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RASSF1A deficiency enhances RAS driven lung tumorigenesis

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Abstract

Mutant K-RAS has been shown to have both tumor promoting and suppressing functions and growing evidence suggests that the RASSF family of tumor suppressors can act as RAS apoptosis and senescences effectors. It has been hypothesized that inactivation of the RASSF1A tumor suppressor facilitates K-RAS-mediated transformation by uncoupling it from apoptotic pathways such as the Hippo pathway. In human lung tumors, combined activation of K-RAS and inactivation of RASSF1A is closely associated with the development of the most aggressive and worst prognosis tumors. Here we describe the first transgenic mouse model for activation of K-RAS in the lung in a RASSF1A-defective background. RASSF1A deficiency profoundly enhanced the development of K-RAS driven lung tumors in vivo. Analysis of these tumors showed loss of RASSF1A uncoupled RAS from the pro-apoptotic Hippo pathway as expected. We also observed an upregulation of AKT and RalGEF signaling in the RASSF1A− tumors. Heterozygosity of RASSF1A alone mimicked many of the effects of RAS activation on mitogenic signaling in lung tissue, yet no tumors developed, indicating that non-standard Ras signaling pathways may be playing a key role in tumor formation in vivo. Additionally, we observed a marked increase in inflammation and IL-6 production in RASSF1A-deficient tumors. Thus, RASSF1A loss profoundly impacts RAS-driven lung tumorigenesis and mitogenic signaling in vivo. Deregulation of inflammatory pathways due to loss of RASSF1A may be essential for RAS-mediated tumorigenesis. These results may have considerable ramifications for future targeted therapy against RAS+/RASSF1A− tumors.

Introduction

There are three members of the RAS oncoprotein family, K, H and N-RAS (1). Activating mutations in the *Ras* oncogenes, primarily *K-ras*, are the most common mutations identified in human cancer (2). Between 20-40% of tumors overall contain such mutations (3). However, in some tumor types, notably pancreatic cancer, the levels approach 100%. In experimental systems, both *in vivo* and *in vitro*, activated forms of RAS are powerfully transforming (1, 4). Thus, *K-Ras* may be the most important oncogene in human cancer.

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Activated RAS is thought to function primarily by binding and activating three main types of mitogenic effector proteins: the RAF kinases, PI-3 kinase and the RALGEF family, to stimulate pro-growth and survival pathways (4). The RAF kinases stimulate the MAPK cascade by phosphorylating and activating MEK1 kinase, which in turn phosphorylates and activates the ERK1 and ERK2 kinases. These kinases can phosphorylate and regulate a broad range of targets, including transcription factors (5). Activation of PI-3 kinase leads to the stimulation of mitogenic lipids and the activation of the AKT pathway (6). RALGEFs are exchange factors for the small. RAS-related GTPases RALA and RALB. The function of the Ral proteins remains only partially understood. When activated by RALGEFs, they bind and activate a series of effector proteins: RALBP1 (endocytosis and drug resistance), the exocyst components Sec5 and Exo84 (inflammation and autophagy), filamin, the Y box transcription factor, ZONAB and to modulate the NFkB pathway via TBK1 kinase (7). Thus, RALs modulate multiple biological pathways, many of which are known to be vital for the development of tumorigenesis and metastasis.

These three mitogenic pathways synergize to drive malignant transformation (8). Moreover, specific inhibitors of the RAF and the PI-3 kinase pathways can act synergistically to suppress the transforming effects of K-RAS (9). However, activated K-RAS can also induce growth inhibition and apoptosis (10) and K-RAS driven tumors often exhibit high levels of apoptosis or senescence. These effects decrease as the tumor progresses to malignancy (11). The growing evidence that the RASSF family tumor suppressors can act as RAS apoptosis and senescence effectors (12) could provide a mechanistic explanation for these paradoxical activities of mutant K-RAS.

RASSF1A is the best-studied member of the RASSF family. It may be the most frequently epigenetically inactivated tumor suppressor in human tumors (12). *In vivo*, knockout of RASSF1A or even haploinsufficiency is sufficient to promote a modestly enhanced rate of spontaneous tumor formation in mice (13). However, tumors only manifest after 18 months of age. Thus, the effects of RASSF1A loss as a single event are relatively modest.

RASSF1A has a RAS Association domain and can form an endogenous complex with activated K-RAS (14, 15). RASSF1A has no enzymatic activity, but appears to act as a K-RAS regulated scaffold. It serves to connect K-RAS to the regulation of multiple pro-apoptotic signaling pathways. Under the influence of K-RAS, RASSF1A binds the protein MOAP-1, which in turn can bind and activate the pro-apoptotic executor BAX (16, 17). RASSF1A also binds the MST (Hippo) kinases to connect K-RAS to the regulation of the Hippo pathway (15). This pathway is a kinase cascade ultimately regulating the activity of the transcriptional co-activating oncoproteins YAP and TAZ. Phosphorylation of YAP and TAZ by a RASSF1A/MST/LATS kinase cascade results in their cytoplasmic retention and proteolytic degradation (18), promoting apoptosis. The binding of K-Ras to RASSF1A stimulates these pathways, whereas suppression of RASSF1A serves to uncouple K-RAS from these pathways, reducing its apoptotic activity (19, 20).

However, the relationship between K-RAS and its RASSF1A effector may be more complex than simple upstream/downstream. As well as connecting K-RAS to pro-apoptotic pathways, RASSF1A also has the potential to influence the activity of pro-mitogenic RAS signaling

pathways indirectly. Romano et al (21) reported that MST2 binds RAF-1 kinase, suppressing its activity, and that RASSF1A competes with RAF-1 for MST2. Thus, reduced levels of RASSF1A can result in enhanced association between MST2 and RAF-1, reducing RAF-1 activity. In contrast Kilili et al suggested loss of RASF1A enhances RAF/MAPK signaling (22). In addition, RASSF1A can indirectly modulate AKT activity (23). Thus, RASSF1A may have a complicated role in K-RAS biology, both directly connecting K-RAS to apoptotic pathways and indirectly modulating K-RAS activation of its mitogenic effectors (12).

RASSF1A is also implicated in the control of inflammation (24), as transgenic mouse studies observed enhanced inflammation in chemically induced colitis in RASSF1A knockout mice. It has been known for over a decade that activated K-RAS can promote inflammatory processes, and inflammation has long been implicated as a key role component in cancer development (25). The role of RASSF1A in RAS mediated inflammation has not been explored.

Examination of the frequencies of mutant *Ras* in lung tumors have suggested that approximately 22%-40% are positive, depending upon the study (3). *Rassf1a* exhibits evidence of epigenetic inactivation in 40-80% of lung tumors (26–28). Multiple studies have sought to detect a relationship between K-RAS activation and RASSF1A inactivation in a variety of cancers. Most of these studies failed to find any particular correlation (26, 29, 30). However, a larger study focused on the group of lung tumors with the most aggressive phenotype and poorest prognosis found that this sub-set of tumors almost always exhibits both activation of RAS and suppression of RASSF1A (31). This supports the idea that RASSF1A inactivation, whilst not essential for RAS transformation, may have a powerful effect on enhancing its potency *in vivo*.

We sought to determine, for the first time, if RASSF1A suppression has an effect on K-RAS driven tumorigenesis *in vivo*, and whether this might involve alterations in K-RAS induced apoptosis, signaling and inflammation. We generated transgenic mice with a lung inducible mutant *K-Ras 12D* transgenic system (32) in a *Rassf1a* haploinsufficient background (13).

We found that even haploinsufficiency of *Rassf1*a was sufficient to dramatically enhance the tumorigenicity of *K-Ras* in the system. Almost three times as many tumors arose in the *K-Ras12D* induced/*Rassf1a* haploinsufficient (RAS12D+/RASSF1A−) mice as in K-*Ras12D* induced mice with wild type *Rassf1a* (RAS12D+/RASSF1A+). We observed the expected reduction in Hippo pathway activity in the RAS12D+/RASSF1A− tumors. We also observed the predicted upregulation of inflammation in the RAS12D+/RASSF1A− tumors. However, surprisingly, we found suppression of RASSF1A also caused the upregulation of RAS mitogenic signaling pathways in tumors and even in normal tissue with no activated RAS.

Thus, we confirm for the first time the importance of RASSF1A inactivation in the development of K-RAS lung tumors *in vivo*, and show that the molecular characteristics of such tumors are markedly different to RASSF1A wild type K-RAS tumors. These findings may have considerable ramifications for the employment of targeted therapies against Ras driven tumors in the clinic. Furthermore, the failure of the activation status of the three main

classic RAS effector pathways to precisely correlate with tumor formation suggests that, *in vivo*, there are non-standard RAS signaling pathways, that are essential for RAS driven tumorigenesis. Their identification could open new avenues for therapeutic intervention.

Materials and Methods

Transgenic Mice

Generation of K-RAS12D+/RASSF1A− transgenic mice-Transgenic mice which have *K-Ras4b* ^{G12D} under the control of doxycycline in type II pneumocytes were obtained as a generous gift from Harold Varmus (NCI) (strain FVB/N (32)). *Rassf1a* knockout mice (essentially C57Bl6) were obtained as a generous gift (Gerd Pfeifer, Caltech) (Tommasi et al (13)). The animals were crossed and geno-typed essentially according to the previously published protocols (13, 32). Modifications to the *Rassf1a* genotyping protocol were as follows : 2μL of isolated genomic DNA (approximately 50ng) was added to a PCR reaction containing 100ng each of three primers (UMIOAI – 5′TTGTGCCGTGCCCCGCCCA3′, LMIIAA – 5′TGACCAGCCCTCCACTGCCGC3′, and Neo48U – 5′GGGCCAGCTCATTCCTCCCAC3′), 2.5μL of 10mM dNTP's, 3μL of 50mM $MgCl_2$, 10μL of 10× PCR Buffer (Life Technologies), 30μL 5M Betaine (Sigma Aldrich Cat# B0300), 49μL H_2O, and 0.5μL Platinum Taq Polymerase (Life Technologies Cat# 10966034). The reaction was run on a standard thermocycler at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 1 minute finishing with a hold at 72°C for 5 minutes. The reaction was analyzed on a 1% agarose gel. Modifications to the K-Ras/Tet Genotyping protocol were as follows: Each genomic sample was run in parallel with each reaction texting for the presence of the K-Ras transgene or the Tet transgene. 2μL of isolated genomic DNA (approximately 50ng) was added to a PCR reaction containing 100ng of each of the paired K-Ras primers (K-RasFWD – 5′GGGAATAAGTGTGATTTGCCT3′, K-RasREV – 5′GCCTGCGACGGCGGCATCTGC3′) or Tet primers (rtTAFWD – 5′AAGGTTTAACAACCCGTAAACTCG3′, rtTAREV – 5′GTGCATATAACGCGTTCTCTAGTG3′), 1μL of 10mM dNTP's, 1μL of 50mM $MgCl_2$, 5uL 10× PCR Buffer (Life Technologies), 38.5μL H_2O, and 0.5μL Platinum Taq Polymerase (Life Technologies Cat# 10966034). The reaction was run on standard thermocycler at 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, finishing with a hold at 72°C for 5 minutes. The reaction was then analyzed on a 1% agarose gel.

Resultant animal groups were RAS12D+/RASSF1A− (RAS12D + CCSP-rtTA with hz RASSF1A), RAS12D/RASSF1A+ (RAS12D + CCSP-rtTA + wild type RASSF1A) and control animals (CCSP-rtTA + wild type RASSF1A). Animals of approximately 4 months of age were induced by feeding of doxycyclin chow (Harlan's Telklad 200mg/kg doxycycline food) for three months. All animal experiments were pre-approved by the institutional animal care and use committee (IACUC protocol number 15015).

Lung Tissue Preparation

Lung tissue to be analyzed by Western blot was removed by surgical procedure from the mouse. Approximately equal amounts of normal or tumor bearing tissue was excised and lysed in either a modified RIPA buffer (NP-40) or lysis buffer in the Millipore RALA Activation kit. Tissue was also re-genotyped (as above) to confirm no further gene loss in the tumors.

Plasmids and tissue culture

NIH-H1792 and A549 lung tumor cells (ATCC) cells contain an activating Ras mutation and were grown in 10% FCS/DMEM. The RASSF1A+/− knockdown matched pair has been described previously (Vos et al (33)). NIH 1792 and A549 cell identity was confirmed by STR profiling and tested negative for mycoplasma by the ATCC. HEK-293 cells were obtained from the ATCC. They were used only for exogenous expression/protein interaction studies and were not genetically validated. For luciferase assays, cells were split into six well plates and each well transfected with 50 ng of IL-6 luciferase reporter (Switchgear Genetics Cat# S721728). Luciferase assays were performed as described in the manufacturers protocol. Assays were performed at least twice in duplicate.

Antibodies

Antibodies against IL-6 were obtained from Ab Cam (Cat# 191194), CD11B were acquired from Novus Biologicals (Cat# NB110-89474), and RALGDS from Abiocode (Cat# R1857-vp). Antibodies for Phospho-ERK (Cat# 9101), ERK (Cat# 9102), Phospho-AKT-Ser473 (Cat# 9271), Phospho-YAP-S127 (Cat# 4911), YAP (Cat# 4912), and Mouse and Rabbit secondary antibodies (Cat# 7076 and 7074) were obtained from Cell Signaling Technologies (Boston, MA). Epitope tag antibodies detecting GFP were purchased from Santa Cruz (Cat# SC-9996) and HA from Covance (Cat# MMS-101P). RASSF1A rabbit antibody was generated by Prosci (Poway CA) against the peptide “ELRELAPAGRAGKGRTRLER”.

Western blotting, Immunoprecipitations, and Immuno-Histo-Chemistry

Cells for protein analysis and immunoprecipitations were lysed in either RIPA buffer (Sigma Aldrich Cat# R0278-500ML) or a modified RIPA buffer (1% NP-40, 50mM Tris-HCL, 150mM NaCl). Cell lysates were analyzed using NOVEX SDS-PAGE Bis-Tris gels (Life Technologies Cat# NP0322BOX) and transferred onto nitrocellulose membranes (Life Technologies Cat# LC2000). The membranes were then probed with antibodies and detected using Pierce West-Pico ECL Substrate (Life Technologies Cat# 34080). Immunoprecipitations were performed using GFP nano-antibodies conjugated to agarose gel from Allele Biotechnology (Cat# ABP-nAb-GFPA050) and analyzed by Western blot. Tissue blots were probed no more than twice before running fresh lysates from the same stock. For IHC experiments, excised lung tissue was formalin fixed then sent to University of Louisville Special Procedures lab for paraffin embedding and sectioning (5um). Once tissue was rehydrated, sections were placed in a 10mM citrate buffer (pH 6.0-6.5) and placed in the Retriever for 30 minutes and permitted to cool to room temperature. Sections were blocked in 10% goat serum for 1hr at room temperature and then then incubated overnight at 4°C with IL-6 antibody at a 1:500 dilution. Sections were washed and incubated at room

temperature with HRP secondary at a 1:1000 dilution for 30 minutes and then incubated with DAB per kit instructions (Cell Signaling Technology Cat# 8059S). Sections were washed and then mounted.

Image Acquisition and Processing—Images were captured with an Olympus IX50-FLA inverted fluorescent microscope (Optical Elements Corp., Dulles, VA) with an attached SPOT Junior digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). A Pharos FX plus Molecular Imager (Biorad, Hercules, CA) was used to digitize images prior to quantification using Quantity One software (Biorad). Figures were compiled using Photoshop software (Adobe).

RALA-GTP Activation Assays

Levels of activated RALA (RALA-GTP) were assayed using a non-radioactive RalA Activation Kit from Millipore (Cat#17-300) as described by manufacturer's protocol. The immuno-precipitation was then analyzed by Western blot using the kit-supplied anti-RALA.

RASSF1A Promoter Methylation Analysis

Mouse RASSF1A promoter methylation status was analyzed using the CpGenome Universal DNA Modification Kit (Millipore Cat# S7820). DNA was isolated from mouse lung tissue using a Blood and Tissue DNA Extraction Kit (Qiagen Cat# 69506). 1 mm^3 of lung tissue was processed for DNA isolation. Following DNA isolation, the purified DNA was modified using bisulfite modification per the kit and manufacturer's protocol. Modified DNA samples were then run on a standard PCR using the following primers: RASSF1A Unmeth #1 5′-GGTGTTGAAGTTGTGGTTTG-3′ and RASSF1A Unmeth #2 5-TATTATACCCAAAACAATACAC-3′ and methylated promoters were probed using RASSF1A Meth #1 5′-TTTTGCGGTTTCGTTCGTTC-3′ and RASSF1A Meth #2 5′-CCCGAACGTACTACTATAAC-3′. The PCR products were analyzed on a 1% agarose gel searching for the presence or absence of products in the methylated and unmethylated reactions.

Results

RASSF1A suppression enhances K-RAS12D driven tumorigenesis

To confirm definitively the hypothesis that RASSF1A suppression enhances mutant K-RAS driven tumorigenesis, we generated transgenic mice with lung specific expression of mutant K-RAS protein in a *Rassf1a* heterozygous background to mimic the effects of epigenetic inactivation. We used *CCSP-rtTA*/*Tet-op-K-Ras4b*^{G12D} bi-transgenic mice (32), crossed with either RASSF1A knockout mice (13) or wild type controls to generate an *in vivo Rassf1a* +/−, *K-Ras* inducible tumor system where the RAS mutant is expressed specifically in Clara cells, one of the major sites of origin of NSCLC (32). Animals positive for the inducible mutant *K-Ras* system and haploinsufficient for *Rassf1a* will be referred to as RAS12D/F1A-, animals positive for inducible mutant *K-Ras* with a wild type *Rassf1a* status will be referred to as RAS12D/F1A+.

Six animals from each system were fed tetracycline in their chow. After 12 weeks of induction, the animals were sacrificed and lung tissue taken for analysis. *Rassf1a* Hz mice with no mutant *K-Ras* showed no tumors, as expected (13). Lung tumors were apparent in all (RAS12D/F1A+) animals, and exhibited the pathological features of adenocarcinomas. However, those in the RAS12D/F1A− mice were approximately three times more frequent (Figure 1A). No overt evidence of metastasis to other organs was apparent at this time point. Further pathological analysis showed that although the increase in tumor number was statistically significant (Figure 1B), there was no statistically significant difference in the average size of the tumors (Figure 1B). Tumor tissue from three separate mice from each experimental group was analyzed for RASSF1A promotor methylation (Figure 1C top) and also genotyped (Figure 1C bottom). Results showed that no additional genetic defects had occurred in the *Rassf1a* gene.

Suppression of RASSF1A alters Hippo pathway activation by K-RAS12D in vivo

YAP is an oncogenic transcriptional co-activator that can promote mitogenesis, induce the stem cell phenotype and induce resistance to apoptosis (34). K-RAS can suppress YAP levels by binding to RASSF1A and using it to stimulate the Hippo pathway. This results in the phosphorylation and subsequent degradation of YAP (12). The inhibition of YAP has been identified as a synthetic lethal event for RAS transformed cells, suggesting that sustained YAP activity may be a key requirement for RAS mediated transformation (35). Examination of YAP levels in the tissue showed that there was a marked and statistically significant decrease in basal levels of total *YAP* protein in the RAS12D/RASSF1A+ tumors compared to the RASwt/RASSF1A+ control. This was completely abrogated by the loss of one RASSF1A allele (Figure 2A). This confirms the RAS/RASSF1A/Hippo link *in vivo* in tumors for the first time and shows that even haploinsufficiency of RASSF1A is enough to uncouple RAS from the Hippo pathway. Examination of tissue from the lungs of RASSF1A − animals with no mutant K-RAS and no tumors showed a similar level of YAP as the RAS12D/RASSF1A− tumor tissue.

RASSF1A levels modulate RAS mitogenic signaling pathways

Activated RAS stimulates three main classes of mitogenic effector to mediate transformation: RAF, PI-3K and RALGEFs (36). To determine if the reduced RASSF1A expression could be altering any of these effector pathways *in vivo*, and thus contributing to the differential transformation, we sequentially assayed their status in the tumors. We first selected the RAF/MAPK pathway. When we examined the status of phospho ERK as a MAPK pathway readout, we found that it was upregulated to an equal level, on average in the K-RAS tumors irregardless of RASSF1A status (Figure 2B) compared to the wild type control. Surprisingly, the pathway was also activated by the suppression of RASSF1A in lung tissue even in the absence of any activated *Ras* transgene (Figure 2B). Thus, differential MAPK activation does not correlate with differential tumorigenesis and downregulation of RASSF1A on its own enhances the MAPK pathway activity. Previously, based on tissue culture experiments, it has been suggested that downregulation of RASSF1A frees up MST2 protein which can bind and inhibit Raf-1 kinase activity (21). Others have suggested that MST2 can support Raf/MAPK activation (22). It appears that *in vivo*, the latter observation may be more accurate.

RAS also activates PI-3 kinase which results in the activation of AKT (37). AKT activation can be measured by phospho-antibody. Western analysis of the samples showed a statistically significant increase (about twofold) steady state activation of AKT in the RAS12D/RASSF1A− tumors compared to the RAS12D/RASSF1A+ tumors (Figure 2C). However, AKT phosphorylation was also activated over wildtype/wild type background in the non-tumor lungs from the RASWT/RASSF1A− control animals. RASSF1A has previously been reported to impact the AKT pathway (23). The difference between RAS12D + tumors and non-mutant RAS normal tissue from the RASSF1A haploinsufficient animals was not statistically significant. Therefore, enhanced activation of AKT is observed in RAS tumors with suppressed RASSF1A, but is does not correlate with the development of tumors.

Analysis of total K-RAS levels did not show large differences (Figure 2D). A combined Western blot of signaling pathway status in the tissue lysates is shown in Figure 2E. As we cannot distinguish induced mutant RAS from the total K-RAS background, we cannot be sure that there are not variations in the induction of the mutant K-RAS protein. However, as we did not observe significant variations in phospho ERK between the different individual RAS induced tumors (Figure 2E), we anticipate that the amount of activated RAS present was similar in each.

The third major mitogenic RAS signaling pathway that identified is the RALGEF pathway (7). RAS binds RALGEFs to stimulate the RAS-related RAL oncoproteins (RALA and RALB). RALs modulate multiple proteins implicated in tumorigenesis, metastasis and malignancy but appear to be particularly important for the regulation of the exocyst system (7). Although the least studied of the major RAS effector pathways, a strong case can be made for the importance of this pathway in Ras transformation *in vivo* (7, 38). RAL pathway activation can be assayed by measuring the ratio of total RAL to RAL in the active, GTP bound, state using a pull-down assay. The ability of activated RAS to stimulate the RAS pathway may be dependent upon the particular amino acid change at residue 12 in RAS. The K-RAS G12D mutant protein present in the transgenic mouse system appears to be quite weak for activating the RALGEF pathway *in vivo* (39).

Examination of the tissue samples showed that, as expected, RAS12D/F1A+ tumors showed little obvious activation of RAL and were comparable to the wild type/wild type control animals. However, RAS12D/RASSF1A− tumors did exhibit a marked elevation of active RAL (Figure 2E). Once again, *Rassf1a* Hz non-tumor tissue, even in the absence of mutant RAS, exhibited a similar increased level of RAL activation that is statistically significant (Figure 3A). Thus, it would appear that RASSF1A can modulate RAL independently of RAS. Moreover, as with the MAPK and AKT pathway, enhanced RAL pathway activation does not correlate with tumor formation *per se*.

To confirm the unanticipated effects of RASSF1A levels on RAL signaling, we repeated the RAL pull down experiment on a matched pair of RAS driven human lung tumor cells which have been knocked down for RASSF1A (14). RASSF1A knockdown in these human cells also enhanced RALA activity (Figure 3B). Knockdown of RASSF1A in the cells was confirmed by Western blot (Figure 3C). To try to determine the mechanism of this effect, we

co-transfected RASSF1A with the RALGEF family member RALGDS, and performed co-immunoprecipitations. We found that RASSF1A and RALGDS formed a complex, and therefore could be acting on RALGDS directly (Figure 3D).

RASSF1A levels modulate inflammation and IL-6

K-RAS lung tumors are known to be inflammatory (25), and inflammation can play a major role in supporting tumor development (40). RASSF1A loss has been implicated in promoting inflammatory processes and tissue from RASSF1A knockout mice shows elevated levels of inflammatory cytokines, including IL-6 (24). Transgenic studies have shown that IL-6 is essential for K-RAS mediated transformation (41). We examined the tumors for the presence of inflammatory cells using Cd11b as a marker. We noticed a striking increase in the RAS12D/RASSF1A− tumors compared to the tumors with normal levels of RASSF1A. (Figure 4A). Quantification (Figure 4B) showed an approximately eightfold increase. As knockout of RASSF1A has been shown to promote IL-6 expression (24), we examined the levels of IL-6 protein in the tumors by IHC, we found that they were enhanced in the RASSF1A deficient RAS tumors (Figure 5A), as expected.

In further studies, we used transfection experiments in RASSF1A+/− matched pairs of human lung tumor cell lines (16) to show that RASSF1A negatively impacts the ability of activated K-RAS to stimulate the IL-6 promoter. RASSF1A negative cells with expression restored by stable transfection exhibited reduced IL-6 promoter activity by K-RAS (Figure 5B). RASSF1A knockdown cells exhibit enhanced IL-6 promoter activity due to K-RAS (Figure 5C). Thus, RASSF1A can modulate IL-6 expression in both murine and human systems.

Discussion

Although mutant K-RAS is a highly potent oncoprotein, K-RAS driven tumors often exhibit high levels of apoptosis or senescence, which decrease as the tumor progresses to malignancy (11). RASSF family tumor suppressors can act as direct RAS apoptosis and senescence effectors (12), thus providing a mechanistic explanation for these paradoxical effects of K-RAS. RASSF1A is frequently downregulated in human tumors via epigenetic mechanisms (42) and experimentally, suppression of RASSF1A reduces the pro-apoptotic effects of K-RAS (14, 43).

Although no simple correlation between *Ras* mutation and loss of RASSF1A function has been identified in cancer, the presence of both genetic defects is closely associated with the most malignant and therapy resistant lung tumors (31). This suggests that although RASSF1A suppression is not essential for RAS mediated tumor formation, its loss enhances the power of oncogenic RAS to promote the tumorigenic phenotype *in vivo*.

In this study, we sought to determine if RASSF1A inhibition really affects K-RAS transformation *in vivo*. We induced the expression of activated K-RAS in the lungs of animals with a wild type or *Rassf1a* haploinsufficient genetic background. The haploinsufficiency mimics the effects of reduced expression of RASSF1A due to epigenetic inactivation found in human tumors. Even haploinsufficiency of *Rassf1a* strongly enhanced

the tumorigenic effects of the mutant K-RAS. Therefore, this study finally confirms *in vivo* the hypothesis that RASSF1A suppresses K-RAS transformation and that reduced expression of RASSF1A enhances K-RAS mediated transformation. The main effect manifested as an increased frequency of tumors, with no apparent increase in average size. Whether this reflects an effect primarily on enhanced tumor initiation, or if it also represents enhanced malignancy is not clear at this point. .

RASSF1A has been shown to connect K-RAS to the pro-apoptotic Hippo pathway, to the pro-apoptotic BAX pathway and to inactivate the anti-apoptotic BCL-X protein (12). Hippo pathway activation status can be measured by its effects on YAP protein levels. In wild type control tissue, YAP levels were quite high, indicating low activity for the pathway. In RAS12D+/RASSF1A+ tumors the YAP virtually disappeared in 4/5 samples as RAS used RASSF1A to activate the Hippo pathway. This effect was lost in the RASSF1A hz tumors where YAP levels slightly exceeded those in the normal control. Therefore, suppression of RASSF1A appears to disconnect K-RAS from the pro-apoptotic Hippo pathway allowing the YAP oncoprotein to persist in the presence of activated K-RAS.

Activated RAS stimulates three main classes of mitogenic effector: RAF, PI-3K and RALGEFs (36). In wild type control animals, the activation status of these pathways was essentially undetectable. To determine if the reduced RASSF1A expression could be altering any of these effector pathways and thus explaining the differential transformation we sequentially assayed their status in the tumors. We first selected the RAF/MAPK pathway. This is perhaps the best-known RAS mediated mitogenic signaling pathway and activation of this pathway alone in simple systems seems to be sufficient to reiterate the transforming effects of RAS (44). When we examined the status of phospho ERK as a MAPK pathway readout, we found that it was upregulated by K-RAS activation (compared to the wild type control) with the levels of RASSF1A making no difference. However, RASSF1A suppression alone, in the absence of a *Ras* mutation, in non-tumor tissue showed similar levels of MAPK pathway activation. This was a somewhat unexpected finding. Previously it has been suggested that downregulation of RASSF1A frees up MST2 protein which can bind and inhibit RAF-1 kinase activity (21). Others have suggested that MST2 can support Raf/MAPK activation (22). The results from our in vivo system suggests that the latter findings are more physiological. Moreover, the similar levels of MAPK pathway activation in tumor and non-tumor tissue samples meant that MAPK pathway activation is not enough for tumorigenesis in this system.

We also examined the status of the PI-3K pathway by measuring phospho-AKT. In contrast to the MAPK result, here we saw a clear upregulation of signaling in the tumors with suppressed RASSF1A. However, again we saw significant activation simply by RASSF1A suppression even in the absence of a *Ras* mutation. This effect of RASSF1A suppression has previously been reported in tissue culture (23). Therefore, deregulation of the PI-3K pathway does not clearly correlate with tumor development.

Finally, we examined the least studied RAS mitogenic pathway, the RALGEF/RAL pathway. Unlike other activating mutations of RAS, the K12D variant is reported to be only weakly activating for the RALGEF pathway (39). When we examined tumor tissue from our

transgenic mice, we found that we saw no detectable active RAL in the normal tissue control or the K-RAS12D/F1A+ tumor tissue. However, suppression of RASSF1A caused a marked upregulation of activated RAL. Thus, downregulation of RASSF1A changes the internal signaling balance of the RAS12D driven tumors, making them much more RAL centric. Yet, once again, a similar effect was observed in the non-tumor tissue suppressed for RASSF1A that did not have mutant K-RAS in it. When we began to examine how RASSF1A might influence RAL pathway, we found that we could detect RASSF1A in complex with RALGDS, so the regulation could be direct.

Thus, we found that RASSF1A haploinsufficient lung tissue from wild type RAS animals exhibited a very similar YAP/MAPK/PI-3K/RALGDS signaling pathway profile to that of the RAS12D/RASSF1A− tumors. Yet, there were no tumors. This highlights the complex role of RASSF1A expression in modulating RAS mitogenic signaling pathways. It also implies that there is some other, non-classic RAS signaling pathway, activated by RAS mutation but not RASSF1A down-regulation alone that is essential to tumorigenesis *in vivo*.

K-RAS lung tumors are known to be inflammatory (45), and inflammation can play a major role in supporting tumor development (40). When we examined the tumors, we detected a marked increase in the levels of macrophage marker positive cells in the RASSF1A deficient tumors. RASSF1A plays a role in suppressing inflammation in an *in vivo* colitis model (24). Here, it appears to play a significant role in suppressing RAS driven inflammation. One of the key inflammatory cytokines is IL-6 and transgenic studies have shown that IL-6 is essential for K-RAS mediated transformation (41). Furthermore, sensitization of the IL-6 signaling pathway leads to enhanced tumor formation in the same K-RAS transgenic mice used in this study (46). Zhang et al have shown that RASSF1A negatively regulates the NFkB pathway by binding TBK1 (47). One of the transcriptional targets of NFkB is IL-6 (48) and Gordon et al have shown that IL-6 levels are elevated in RASSF1A deficient transgenic mice (24). When we examined the levels of IL-6 in the tumors, we found that they were strongly enhanced in the RASSF1A deficient RAS tumors compared to the tumors with wild type RASSF1A. It has recently been shown that IL-6 signaling plays a key role in YAP induced stemness (49). Therefore, enhanced IL-6 expression without suppressing YAP, as is the case in the RAS12D/RASSF1A− tumors, may enhance the cancer stem cell population to give rise to the higher rate of tumor formation. As macrophage are attracted by IL-6 and then establish a positive feedback loop for IL-6 production, it is not yet clear which is the major source of IL-6 production in the tumors. However, the elevated levels of IL-6 suggest that anti-IL-6 agents might be particularly suitable for treating highly malignant, treatment resistant (31), mutant Ras/RASSF1A− lung tumors.

The overall implications of these studies are that RASSF1A status of the mutant *ras* tumor may play a critical role in determining the activity of RAS mitogenic signaling pathways, that non-canonical RAS signaling pathways may be essential for full transformation, and that anti-IL-6 approaches may have considerable potential as novel therapeutics in RAS+/RASSF1A− tumors.

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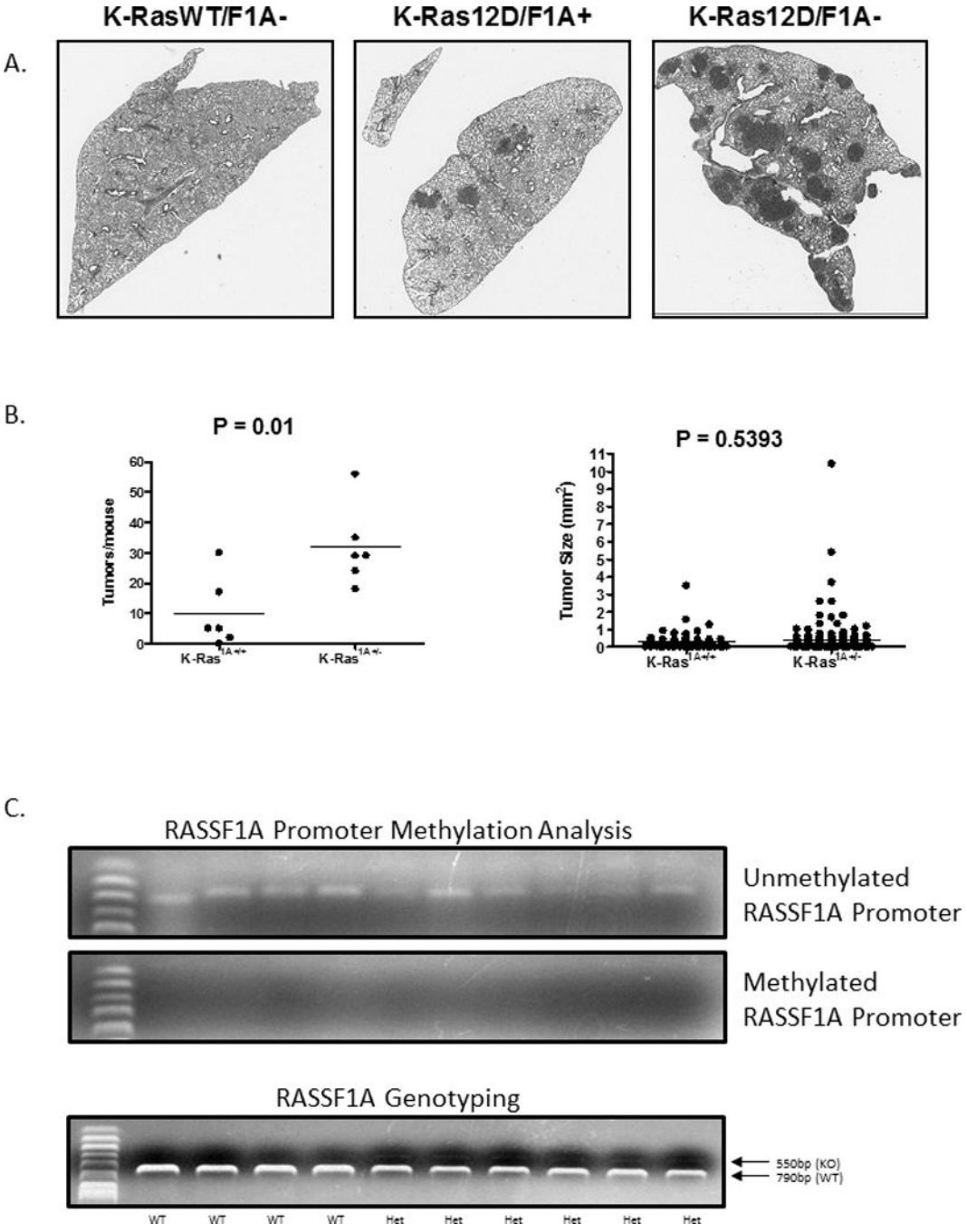

Figure 1. Effects of RASSF1A heterozygosity on Ras driven tumorigenesis

a. Representative examples of lung tissue H&E slides from control and mutant RAS induced animals showing tumors. Left panel is wild type RASSF1A and wild type RAS. Middle panel is mutant RAS12D and wild type *Rassf1a*, right panel is mutant RAS12D and *Rassf1a* hz.

b. Quantification of tumor numbers/mouse and average tumor size. Left panel shows tumors per mouse (n = 6), p value .01. Right panel shows no significant difference in average tumor size.

c. Analysis of the *Rassf1a* locus in tumors that arose. Mouse *Rassf1a* promoter methylation analysis was performed using bisulfite treatment. No methylation was detected in the RASSF1A promoter region. To confirm no additional genetic modifications had occurred at the *Rassf1a* locus in the lung tissue, DNA from the tissue samples was also analyzed by genotyping. 550bp band indicating hz is partially obscured by the dye front. Three samples were randomly chosen from the RASSF1A+/RAS12D+, the RASSF1A hz/wild type RAS and RASSF1Ahz/RAS12D groups for testing.

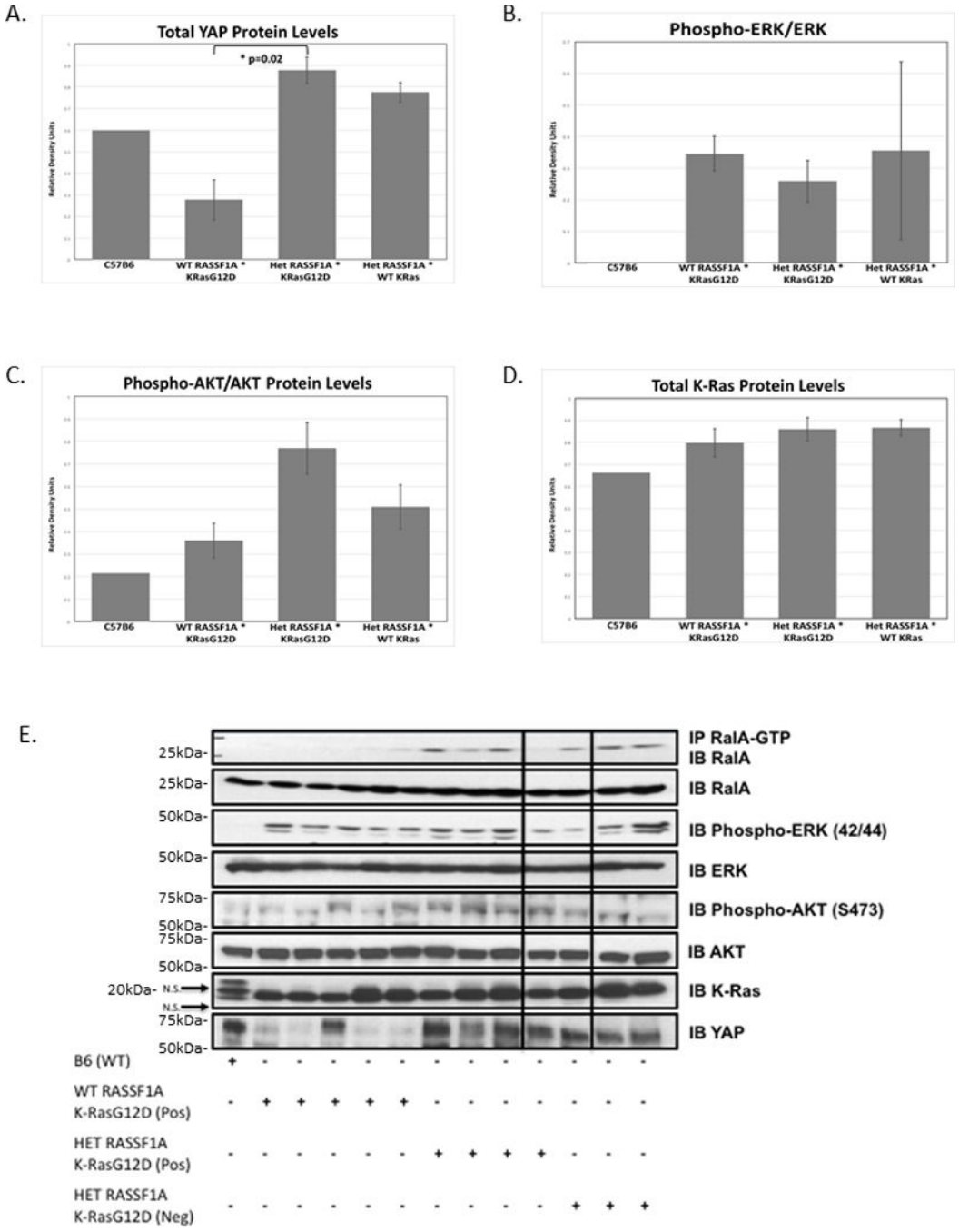

Figure 2. Effects of RASSF1A suppression on RAS signal transduction

Western analysis of protein expression and signaling activity control and tumor tissue. Densitometric quantification of: a. YAP, b. phospho ERK/total ERK, c, Phospho AKT, d, total K-RAS in the transgenic tissue. e. Shows a compilation of the Western blot data for YAP, phospho ERK, phospho AKT, RAL-GTP and total K-RAS. Samples were quantified prior to loading and equal amounts of protein added. NS- non-specific band derived from standards co-run. Vertical lines indicate a gel splice where we removed samples that began to lose quality during the sequential blotting process.

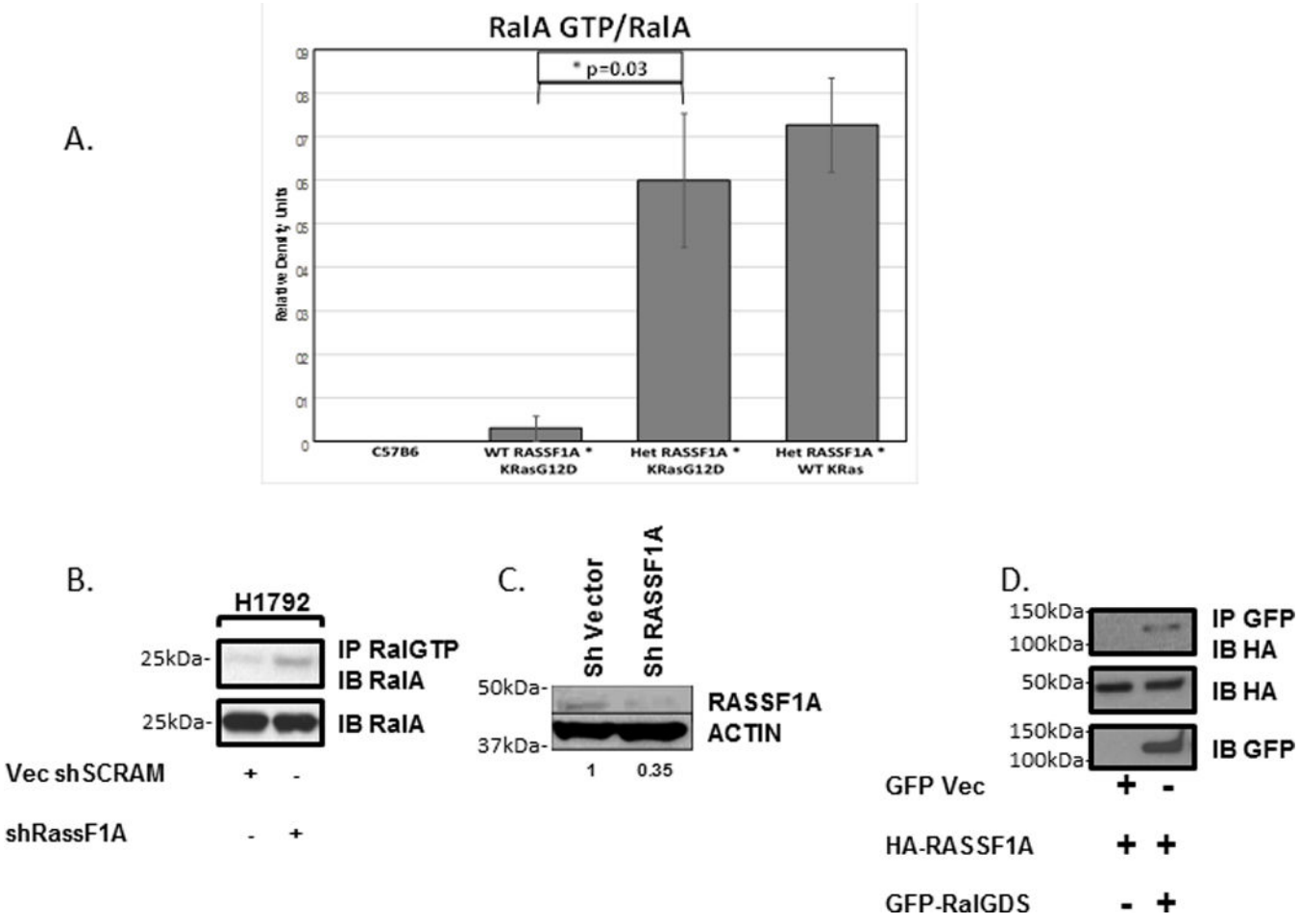

Figure 3. Effects of RASSF1A on the Ral pathway

a. Levels of active RALA *in vivo* in control and tumor tissue. b. Relative levels of active RALA in a RASSF1A +/− matched set of human lung tumor cell lines., p value < .05. c. Western blot of levels of RASSF1A in the vector/knockdown cell lines. d. Co-immunoprecipitation of HA tagged RASSF1A and GFP tagged RALGDS from transfected HEK-293 cells.

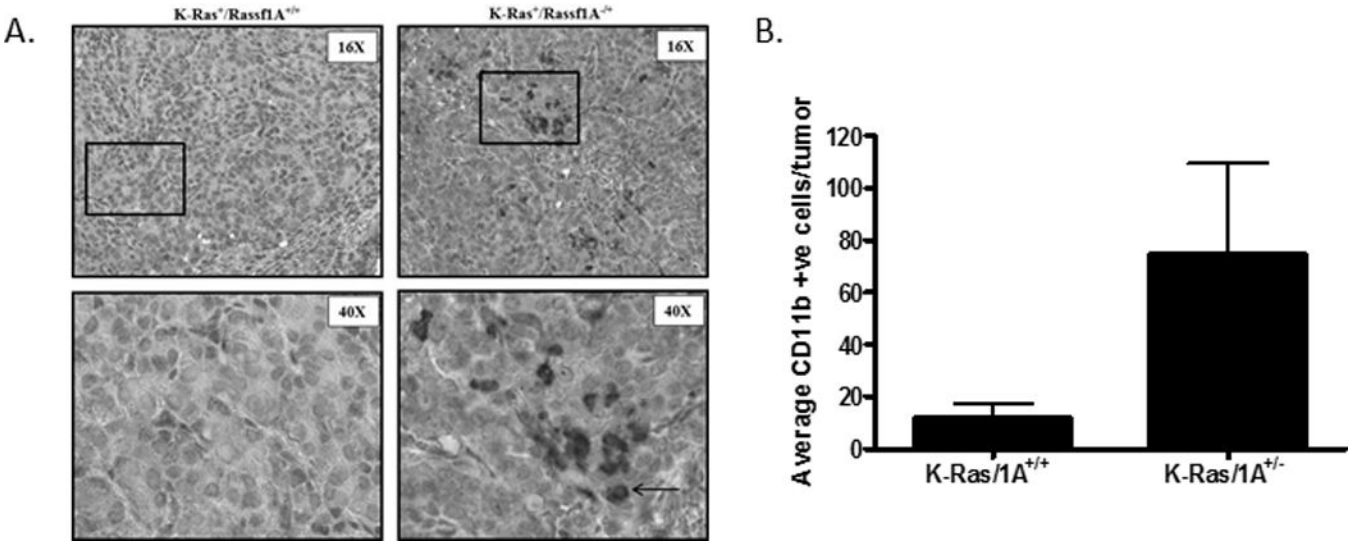

Figure 4. Effects of RASSF1A suppression on Immune infiltration of tumor tissue

a. Representative IHC staining of tumor tissue for CD11b. left panels show RAS12D/RASSF1A wild type tissue at two different magnifications. Right panels show an example of RAS12D/RASSF1A− tumor tissue at two different magnifications.

b. Quantification of staining derived from 5 different animals. P = < .05.

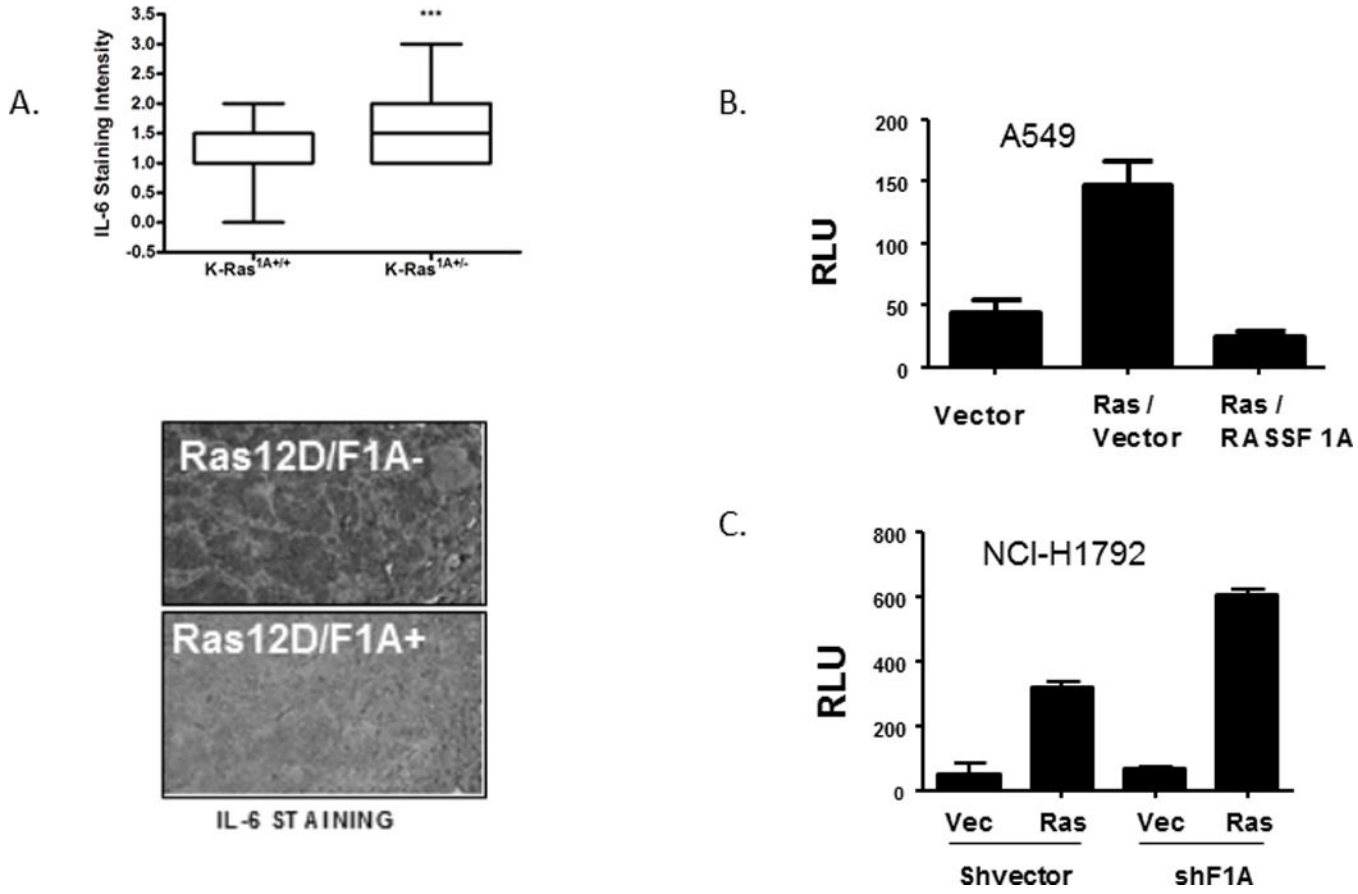

Figure 5. Effects of RASSF1A levels on IL-6 in vivo and in vitro

a. Upper panel, quantification of average intensity of staining of lung tumor sections from 5 different animals (in arbitrary units) that were RAS12D/RASSF1A+ or RAS12D/RASSF1A − for IL-6 expression. Lower panel, shows a representative example of staining.

b. Relative activity of an IL-6 promoter luciferase reporter in A549 lung tumor cells co-transfected with activated K-RAS 12V or activated K-RAS 12V and RASSF1A. n = 2, p = < .05.

c. Relative levels of IL-6 promoter activity in a matched set of RASSF1A+/− cells co-transfected with the reporter and activated K-RAS 12V or vector. n = 2, p = < .05.