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Mouse tissues that undergo neoplastic progression after K-Ras activation are distinguished by nuclear translocation of phospho-ERK1/2 and robust tumor suppressor responses

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Abstract

Mutation of K-Ras is a frequent oncogenic event in human cancers, particularly cancers of lungs, pancreas, and colon. It remains unclear why some tissues are more susceptible to Ras-induced transformation than others. Here, we globally activated a mutant oncogenic K-Ras allele (K-Ras^{G12D}) in mice and examined the tissue-specific effects of this activation on cancer pathobiology, Ras signaling, tumor suppressor, DNA damage, and inflammatory responses. Within 5–6 weeks of oncogenic Ras activation, mice develop oral and gastric papillomas, lung adenomas and hematopoietic hyperproliferation and turn moribund. The oral, gastric and lung pre-malignant lesions display activated Erk1/2 and NF-κB signaling as well as activated tumor suppressor and DNA damage responses. Other organs such as pancreas, liver and small intestine do not exhibit neoplastic progression within six weeks following K-ras^{G12D} activation and do not show a potent tumor suppressor response. Even though robust Erk1/2 signaling is activated in all the tissues examined, the pErk1/2 distribution remains largely cytoplasmic in K-Ras^{G12D} refractory tissues (pancreas, liver and intestines) as opposed to a predominantly nuclear localization in K-Ras^{G12D} induced neoplasms of lung, oral, and gastric mucosa. The downstream targets of Ras signaling, pElk-1 and c-Myc, are elevated in K-Ras^{G12D} induced neoplastic lesions but not in K-Ras^{G12D} refractory tissues. We propose that oncogenic K-Ras refractory tissues delay oncogenic progression by spatially limiting the efficacy of Ras/Raf/Erk1/2 signaling, whereas K-Ras responsive tissues exhibit activated Ras/Raf/Erk1/2 signaling, rapidly form pre-malignant tumors, and activate potent anti-tumor responses that effectively prevent further malignant progression.

Keywords

K-Ras; ERK1/2; Elk-1; nuclear translocation; p53

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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 three 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-MAPK (mitogen-activated protein kinase) signaling that leads to uncontrolled proliferation. Key downstream targets in Ras signaling are extracellular signal regulated protein kinases (Erk1/2), MAP kinases that are phosphorylated by the Ras-activated Raf and MEK kinases (5). Phosphorylated Erks (pErk1/2) efficiently translocate to the nucleus via a non-canonical 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 while other tissues remain refractory. Some oncogenic K-Ras mouse models revealed induction of tumor suppressors such as p16^{Ink4a} and p19^{Arf} in early stage lesions (19, 20), presumably as a key defense mechanism that blocks further progression of the pre-malignant 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 pathway (25, 26). The DNA damage response (DDR) is believed to be induced by DNA replication stress that promotes anti-proliferative 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 pre-malignant 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. In order to investigate the response of different tissues to an oncogenic stimulus, we crossed Rosa26-CreERT2 and LSL-K-Ras^{G12D} mouse lines to generate bi-allelic progeny that allowed us to conditionally induce an activated form of K-Ras (K-Ras^{G12D}) in adult mouse tissues in a global manner. We examined the pathobiology of tumor formation in multiple tissues and employed molecular approaches to examine the tissue-specific differences in the activated signaling pathways. Activation of mutant K-Ras led to potent Ras signaling in most mouse tissues within one week. Mice developed oral,

gastric, and lung neoplastic lesions along with myeloproliferative disease and leukemia within four weeks and were moribund by six weeks. However, despite exhibiting activated Ras signaling, tissues such as liver, intestine, and pancreas failed to display significant cancer-related pathobiology in the six week time period following K-Ras^{G12D} activation. Interestingly, these K-Ras-refractory tissues upregulated the Ras downstream target phospho-Erk1/2 but retained it primarily in the cytoplasm, in contrast to oncogenic K-Ras induced neoplasms where pErk1/2 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, DNA damage response pathways, and NF-κB signaling was observed in the K-Ras^{G12D} susceptible pre-malignant 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 CreERT2 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. All research with mice was conducted in compliance with the Baylor Animal Protocol Committee (Baylor College of Medicine Animal Protocol AN336) and 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 CreERT2 gene was verified using the primers Cre-F: 5'-AAG AAC CTG ATG GAC ATG 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 performed using the Forward primer, 5' AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC 3' and Reverse primer 5' CCT TTA CAA GCG CAC GCA 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 performed with the primers Forward 5' GTC TTT CCC CAG CAC AGT GC 3' and Reverse 5' CTC TTG CCT ACG CCA CCA GCT C 3'. These primers amplify the wildtype 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.

Wildtype and mutant K-Ras allele expression

Total RNA was isolated from tissues using the TRIzol reagent (Life Technologies, Carlsbad, CA). RNA expression of wildtype 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, Valencia, CA), primers 5' GCCATTTTCGGACCCGGAGCGA 3' and 5' CCTACCAGGACCATAGGCACATC 3'. The mutant K-Ras^{G12D} allele contains a *HindIII* restriction site engineered in exon 1, which

is absent in the wildtype 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^{G12D} allele induction

Tamoxifen (Sigma Aldrich, St. Louis, MO) was prepared at a concentration of 10 mg/ml in corn oil. Cre-ERT2 allele-containing mice were injected intraperitoneally with 100 μ l tamoxifen solution (1 mg tamoxifen per injection) daily for five consecutive days at the ages of 3–4 months.

BrdU injection

BrdU stock (10 mg/ml) was made in sterile PBS and stored in aliquots at -20°C . Mice were intraperitoneally injected with 100 μ g BrdU/gm body weight 5 hours prior to 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 μ l NP40 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, Indianapolis, IN). After an hour of incubation at 4°C , tissue lysates were centrifuged at 12,000 g for 10 min, and supernatants stored at -80°C until use. For detection of γ H2AX, 0.75% NP-40 was replaced with 1% SDS in the lysis buffer and lysates were sonicated three times for 10 seconds each with intermittent incubations on ice. 35–40 μ g tissue lysates were loaded per SDS polyacrylamide gel and transferred to PVDF membrane. Blots were incubated with antibodies for pErk1/2 (Cat #4370), Erk1/2 (Cat #4695), pSTAT3 (Cat # 9145), STAT3 (Cat #9139) and pIKK α/β (Cat #2681) from Cell Signaling Technology (Danvers, MA); p53 (R-19), p21 (M-19 and C-19), p16^{Ink4a} (M-156), IKK α/β (Cat # sc-7607) and RelA (H-286) from Santa Cruz Biotechnology (Santa Cruz, CA); p19^{Arf} (Cat # ab26696) from Abcam (Cambridge, MA); γ H2AX (Cat# 05–636), H2AX (Cat. # 07–627) and GAPDH (Cat #MAB374) from Millipore (Billerica, MA). Signal was detected using SuperSignal West Pico and Femto Chemiluminescent substrates (Pierce Thermo Fisher scientific, Rockford, IL)

Histologic Analysis and Immunohistochemistry

All animals showing overt tumors or other signs of distress were humanely sacrificed and subjected to full necropsy. For histological analysis, tissues and tumors were fixed in 10% neutral buffered formalin, paraffin processed, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed using the Vectastain ABC Kit Rabbit (Vector Labs Cat #6101), Vector M.O.M. Immunodetection Kit (Vector Labs Cat #2202) and BrdU Detection Kit (BD Biosciences, Cat #550803) as per manufacturer instructions. Citrate-based Vector Antigen Unmasking Solution (Vector Labs, Burlingame, CA) 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 (NLS) (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 h in a TSA kit blocking buffer (PerkinElmer, Waltham, MA) and incubated overnight at 4°C or room temperature with the following antibodies: anti-pErk1/2, anti-pSTAT3, anti-p19^{Arf}, anti-p53 (Novocastra, clone CM5), anti-pATM (Rockland, Cat #200–301–500), anti-

p16^{Ink4a} (Santa Cruz Biotechnology, F-12), 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 based on the manufacturer's instructions. Ki67 (Vector Labs, Cat #VP-RM04), BrdU (BD Biosciences, Cat # 555627), K5 (Covance, Cat #PRB-160P), K13 (abcam, Cat #ab16112) and γ H2AX proteins were probed using Alexa Fluor conjugated secondary antibodies (Life Technologies). Sections were counterstained with DAPI and mounted with ProLong Gold antifade reagent (Life Technologies).

Microscopy

Bright field images were captured using an inverted microscope (BX50, Olympus, Center Valley, PA) equipped with Olympus DP11 camera. Fluorescent images were captured at 40X with a Zeiss Axioplan II upright microscope and images processed using MetaVue software version 6.3r7 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 instructions. Real-time PCR was performed in Corbett Rotor-Gene, RG-3000 thermal cycler (Qiagen) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The following primers designed using Primer-BLAST software were used: IL-6 Fwd 5' CCA TCC AGT TGC CTT CTT G 3' and IL-6 Rev 5' AAG TGC ATC ATC GTT GTT CAT AC 3'; IL-6R Fwd 5' GCA CGT GGT CCA GGT CCG TG 3' and IL-6 Rev 5' TGC ACT GGG GCG AGG ACA CT 3'; MIP-1 Fwd 5' GCAGCAGCGAGTACCAGTCCC 3' and MIP-1 Rev 5' AGCAGGCAGTCGGGGTGTCA 3'; CXCR2 Fwd 5' CAG CCC TGA CAG CTC CCA AGC 3' and CXCR2 Rev 5' GGC ATG GGA CAG CAT CTG GCA 3'; GM-CSF Fwd 5' TCGAGCAGGGTCTACGGGGC 3' and GM-CSF Rev 5' GTCCGTTTCCGGAGTTGGGGG 3'; MCP-1 Fwd 5' CACAGTTGCCGGCTGGAGCA 3' and MCP-1 Rev 5' GGGGTCAGCACAGACCTCTCTC 3'; IL-1 α Fwd 5' ACCAGCCCGTGTGCTGAAG and IL-1 α Rev 5' TAGAGGGCAGTCCCCGTGCC 3'. Each sample was tested in replicates and the real-time PCR performed 2–3 times for each sample and primer pair. Differences between tumors and control tissues were normalized to expression of β -Actin using the $\Delta\Delta$ Ct method.

Results

Mutant Ras is activated in most tissues of Cre-ERT2; K-Ras^{G12D} mice

In order to achieve global expression of oncogenic K-Ras, we generated Cre-ERT2; K-Ras^{G12D} mice by crossing the knock-in Rosa26-Cre-ERT2 mice with mice carrying a knock-in Lox-Stop-Lox(LSL)-K-Ras^{G12D} allele containing an oncogenic point mutation in exon 1 (G12D) (30, 33). The Cre-ERT2 allele, which encodes for a tamoxifen-responsive modified Cre recombinase, is expressed by the ubiquitous Rosa26 promoter while the LSL-K-Ras^{G12D} 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 bi-allelic Cre-ERT2; LSL-K-Ras^{G12D} mice (Fig. 1A). All tissues examined other than the brain showed extensive deletion of the LSL cassette following tamoxifen injection in Cre-ERT2; K-Ras^{G12D} mice as assessed by PCR (Fig. 1B). In order to test the expression of the K-Ras^{G12D} allele in various tissues, we employed reverse transcription-PCR followed by *Hind*III digestion specific for the Ras^{G12D} allele (32). K-Ras^{G12D} mRNA was detected in most tissues, including brain tissue where we did not detect the recombined K-Ras^{G12D} allele by DNA PCR (Fig. 1C).

K-Ras^{G12D} expression results in early oral, gastric, and lung neoplasms

In order 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 five consecutive days and monitored for morbidity or neoplastic lesions. Over 90% of K-Ras^{G12D} mice developed oral papillomas at the mucocutaneous junction within 4 weeks post tamoxifen treatment (Fig. 2A). By 5–6 weeks, Cre-ERT2; K-Ras^{G12D} mice exhibited a mean 30% reduction in body mass compared to mono-allelic mice. Mice were humanely sacrificed at the moribund stage (5–6 weeks post tamoxifen) and subjected to necropsy.

Histopathological analysis indicated the oral tumors to be benign in nature as reported previously (17, 32, 34) (Fig. 2B). In addition, stomachs from Cre-ERT2; K-Ras^{G12D} mice were enlarged and exhibited raised irregular masses on the inner stomach surface (Fig. 2C). Histopathological analysis revealed the transformation of the non-glandular part of the stomach into gastric papillomas in all K-Ras^{G12D} expressing mice (Fig. 2D). Gross examination of lungs from Cre-ERT2; K-Ras^{G12D} mice revealed multifocal lesions on the lung surface (Fig. 2E). Histopathological analysis showed the development of epithelial hyperplasia and benign adenoma in K-Ras^{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^{G12D} expressing mice (Supplementary Fig. S1A). The spleens were markedly enlarged in Cre-ERT2; K-Ras^{G12D} mice (Supplementary Fig. S1B) by five-six weeks after injection. Lymphoid infiltration was observed in livers from Cre-ERT2; K-Ras^{G12D} mice with an otherwise normal histology (Fig. 2G). Pancreas and small intestines from K-Ras^{G12D} expressing mice also displayed normal histology (Fig. 2H and I). Thus, global expression of K-Ras^{G12D} resulted in oral papilloma, lung adenoma, gastric papilloma and hematopoietic hyperproliferation, as described previously (15, 18, 32, 35). The global expression of K-Ras^{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 hyperproliferation resulting in a moribund state within 5–6 weeks of oncogenic K-Ras induction may have precluded the observation of more slowly arising neoplasms.

K-Ras^{G12D} expression activates ERK1/2 signaling in both K-Ras^{G12D} susceptible and refractory tissues

Expression of the K-Ras^{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^{G12D} susceptible oral, gastric and lung tumor (Fig. 3A–C, Supplementary Fig. S2). Likewise K-Ras^{G12D} refractory tissues liver, pancreas and small intestine from bi-allelic mice showed upregulation of pErk1/2 in comparison to littermate controls (Fig. 3D and E). Thus the levels of activated pErk1/2 and pSTAT3 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^{G12D} susceptible and refractory tissues from tamoxifen-treated Cre-ERT2; K-Ras^{G12D} mice.

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

Since there were tissue-dependent differences in responsiveness to oncogenic K-Ras in the 5–6 weeks after tamoxifen injection, we investigated Ras-activated MAPK signaling in the K-Ras refractory tissues pancreas, liver and small intestine. We reasoned that these tissues may exhibit a blunted Ras signaling to prevent neoplastic progression. The extracellular signal regulated protein kinases (Erk1/2) are key signal transducing kinases that are phosphorylated in response to activated Ras signaling. The nuclear translocation of pErk1/2 kinases 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^{G12D} mice, it was largely localized to the cytoplasm in these tissues (Fig. 4A). In contrast, K-Ras^{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 is a nuclear transcription factor phosphorylated by pErk1/2. In contrast to K-Ras^{G12D} induced oral, gastric and lung neoplasms that expressed pElk-1 in the nuclei, there was negligible detection of pElk-1 in the K-Ras^{G12D} 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-Ras^{G12D} susceptible oral and gastric tumors but not in K-Ras^{G12D} 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 if the nuclear accumulation of pErk1/2 in K-Ras^{G12D} susceptible tissues is an early event leading to neoplastic transformation, we harvested oral mucosa and stomach at an earlier time point of 1 week post 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-Ras^{G12D} mice. As expected, pancreas harvested at 1 week post tamoxifen injection showed predominantly cytoplasmic distribution of pErk1/2 (Supplementary Fig. S3). Collectively, these data show differential subcellular distribution of pErk1/2 in K-Ras^{G12D} 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-Ras^{G12D} induced neoplastic progression in the 6 week time frame following K-Ras^{G12D} expression.

K-Ras^{G12D} expression induces tumor suppressor proteins in K-Ras^{G12D} susceptible tissues

The Ki67 staining for proliferating cells indicated hyperproliferation in the K-Ras^{G12D} 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-Ras^{G12D} induced oral and gastric papillomas. We hypothesized that the well differentiated and benign nature of K-Ras^{G12D} induced tumors was the result of a strong global tumor suppressor response. Indeed, Western blot analysis of oral tumor lysates from Cre-ERT2; K-Ras^{G12D} mice showed an upregulation of tumor suppressors p16^{Ink4a}, p19^{Arf} and p53 (Fig 6B). Similarly gastric papillomas showed an upregulation of tumor suppressors p16^{Ink4a}, p53, p21 (Supplementary Fig. S5A) and p19^{Arf} (data not shown). The relative expression of p16^{Ink4a} and p19^{Arf} 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. PML (promyelocytic leukemia), 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 p19^{Arf}, p53 and p16^{Ink4a} across multiple layers of oral papilloma in comparison to limited Ki67/BrdU 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 p19^{Arf} and p53 in the K-Ras^{G12D} 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-Ras^{G12D} induced pre-malignant tumors.

However K-Ras^{G12D} 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- κ B responses are elicited in K-Ras^{G12D} induced neoplasms

Some human pre-malignant tumors elicit a DNA damage response (DDR) following oncogenic stress (25). However, in mouse tumor models, the DNA damage response 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 5–6 weeks post tamoxifen injection. Histone variant γ H2AX, a key DDR marker associated with DNA strand breaks, was found to be upregulated in oral, gastric and lung benign lesions (Fig. 7A). γ H2AX 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 shows that K-Ras^{G12D} induced tumors in mice elicit a widespread DNA damage response.

Mutant Ras can induce cell senescence accompanied by a senescence-associated secretory phenotype (SASP)(42). We tested for the senescence-associated secretory phenotype (SASP) in the K-Ras-induced tumors. Quantitative PCR on RNA from K-Ras^{G12D} induced oral and gastric tumors was used to assay Interleukin (IL)-6, MIP-2 (functional homolog of human IL-8 in mice), GM-CSF, MCP-1 and IL-1 α expression. We also examined IL-6R and CXCR2, receptors for IL-6 and MIP-2, respectively. Realtime PCR analysis indicated elevated mRNA levels of several proteins associated with the SASP response for oral and gastric papillomas (Fig. 7D and Supplementary Fig. S7A). Based on the induction of several inflammatory cytokines, we assayed for NF- κ B signaling in K-Ras^{G12D} induced tumors. Phosphorylation of inhibitor of NF- κ B kinases (IKK α / β) relieves cytoplasmic sequestration and induces nuclear translocation of NF- κ B proteins. Western blot analysis showed upregulation of pIKK β (upper band) in oral, gastric (Fig. 7E) and lung tumors (not shown), indicating activation of the canonical NF- κ B pathway. NF- κ B p65 (RelA), the predominant form of NF- κ B in cells was elevated in tumors (Fig. 7E and F). Further examination using an antibody that recognizes the nuclear localization signal (NLS) of NF- κ B p65 showed nuclear distribution of p65 in oral papilloma across several cell layers (Supplementary Fig. S7B). These data demonstrate the activation of the SASP response in K-Ras^{G12D}-induced neoplastic lesions *in vivo*. There is also significant activation of NF- κ B signaling in K-Ras^{G12D} induced pre-malignant 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, myelodysplasia) (16, 17). Our data is more consistent with the latter study, as we observed pre-malignant neoplasms in the hematopoietic tissues, oral mucosa, stomach and lungs (Fig. 2A–F, Supplementary Fig. S1). In the 5–6 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, demonstrate tissue specificity in cellular responses to oncogenic Ras activation. Some tissues undergo immediate hyperproliferation, while 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 retain upregulated pErk1/2 largely in the cytoplasm (Fig. 4A). Cell culture-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 shows the induction of tumor suppressors such as p53, p19^{Arf}, p21 and p16^{Ink4a} in oncogenic K-Ras^{G12D} 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 DNA damage response was elicited across oral papillomas (Fig. 7B and Supplementary Fig. S6). The widespread induction of several tumor suppressors and DNA damage response proteins in response to oncogenic K-Ras^{G12D} likely explains the lack of malignant tumor formation in oral epithelium in our study and elsewhere (32, 34). Interestingly the K-Ras^{G12D} refractory tissues did not elicit a potent tumor suppressor response (Fig. 6F,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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cre-mediated allelic recombination and K-Ras^{G12D} expression occurs in multiple tissues. **A**, Schematic representation of the generation of Cre-ERT2; K-Ras^{G12D} mice and tamoxifen-mediated expression of K-Ras^{G12D}. **B**, PCR analysis of genomic DNA extracted from various tissues. In the schematic, arrows indicate the location of the primers used to detect excision of the stop cassette; first 2 exons in K-Ras allele are indicated in boxes; triangles indicate LoxP sites and the asterisk indicates the mutation in codon 12 of K-Ras^{G12D}. Indicated tissues were harvested from Cre-ERT2; K-Ras^{G12D} mice two weeks post tamoxifen treatment. DNA from tail prior to tamoxifen injection [Tail Pre Tam] served as a control. Both the wild type (~620 bp) and Cre recombined mutant Ras (~650 bp) alleles are detected using the primers indicated. **C**, Mutant K-Ras^{G12D} RNA expression in various tissues. Following *HindIII* restriction digestion, the cDNA for mutant K-Ras generates two smaller fragments, indicated as 'Mut' (lanes 3–8) whereas cDNA for wild type K-Ras gives one single product indicated as 'Wt' (lanes 1, 2) (as described in methods).

Figure 2. K-Ras^{G12D} expression generates rapid oral, gastric and lung neoplasms, but little tumorigenic effect in liver, pancreas and small intestine. A, Cre-ERT2; K-Ras^{G12D} mice develop oral papillomas within 4–6 weeks post tamoxifen injection. B, H&E staining of oral tumors indicates benign papilloma formation in tamoxifen-treated Cre-ERT2; K-Ras^{G12D} mice. C, Induction of K-Ras^{G12D} results in gastric papillomas. An average sized stomach and inner surface of stomach from tamoxifen treated Cre-ERT2 mouse is shown. Stomachs obtained from Cre-ERT2; K-Ras^{G12D} mice are enlarged and reveal growths on the inner surface. D, H&E staining of a non-glandular region of normal stomach and gastric tumor. E, Lungs expressing oncogenic K-Ras^{G12D} show multiple lesions (arrows) on the surface. F,

H&E staining of lungs from tamoxifen-treated control (Cre) and bi-allelic mice G, H&E stain of liver from tamoxifen-treated Cre-ERT2; K-Ras^{G12D} mice illustrate lymphocytic (arrows) infiltration with otherwise normal histology. H, Pancreas from Cre-ERT2; K-Ras^{G12D} mice is histologically similar to controls (Cre-ERT2) I, Ki67 (brown) staining of small intestines (jejunum) from Cre-ERT2 and Cre-ERT2; K-Ras^{G12D} mice show no significant difference (counterstained with hematoxylin).

Figure 3. K-Ras^{G12D} expression results in activation of Erk signaling in various tissues. A and B, Western blot analysis show upregulation of pErk1/2 and pSTAT3 in oral tumors (A, lanes 4–6) and gastric tumors (B, lanes 3–5), from Cre-ERT2; K-Ras^{G12D} mice as compared to normal tissues derived from Cre-ERT2 mice. Total STAT3, Erk1/2, Tubulin or GAPDH served as loading controls. C, IHC staining (brown) shows upregulation of pErk1/2 and pSTAT3 in hyperplastic lung lesions as compared to normal lungs from tamoxifen-treated littermate controls. Sections are counterstained with hematoxylin. D, IF staining for pErk1/2 shows upregulation in pancreas (green), small intestine (green) and liver (red) from Cre-ERT2; K-Ras^{G12D} mice as compared to littermate controls. For small intestine, pErk1/2 expression was largely restricted to the base of the crypts as depicted. Nuclei are marked with DAPI (blue). E, Western blot analyses for pErk1/2 and pSTAT3 in pancreas, liver, small intestines, oral tumor and gastric tumor from Cre-ERT2; K-Ras^{G12D} mice as compared to littermate Cre-ERT2 controls. Total Erk1/2 and GAPDH served as loading controls.

Figure 4.

Activated pErk1/2 is retained in the cytoplasm of oncogenic K-Ras refractory tissues. A, Immunofluorescence staining reveals largely cytoplasmic distribution of upregulated pErk1/2 (red) in pancreas, liver and small intestine from Cre-ERT2; K-Ras^{G12D} mice. CreERT2 littermate controls display virtually no pERK1/2 staining. Nuclei are marked by blue DAPI staining. B, Oral, gastric and lung tumors from Cre-ERT2; K-Ras^{G12D} mice show predominantly nuclear pErk1/2 (shown as red in oral and gastric tumors and brown stain in lung lesion). Oral and gastric tissues are counterstained with DAPI and the lung sections with hematoxylin.

Figure 5. Cytoplasmic pErk1/2 is unable to activate downstream mitogenic targets in K-Ras^{G12D} refractory tissues. **A**, Immunohistochemistry staining shows minimal pElk-1 detection in oncogenic K-Ras refractory tissues pancreas, liver and small intestine, compared to marked pElk-1 nuclear detection in K-Ras^{G12D} induced neoplastic lesions from K-Ras susceptible tissues (lower panel). **B**, Immunofluorescence staining for c-Myc (red) shows marked elevation in K-Ras^{G12D} induced oral and gastric tumors as compared to K-Ras^{G12D} refractory tissues pancreas, liver and small intestines from Cre-ERT2; K-Ras^{G12D} mice. Scale bar: 10 μ m.

Figure 6.

Tumor suppressor proteins are upregulated in oral papillomas while K-Ras^{G12D} refractory tissues do not elicit any significant tumor suppressor response. A, Ki67 staining (brown) of normal oral mucosa (upper panel) and oral papilloma (lower panel) shows hyperproliferation in the oral tumor. Basal (b) and suprabasal (sb) cell layers are indicated in normal oral mucosa. B, Western blot analysis shows upregulation of tumor suppressors (as indicated) in oral papillomas (lanes 4–6) from Cre-ERT2; K-Ras^{G12D} mice. C–E, Co-immunofluorescence staining for proliferation markers, Ki67 and BrdU (green) with the tumor suppressors: p19^{Arf}, p53 and p16^{Ink4a} (red) in oral papillomas. Basal (b) cell layer, suprabasal (sb) cell layer and dermis (D) are indicated. F and G, Immunofluorescence staining for p19^{Arf} (F, in green) and p53 (G, in red) shows sparse induction of tumor suppressors in pancreas, liver and small intestine from Cre-ERT2; K-Ras^{G12D} mice compared to oral tumors from Cre-ERT2; K-Ras^{G12D} mice. Scale bar: 10μm.

Figure 7.

DNA damage response and NF- κ B signaling is activated in K-Ras^{G12D}-induced tumors. A, Western blot analysis for γ H2AX reveals upregulation in tumors derived from tamoxifen-treated Cre-ERT2; K-Ras^{G12D} mice. Total H2AX levels stayed constant. B, Immunofluorescence staining for Ki67 (red) and γ H2AX (green) indicate marked upregulation of γ H2AX in oral tumors from Cre-ERT2; K-Ras^{G12D} mice. Arrowheads indicate cells with ≥ 3 DNA damage foci marked with γ H2AX. Basal (b) and suprabasal (sb) cell layers are indicated. Scale bar: 10 μ m. C, Percentages of γ H2AX positive cells in oral tumor from Cre; K-Ras^{G12D} mice versus oral mucosa from Cre-ERT2 littermates. Columns represent Mean $\pm$ S.E.M. * $p < 0.001$. D, Real time quantitative PCR analysis of oral tumors for indicated cytokines and receptors. Asterisks indicate transcriptional target genes for NF- κ B signaling. E, Western blot for pIKK α / β and NF- κ B p65 (RelA) in oral and gastric tumors. Total IKK α / β and GAPDH served as loading controls. F, Immunohistochemistry staining for p65 RelA shows upregulation in oral tumor (Cre-ERT2; K-Ras^{G12D}).