissue sections immunostained with anti-BrdUrd were analyzed, scoring the number of BrdUrd-positive cells per a minimum of 250 basal cells for each sample.

To determine the potential effect of p38δ gene disruption on skin tumor formation, we monitored tumorigenesis in mice of both genotypes. In the pilot experiment 1, groups of p38δ$^{+/+}$ and p38δ$^{-/-}$ mice were initiated with a single 10 μg dose of DMBA and promoted with twice weekly application of 10 μg TPA for 21 weeks (Supplementary Fig. S1). Under these treatment conditions, p38δ$^{+/+}$ mice started to develop the first papillomas within 10 weeks of promotion. By 14 weeks, ~33% of p38δ$^{+/+}$ mice had developed papillomas (Supplementary Fig. S1*A*). On average, p38δ$^{+/+}$ mice developed 3.3 tumors per mouse by the end of the promotion phase (Supplementary Fig. S1*B*). Notably, p38δ$^{-/-}$ mice failed to develop tumors under these conditions.

A low overall tumor incidence even in the wild-type group observed in this initial experiment is likely due to a relative resistance of mice of C57BL/6 genetic background to two-stage chemical skin carcinogenesis (12, 27). To assess whether p38δ-null mice can be induced to develop tumors, we performed an identical experiment at increased DMBA and TPA levels: in experiment 2, groups of p38δ$^{+/+}$ and p38δ$^{-/-}$ mice were initiated by an application of a 100 μg DMBA and promoted by treatment with 12.5 μg TPA twice weekly for 25 weeks. These studies show that p38δ-null mice do develop skin tumors; however, DMBA/TPA-induced tumorigenesis is different in mice lacking p38δ.

As shown in Fig. 2*A*, tumors occurred later and less frequently in p38δ$^{-/-}$ than in p38δ$^{+/+}$ mice ($P < 0.0001$, log-rank test). Tumors began to appear in p38δ$^{-/-}$ mice at week 12 of the promotion compared with week 10 for p38δ$^{+/+}$ mice. Tumor incidence was significantly lower in p38δ$^{-/-}$ than in p38δ$^{+/+}$ mice at weeks 10 to 25 of the tumor promotion period ($P < 0.01$). At week 25, 24.2% of mutant mice developed tumors compared with 71.0% of wild-type animals ($P = 0.0002$; Fig. 2*A*). Overall, p38δ-null mice developed significantly fewer tumors per animal than wild-type mice ($P = 0.0003$; Fig. 2*B*). On a weekly basis, p38δ$^{-/-}$ mice had a significantly smaller number of tumors per animal than p38δ$^{+/+}$ mice each week from weeks 14 to 25 ($P < 0.02$). Thus, by the end of the promotion phase, at week 25, wild-type animals developed on average 4.1 tumors per mouse, whereas p38δ-null animals developed 1.2 tumors per mouse ($P = 0.0008$; Fig. 2*B*). The size of tumors that developed in p38δ-null mice was markedly reduced in comparison with the size of tumors in control mice (Fig. 2*C*). In addition, tumor-bearing p38δ$^{-/-}$ mice had a significantly lower total tumor volume than tumor-bearing p38δ$^{+/+}$ mice ($P = 0.01$; Fig. 2*D*). Statistical analyses of the data for males and females separately showed that p38δ$^{-/-}$ mice of both genders were significantly more resistant to DMBA/TPA-mediated induction of skin tumors than their p38δ$^{+/+}$ counterparts (data not shown).

At the end of the promotion stage, we detected a total of 90 tumors in the p38δ$^{+/+}$ cohort and 10 tumors in the p38δ$^{-/-}$ cohort. Thirty-five p38δ$^{+/+}$ tumors and 5 p38δ$^{-/-}$ tumors were examined histopathologically. The majority of p38δ$^{+/+}$ tumors (30 of 35) and all p38δ$^{-/-}$ tumors (5 of 5) were characterized as squamous cell papillomas. With the exception of size differences, the overall histologic appearance of papillomas was not obviously dissimilar between p38δ$^{+/+}$ and p38δ$^{-/-}$ mice (Fig. 2*C*). Notably, 2 of the 35 p38δ$^{+/+}$ tumors examined were classified as well-differentiated SCC. In addition, histopathologic examination revealed three sebaceous adenomas among the 35 p38δ$^{+/+}$ tumors inspected.

Skin tumors of both genotypes harbor activating H-Ras mutations. A carcinogen-induced activating *H-Ras* mutation is frequently the initiating event in the multistage mouse skin carcinogenesis (29). We therefore examined papillomas isolated from wild-type and p38δ-null mice for the presence of Ras mutations. Genomic DNA analysis from five p38δ$^{+/+}$ and four p38δ$^{-/-}$ papillomas revealed the signature codon 61 activating mutation in the *H-Ras* gene in all lesions sampled (data not shown). These data indicate that lack of p38δ did not alter the mechanism of DMBA-induced mutagenesis in the initiation stage of skin tumor formation.

p38δ deficiency reduces TPA-dependent epidermal hyperplasia and cell proliferation. To gain insight into the mechanism underlying resistance to carcinogenesis in p38δ-null mice, we examined the effect of TPA treatment on epidermal cell proliferation. As shown in Fig. 3, p38δ-null skin exhibits reduced hyperplasia (epidermal thickening) following TPA challenge relative to similarly treated wild-type skin (Fig. 3*A*). Moreover, a single topical application of TPA produces markedly reduced BrdUrd incorporation in p38δ-null epidermis compared with wild-type (Fig. 3*B*). Thus, resistance to tumor development in p38δ-null skin is, at least in part, due to a decrease in epidermal proliferation during tumor promotion. These results indicate that p38δ-null epidermis is proliferation-deficient. We next sought to assess whether this deficiency would translate into reduced cell proliferation of keratinocytes in TPA-induced papillomas. Indeed, we observed a marked reduction in the number of proliferating

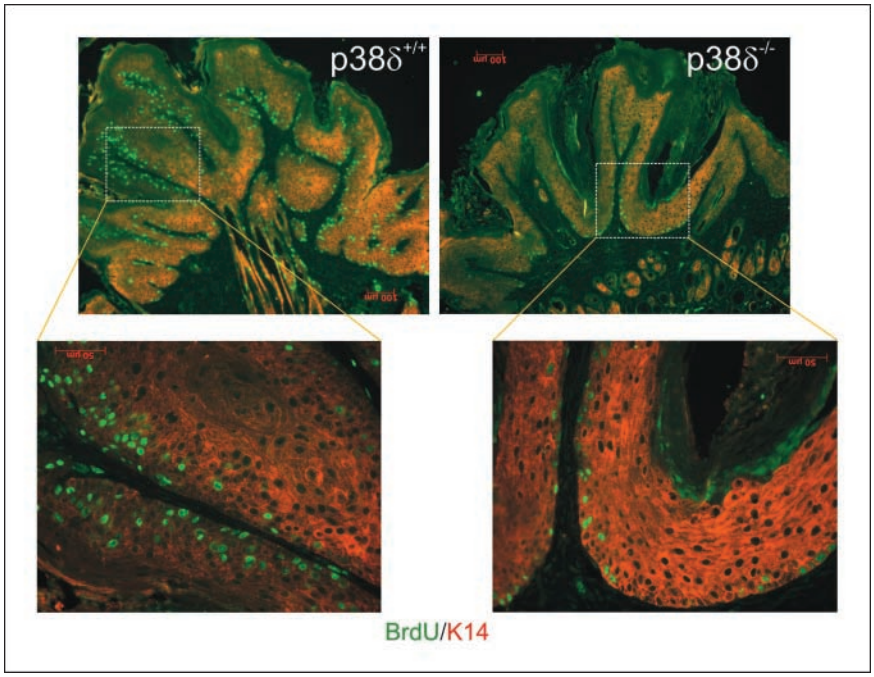

Figure 4. Papillomas from p38δ$^{-/-}$ mice show reduced proliferation. Proliferation was assessed by monitoring BrdUrd incorporation after 25 weeks of twice weekly TPA administration. TPA treatment was terminated 2 weeks before harvest of tumors. BrdUrd was injected 1 h before sacrifice. A keratin 14 antibody stains the epithelial compartment of the tumors. Four p38δ$^{+/+}$ and three p38δ$^{-/-}$ papillomas were examined. Representative tissue fields. *Bar,* 100 μm (*top*) and 50 μm (*bottom*).

BrdUrd-positive cells in tumors from p38δ-null mice compared with tumors isolated from the wild-type mice (Fig. 4).

Lack of p38δ does not alter TPA-induced apoptosis. To determine whether p38δ deficiency influences the apoptotic process in response to TPA, we examined the levels of cell death in TPA-treated wild-type and p38δ-null adult mouse skin and in papillomas isolated from animals of both genotypes by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. As shown in Supplementary Fig. S2, the number of TPA-induced apoptotic cells in the epidermis of p38δ$^{-/-}$ mice was not increased compared with the number of apoptotic cells in p38δ$^{+/+}$ skin. Apoptosis levels were negligible in papillomas harvested from mice of either genotype (data not shown). These results indicate that increased apoptosis does not account for reduced sensitivity of p38δ-null mice to skin carcinogenesis.

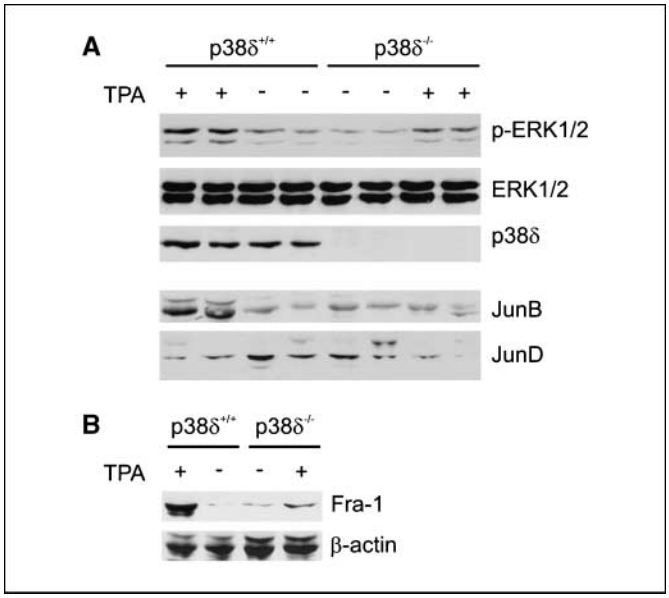

Figure 5. Reduced ERK1/2 activity and impaired JunB and Fra-1 expression in TPA-treated skin of p38δ-null mice. p38δ-null and control mice were treated topically with a single application of acetone (-) or 12.5 μg TPA (+) and sacrificed 3 h later. ERK1/2 activation in epidermal lysates was assessed by immunoblot using phospho-ERK1/2 antibodies. Total ERK1/2 level was measured to ensure equal protein loading. p38δ status was verified by assaying p38δ levels. JunB, JunD (*A*), and Fra-1 (*B*) levels were determined by immunoblot using the corresponding specific antibodies. β-Actin level (*B*) was assayed to ensure equal protein loading. Representative of four independent experiments.

Reduced ERK1/2 activity and impaired expression of JunB and Fra-1 in TPA-treated skin of p38δ-null mice. To establish the molecular mechanisms underlying p38δ$^{-/-}$ skin resistance to TPA-induced epidermal hyperplasia and decreased rate of cell proliferation (Fig. 3), we examined the effect of p38δ deficiency on ERK1/2 activation and expression of activator protein 1 (AP1) family members. The ERK1/2-AP1 pathway has been implicated in promoting cell cycle progression and cell proliferation (29). We observed that TPA-mediated activation of ERK1/2 was diminished in p38δ-null epidermis compared with wild-type epidermis (Fig. 5*A*). In addition, we found that TPA-stimulated increase in protein levels of AP1 factors JunB and Fra-1 was blocked in p38δ-null epidermis compared with wild-type epidermis, whereas expression of AP1 factor JunD was decreased in TPA-treated skin of both genotypes compared with acetone vehicle-treated skin from each group (Fig. 5*A* and *B*). These results indicate that eliminating p38δ impairs the ERK1/2-AP1 pathway critically linked to control of cell proliferation and tumorigenesis.

p38δ deficiency results in inhibition of Stat3 activation in skin papillomas. Stat3 transcription factor is constitutively activated in many human malignancies and has been suggested to play an important role in carcinogenesis (30, 31). Recent studies show an essential role of the Stat3 in mouse skin tumorigenesis (32–35). We therefore examined Stat3 activity in wild-type and p38δ-null papillomas induced by DMBA/TPA regimen. Immunohistochemical analyses revealed markedly reduced levels of phosphorylated activated Stat3 in tumors isolated from mice lacking p38δ in comparison with those in tumors derived from wild-type mice (Fig. 6), indicating that p38δ function is required for Stat3 activation during the process of skin tumorigenesis. These

data suggest that p38δ plays a role as an *in vivo* regulator of Stat3 in epidermis during multistage skin carcinogenesis.

p38δ gene disruption attenuates K-Ras–driven lung carcinogenesis. We next investigated the requirement for p38δ in another mouse model of Ras-induced tumor formation. To examine whether the endogenous p38δ plays a role in lung carcinogenesis caused by oncogenic mutation of the K-Ras gene, the p38δ-null mice were bred with K-Ras LA1 mice (36). In this strain, a sporadically activated single K-Ras allele ($K\text{-}Ras^{G12D}$) that is expressed at endogenous levels causes lung adenocarcinoma development at high frequency. These mice die by age 300 days due to an extensive tumor burden (36). $p38\delta^{-/-}$;$K\text{-}Ras^{G12D+/-}$ mice exhibited reduced number of tumors per lung (tumor incidence; $P = 0.03$) and significantly reduced average tumor volume ($P = 0.002$) and total tumor volume per lung ($P = 0.005$; tumor promotion) compared with $p38\delta^{+/+}$;$K\text{-}Ras^{G12D+/-}$ mice at age 16 weeks (Supplementary Fig. S3). These results indicate that loss of p38δ reduces susceptibility to K-Ras–driven lung tumor development.

Discussion

In the present study, we analyzed the potential *in vivo* role of p38δ MAPK in mouse skin carcinogenesis using p38δ knockout mice.

Consistent with the reported lack of overt phenotype (24), p38δ-null mice displayed normal epidermal morphology and normal epidermal growth and differentiation (Fig. 1*C* and *D*). These data indicate that p38δ is not essential for epidermal homeostasis. This is surprising given that *in vitro* studies have suggested a role for p38δ in regulating keratinocyte differentiation (11, 16–18). The lack of obvious effect of p38δ deficiency on epidermal differentiation could be explained by possible functional compensatory redundancy of the remaining p38 isoforms (8, 24). Thus, a compensatory increase in p38α activity has been documented in p38γ-/p38δ-null setting (24). Notably, although the compensatory actions of other p38 MAPK members appear to counterbalance the effects of p38δ deficiency on skin morphogenesis and differentiation, our present data argue that the remaining p38 isoforms cannot compensate for p38δ elimination during the process of skin tumorigenesis.

We show that the susceptibility to skin tumor development in the chemical skin carcinogenesis model associated with mutations of the *H-Ras* oncogene is greatly reduced in the absence of p38δ. Mice with targeted deletion of *p38δ* exhibited longer tumor latency and significantly reduced incidence, multiplicity, and size of skin tumors compared with wild-type mice. Our data suggest that the underlying mechanism for reduced susceptibility to carcinogens in p38δ-null mice is a defect in proliferative response associated with aberrant signaling through the two major transformation-promoting pathways, ERK1/2-AP1 and Stat3. These findings strongly suggest an *in vivo* role for p38δ in enhancing cell proliferation and tumor formation in epidermis, a role further supported by a recent finding that p38δ expression is increased in human SCC (13). Our data also show that elimination of p38δ decreases lung tumor development in mice heterozygous for oncogenic mutation of the K-Ras gene.

Activating Ras mutations have been identified in 30% of all human cancers, including skin cancer (37). In DMBA/TPA chemical skin carcinogenesis model, codon 61 activating mutation [CAA(Gln)-to-CTA(Leu) transversion] in the *H-Ras* gene is the initiating event (29). Our data show that tumors of both $p38\delta^{+/+}$ and $p38\delta^{-/-}$ genotypes harbored this signature oncogenic H-Ras mutation, indicating that p38δ depletion did not influence the mechanism of DMBA-induced mutagenesis during initiation stage of skin tumor formation.

We found that p38δ-deficient mice exhibited significantly reduced both the overall number of tumors that developed in

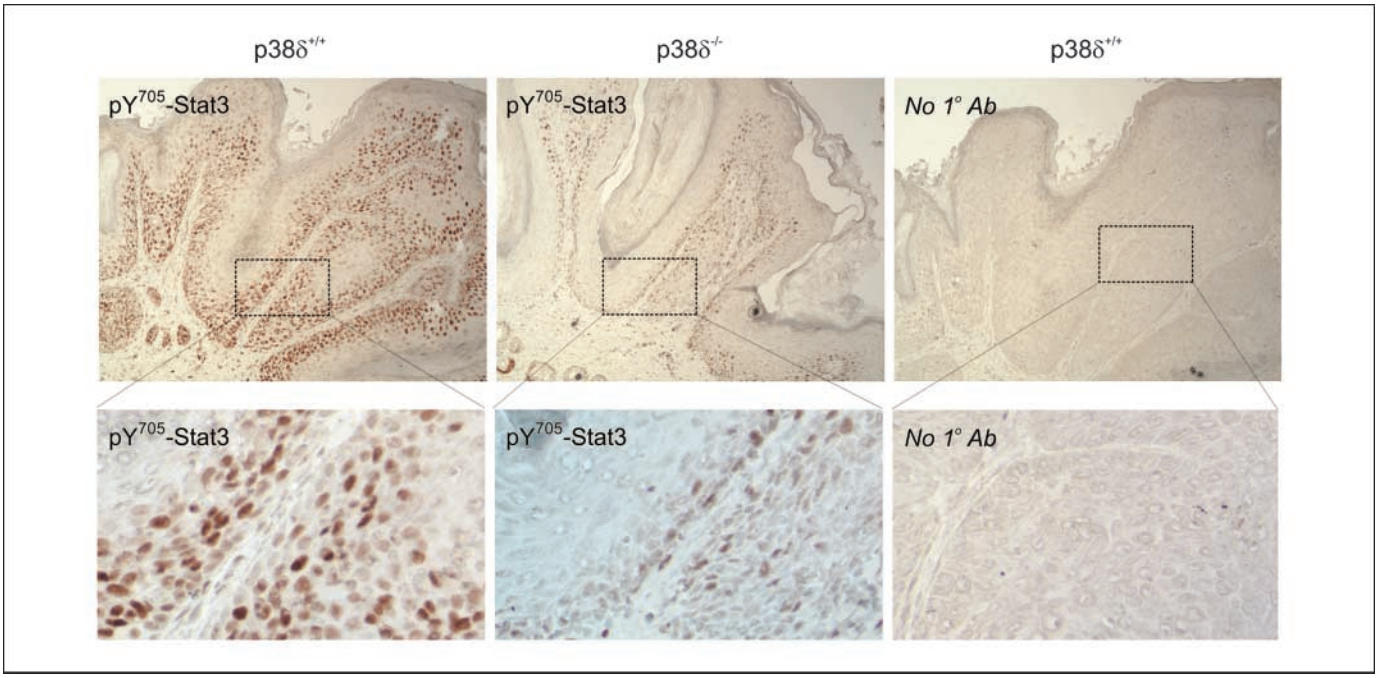

Figure 6. Reduced Stat3 activation in p38δ-null papillomas. Detection of phospho-Tyr^{705}-Stat3 (*pY^{705}-Stat3*) using anti-phospho-Tyr^{705}-Stat3 of representative papillomas of the indicated genotype. Positive staining is shown in keratinocyte nuclei by brown staining. The sections were counterstained with hematoxylin. Three $p38\delta^{+/+}$ and four $p38\delta^{-/-}$ papillomas were examined. Representative tissue fields. Magnification, ×10 (*top*) and ×20 (*bottom*).

response to DMBA/TPA regimen and the size the tumors attained. These data suggest that p38δ is required for both the initiation and the promotion stages of skin carcinogenesis. The timeframe of our present study (Fig. 2*A*) was not designed to ascertain the contribution of p38δ to malignant conversion of papillomas to SCC. Because this process typically requires a latency period of >6 months (29), additional extended studies will be necessary to investigate whether malignant conversion is influenced by p38δ deficiency.

Our data show that p38δ depletion markedly attenuated a major cancer-promoting cascade, ERK1/2-AP1 pathway (Fig. 5). This pathway has been implicated in regulating cyclin D1 expression and promoting cell cycle progression, cell proliferation, and survival (29). Indeed, we found that keratinocyte proliferation was inhibited in TPA-treated p38δ-null epidermis and DMBA/TPA-induced p38δ-null papillomas (Figs. 3 and 4). Notably, both ERK1-null mice and transgenic mice expressing dominant-negative AP1 construct targeted to epidermis are resistant to developing skin tumors (38, 39). JNK2-null mice exhibit a repressed ERK1/2-AP1 signaling and a resistance to chemical skin carcinogenesis, whereas JNK1-null mice show enhanced DMBA/TPA skin carcinogenesis and increased ERK1/2-AP1 signaling (40, 41). Hence, ERK1, JNK2, or p38δ deficiency in mice protects against skin carcinogenesis by reducing proliferation and thus limiting intensive tumor growth.

Our results show that p38δ is required for activation of Stat3 transcription factor in papillomas generated by the DMBA/TPA skin carcinogenesis regimen (Fig. 6). These data imply that Stat3 is a target of p38δ-mediated signal transduction in skin tumor development. Recent studies using skin-specific gain-of-function and loss-of-function transgenic mouse models indicate that Stat3 activation plays an essential role in all three stages of skin carcinogenesis, albeit Stat3 is dispensable for normal skin morphogenesis (reviewed in ref. 31). Tumor promoters, including TPA, activate Stat3 in mouse epidermis and Stat3 is constitutively active in UVB- and DMBA/TPA-induced papillomas (32, 34). During tumor initiation, Stat3 is necessary for keratinocyte stem cell survival following carcinogen-induced DNA damage. During promotion, Stat3 drives cell cycle progression regulating genes involved in survival and proliferation (31, 32). Moreover, Stat3 plays a role in malignant conversion of skin tumors by regulating genes involved in angiogenesis and invasion (31). Because our data argue that p38δ is essential for Stat3 activation during epithelial carcinogenesis, it is tempting to speculate that p38δ exerts its effects on skin tumor development at least in part through regulation of Stat3 pathway. The molecular basis for this regulation and specific aspects of Stat3 signaling modulated by p38δ in epithelial carcinogenesis remain to be determined.

Our results suggest that p38δ is essential for development of skin cancer. In contrast, p38α may negatively regulate cell proliferation and tumorigenesis in some tissues, although the *in vivo* roles of p38α signaling in skin carcinogenesis have not been analyzed. A role for p38α as a potential tumor suppressor has been suggested (42, 43). p38α prevents tumor formation in liver and lungs (44, 45). *p38α* deletion sensitizes p38α-null animals to activated K-Ras-induced lung tumorigenesis (45), whereas deletion of *p38δ* renders mice more resistant to the development of lung tumors in the similar mouse lung cancer model (Supplementary Fig. S3). These findings imply that p38α and p38δ isoforms may play opposing roles in regulating Ras-driven lung carcinogenesis. However, in human SCC, both *p38α* and *p38δ* genes are up-regulated (13). Moreover, both p38α and p38δ promote malignant phenotype of head and neck SCC cell lines (46). Further studies using mice lacking p38 isoforms α, β, and γ will be required to define the potential roles of these kinases in skin tumorigenesis.

Cancer cells depend on stress response pathways for survival and proliferation, as these cells must be able to adapt effectively to numerous stresses, such as high level of reactive oxygen species, DNA damage, aneuploidy/heat shock response, metabolic/energy stress, endoplasmic reticulum, mitochondrial, and membrane stress (47). Recent evidence, as well as our present data, suggests that inactivating certain components of the stress response pathways (e.g., stress-activated kinases JNK2 or p38δ or transcription factor heat shock factor 1, a regulator of stress-inducible gene expression) offer a powerful protection against carcinogenesis (40, 47, 48). Hence, p38δ, among other molecular constituents of the stress response machinery, may function to promote carcinogenesis by enabling effective cellular adaptation to the malignant state through modulation of key cellular responses including survival, growth, proliferation, metabolism, etc. (47, 48). Additional studies are required to determine how p38δ regulate these responses to influence tumor development. Further investigation will also address the issue of a potential contribution of nonepithelial components, that is, systemic or stromal, to altered tumor development in p38δ-null mice.

In conclusion, out findings suggest that p38δ functions as a positive regulator of skin tumorigenesis. We propose that targeting p38δ could be effective at treating cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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