

# Endogenous oncogenic *K-ras*<sup>G12D</sup> stimulates proliferation and widespread neoplastic and developmental defects

David A. Tuveson,<sup>1</sup> Alice T. Shaw,<sup>2,3,4</sup> Nicholas A. Willis,<sup>2,3</sup> Daniel P. Silver,<sup>4,5</sup> Erica L. Jackson,<sup>2</sup> Sandy Chang,<sup>6</sup> Kim L. Mercer,<sup>2,3</sup> Rebecca Grochow,<sup>2</sup> Hanno Hock,<sup>4</sup> Denise Crowley,<sup>2,3</sup> Sunil R. Hingorani,<sup>1</sup> Tal Zaks,<sup>1</sup> Catrina King,<sup>1</sup> Michael A. Jacobetz,<sup>1</sup> Lifu Wang,<sup>1</sup> Roderick T. Bronson,<sup>7</sup> Stuart H. Orkin,<sup>8,9</sup> Ronald A. DePinho,<sup>4</sup> and Tyler Jacks<sup>2,3,\*</sup>

<sup>1</sup>Abramson Family Cancer Research Institute, Abramson Cancer Center and Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

<sup>2</sup>MIT Cancer Center and Department of Biology, Cambridge, Massachusetts 02139

<sup>3</sup>Howard Hughes Medical Institute at MIT, Cambridge, Massachusetts 02139

<sup>4</sup>Department of Adult Oncology

<sup>5</sup>Department of Cancer Biology

Dana-Farber Cancer Institute, Boston, Massachusetts 02115

<sup>6</sup>Department of Molecular Genetics, MD Anderson Cancer Center, Houston, Texas 77030

<sup>7</sup>Department of Pathology, Tufts University School of Medicine and Veterinary Medicine, Boston, Massachusetts 02111

<sup>8</sup>Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115

<sup>9</sup>Howard Hughes Medical Institute at Children's Hospital, Boston, Massachusetts 02115

\*Correspondence: tjacks@mit.edu

## Summary

**Activating mutations in the *ras* oncogene are not considered sufficient to induce abnormal cellular proliferation in the absence of cooperating oncogenes. We demonstrate that the conditional expression of an endogenous *K-ras*<sup>G12D</sup> allele in murine embryonic fibroblasts causes enhanced proliferation and partial transformation in the absence of further genetic abnormalities. Interestingly, *K-ras*<sup>G12D</sup>-expressing fibroblasts demonstrate attenuation and altered regulation of canonical Ras effector signaling pathways. Widespread expression of endogenous *K-ras*<sup>G12D</sup> is not tolerated during embryonic development, and directed expression in the lung and GI tract induces preneoplastic epithelial hyperplasias. Our results suggest that endogenous oncogenic *ras* is sufficient to initiate transformation by stimulating proliferation, while further genetic lesions may be necessary for progression to frank malignancy.**

## Introduction

Oncogenes were originally characterized as transforming genetic elements contained within tumorigenic retroviruses (Maulmbs and Barbacid, 2003; Varmus and Bishop, 1986). However, the role of oncogenes in the initiation of cancer has remained ambiguous. Indeed, ectopic expression of individual oncogenes in primary cells does not promote transformation without cooperating genetic events (Kamijo et al., 1997; Land et al., 1983; Ruley, 1983; Serrano et al., 1996; Tanaka et al., 1994). Furthermore, retroviral transduction of oncogenes in primary rodent or human cells does not stimulate proliferation, but instead causes either growth arrest or apoptosis through activation of the p19ARF (p14ARF)/p53 pathway (de Stanchina

et al., 1998; Lowe and Sherr, 2003; Serrano et al., 1997; Zindy et al., 1998). The inability of ectopically expressed oncogenes to transform primary cells in culture is in contrast to several clinical observations in which preneoplastic cells harbor single oncogenic mutations in the absence of obvious cooperating genetic events. For example, the *Bcr-Abl* translocation in stable phase chronic myelogenous leukemia (Shet et al., 2002) and *K-ras* mutations in pancreatic ductal hyperplasias (Moskaluk et al., 1997) occur in cell populations that apparently lacked additional oncogenic or *p14ARF/p53* mutations. These findings suggest that aberrant expression of ectopically introduced oncogenes confers different biological effects than endogenously expressed oncogenes.

Investigations into the role of oncogenes in tumorigenesis

## SIGNIFICANCE

Although the *K-ras* oncogene is mutated in a significant proportion of pancreatic, colon, and lung tumors, its role in the earliest stages of neoplasia is unclear. Indeed, recent findings have demonstrated that the widespread expression of an endogenous *K-ras*<sup>G12V-ires-BGeo</sup> allele had no overt consequences in most tissues, supporting the premise that the role of oncogenic *ras* is restricted to tumor progression. Using a different gene-targeting strategy, we generated a mutant mouse that harbors a conditional *K-ras*<sup>G12D</sup> allele and found that widespread expression of *K-ras*<sup>G12D</sup> causes embryonic lethality, whereas directed expression stimulates abnormal proliferation in tissues that harbor *K-ras* mutations in human cancer. Therefore, *ras* mutation may be a primary event in tumorigenesis, highlighting the need to pursue anti-Ras therapies in preneoplastic conditions as well as in advanced malignancies.

have often employed the *ras* gene family, due to the frequency of *ras* mutations in human epithelial cancers (Bos, 1989). Genetic and biochemical analyses have established that Ras proteins function as plasma membrane-bound GTPases that are stimulated by growth factor receptor tyrosine kinases and activate downstream effector pathways when bound to GTP (Scolnick et al., 1979). Several Ras effector pathways, including the Raf/MEK/ERK (MAPK) and PI3K/AKT kinase cascades, promote cell proliferation, differentiation, and survival (Vojtek and Der, 1998). It has been proposed that the molecular basis of *ras* oncogenicity is constitutive effector pathway stimulation due to *ras* missense mutations that stabilize the GTP-bound configuration of Ras by decreasing the intrinsic Ras-GTPase activity and by conferring resistance to cellular proteins that allosterically stimulate Ras GTPase activity (Malumbres and Barbacid, 2003).

Studies examining *ras* mutation as an initiating event in cellular transformation have been hampered by the observation that mutant *ras* induces growth arrest in primary cells unless accompanied by cooperating oncogenes or coincident loss of functional p53 or p19ARF pathways (Kamijo et al., 1997; Land et al., 1983; Ruley, 1983; Serrano et al., 1997; Tanaka et al., 1994; Zindy et al., 1998). The growth arrest triggered by overexpression of oncogenic *H-ras* has been termed "premature senescence" because it mimics many aspects of cellular replicative senescence (Serrano et al., 1997). Premature senescence has been attributed to chronic hyperstimulation of the MAPK cascade (Lin et al., 1998; Zhu et al., 1998). However, most studies of oncogenic *ras* function have utilized *ras* cDNA constructs that direct supraphysiological expression levels. Additionally, although *K-ras* is the *ras* family member most often mutated in human cancer (Bos, 1989), mutant *H-ras* alleles have been used in most studies.

To examine the signal transduction pathways, cellular properties, and in vivo consequences conferred by physiological expression levels of oncogenic *K-ras*, we have extended our analysis of a conditional *K-ras*<sup>G12D</sup> mutant mouse to primary mouse embryonic fibroblasts (MEFs) and epithelial tissues. An analogous approach that utilized a bicistronic *K-ras*<sup>G12V-IRE5-BGeo</sup> endogenous allele was recently reported (Guerra et al., 2003). The conditional *K-ras*<sup>G12D</sup> mice develop epithelial neoplasms upon activation of endogenous *K-ras*<sup>G12D</sup> expression in the lung (Jackson et al., 2001) and pancreas (Hingorani et al., 2003). Here we find that *K-ras*<sup>G12D</sup>-expressing MEFs demonstrate enhanced proliferative properties, lack contact inhibition, and are immortal despite having functional p19ARF and p53 pathways. Moreover, widespread embryonic expression of *K-ras*<sup>G12D</sup> is uniformly lethal, whereas the spatially controlled expression of *K-ras*<sup>G12D</sup> induces epithelial hyperplasias in vivo. Strikingly, *K-ras*<sup>G12D</sup>-expressing fibroblasts and epithelial cells exhibit attenuation of the MAPK pathway, which may explain why they do not undergo premature cellular senescence. Furthermore, and in contrast to primary cells overexpressing oncogenic *ras*, MEFs expressing endogenous levels of *K-ras*<sup>G12D</sup> cooperate with *E1a*, but not *c-Myc*, to achieve full transformation. Collectively, these results question the relevance of oncogenic *ras*-induced premature senescence and challenge the model of oncogene cooperation in the initiation of cellular transformation.

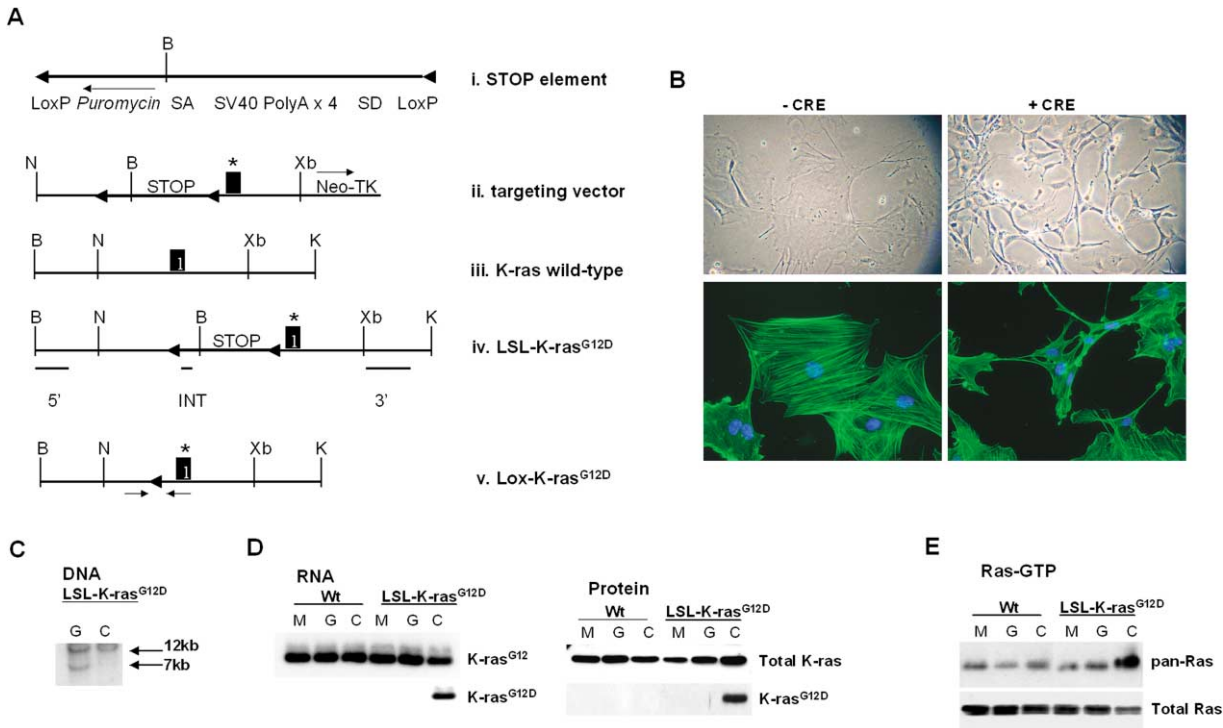
## Results

### **K-ras<sup>G12D</sup>-expressing fibroblasts exhibit elevated Ras-GTP levels and enhanced proliferative properties, and not premature senescence**

We recently described the construction of a conditionally expressing *K-ras*<sup>G12D</sup> mutant mouse using the Cre-Lox system (Jackson et al., 2001). Germline transmission of the conditional *K-ras* allele (*LSL-K-ras*<sup>G12D</sup>; Figure 1A) was confirmed by Southern blotting, and conditional heterozygous *LSL-K-ras*<sup>G12D/+</sup> murine embryonic fibroblasts (MEFs) and littermate wild-type MEFs were prepared. Molecular and cellular properties were examined 2 days after infecting early passage wild-type and littermate *LSL-K-ras*<sup>G12D/+</sup> MEFs with recombinant retroviruses encoding green fluorescent protein (GFP) or "self-excising" cre recombinase (Silver and Livingston, 2001). The *LSL-K-ras*<sup>G12D</sup> MEFs demonstrated striking morphological alterations, including increased refractility and disruption of the actin cytoskeleton, within 2–3 days of viral-cre infection, when compared to uninfected conditional MEFs or uninfected and viral-cre infected wild-type MEFs (Figure 1B and data not shown). Transduction with a virus expressing GFP revealed greater than 95% infection efficiency, without accompanying morphological changes (data not shown). Examination of DNA, mRNA, and protein from the viral-cre infected *LSL-K-ras*<sup>G12D</sup> MEFs demonstrated that the conditionally mutant *K-ras* allele was efficiently recombined and expressed at levels similar to the wild-type allele (Figures 1C and 1D) (Johnson et al., 2001). This *K-ras*<sup>G12D</sup>-expressing MEF population will hereafter be designated *Lox-K-ras*<sup>G12D</sup> MEFs. *Lox-K-ras*<sup>G12D</sup> MEFs demonstrated elevated Ras-GTP levels in a Raf-GST pulldown assay (Figure 1E) (Taylor and Shalloway, 1996). To exclude the possibility that these results were due to genomic damage caused by the expression of cre recombinase (Loonstra et al., 2001; Silver and Livingston, 2001), we performed spectral karyotyping (SKY) on MEFs infected with self-excising retroviral-cre. SKY analysis demonstrated no cytogenetic abnormalities after self-excising retroviral-cre infection (N = 10, data not shown), consistent with prior observations (Silver and Livingston, 2001).

The cellular properties of *Lox-K-ras*<sup>G12D</sup>, *LSL-K-ras*<sup>G12D</sup>, and wild-type MEFs, passaged in parallel, were further evaluated in standard proliferation assays. *Lox-K-ras*<sup>G12D</sup> MEFs demonstrated enhanced proliferation compared to control MEFs, particularly at high cell densities (Figure 2A). Additionally, although none of the MEF populations proliferated in 0.5% serum, *Lox-K-ras*<sup>G12D</sup> MEFs proliferated in 2% serum, whereas the other MEFs did not (Figure 2B). Cell cycle analysis with BrdU incorporation of early-passage, asynchronously growing MEFs demonstrated an S phase content of 27% in *Lox-K-ras*<sup>G12D</sup> MEFs and 15% in *LSL-K-ras*<sup>G12D</sup> MEFs (Figure 2C). These findings contrast with the ectopic overexpression of oncogenic *H-ras* in MEFs, which has been shown to cause a decreased S phase fraction and cell cycle arrest (Serrano et al., 1997).

Since much of the work describing Ras-induced senescence was performed with cDNA constructs expressing high levels of the *H-ras* oncogene, we wished to determine whether the discrepancies between the earlier results and those observed here were attributable to the type of *ras* oncogene used. Accordingly, MEFs were evaluated following transduction with retroviruses encoding *H-ras*<sup>G12V</sup> and the 4A and 4B splice forms of *K-ras*<sup>G12D</sup> (George et al., 1985). In contrast to the increased prolif-



**Figure 1.** Conditional *K-ras*<sup>G12D</sup> mouse strain and MEFs

**A:** Schematic of (i) STOP element, (ii) targeting vector, (iii) wild-type allele, (iv) *LSL-K-ras*<sup>G12D</sup> allele, and (v) *Lox-K-ras*<sup>G12D</sup> allele. B, BamH1; K, Kpn1/Acc651; N, Not1; Xb, Xba1; SA, adenoviral splice acceptor site; \*, GGT to GAT mutation at codon 12. The position of the probes for Southern blotting is shown (5', INT = internal, 3'). Arrows in v denote PCR primer sites for the recombination assay.

**B:** Morphological alterations and increased refractility in *LSL-K-ras*<sup>G12D</sup> MEFs 72 hr after infection with self-excising retroviral-cre. Phase contrast (upper panels) and actin cytoskeleton (lower panels, phalloidin Oregon-green + DAPI) are shown.

**C:** Efficient excision of the STOP cassette in cell culture. Southern blot analysis of Bam H1 + Acc651 digested genomic DNA prepared from *LSL-K-ras*<sup>G12D</sup> MEFs 72 hr after infection with retroviral-GFP (G) or retroviral-cre (C), using the 5' external probe. The wild-type allele and recombined *Lox-K-ras*<sup>G12D</sup> allele are denoted by the 12 kb fragment, and the intact conditional *LSL-K-ras*<sup>G12D</sup> allele migrates as a 7 kb fragment.

**D:** The mutant *K-ras*<sup>G12D</sup> mRNA and protein is expressed only after infection of conditional MEFs with retroviral-cre. Wild-type and *LSL-K-ras*<sup>G12D</sup> MEFs were either mock infected (M), infected with retroviral-GFP (G), or infected with retroviral-cre (C).

**E:** Ras-GTP levels are specifically elevated in *Lox-K-ras*<sup>G12D</sup> MEFs. Ras-GTP was recovered from MEFs with Raf-GST and glutathione-agarose. A loading control for total Ras is shown below.

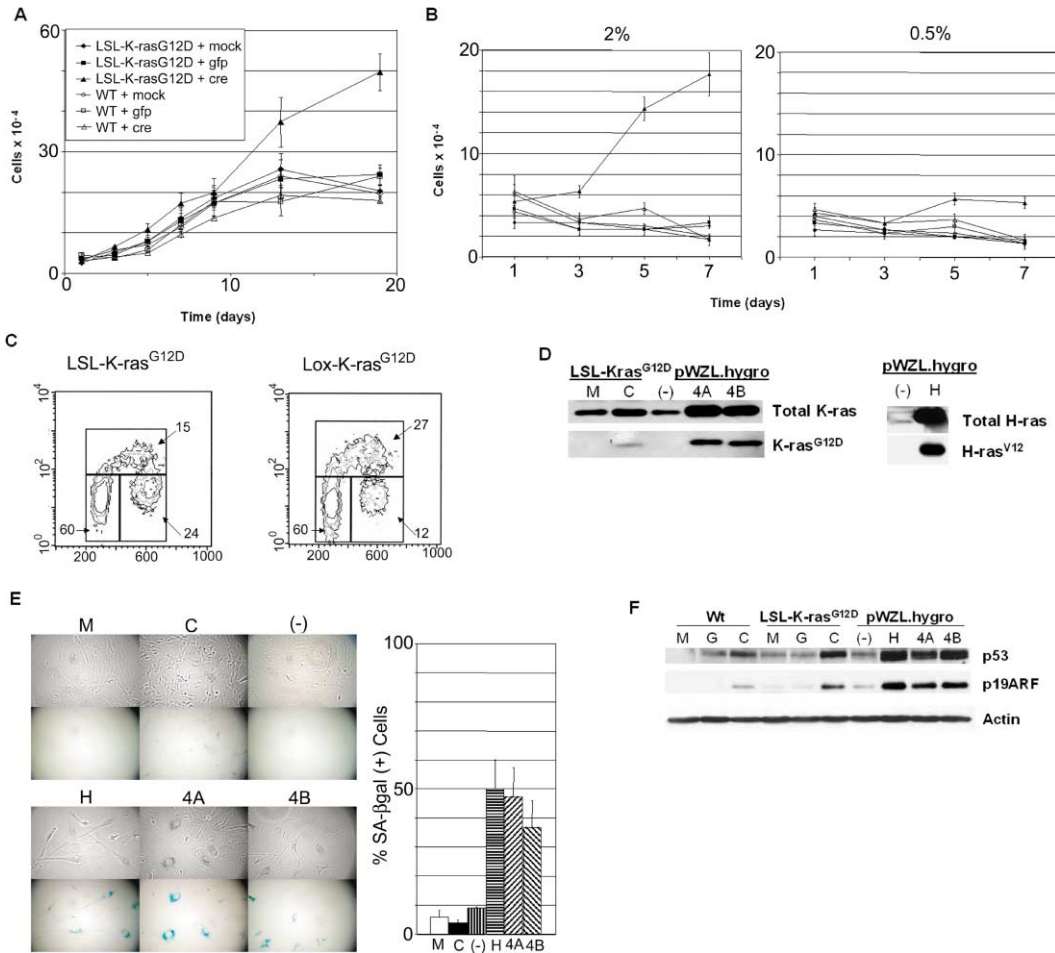
erative properties of *Lox-K-ras*<sup>G12D</sup> MEFs, growth inhibition was observed in MEFs transduced with either *H-ras*<sup>G12V</sup>, *K-ras* 4A<sup>G12D</sup>, or *K-ras* 4B<sup>G12D</sup> (data not shown). These constructs expressed approximately 20, 5, and 5 fold more H-Ras, K-Ras 4A, and K-Ras 4B, respectively, than control and *Lox-K-ras*<sup>G12D</sup> MEFs (Figure 2D). Furthermore, while MEFs ectopically overexpressing either oncogenic *H-ras* or *K-ras* demonstrated a premature senescent morphology and contained elevated levels of senescence-associated  $\beta$  galactosidase (SA $\beta$ -gal) activity, *Lox-K-ras*<sup>G12D</sup> MEFs did not (Figure 2E). Consistent with these results, the levels of p19ARF and p53, two proteins known to mediate oncogenic *ras*-induced growth arrest, were both noticeably higher in MEFs overexpressing oncogenic *ras* compared to control and *Lox-K-ras*<sup>G12D</sup> MEFs (Figure 2F). Hence, the difference between the earlier results and those represented here cannot be attributed to the use of different *ras* oncogenes.

#### ***Lox-K-ras*<sup>G12D</sup> MEFs are partially transformed**

*Lox-K-ras*<sup>G12D</sup> MEFs were further characterized with standard immortalization and transformation assays. Serial passaging revealed that multiple lines of *Lox-K-ras*<sup>G12D</sup> MEFs (3/3) were im-

mortal, whereas *LSL-K-ras*<sup>G12D</sup> and wild-type MEFs senesced after 5–10 passages (Figure 3A). The replicative immortality of *Lox-K-ras*<sup>G12D</sup> MEFs contrasts with the premature senescence observed in MEFs overexpressing oncogenic *H-ras* (Serrano et al., 1997) and *K-ras* (not shown). Additionally, confluent cultures of *Lox-K-ras*<sup>G12D</sup> MEFs grew to an extremely high density and readily demonstrated loss of contact inhibition and focus formation after two to three weeks of continuous culture (Figure 3B). Nevertheless, *Lox-K-ras*<sup>G12D</sup> MEFs were unable to form colonies when cultured at very low density or grown in semisolid media, and were not tumorigenic in nude mice (data not shown).

Since ectopic expression of oncogenic *ras* cooperates with several other oncogenes and p53 pathway deficiency to promote the transformation of primary cells (Land et al., 1983; Ruley, 1983; Tanaka et al., 1994), we assessed *Lox-K-ras*<sup>G12D</sup> MEFs for similar effects. Notably, the proliferation of *Lox-K-ras*<sup>G12D</sup> MEFs was increased only slightly following retroviral transduction of *c-Myc* (Figure 3C), but was greatly increased when transduced with adenoviral *E1a* (Figure 3D) or by p53 deficiency (Figure 3E). p53 absence, and to a lesser extent *E1a* expression, permitted anchorage-independent growth of *Lox-*



**Figure 2.** *Lox-K-ras*<sup>G12D</sup> MEFs have enhanced proliferation and are not senescent

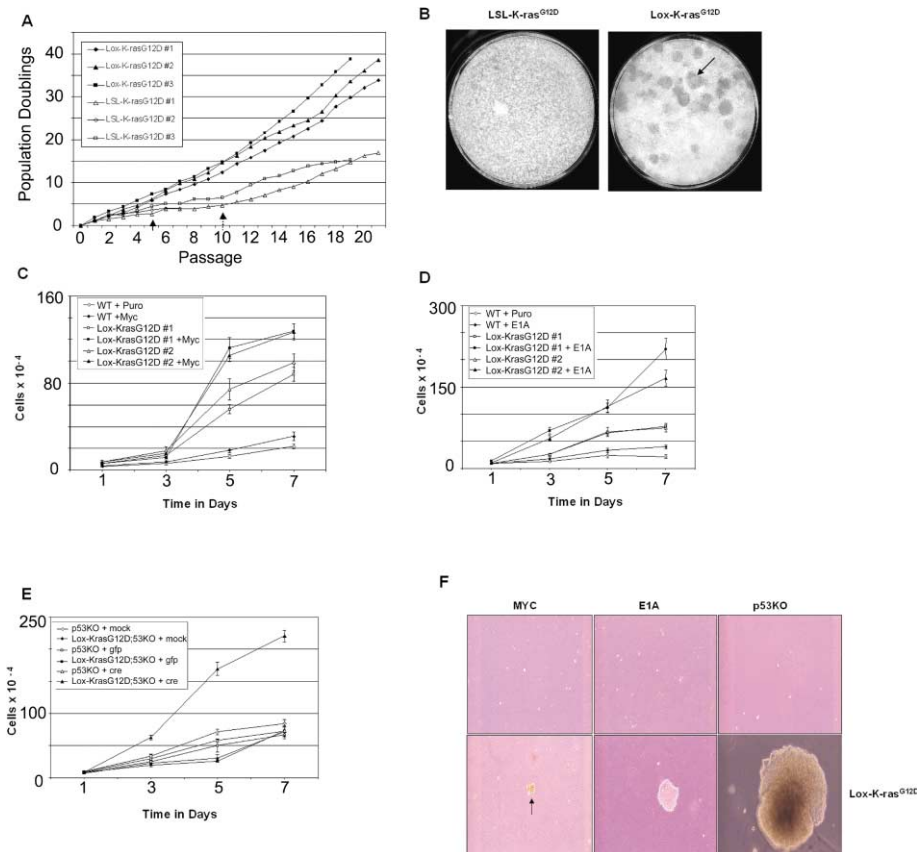
**A:** Enhanced proliferation in MEFs expressing endogenous levels of oncogenic *K-ras*. Wild-type (open symbols) and *LSL-K-ras*<sup>G12D</sup> (filled symbols) MEFs were mock infected (diamonds), viral-GFP infected (squares), or viral-cre infected (triangles).  
**B:** *Lox-K-ras*<sup>G12D</sup> MEFs proliferate in limiting serum. MEFs were as described in **A**, and cultured in the presence of media supplemented with 2% or 0.5% serum.  
**C:** Increased S phase by BrdU incorporation and FACS analysis in asynchronously proliferating *Lox-K-ras*<sup>G12D</sup> MEFs compared to *LSL-K-ras*<sup>G12D</sup> MEFs.  
**D:** Relative Ras protein levels following transduction of *LSL-K-ras*<sup>G12D</sup> MEFs with no virus (M), viral-cre (C), empty vector (-), oncogenic *H-ras*<sup>G12V</sup> (H), *K-ras* 4A<sup>G12D</sup> (4A), and *K-ras* 4B<sup>G12D</sup> (4B).  
**E:** Ectopic but not endogenous expression of oncogenic *ras* leads to premature senescence. SA $\beta$ -gal levels are increased in MEFs ectopically expressing oncogenic *ras*, but not *Lox-K-ras*<sup>G12D</sup> MEFs. Phase contrast and bright field micrographs are shown at 200 $\times$  magnification. SA $\beta$ -gal positive cells were counted ( $\pm$  SEM) 5 days after transduction. Symbols are as in **D**.  
**F:** Elevated levels of p19ARF and p53 in oncogenic *ras* overexpressing MEFs compared to *Lox-K-ras*<sup>G12D</sup> MEFs and control MEFs. Wild-type and *LSL-K-ras*<sup>G12D</sup> MEFs were either mock treated (M) or infected with GFP-virus (G) or cre-virus (C). Alternatively, *LSL-K-ras*<sup>G12D</sup> MEFs were transduced with empty (-), *H-ras*<sup>G12V</sup> (H), *K-ras*4A<sup>G12D</sup> (4A), or *K-ras*4B<sup>G12D</sup> (4B) viruses.

*K-ras*<sup>G12D</sup> MEFs, whereas *c-Myc* expression did not (Figure 3F). Unexpectedly, and in contrast to the cotransfection of primary cells with *ras* and *myc*, examination of the soft agar cultures of *Lox-K-ras*<sup>G12D</sup> MEFs transduced with *c-Myc* revealed evidence of adipocyte differentiation as indicated by the Oil Red O staining of refractile, lipid-laden cells (Figure 3F). Lastly, *Lox-K-ras*<sup>G12D</sup> MEFs lacking *p53* were tumorigenic in vivo (N = 6/6), whereas *Lox-K-ras*<sup>G12D</sup> MEFs transduced with *E1a* or *c-Myc* were not (N = 0/6, data not shown). Thus, endogenous *K-ras*<sup>G12D</sup> expression did not cooperate with *c-Myc* for cellular transformation, and cooperated to a limited extent with ectopic *E1a* expression and fully with *p53* deficiency to transform MEFs, in contrast to genetic cooperation experiments with primary cells that ectopically express oncogenic *ras*. These results contrast with the

inability of *Lox-K-ras*<sup>G12V-IRES-BGeo</sup> MEFs to form foci or cooperate with *E1a* in standard transformation assays (Guerra et al., 2003).

### Cell cycle activation in *Lox-K-ras*<sup>G12D</sup> MEFs

To investigate the molecular pathways responsible for the markedly different proliferative properties of the MEF populations described above, protein lysates from early passage cells were prepared and immunoblotted with antibodies against specific cell cycle regulatory components (Figure 4A). *Lox-K-ras*<sup>G12D</sup> MEFs contained elevated Cdk2, cyclin A, cyclin E, cyclin D1, p21, and p16 protein levels compared to control MEFs, whereas oncogenic *ras* overexpressing MEFs contained increased cyclin D1, p21, and p16 levels, but decreased cyclin A and cyclin E levels. The levels of cyclin D2, Cdk4, and p27 were not signifi-



**Figure 3.** *Lox-K-ras<sup>G12D</sup>* MEFs are partially transformed

**A:** *Lox-K-ras<sup>G12D</sup>* MEFs are immortal in a 3T3 assay. 3T3 assay of three independent *Lox-K-ras<sup>G12D</sup>* MEF lines (filled symbols) and the corresponding *LSL-K-ras<sup>G12D</sup>* MEFs (open symbols), with population doublings (ordinate) plotted against the passage number (abscissa). The onset of senescence (arrow) and immortalization (dashed arrow) are denoted in the *LSL-K-ras<sup>G12D</sup>* MEFs.

**B:** Loss of contact inhibition in *Lox-K-ras<sup>G12D</sup>* MEFs. MEFs were grown to confluency, and the media was replenished each day until foci were evident (14–21 days). Foci were demonstrated by fixation and staining with Giemsa (arrow).

**C:** C-Myc slightly increases the proliferation of *Lox-K-ras<sup>G12D</sup>* MEFs. Wild-type MEFs (diamonds) and two independent lines of *Lox-K-ras<sup>G12D</sup>* MEFs (squares and triangles) were transduced with empty vector (open symbols) or c-Myc (filled symbols).

**D:** E1a greatly increases proliferation of *Lox-K-ras<sup>G12D</sup>* MEFs. Wild-type MEFs (diamonds) and two independent lines of *Lox-K-ras<sup>G12D</sup>* MEFs (squares and triangles) were transduced with empty vector (open symbols) or E1a (filled symbols).

**E:** P53 loss greatly increases proliferation of *Lox-K-ras<sup>G12D</sup>* MEFs. *P53<sup>-/-</sup>* MEFs (open symbols) and *p53<sup>-/-</sup>;LSL-K-ras<sup>G12D</sup>* MEFs were either mock infected (diamonds) or transduced with viral-GFP (squares) or viral-cre (triangles).

**F:** P53 loss and E1a synergize with *K-ras<sup>G12D</sup>* for anchorage-independent growth in MEFs. Soft agar assay demonstrates effects of c-Myc, E1a, and p53 loss alone (upper panels) and in the setting of *K-ras<sup>G12D</sup>* expression (lower panels). Adipocyte differentiation in c-Myc-transduced *Lox-K-ras<sup>G12D</sup>* MEFs is demonstrated by Oil Red O staining (arrow).

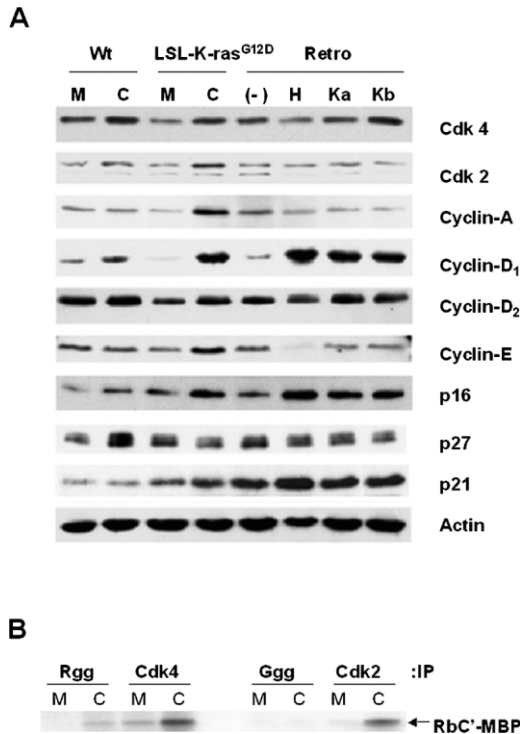
cantly different between the cell types. To determine whether the alterations in the cell cycle machinery in *Lox-K-ras<sup>G12D</sup>* MEFs conferred increased enzymatic activity in cyclin dependent kinase complexes, we assessed Cdk2 and Cdk4 in vitro kinase activities in asynchronously proliferating MEFs (Matsushima et al., 1994). Notably, *Lox-K-ras<sup>G12D</sup>* MEFs had 2–4 fold increases in Cdk2 and Cdk4 in vitro kinase activities compared to *LSL-K-ras<sup>G12D</sup>* MEFs (Figure 4B). Importantly, the increased Cdk2 and Cdk4 in vitro kinase activity in *Lox-K-ras<sup>G12D</sup>* MEFs contrasts with the decreased Cdk2 activity demonstrated in MEFs overexpressing oncogenic *H-ras* (Serrano et al., 1997), and provides a potential explanation for the distinct proliferative characteristics among the cell populations assessed here.

#### Attenuation of Ras effector pathway signaling in *Lox-K-ras<sup>G12D</sup>* MEFs

The activation of the well-characterized Ras effector pathways, MAPK and PI3-kinase (PI3K), was assessed by preparing whole cell lysates from subconfluent MEFs and immunoblotting with phosphorylation state-specific anti-ERK 1/2 and anti-AKT antibodies. While MEFs ectopically overexpressing oncogenic *ras* had elevated levels of phosphorylated ERK 1/2 and phosphorylated AKT, *Lox-K-ras<sup>G12D</sup>* MEFs unexpectedly contained equal or even decreased levels compared to *LSL-K-ras<sup>G12D</sup>* and wild-type MEFs (Figure 5A). Despite this observation, the use of

pharmacological inhibitors of the MAPK and PI3K pathways, U0126 and LY294002, respectively, indicated that these pathways contribute to the morphological changes seen in *Lox-K-ras<sup>G12D</sup>* MEFs (Figure 5B). Additionally, the DNA binding activity of ELK-1, a transcription factor activated by the MAPK cascade, was increased in nuclear extracts of *Lox-K-ras<sup>G12D</sup>* MEFs compared to control MEFs (Figure 5C). Therefore, the MAPK and PI3K cascades can be found to be activated in *Lox-K-ras<sup>G12D</sup>* MEFs and may be important for some aspects of their phenotype. However, in exponentially growing cells, the steady state levels of the activated components of these pathways are not increased.

To determine whether the activation of the MAPK and PI3K cascades by wild-type Ras-GTP was augmented in cells that express endogenous levels of *Lox-K-ras<sup>G12D</sup>*, *LSL-K-ras<sup>G12D</sup>* MEFs and *Lox-K-ras<sup>G12D</sup>* MEFs were grown to confluency, serum starved, and restimulated, and protein lysates prepared. Consistent with the observations from exponentially growing cells, phosphorylation of MEK and AKT in response to serum stimulation was found to be attenuated in *Lox-K-ras<sup>G12D</sup>* MEFs compared to *LSL-K-ras<sup>G12D</sup>* MEFs (Figure 5D). Similar results were obtained with phosphorylated ERK 1/2 (data not shown). Additionally, the ERK (Figure 5E) and AKT (Figure 5F) in vitro enzymatic activities were also diminished following serum stimulation in *Lox-K-ras<sup>G12D</sup>* MEFs compared to *LSL-K-ras<sup>G12D</sup>* MEFs.



**Figure 4.** Cell cycle pathway activation in *Lox-K-ras*<sup>G12D</sup> MEFs

**A:** Levels of cell cycle associated proteins in whole cell lysates. Wild-type (Wt) and *LSL-K-ras*<sup>G12D</sup> MEFs were either mock infected (M) or transduced with viral-cre (C). Additionally, MEFs were transduced with empty vector (-), *H-ras*<sup>G12V</sup> (H), murine *K-ras* 4A<sup>G12D</sup> (4A), or murine *K-ras* 4B<sup>G12D</sup> (4B).

**B:** Elevated Cdk2 and Cdk4 in vitro kinase activities. Rabbit  $\gamma$  globulin (Rgg), goat  $\gamma$  globulin (Ggg), and Cdk2 and Cdk4 immunoprecipitates were prepared from *LSL-K-ras*<sup>G12D</sup> MEFs that were either mock infected (M) or viral-cre (C) infected. In vitro kinase activities were evaluated with recombinant pRb as the substrate.

Similar analyses of MEFs that ectopically overexpressed *H-ras*<sup>G12V</sup> did not demonstrate any attenuation of Ras effector pathway signaling (Supplemental Figure S1 at <http://www.cancer.org/cgi/content/full/5/4/375/DC1>). Thus, the activation of the PI3K and MAPK cascades by serum stimulation is regulated differently in *Lox-K-ras*<sup>G12D</sup> MEFs as compared to control MEFs and MEFs overexpressing oncogenic *ras*, perhaps reflecting the adaptation of cells that harbor endogenous alleles of oncogenic *K-ras*. These results are particularly interesting as the MAPK cascade had been previously implicated as the Ras effector pathway responsible for premature senescence (Lin et al., 1998; Serrano et al., 1997; Zhu et al., 1998), and suggest that ectopic expression of oncogenic *ras* generates constitutive, high-level MAPK cascade activation that cannot be appropriately modulated in primary cells.

#### Intact p19ARF/p53 pathway in *Lox-K-ras*<sup>G12D</sup> MEFs

The p19ARF/p53 pathway is commonly inactivated during immortalization in murine cells (Harvey and Levine, 1991; Kamijo et al., 1997), and the overexpression of oncogenic *ras* can transform cells with mutations in this pathway (Kamijo et al., 1997; Serrano et al., 1996; Tanaka et al., 1994). Thus, it was important to know the state of this pathway in *Lox-K-ras*<sup>G12D</sup> MEFs. In order to stimulate p19ARF expression and evaluate whether the

*Lox-K-ras*<sup>G12D</sup> MEFs were sensitive to the effects of *ras* overexpression, *Lox-K-ras*<sup>G12D</sup> MEFs and *p53*<sup>-/-</sup> MEFs were transduced with oncogenic *K-ras* and *H-ras* and examined for proliferative capacity and signaling pathways. Following transduction with oncogenic *ras*, *Lox-K-ras*<sup>G12D</sup> MEFs demonstrated a decreased growth rate and a premature senescent morphology; in contrast, *p53*<sup>-/-</sup> MEFs continued to proliferate (Figure 6A and data not shown), as previously described (Serrano et al., 1997; Tanaka et al., 1994). Additionally, protein lysates from oncogenic *ras* overexpressing *Lox-K-ras*<sup>G12D</sup> MEFs contained increased p19ARF, p53, and phosphorylated ERK 1/2 levels (Figure 6B). This result confirms that the cellular characteristics of *Lox-K-ras*<sup>G12D</sup> MEFs are dependent upon the lower endogenous expression level of oncogenic *K-ras* than the levels directed by retroviral transduction.

To assess the function of the p53 pathway, *Lox-K-ras*<sup>G12D</sup> MEFs and *LSL-K-ras*<sup>G12D</sup> MEFs were incubated with 0.2  $\mu$ g/ml adriamycin, and cells and protein lysates were serially collected over a 20 hr time course. Following 20 hr of treatment, both *Lox-K-ras*<sup>G12D</sup> MEFs and *LSL-K-ras*<sup>G12D</sup> MEFs arrested in G1 and G2/M with no cells remaining in the S phase of the cell cycle (Figure 6C). As previously demonstrated (Figures 2F and 4A), the p53 and p21 protein levels were higher in the *Lox-K-ras*<sup>G12D</sup> MEFs than the *LSL-K-ras*<sup>G12D</sup> MEFs prior to adriamycin incubation (Figure 6D). Following adriamycin treatment, the magnitude of increase of p53 and p21 protein levels was similar between the two populations of MEFs, suggesting a similar sensitivity to genotoxic stress (Figure 6D). Thus, the p19ARF/p53 pathway appears to be intact in the *Lox-K-ras*<sup>G12D</sup> MEFs, as has been demonstrated in *Lox-K-ras*<sup>G12V-IRES-BG60</sup> MEFs (Guerra et al., 2003).

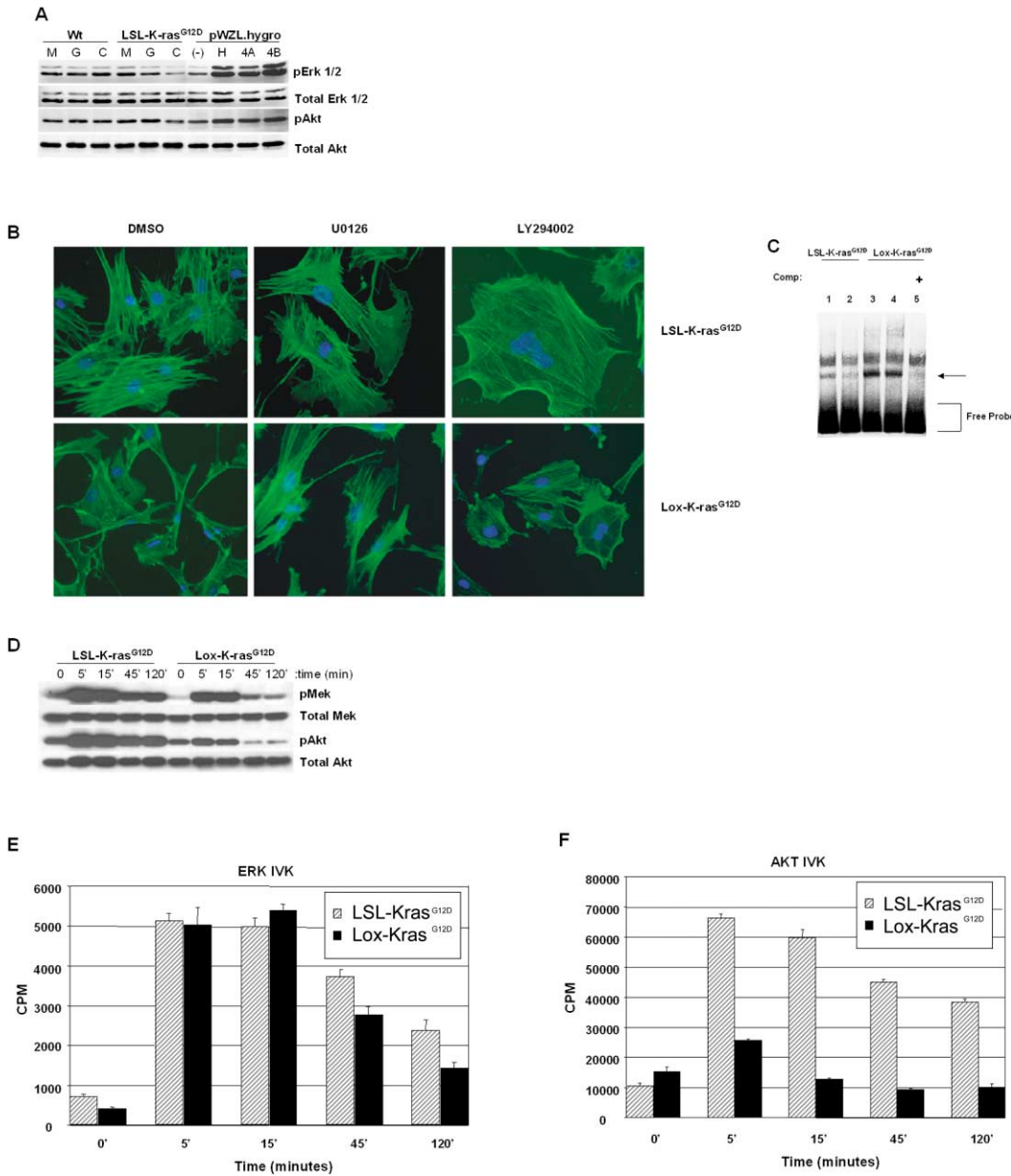
#### Expression of endogenous oncogenic *K-ras*<sup>G12D</sup> during murine development results in widespread morphological aberrations and early embryonic lethality

The effects of embryonic expression of *K-ras*<sup>G12D</sup> were assessed in mice by interbreeding the conditional *LSL-K-ras*<sup>G12D</sup> mice with *Protamine-Cre* (PrmCre) and *CMV-cre* mice.

The mouse protamine 1 promoter is active in haploid sperm, leading to efficient cre-mediated recombination in the male germ line (O'Gorman et al., 1997), and the CMV-cre transgene is active in mosaic fashion during development (Schwenk et al., 1995). Male offspring bearing both the PrmCre transgene and the conditional *LSL-K-ras*<sup>G12D</sup> allele were thus mated to wild-type females, and genotyping revealed no viable *Lox-K-ras*<sup>G12D</sup> progeny. An analysis of over 100 embryos ranging in age from 8.5 days old (E8.5) to E18.5 identified 30 which harbored the recombined allele; however, none were viable past E11.5. Mutant embryos were characteristically small and pale (Figures 7A and 7B), and microscopic analysis of E9.5 mutant embryos showed cardiomegaly and abnormal brain development (Figures 7C and 7D). At the cellular level, neuroepithelial sections revealed extensive areas of architectural distortion and apoptosis (Figures 7E and 7F). Additionally, and in contrast to Guerra and colleagues, we were unable to produce any *CMV-cre;LSL-K-ras*<sup>G12D</sup> mice, or *CMV-cre;Lox-K-ras*<sup>G12D</sup> mice, and an embryological assessment of this cross is in progress. These findings demonstrate that widespread or germline embryonic expression of an endogenous *K-ras*<sup>G12D</sup> allele is uniformly lethal.

#### Induction of epithelial hyperplasias by *K-ras*<sup>G12D</sup>

To extend our analysis of endogenous *K-ras*<sup>G12D</sup> function to specific epithelial tissues in vivo, the *LSL-K-ras*<sup>G12D</sup> allele was condi-



**Figure 5.** Modulation of Ras effector pathways in *Lox-K-ras<sup>G12D</sup>* MEFs

**A:** Levels of phosphorylated ERK and AKT in control MEFs, *Lox-K-ras<sup>G12D</sup>* MEFs, and oncogenic *ras*-overexpressing MEFs.

**B:** Reversion of morphological alterations in *Lox-K-ras<sup>G12D</sup>* MEFs by MEK and PI3K inhibition. MEFs were cultured in the presence of either 0.1% DMSO, 10  $\mu$ M U0126, or 50  $\mu$ M LY294002 for 24 hr before fixation and staining with phalloidin-Oregon green and DAPI.

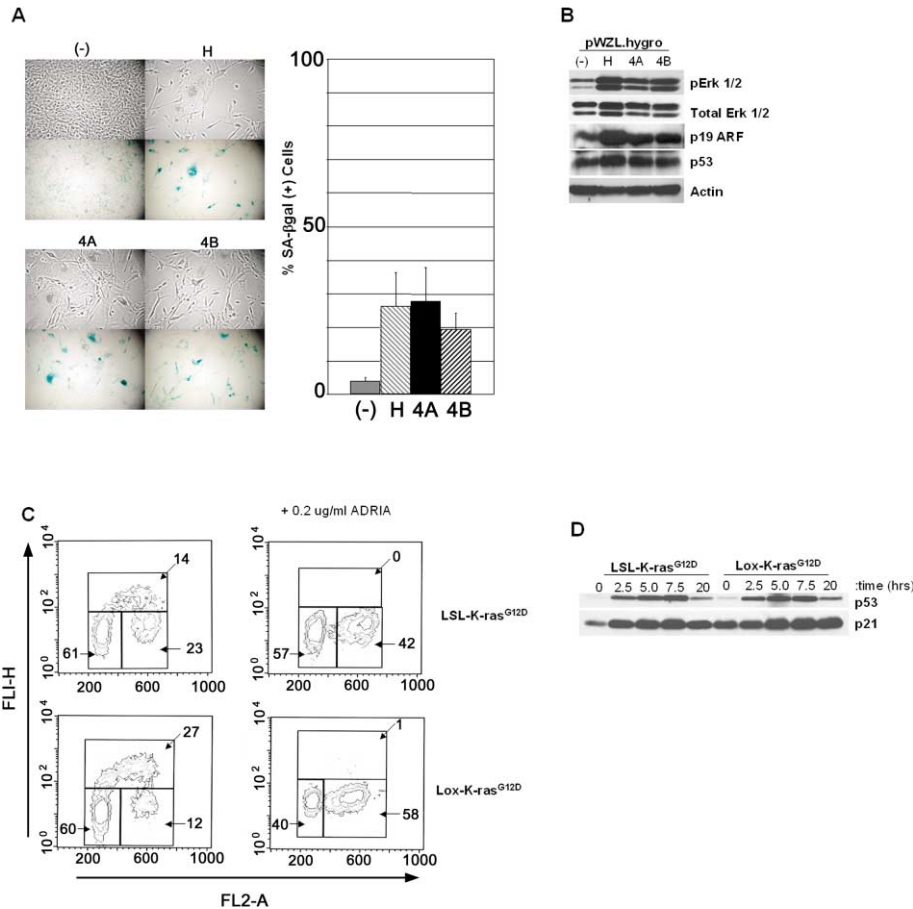
**C:** ELK-1 activity is increased in *Lox-K-ras<sup>G12D</sup>* MEFs. Nuclear extracts were prepared from multiple lines of *LSL-K-ras<sup>G12D</sup>* MEFs (lanes 1 and 2) and *Lox-K-ras<sup>G12D</sup>* MEFs (lanes 3–5), and EMSA performed. The retarded migration of the ELK-1 probe and migration of the free probe is shown. Incubation with cold competitor ELK oligonucleotide is shown in lane 5.

**D:** Diminished activation of MEK and AKT following serum stimulation of *Lox-K-ras<sup>G12D</sup>* MEFs compared to *LSL-K-ras<sup>G12D</sup>* MEFs. MEFs were grown to confluency, serum starved for 24 hr, and stimulated for various times with normal media before preparation of protein lysates and immunoblotting. Anti-phosphorylated and total MEK and AKT antibodies were used. Similar results were obtained with anti-phospho ERK (data not shown).

**E and F:** ERK (**E**) and AKT (**F**) *in vitro* kinase reactions from cells treated as described in **D**.

tionally activated in the lung and gastrointestinal tract. Pulmonary hyperplasias were induced by the nasal instillation of adenoviral-cre (Jackson et al., 2001) within 4 days of infection (Supplemental Figure S2 at <http://www.cancer.org/cgi/content/full/5/4/375/DC1>, Figure 8A), demonstrating that proliferation *in vivo* closely parallels *K-ras<sup>G12D</sup>* expression and sug-

gesting that other genetic events are not required. The proliferative nature of these lesions was supported by the elevated nuclear immunostaining with Ki-67 ( $17.5 \pm 2.5\%$ , compared with  $2.6 \pm 0.6\%$  in control pulmonary tissue). Additionally, intranuclear cyclin D1 was detected in the hyperplasias, consistent with its postulated role in stimulating proliferation. As a positive



**Figure 6.** Intact p19ARF and p53 pathways in Lox-K-ras<sup>G12D</sup> MEFs

**A:** Overexpression of oncogenic *ras* causes premature senescence of Lox-K-ras<sup>G12D</sup> MEFs. Lox-K-ras<sup>G12D</sup> MEFs were transfected with empty vector (-), H-ras<sup>G12V</sup> (H), K-ras 4A<sup>G12D</sup> (4A), or K-ras 4B<sup>G12D</sup> (4B) and assessed for SA- $\beta$ -gal positivity ( $\pm$  SEM) as in Figure 2E.

**B:** Overexpression of oncogenic *ras* causes hyperactivation of ERK and elevated p19ARF and p53 protein levels in Lox-K-ras<sup>G12D</sup> MEFs.

**C:** Intact p53 response in LSL-K-ras<sup>G12D</sup> and Lox-K-ras<sup>G12D</sup> MEFs. MEFs were incubated with 0.2  $\mu$ g/ml adriamycin for 20 hr, and cell cycle analysis was performed.

**D:** p53 and p21 induction from genotoxic stress. LSL-K-ras<sup>G12D</sup> and Lox-K-ras<sup>G12D</sup> MEFs were incubated with 0.2  $\mu$ g/ml adriamycin (Sigma) for various lengths of time, and p53 and p21 protein levels were assessed by immunoblotting.

control for ERK activation in vivo, phosphorylated ERK1/2 was detectable in primitive spermatogonia and primary spermatocytes—cells known to contain elevated phosphorylated ERK 1/2 levels (Lu et al., 1999). However, phosphorylated ERK 1/2 was not detectable by this method in the pulmonary hyperplasias. Pulmonary hyperplasias and neoplasms were also reported with the bitransgenic *CMV-cre;LSL-K-ras<sup>G12V-IRES-BGeo</sup>* mice (Guerra et al., 2003), although the latency between expression of the endogenous *K-ras<sup>G12V-IRES-BGeo</sup>* allele and pulmonary epithelial hyperplasia was not reported.

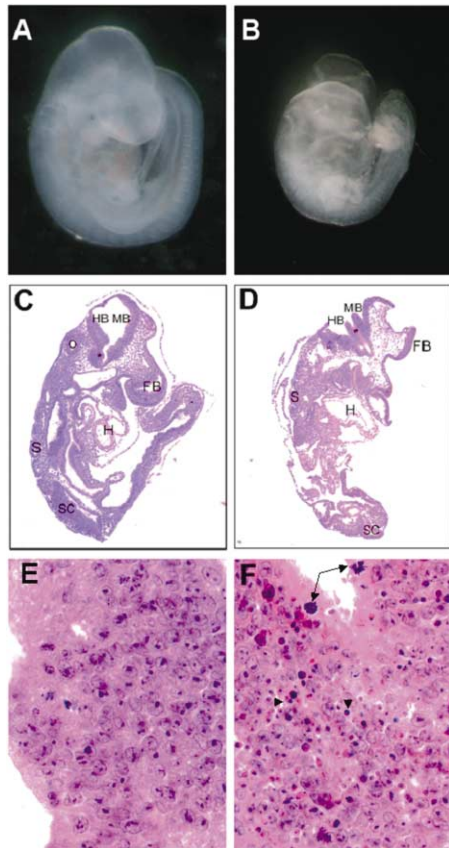
The effects of endogenous *K-ras<sup>G12D</sup>* expression in colonic epithelial cells were examined by interbreeding *LSL-K-ras<sup>G12D</sup>* mice with fatty acid binding protein (*Fabp*-*cre*) transgenic mice (Saam and Gordon, 1999). All *LSL-K-ras<sup>G12D</sup>;Fabp-cre* compound mice examined (14/14) had diffuse hyperplasia and dysplasia of the colonic crypts that was obvious by 4 weeks of age, whereas the parental strains did not (Figure 8B and Supplemental Figure S3). Evidence of increased proliferation in the hyperplastic and dysplastic colonic epithelium was demonstrated by Ki-67 immunostaining in nuclei located distant from the base of the colonic crypts (Figure 8B and Supplemental Figure S3). Consistent with the results of *K-ras<sup>G12D</sup>* expression in pulmonary hyperplasias, phosphorylated ERK 1/2 was again not detectable. In contrast to the pulmonary hyperplasias, cyclin D1 could not be detected in these colonic hyperplasias for unknown reasons. These epithelial hyperplasias and dyspla-

sias are markedly different from the reported lack of effect of an expressed *K-ras<sup>G12V-IRES-BGeo</sup>* allele in colonic epithelial cells (Guerra et al., 2003).

We have recently reported that the expression of endogenous *K-ras<sup>G12D</sup>* in the exocrine pancreas induces proliferative pancreatic intraepithelial neoplasms (PanIN) as early as two weeks of age (Hingorani et al., 2003). These findings significantly differ from the lack of effect of an expressed *K-ras<sup>G12V-IRES-BGeo</sup>* allele in pancreatic epithelial cells unless a concomitant *CDK4<sup>R24C</sup>* mutation is present (Guerra et al., 2003). Additional studies with other transgenic-*cre* mice have revealed skin papillomas in the background of a K14-*creER* allele (Vasioukhin et al., 1999), submandibular gland hyperplasia (Supplemental Figure S4) in the background of an MMTV-*cre* allele (Wagner et al., 1997), and a fatal myeloproliferative disorder in the setting of an Mx1-*cre* allele (Braun et al., 2004; Chan et al., 2004) (see Supplemental Data at <http://www.cancercell.org/cgi/content/full/5/4/375/DC1>).

To investigate whether *ras*-induced senescence occurs following *K-ras<sup>G12D</sup>* expression in vivo, lung, colonic, and pancreatic tissue was immunohistochemically evaluated for the levels of p53, p16Ink4a, p19ARF, and p21 proteins. Neither normal nor hyperplastic portions of epithelial tissue contained detectable levels of these proteins, suggesting that *ras*-induced senescence was not occurring by these criteria in vivo (D.A.T., unpublished data). An additional approach was undertaken





**Figure 7.** Embryologic consequences of expressing endogenous levels of oncogenic *K-ras*<sup>G12D</sup> in the mouse germline

**A:** Normal external appearance of wild-type 9.5 day old (E9.5) embryos. Original magnification 60 $\times$ .

**B:** Marked developmental abnormalities of *Lox-K-ras*<sup>G12D</sup> E9.5 embryos. Compared to its littermate in **A**, the mutant embryo is smaller and pale, with an open neural tube and cardiomegaly. Same magnification as in **A**.

**C:** Normal histology of wild-type E9.5 embryos. Shown is an H&E stained parasagittal section. Original magnification 40 $\times$ .

**D:** Histological analysis of *Lox-K-ras*<sup>G12D</sup> E9.5 mouse embryos. H&E staining of parasagittal sections reveals areas of gross morphologic changes in the heart and developing neuraxis. Same magnification as in **C**.

**E:** High-power magnification (1000 $\times$ ) of neuroepithelium from the wild-type E9.5 embryo shown in **C**.

**F:** High-power magnification (1000 $\times$ ) of neuroepithelium from the mutant E9.5 embryo shown in **D**. Note the abundant mitotic figures (arrows) and numerous pyknotic and fragmented nuclei characteristic of apoptotic cell death (arrow heads).

Abbreviations: H, heart; FB, forebrain; MB, midbrain; HB, hindbrain; O, otic pit; S, somites; SC, spinal cord.

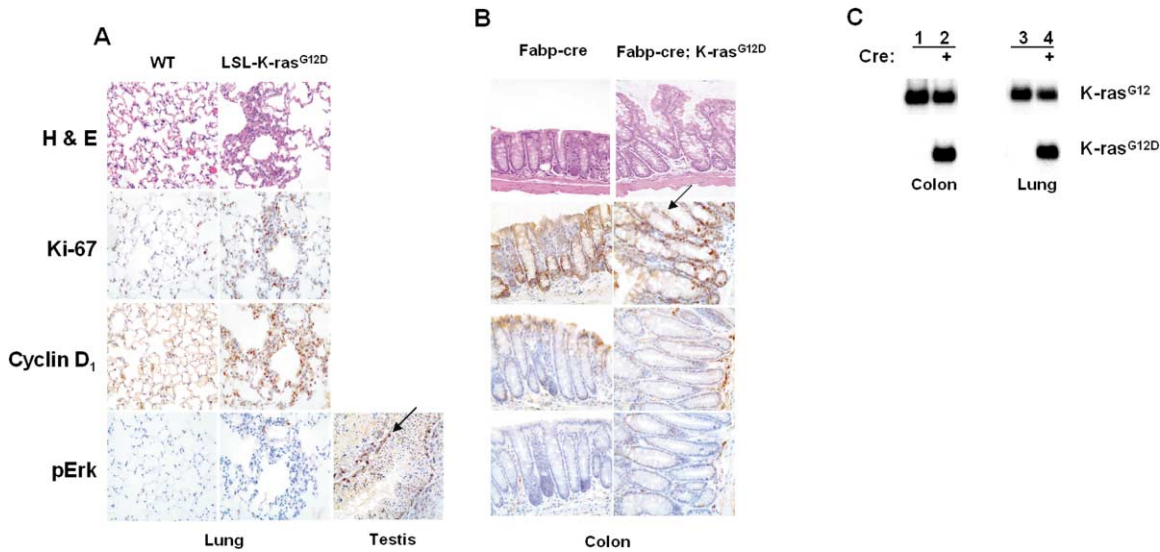
with pancreatic lysates prepared from bitransgenic *LSL-K-ras*<sup>G12D</sup>;*p48*<sup>+cre</sup> mice. These mice express oncogenic *K-ras* in every epithelial cell of the pancreas, and the direct assessment of p53, p16Ink4a, and p21 levels failed to reveal any increased levels of these proteins (Supplemental Figure S5), suggesting that *ras*-induced senescence is not a prominent feature of this model. Collectively, these results demonstrate that the endogenous expression of *K-ras*<sup>G12D</sup> *in vivo* confers significantly different properties in most tissues than the endogenous expression of *K-ras*<sup>G12V-IRES-BGeo</sup>, even in tissues that do not normally harbor *K-ras* mutations in human tumors, such as the salivary gland.

## Discussion

### Enhanced proliferation and partial transformation by endogenous *K-ras*<sup>G12D</sup>

We show that the endogenous expression of *K-ras*<sup>G12D</sup> stimulates the proliferation of MEFs in contrast to the growth arrest observed in primary cells expressing ectopic oncogenic *ras* (Serrano et al., 1997). *Lox-K-ras*<sup>G12D</sup> MEFs have elevated levels of Cdk2, cyclin A, cyclin E, and cyclin D1, and increased Cdk2 and Cdk4 activities *in vitro*, consistent with the augmented proliferation of these cells. Conversely, the growth arrest induced in primary cells by ectopically expressed oncogenic *ras* is characterized by decreased Cdk2 activity in the setting of decreased cyclin A levels and elevated cyclin D1, p21, and p16 levels (Serrano et al., 1997). However, overexpressed *H-ras*<sup>G12V</sup> also induces growth arrest in p21<sup>-/-</sup>, p21<sup>-/-</sup>;p27<sup>-/-</sup>, and p16<sup>-/-</sup> primary MEFs (Groth et al., 2000; Pantoja and Serrano, 1999; Sharpless et al., 2001), suggesting the involvement of still other mediators in oncogenic *ras*-induced growth arrest. Indeed *p19ARF*, a gene expressed in response to ectopically introduced mutant *ras* and other oncogenes (de Stanchina et al., 1998; Zindy et al., 1998), activates p53 through the inhibition of mdm2 (Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998) and causes growth arrest in both cyclin D/Cdk4-dependent and -independent fashions (Groth et al., 2000). Furthermore, *p19ARF* is required for ectopic oncogenic *ras*-induced senescence (Kamijo et al., 1997; Lowe and Sherr, 2003). Notably, *Lox-K-ras*<sup>G12D</sup> MEFs have lower p19ARF and p53 levels compared to MEFs overexpressing oncogenic *ras*, and *Lox-K-ras*<sup>G12D</sup> MEFs are not growth arrested but rather demonstrate increased proliferation in limiting serum and at high cell density.

The loss of contact inhibition is an archetypal characteristic of fully transformed cells (Stoker and Rubin, 1967), and is well documented in both immortal cell lines following the introduction of oncogenic *ras* (reviewed in Malumbres and Barbacid, 2003) and primary cells cotransfected with oncogenic *ras* and *c-Myc* (Land et al., 1983). The *Lox-K-ras*<sup>G12D</sup> MEFs described here support focus formation, yet are very different from previously described *ras*-transformed cells in several ways. First, in contrast to ectopic oncogenic *ras*-transformed cells, *Lox-K-ras*<sup>G12D</sup> MEFs cannot form colonies when cultured at low density or when grown in semisolid media, and they are not tumorigenic. Second, the subculturing of "foci" from cell layers did not reveal any subpopulations of cells that were fully transformed (data not shown) and that could represent clonal events such as biallelic *p19ARF* loss or *p53* mutation (Zindy et al., 1998). Finally, since *Lox-K-ras*<sup>G12D</sup> MEFs grew to very high densities, we hypothesized that focus formation was an intrinsic characteristic of all MEFs that express endogenous levels of *K-ras*<sup>G12D</sup> in the presence of functional p19ARF and p53 pathways, and assessed the frequency of focus formation through the serial dilution of *Lox-K-ras*<sup>G12D</sup> MEFs in coculture experiments with a fixed number of wild-type MEFs. These mixing experiments revealed a frequency of focus formation of 1%–2% for *Lox-K-ras*<sup>G12D</sup> MEFs, and an only slightly higher frequency of 4%–5% for fully transformed *Lox-K-ras*<sup>G12D</sup>;p53<sup>-/-</sup> MEFs (data not shown). Taken together, these results suggest that the formation of foci is a cell autonomous property of *Lox-K-ras*<sup>G12D</sup> MEFs and does not represent individual clonal events.



**Figure 8.** Proliferative and signaling properties of *K-ras*<sup>G12D</sup>-expressing hyperplasias

**A:** Pulmonary hyperproliferative lesions (hematoxylin & eosin, 200 $\times$ ) in *LSL-K-ras*<sup>G12D</sup> mice 2 weeks after infection with adenoviral-cre demonstrate increased intranuclear Ki-67 and cyclin D1 levels but not phosphorylated ERK 1/2 compared to similarly treated wild-type mice. Testicular tissue demonstrates primitive spermatogonia and primary spermatocytes with elevated phosphorylated ERK 1/2 (arrow).

**B:** Hyperplastic and dysplastic colonic epithelium in bitransgenic *Fabp-cre;LSL-K-ras*<sup>G12D</sup> mice but not *Fabp-cre* mice. Increased numbers of Ki-67 positive nuclei are found away from the crypts of the bitransgenic mice (arrow). Neither cyclin D1 nor phosphorylated ERK 1/2 could be detected in either genetic background.

**C:** Molecular evidence of *K-ras*<sup>G12D</sup> mRNA expression in target tissues. Colonic tissue was isolated from *LSL-K-ras*<sup>G12D</sup> (lane 1) and *LSL-K-ras*<sup>G12D</sup>;*Fabp-cre* mice (lane 2), and lung tissue was isolated from *LSL-K-ras*<sup>G12D</sup> mice before (lane 3) and 4 weeks after (lane 4) adenoviral-cre infection. RNA was prepared, reverse transcribed, amplified by PCR, and probed with *K-ras*<sup>G12</sup> or *K-ras*<sup>G12D</sup> probes.

### Absence of premature senescence and Ras effector pathway modulation in *Lox-K-ras*<sup>G12D</sup> MEFs

Oncogenic *ras*-induced premature senescence is caused by the sustained hyperactivation of the MAPK pathway (Lin et al., 1998; Zhu et al., 1998). In this study, we demonstrate that *K-ras*<sup>G12D</sup>-expressing MEFs and epithelial hyperplasias exhibit neither features of premature senescence nor evidence of MAPK hyperactivation, but rather MAPK cascade attenuation. Additionally, although *Lox-K-ras*<sup>G12D</sup> MEFs are "immortal" when assessed by serial passaging, they remain susceptible to premature senescence when transduced with oncogenic *H-ras* or *K-ras*, and such senescence is accompanied by hyperactivation of the MAPK pathway. The dependency of both premature senescence and hyperactivation of the MAPK pathway on supraphysiological expression levels of oncogenic *ras* suggests that neither may be important aspects of endogenous oncogenic *ras* function (Lowe and Sherr, 2003). Finally, the lack of increased levels of p53, p16Ink4a, p19ARF, and p21 in lung, colon, and pancreatic tissues suggests that the predominant response in epithelial cells that express the *K-ras*<sup>G12D</sup> allele is to initiate proliferation and not senescence in vivo.

The Ras effector pathways have previously been examined almost exclusively in the context of transient transfection experiments that employed supraphysiological levels of oncogenic Ras. In contrast with the ease of identifying such signaling events following the ectopic expression of oncogenic *ras*, evidence of Ras effector pathway activation in *Lox-K-ras*<sup>G12D</sup> MEFs was detected only after employing specific pharmacological inhibitors of MEK and PI3K and assessing nuclear extracts for ELK-1 activity. Interestingly, the activation of ERK and AKT

following serum starvation and restimulation was suppressed in *Lox-K-ras*<sup>G12D</sup> MEFs, but not in MEFs ectopically overexpressing *H-ras*<sup>G12V</sup>, suggesting that the MAPK and PI3K cascades can be attenuated only when oncogenic *ras* is expressed at physiological levels and offering an explanation for the previous difficulty in detecting pathway activation in *Lox-K-ras*<sup>G12D</sup> MEFs. The inability to detect elevations of phosphorylated ERK 1/2 levels was not restricted to *Lox-K-ras*<sup>G12D</sup> MEFs, as *K-ras*<sup>G12D</sup>-expressing pulmonary and colonic epithelial hyperplasias also lacked immunohistochemical evidence of this activated signaling intermediate. Currently, the mechanisms of Ras effector pathway attenuation are unclear, and preliminary investigations have not revealed any differences in the protein levels of Raf, MEK, ERK, PI3-kinase, or AKT. Collectively, our data demonstrate that Ras effector pathway signaling that accompanies endogenous expression levels of oncogenic *K-ras*<sup>G12D</sup> is not reliably assessed by the isolated analysis of phosphorylated ERK or phosphorylated AKT levels, a finding that may be relevant to the evaluation of Ras pathway inhibitors in clinical trials.

### Initiation of transformation by endogenous *K-ras*<sup>G12D</sup> may not require additional genetic events

Transformation of primary murine fibroblasts with ectopically expressed oncogenic *ras* requires a cooperating oncogene (Land et al., 1983; Ruley, 1983). Our evidence, however, suggests that the endogenous expression of *K-ras*<sup>G12D</sup> alone is sufficient to initiate the transformation of MEFs in cell culture and to stimulate epithelial proliferation in vivo within several days after the action of cre recombinase. First, the onset of *K-ras*<sup>G12D</sup> expression coincides with morphological alterations and en-

hanced proliferation in MEFs and alveolar epithelial cells (Figure 1 and Supplemental Figure S2). Second, spectral karyotyping (SKY) analysis was undertaken on *Lox-K-ras<sup>G12D</sup>* MEFs, since karyotypic alterations such as *c-Myc* translocation have been described in other immortal cell lines (Elenbaas et al., 2001). Importantly, SKY revealed normal cytogenetic profiles in all ten metaphase spreads examined from *Lox-K-ras<sup>G12D</sup>* MEFs (data not shown). Third, because ectopically expressed oncogenic *ras* is known to cooperate with *p19ARF* loss (Kamijo et al., 1997) or *p53* mutation (Tanaka et al., 1994), the integrity of these pathways was assessed in *K-ras<sup>G12D</sup>* MEFs. Examination of *Lox-K-ras<sup>G12D</sup>* MEFs failed to reveal functional deficiencies in *p19ARF* or *p53* in response to oncogenic *ras* overexpression or genotoxic stress, respectively. Although we cannot exclude the possibility that undetectable genomic damage due to cre recombinase contributes to the cellular phenotypes we describe here, this is unlikely due to the absence of heterogeneous behavior in the *Lox-K-ras<sup>G12D</sup>* MEFs, and the lack of similar phenotypes in control MEFs infected with viral-cre.

### Oncogene cooperativity and tumorigenesis

The premise that *ras* gene mutations play a pivotal role in cellular transformation and tumorigenesis is based upon the high frequency of *ras* mutations in human cancer and the transforming capability of mutant *ras* in immortal cell lines. The landmark studies describing oncogene cooperativity demonstrated a requirement for both *ras* and either *c-Myc* or *E1A* to transform primary cells (Land et al., 1983; Ruley, 1983). However, *c-Myc* appears to select for *p53* deficiency and thereby enables overexpressed *ras* to induce proliferation rather than growth arrest (Zindy et al., 1998); *E1A* may function similarly (de Stanchina et al., 1998). Indeed, in the presence of an intact *p19ARF/p53* pathway, high levels of *ras* expression induce senescence (Serrano et al., 1997). We find that the lower, physiological levels of mutant Ras do not activate the *p19ARF/p53* pathway to the same extent as primary cells overexpressing oncogenic *ras*, and can instead promote proliferation in primary fibroblasts in culture and epithelial cells in vivo despite a lack of obvious cooperating events. Thus, cooperativity may be a requirement only in the context of *ras* overexpression. These findings explain how point mutated *ras* can serve as an initiating event in human malignancy, and, while less common, *ras* gene amplification or overexpression would occur later in tumor progression, as indeed appears to be the case (Bos, 1988).

While this manuscript was in preparation, Guerra and colleagues reported that an endogenous *K-ras<sup>G12V-IRES-BGeo</sup>* allele could immortalize MEFs and stimulate the production of pulmonary hyperplasias and neoplasias (Guerra et al., 2003). Significant differences exist between our findings and the report of Guerra and colleagues. First, we describe morphological alterations, focus formation, and cooperation with *E1a* in *K-ras<sup>G12D</sup>*-expressing MEFs, whereas these phenotypes were absent in *K-ras<sup>G12V-IRES-BGeo</sup>*-expressing MEFs. Second, the *Lox-K-ras<sup>G12D</sup>* MEFs have enhanced proliferative properties with accompanying Cdk2 and Cdk4 cyclin-dependent kinase complex activation, whereas these properties were not demonstrated by Guerra and colleagues. Third, *Lox-K-ras<sup>G12D</sup>* MEFs demonstrate the attenuation of MAPK and PI3K activation following serum stimulation, whereas *K-ras<sup>G12V-IRES-BGeo</sup>* MEFs demonstrate activation of these signaling pathways in response to EGF. Finally, a thematic message of Guerra and colleagues is that endogenous

*K-ras<sup>G12V</sup>* expression alone is tolerated with no overt consequences in most tissues, including the colonic and pancreatic epithelium. While we cannot exclude that oncogenic *K-ras* expression may be tolerated in certain tissues, we find that widespread expression of *K-ras<sup>G12D</sup>* during embryogenesis is not tolerated, whereas the expression of *K-ras<sup>G12V-IRES-BGeo</sup>* does not preclude embryonic development. Also, we demonstrate that expression of the *K-ras<sup>G12D</sup>* allele in colonic epithelium causes epithelial hyperplasia and dysplasia with total penetrance, and recently reported that expression of *K-ras<sup>G12D</sup>* in pancreatic epithelium induces pancreatic intraepithelial neoplasms (Hingorani et al., 2003), whereas Guerra and colleagues could only show pancreatic ductal metaplasia in cooperation with the *CDK4<sup>R24C</sup>* mutation. Finally, the analysis of the expression of *K-ras<sup>G12D</sup>* in multiple other tissue types suggests that this allele has proliferative effects in additional tissues such as the salivary gland, skin, and hematopoietic system (Braun et al., 2004; Chan et al., 2004). Possible explanations for the discrepancy between our two studies include different molecular properties between the *K-ras<sup>G12V</sup>* and *K-ras<sup>G12D</sup>* proteins, and differences in genetic backgrounds. An additional possibility is the altered regulation of expression and splicing of the *K-ras<sup>G12V-IRES-BGeo</sup>* allele as compared to the *K-ras<sup>G12D</sup>* allele, since the former allele is a bicistronic allele and contains a 3' *BGeo* reporter transgene. Future investigations will be needed to address these possibilities.

Numerous models of cellular transformation and cancer have been developed through the introduction of oncogenes into cells and animals. The ability of these systems to faithfully model human disease, however, depends critically on recapitulating physiological conditions as closely as possible. To this end, systems relying on ectopic overexpression of oncogenes may activate or suppress pathways not normally involved in cognate human conditions harboring the very same endogenous mutations and may therefore be more obfuscatory than revealing. In this study, we have found that physiological expression of an activating *K-ras<sup>G12D</sup>* mutation can by itself partially transform MEFs and initiate tumorigenesis in animals. Our results contrast both with the presumed requirement for oncogene cooperativity to achieve such effects, and the reported induction of senescence in primary cell cultures by ectopic expression of activated *ras*. We propose, therefore, that the specific patterns of activated and suppressed signaling pathways in this model will more closely resemble those occurring in human malignancies containing *K-ras* mutations. Moreover, the lessons learned here may also be applicable to studies of other oncogenes.

### Experimental procedures

#### Mouse strains and tumor models

The *LSL-K-ras<sup>G12D</sup>* strain was interbred to Prmc1 mice (O'Gorman et al., 1997) and *CMV-cre* mice (Schwenk et al., 1995) to evaluate the effects of *K-ras<sup>G12D</sup>* expression during development. Also, *LSL-K-ras<sup>G12D</sup>* mice were infected with aerosolized adenoviral-cre to produce pulmonary epithelial hyperplasias (Jackson et al., 2001), and interbred to *Fabp-cre* mice (Saam and Gordon, 1999), *K14-cre* and *K14-creER* mice (Vasioukhin et al., 1999), and *MMTV-cre* mice (Wagner et al., 1997) to generate epithelial hyperplasias in bitransgenic mice.

#### MEFs

Early passage MEFs (p3-4) were used at the initiation of all experiments. *LSL-K-ras<sup>G12D</sup>* mice were successively crossed to *p53<sup>+/-</sup>* mice to generate *LSL-K-ras<sup>G12D</sup>;p53<sup>-/-</sup>* MEFs. Mice and MEFs were on a mixed 129 Sv/C57BL/6 background. MEFs were grown in 10% FCS/DMEM/25 mM HEPES

and serum starved overnight in the same media supplemented with 0.1% FCS.

#### Proliferation, transformation, and senescence assays

Proliferation, 3T3, focus formation, soft agar, and nude mouse tumorigenesis assays were as described (Sage et al., 2000). SA $\beta$ -gal assays were as described (Dimri et al., 1995), with over 300 cells evaluated in total for each case. Cell cycle analysis was performed on subconfluent early passage MEFs as described (Serrano et al., 1997). Mixing experiments to determine the frequency of focus formation were performed by mixing serial dilutions of *Lox-K-ras*<sup>G12D</sup> MEFs with 300,000 lethally irradiated wild-type MEFs and culturing on 6 cm dishes for 3 weeks before staining with Giemsa and counting the number of foci.

Please see the Supplemental Data at <http://www.cancer.org/cgi/content/full/5/4/375/DC1> for details on immunoblotting, immunohistochemistry and cytochemistry, biochemical assays, cDNA clones, Southern blotting and modified Northern blotting analysis of rearranged LSL-K-ras allele, and SKY analysis.

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