Introduction

The evolution of a cancer cell is accompanied by the dysregulation of growth signaling pathways that are often the result of altered structure or expression of proto-oncogenes or tumor suppressor genes (1). Among the most frequently mutated proto-oncogenes in human cancers are the Ras family of small GTPases. Ras isoforms are mutated in approximately 30% of human cancers, leading to dysregulation of cellular proliferative functions. Of the 3 oncogenic Ras alleles, K-Ras, H-Ras, and N-Ras, K-Ras is the most frequently mutated isoform in human cancers (2–4). Oncogenic K-Ras–induced neoplasms result in part from constitutive activation of Ras/Raf/MEK/ERK (mitogen-activated protein kinase) signaling that leads to uncontrolled proliferation. Key downstream targets in Ras signaling are extracellular signal–regulated kinases (Erk1/2), MAPKs that are phosphorylated by the Ras-activated Raf, and MEK kinases (5). Phosphorylated Erks (pErk1/2) efficiently translocate to the nucleus via a noncanonical nuclear localization signal (6–8). In the nucleus, pErk1/2 phosphorylate and activate a number of transcription factors such as Elk-1 and c-Myc that induce genes important for cell proliferation. The nuclear translocation of pErk1/2, which is rapid and reversible, is critical for the activation of growth factor–induced mitogenic signaling (9–13). The pErks also phosphorylate a number of cytoplasmic substrates, some of which provide a negative feedback loop to the Erk pathway (14). A number of studies in cell lines have shown that spatial regulation of Erks control their function. However, clear in vivo correlates of differential Erk subcellular distribution and tumorigenic outcome have been lacking.

Despite the association of Ras mutations with human cancers such as pancreatic, colon, and lung carcinoma, other tumor types are largely devoid of Ras lesions (2–4). Moreover, genetically programmed Ras activation in mice shows
variable efficacy in inducing rapid neoplasias that are, in part, tissue-dependent (15–18). It remains largely unclear why some tissues are susceptible to oncogenic K-Ras–induced tumors whereas other tissues remain refractory. Some oncogenic K-Ras mouse models revealed induction of tumor suppressors such as p16\(^{\text{Ink4a}}\) and p19\(^{\text{ARF}}\) in early-stage lesions (19, 20), presumably as a key defense mechanism that blocks further progression of the premalignant tumors to malignant tumors. However, the robustness of the tumor suppressor response may depend on many variables, including, but not limited to, the type of tissue, lesion, and nature of oncogenic stimulus (21–24). For example, the Ink4a/Arf (Cdkn2a) locus was found to be repressed in K-Ras\(^{G12D}\)–induced lung tumors in contrast to K-Ras\(^{G12D}\)–induced sarcomas in mice (21). Another component of the anti-oncogenic response may be provided by activation of the DNA damage response (DDR) pathway (25, 26). The DDR is believed to be induced by DNA replication stress that promotes antiproliferative responses such as cell-cycle arrest, senescence, or apoptosis (26). Recent mouse cancer models have also implicated NF-κB signaling as an important component of the Ras-initiated premalignant lesions, a chemotherapeutic target or a promoter of senescence (27–29). Thus, the outcome of oncogene-induced hyperproliferation may depend on the interplay between oncogenic signaling and tumor suppressor pathways.

Understanding the mechanisms by which certain mutational events promote cancer phenotypes in some tissues but not in others remains a major challenge and requires systematic analyses. To investigate the response of different tissues to an oncogenic stimulus, we crossed Rosa26-Cre–LSL-K-Ras\(^{G12D}\) mouse lines to generate biallelic progeny that allowed us to conditionally induce an activated form of K-Ras (K-Ras\(^{G12D}\)) in adult mouse tissues in a global distribution was largely nuclear. Also, in contrast to K-Ras\(^{G12D}\)–susceptible tissues, there was minimal detection of the pErk1/2 nuclear targets phospho-Elk-1 and c-Myc in K-Ras\(^{G12D}\)–refractory tissues. Robust activation of tumor suppressor, DDR pathways, and NF-κB signaling was observed in the K-Ras\(^{G12D}\)–susceptible premalignant tumors. In contrast, tumor suppressor responses were not noted in the Ras refractory tissues. In the mouse model of K-Ras\(^{G12D}\) activation presented here, the restriction of pErk1/2 signaling may represent a critical failsafe mechanism that protects some tissues from developing Ras-induced early preneoplastic lesions. Such a mechanism may explain some of the tissue-specific differences in susceptibility to Ras transformation.

Materials and Methods

Mice

B6.129 background LSL–K-Ras\(^{G12D}\) mice (donated by Dr. Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA) were obtained from the National Cancer Institute Mouse Models of Human Cancers Consortium Repository. These mice were crossed into C57BL/6 Cre–ERT2 mice obtained from Artemis Pharmaceuticals GmbH (30). All mice were bred and maintained in a specific pathogen-free animal facility at Baylor College of Medicine (Houston, TX). All research with mice was conducted in compliance with the Baylor Animal Protocol Committee (Baylor College of Medicine Animal Protocol AN336) and Association for Accreditation and Accreditation of Laboratory Animal Care International (AAALAC) recommendations as published in The Guide for the Care and Use of Laboratory Animals (NRC1996).

Allele genotyping

Genomic DNA was prepared from 5-mm tail tips or 50 mg tissue as described (31). The presence of the Cre–ERT2 gene was verified using the primers Cre-F: 5’-AAG AAC CTG ATG GAC CATG TTC AGG G-3’ and Cre-R: 5’-CCA GAC CAG GCC AGG TAT CTC T-3’, which produce a 790-bp product following standard PCR. Genotyping for the LSL–K-Ras\(^{G12D}\) allele was carried out using the forward primer, 5’ AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC 3’ and reverse primer 5’ CCT TTA CAA GGC CAC GAC GAC TGT AGA 3’ giving a 550-bp PCR product for the LSL allele. PCR analysis to detect Cre-mediated recombination in various tissues was carried out with the primers forward 5’ GTC TTT CCC CAG CAC AGT GC 3’ and reverse 5’ TTC TTT CCT AGG CCA CCA GCT C 3’. These primers amplify the wild-type K-Ras (~620 bp) and LoxP-recombined K-Ras\(^{G12D}\) allele (~650 bp). LSL–K-Ras\(^{G12D}\) is too big (~6 kb) to be amplified under the conditions used for PCR.

Wild-type and mutant K-Ras allele expression

Total RNA was isolated from tissues using the TRIzol reagent (Life Technologies). RNA expression of wild-type K-Ras and mutant K-Ras\(^{G12D}\) was determined as described (32). Briefly, cDNA was generated and PCR amplified using the One-Step Reverse Transcription PCR Kit (QIAGEN), primers 5’ GCCATTTCCGACCCGGACGGA 3’ and 5’ CCTACCCAGGCACATGGACCATG 3’. The mutant K-Ras\(^{G12D}\) allele contains a HindIII restriction site engineered in exon 1, which is absent in the wild-type allele, thus generating 300-bp and 148-bp restriction fragments in the mutant but not in the wild-type 448-bp PCR product.
K-Ras<sup>G12D</sup> allele induction
Tamoxifen (Sigma-Aldrich) was prepared at a concentration of 10 mg/mL in corn oil. CreERT2 allele-containing mice were injected intraperitoneally with 100 µL tamoxifen solution (1 mg tamoxifen per injection) daily for 5 consecutive days at the ages of 3 to 4 months.

Bromodeoxyuridine injection
Bromodeoxyuridine (BrdUrd) stock (10 mg/mL) was made in sterile PBS and stored in aliquots at −20°C. Mice were intraperitoneally injected with 100 µg BrdUrd/g body weight 5 hours before sacrificing.

Tissue lysate preparation and Western blot analysis
Freshly excised tissue samples were harvested, snap-frozen, and stored at −80°C until use. Approximately 50 mg tissue was homogenized in 500 µL NP-40 lysis buffer: 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.75% NP-40 + 1 mini EDTA protease inhibitor tablet/10 mL (Roche Applied Science). After an hour of incubation at 4°C, tissue lysates were centrifuged at 12,000 × g for 10 minutes, and supernatants stored at −80°C until use. For detection of γH2AX, 0.75% NP-40 was replaced with 1% SDS in the lysis buffer and lysates were sonicated 3 times for 10 seconds each with intermittent incubations on ice. Thirty-five to 40 µg tissue lysates were loaded per SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were incubated with antibodies for pErk1/2 (Cat #4370), Erk1/2 (Cat #4695), pSTAT3 (Cat #9145), STAT3 (Cat #9139), and pIKKα/β (Cat #2681) from Cell Signaling Technology; p53 (Novo-Roche; Cat #2524), anti-pErk1/2, anti-pSTAT3, anti-p19Arf, anti-p53 (Novo-Roche, clone CM5), anti-pATM (Rockland, Cat #200-301-500), anti-p16<sup>INK4a</sup> (Santa Cruz Biotechnology, F-12), and anti-c-Myc (Santa Cruz Biotechnology, Cat # sc-764). The secondary biotinylated antibodies were from Santa Cruz Biotechnology. The immunofluorescence was developed using TSA kits on the basis of the manufacturer’s instructions. Ki67 (Vector Labs, Cat #VP-RM04), BrdU (BD Biosciences, Cat # 550803), K5 (Covance, Cat #PRB-160P), K13 (Abcam, Cat #ab16112), and γH2AX proteins were probed using Alexa Fluor–conjugated secondary antibodies (Life Technologies). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted with ProLong Gold Antifade Reagent (Life Technologies).

Histologic analysis and immunohistochemistry
All animals showing overt tumors or other signs of distress were humanely sacrificed and subjected to full necropsy. For histologic analysis, tissues and tumors were fixed in 10% neutral-buffered formalin, paraffin-processed, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Immunohistochemistry was conducted using the VECTASTAIN ABC Kit Rabbit (Vector Labs Cat # SK-4800), Vector M. O.M. Immunodetection Kit (Vector Labs Cat #2202) and BrdU Detection Kit (BD Biosciences, Cat #508083) as per manufacturer’s instructions. Citrate-based Vector Antigen Unmasking Solution (Vector Labs) was used for antigen retrieval. The sections were incubated with following primary antibodies (4°C overnight): anti-pErk1/2, anti-pSTAT3, anti-RelA (Cell Signaling, Cat #4764), anti-RelA (NEL; Millipore, Cat #17-10060), anti-PML (Santa Cruz Biotechnology, Cat #sc-966), anti-pElk-1 (Santa Cruz Biotechnology, Cat #sc-8406), and anti-Ki67 (Vector Labs, Cat #VP-RM04). Vector NovaRED Substrate Kit (Vector Labs, Cat #SK-4800) was used for color development followed by hematoxylin counterstaining.

Immunofluorescence
For immunofluorescence, paraffin sections were blocked at room temperature for 1 hour in a TSA kit blocking buffer (PerkinElmer) and incubated overnight at 4°C or room temperature with the following antibodies: anti-pErk1/2, anti-pSTAT3, anti-p19Arf, anti-p53 (Novo- rocastra, clone CM5), anti-pATM (Rockland, Cat #200-301-500), anti-p16<sup>INK4a</sup> (Santa Cruz Biotechnology, F-12), and anti-c-Myc (Santa Cruz Biotechnology, Cat # sc-764). The secondary biotinylated antibodies were from Santa Cruz Biotechnology. The immunofluorescence was developed using TSA kits on the basis of the manufacturer’s instructions. Ki67 (Vector Labs, Cat #VP-RM04), BrdU (BD Biosciences, Cat # 550803), K5 (Covance, Cat #PRB-160P), K13 (Abcam, Cat #ab16112), and γH2AX proteins were probed using Alexa Fluor–conjugated secondary antibodies (Life Technologies). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted with ProLong Gold Antifade Reagent (Life Technologies).

Microscopy
Bright field images were captured using an inverted microscope (BX50, Olympus) equipped with Olympus DP11 camera. Fluorescent images were captured at ×40 with a Zeiss Axiosplan II upright microscope and images processed using MetaVue software version 6.3/7 at the Integrated Microscopy Core facility at Baylor College of Medicine.

Quantitative real-time PCR
Total RNA extracted from mouse tissues was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) as per manufacturer’s instructions. Real-time PCR (RT-PCR) was carried out in Corbett Rotor-Gene, RG-3000 thermal cycler (Qiagen) using iQ SYBR Green Supermix (Bio-Rad Laboratories). The following primers designed using Primer-BLAST software were used: IL-6 forward 5′-CGG ATG GGA CAG CAT CTG GCA-3′; IL-6 reverse 5′-GGC ATG GGA CAG CAT CTG GCA-3′; MCP-1 forward 5′-GGG CCG AGG ACA CT-3′; MCP-1 reverse 5′-GGG CCG AGG ACA CT-3′; GAGCG CGT GGT-3′. Each sample was tested in triplicates and the RT-PCR carried out 2 to 3 times for each sample and primer pair. Differences between tumors
and control tissues were normalized to expression of β-actin using the ΔΔCt method.

**Results**

**Mutant Ras is activated in most tissues of Cre-ERT2;K-RasG12D mice**

To achieve global expression of oncogenic K-Ras, we generated Cre-ERT2;K-RasG12D mice by crossing the knock-in Rosa26-Cre-ERT2 mice with mice carrying a knock-in Lox-Stop-Lox(LSL)-K-RasG12D allele containing an oncogenic point mutation in exon 1 (G12D; refs. 30, 33). The Cre-ERT2 allele, which encodes for a tamoxifen-responsive–modified Cre recombinase, is expressed by the ubiquitous Rosa26 promoter, whereas the LSL-K-RasG12D allele is downstream of the endogenous K-Ras promoter. Administration of tamoxifen induces nuclear translocation of Cre-ERT2 resulting in deletion of the LSL cassette in biallelic Cre-ERT2;LSL-K-RasG12D mice (Fig. 1A). All tissues examined other than the brain showed extensive deletion of the LSL cassette following tamoxifen injection in Cre-ERT2;K-RasG12D mice as assessed by PCR (Fig. 1B).

To test the expression of the K-RasG12D allele in various tissues, we used reverse transcription PCR followed by HindIII digestion specific for the K-RasG12D allele (32). K-RasG12D mRNA was detected in most tissues, including brain tissue where we did not detect the recombined K-RasG12D allele by DNA PCR (Fig. 1C).

**K-RasG12D expression results in early oral, gastric, and lung neoplasms**

To investigate the differential effects of mutant K-Ras expression on signaling and tumorigenic progression in various tissues, mice were injected with 1 mg/mL tamoxifen for 5 consecutive days and monitored for morbidity or neoplastic lesions. More than 90% of Cre-ERT2;K-RasG12D mice developed oral papillomas at the mucocutaneous junction within 4 weeks after tamoxifen treatment (Fig. 2A). By 5 to 6 weeks, Cre-ERT2;K-RasG12D mice exhibited a mean 30% reduction in body mass compared with monoallelic mice. Mice were humanely sacrificed at the moribund stage (5–6 weeks after tamoxifen) and subjected to necropsy.
K-Ras\textsuperscript{G12D} expression activates Erk1/2 signaling in both K-Ras\textsuperscript{G12D}-susceptible and refractory tissues

Expression of the K-Ras\textsuperscript{G12D} allele resulted in activation of Ras/Raf/MAPK signaling in several tissues. Thus, elevated levels of Ras signaling markers pErk1/2 and pSTAT3 were detected in K-Ras\textsuperscript{G12D}-susceptible oral, gastric, and lung tumors. The nuclear translocation of pErk1/2 culminate in unlimited proliferation via phosphorylation and activation of proteins do not explain the tissue-specific differences in susceptibility to tumor formation. Collectively, these data show robust activation of Erk1/2 signaling in both K-Ras\textsuperscript{G12D}-susceptible and -refractory tissues from tamoxifen-treated Cre-ERT2;K-Ras\textsuperscript{G12D} mice.

The oncogenic K-Ras\textsuperscript{G12D}-refractory tissues retain upregulated pErk1/2 in the cytoplasm

Because there were tissue-dependent differences in responsiveness to oncogenic K-Ras in the 5 to 6 weeks after tamoxifen injection, we investigated Ras-activated MAPK signaling in the K-Ras–refractory tissues such as pancreas, liver, and small intestine. We reasoned that these tissues may exhibit a blunted Ras signaling to prevent neoplastic progression. The Erk1/2 are key signal transducing kinases that are phosphorylated in response to activated Ras signaling. The nuclear translocation of pErk1/2 culminate in uncontrolled proliferation via phosphorylation and activation of several downstream targets including transcription factors such as Elk-1 and c-Myc (9–13). Interestingly, even though pErk1/2 staining was greatly elevated in pancreas, liver, and small intestine in Cre-ERT2;K-Ras\textsuperscript{G12D} mice, it was largely localized to the cytoplasm in these tissues (Fig. 4A). In contrast, K-Ras\textsuperscript{G12D}-induced oral, gastric, and lung neoplasms displayed intense nuclear distribution of pErk1/2 (Fig. 4B). Next, we looked at the expression of pElk-1, which

Histopathologic analysis indicated the oral tumors to be benign in nature as reported previously (refs. 17, 32, 34; Fig. 2B). In addition, stomachs from Cre-ERT2;K-Ras\textsuperscript{G12D} mice were enlarged and exhibited raised irregular masses on the inner stomach surface (Fig. 2C). Histopathologic analysis revealed the transformation of the nonglandular part of the stomach into gastric papillomas in all K-Ras\textsuperscript{G12D}-expressing mice (Fig. 2D). Gross examination of lungs from Cre-ERT2;K-Ras\textsuperscript{G12D} mice revealed multifocal lesions on the lung surface (Fig. 2E). Histopathologic analysis showed the development of epithelial hyperplasia and benign adenoma in K-Ras\textsuperscript{G12D} lungs (Fig. 2F). Finally, complete blood counts showed hyperplasia in myeloid and lymphoid compartments indicative of development of myeloproliferative disease (MPD) and leukemia in K-Ras\textsuperscript{G12D} expressing mice (Supplementary Fig. S1A). The spleens were markedly enlarged in Cre-ERT2;K-Ras\textsuperscript{G12D} mice (Supplementary Fig. S1B) by 5 to 6 weeks after injection. Lymphoid infiltration was observed in livers from Cre-ERT2;K-Ras\textsuperscript{G12D} mice with an otherwise normal histology (Fig. 2G). Pancreas and small intestines from K-Ras\textsuperscript{G12D}-expressing mice also displayed normal histology (Fig. 2H and I). Thus, global expression of K-Ras\textsuperscript{G12D} resulted in oral papilloma, lung adenoma, gastric papilloma, and hematopoietic hyperplasia, as described previously (15, 18, 32, 35). The global expression of K-Ras\textsuperscript{G12D} did not result in tumor formation in intestines, pancreas, liver (Fig. 2G–I), or mammary gland (data not shown), as reported previously in some tissue-specific expression studies (36–38). The rapid appearance of oral, lung, gastric tumor, and hematopoietic hyperplasia, resulting in a moribund state within 5 to 6 weeks of oncogenic K-Ras induction, may have precluded the observation of more slowly arising neoplasms.
is a nuclear transcription factor phosphorylated by pErk1/2. In contrast to K-RasG12D–induced oral, gastric, and lung neoplasms that expressed pElk-1 in the nuclei, there was negligible detection of pElk-1 in the K-RasG12D–expressing liver, pancreas, and small intestine (Fig. 5A). In addition, c-Myc protein, which is stabilized following phosphorylation by activated Ras/Erk signaling, was significantly elevated in the K-RasG12D–susceptible oral and gastric tumors but not in K-RasG12D–refractory tissues (Fig. 5B). The percentages of Myc-positive cells in oral and gastric papilloma were 45.4% and 33%, respectively, whereas no nuclear staining for Myc was observed in pancreas, liver, and small intestine. To test whether the nuclear accumulation of pErk1/2 in K-RasG12D–susceptible tissues is an early event leading to neoplastic transformation, we harvested oral mucosa and stomach at an earlier time point of 1 week after tamoxifen injection. At this time point, there were no obvious neoplastic lesions in these tissues. The immunostaining revealed largely nuclear distribution of pErk1/2 in oral and gastric mucosa from Cre-ERT2;K-RasG12D mice. As expected, pancreas harvested at 1 week after tamoxifen injection showed predominantly cytoplasmic distribution of pErk1/2 (Supplementary Fig. S3). Collectively, these data show differential subcellular distribution of pErk1/2 in K-RasG12D–susceptible and -refractory tissues. The largely cytoplasmic distribution of activated Erk kinases correlates with restricted pErk1/2 signaling in tissues that are refractory to K-RasG12D–induced neoplastic progression in the 6-week time frame following K-RasG12D expression.

K-RasG12D expression induces tumor suppressor proteins in K-RasG12D–susceptible tissues

The Ki67 staining for proliferating cells indicated hyperproliferation in the K-RasG12D–induced oral (Fig. 6A), gastric, and lung tumors (not shown). Despite hyperproliferation, the differentiation pattern in the oral tumors was similar to normal oral mucosa as marked by distribution of the basal and differentiated cell markers, keratin 5 (K5) and keratin 13 (K13; Supplementary Fig. S4). Previous studies indicate that the oncogenic K-Ras–induced tumor
suppressor response can vary depending on the tissue type and stage of tumor (21–23). The tumor suppressor response has not been clearly defined in K-RasG12D–induced oral and gastric papillomas. We hypothesized that the well-differentiated and benign nature of K-RasG12D–induced tumors was the result of a strong global tumor suppressor response. Indeed, Western blot analysis of oral tumor lysates from Cre-ERT2;K-RasG12D mice showed an upregulation of tumor suppressors p16ink4a, p19Arf, and p53 (Fig. 6B). Similarly, gastric papillomas showed an upregulation of tumor suppressors p16ink4a, p19Arf, and p53 (data not shown). The relative expression of p16ink4a and p19Arf in lungs was much lower than in oral and gastric papillomas (data not shown). This is in accordance with the recent report where the Cdkn2a (Ink4a/Arf) locus was shown to be downregulated in lung tumors (21).

We further assessed the relative distribution of proliferation markers and tumor suppressors using the oral papilloma model. Promyelocytic leukemia (PML), a marker of senescence induced by oncogenic Ras (39, 40), was upregulated in the suprabasal cell layers of oral papillomas (Supplementary Fig. S5B). Immunofluorescence staining for tumor suppressors indicated more widespread induction of p19Arf, p53, and p16ink4a across multiple layers of oral papilloma in comparison with limited Ki67/BrdUrd expression in the basal and lower suprabasal cell layers of oral tumors (Fig. 6C–E). In contrast to oral papillomas, we did not detect a significant induction of tumor suppressors p16ink4a and p53 in the K-RasG12D–refractory pancreas, liver, and small intestines (Fig. 6F and G; Supplementary Fig. S5C and S5D). These results indicate a strong and widespread induction of tumor suppressors in K-RasG12D–refractory tissues that do not form neoplasms within 6 weeks of tamoxifen treatment do not elicit a potent tumor suppressor response.

DNA damage, senescence, and NF-κB responses are elicited in K-RasG12D–induced neoplasms

Some human premalignant tumors elicit a DDR following oncogenic stress (25). However, in mouse tumor models, the DDR is not invariably observed in oncogene-activated tissues (17, 41). We examined whether the DDR pathway is activated in K-Ras–induced oral, gastric, and lung tumors within the time frame of 5 to 6 weeks after tamoxifen injection. Histone variant γH2AX, a key DDR marker associated with DNA strand breaks, was found to be upregulated in oral, gastric, and lung benign lesions (Fig. 7A). γH2AX was markedly upregulated in oral tumors across all cell layers (Fig. 7B and C). Phosphorylated pATM, another marker of an activated DDR, along with proliferation marker Ki67 were elevated in oral tumors across basal and suprabasal cell layers (Supplementary Fig. S6). Collectively, our data show that K-RasG12D–induced neoplastic lesions in mice elicit a widespread DDR.

Mutant Ras can induce cell senescence accompanied by a senescence-associated secretory phenotype (SASP; ref. 42). We tested for the SASP in the K-Ras–induced tumors. Quantitative PCR on RNA from K-RasG12D–induced oral and gastric tumors was used to assay interleukin (IL)-6, MIP-2 (functional homolog of human IL-8 in mice), granulocyte macrophage colony-stimulating factor (GM-CSF), MCP-1, and IL-1t expression. We also examined IL-6R and CXCR2, receptors for IL-6 and MIP-2, respectively. RT-PCR analysis indicated elevated mRNA levels of several proteins associated with the SASP response for oral and gastric papillomas (Fig. 7D; Supplementary Fig. S7A). On the basis of the induction of several inflammatory cytokines, we assayed for NF-κB signaling in K-RasG12D–induced tumors. Phosphorylation of inhibitor of NF-κB kinases (IKKβ/ε) relieves cytoplasmic sequestration and induces nuclear translocation of NF-κB proteins. Western blot analysis showed upregulation of pIKKβ (upper band) in oral, gastric (Fig. 7E), and lung tumors (not shown), indicating activation of the canonical NF-κB pathway. NF-κB p65 (RelA), the predominant form of NF-κB in cells, was elevated in tumors (Fig. 7E and F). Further examination using an antibody that recognizes the nuclear localization signal (NLS) of NF-κB p65 showed nuclear distribution of p65 in oral papilloma across several...
cell layers (Supplementary Fig. S7B). These data show the activation of the SASP response in K-Ras\(^{G12D}\)-induced neoplastic lesions in vivo. There is also significant activation of NF-κB signaling in K-Ras\(^{G12D}\)-induced premalignant tumors that does not require a concomitant inactivation of p53 as reported previously (27).

**Discussion**

Mouse models of oncogenic K-Ras have revealed the tumorigenic potential of K-Ras in multiple organs. In one study, induction of K-Ras\(^{G12V}\) driven by CMV-Cre and RERTn-Cre-ERT2 resulted primarily in lung adenoma, whereas in another study, expression of K-Ras\(^{G12D}\) driven by Rosa26-Cre-ERT2 resulted in multiple tumor types (oral, lung, and myelodysplasia; refs. 16, 17). Our data are more consistent with the latter study, as we observed premalignant neoplasms in the hematopoietic tissues, oral mucosa, stomach, and lungs (Fig. 2A–F; Supplementary Fig. S1). In the 5- to 6-week time frame after K-Ras activation, we did not find any obvious tumor formation in intestine, liver, pancreas (Fig. 2G–I), and mammary gland (data not shown) as has been reported in tissue-specific expression studies for some of these organs (36–38). It is possible that if these mice were to live longer, they may have developed tumors in the refractory tissues. Recently, K-Ras\(^{G12D}\) driven by epithelial promoter CK19 resulted in tumors in the oral cavity, stomach, lungs, and pancreas (18). This study did not find any hyperproliferation in intestines, liver, and kidney where the CK19 promoter is active. Such results, coupled with our results, show tissue specificity in cellular responses to oncogenic Ras activation. Some tissues undergo immediate hyperproliferation, whereas others are unaffected or affected in a more delayed manner. The mechanisms for these differential effects remain unclear (16, 18) and could be due to several undetermined factors such as tissue-specific differences in Ras signaling levels, differential activation of downstream Ras targets, or tissue-specific differences in Ras anti-oncogenic responses.

Our study, designed to explore the mechanisms by which tissues respond differently to an oncogenic stimulus, reveals that a tissue-specific difference in the subcellular distribution of downstream Ras targets is one mechanism that might determine the tumorigenic response to mutant K-Ras. Thus, K-Ras\(^{G12D}\)-induced oral, stomach, and lung neoplasms show predominantly nuclear pErk1/2 distribution indicative of activated mitogenic signaling (Fig. 4B). In contrast, pancreas, liver, and small intestine that do not develop early neoplasms based studies have shown that growth factor-induced nuclear translocation of pErk1/2 is essential for mitogenic signal transduction and cell-cycle entry (9–11). But the effects of subcellular pErk1/2 distribution on tissue-specific cancer induction have not been reported earlier. It remains largely unclear why some tissues succumb to certain oncogenic mutations whereas others remain refractory. We propose that inadequate nuclear translocation of pErk1/2 results in limiting the mitogenic stimulus in oncogenic K-Ras–refractory tissues (Supplementary Fig. S8). The failure
to observe phosphorylated Elk-1 and c-Myc (downstream nuclear targets of pErk1/2 and transcriptional inducers of mitogenic genes) in the nuclei of K-Ras–refractory tissues provides functional confirmation of the block in Ras/MAPK signaling in these tissues (Fig. 5). Several proteins such as PEA-15, Sef, and MKP-3 have been identified that restrict the nuclear localization and activity of Erks (14, 43–45). One or more of these potential anchoring proteins may limit the nuclear accumulation of pErk1/2 in the oncogenic K-Ras–refractory tissues.

The oncogene-induced tumor suppressor response is another key mechanism that restricts tumor progression. The tumor suppressor responses elicited in oncogenic K-Ras–driven oral and gastric tumors have not been investigated before (18, 32, 34). Our data show the induction of tumor suppressors such as p53, p19(ARF)m, p21, and p16(INK4a) in oncogenic K-RasG12D–driven oral and gastric tumors (Fig. 6B; Supplementary Fig. S5A). Further examination of the spatial expression of tumor suppressors indicated their expression across all cell layers in the oral tumors (Fig. 6C–E). Similarly, the DDR was elicited across oral papillomas (Fig. 7B; Supplementary Fig. S6). The widespread induction of several tumor suppressors and DDR proteins in response to oncogenic K-RasG12D likely explains the lack of malignant tumor formation in oral epithelium in our study and elsewhere (32, 34). Interestingly, the K-RasG12D–refractory tissues did not elicit a potent tumor suppressor response (Fig. 6F and G; Supplementary Fig. S5C and S5D). Thus, uncontrolled replication following an oncogenic mutation appears to be an essential stimulus to induce a potent tumor suppressor response in tissues. Collectively, our in vivo data reveal a novel mechanism by which tissues may differentially respond to an oncogenic stimulus such as mutant K-Ras. The pErks are largely retained in the cytoplasm in tissues that remain refractory to mutant K-Ras transformation. Other tissues that develop early neoplasms have high nuclear pErk1/2 levels and full activation of nuclear targets of Ras/Erk signaling (Supplementary Fig. S8). The spatial regulation of Erks may be one of the mechanisms that determine the tissue-specific response to an oncogenic mutation. We speculate that, apart from Mek/Erk inhibitors, spatial control of Erks could serve as another node for therapeutic intervention in tissues with deregulated Ras/MAPK signaling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
**Authors' Contributions**

Conception and design: N. Parikh, L.A. Donehower
Development of methodology: N. Parikh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Parikh, R.L. Shuck, T.-A. Nguyen, A. Herron
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Parikh, T.-A. Nguyen, A. Herron, L.A. Donehower
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Parikh, T.-A. Nguyen
Study supervision: A. Herron, L.A. Donehower

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