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Interactions Between Wildtype and Mutant *Ras* Genes in Lung and Skin Carcinogenesis

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Abstract

Ras oncogenes (*Hras*, *Kras*, and *Nras*) are important drivers of carcinogenesis. However, tumors with *Ras* mutations often show loss of the corresponding wildtype (*WT*) allele, suggesting that proto-oncogenic forms of *Ras* can function as a suppressor of carcinogenesis. *In vitro* studies also suggest that WT Ras proteins can suppress the tumorigenic properties of alternate mutant Ras family members, but *in vivo* evidence for these heterologous interactions is lacking. We have investigated the genetic interactions between different combinations of mutant and *WT Ras* alleles *in vivo* using carcinogen-induced lung and skin carcinogenesis in mice with targeted deletion of different *Ras* family members. The major suppressor effect of *WT Kras* is observed only in mutant *Kras*-driven lung carcinogenesis, where loss of one *Kras* allele led to increased tumor number and size. Deletion of one *Hras* allele dramatically reduced the number of skin papillomas with *Hras* mutations, consistent with *Hras* as the major target of mutation in these tumors. However, skin carcinoma numbers were very similar, suggesting that WT *Hras* functions as a suppressor of progression from papillomas to invasive squamous carcinomas. In the skin, the *Kras* proto-oncogene functions cooperatively with mutant *Hras* to promote papilloma development, although the effect is relatively small. In contrast, the *Hras* proto-oncogene attenuated the activity of mutant *Kras* in lung carcinogenesis. Interestingly, loss of *Nras* increased the number of mutant *Kras*-induced lung tumors but decreased the number of mutant *Hras*-induced skin papillomas. These results show that the strongest suppressor effects of WT Ras are only seen in the context of mutation of the cognate Ras protein, and only relatively weak effects are detected on tumor

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development induced by mutations in alternative family members. The data also underscore the complex and context-dependent nature of interactions between proto-oncogenic and oncogenic forms of different *Ras* family members during tumor development.

Keywords

Ras; Hras; Kras; Nras; skin carcinogenesis; lung carcinogenesis

Introduction

Mutational activation of the *RAS* family of genes is one of the most common oncogenic events in cancer, occurring in ~30% of human solid tumors. Studies in the mouse have demonstrated that expression of mutant *Ras* results in tumor development in a range of tissues, underscoring the potency of *Ras* oncogenes as drivers of tumorigenesis. There is, however, a high degree of specificity with respect to which *RAS* gene family member is mutated in different tumor types[1]. While mutations in *KRAS* are common in lung, colon and pancreatic cancers, those in *NRAS* predominate in melanoma, and *HRAS* mutations are commonly seen in bladder, head and neck, and skin cancers [1]. Strong tissue-specific mutation patterns are also seen in mouse models of cancer. Chemically induced tumors in mouse lung and skin show complete specificity for mutations involving *Kras* and *Hras*, respectively [2, 3]. Differences in the regulation of expression play an important role in determining *Ras* mutation specificity, as insertion of *Hras* into the *Kras* locus in *Hras* knock-in mice demonstrated the capability of *Hras* to induce lung tumors *in vivo*, in spite of the complete specificity for *Kras* mutations in lung tumors from *WT* mice [4, 5].

RAS genes encode small GTPases that cycle between active (GTP-bound) and inactive (GDP-bound) states in response to extracellular cues. In their active conformations RAS proteins engage and activate effectors that include RAF, phosphatidylinositol 3-kinase (PI3K) and Ral guanine-dissociation stimulator (RalGDS), to regulate diverse cellular functions including cell growth, proliferation, and differentiation. *RAS* activating mutations found in tumors occur predominantly at codons 12, 13 and 61, and have historically been viewed as functionally dominant because they render RAS proteins constitutively active. However, the status of the *WT RAS* allele may also play a role in tumors carrying mutant *RAS* genes. Studies in the mouse showed that skin tumors initiated by somatic activating mutations in *Hras* are frequently accompanied by an increase in copy number of the mutant allele or loss of the *WT* allele [6]. Furthermore, these genomic events contribute to the progression of squamous carcinomas to more invasive tumors [7]. Mutant *KRAS* alleles are also often expressed at higher levels compared to the *WT* allele in human lung tumors, likely as a result of preferential amplification of the mutant copy of the gene [8]. We and others have shown that copy number alteration involving the *Kras* locus on chromosome 6 is the earliest and most common somatic genetic event in mouse lung tumors initiated by oncogenic *Kras* [9, 10]. Finally, genetic and *in vitro* functional studies have shown that WT Kras can functionally suppress the oncogenic activity of mutant Kras through mechanisms that remain to be elucidated [4, 11, 12]. Therefore, the imbalance in favor of mutant *Ras* alleles in tumors is

compatible with the requirement of tumor cells to overcome the suppressor effects of the respective WT *Ras*.

Members of the *Ras* family of genes share extensive sequence identity with one another, and are ubiquitously expressed, albeit at varying levels. In addition to the respective WT counterpart, the oncogenic activity of one mutant Ras protein may be further modulated by other members of this gene family. Data from *in vitro* studies suggest complex interactions among *Ras* oncogenes and proto-oncogenes [13], but these studies involved ectopic overexpression of *Ras* alleles and relied on reporter systems as functional readouts. Therefore, it is not clear to what extent these interactions actually contribute to the cancer phenotype. To address this question, we took advantage of mouse models of lung and skin cancers to study the *in vivo* effects of *Ras* proto-oncogenes on mutant *Ras*-driven carcinogenesis. We found that the same *Ras* proto-oncogene could have positive or negative effects on mutant *Ras*-driven carcinogenesis, depending on the tumor type and/or the mutant *Ras* oncogene. These findings suggest that the interactions between *Ras* oncogenes and proto-oncogenes during carcinogenesis are complex and context-dependent.

Results

Chemical carcinogenesis has been widely used to study tumorigenesis and to identify important genetic determinants of this process. Mice treated with a single dose of urethane by intra-peritoneal injection develop multiple lung tumors, the majority of which contain an activating mutation at codon 61 of the *Kras* gene [4, 12]. On the other hand, topical application of a single dose of 7,12-dimethylbenz(a)anthracene (DMBA) to dorsal skin followed by promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) results in the development of skin tumors, the majority of which harbor a codon 61 activating mutation in the *Hras* gene [2]. We have used these chemical carcinogenesis models to assess the effects of targeted deletions of *Ras* gene family members on lung and skin tumor development. All studies were conducted in the *FVB/N* strain of mice, and on this background, *Hras*, *Kras* and *Nras* are highly expressed in both skin and lung (Supplementary Table 1). While levels of *Nras* are similar in both tissues, *Kras* is more highly expressed in the lung and *Hras* in the skin. These differences in expression levels could potentially account, at least in part, for the specificity of *Ras* mutations in tumors of these tissues.

Deletion of Kras enhances lung but attenuates skin tum,or development

Homozygous deletion of *Kras* results in embryonic lethality, but mice with one functional copy of the *Kras* gene are viable and have no apparent developmental defects [14]. As a surrogate for a conventional *Kras* null allele, we used the *LSL-Kras*^{G12D} allele, which we backcrossed into the *FVB/N* genetic background. The *LSL-Kras*^{G12D} allele contains a transcriptional termination STOP element which renders the allele nonfunctional until removed by Cre recombinase [15, 16]. We found *Kras* levels in mice heterozygous for the *LSL-Kras*^{G12D} (*Kras*$^{LSL/WT}$) allele reduced by approximately 2-fold, both in RNA (Figure 1a) and protein (Figure 1b), compared to *WT* animals. While we cannot completely rule out the possibility of some level of transcriptional leakage, we noted that animals containing the

LSL-Kras^{G12D} allele did not develop any lung tumor, or tumors in other tissues, without administration of adeno-Cre.

Kras is the major mutational target in carcinogen-induced lung tumors [4, 12], but *Kras*$^{LSL/WT}$ mice containing only one functional *Kras* allele developed almost 2-fold more lung tumors (18.4 ± 6.0, mean ± s.d.) than *WT* littermates (10.3 ± 3.0) with two functional *Kras* alleles ($P = 1.4 \times 10^{-5}$) (Figure 1c). We also observed that tumors from *Kras*$^{LSL/WT}$ animals were larger in size (data not shown). As expected, more than 95% of lung tumors from mice of both genotypes contained *Kras* codon 61 activating mutations (Supplementary Table 2). These codon 61 mutations must occur on the functional *Kras* allele in tumors from heterozygous mice, given that the *LSL-Kras*^{G12D} allele is nonfunctional. These observations in the *LSL-Kras*^{G12D} model are in concordance with previous studies using conventional *Kras* null alleles [11], emphasizing the suppressor function of *WT Kras* during lung carcinogenesis.

While *WT Kras* can suppress the activity of mutant *Kras*, it is not known whether WT *Kras* exerts a similar effect on cancers driven by mutant forms of other *Ras* gene family members. To address this question we treated *Kras*$^{LSL/WT}$ and *WT* mice with DMBA/TPA to induce skin tumor development. Skin papillomas that developed in both groups of mice contained the codon 61 activating mutation in *Hras*, as expected (Supplementary Table 3). There was however a ~30% reduction in number of papillomas in *Kras*$^{LSL/WT}$ mice compared to *WT* littermates (17.1 ± 8.9 vs. 23.7 ± 6.9, P = 0.012) (Figure 1d). These data suggest that in the skin *Kras* functions cooperatively with mutant *Hras* to drive the formation of papillomas.

Deletion of Hras suppresses skin but potentiates lung tumor development

Hras is the major target of mutation in skin tumors induced by DMBA/TPA treatment [2]. A previous study used mice of a mixed genetic background (*129/Sv*, *C57BL/6*, and *DBA/2*) to study the effect of *Hras* deletion on skin tumor development [17]. Although *C57BL/6* is highly resistant to DMBA/TPA skin carcinogenesis and developed only a modest number of papillomas in *WT* mice (~16 tumors/mouse), it was found that *Hras*$^{KO/KO}$ animals developed significantly fewer tumors (~3 tumors/mouse) [17]. Because genetic background can affect tumor development as well as pattern of genetic alterations in tumors [10, 18-20], we backcrossed the *Hras*^{KO} allele into the *FVB/N* background for more than 15 generations. *FVB/N* mice are highly susceptible to the development of epithelial tumors, and particularly skin tumors [21]. In this genetic background, the difference in papilloma number between *WT* and *Hras*$^{KO/KO}$ littermates was much more dramatic (Figure 2a). Whereas *WT* mice developed between 18-41 papillomas (29.3 ± 6.0), *Hras*$^{KO/KO}$ mice developed almost 30-fold fewer papillomas (1.3 ± 1.3, $P = 1.3 \times 10^{-19}$), compared to the ~6-fold difference in the previous study, and with more than 65% of animals having only one or no tumors. Mice heterozygous for *Hras* (*Hras*$^{KO/WT}$) showed skin tumor numbers (15.3 ± 5.4) intermediate to those of *WT* and *Hras*$^{KO/KO}$ animals, indicating a clear gene-dosage effect on skin papilloma development.

As expected, papillomas from *WT* mice had a 100% incidence of *Hras* mutations at codon 61 (Supplementary Table 3). The majority of papillomas (8 of 10) from *Hras*$^{KO/WT}$ mice

also contained the codon 61 activating *Hras* mutation. In contrast, of the 6 papillomas from $Hras^{KO/KO}$ mice that were available for analysis, 5 (83%) had activating mutations in *Kras*, at codons 13 or 61. We found levels of Kras protein, but not Nras protein, to be higher in the skin of $Hras^{KO/KO}$ mice compared to *WT* animals (Figure 2b), possibly contributing to the preference for *Kras* mutations in papillomas from $Hras^{KO/KO}$ mice. These data demonstrate that while oncogenic *Kras* can functionally replace mutant *Hras* during skin carcinogenesis, the preference is clearly in favor of *Hras* in mice with functional *Hras* alleles.

In the lung, mutational activation of *Kras* occurs in the majority of tumors induced by urethane [4, 12]. To determine whether *Hras* levels influence lung tumor development, we treated *WT*, $Hras^{KO/WT}$, and $Hras^{KO/KO}$ littermates with urethane and determined their lung tumor number after 20 weeks. Mice of all three genotypes developed multiple lung tumors, and molecular analyses showed that *Kras* mutations are common in these tumors and occur at similar frequencies in all three genotype groups (Supplementary Table 2). However, we observed a relatively modest but statistically significant increase in lung tumor number in $Hras^{KO/WT}$ (10.2 ± 3.6, P = 0.04) and $Hras^{KO/KO}$ (10.5 ± 3.0, P = 0.01) mice compared to *WT* (8.0 ± 3.0) animals (Figure 2c). Unlike in the skin, we did not detect a change in the level of Kras protein in the lungs of $Hras^{KO/KO}$ mice that could potentially have accounted for the increase in lung tumor number (Figure 2d). Nevertheless, these data suggest that *Hras* negatively modulates the oncogenic activity of mutant *Kras* during lung carcinogenesis.

Hras heterozygosity increases progression rate of papillomas to squamous carcinomas

Genomic imbalances favoring the mutated *Ras* allele are observed in lung and skin tumors, suggesting that the WT counterpart of the mutant Ras protein functions as a suppressor of carcinogenesis in both tissues. Heterozygosity at the *Kras* locus indeed resulted in a significant increase in number of carcinogen-induced lung tumors (Figure 1c). In contrast, mice heterozygous for *Hras* developed fewer papillomas than WT mice (Figure 2a). Because genomic imbalances at the *Hras* locus are common in invasive squamous carcinomas [6, 7], we speculated that these genetic events might be contributing to skin tumor progression. Papillomas are thought to be precursors to invasive carcinomas [22], and previous studies have used the ratio of carcinomas to papillomas to measure the rate of malignant progression [23]. We therefore monitored $Hras^{KO/WT}$ and *WT* mice up to one year for the occurrence of carcinomas. Although $Hras^{KO/WT}$ mice developed almost 2-fold fewer papillomas than *WT* mice, we found the incidence and time of onset of carcinomas to be very similar between both groups (P = 0.18, Kaplan-Meier analysis). In addition, carcinomas from both *WT* and $Hras^{KO/WT}$ mice have the activating mutation at codon 61 of *Hras* (Supplementary Table 4). However, $Hras^{KO/WT}$ mice had a significantly higher rate of malignant progression compared to *WT* mice (Table 1, P = 0.046, Fisher's exact test). These data suggest that WT Hras functions as a suppressor of skin tumor progression rather than initiation, and that its loss, either through genetic manipulation of the mouse germline or somatic genetic alterations in tumor cells, promotes the conversion of benign skin papillomas to invasive squamous carcinomas.

Opposing effects of Nras deletion on lung and skin tumor development

Genetic studies in mice have shown that the *Nras* proto-oncogene can suppress the malignant phenotype of thymic lymphomas driven by its oncogenic counterpart [24]. However, it is not known whether *Nras* exerts similar effects on the development of tumors driven by mutant *Kras* or mutant *Hras*. To address these questions, we subjected $Nras^{WT/WT}$, $Nras^{KO/WT}$, and $Nras^{KO/KO}$ littermates to the urethane protocol to induce lung tumors, and to the DMBA/TPA protocol to induce skin tumors.

Urethane induced lung tumors from $Nras^{WT/WT}$, $Nras^{KO/WT}$, and $Nras^{KO/KO}$ mice all contained activating mutation at codon 61 of *Kras* (Supplementary Table 2). However, we observed an increase in lung tumor number in both $Nras^{KO/WT}$ (11.1 ± 2.9, P = 0.015) and $Nras^{KO/KO}$ (12.5 ± 5.0, P = 0.008) mice compared to $Nras^{WT/WT}$ mice (8.4 ± 3.3) (Figure 3a). $Nras^{KO/KO}$ developed more tumors than $Nras^{KO/WT}$, but the difference was not statistically significant. These data suggest that the *Nras* proto-oncogene attenuates the oncogenic activity of mutant *Kras* during lung tumor development.

The status of *Nras* had no effect on the frequency of *Hras* mutations in papillomas, as all tumors analyzed contained the codon 61 *Hras* mutation (Supplementary Table 3). However, both $Nras^{KO/WT}$ (24.6 ± 6.9, P = 0.002) and $Nras^{KO/KO}$ (24.3 ± 7.5, P = 0.02) and mice developed fewer papillomas compared to *WT* mice (31.7 ± 6.8) (Figure 3b). While *Nras* has a negative role in lung carcinogenesis, these data show that in the skin it has a positive effect on mutant *Hras*-induced carcinogenesis.

Discussion

We have investigated the genetic interactions between proto-oncogenic and oncogenic forms of *Ras* family members during the process of tumor development in the lung and the skin. Several studies have noted that *WT* alleles of various members of the *Ras* family are lost in tumors carrying mutations of the same *Ras* family member. The observation that *Ras* genes appear to be co-expressed, albeit at different levels, in mouse and human tissues raised the possibility that WT proteins of one isoform may suppress mutant Ras activity driven by a different isoform. Previous studies aimed at addressing this question were carried out using transfection assays with resultant expression of Ras proteins at non-physiological levels [13]. We therefore initiated this study to investigate interactions between the different *Ras* family members in an *in vivo* context. The use of chemical carcinogenesis to induce tumor formation in the mouse mimics the effects of carcinogen exposure in human cancer development and also recapitulates the specificity of *Ras* mutations observed in human cancers. Our data show that the major effect of loss of *WT Kras* is seen only in lung tumors driven by mutant *Kras*, and only marginal effects are seen due to altered gene dosage of other *Ras* family members.

Mice with only one functional copy of *Kras* developed more and larger lung tumors than *WT* littermates, consistent with the notion that the remaining *WT* copy of *Kras* is a potent suppressor of lung tumor formation and progression [11]. This is compatible with the observation that human and murine lung tumors with *Kras* mutations frequently have copy number alterations involving the *Kras* locus [8-10], likely as a mechanism to shift the balance

in favor of mutant *Kras*. The molecular mechanism that underlies the suppressor function of WT Kras is not known, but could potentially involve competition for common downstream effectors [11]. Alternatively, signaling through WT Kras could occur independently of mutant Kras in the lung to trigger cellular functions that have evolved as protective mechanisms against oncogenic conditions. We recently showed that mutational activation of *Kras4A*, the minor isoform of *Kras*, is necessary for lung tumor development, and that this isoform is also responsible for the suppressor function of WT Kras [4]. Detailed functional analyses of Kras4A could provide important mechanistic insights into the oncogenic and suppressor functions of Kras.

We found papilloma numbers to directly correlate with the number of functional *Hras* alleles, compatible with *Hras* as the target of mutation in these tumors. As previously noted [17], animals that completely lack *Hras* developed few skin papillomas with the majority having activating mutations in *Kras*, indicating that mutational activation of *Kras* can induce papilloma formation *in vivo*. These observations agree with the fact that *Kras* mutations occur in a broad range of tumor types in different tissues, perhaps due to the unique function of *Kras* in stem cell expansion [25]. In the skin, the preference for *Hras* mutations in papillomas may be attributed, at least in part, to the higher levels of *Hras* compared to *Kras* in this tissue. Alternatively, the preference for *Hras* mutations may be due to the particular signaling networks involving these two Ras proteins in skin. In $Hras^{KO/KO}$ animals, levels of Kras are upregulated in the skin, and this could potentially render signaling through Kras more conducive for skin carcinogenesis. Interestingly, mice carrying the $Kras^{LA2}$ allele, which undergoes spontaneous somatic recombination that results in oncogenic activation of *Kras*, develop skin papillomas but only on a mixed genetic background and not on the *FVB/N* inbred strain [26], suggesting that genetic background may also contribute to the specificity of *Ras* mutations.

Hras and *Kras* are major targets of mutation in skin and lung tumors respectively, but targeted deletions of these genes showed opposite effects on tumor number in the respective tissues. In the lung, the suppressor function of WT Kras is a major determinant of lung tumor formation, as targeted deletion of one *Kras* allele resulted in an increase in tumor number. This is in agreement with genetic studies showing that the balance between levels of mutant and WT *Kras* regulates lung cancer susceptibility [12]. On the other hand, deletion of one *Hras* allele resulted in a decrease in number of papillomas despite the fact that *Hras* also undergoes loss of *WT* allele or gain of mutant allele during skin tumor development [6, 7]. However, $Hras^{KO/WT}$ mice demonstrated a significantly higher rate of malignant progression from papillomas to carcinomas, suggesting that WT Hras functions as a suppressor of skin tumor progression rather than skin tumor initiation. This is consistent with the high frequency of genomic imbalance at the *Hras* locus in invasive squamous carcinomas with *Hras* mutations [7]. Furthermore, the effect on skin tumor progression appeared to be specific to WT Hras, as reduced Nras or Kras level had no effect on this process (data not shown).

We also found that *Ras* proto-oncogenes are capable of influencing tumor formation driven by mutant forms of other *Ras* family members, albeit on a modest level. These results are summarized in Figure 4. Deletion of *Hras* or *Nras* increased the number of mutant Kras-driven lung tumors, suggesting that *Hras* and *Nras* attenuate the ability of mutant *Kras* to

promote lung carcinogenesis. In contrast, deletion of *Kras* or *Nras* reduced the number of mutant *Hras*-driven skin papillomas, suggesting that Kras and Nras signaling function cooperatively with oncogenic Hras during skin carcinogenesis. These findings, particularly those involving *Nras*, demonstrate that the effects of *Ras* proto-oncogenes on mutant *Ras*-driven carcinogenesis are context-dependent. It is not clear whether this is due to differences in oncogene function (*Hras* vs. *Kras*) or tissue-specific signaling networks (*skin* vs. *lung*). One possible explanation for the data from our skin carcinogenesis studies is that signaling through WT Kras and WT Nras has additive contributions to the oncogenic activity of mutant Hras. However, *in vitro* studies have shown that expression of *Nras* has little to no effect on the activity of mutant *Hras* [13], suggesting that the interactions between *Nras* and mutant *Hras*, as well as other Ras proto-oncogene and oncogene pairs, may have a non-cell autonomous mechanistic basis. For example, deletion of *Ras* proto-oncogenes could affect aspects of tissue physiology that are relevant to carcinogenesis, such as the inflammatory response, which has been shown to play an important role in mutant *Ras*-driven cancer development [27]. Additive effects of Kras and Nras deletion on vascular development and haemaotpoiesis during embryogenesis [14] may also reflect complex non cell-autonomous roles in tumorigenesis. Future work incorporating *in vitro* and *in vivo* studies are necessary in order to elucidate the mechanisms that underlie the interactions between proto-oncogenic and oncogenic forms of *Ras* family members, and to understand their contributions to cancer development.

Materials and Methods

Animals

The $Hras^{KO}$, $Nras^{KO}$, and $LSL\text{-}Kras^{G12D}$ alleles have been backcrossed into the *FVB/N* background over multiple generations to minimize the effects of genetic heterogeneity on tumor development. For the $Hras^{KO}$ and $Nras^{KO}$ alleles, heterozygous animals were bred to generate littermates of all three possible genotypes. Because $LSL\text{-}Kras^{G12D}$ is a non-functional allele until activated by Cre recombinase, it can effectively be used as a functional knockout allele of *Kras*. $LSL\text{-}Kras^{G12D}$ heterozygous mice were bred with *FVB/N* animals to generate *WT* and heterozygous mice.

Lung Carcinogenesis

Male mice were treated with a single dose of urethane (in PBS; at 1g/kg body mass) by intraperitoneal injection at 5-8 weeks of age. Animals were sacrificed twenty weeks after the injection, and lungs were collected, fixed overnight in formalin, and stored in 70% ethanol. Lung tumor numbers were counted under a dissecting microscope.

Skin Chemical Carcinogenesis

Skin tumor development was initiated with a single dose of DMBA on the dorsal skin at 8 weeks of age, followed by biweekly promotion with TPA for twenty weeks as previously described [2]. Because male animals often fight, causing skin wounding, only female mice were used in this assay. The number of skin tumors that developed on individual animals was counted at different time points during the course of the study.

RNA and Protein Analysis

Frozen skin and lung tissues were ground up in liquid nitrogen and processed for RNA and protein as previously described [28]. Gene expression was measured on the Affymetrix M430 2.0 platform, and by real-time PCR using Mm00517491_m1 (*Kras2*) and Mm00607939_s1 (*β-Actin*) assays on demand from Applied Biosystems. Ras proteins were detected using antibodies against Hras (C-20), Kras (F234), and Nras (C-20), purchased from Santa Cruz Biotechnology.

Mutational Analysis

Tumor tissues were incubated overnight at 55 °C with proteinase K, and DNA was purified by phenol/chloroform extraction. The status of *Hras* codon 61 was determined using a digestion assay as previously described [29]. *Kras* mutations were identified by direct DNA sequencing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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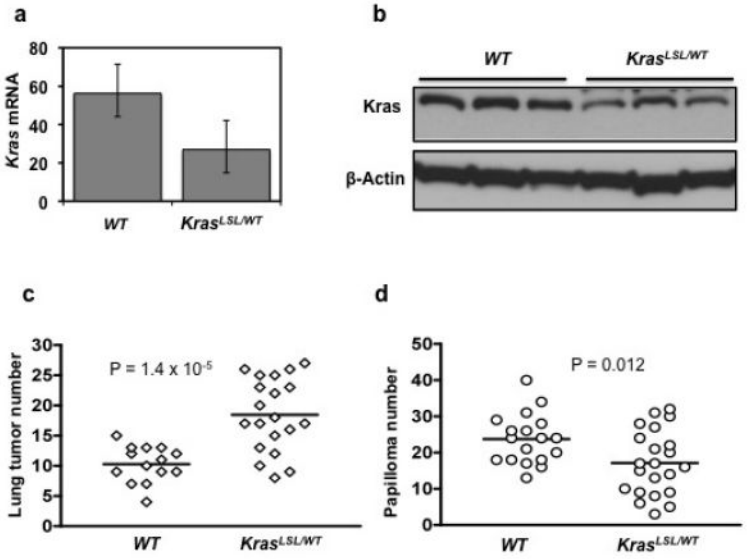

Figure 1.

Lung and skin carcinogenesis in mice with one or two functional copies of *Kras*. The *LSL-Kras*^{G12D} allele contains a transcriptional STOP element, and is used as a surrogate *Kras* knockout allele. *Kras* expression is reduced approximately 2-fold in lungs of *Kras*$^{LSL/WT}$ mice compared to *WT* mice, both at (a) RNA and (b) protein levels. Error bars indicate s.d. (c) Lung tumor number at 20 weeks after treatment with urethane. (d) Skin papilloma number at 20 weeks after initiation with DMBA. Data points correspond to tumor number of individual mice of the indicated genotypes. Horizontal line indicates the average tumor number for each genotype. Statistics were calculated using the Wilcoxon-Mann-Whitney test.

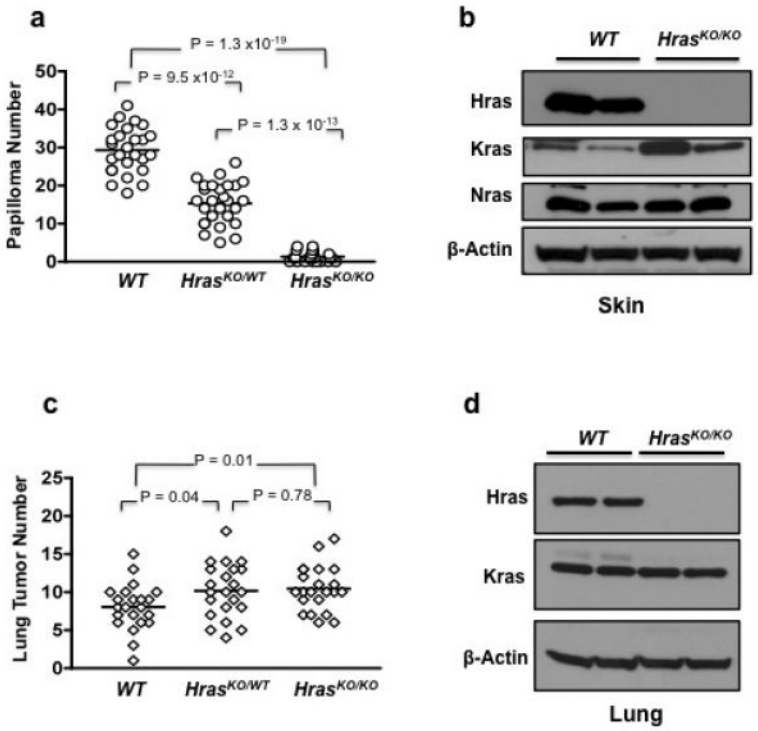

Figure 2.

Papilloma and lung tumor development in mice with targeted deletion of *Hras*. (a) Skin papilloma numbers at 20 weeks after initiation with DMBA. (b) The level of Kras protein is elevated in the skin of $Hras^{KO/KO}$ mice compared to *WT* animals. (c) Lung tumor numbers at 20 weeks after treatment with urethane. (d) In the lung, there is no difference in level of Kras protein between $Hras^{KO/KO}$ and *WT* mice.

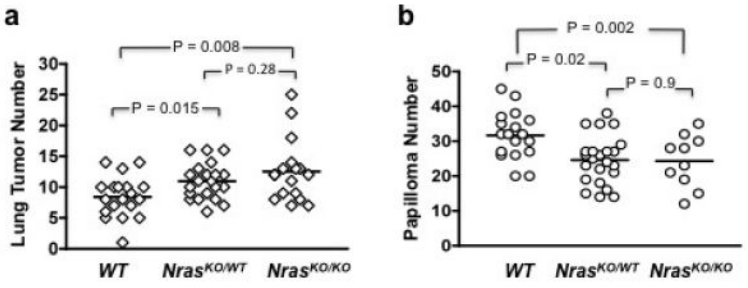

Figure 3.

Lung tumor and papilloma development in mice with targeted deletion of *Nras*. (a) Lung tumor number at 20 weeks after IP injection of urethane. (b) Skin papilloma number at 20 weeks after initiation with DMBA.

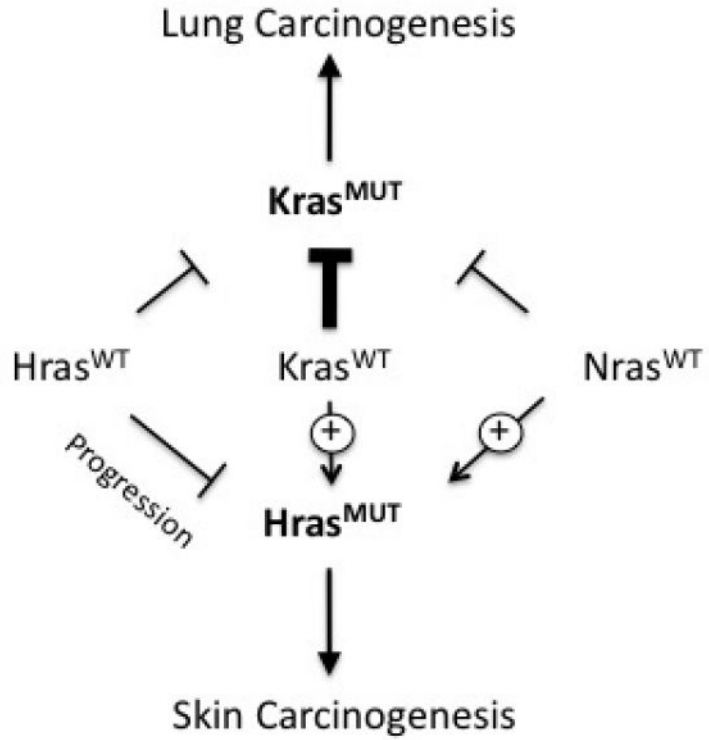

Figure 4.

Genetic interactions between *Ras* proto-oncogenes and oncogenes during lung and skin carcinogenesis. Thickness of the lines indicates the strength of interactions. The *Kras* proto-oncogene is a strong suppressor of lung tumor development driven by oncogenic *Kras*. Both *Nras* and *Hras* proto-oncogenes also suppress mutant *Kras*-driven lung carcinogenesis, but the effects were relatively modest. In the skin, *Nras* and *Kras* proto-oncogenes had positive effects on the development of mutant *Hras*-driven papillomas. The *Hras* proto-oncogene also function as a suppressor of skin carcinogenesis, but at the level of progression rather than initiation.

Table 1

Hras heterozygosity increases rate of malignant progression of skin tumors.

Genotype	No. of Mice	Cumulative No. of Papillomas	Cumulative No. of Carcinomas	Conversion Rate[a]
$Hras^{WT/WT}$	24	700	29	4.1%
$Hras^{KO/WT}$	26	393	28	7.1%

[a]Ratio of cumulative number of carcinomas (at 52 weeks) to cumulative number of papillomas (at 20 weeks).