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κB-Ras proteins regulate both NF-κB-dependent inflammation and Ral-dependent proliferation

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Abstract

Transformation of cells generally involves multiple genetic lesions that undermine control of both cell death and proliferation. We now report that κB-Ras proteins act as regulators of NF-κB and Ral pathways, which control inflammation/cell death and proliferation, respectively. Cells lacking κB-Ras therefore not only show increased NF-κB activity, that results in increased expression of inflammatory mediators, but also exhibit elevated Ral activity, that leads to enhanced anchorage-independent proliferation (AIP). κB-Ras deficiency consequently leads to significantly increased tumor growth that can be dampened by inhibiting either Ral or NF-κB pathways, revealing the unique tumor suppressive potential of κB-Ras proteins. Remarkably, numerous human tumors show reduced levels of κB-Ras, and increasing the level of κB-Ras in these tumor cells impairs their ability to undergo AIP, thereby implicating κB-Ras proteins in human disease.

Introduction

Transformation of cells is a multi-step process that culminates in dysregulated proliferation and survival. The classical Ras GTPases, H-Ras, K-Ras and N-Ras, are critical regulators of proliferation and it is thus not surprising that they represent the most frequently mutated human oncogenes (Cox and Der, 2010). GTPases of the Ras superfamily function as cellular switches by cycling through guanine nucleotide-dependent conformational transitions

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Author Contributions

AO performed experiments. AO and SG designed the overall research and wrote the manuscript. TP, YG, LK, VS, HS and PR contributed to experimental work. CG performed the mass-spectrometry analysis. GL provided reagents and experimental support.

(Wennerberg et al., 2005). Signal transduction through interaction with their effectors requires GTP-binding, which is promoted by guanine nucleotide exchange factors (GEFs). The reverse process is catalyzed by GTPase activating proteins (GAPs) which enhance the intrinsic GTP hydrolysis activity of GTPases and thus accelerate transition to the GDP-bound OFF-state (Bos et al., 2007; Cherfils and Zeghouf, 2013; Colicelli, 2004). In over 30% of human tumors, classical Ras proteins are mutated at positions G12, G13 and Q61, which inhibits GTP hydrolysis and locks the Ras-proteins in a constitutively active, GTP-bound form (Karnoub and Weinberg, 2008; Young et al., 2009). These mutated Ras proteins trigger aberrant activation of several effector pathways such as Raf kinase and Ral GTPases that collaboratively contribute to tumorigenesis (Karnoub and Weinberg, 2008; Young et al., 2009).

The Ral GTPases, RalA and RalB, are members of the Ras superfamily and have been shown to regulate diverse cellular processes, such as vesicular transport, cytoskeletal organization and cell proliferation (Feig, 2003). Ral GTPases can be activated through several GEFs, many of which are *bona fide* Ras effectors (de Bruyn et al., 2000; Rebhun et al., 2000; Shao and Andres, 2000; Wolthuis and Bos, 1999). More recent studies have further suggested that inactivation of the two described heterodimeric Ral-GAP complexes, Ral-GAP1 and Ral-GAP2, is sufficient to elicit Ral activation (Chen et al., 2011; Leto et al., 2013; Saito et al., 2013). The Ral-GAP complexes contain the regulatory subunit Ral-GAPβ and the catalytic subunits Ral-GAPα1 or Ral-GAPα2, respectively (Gridley et al., 2006; Shirakawa et al., 2009). Association of the α subunits with Ral-GAPβ has been shown to be essential for Ral-GAP activity and both Ral-GAP complexes have activity towards RalA and RalB, *in vitro* (Chen et al., 2011; Gridley et al., 2006; Shirakawa et al., 2009). In recent years Ral GTPases have emerged as critical effectors of Ras-driven tumorigenesis: chronic Ral activity has been detected in a variety of tumor types and genetic ablation of RalA and RalB blocks K-Ras driven tumor formation in mice (Peschard et al., 2012). In this context, Ral GTPases are key regulators of anchorage-independent proliferation (AIP) which is a hallmark of cancer cells as it allows tumors to expand and spread (Bodemann and White, 2008; Chien and White, 2003; Lim et al., 2005; Lim et al., 2006; Martin et al., 2011; Peschard et al., 2012; Smith et al., 2007).

Maintenance of cellular homeostasis however depends not only on control of proliferative pathways, but also of cell death. In addition, recent studies have revealed a strong connection between inflammation and cancer development (Ben-Neriah and Karin, 2011; Karin, 2006). A major regulator of both of these processes is the transcription factor NF-κB which can induce the expression of pro-inflammatory cytokines, chemokines, adhesion molecules, as well as pro-survival, anti-apoptotic molecules (Hayden and Ghosh, 2012). NF-κB activity is regulated through cytoplasmic sequestration by the inhibitory IκB proteins (Hayden and Ghosh, 2008). Degradation of IκB proteins is triggered upon phosphorylation by the IKK complex, and releases the bound NF-κB, which translocates to the nucleus and induces transcription. The two major IκB proteins, IκBα and IκBβ, exhibit distinct regulation and function. IκBα is degraded rapidly and then resynthesized, allowing it to suppress activated nuclear NF-κB. Degradation of IκBβ is slower and more persistent (Hoffmann et al., 2002; Tam and Sen, 2001; Thompson et al., 1995; Tran et al., 1997).

Importantly, newly synthesized IκBβ, characterized by its hypophosphorylated state, acts in the nucleus at promoters/enhancers of particular inflammatory genes, to ensure their persistent expression (Rao et al., 2010; Scheibel et al., 2010). Therefore, cells lacking IκBβ show reduced expression of critical pro-inflammatory genes such as TNFα and a consequent reduction in inflammation in animal models of septic shock or collagen-induced arthritis (Rao et al., 2010).

In an effort to understand the unique biological properties of IκBβ, we identified a novel class of Ras-like proteins that we named κB-Ras1 (*NKIRAS1*) and κB-Ras2 (*NKIRAS2*) as IκBβ-interacting proteins (Fenwick et al., 2000). While these evolutionarily conserved proteins display a high degree of homology with Ras GTPases, they differ in two key respects. First, the κB-Ras proteins have amino acids at positions 12, 13 and 61 that predict a constitutively GTP-bound state. Second, they lack sequences at the C-terminus necessary for farnesylation and membrane attachment. Consequently, κB-Ras proteins are not generally membrane-associated, but rather display cytoplasmic and nuclear distribution (unpublished observations and (Tago et al., 2010)). While overexpression experiments demonstrated that κB-Ras could inhibit NF-κB activation (Chen et al., 2004; Chen et al., 2003; Fenwick et al., 2000; Tago et al., 2010), studies on the endogenous proteins were lacking.

To better understand the biological role of κB-Ras proteins, we created mice lacking both κB-Ras isoforms. These mice reveal an unexpected role for κB-Ras proteins in lung development and confirm κB-Ras proteins as critical regulators of IκBβ-dependent NF-κB activation and consequent inflammatory responses *in vivo*. We also report that κB-Ras proteins are associated with Ral-GAP proteins and are essential for their GAP activity towards the Ral GTPases. κB-Ras deficient cells demonstrate a striking increase in Ral-GTP levels and consequently AIP *in vitro*, and tumor growth *in vivo*. Furthermore, decreased κB-Ras expression, detected in several human cancers, provides a growth advantage to tumor cells, strongly implicating κB-Ras proteins as novel tumor suppressors.

Results

Deficiency of κB-Ras1 and κB-Ras2 causes perinatal lethality

κB-Ras1 (1SKO) and κB-Ras2 (2SKO) deficient mice were generated using conventional strategies (Figure S1A–D). Both mouse strains were viable without obvious phenotypes (Figure S1E). However, κB-Ras1/κB-Ras2 double knock-out (DKO) led to perinatal lethality. DKO pups died within hours after birth exhibiting a hunched posture and central cyanosis, suggesting a ventilatory defect. (Figure 1A and S1F).

While histopathological examination of all other major organs revealed no gross defects, sections of the lungs of κB-Ras DKO pups displayed hypercellularity and reduced alveolar inflation in comparison to littermate controls (Figure 1B). Quantification of immunohistochemical PCNA staining confirmed an increase in proliferating cells in lungs from newborn DKO mice (Figure 1C). Insufficient alveolar inflation causing respiratory failure is often linked to disturbed pulmonary surfactant production. The surfactant proteins SP-A, -B, -C and -D are critical for surface maintenance, lung inflation at birth and general

lung function (Frerking et al., 2001; Weaver and Conkright, 2001). We found that mRNA levels for all four surfactant proteins were significantly reduced in lungs of late-stage DKO embryos (Figure S1G) and newborn DKO offspring (Figure 1D), likely explaining the observed reduction in lung inflation. These findings suggest that lung hypercellularity and inadequate alveolar inflation lead to perinatal lethality of κB-Ras DKO mice (Figure 1C) and thereby reveal an unexpected role for κB-Ras proteins in lung development.

κB-Ras2 knock-out mice show enhanced IκBβ-dependent inflammatory responses

We first analyzed the effects of complete κB-Ras deficiency on NF-κB signaling in mouse embryonic fibroblasts (MEFs). We found that IκBβ, but not IκBα, degradation was enhanced (Figure 2A). The analysis of IκBβ knock-outs had shown that IκBβ regulated only a subset of NF-κB-dependent genes, including TNFα but not other well-characterized NF-κB target genes such as IL-6 (Rao et al., 2010). Interestingly, expression of TNFα but not of IL-6 was increased in DKO MEFs in response to LPS when compared to wild-type cells (Figure 2B). The lethality of DKO mice prevented direct investigation of dysregulation of the inflammatory response in mice lacking both κB-Ras isoforms. However, we found that in macrophages κB-Ras2 was expressed at a significantly higher level than κB-Ras1 (Figure 2C). Thus, we investigated whether κB-Ras2-deficient macrophages (2SKO) might behave like kB-Ras1/kB-Ras2 double knock-outs (DKO) and demonstrate enhanced NF-κB dependent transcription.

Consistent with the experiments carried out in DKO MEFs, we found that 2SKO macrophages showed enhanced expression of TNFα, but not IL-6 (Figure 2D). A miniarray containing 92 NF-κB target genes confirmed that absence of κB-Ras2 affects only a subset of NF-κB target genes (Figure S2A). Macrophages that also lacked IκBβ, in addition to κB-Ras2, failed to show increased levels of TNFα (Figure 2D and S2B, C), thereby establishing κB-Ras2 as a regulator of IκBβ-dependent NF-κB activity. In agreement with these findings, κB-Ras2 deficient mice were significantly more susceptible in a LPS-induced septic shock model where sensitivity largely depends on the NF-κB induced production of pro-inflammatory cytokines by macrophages (Figure 2E). As predicted by the results on isolated macrophages, the enhanced septic shock lethality of κB-Ras2 knock-outs was nearly abolished when IκBβ was also deleted from these mice (Figure 2F). These results suggest that κB-Ras proteins indeed regulate NF-κB activity in primary cells in an IκBβ-dependent fashion and that κB-Ras function is required for regulating inflammation *in vivo*.

We next asked whether increased cytokine production might play a role in the perinatal lethality of DKO pups as TNFα can act as a potent inhibitor of surfactant protein expression (Bachurski et al., 1995; Pryhuber et al., 1996; Wispe et al., 1990). Analysis of lung tissues of newborn κB-Ras DKO mice revealed enhanced levels of TNFα (Figure 2G). Importantly, concomitant deletion of TNF receptor 1 (TNFR1, tnfrsf1a) could partially rescue perinatal lethality of DKO mice with about 15% of the expected number of pups surviving (Figure S2D). The triple knock-out mice (*NKIRAS1*$^{-/-}$*NKIRAS2*$^{-/-}$*TNFR1*$^{-/-}$) were runted and mostly obtained when litter size was small, implying that sibling competition might decrease the survival of these weaker mice. Nevertheless, the surviving triple knock-outs were viable (Figure 2H). The rescue from lethality through TNFR1 ablation strongly suggests that the

completely penetrant lethality of κB-Ras DKO mice are due to increased levels of TNFα. κB-Ras proteins interact with Ral-GAP complexes.

To identify potential, novel κB-Ras-interacting proteins, we performed tandem-affinity purification (TAP) with tagged κB-Ras2, followed by mass-spectrometry (Figure 3A). Interestingly, we detected subunits of the Ral-GAP complexes in our purified samples (Figure 3B). Confirming these results, we detected binding of endogenous Ral-GAP to TAP-tagged κB-Ras2, as well as association of endogenous κB-Ras proteins with both Ral-GAP complexes by immunoblot analysis (Figure 3C, D). Furthermore, gel-filtration experiments demonstrated that a large fraction of endogenous κB-Ras forms a high molecular weight complex with Ral-GAP under resting cellular conditions (Figure 3E).

To characterize the architecture of the κB-Ras:Ral-GAP complexes, we overexpressed κB-Ras1 or κB-Ras2, and different combinations and deletions of Ral-GAP subunits in HEK293 cells, which possess very low levels of endogenous Ral-GAP (unpublished observations and (Leto et al., 2013)). We found that both κB-Ras1 and κB-Ras2 interacted with the catalytic subunits Ral-GAPα1 or Ral-GAPα2, and that this interaction was independent of co-expression of the regulatory subunit, Ral-GAPβ (Figure 3F, G). In contrast, co-precipitation of Ral-GAPβ with κB-Ras1 or κB-Ras2 required the presence of Ral-GAPα1 or Ral-GAPα2 demonstrating that κB-Ras proteins bind to the Ral-GAPα catalytic subunits (Figure 3H, I). Progressive deletions from the N-terminus or C-terminus of Ral-GAPα revealed that κB-Ras proteins did not bind to the C-terminal GAP domain; instead the κB-Ras binding domain was located within the N-terminal 150 amino acids, a region of undefined structure and function, which is highly homologous between Ral-GAPα1 and Ral-GAPα2 (Figure S3A–D). In agreement with these findings, co-expression of κB-Ras did not compete with Ral for binding to Ral-GAP, which occurs through the C-terminal GAP domain (Fig. S3E). Next, we introduced mutations in κB-Ras2 that force a GDP-bound state (κB-Ras2 T18D and D61A) (Tago et al., 2010). Binding of these κB-Ras2 mutants to Ral-GAPα2 was significantly reduced compared to wild-type κB-Ras, demonstrating that constitutive GTP-binding is required for association of κB-Ras with Ral-GAP (Figure 3J). Interestingly, the κB-Ras T18N mutant has previously been shown to retain the inhibitory effect of κB-Ras on NF-κB activity (Tago et al., 2010), suggesting that regulation of Ral-GAP and NF-κB activity are independent and differentially regulated functions of κB-Ras proteins. Together these results reveal a remarkable role for GTP-bound κB-Ras proteins as constitutive cofactors of Ral-GAPs, with the interaction occurring through a novel GTPase-binding region in the Ral-GAPα subunits.

κB-Ras proteins regulate the Ral pathway by activating the Ral-GAP complex

Based on the identification of the Ral-GAP-κB-Ras interaction, we decided to investigate a possible role of κB-Ras proteins in the Ras/Ral signaling network. To detect Ral activity in cells, we used immobilized, recombinant GST-tagged Ral-binding domain of the Ral effector Sec5 (aa 1–99) to specifically pull-down GTP-loaded forms of Ral GTPases from cell lysates. We found that the levels of GTP-bound RalA and RalB were dramatically increased in κB-Ras DKO cells, under resting conditions and in response to EGF (epidermal growth factor) (Figure 4A, B and S4A). Absence of κB-Ras1 or κB-Ras2 alone did not

affect Ral-GTP levels, and increased levels of Ral-GTP in the DKO cells could be reversed by reintroduction of κB-Ras1 or κB-Ras2 (Figure 4A, B, C and S4B, C). This demonstrates that both κB-Ras proteins are important in Ral signaling and that, in cell types that express both κB-Ras isoforms, absence of both is required to increase Ral-GTP levels. Ras-GTP levels were unaltered in κB-Ras deficient cells, demonstrating that Ral deregulation in κB-Ras DKO MEFs does not occur through an effect on Ras-induced Ral-GEF activation (Figure 4D). Furthermore, the Raf/MAPK and PI3K/Akt signaling pathways were unaffected in κB-Ras DKO cells as assayed by phosphorylation of Erk and Akt, revealing that κB-Ras deficiency specifically affects Ral signaling downstream of Ras (Figure 4A, B).

The fact that κB-Ras proteins interacted with Ral-GAP complexes, and that Ral-GTP levels were upregulated in κB-Ras DKO cells while Ras-GTP levels were unaltered, suggested that κB-Ras proteins might positively regulate the activity of Ral-GAP complexes. Absence of κB-Ras proteins would consequently lead to a reduction of GAP activity and increased levels of active Ral. Furthermore, as levels of Ral-GTP were augmented in unstimulated κB-Ras DKO cells, we hypothesized that constitutive Ral-GAP activity might be required to prevent aberrant Ral signaling under resting conditions. Indeed, abrogation of Ral-GAP activity through stable knock-down of Ral-GAPβ resulted in increased Ral-GTP levels comparable to those seen in κB-Ras DKO MEFs (Figure 4E and S4D). Next, we wished to directly demonstrate that κB-Ras regulates Ral signaling through an effect on Ral-GAP. Since expression and heterodimer formation of Ral-GAP subunits were unaltered in κB-Ras DKO cells (Figure S4E, F, G), we tested whether binding of κB-Ras to Ral-GAP could alter the function of the complex in cells. As expected, overexpression of Ral-GAP complex subunits decreased Ral-GTP levels in HEK293 cells. Co-expression of κB-Ras however enhanced Ral-GAP activity and consequently further lowered Ral-GTP levels demonstrating that κB-Ras association boosts GAP activity (Figure 5A). Next, we generated a fast-exchanging RalA mutant F39L, which is activated largely independently of GEFs but is still responsive to GAP function, and has previously been used to distinguish between GEF- and GAP-dependent Ral-activating events (Chen et al., 2011). We found that RalA F39L-GTP levels were much higher in κB-Ras DKO cells than in wildtype cells demonstrating that Ral-GAP, but not Ral-GEF, activity is deregulated in the absence of κB-Ras proteins (Figure 5B). In addition, we overexpressed a dominant-negative RalA mutant, RalA S28N that has previously been demonstrated to inhibit Ral-GEF dependent activation of Ral GTPases (Bodempudi et al., 2009; Urano et al., 1996; Vigil et al., 2010). Expression of RalA S28N did not alter Ral-GTP levels in κB-Ras DKO MEFs (Figure 5C), confirming that κB-Ras regulates Ral activity through a GEF-independent mechanism. Together these results reveal that κB-Ras proteins are essential for Ral-GAP activity and consequently for controlling Ral signaling in cells.

κB-Ras proteins regulate AIP by affecting the Ral-pathway

Ral GTPases have been implicated in the regulation of various cellular processes including cytoskeletal rearrangements, protein trafficking and autophagy. Furthermore RalB activity has been demonstrated to help mounting immune responses to viral infections through promoting production of IRF3 target genes such as IFNβ and ISG56/IFIT1(Chien et al., 2006). Importantly, Ral GTPases regulate cell survival and proliferation under anchorage-

independent growth conditions in an oncogenic context (Bodemann and White, 2008; Feig, 2003). We therefore next examined the proliferation characteristics of κB-Ras deficient cells. Comparing the growth of primary as well as SV40-immortalized wildtype, κB-Ras1 SKO, κB-Ras2 SKO and the κB-Ras1/2 DKO MEFs under adherent conditions failed to show any significant difference (Figure 5D and data not shown). However, when we performed colony formation assays under anchorage independent conditions, we observed that absence of both κB-Ras proteins greatly enhanced AIP of immortalized MEFs (Figure 5E). In agreement with the previously demonstrated redundancy between κB-Ras proteins in Ral regulation in MEFs, re-expression of κB-Ras2 in DKO MEFs to a level equivalent to the sum of total endogenous κB-Ras1 and κB-Ras2 reduced colony formation to that of wildtype MEFs (Figure 5E).

Several earlier studies had suggested that Ral activation does not drive transformed growth in rodent fibroblasts, as overexpression of GTP-locked Ral did not cause potent transformation (Collette et al., 2004; Hamad et al., 2002). Thus, we wished to confirm that activation of Ral GTPases in our immortalized MEFs indeed contributes to transformed growth. We examined MEFs in which Ral activation was triggered by stable knock-down of Ral-GAPβ, which - like κB-Ras deficiency - specifically affects the Ral, but not the Raf and PI3K pathways. We found that Ral-GAP depletion resulted in a significant increase in AIP, confirming the importance of Ral-GAP/Ral activity for this process in our system (Figure 5F). To test whether increased Ral activity was responsible for the increased AIP in κB-Ras DKOs, we next blocked activity of both Ral GTPases by stable overexpression of the Ral binding domain of Ral-BP1 (RBD-RBP1), which acts as a Ral super-repressor (Chien and White, 2003). Inhibition of Ral GTPases by RBD-RBP1 diminished AIP of κB-Ras DKO cells, demonstrating that κB-Ras proteins can counteract AIP through their effect on Ral signaling (Figure 5G).

We also analyzed whether κB-Ras proteins affect other Ral-dependent processes. We found that autophagy was enhanced in κB-Ras DKO MEFs, which could be counteracted by knocking-down RalB, thereby demonstrating Ral dependence (Figure S5A, B). Furthermore, in agreement with our earlier findings that κB-Ras2 is the major isoform in macrophages we detected increased Ral-GTP levels and enhanced production of IFNβ and ISG56/IFIT1 in κB-Ras2 SKO macrophages upon poly-IC stimulation (Figure S5C, D). This enhancement was Ral-dependent as RalB knock-down reduced ISG56/IFIT1 production (Figure S5E). Concordantly, we also detected increased expression of ISG56/IFIT1 in Raw cells, in which Ral activity was increased through Ral-GAPβ knock-down (Figure S5F). However, in contrast to κB-Ras deficiency, Ral-GAP knock-down did not alter TNFα production in response to LPS stimulation, confirming that regulation of NF-κB and Ral-GAP are independent functions of κB-Ras proteins (Figure S5G).

κB-Ras deficiency promotes Ras-driven tumorigenesis

Chronic Ral activity is particularly relevant in cancers expressing mutated classical Ras proteins, which have been shown to trigger Ral activity through recruitment of Ral-GEFs as a critical part of their tumorigenic program (Bodemann and White, 2008). Therefore, we tested whether κB-Ras proteins, through their effect on Ral-GAP, might be able to curtail

Ras-induced Ral activation and counteract the tumorigenic potential of mutated Ras oncogenes. We stably expressed an oncogenic H-Ras mutant (H-Ras G12V) in DKO and Ral-GAPβ knock-down cells and the respective control cells (Figure S6A). As in immortalized MEFs, activation of Raf/MAPK and PI3K/Akt pathways did not differ between Ras12V-expressing WT and DKO cells as determined by immunoblot of Erk and Akt phosphorylation (Figure S6B). However, absence of either κB-Ras or Ral-GAP led to further elevation of Ral-GTP levels when compared to wildtype cells expressing H-Ras G12V, demonstrating that abrogation of Ral-GAP activity further augments oncogenic Ras-induced Ral activity (Figure 6A and S6C).

We next wanted to test whether absence of κB-Ras affects oncogenic Ras-induced tumorigenesis. Foci formation assays showed that absence of κB-Ras proteins promoted transformation of spontaneously immortalized MEFs (3T3 MEFs) through oncogenic H-Ras G12V (Figure 6B). We then subcutaneously injected Ras G12V expressing wild-type and κB-Ras DKO cells into the flanks of Scid-beige mice. Tumors devoid of κB-Ras proteins showed significantly accelerated tumor growth as monitored by tumor size and tumor weight (Figure 6C and S6D). κB-Ras deficiency seemed to mainly affect proliferation, while only mildly reducing apoptosis rates (Figure S6E, F). Together these findings reveal that κB-Ras proteins can function as tumor suppressors in Ras G12V-driven cancer, counteracting oncogenic Ras-induced cellular transformation and transformed growth *in vivo*,

In agreement with the assumption that enhanced Ral activity can promote tumor growth in our system, knock-down of Ral-GAP activity also led to increased tumor growth of Ras G12V-expressing cells *in vivo* (Figure 6D and S6G, H). However, κB-Ras proteins can regulate both Ral- and NF-κB signaling and increased NF-κB activity could also contribute to enhanced tumor growth by direct effects on tumor cell survival and through generation of a pro-inflammatory tumor environment that further promotes proliferation (Barbie et al., 2009; Ben-Neriah and Karin, 2011; Meylan et al., 2009). We therefore overexpressed the Ral-superrepressor RBD-RBP1 or the IκB superrepressor in Ras12V-transformed DKO MEFs and tested the resulting effects on the increase of AIP *in vitro* and of Ras-driven tumorigenesis *in vivo* (Figure 6E, F and S6I). In agreement with our previous results, expression of RBD-RBP1 strongly decreased tumor growth of DKO MEFs. Expression of the IκB superrepressor led to a milder but still significant reduction in AIP and tumor growth. These results are in line with our finding that κB-Ras proteins affect a limited, mostly pro-inflammatory subset of NF-κB target genes. In agreement, we found that pro-inflammatory NF-κB target genes, such as TNF and KC (murine IL-8 homologue) were significantly increased in κB-Ras DKO tumors and were reduced by overexpression of the IκB superrepressor (Figure 6G and S7A, B). Anti-apoptotic NF-κB target genes such as Bcl-XL and the Bcl2 family member Bfl/A1 were also upregulated in DKO tumors but more modestly, which likely reflects a secondary effect of increased production of proinflammatory cytokines by DKO tumors (Figure S7A). While TNFα was increased in κB-Ras DKO tumors only, known Ral-dependent genes, such as MMP-1 (Smith et al., 2012) or CD24 (Smith et al., 2006), were upregulated in both κB-Ras deficient and Ral-GAPβ depleted tumors (Figure 6G, H and S7C). These results confirm that κB-Ras and Ral-GAP

function through a common pathway and highlight that the regulation of NF-κB activity through κB-Ras proteins is independent of their effect on Ral-GAP/Ral activity. Importantly, these findings demonstrate that κB-Ras effects on both proliferation and inflammation/cell death, through the Ral and NF-κB pathways respectively, contribute to the tumor suppressor potential of these proteins.

κB-Ras proteins are decreased in human tumors and affect the tumorigenic capacity of cells derived from such tumors

Data mining revealed that κB-Ras protein or mRNA levels are reduced in a variety of human cancers including breast cancer (Finak et al., 2008; Karnoub et al., 2007), lung cancer (Garber et al., 2001), glioblastoma (Murat et al., 2008; Sun et al., 2006), glioma (Lin et al., 2012), renal cell carcinoma (Gerashchenko et al., 2010), oral cancer (Jou et al., 2010) and esophageal adenocarcinoma (Kim et al., 2010) (Figure S7D, E). While the underlying mechanisms and resulting consequences had not been addressed, these studies implied that downregulation of κB-Ras might support tumorigenesis in different oncogenic contexts. To further support this hypothesis, we analyzed samples from patients with pancreatic ductal adenocarcinoma (PDAC), a highly aggressive cancer characterized by the presence of mutated K-Ras in >90% of cases. We found that mRNA levels for both κB-Ras proteins were significantly decreased compared to matched normal pancreas controls (Figure 7A). As κB-Ras levels had been reported to be low in lung cancer, we next examined expression of κB-Ras in mutant or wild-type K-Ras expressing lung cancer cell lines. Remarkably, we observed that the κB-Ras protein level was significantly lower in the mutant K-Ras expressing tumor cells (Figure 7B). These mutant K-Ras expressing cell lines have been demonstrated to critically depend on Ral activity for survival (Barbie et al., 2009), suggesting that downregulation of κB-Ras might be especially advantageous for tumor growth under conditions of Ral-dependence. Raising κB-Ras levels by stable lentiviral transduction significantly diminished their potential to form colonies under anchorage-independent growth conditions (Figure 7C, D). Hence, lowering of κB-Ras levels represents a tumorigenic advantage in human cancer cell lines, strongly implicating κB-Ras-mediated regulation in human tumorigenesis.

Discussion

Tumorigenesis is a multi-factorial process that leads to the loss of normal controls on cell proliferation and cell death. A pro-inflammatory microenvironment can also potently support tumor growth. The cellular pathways that trigger these biological processes generally initiate from distinct regulatory molecules. We have now found that κB-Ras proteins negatively regulate the transcription factor NF-κB through IκBβ *and* Ras/Ral signaling through the Ral-GAP complexes. Hence κB-Ras deficiency leads to upregulation of both the NF-κB and Ral pathways, which have been shown to control survival/inflammation as well as proliferation (AIP) in the context of tumorigenesis. Consequently, κB-Ras deficiency leads to significantly enhanced AIP and tumorigenesis of grafted cells, as well as a concomitant increase in inflammatory gene expression in tumors.

Due to the perinatal lethality of κB-Ras DKO mice, we tested the tumorigenic potential of transformed κB-Ras deficient cells by grafting them subcutaneously. In this model, blocking the NF-κB or Ral pathways leads to decreased tumor growth, implicating both pathways in tumor formation. Inhibition of NF-κB signaling had a more modest effect but the tumors were grown in immune-deficient mice, in which NF-κB dependent inflammatory tumor-promoting effects are likely underemphasized. Unfortunately, the perinatal lethality of κB-Ras deficient mice impeded analysis of the effect of complete κB-Ras deficiency on tumorigenesis in a mouse model of cancer. Nevertheless, our results establish that κB-Ras proteins function in two critical pathways known to affect inflammation, cell proliferation, and cell survival, to counteract tumorigenesis, and therefore appear to possess a unique tumor suppressive potential. Clinical relevance of κB-Ras proteins is indicated through the observed reduction of κB-Ras levels in several human cancers and the fact that reduction of κB-Ras levels in cells derived from patients promotes potent transformed growth.

The perinatal lethality of κB-Ras DKO mice was unexpected, as knock-outs of most other inhibitors of NF-κB lead to later postnatal death. However, mice expressing catalytically inactive CYLD also die soon after birth due to a lung phenotype, with less inflated, hypercellular lungs (Trompouki et al., 2009). While the basis of the CYLD-related lung phenotype remains uncharacterized, it is intriguing that abolishing function of another negative regulator of the NF-κB pathway leads to a phenotype similar to the κB-Ras knock-outs. The rescue from lethality observed upon deleting TNFR1 in κB-Ras DKO mice clearly implicates TNF in the lung phenotype and early death, possibly through reduction of surfactant protein production. However the low numbers and the runted phenotype of the rescued mice suggest other pathways must be dysregulated as well. Whether disturbance of Ral homeostasis contributes to lethality remains to be investigated, though RalA, RalB, or Ral-GAP complexes have not been linked to lung development or function. Determining the exact reasons underlying the lethality of κB-Ras DKO mice remains a topic for further research.

In addition to their function in IκBβ/NF-κB regulation, our studies revealed interaction of κB-Ras proteins with Ral-GAP complexes and a novel role for κB-Ras in the Ral pathway. Interestingly, interaction of the *Drosophila* homologues of κB-Ras and Ral-GAP (CG5521-PA) has recently been detected in a high-throughput screen, suggesting evolutionary conservation of the κB-Ras:Ral-GAP interaction (Guruharsha et al., 2011). While our studies demonstrate that κB-Ras proteins affect the activity of Ral-GAP complexes in cells, the mechanism by which κB-Ras binding alters Ral-GAP activity remains to be determined. κB-Ras binding to the Ral-GAP complex does not change its activity *in vitro* (unpublished observation), suggesting that κB-Ras proteins regulate GAP activity in conjunction with unknown cellular factors or affect subcellular localization of Ral-GAP complexes. Regulation of GAP activity by a constitutively associated, GTP-locked GTPase of the same family represents a unique scenario. A similar situation has only been suggested for the regulation of p190-B Rho-GAP activity through direct interaction with the GTPase Rnd3 (Wennerberg et al., 2003). Like κB-Ras, Rnd3 is found GTP-bound under resting cellular conditions. However, it remains unclear whether Rnd proteins affect Rho GEF activity and how Rnd proteins affect GAP activity, though recent studies have suggested they might

target p190-B to lipid rafts (Oinuma et al., 2012). An analogous scenario could be imagined for the regulation of Ral-GAP by κB-Ras, however κB-Ras proteins lack membrane targeting sequences and we do not observe localization changes of ectopically expressed Ral-GAP subunits in the absence of κB-Ras proteins (unpublished observation). Elucidating the exact molecular mechanism by which κB-Ras proteins affect Ral-GAP activity, and whether this interaction allows for stimulus-induced Ral activation under specific conditions remains an important future objective and will require generation of improved biochemical and genetic tools to study Ral-GAP complexes.

In this study, we demonstrate that in the absence of κB-Ras, upregulation of the Ral pathway leads to increased AIP of immortalized MEFs. Although Ral GTPases have been linked to AIP of human cancer cells by several independent studies, their involvement in AIP regulation in murine cells had been controversial. This was largely due to earlier studies demonstrating that ectopic expression of activated Ral mutants, or Ras mutants that specifically trigger Ral, in murine cells did not lead to transformation (Collette et al., 2004; Hamad et al., 2002; Rangarajan et al., 2004). However, active Ral proteins enhance transformation of NIH3T3 cells expressing activated forms of Raf, and dominant-negative Ral mutants can suppress their Ras-induced transformation (Urano et al., 1996; White et al., 1996). Importantly, recent studies on conditional RalA and RalB knock-out mice have clearly demonstrated the importance of both Ral GTPases for K-Ras-driven tumorigenesis in murine cells (Peschard et al., 2012). By investigating κB-Ras deficient and Ral-GAPβ knock-down murine fibroblasts as well as human cancer cell lines we confirm the importance of the κB-Ras/Ral-GAP/Ral signaling node for AIP in both mouse and human systems. It is likely that the reported differences in the effects of Ral on transformation of human versus mouse cells are due to the different cell systems and immortalization methods used or might be explainable by the fact that GTP-locked Ral mutants cannot mimic all facets of chronic Ral activation.

Deficiency of κB-Ras leads to upregulation of both RalA- and RalB-GTP levels. Knock-down studies in cancer cell lines had originally suggested distinct functions of the two Ral GTPases in tumor growth, with RalA promoting proliferation and RalB enhancing cell survival. In our κB-Ras deficient cells the main effect of increased Ral activity seems to be on proliferation which is in line with the recently published effects of RalA/B knock-out on K-Ras driven tumorigenesis *in vivo* (Peschard et al., 2012). We also investigated some of the described RalB specific functions. We find expression of the IRF3 target genes IFIT1/ISG56 and IFNβ increased in κB-Ras2 deficient macrophages in response to poly(I:C) stimulation. This may suggest that during a viral infection, both deregulated NF-κB and Ral signaling would contribute to an increased immune response in the case of κB-Ras deficiency. Furthermore, RalB has been shown to upregulate autophagy and congruently we found autophagy to be enhanced in κB-Ras DKO MEFs. Interestingly, autophagy has been demonstrated to support tumorigenesis as it can ensure cell survival by providing cellular energy during starvation periods.

We, and a number of independent groups, observed that κB-Ras levels are reduced in tumors (Finak et al., 2008, Karnoub et al., 2007, Garber et al., 2001, Murat et al., 2008, Sun et al., 2006, Gerashchenko et al., 2010, Jou et al., 2010, Kim et al., 2010). One study

reported a correlation between κB-Ras protein level and clinical outcome of glioma patients (Lin et al., 2012). The mechanism responsible for the reduction in κB-Ras levels remains to be determined. As the reduction is observed at the mRNA level, the most likely explanations are either down-regulation of κB-Ras transcription, or a post-transcriptional mechanism that leads to enhanced degradation of the κB-Ras mRNA. The κB-Ras1 and κB-Ras2 genes in humans are located on chromosomes 3 and 17, respectively, and analysis of their unique promoter sequences failed to reveal an obvious pathway that would explain decreased expression of both κB-Ras isoforms in tumors. We are thus actively studying whether κB-Ras downregulation occurs through transcriptional regulation or through effects on mRNA stability. Interestingly, we found that κB-Ras levels are preferentially reduced in mutant K-Ras expressing human lung cancer cell lines, suggesting that in these cells, which critically depend on Ras and Ral activity for survival, a reduction of κB-Ras levels might be advantageous for tumor growth as it further boosts Ral activity.

In summary, we have identified κB-Ras proteins as regulators of both NF-κB and Ral pathways. Suppression of both of these pathways contributes to the ability of κB-Ras proteins to suppress Ras-driven tumorigenesis *in vivo*. Our studies place κB-Ras proteins at the interface of cancer and inflammation and raise the possibility that modulating κB-Ras function could help counteract the proliferative and survival processes, as well as the tumor-promoting inflammatory processes, that characterize mutant Ras-driven tumorigenesis (Figure 7E).

Experimental Procedures

Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were generated from E12.5 embryos. MEFs were either immortalized following a standard 3T3 procedure(Todaro and Green, 1963) or through transfection with SV40 large T antigen. Immortalized MEF lines, HEK293 and HEK293FT cell lines were cultured in DMEM containing 5 % fetal bovine serum (FBS). Raw cells were cultured in RMPI medium containing 10 % FBS. For co-immunoprecipitation experiments, HEK293 cells were transfected using Lipofectamine2000 transfection reagent (Invitrogen) and lysed 24 h after transfection. For retrovirus production, 3×10^6 HEK293FT cells were seeded per 10 cm dish and transfected using Fugene 6 (Roche). Virus was harvested 48 h after transfection and used to infect MEFs that had been seeded the previous day at 1×10^5 cells per 10cm dish. Antibiotic selection (Puromycin 15 μg/ml, Zeocin 0.3 μg/ml) was started 48h after infection to achieve stable expression.

Immunoprecipitation, immunoblotting and gelfiltration

For all co-immunopreciptations, cells were lysed in Co-IP buffer. Protein complexes were precipitated with the respective antibodies for 2 h to overnight at 4 °C. Precipitates were washed 3 times and then subjected to SDS-PAGE. Proteins were transferred onto PVDF membranes. Membranes were blocked using 5 % dry milk powder/TBS-T or 3 % BSA/ TBS-T and incubated with primary antibody overnight. For gel filtration, cells were lysed in Co-IP buffer without glycerol. Lysates were fractionated (0.5 ml fraction size) on Superose 6 or Superdex 200 columns and every second fraction was analyzed by immunoblotting.

Analysis of Ral-GTP levels

SV40-immortalized mouse embryonic fibroblasts (MEFs) were seeded at 1×10^6 cells per 10cm culture dish and starting on the next day starved (DMEM medium without serum) for 24h. MEFs were then stimulated with EGF (50 ng/ml) and lysed in Ral lysis buffer (50 mM Tris pH7.5, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM EGTA, 1 % Triton-X-100). GTP-bound RalA was precipitated from the lysate with 20ug GST-Sec5 bound to glutathione sepharose for 20 min. Precipitates were washed with Ral lysis buffer and analyzed by SDS-PAGE and immunoblotting using RalA or RalB antibodies.

Tumorigenesis assay

SV40-immortalized and H-Ras G12V-transformed MEFs at a density of 2×10^7 cells/ml in cold PBS were mixed with Matrigel HC (BD Biosciences) at a 1:1 ratio. 1 million cells (100ul volume) were injected subcutaneously into both flanks of Scid-beige mice (C.B-17, Taconic). Tumor volumes were determined every 2–3 days using a caliper and calculated as ½ × $length^2$ × width in mm^3. At the end of the study, tumors were resected, weighed and snap-frozen for RNA preparation.

BrDU, colony formation and foci formation assays

Proliferation of fibroblasts was measured using a BrDU cell proliferation assay kit (Calbiochem) according to manufacturer's protocol. For colony formation assays, 3000 SV40-immortalized MEFs were suspended in 1.5 ml 1:1 Matrigel LC (BD Biosciences)/ DMEM and seeded on 3cm dishes coated with 1.5ml of 1% soft agar/DMEM. After solidification, the Matrigel layer was overlaid with 0.5ml growth medium which was replenished once a week. After 14 days, pictures were taken using a Zeiss Imager M2 microscope with motorized stage and colonies larger than 50 cells were scored using ImageJ software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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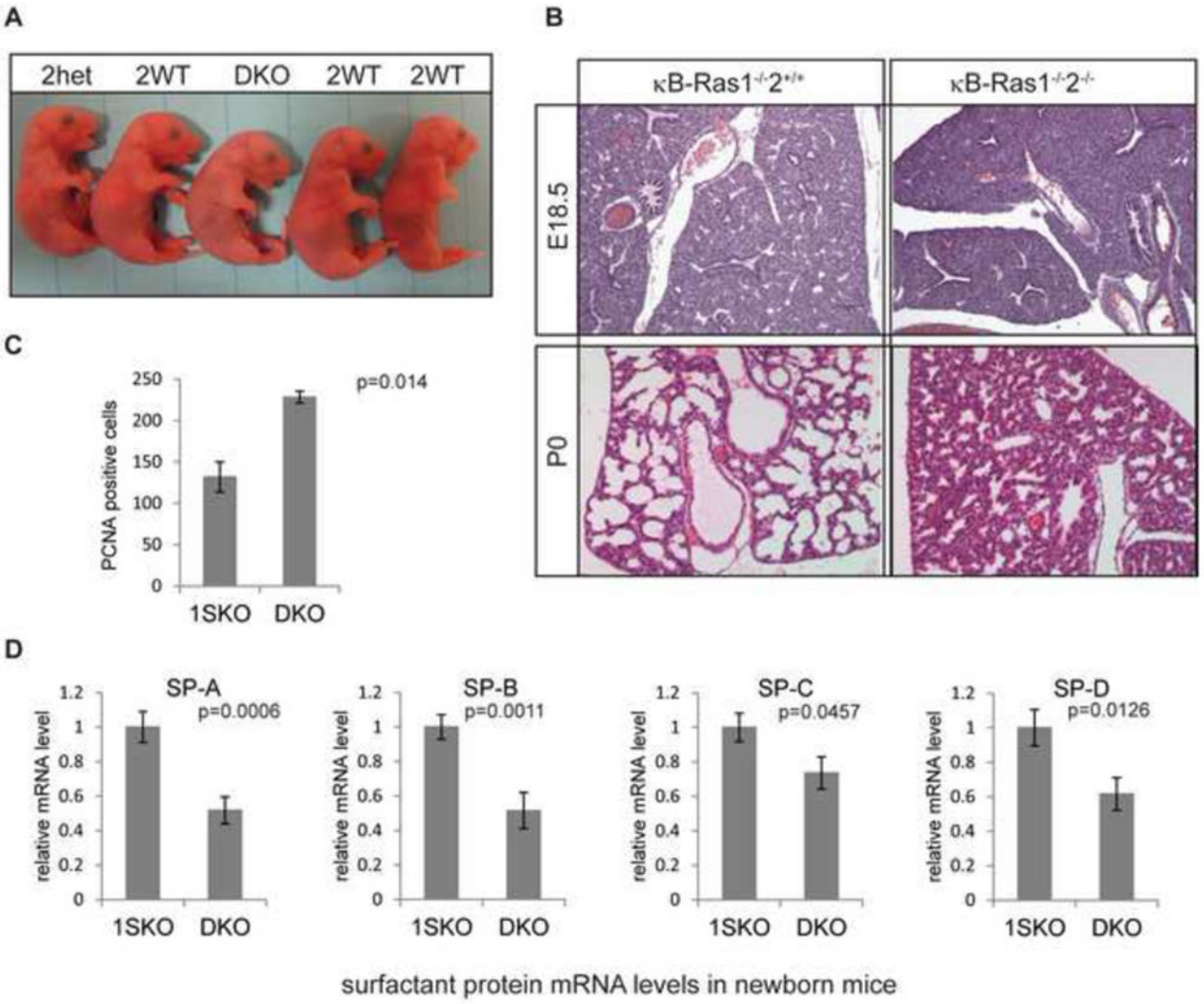

Figure 1. Genetic ablation of κB-Ras1 and κB-Ras2 leads to perinatal lethality

(**A**) New born *κB-Ras1*$^{-/-}$*2*$^{+/-}$ (2het), *κB-Ras1*$^{-/-}$*2*$^{+/+}$ (2WT) and *κB-Ras1*$^{-/-}$*2*$^{-/-}$ (DKO) pups 1h after birth. (**B**) Lung sections of κB-Ras DKO and control littermates were prepared on gestational day E18.5 and right after birth (P0) and stained with Hematoxylin and Eosin. (**C**) Lung sections were prepared and stained for PCNA. PCNA positive cells were counted per field of view. Data are represented as mean ±SD (Student's t-test: p=0.014; n=4). (**D**) qPCR analysis of surfactant protein mRNA levels in lungs from newborn κB-Ras DKO and control littermates (*κB-Ras1*$^{-/-}$*2*$^{+/+}$). Data are represented as mean ±SD (Student's t-test; n=12). (See also Figure S1).

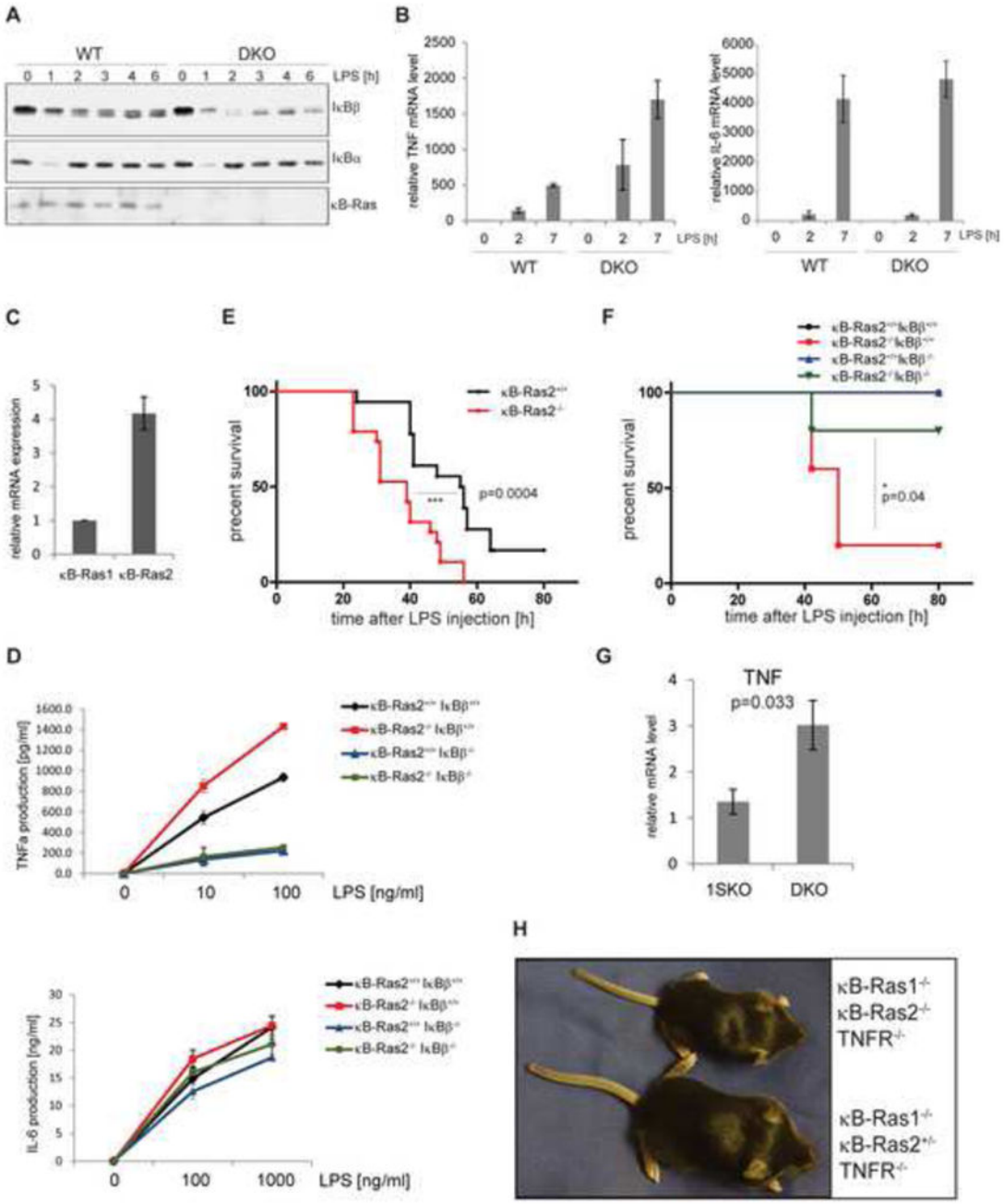

Figure 2. κB-Ras deficiency leads to upregulation of IκBβ-dependent NF-κB activity

(**A**) Wildtype and DKO primary embryonic fibroblasts (MEFs) were stimulated with LPS and IκB degradation was analyzed by immunoblot. (**B**) Wildtype and DKO MEFs were stimulated with LPS and mRNA levels of TNFα and IL-6 were analyzed by qPCR. Data are represented as mean ±SD (n=3). (**C**) Bone marrow-derived macrophages (BMDMs) from wildtype mice were analyzed for κB-Ras1 and κB-Ras2 mRNA levels by qPCR. Data are represented as mean ±SD (n=3). (**D**) BMDMs of the indicated genotypes were stimulated with different doses of LPS. TNFα and IL-6 mRNA levels were analyzed by ELISA. Data are represented as mean ±SD (n=3) (**E**) A total of 20 κB-Ras2 deficient mice and 20 control

wildtype littermates were intraperitoneally injected with LPS (18ug LPS/g body weight) in three independent experiments and survival was monitored. For statistical analysis a Mantel-Cox Test was performed. (**F**) Mice with the indicated genotypes were injected with LPS (18ug LPS/g body weight) and monitored for survival. For statistical analysis a Mantel-Cox Test was performed (n=5). (**G**) TNFα mRNA levels in lungs of newborn DKO mice were analyzed by qPCR. Data are represented as mean ±SEM (Student's t-test; n=11). (**H**) *κB-Ras1*$^{-/-}$ *κB-Ras2*$^{-/-}$*TNFR*$^{-/-}$ and *κB-Ras1*$^{-/-}$ *κB-Ras2*$^{+/-}$*TNFR*$^{-/-}$ at the age of 21 days. (See also Figure S2).

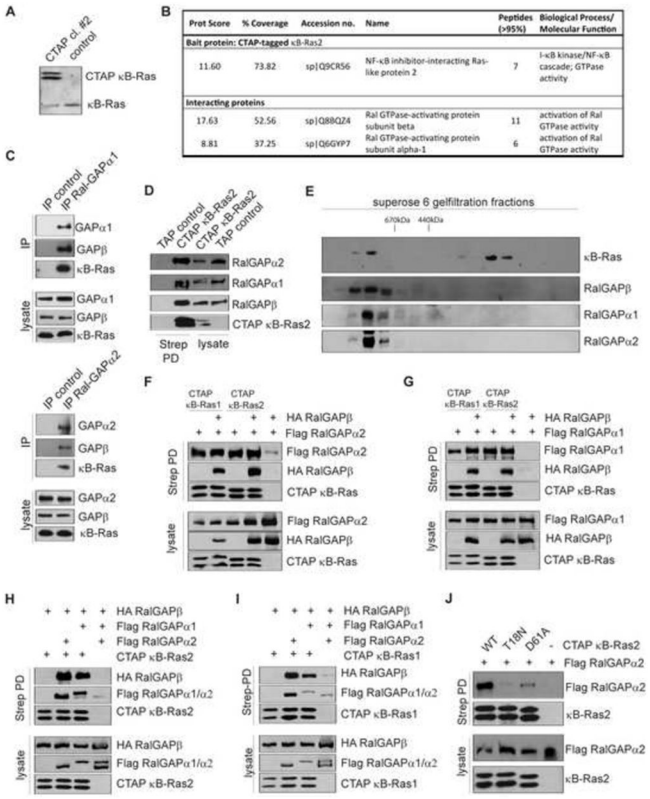

Figure 3. κB-Ras proteins interact with Ral-GAP complexes

(**A**) CTAP-κB-Ras2 was stably expressed in Raw 264.7 cells. Clone #2 was used for large scale purifications and all further procedures. (**B**) Interacting proteins of CTAP κB-Ras2 were identified by TAP proteomics. UniProtKB database (*Mus muculus*) analysis of the MS/MS data was performed using Protein Pilot™ and the Paragon algorithm. (**C**) Resting WT MEFs were lysed and endogenous Ral-GAPα1 or Ral-GAPα2 immunoprecipitated. Association of κB-Ras and Ral-GAPβ was analyzed by immunoblotting. (**D**) Raw cells stably expressing CTAP κB-Ras2 or TAP-Tag alone were lysed and CTAP κB-Ras2 precipitated with Streptavidin agarose. Association of Ral-GAP subunits was analyzed by immunoblotting. (**E**) Raw 264.7 cell lysate was fractionated on a Superose 6 gel-filtration column. Elution fractions were analyzed by immunoblotting. (**F, G**) HEK293 cells were transfected with the indicated plasmids. κB-Ras:Ral-GAPα2 or Ral-GAPα1 binding was determined by precipitation of CTAP κB-Ras proteins with Streptavidin agarose (Strep PD) and immunoblotting. (**H, I**) HEK293 cells were transfected with the indicated plasmids. κB-Ras:Ral-GAPβ binding was determined by precipitation of CTAP κB-Ras as in **F/G**. (**J**). CTAP κB-Ras2 wild-type, T18N and D61A were co-expressed with Flag Ral-GAPα2 in HEK293 cells. κB-Ras-associated proteins were analyzed as in **F/G**. (See also Figure S3).

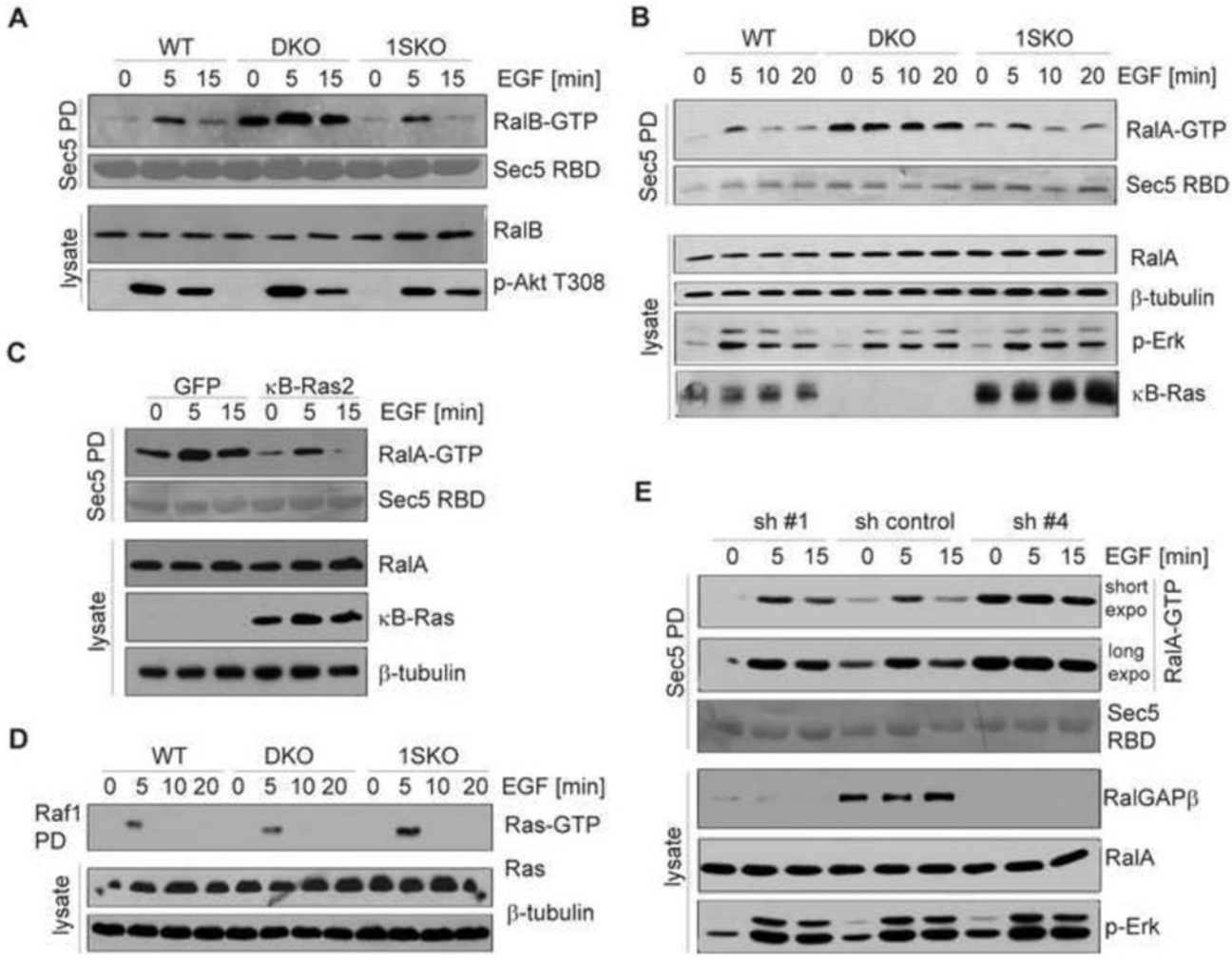

Figure 4. κB-Ras proteins regulate Ral activity

(**A**) SV40-transformed WT, 1SKO and DKO MEFs were stimulated with EGF for the indicated times. RalB-GTP levels were determined by pull-down using immobilized recombinant GST-Sec5 RBD (aa 1–99). Precipitates were analyzed by immunoblotting for RalB. GST-Sec5 levels were determined by Coomassie staining of PVDF membrane. p-Akt: phosphorylation of Akt at Thr308. (**B**) Experiments were performed as in **A** and analyzed by immunoblotting for RalA. pErk: phospho-Erk. (**C**) DKO MEFs were stably transduced with either GFP or κB-Ras2. Cells were stimulated with EGF and RalA-GTP levels determined as in **A**. (**D**) SV40-transformed WT, 1SKO and DKO MEFs were stimulated with EGF. After cell lysis, GTP-bound Ras was pulled-down by immobilized, recombinant GST-Raf1-RBD (Ras-binding domain) and analyzed by immunoblotting for Ras. (**E**) SV-40 transformed MEFs were stably transduced with two different shRNAs (sh#1 and sh#4) targeting Ral-GAPβ. RalA-GTP levels were determined by GST-Sec5 RBD pull-down and immunoblotting. (See also Figure S4).

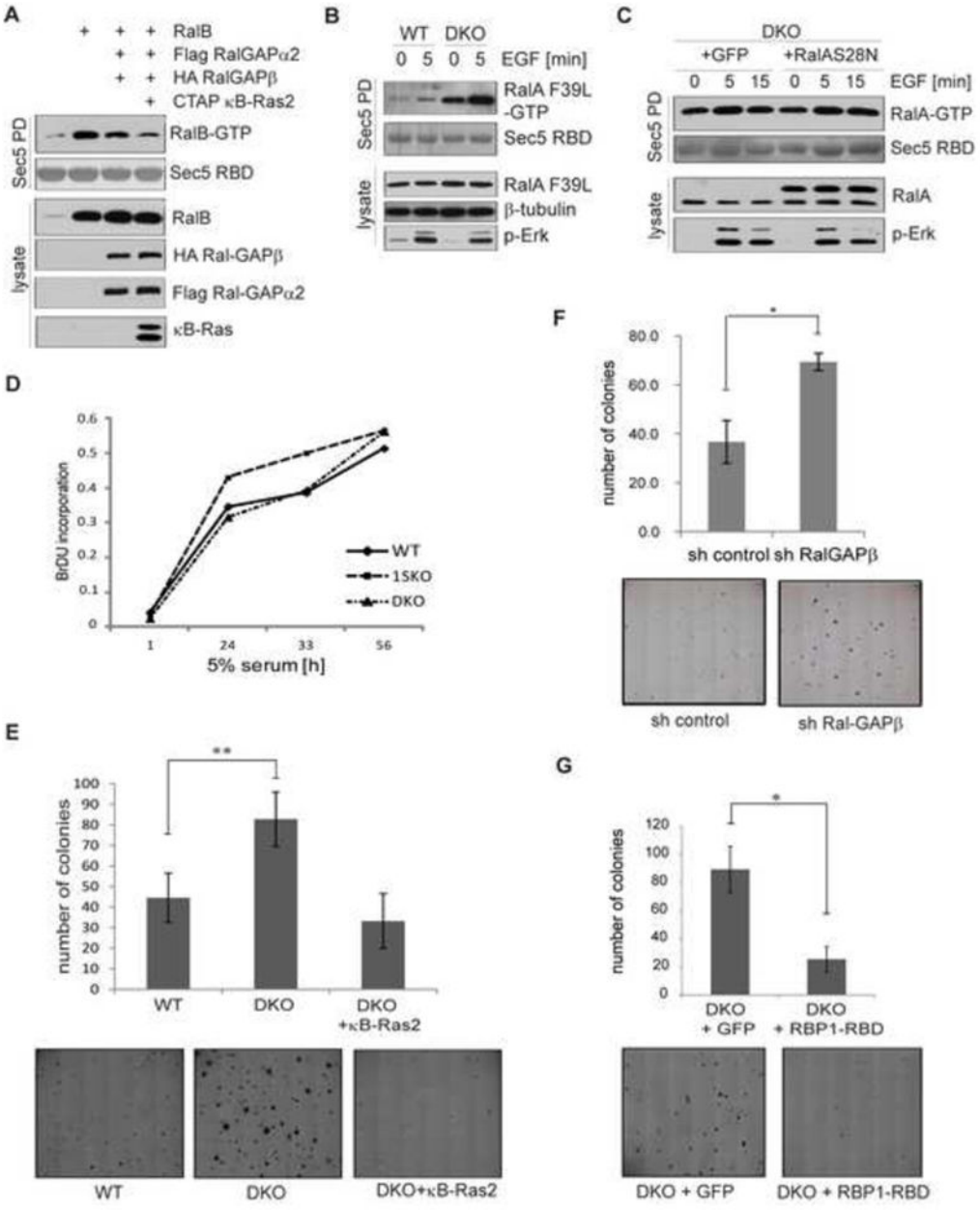

Figure 5. κB-Ras proteins inhibit Ral signaling through activating Ral-GAP activity and thus counteract AIP

(**A**) HEK293 cells were transfected with the indicated plasmids. Levels of RalB-GTP were examined by immunoblot after GST Sec5 pull-down. (**B**) SV40-transformed WT or DKO MEFs were stably transduced with the fast-cycling RalA F39L mutant. Cells were stimulated with EGF and subjected to GST-Sec5 pull-down. (**C**) SV40-transformed DKO MEFs were stably transduced with GFP or the dominant-negative RalA S28N mutant. Cells were stimulated with EGF and subjected to GST-Sec5 RBD pull-down. (**D**) BrDU incorporation of SV40 transformed WT, 1SKO and DKO MEFs during proliferation. (**E**) The ability of SV40 transformed WT, DKO MEFs and DKO MEFs, in which κB-Ras2 was stably reintroduced, to grow under anchorage-independent conditions was tested in Matrigel

colony formation assays. Data are represented as mean ±SD (n=3; Student's t-test, p=0.00022). (**F**) SV40 transformed MEFs, in which Ral-GAPβ was stably knocked-down (shRNA #4), and control MEFs expressing a scrambled shRNA were analyzed for their growth ability under anchorage-independent conditions in Matrigel colony formation assays. Data are represented as mean ±SD (n=3; Student's t-test, p=0.01013). (**G**). The ability of SV40 transformed DKO MEFs stably expressing GFP or RBP1-RBD (Ral binding domain of Ral-BP1, aa 393–499) to grow under anchorage-independent conditions was tested in Matrigel colony formation assays. Data are represented as mean ±SD (n=3; Student's t-test, p=0.032). (See also Figure S5).

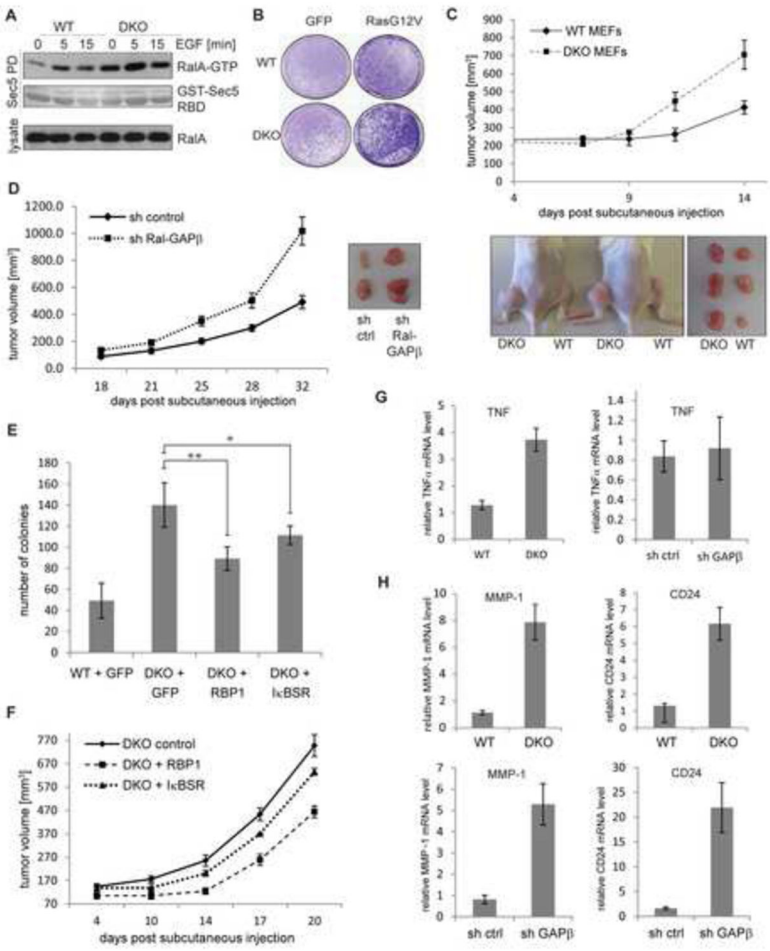

Figure 6. Enhanced Ral and NF-κB signaling in κB-Ras deficient cells contribute to their tumorigenicity

(**A**) SV40/Ras12V-transformed WT and DKO MEFs were stimulated with EGF. RalA-GTP levels were determined by GST-Sec5 pull-down and immunoblotting for RalA. (**B**) 3T3-immortalized WT or DKO MEFs were retrovirally infected with either GFP or H-Ras G12V. 14 days after infection foci formation was analyzed. A representative picture of three independent experiments is given. (**C**) SV40/RasG12V transformed WT and DKO MEFs were subcutaneously injected into the flanks of Scid-beige mice. Tumor volume −/+ SEM [mm^3] was determined by caliper measurements. (n= 9). In addition, representative pictures of subcutaneous tumors in mice (left panel) and resected tumors (right panel) are shown. (**D**) SV40/RasG12V-transformed MEFs, in which Ral-GAPβ was stably knocked-down (shRNA #4) and control MEFs expressing a scrambled shRNA were subcutaneously injected into the flanks of Scid-beige mice. Tumor volume (mm^3) −/+ SEM is given (n = 11). In addition, representative pictures of resected tumors are shown. (**E**) The ability of SV40/RasG12V transformed DKO MEFs, stably expressing GFP, the Ral superrepressor RBP1-RBD or the IκB superrepressor (NF-κB inhibition) to grow under anchorage-independent conditions were tested in Matrigel colony formation assays. Data are represented as mean ±SD (Student's t-test; n=3). (**F**) SV40/RasG12V transformed DKO MEFs, stably expressing GFP, the Ral superrepressor RBP1-RBD or the IκB superrepressor (NF-κB inhibition) were

injected in the flanks of Scid-beige mice. Tumor volume (mm^3) −/+ SEM is given (n = 7). (**G**) and (**H**) SV40/RasG12V transformed WT and DKO MEFs or sh control and sh Ral-GAPβ expressing MEFs were subcutaneously injected into the flanks of Scid-beige mice. After resection, mRNA levels for the indicated Ral- or NF-κB dependent genes were analyzed by quantitative PCR and normalized to actin mRNA levels. Data are represented as mean ±SD (n=5). (See also Figure S6).

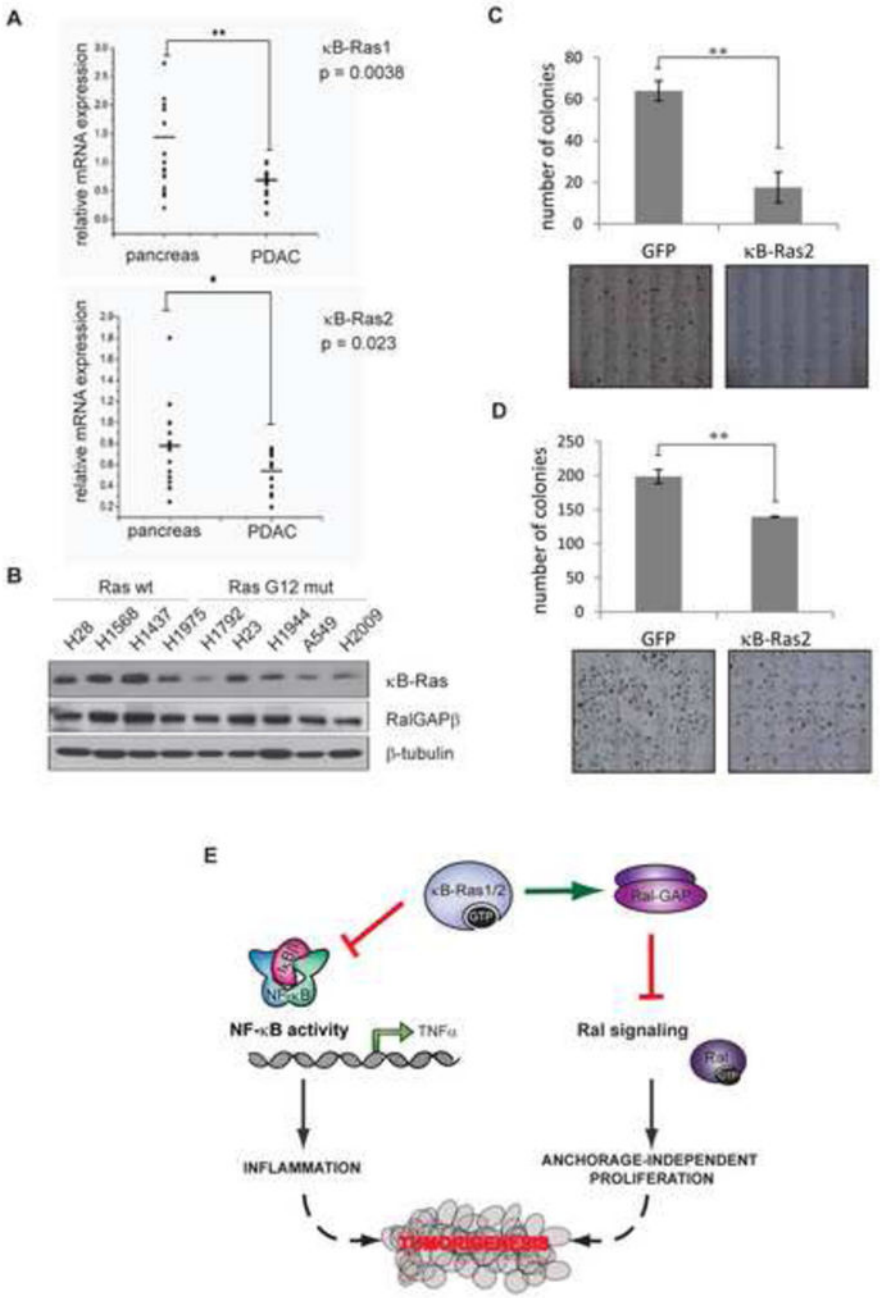

Figure 7. κB-Ras levels are decreased in human tumors and affect the tumorigenic capacity of cells derived from these tumors

(**A**) Changes in expression levels of κB-Ras1 and κB-Ras2 mRNA between pancreatic adenocarcinoma samples and matched healthy pancreatic tissue from 15 patients was analyzed by quantitative PCR and normalized to GAPDH mRNA levels. Statistical significance was evaluated by Wilcoxon rank test (κB-Ras1: p = 0.0038; κB-Ras2: p = 0.023). (**B**) The expression level of κB-Ras proteins was analyzed in four wild-type Ras expressing and five mutant K-Ras expressing cell lines by immunoblotting. (**C, D**) The ability of NCI-23 (C) or NCI-1792 (D) human lung cancer cell lines stably transduced with

GFP or κB-Ras2 to grow under anchorage-independent conditions was tested in Matrigel colony formation assays. Data are represented as mean ±SD (Student's t-test; n=3). (**E**) κB-Ras proteins regulate both NF-κB dependent inflammation and Ral-dependent proliferation. κB-Ras proteins negatively regulate certain NF-κB target genes such as TNFα through their specific effect on the NF-κB inhibitor protein IκBβ. As a consequence, genetic ablation of κB-Ras leads to enhanced production of pro-inflammatory cytokines and deregulated inflammation. In addition, κB-Ras proteins are required to maintain Ral-GAP activity in cells. Absence of κB-Ras proteins consequently results in upregulated Ral-GTP levels and enhanced AIP. Both, deregulation of inflammation and proliferation contribute to the progression of tumorigenesis, (See also Figure S7).