Cancer Cell
Article

The Signaling Adaptor p62 Is an Important NF-κB Mediator in Tumorigenesis

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DOI 10.1016/j.ccr.2008.02.001

SUMMARY

The balance between cell death and survival, two critical aspects of oncogenic transformation, determines the outcome of tumorigenesis. Nuclear factor-κB (NF-κB) is a critical regulator of survival; it is induced by the oncogene Ras and, when inhibited, accounts for the cell death response of Ras-transformed cells. Here, we show that the signaling adaptor p62 is induced by Ras, its levels are increased in human tumors, and it is required for Ras-induced survival and transformation. *p62*$^{-/-}$ mice are resistant to Ras-induced lung adenocarcinomas. p62 is necessary for Ras to trigger IκB kinase (IKK) through the polyubiquitination of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), and its deficiency produces increased reactive oxygen species (ROS) levels, which account for the enhanced cell death and reduced tumorigenicity of Ras in the absence of p62.

INTRODUCTION

Cancer arises as a consequence of sequential mutations in genes that play essential roles in the control of cell growth and proliferation (Hanahan and Weinberg, 2000). These mutations involve the loss of tumor suppressor function and the gain of function in proto-oncogenes, making tumor cells autonomous of external proliferation and survival cues (Hanahan and Weinberg, 2000). Among the proto-oncogenes most frequently altered in human tumors are the small GTPases of the Ras family, which have been found mutated in at least 25% of human cancers (Bos, 1989). The Ras oncogene family includes three members—H-Ras, K-Ras, and N-Ras—and their activation triggers a myriad of signaling pathways, only a few of which have been characterized in detail, such as the Raf-MEK (mitogen-activated protein kinase [MAPK]/extracellular signal-regulated kinase) and phosphoinositide (PI) 3-Kinase-Akt signaling cascades (Downward, 2003; Malumbres and Barbacid, 2003).

Previous studies showed that Ras activates NF-κB, which is important for cell survival and tumor transformation because it suppresses p53-independent Ras-induced cell death (Mayo et al., 1997). This is a key event because the final outcome of the oncogenic process is determined by a finely tuned balance between cell survival and death (Luo et al., 2005). Recent in vivo data strongly support the role of NF-κB in tumor initiation and progression in a cell-type dependent manner (for recent reviews, see Karin, 2006). Thus, the selective knockout of the NF-κB pathway in epithelial colon cells prevents tumor progression in a model of inflammation-modulated colon cancer (Greten et al., 2004). In contrast, the inactivation of NF-κB in the epidermis increases tumorigenesis in skin in the apparent absence of inflammation, likely as the result of increased c-Jun N-terminal kinase (JNK) activation (Khavari, 2006). On the other hand, NF-κB inhibition in hepatocytes does not prevent, but rather enhances, liver cancer in a model of diethylnitrosamine-induced hepatocarcinogenesis in mice (Maeda et al., 2005). A potential

SIGNIFICANCE

The Ras proto-oncogene has been found mutated in at least 25% of human cancers and is a potent activator of NF-κB, which is important for cell survival and tumor transformation. However, the mechanism whereby Ras activates NF-κB is not yet understood. We identify here the signaling adaptor p62 as a critical target of Ras-induced tumorigenesis and NF-κB activation in vitro and in vivo in a model of Ras-induced lung adenocarcinoma. We show that p62 is induced by Ras, activates IKK through TRAF6 polyubiquitination, is overexpressed in human tumors, and is necessary for survival of human lung adenocarcinoma cells. These findings establish the mechanism for NF-κB activation by Ras and reveal p62 as a key target in human cancer.

interpretation of this intriguing observation is the existence of compensatory proliferation to counteract the increased cell death in NF-κB-deficient hepatocytes. This cell death activates an inflammatory response orchestrated by liver macrophages (Kupffer cells) that secrete proliferative cytokines. This results in conditions that support the growth and development of surviving hepatocytes, leading to increased tumorigenesis in the liver (Maeda et al., 2005). In this case, JNK that is amplified because of the lack of NF-κB (Chang et al., 2006) is essential for the enhanced tumorigenicity (Sakurai et al., 1999). This is very interesting considering that Ras-induced apoptosis in fibroblasts, an important mechanism to restrain tumor development, relies on the activation of JNK to act as a brake in cell transformation induced by that oncogene (Kennedy et al., 2003). Therefore, the mechanism whereby the loss of NF-κB leads to increased cell death in transformed cells could be accounted for by increased JNK activation in the absence of NF-κB. This model has been very well studied in TNF-α-activated cells, but whether it is also valid in Ras transformants is unclear. However, very recent results show that p38α, another stress-activated MAPK, is, like JNK, an ROS sensor that negatively regulates Ras-induced tumorigenesis (Dolado et al., 2007). Therefore, JNK regulation by NF-κB-mediated ROS production may be considered a critical mechanism for the control of oncogenic transformation (Kennedy et al., 2003). However, a critical aspect of Ras-induced transformation that, as yet, is not fully understood is the mechanism whereby Ras regulates NF-κB.

The IKK complex is central to the activation of NF-κB. It is composed of a regulatory subunit, termed IKKγ or Nemo, and two catalytic subunits, IKKα and IKKβ (Ghosh and Karin, 2002). The genetic inactivation of IKKγ or IKKβ severely inhibits the canonical NF-κB pathway in vitro and in vivo, whereas that of IKKα reveals its role in the alternative NF-κB cascade (Ghosh and Karin, 2002). The canonical and alternative pathways play different functions in vivo. Thus, activation of the canonical pathway results in the phosphorylation and ubiquitination of IκBα, which is then degraded through the proteasome system (Ghosh and Karin, 2002). This releases the RelA(p65)/p50 complex that controls the expression of several genes, including those that code for ROS scavengers and those that are essential to restrain JNK and apoptosis (Pham et al., 2004). Previous studies have implicated the Phox and Bem1p (PB1) domain-containing signaling adaptor p62 in the activation of NF-κB in several cell systems (Moscat et al., 2006). Studies in knockout (KO) mice reveal that p62 is induced during the differentiation of osteoclasts and T cells (Duran et al., 2004; Martin et al., 2006) and is required for the sustained activation of NF-κB necessary for cell survival (Martin et al., 2006). Interestingly, p62 has been proposed to regulate the IκB kinase complex at two levels. First, through its ability to bind the atypical PKCs (aPKC), PKCζ and PKCλ/ι, p62 probably favors the phosphorylation of IKKβ by the aPKCs (Lallena et al., 1999). Second, its ability to oligomerize is important for the activation and polyubiquitination of TRAF6 (Martin et al., 2006; Wooten et al., 2005), a relevant event in the activation of the NF-κB pathway (Chen, 2005). Adapters are critical components of cell signaling cascades because they confer specificity to the function of otherwise promiscuous kinases, such as the aPKCs (Moscat and Diaz-Meco, 2000); therefore, understanding the role and mechanisms of action of p62 in NF-κB activation and its potential involvement in cancer is of great interest.

Although the expression of oncogenic Ras chronically activates NF-κB, the mechanisms that govern the Ras effect are far from clear. For example, oncogenic Ras has been found to activate the expression of transfected κB-dependent reporter constructs (Finco et al., 1997; Mayo et al., 1997), but what has not been established yet is whether this is due to Ras actions on the IKK complex (Arsura et al., 2000) or whether Ras triggers a mechanism that directly targets the phosphorylation of RelA, the transcriptionally active subunit of NF-κB (Madrid et al., 2001). Because p62 is important for IKK activation, at least in some cell systems (Duran et al., 2004; Martin et al., 2006), it is possible that it may orchestrate a signaling complex that facilitates NF-κB activation by Ras and, therefore, may be necessary for Ras-induced transformation. Here, we show that the loss of p62 in embryo fibroblasts enhances Ras-activated cell death, reducing oncogenic transformation through impaired NF-κB activation. Importantly, the reduced activation of NF-κB observed in the absence of p62 results in enhanced ROS production, a critical step for the induction of apoptosis. Ras also promotes the accumulation of p62 mRNA and protein in embryo fibroblasts (EFs). A similar increase in p62 levels is observed in several human tumor tissues and in cell lines where it is essential for proliferation. We provide evidence of the in vivo functional relevance of this phenomenon in experiments with KO mice demonstrating that the lack of p62 inhibits the induction of lung adenocarcinomas by Ras.

RESULTS

Cell-Autonomous Role of p62 in the Survival of Ras-Transformed Cells

Oncogenic Ras mutations are detected in numerous human cancers and are known to play a critical role in tumorigenesis (Bos, 1989). The aPKCs have been proposed to be relevant targets of Ras in some cell model systems (Diaz-Meco et al., 1994). In addition, these kinases, along with their adaptor p62, have been shown to be important regulators of NF-κB, a critical modulator of cell survival and tumor transformation (Moscat et al., 2006). These findings led us to address the question of whether the genetic ablation of p62 would affect tumor transformation of mouse embryo fibroblasts (MEFs) by oncogenic Ras. Toward this end, we generated immortal EFs from wild-type (WT) and p62-deficient mice by infection with a simian virus (SV)-40 large T retroviral construct. These immortal EFs were infected with a control retroviral vector or a vector expressing the oncogenic Ha-RasV12 mutant. Because these vectors contain a hygromycin-selectable marker, infected cells were incubated in the presence of hygromycin to select Ras-expressing cells for 3 weeks. The first striking observation was that, although Ras-expressing WT cells were easily obtained (Figure 1A) with clear characteristics of a transformed phenotype (data not shown), we did not find $p62^{-/-}$ cells with Ras levels similar to those of the $p62^{+/+}$ cells. This result suggested negative selection against high Ras-expressing cells in the absence of p62. This could mean that Ras transformation in the absence of p62 induces more cell death than does Ras in the presence of p62, or that it activates cell cycle progression less efficiently, which would prevent the generation of p62-deficient high Ras-expressing cells. To explore

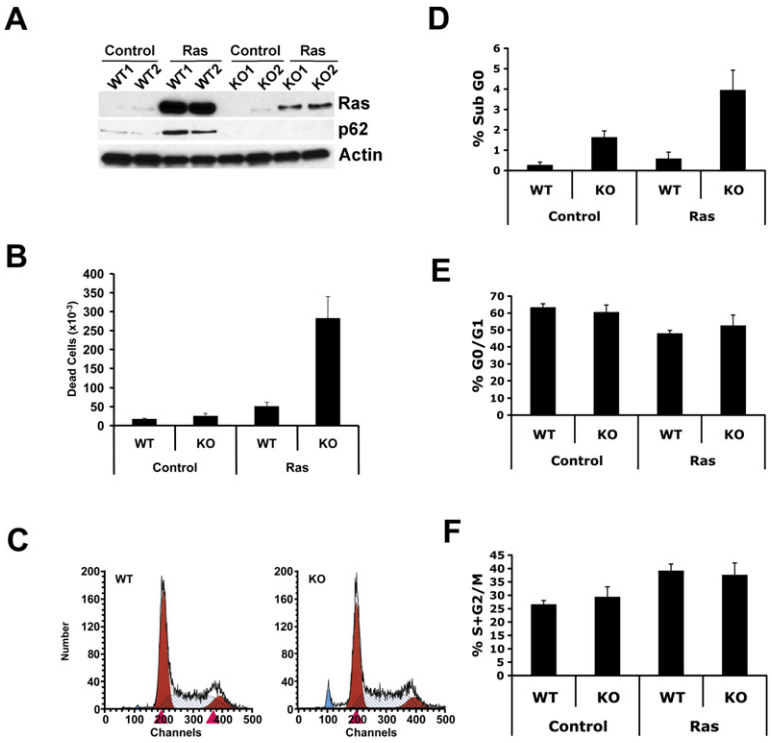

Figure 1. Ras-Induced Cell Survival Requires p62

(A) EFs from WT or p62-deficient mice immortalized with an SV-40 large T retroviral construct were infected with a control retroviral vector or a vector expressing the oncogenic Ha-RasV12 mutant and selected for hygromycin resistance to identify Ras-expressing cells. Extracts were prepared, and the levels of Ras, p62, and actin, as a loading control, were determined by immunoblotting. This represents an experiment of at least other two with similar results.

(B–E) In another set of experiments, the number of dead cells was determined in WT or p62-deficient cells infected with Ha-RasV12 at 7 d postinfection without antibiotic selection by trypan blue exclusion (B) and flow cytometry (sub G0; [C and D]). The percentage of cells in G0/G1 (E) and S+G2/M (F) was also determined by flow cytometry in these cultures; results of (B) and (D–F) are the mean ± SD of triplicates.

these possibilities, we compared cell death and cell cycle properties of WT and p62 KO immortalized EFs infected with the Ras-expressing retroviral vector. We measured the number of dead cells recovered at 7 d postinfection, without antibiotic selection, a time at which Ras expression reaches a maximum plateau (data not shown), which is similar in both WT and KO cells (Figure 2A). Results shown in Figure 1B demonstrate that the number of dead cells in the $p62^{-/-}$ Ras-transformed EF cultures is significantly higher (10-fold) than in the Ras WT controls (12 ± 2% in KO versus 1.2 ± 1% in WT cells). These results suggest that cell viability during Ras-induced transformation is compromised in the absence of p62, which would explain why we are unable to select $p62^{-/-}$ cells with levels of Ras expression comparable to those of $p62^{+/+}$ cells, after a 3-week hygromycin selection period. To confirm this observation, we performed flow cytometric cell cycle analysis of $p62^{+/+}$ and $p62^{-/-}$ cells transformed with Ras as above (Figure 2A). Interestingly, the percentage of cells with SubG0 DNA content was significantly increased in the Ras-transformed $p62^{-/-}$ cells, compared with the identically treated $p62^{+/+}$ controls (Figures 1C and 1D). However, there were no differences between both genotypes in terms of the percentage of cells in G0/G1, S, or G2/M, although the Ras transformants displayed a higher proliferative potential (Figures 1E and 1F). These observations suggest that the loss of p62 prevents some sort of survival signal required for Ras to efficiently induce cell transformation. We also used the WT and p62-deficient cells infected with the control vector or the Ras-expressing construct for 7 d without hygromycin selection to determine the potential role of p62 in other critical aspects of Ras-induced transformation. Even though this protocol generates p62-deficient cells that express oncogenic Ras at levels comparable to those of the Ras-transduced WT cells (Figure 2A), and cell death was increased in the Ras-expressing p62-deficient cells (Figure 1B), no morphological differences were observed between the two types of Ras transformants, which displayed disorganized multilayered growth with spindle-shaped, refractile morphology (Figure 2B). These results indicate that p62 is selectively involved in the control of cell survival during Ras transformation without affecting the morphological changes associated with the Ras-induced transformation process. Nevertheless, the lack of p62 severely impaired the ability of Ras-transformed cells to grow in soft agar (Figure 2C), a key characteristic of the transformed phenotype—that is, the size of the colonies obtained in the Ras-transformed p62 KO cultures were significantly smaller than those obtained in the Ras-transformed WT cells (Figure 2C, inset), and the number of colonies was also dramatically reduced in the KO cells, compared with the WT controls (Figure 2C). Of note, culturing these cells for long periods led to loss by apoptosis of the $p62^{-/-}$ Ras-expressing cells (data not shown), consistent with what was observed in the hygromycin-selected clones (Figure 1A). Collectively, these results demonstrate that Ras requires p62-derived survival signals to efficiently generate oncogenically transformed cells.

Requirement of p62 for Ras-Induced Lung Cancer in Mice

To address the physiological relevance of p62 for Ras-induced transformation in vivo, we tested whether p62 is required for Ras-induced transformation in an in vivo model of carcinogenesis. Because oncogenic Ras mutations have been detected in at least 25% of human lung cancers, and because we observed p62 overexpression in human lung carcinomas, we tested the requirement for p62 in a physiologically relevant mouse in vivo model of Ras-induced lung cancer (Fisher et al., 2001). In these experiments, we crossed WT and p62 KO mice with bitransgenic mice that develop pulmonary adenocarcinomas as consequence of the inducible expression of oncogenic Ras in type II alveolar epithelial cells in response to the presence of doxycycline in the diet (Fisher et al., 2001). These mice are based on a line of transgenic mice (Clara cell secretory protein [CCSP]-rtTA) that express the reverse tetracycline transactivator protein (rtTA) in type II alveolar epithelial cells. The expression of the

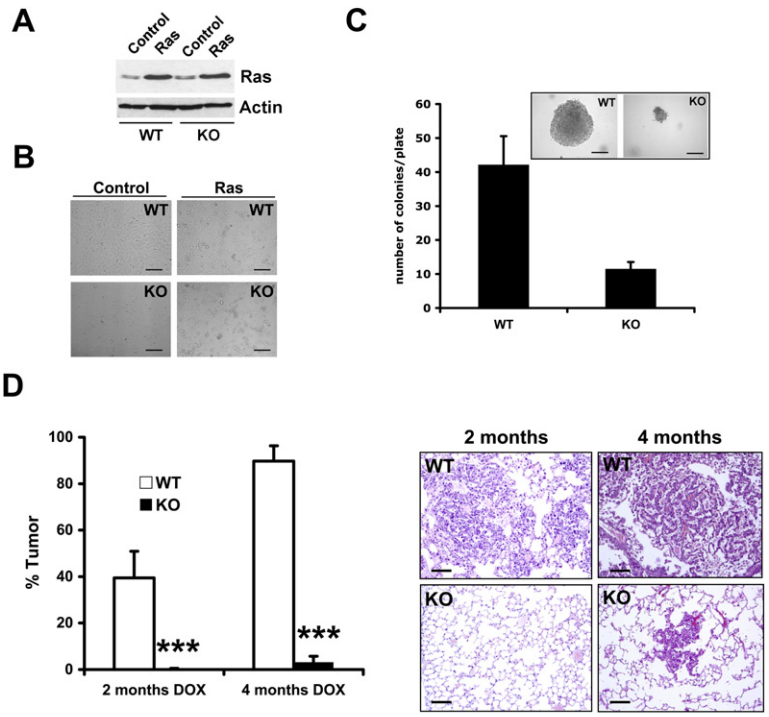

Figure 2. Ras-Induced Transformation and Tumorigenesis Are Inhibited in the Absence of p62

(A) WT and p62-deficient cells were infected with the control vector or the Ras-expressing construct for 7 d without hygromycin selection to generate p62-deficient cells that express oncogenic Ras at levels comparable to those of the Ras-transduced WT cells.

(B) There were no apparent morphological differences between WT and p62-deficient Ras transformants. Scale bar, 100 μm.

(C) Lack of p62 severely impaired the ability of Ras-transformed cells to grow in soft agar. The total number of colonies per plate was scored by counting and represented as mean ± SD of six plates of two independent experiments; representative colonies of the two genotypes (inset). Scale bar, 100 μm.

(D) WT and p62 KO mice were crossed with mice that developed pulmonary adenocarcinomas through inducible expression of oncogenic Ras in type II alveolar epithelial cells in response to the presence of doxycycline for 2 or 4 months (n = 6 per treatment and genotype). Overall tumor burden was determined by quantification of the tumor area as percentage of total area of H&E stainings (left panel). Results are the mean ± SD. Right panels show representative H&E stainings. Scale bar, 100 μm.

(E) Positive staining of Ras by IHC in WT and p62-deficient lungs after 2 months of doxycycline induction (left panel; scale bar, 50 μm) and Q-PCR quantification of oncogenic Ras expression (right panel) are shown as mean ± SD. ***$p < 0.0001$.

transgene in this particular cell type is presumably due to the site of insertion or alterations in the transgene during insertion, because the CCSP promoter was expected to drive expression of the tetracycline activator in Clara cells. When we bred these mice with mice carrying a transgene containing the coding sequence for an oncogenic mutant of K-Ras that can be regulated by rtTA and doxycycline, the result was a bitransgenic mouse that developed alveolar lesions and pulmonary tumors when given doxycycline in the drinking water. Hyperplasia of type II pneumocytes occurred at 14 d after induction with doxycycline in the WT mice (see Figure S1A available online), whereas solid-type adenomas and adenocarcinomas were reproducibly detected when doxycycline induction was prolonged for 2 months (Figures S1B and S1C). This is a physiologically relevant model for human cancer, because it has been reported that the likely precursors of lung adenocarcinomas include the type II alveolar epithelial cells and the Clara cells (Malkinson, 1991). Also, recent studies demonstrate that expression of oncogenic K-Ras in an endogenous manner in mice leads to lung adenomas and adenocarcinomas in which the likely target cell is the type II pneumocyte, evidenced by the fact that these tumor cells express surfactant protein C, a clear marker of this type of cells (Guerra et al., 2003; Tuveson et al., 2004). Importantly, when this experiment was performed in a p62 KO background, no tumor was detected in mice treated up to 2 months with doxycycline (Figure 2D), indicating that p62 is important for Ras-induced lung cancer. To determine whether a longer Ras expression could lead to the generation of lung tumors in the KO mice, we treated these and WT controls with doxycycline for 4 months, after which mice were killed and the tumor incidence was determined by hematoxylin and eosin (H&E) staining of lung sections as above. As expected, the overall tumor burden and the number of fully developed adenocarcinomas in the WT mice was higher than in the 2-month treated WT mice (Figure 2D). Importantly, the tumor incidence in the KO mice was still dramatically reduced in the 4-month treatment regimen, and the few tumors detected in the p62-deficient mice never went beyond the adenoma stage (Figure 2D, right panels). These are particularly relevant observations because they demonstrate the importance of p62 for Ras-induced tumorigenesis in vivo even after a long chronic expression of Ras. Results shown in Figure 2E demonstrate that Ras was actually expressed upon doxycycline treatment and that Ras expression levels in the KO lungs were not lower than in WT lungs. This control experiment demonstrates that the inhibited tumor incidence observed in the p62 KO mice cannot be explained by lower Ras expression in the mutant lungs.

Effect of p62 Loss in Ras-Induced Cell Signaling

To discern the mechanism underlying the p62 requirement for survival of Ras-transformed cells, we asked whether the lack

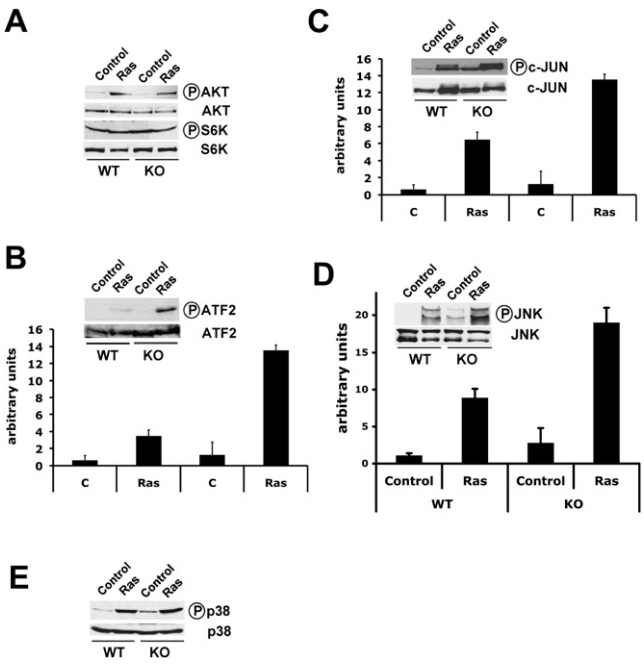

Figure 3. Lack of p62 Selectively Alters Ras-Induced Cell Signaling

The activation of Akt (A), S6K (A), ATF2 (B), c-Jun (C), JNK (D), and p38 (E) was determined in WT and p62 KO EFs expressing similar levels of Ras (Figure 2A). The activation of JNK and its substrates c-Jun and ATF2 were enhanced in p62-deficient cells transformed by Ras, compared with their WT controls. Blots are representative of other two with similar results. Graphs are mean ± SD of triplicates.

of p62 affects Ras signaling. Many studies have documented the signaling pathways activated by oncogenic Ras (reviewed in Downward, 2003; Malumbres and Barbacid, 2003). With this information, we were able to compare the status of a number of key signaling intermediaries in extracts from WT or p62 KO cells, with or without transformation by infection with the oncogenic Ras retroviral vector. These experiments were performed with WT and p62 KO cells generated as described previously, without hygromycin selection, and were analyzed 7 d postinfection, which leads to comparable Ras levels in both genotypes (Figure 2A). Under these conditions, Akt activation, as determined by immunoblotting with an antiphosphoserine 473 Akt/PKB antibody, was robustly induced by Ras expression in both WT and p62-deficient cells (Figure 3A). On the other hand, S6K activity was not detectably induced by Ras in either WT or p62-deficient cells (Figure 3A). Ras has also been shown to efficiently activate the stress-regulated MAPKs, JNK and p38 (Dolado et al., 2007; Ventura et al., 2004). These two kinases are particularly relevant because they have been shown to mediate Ras-induced cell death and, therefore, to have tumor suppressor properties, at least in some systems (Dolado et al., 2007; Ventura et al., 2004). We found that Ras-induced activation of activating transcription factor 2 (ATF2) and c-Jun phosphorylation, two bona fide substrates for JNK and p38, was increased in p62-deficient cells, compared with similarly treated WT cells (Figure 3B and 3C). These results suggest that the loss of p62 selectively augments the ability of Ras to activate the phosphorylation of substrates of the stress-activated MAPKs. This is potentially important because these kinases have been genetically and physiologically implicated in Ras-induced cell death (Dolado et al., 2007; Ventura et al., 2004). Results shown in Figure 3D demonstrate that Ras-induced JNK phosphorylation was significantly increased in p62 KO cells, compared with the WT controls, whereas the loss of p62 had little or no effect on the activation of p38 by Ras (Figure 3E). These results demonstrate that the lack of p62 enhances the ability of Ras to activate JNK.

Sustained JNK activation has also been implicated in the induction of cell death in TNF-α-treated cells with deficiencies in the NF-κB pathway. Specifically, *RelA*$^{-/-}$ and *IKKβ*$^{-/-}$ cells are sensitive to TNF-α-induced apoptosis, whereas WT cells are not. This is, at least in part, due to the fact that NF-κB ablation allows a sustained activation of JNK (Chang et al., 2006; De Smaele et al., 2001; Kamata et al., 2005; Nakajima et al., 2006; Tang et al., 2001), most likely because of the increased ROS production by TNF-α in the absence of NF-κB (Pham et al., 2004). Similarly, increased JNK leads to enhanced ROS production that further increases JNK activation (Nakano et al., 2006), which creates a feed-forward loop between both parameters. Ras-induced oncogenic transformation also leads to ROS production, which can lead to cell death through the activation of JNK (Ventura et al., 2004) and p38 (Dolado et al., 2007). This would serve to control the survival of transformed cells and the ability of tumors to progress. Taking into account that NF-κB restrains ROS production, that ROS are important activators of JNK, that Ras is a potent inducer of NF-κB, and that p62 has been shown to be important for the sustained activation of NF-κB, we reasoned that Ras might induce higher levels of JNK activity in the absence of p62 through higher ROS production in p62-deficient cells as a consequence of impaired NF-κB activation. To test this hypothesis, we first measured the levels of ROS in Ras-transformed WT and *p62*$^{-/-}$ EFs using both fluorimetric and flow cytometric procedures. Indeed, we found that ROS levels were significantly higher in p62-deficient EFs than in controls (Figures 4A and 4B). Therefore, we can conclude that the loss of p62 leads to increased ROS production in Ras-transformed cells, which could account for the higher JNK and apoptosis levels in p62 KO cells. To determine whether the increased JNK activation detected in the Ras-transformed KO cells is actually due to enhanced ROS production, we incubated Ras-transformed or nontransformed WT and KO cells in the presence or absence of the ROS scavenger N-acetyl cysteine (NAC). Results shown in Figure 4C clearly demonstrate that NAC addition reduces the increase in JNK activity in Ras-transformed KO cells, compared with WT cells. Similar results were obtained when cells were treated with butylated hydroxyanisole (BHA; Figure S2). These results indicate that the enhanced JNK activation induced by Ras in the absence of p62 is due, at least in part, to increased ROS production in the KO cells. If this model is correct, we can predict that JNK activation by ROS induced by adding H_2O_2 to the cell cultures would not be affected by the loss of p62. Results shown in Figure 4D demonstrate that this is, in fact, the case.

To determine whether increased ROS account for the reduced viability of the p62 KO cells in the presence of Ras, we incubated these cells in the absence or in the presence of the ROS scavenger BHA for 3 d, after which the percentage of dead cells was

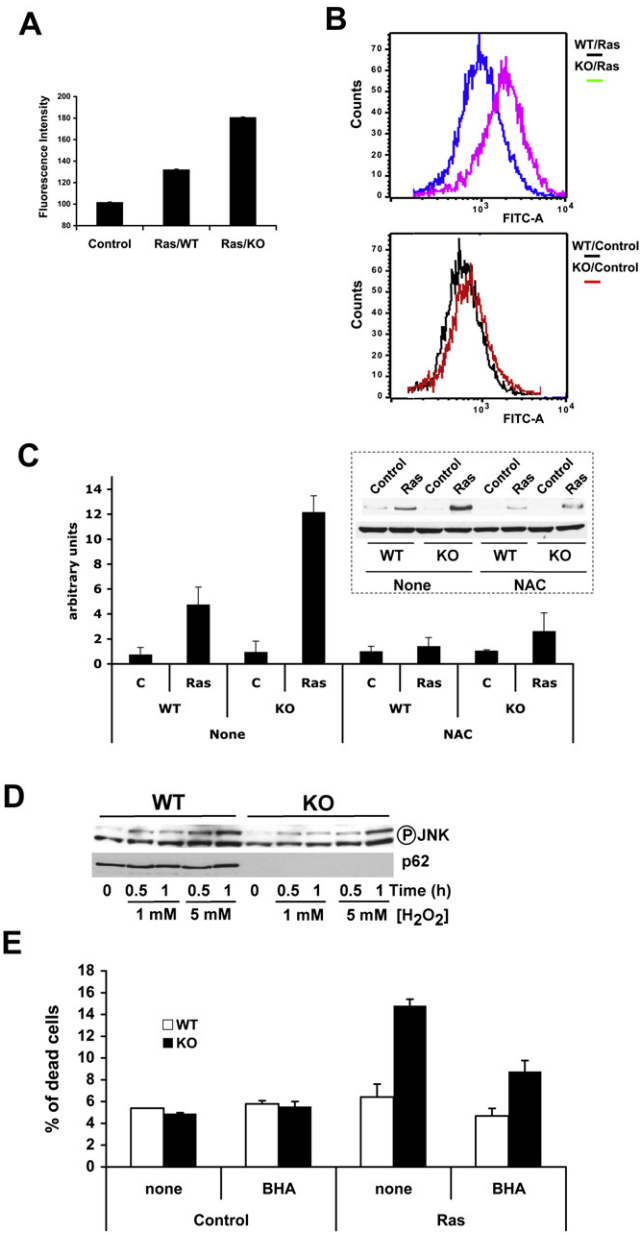

Figure 4. Lack of p62 Enhances Ras-Induced ROS Production

(A and B) ROS levels in Ras-transformed WT and *p62*$^{-/-}$ EFs were measured by using both fluorimetric and flow cytometric (B) procedures.

(C) Phospho-c-Jun levels were determined by immunoblotting of extracts of WT and p62 KO EFs in the presence or absence of Ras expression and NAC treatment. Immunoblotting of the lower panel is an actin loading control.

(D) JNK phosphorylation was determined by immunoblotting in WT and KO cells in response to different doses of H_2O_2.

(E) Cells were incubated or not with 100 μM BHA for 3 d, after which cell death was determined by trypan blue exclusion. Experiments shown in (B) and (D) are representative experiments of at least other two with similar results. Results in panels (A), (C), and (E) are the mean ± SD of three independent experiments. Inset in (C) shows one of the three experiments.

determined by trypan blue staining. The data shown in Figure 4E clearly demonstrate that BHA blocks the increased cell death observed in the Ras-expressing *p62*$^{-/-}$ EFs, lending support to the notion that the inability of p62-deficient cells to restrain ROS production during Ras transformation explains, at least in part, the lower viability of these cells.

Because ROS levels are normally kept under control by the products of NF-κB-inducible genes, we next determined whether the loss of p62 would affect NF-κB activation by Ras. Figures 5A and 5B show that the expression of bona fide NF-κB-dependent genes in Ras-expressing EFs was severely impaired by the lack of p62. Of particular relevance are the levels of ferritin heavy chain (FHC; Figure 5B), which has been reported to be an ROS scavenger whose expression is regulated by NF-κB (Pham et al., 2004). Collectively, these results demonstrate that p62 is important for the expression of NF-κB-dependent genes by Ras, including the ROS scavenger FHC. To determine whether p62 is required for the activation of NF-κB, we transfected HeLa cells with a κB-dependent luciferase reporter, along with a plasmid control or a Ras-expressing vector, with or without a p62 antisense construct that was previously described by our laboratory (Sanz et al., 1999, 2000). Transfection of the p62 antisense construct severely impaired NF-κB activation by Ras (Figure 5C). Figure 5D shows the impairment in Ras-induced κB-dependent reporter activity in KO EFs, compared with that in the WT controls. Of note, reintroduction of p62 into the KO cells restores the ability of Ras to activate FHC (Figure 5B and Figure S3) and κB-dependent transcription (Figure 5D and Figure S3). These observations clearly suggest that p62 is required for Ras-induced NF-κB activation, which promotes cell survival by reducing ROS levels and JNK activity. Recent data demonstrate that Ras activates NF-κB through IKK activation (Arsura et al., 2000). Because p62 is important for the stimulation of NF-κB in response to Ras (Figures 5A–5D), we tested the hypothesis that p62 is the functional link between Ras and activation of the IKK complex. For this, extracts from Ras-transformed or nontransformed WT and KO EFs were immunoprecipitated with an anti-IKKγ antibody, after which IKK activity was determined in the immunoprecipitates. The presence of Ras resulted in activated IKK in WT EFs, but this was not the case in p62 KO cells, where the level of Ras-activated IKK was inhibited (Figure 5E). This indicates that p62 is a relevant functional link between Ras and NF-κB at the level of IKK activation. Collectively, these results, in conjunction with those shown in Figures 3 and 4, demonstrate that the lack of p62 impairs NF-κB activation in Ras-transformed cells, which leads to higher ROS levels and increased cell death, which accounts for the lower tumorigenic potential of the *p62*$^{-/-}$ cells (Figures 1 and 2). We next determined whether the loss of p62 impairs the ability of Ras to promote the nuclear translocation of NF-κB. Results shown in Figure 5F clearly demonstrate the inhibition of Ras-induced p65 (RelA) nuclear translocation in KO EFs, compared with the WT control. To determine whether NF-κB is actually activated in the lungs expressing the oncogenic Ras and to determine whether this needs p62, we stained sections from WT and KO lungs expressing or not Ras (Figure 2E) with an anti-RelA antibody and scored for number of cells showing nuclear staining of RelA. To make a better comparison between WT and KO samples, we focused in the regions free of tumors in the WT lungs (Figure S4). The data shown in Figure 5G demonstrate that Ras actually triggers the nuclear translocation of RelA in lung, as well as that the lack of p62 severely impairs that parameter.

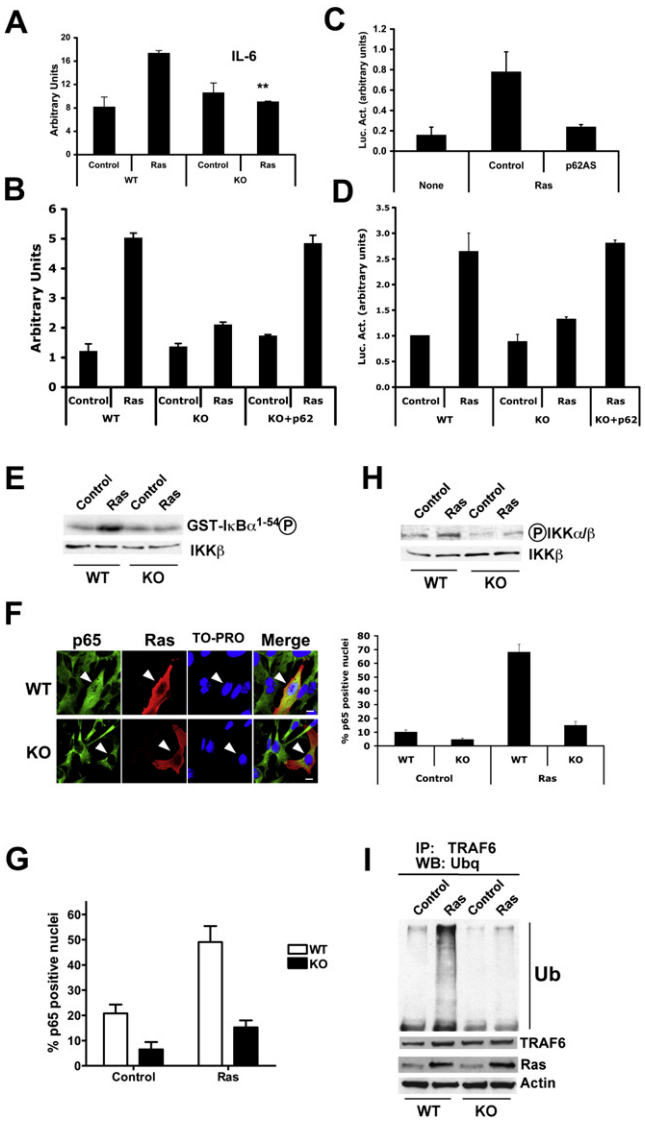

Figure 5. p62 Is Required for Ras-Induced NF-κB Activation

(A and B) IL-6 (A) and (B) FHC mRNA levels were determined by Q-PCR in WT and p62-deficient EFs in the presence or absence of Ras expression. p62-KO EFs were reconstituted with p62 by retroviral transduction.

(C) κB-transcriptional activity was determined in HeLa cells transfected with a κB-dependent luciferase reporter plasmid along with a plasmid control or a Ras expression vector, with or without p62 antisense.

(D) EFs WT, KO, and KO-p62 were transfected with the κB-luciferase reporter as above.

(E) Cell extracts from WT and p62-deficient EFs infected were immunoprecipitated with an anti-IKKγ antibody, and IKK activity was determined.

(F) NF-κB activation was determined in WT or KO EFs either transfected or not with oncogenic HA-tagged Ras, followed by immunofluorescence staining with anti-p65 and anti-HA antibodies, and TO-PRO nuclear staining (left panel; scale bar, 20 μm). The percentage of p65 nuclear-positive cells was determined by counting ten fields of each experimental condition (right panel). Results are the mean ± SD.

(G) Sections from WT and KO lungs expressing or not Ras were stained with an anti-RelA antibody and scored for number of cells showing nuclear staining of RelA. Results are the mean ± SD of five different sections per mouse of a total of six mice in each condition.

(H) The phosphorylation state of IKKα/β was determined in the same extracts as in (E) by immunoblotting with a phosphospecific antibody.

Previous studies from our and other laboratories demonstrated biochemically and genetically that p62 is required for IKK activation (Duran et al., 2004; Martin et al., 2006). The data reported here are important because they represent the identification of p62 as a regulator of Ras signals leading to IKK activation, and they constitute a mechanistic explanation of how Ras regulates NF-κB. A critical question that arises from these studies concerns the precise molecular interactions whereby p62 channels Ras signals for the activation of NF-κB. In this regard, two protein modifications have been reported to be critical in IKK activation in response to other stimuli: IKKβ phosphorylation and the K63 polyubiquitination of IKKγ, receptor-interacting protein 1 (RIP1), and TRAF6 (Chen, 2005; Ghosh and Karin, 2002). RIP1 ubiquitination occurs in response to TNF-α, and TRAF6 ubiquitination is activated by IL-1. IKKγ ubiquitination, on the other hand, has been reported in in vitro overexpression experiments and in response to signals emanating from the T-cell receptor (TCR) in T cells (Chen, 2005; Zhou et al., 2004). To determine which of these parameters are activated by Ras and which require p62, we analyzed the phosphorylation state of IKKβ by using an anti-phospho-IKKα/β antibody in cell extracts from Ras-transformed or nontransformed WT and p62 KO EFs. Figure 5H shows that Ras significantly activates IKKβ phosphorylation in WT EFs. Also, although Ras-induced IKKα/β phosphorylation was detectable in the KO cells, it was significantly reduced, compared with that in WT cells (Figure 5H). Because p62 has been shown, biochemically and genetically, to be linked to TRAF6, we next determined whether the lack of p62 affects the ability of Ras to promote TRAF6 polyubiquitination. WT and KO cells, with or without Ras transformation, were immunoprecipitated with an anti-TRAF6 antibody, and the immunoprecipitates were analyzed with an anti-ubiquitin antibody. Interestingly, although TRAF6 was dramatically polyubiquitinated in the WT Ras transformants, this effect was completely abolished in the p62 KO cells (Figure 5I). These results are consistent with the proposed role of p62 in TRAF6 polyubiquitination in other systems (Martin et al., 2006; Wooten et al., 2005) and demonstrate that p62 is essential for the ability of Ras to activate this parameter. Because TRAF6 polyubiquitination has been implicated in IKK activation, these results could account for the role of p62 in Ras-induced IKK activation. Intriguingly, no RIP1 or IKKγ polyubiquitination was observed in response to Ras in WT or in p62-deficient EFs (data not shown), suggesting that, at least in this system, IKK can be activated without IKKγ polyubiquitination and that RIP1 is probably not a target of Ras signaling.

Increased p62 Levels in Ras-Transformed Cells and Human Tumors

Once the role of p62 in NF-κB activation and transformation by Ras has been established, we sought to gain insights into the mechanism whereby Ras actions are linked to p62. Data shown in Figure 1A and Figure 6A demonstrate that Ras expression

(I) These extracts were also immunoprecipitated with an anti-TRAF6 antibody, and the immunoprecipitates were then analyzed for polyubiquitination with an antiubiquitin antibody. Ras and actin levels were determined by immunoblot. These are representative experiments of at least other two with similar results. Results in (A–D) are the mean ± SD of triplicates.

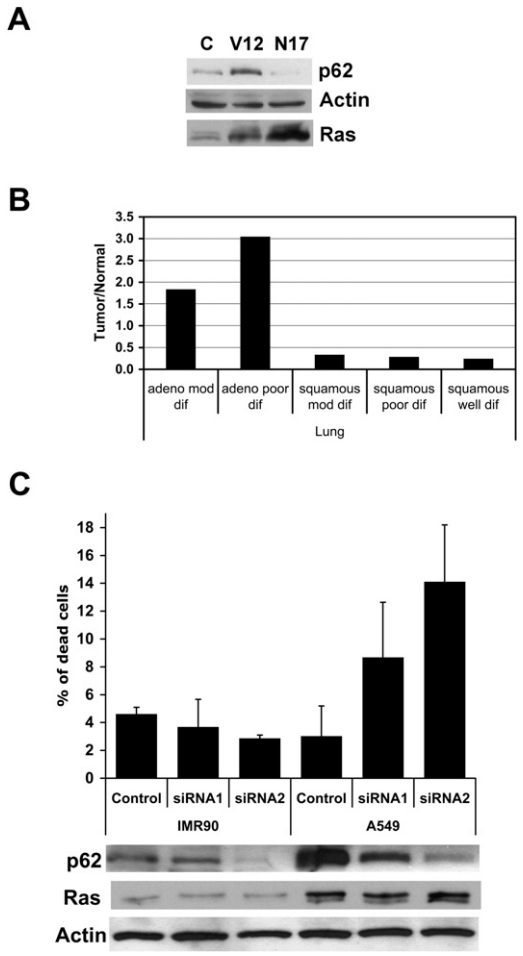

Figure 6. Induction of p62 by Ras and Increased Expression in Human Tumors and Cancer Lines

(A) p62 levels were increased in EFs infected with RasV12 but not with RasN17. Actin and Ras levels were also determined by immunoblot in the same extracts.

(B) Lung adenocarcinomas, but not squamous cell carcinomas, show overexpression of p62, compared with normal lung tissue.

(C) IMR90 and A549 cells, treated with a control siRNA or with two p62 specific siRNAs, were analyzed for viability by trypan blue exclusion and for p62 and Ras levels, as described in Experimental Procedures; results are the mean ± SD of triplicates.

leads to increased p62 protein levels, consistent with the existence of a signaling connection between these two molecules. Interestingly, the expression of a nontransforming dominant negative mutant of Ras not only did not induce p62 but even provoked a significant reduction in the levels of this protein (Figure 6A). To determine whether these observations are relevant to human cancers, we analyzed whether p62 overexpression was a general phenomenon associated with tumor transformation in humans by examining the expression of p62 in a panel of corresponding normal and tumor-derived tissue extracts. We probed the membrane of a protein microarray with a highly specific monoclonal antibody to human p62 and actin as a control. Quantitation of p62 expression in each tumor tissue, compared with their respective normal controls, revealed that p62 protein levels were increased in a highly significant number of cancers (Figure S5). In lung, p62 levels were overexpressed in adenocarcinomas but not in squamous cell carcinomas (Figure 6B and Figure S5). Interestingly, Ras mutations are often associated with the former but rarely to the latter kind of tumors (Bos, 1989). To further address the role of p62 in human lung cancer, we have analyzed p62 levels and its requirement for survival of the human lung cancer cell line A549, compared with nontransformed IMR90 cells. Immunoblot analysis demonstrates that A549 cells display higher p62 levels than do the IMR90 controls (Figure 6C, lower panels). We next used two different siRNA to deplete p62 levels in these cells and determined whether that affects their viability. Interestingly, both siRNAs effectively depleted cellular p62 levels (Figure 6C, lower panels), but they only increased cell death in the tumor cell line (Figure 6C, upper panel). Of note, the siRNA2 was the most efficient in depleting p62 levels and, interestingly, was the one that also more efficiently induced cell death (Figure 6C). On the basis of all these observations, it seems that p62 overexpression is a phenomenon associated with cancer and, in the case of lung cancer, is likely linked to Ras mutations. Although a more comprehensive analysis of p62 expression in human cancer samples is necessary to definitively assess the prevalence of p62 expression in human tumors, these data strongly suggest that our results in Ras-transformed EFs and mice are of physiological relevance.

Mechanism of p62 Induction by Ras

To investigate the mechanism of p62 induction by Ras, we initially determined whether Ras induction of p62 is at the mRNA level. WT EFs, both expressing and not expressing Ras, were lysed; RNA was extracted and analyzed for levels of p62 mRNA by Q-PCR. Results shown in Figure 7A demonstrate that Ras expression leads to increased p62 mRNA levels, interestingly, in a manner that depends on the MEK and PI 3-kinase pathways. These data are of special relevance because Ras-induced activation of IKK has previously been reported to rely on these same two pathways (Arsura et al., 2000), further supporting the notion that p62 is the previously unidentified link between Ras and NF-κB activation. Consistently, inhibition of transcription by treating cells with DRB (5,6-dichloro-1-β-D-ribobenzimidazole) completely reduced p62 mRNA levels (Figure 7B), strongly suggesting that Ras induction of p62 mRNA is at a gene transcriptional level. To determine the precise mechanism whereby Ras regulates p62 transcription, we cloned 1808 bp of the p62 promoter (Figure S6), which were subcloned into a luciferase reporter plasmid to further establish the role of transcription in Ras-induced p62 mRNA levels (Figure 7C). Interestingly, Ras expression provokes a dramatic induction of luciferase activity (Figure 7D), indicating that Ras induces the activity of the p62 promoter. This demonstrates that the p62 increased levels in Ras-expressing cells can be accounted for by changes at a gene transcriptional level as consequence of Ras-induced signals acting on the p62 promoter. To identify the enhancer elements in the p62 promoter responsible of the induction by Ras, we next constructed different 5′ deletions of the p62 promoter (Figure 7C and Figure S6). Experiments with deletions 1, 2, and 3 (Figure 7C) demonstrate that the region encompassing nucleotides −1808 to −1428 contains potential Ras-responsive regulatory elements (Figure 7D). That region contains one activator protein (AP)-1 and one κB element (Figure S6). To establish

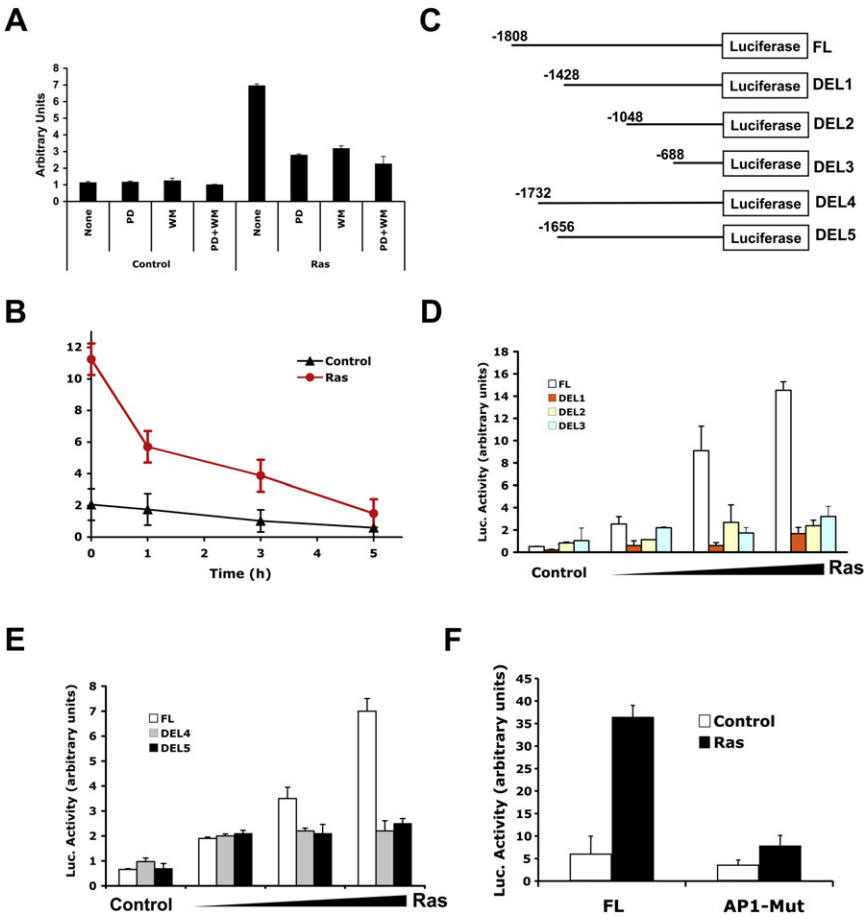

Figure 7. Transcriptional Mechanism of Ras-Induced p62 Levels

(A) Control and Ras-expressing cells were incubated for 5 hr with or without PD98059 (PD) or wortmannin (W), and p62 mRNA levels were then determined by RT-PCR.

(B) Control and Ras-infected cells were incubated in the absence or in the presence of DRB for different times to block transcription, and p62 mRNA levels were determined as above.

(C) Schematic representation of the different luciferase reporter constructs of the p62 promoter.

(D and E) Analysis of p62 promoter in response to Ras. HeLa cells were transfected with the different luciferase reporter constructs with different doses of control plasmid or Ras-expressing vector, and luciferase activity was measured as described in Experimental Procedures.

(F) A point mutation was introduced in the AP-1 enhancer element of the p62 promoter, and its activity in response to Ras was determined as described above. These are mean ± SD of triplicates.

which of these elements is required for Ras-induced p62 promoter activity, we transfected control and Ras-expressing cells with luciferase reporter vectors containing p62 promoter deletions 4 and 5 starting at nucleotides −1732 and −1656 (Figure 7C). Importantly, the simple deletion of the region encompassing nucleotides −1808 to −1732, which contains the AP-1 enhancer element, was sufficient to abolish the activation of the p62 promoter by Ras (Figure 7E). A point mutant in the AP-1 enhancer element severely abolished the p62 promoter activation by Ras (Figure 7F). This indicates that the AP-1 enhancer element is responsible of the Ras ability to induce p62 promoter activity.

DISCUSSION

The balance between survival and death is critical for the control of tumor progression. The ability of Ras to promote cell survival is essential for the suppression of apoptosis associated with Ras-induced transformation and, therefore, for cancer initiation and progression (Hanahan and Weinberg, 2000). The NF-κB pathway is central to this prosurvival function of Ras (Mayo et al., 2001). This, along with the Akt-initiated cascade (Downward, 2003), provides independent signals that inhibit cell death. Several mouse models have demonstrated the in vivo role of NF-κB in cell survival and in cancer initiation and progression (Greten et al., 2004; Rakoff-Nahoum and Medzhitov, 2007), as well as the role of PI 3-kinase-derived signals in Ras-induced lung cancer (Gupta et al., 2007). Our own results in p62 KO mice demonstrate that p62 is required for Ras-induced lung cancer. Additional non-cell-autonomous factors could, at least theoretically, come into play in the in vivo environment. However, our cell culture experiments reported here clearly demonstrate a role for p62 in NF-κB and cell survival during Ras-induced transformation and strongly suggest that the simplest explanation for the failure of tumors to develop in p62 KO mice is that lung epithelial cells expressing activated Ras fail to activate NF-κB, which is directly required for their survival. Our data in Figure 5G demonstrate that Ras-induced RelA nuclear translocation in lung cells is severely inhibited in p62 KO mice and are consistent with, and supportive of, this notion. Moreover, the fact that we see the same effect in EFs (Figure 5F) strongly supports the concept that there is a major cell-autonomous component to the ability of p62 deficiency to inhibit NF-κB and Ras-induced lung cancer in vivo.

The activation of NF-κB by Ras has long been recognized. However, the mechanisms controlling this important event remained unclear. Interestingly, recent studies presented convincing evidence indicating that IKK activation by Ras requires the combined action of extracellular signal-regulated kinase (ERK) and Akt signals, although how these translate into IKK activation was not determined (Arsura et al., 2000). Here, we show that the signaling adaptor p62 is required for Ras-induced transformation and NF-κB activation both in vitro and in an in vivo model of inducible lung tumorigenesis. Interestingly, p62 is induced by Ras at the mRNA level through an ERK- and Akt-dependent pathway, by a mechanism that involves the transcriptional regulation of the p62 promoter. Specifically, we show that a fragment encompassing an AP-1 enhancer element is required for p62 transcriptional activation by Ras. These observations are clearly consistent with the notion that p62 is the missing link between Ras and IKK activation, because IKK activation has also been

reported to require Ras-induced ERK and Akt and, more importantly, because we show here that p62 is actually necessary for Ras-induced IKK activation. Therefore, the mechanism whereby Ras induces NF-κB clearly involves its ability to activate IKK through p62. This explains why p62 is necessary for Ras-induced transformation, because the absence of NF-κB is known to impair the transforming ability of Ras as the result of the loss of an important survival pathway that antagonizes Ras induction of cell death. Whether the induction of p62 is required for Ras to activate NF-κB, or whether basal p62 levels are sufficient for that effect and the induction of p62 is a way to amplify the Ras signal, cannot be concluded from these studies. However, the fact that p62 is upregulated by Ras, together with the fact that p62 levels are increased in human tumors, suggests that the enhanced levels of p62 are likely to be important, if not for tumor initiation, at least for tumor progression.

The critical observations reported here lead to the question of how p62 channels Ras signals toward IKK. We have previously demonstrated that p62 is part of several signaling complexes that are reportedly essential for IKK activation through the signaling adaptor protein TRAF6. Specifically, p62 binds TRAF6 in at least four different cellular settings: (1) the interleukin-1-triggered Myd88/IRAK complex (Sanz et al., 2000); (2) the TCR-activated Bcl10/Malt-1 signalsome cassette (Martin et al., 2006); (3) receptor activatory of NF-κB (RANK) in RANKL-activated osteoclasts (Duran et al., 2004); and (4) the nerve growth factor (NGF) receptor system (Wooten et al., 2005). This is important because TRAF6 is an E3 ubiquitin-ligase that promotes K63-polyubiquitination of many proteins, including itself (Chen, 2005). Although the mechanism is not entirely clear, that ubiquitination process creates a number of docking sites that are necessary for IKK activation. We have shown that p62 is critically involved in the oligomerization of TRAF6 (Sanz et al., 2000; Wooten et al., 2005), which is an essential step for these polyubiquitinations (Chen, 2005). In addition to K63-polyubiquitination, IKK activation correlates with the phosphorylation of both catalytic subunits (IKKα and IKKβ) at their activation loops (Ghosh and Karin, 2002). Recent results suggest that these are separate required events that independently are not sufficient to trigger IKK activity (Grabiner et al., 2007; Misra et al., 2007; Su et al., 2005). We have shown here that the lack of p62 significantly reduces the ability of Ras to induce IKKα/β phosphorylation and completely abolishes the polyubiquitination of TRAF6. The latter finding indicates that p62 induction by Ras activates TRAF6 E3-ubiquitin ligase activity. On the other hand, RIP1 ubiquitination, which is important for TNF-α-induced IKK activation (Ea et al., 2006), was not induced by Ras (data not shown). Our previous results showed that p62 associates with RIP1 in TNF-α-activated cells (Sanz et al., 1999). However, the genetic inactivation of p62 does not seem to affect TNF-α signaling, at least in fibroblasts, indicating that although p62 is essential for TRAF6-mediated signaling toward NF-κB in several cell systems (Duran et al., 2004; Martin et al., 2006), p62 might be redundant for RIP1 function. On the other hand, we did not see any Ras-induced IKKγ polyubiquitination (data not shown). This suggests that Ras regulates IKK activation through a mechanism that most likely involves the TRAF6 E3-ubiquitin ligase activity but not the ubiquitination of IKKγ. However, we cannot completely rule out that IKKγ ubiquitination is induced by Ras at levels below the detection capability of the methods used here. In this regard, it should be noted that IKKγ polyubiquitination is readily detectable in overexpression experiments, but that recent studies illustrate the difficulty of measuring the endogenous activation of this parameter (Grabiner et al., 2007).

The defect in NF-κB activation that we observed in $p62^{-/-}$ cells is relevant to their resistance to transformation by Ras. Among the different antiapoptotic signals delivered by NF-κB is the expression of genes involved in ROS scavenging, such as ferritin heavy chain (FHC; Pham et al., 2004). In this regard, our results strongly demonstrate that the loss of p62 impairs FHC expression and provide a likely explanation for why Ras transformation leads to higher ROS levels in p62 KO cells than in WT cells. This enhanced ROS production could explain why JNK activity is enhanced in the absence of p62, because it has been demonstrated that ROS is capable of inactivating phosphatases that keep JNK under control (Kamata et al., 2005). In addition, elevated JNK activity induces the production of more ROS (Nakano et al., 2006), activating a positive feedback cycle that is deleterious for the viability of the transformed cell, and explains the lack of cell toxicity in JNK-deficient Ras-transformed cells (Ventura et al., 2004). Our data are clear in that the inhibition of ROS restores cell viability in the p62-deficient Ras transformed cells.

In summary, we have identified p62 as target of Ras regulation of cell transformation. This mediation of Ras signaling relies on p62's ability to channel signals toward IKK activation and on the subsequent expression of survival genes, including those involved in the regulation of ROS induction and JNK activation. These are two interconnected elements that promote cell death during oncogenic transformation by Ras. Because p62 has been shown genetically and biochemically to play an essential role in the sustained activation of NF-κB in two settings unrelated to cancer, namely osteoclast differentiation and Th2 cell polarization, these results not only solve an important conundrum with regard to the precise mechanism whereby Ras activates NF-κB but also illustrate how oncogenes might impinge on pathways used by other signaling cascades and take advantage of them to ensure the survival of the transformed cell.

EXPERIMENTAL PROCEDURES

Mice

The p62KO, CCSP-rtTA, and K-Ras (Tet-op-K-Ras^{G12D}) transgenic mice were described elsewhere (Duran et al., 2004; Fisher et al., 2001). All mice were born and kept under pathogen-free conditions. Animal handling and experimental procedures conform to institutional guidelines (University of Cincinnati Institutional Animal Care and Use Committee). All genotyping was done by PCR. Doxycycline was administered via drinking water freshly prepared twice a week, at a concentration of 500 mg/l.

Histological Analysis

Lung were inflated, excised, fixed in 10% neutral buffer formalin for 24 hr, dehydrated, and embedded in paraffin. Sections (5 μm) were cut and stained with H&E. Overall tumor burden was quantitated by measuring tumor area in H&E-stained sections using NIH ImageJ (version 1.38; http://rsb.info.nih.gov/ij/). Immunohistochemistry was performed with anti-Ras (F-132; 1:50 dilution) and anti-p65 (C-20; 1:100 dilution) antibodies from Santa Cruz. Sections were deparaffinized and rehydrated, and antigen retrieval was performed with 10 mM citric acid and 2 mM EDTA (pH 6.2) with boiling for 10 min. After quenching of endogenous peroxidase and blocking in normal serum, tissues were incubated with primary antibody overnight at 4°C, followed by incubation with biotinylated secondary antibody. Antibodies were visualized with

avidin-biotin complex (Vectastain Elite, Vector Labs) using diaminobenzidine as a chromagen. Slides were then counterstained with hematoxylin, dehydrated, and mounted.

Determination of ROS Levels

Proliferating cells were washed once with warm PBS and were incubated with 10 μM 2′-7′-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes) in warm PBS supplemented with 5.5 mM glucose. After 10 min at 37°C, PBS was replaced with complete culture medium, and cells were incubated for an additional 10–15 min, washed once again with warm PBS, trypsinized, and analyzed by FACS (FACS Calibur, BD) or in a fluorimeter (LS55-Luminiscence Spectrometer, Perkin Elmer).

Cell Cycle Analysis

Cell cycle analysis was determined by flow cytometry, as described elsewhere (Barradas et al., 1999). Briefly, cells were seeded in p6-wells in DMEM containing 10% FCS. After different treatments according to the experiments, medium was collected, and cells were trypsinized and fixed in 70% ethanol. After fixation, cells were stained with propidium iodide (50 μg/ml) in a solution containing 0.05% Triton X-100 and 0.1 mg/ml RNase A. Cells were then analyzed in an EPICS XL flow cytometer (Coulter Electronics Inc.) and ModFit LT Software (Verity).

Western Blot

Cell extracts for western blot were prepared in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitors). Lysates were separated by SDS-PAGE and were transferred to nitrocellulose-ECL membranes (GE Healthcare); the immune complex was detected by chemiluminescence (GE Healthcare). Western blot of the commercially available human cancer protein array (Biochain A1235713-1) was performed according manufacturer's instructions with anti-p62 antibody. These studies were approved by the University of Cincinnati Institutional Review Board #1-2.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and six supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/4/343/DC1/.

ACKNOWLEDGMENTS

We thank Jeff Whitsett (Cincinnati Children's Hospital Medical Center) for providing CCSP-rtTA mice, Harold Varmus (Memorial Sloan-Kettering Cancer Center) for providing Tet-op-K-RasG12D mice, Maryellen Daston for editing this manuscript, and Lyndsey Cheuvront and Michael Winstead for technical assistance. We thank Glenn Doerman for preparing the figures. This work was funded in part by the University of Cincinnati-Consejo Superior de Investigaciones Cientificas Research Collaborative Agreement.

Received: August 17, 2007
Revised: November 19, 2007
Accepted: February 1, 2008
Published: April 7, 2008

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