The GTPase KRAS suppresses the P53 tumor suppressor by activating the NRF2-regulated antioxidant defense system in cancer cells

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ABSTRACT

In human cancer cells that harbor mutant KRAS and WT P53 (P53), KRAS contributes to the maintenance of low P53 levels. Moreover, KRAS depletion stabilizes and reactivates P53 and thereby inhibits malignant transformation. However, the mechanism by which KRAS regulates P53 is largely unknown. Recently, we showed that KRAS depletion leads to P53 Ser-15 phosphorylation (P-P53) and increases the levels of P53 and its target P21/wild-type P53-activated fragment 1 (WAF1)/CIP1. Here, using several human lung cancer cell lines, siRNA-mediated gene silencing, immunoblotting, quantitative RT-PCR, promoter-reporter assays and reactive oxygen species (ROS) assays, we demonstrate that KRAS maintains low P53 levels by activating the NFE2-related factor 2 (NRF2)-regulated antioxidant defense system. We found that KRAS depletion led to down-regulation of NRF2 and its targets NAD(P)H quinone dehydrogenase 1 (NQO1) and solute carrier family 7 member 11 (SLC7A11), decreased reduced/oxidized glutathione (GSH/GSSG) ratio, and increased ROS levels. We noted that the increase in ROS is required for increased P-P53, P53, and P21/WAF1/CIP1 levels following KRAS depletion. Downstream of KRAS, depletion of RAS-like proto-oncogene B (RALB) and IκB kinase (IKK)-related TANK-binding kinase 1 (TBK1) activated P53 in a ROS- and NRF2-dependent manner. Consistent with this, the IKK inhibitor BAY11-7085 and dominant-negative mutant IκBαM inhibited NFκB activity and increased P-P53, P53, and P21/WAF1/CIP1 levels in a ROS-dependent manner. In conclusion, our findings uncover an important role for the NRF2-regulated antioxidant system in KRAS-mediated P53 suppression.

The p53 protein exerts its tumor suppressor activity by triggering transient cell cycle arrest, cellular senescence, and apoptosis in response to stresses such as oncogene activation and DNA
damage (1-4). In the absence of stress, normal cells maintain low p53 levels by inducing its degradation. MDM2, an E3 ubiquitin-protein ligase that directly binds p53 and targets it for protein degradation by the proteasome, plays a major role in maintaining low levels of p53 in normal cells (5, 6). The binding of MDM2 to p53 is inhibited by the phosphorylation of p53 on amino acids involved in the MDM2/p53 binding interface. For example, during DNA damage, Ataxia telangiectasia mutated (ATM) kinase is activated to phosphorylate p53 on serine 15 (Ser-15), which leads to its stabilization by inhibiting binding of MDM2 to p53, its ubiquitination, and its degradation (7-9). As such, the stabilized p53 allows the cells to repair the DNA damage by inducing cell cycle arrest at the G1 phase (10), which is mediated at least in part by transcriptional activation of the cyclin-dependent kinase inhibitor p21Waf1/cip1. Thus, one proposed mechanism by which p53 prevents cancer is by arresting or eliminating cells with DNA damage and hence limiting the formation of oncogenic mutations.

Most human cancers protect themselves from the tumor suppressor activity of p53 by inactivating it, leading to genetic instability and increased mutation frequency. About half of human cancers harbor mutations in the TP53 gene, which results in loss of wild-type (WT) p53 function as well as oncogenic gain-of-function (3, 4). In the remaining cancers that harbor WT TP53, several oncogenic pathways lead to the inactivation of the p53 protein. In human cancer cells that harbor mutant (mt) KRas and WT p53, we have shown that KRas and RalB, but not Akt1/2 and c-Raf, are required for maintaining low levels of p53 and that depletion of KRas and RalB stabilizes and reactivates p53 to inhibit malignant transformation (11). Importantly, depletion of KRas and RalB led to ATM kinase activation and phosphorylation of p53 on Ser-15 and the subsequent increase in p53 half-life, which in turn led to a p53-dependent up-regulation of its transcriptional target p21Waf1/cip1 (11). However, the mechanism by which depletion of KRas and RalB leads to activation of ATM and the subsequent activation of p53 is not known.

RESULTS

Depletion of KRas and RalB promotes p53 phosphorylation on serine 15 and p53 activation by a reactive oxygen species (ROS)-dependent mechanism

Because ATM can be activated directly by reactive oxygen species (ROS) (12), we reasoned that depletion of KRas and RalB may lead to the generation of ROS and subsequent phosphorylation of p53. To test this hypothesis, we depleted KRas and RalB from A549 human lung cancer cells, which harbor mt KRas and WT p53, and processed the cells for ROS-level and Western blot analyses as described under Methods. Depletion of KRas and RalB resulted in increased cellular ROS levels (Fig. 1A) and increased levels of the phosphorylation of p53 on Ser-15 (Fig. 1C). This in turn led to increased levels of total p53 and its transcriptional target p21Waf1/cip1 (Fig. 1C). Consistent with the ROS-level increase, Fig. 1B shows that depletion of KRas and RalB decreased the ratio of the reduced form of glutathione (GSH) to its oxidized form (GSSG) (GSH/GSSG). These effects were Ras isoform-specific in that KRas, but not HRas and NRas, depletion increased p53 levels in A549 cells (Supplemental Fig. S1A). Similarly, KRas and RalB but not HRas and NRas depletion increased p53 levels in H460, another human lung cancer cell line with mt KRas and WT p53 (Supplemental Fig. S1B).

We next determined whether the increase in the phosphorylation of p53 and its up-regulation, following KRas and RalB depletion, require ROS, by treating the cells with the ROS scavenger and potent antioxidant N-acetylcysteine (NAC). Figure 1D shows that NAC attenuated the increased phosphorylation of p53 on Ser-15 and total p53 and p21Waf1/cip1 levels following depletion of KRas and RalB in A549 cells. In two other human lung cancer cell lines with mt KRas and WT p53 (H460 and H1944), NAC treatment also prevented increased phosphorylation of p53 on Ser-15 and increased total p53 and p21Waf1/cip1 levels following depletion of KRas and RalB in A549 cells. In two other human lung cancer cell lines with mt KRas and WT p53 (H460 and H1944), NAC treatment also prevented increased phosphorylation of p53 on Ser-15 and total p53 and p21Waf1/cip1 levels following depletion of KRas and RalB (Supplemental Figs. S2A and 2B). These results indicate that depletion of KRas and RalB leads to phosphorylation and up-regulation of p53 by a mechanism requiring ROS. To provide further support for the involvement of ROS in the up-regulation of p53, we treated A549 cells with Erastin, an inhibitor of SLC7A11, the cystine-glutamate antiporter that decreases levels of ROS by increasing cellular levels of cysteine and subsequently the antioxidant GSH (13). Figure 1E and 1F show that treatment with Erastin increased
KRas suppresses p53 by Nrf2 activation

cellular ROS levels and promoted p53 phosphorylation and increased p53 and p21\textsuperscript{Waf1/cip1} levels.

**Depletion of KRas and RalB down-regulates the expression of the master regulator of the antioxidant defense system Nrf2 and its transcriptional targets**

The fact that depletion of KRas and RalB resulted in increased ROS levels suggested the involvement of the antioxidant defense system in p53 induction. To explore this mechanism further, we first determined whether KRas and RalB are required for the expression of the master regulator of the antioxidant defense system, the basic leucine zipper transcription factor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) (14-16). Nrf2 protects cells from oxidative damage by inducing the expression of several antioxidant genes that maintain a redox balance by reducing intracellular ROS levels. These genes include for example NAD(P)H quinone oxidoreductase (NQO1), an enzyme responsible for reduction/detoxification of reactive quinones that cause redox cycling and oxidative stress (17), and the above-mentioned cystine-glutamate antiporter SLC7A11 (13). As shown in Fig. 2A, depletion of KRas and RalB resulted in decreased Nrf2 protein levels. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) demonstrated that depletion of KRas and RalB down-regulated mRNA expression of Nrf2 (Fig. 2B) and its transcriptional target genes NQO1 (Fig. 2C) and SLC7A11 (Fig. 2D) in a time-dependent manner with little effects after 24 hours but with significant effects at 36 and 48 hours.

We next determined whether the decrease in Nrf2 expression mediates p53 up-regulation following KRas and RalB depletion. We reasoned that if this is the case, then a compound able to increase Nrf2 levels could rescue from KRas and RalB siRNA. To this end, we used the clinical agent Bardoxolone-methyl (CDDO-ME), a compound known to greatly increase the cellular levels of NRF2 by binding to and preventing the Nrf2 negative regulator, Keap1, from inducing the ubiquitination and proteasomal degradation of Nrf2. Fig. S3 shows that in control vehicle-treated A549 cells, the levels of P-p53, p53 and p21\textsuperscript{Waf1/cip1} were increased following depletion of KRas and RalB relative to NT siRNA. In contrast, in CDDO-ME-treated cells, Nrf2 levels were greatly increased and the ability of KRas and RalB siRNAs to increase the levels of P-p53, p53 and p21 was blocked (Fig. S3), suggesting that low levels of Nrf2 are required for p53 up-regulation following KRas and RalB depletion.

**IkB kinase-related Tank-binding kinase 1 (TBK1) depletion leads to activation of p53 in a ROS-dependent manner**

To determine the mechanism by which depletion of KRas and RalB down-regulates Nrf2 and activates p53, we focused on the IkB kinase (IKK)-related TBK1, as TBK1 has been shown to bind in a complex with RalB and its effector Sec5, and RalB/TBK1 activation has been shown to contribute to cancer cell survival and oncogenesis (18). Therefore, we reasoned that, if TBK1 mediates the ability of RalB to regulate Nrf2 levels and p53 up-regulation, then TBK1 itself may be required for Nrf2 expression and suppression of p53 up-regulation. To test this hypothesis, we depleted TBK1 from A549 cells and determined whether it is required for the expression of Nrf2 and suppression of p53. Fig. 3A shows that depletion of TBK1 resulted in decreased Nrf2 expression and increased p53 phosphorylation and total p53 and p21\textsuperscript{Waf1/cip1} levels. The decrease in Nrf2 expression was at the transcriptional level, as documented by the reduction of the mRNA levels of Nrf2 (Fig. 3B) and its target genes NQO1 (Fig. 3C) and SLC7A11 (Fig. 3D). Consistent with the decreased expression of Nrf2, depletion of TBK1 also resulted in increased intracellular ROS levels (Fig. 3E). The ROS scavenger NAC prevented TBK1 siRNA from activating p53 (Fig. 3F), suggesting that ROS is required for p53 activation following TBK1 depletion. Taken together, the results shown in Figs. 1, 2, and 3 suggest that KRas, RalB, and TBK1 suppress p53 by activation of the antioxidant defense system.

TBK1 and IkB kinase \(\epsilon\) (IKK\(\epsilon\)) are serine/threonine protein kinases related to the IKK family of kinases and play a key role in coordinating the activation of the NF\(\kappa\)B pathway. TBK1, just like IKK\(\epsilon\), can phosphorylate the NF\(\kappa\)B inhibitor IkB\(\alpha\) and promote its proteasomal degradation, thereby allowing activation and nuclear translocation of the NF\(\kappa\)B complex (19, 20). To provide further support for the involvement of
TBK1 and the NFκB pathway in the regulation of p53 through the antioxidant defense system, we next investigated the effects of inhibiting the NFκB pathway on the Nrf2 anti-oxidant system, ROS levels and p53 activation. To this end, A549 cells were co-transfected with the dominant-negative IκBα mt IκBαM (21) and a NFκB responsive promoter-reporter construct; cells were then treated with vehicle or NAC. Figure 4A shows that expression of the IκBαM results in decreased NFκB promoter activity, confirming that IκBαM inhibited the NFκB pathway in A549 cells. Furthermore, in the absence of NAC, IκBαM expression resulted in decreased Nrf2, Nqo1 and SLC7A11 mRNA (Fig. 4B), decreased Nrf2 protein levels (Fig. 4D), increased ROS levels (Fig. 4C), and increased levels of p53 phosphorylation, p53, and p21Waf1/cip1 (Fig. 4D). The induced ROS and activation of p53 by IκBαM was abrogated by NAC (Figs. 4C and 4D), suggesting that the ability of IκBαM to activate p53 depends on ROS. Further support for the involvement of the NFκB pathway in p53 activation through the antioxidant defense system was provided by experiments using BAY 11-7085, an irreversible inhibitor of IκBα phosphorylation that prevents activation of the NFκB pathway.22 Treatment of A549 cells with BAY 11-7085 resulted in decreased NFκB promoter activity (Fig. 4E), confirming that it inhibited the NFκB pathway, decreased Nrf2, Nqo1, SLC7A11 mRNA (Fig. 4F), decreased Nrf2 protein levels (Fig. 4H), increased ROS levels (Fig. 4G), and increased levels of p53 phosphorylation, p53, and p21Waf1/cip1 in a ROS-dependent manner (Fig. 4H). Taken together, these data suggest the involvement of the NFκB pathway in the regulation of p53 through the antioxidant defense system.

DISCUSSION

The genome guardian tumor suppressor p53 is one of the pivotal gate keepers that prevent normal cells from becoming cancerous; therefore, it is not surprising that about half of cancers contain p53-inactivating mutations and the other half harbor oncogenic events that inactivate WT p53 (3, 4, 23). Consistent with this, we have shown that, in human tumors with WT p53, mt KRas is required for maintaining low p53 levels and that depletion of KRas increases p53 stability by activating ATM to phosphorylate p53 on Ser-15, leading to a 6-fold increase in p53 half-life (11). However, the mechanism by which KRas regulates ATM phosphorylation of p53 Ser-15 to maintain low p53 levels remained unknown. The fact that ROS can directly activate ATM by reacting with ATM Cys2991 and inducing a disulfide cross-linked, oxidized, and active ATM dimer (12), prompted us to test the hypothesis that KRas regulation of p53 is mediated by ROS. In this study, we discovered that KRas regulation of p53 Ser-15 phosphorylation is dependent on ROS cellular levels, and is mediated by the antioxidant program.

In contrast to normal cells that maintain redox homeostasis through an antioxidant defense system tightly regulated by Nrf2, cancer cells protect themselves from oxidative stress by aberrantly up-regulating Nrf2 (24-27). In this study, we demonstrated that KRas depletion decreased expression of Nrf2 and its transcriptional targets the ROS detoxifying/antioxidant enzyme NQO1 and the cysteine-glutamate antiporter SLC7A11 (28, 29). Consistent with this, depletion of KRas decreased the GSH/GSSG ratio and increased ROS levels. While it has been shown that KRas G12D activates Nrf2 and lowers ROS levels (30), whether ROS mediates the ability of mt KRas to maintain low p53 levels is not known. Here, we showed that the ROS scavenger NAC rescued from p53 activation induced by depletion of KRas, suggesting that ROS is required for p53 Ser-15 phosphorylation and up-regulation. Furthermore, CDDO-ME, a compound known to increase the levels of Nrf2 by binding to Keap1 and inhibiting the proteasomal degradation of Nrf2, rescued from KRas siRNA up-regulation of p53, suggesting that low levels of Nrf2 are required for p53 up-regulation. Our results suggest that depletion of KRas leads to down-regulation of Nrf2 and its antioxidant targets, decreased GSH/GSSG ratio, and increased ROS levels, which in turn can activate ATM to phosphorylate Ser-15 p53, preventing its degradation and hence increasing its stability. These results are significant as they suggest that one mechanism by which tumors that harbor mt KRas and WT p53 overcome the ability of WT p53 to induce oxidative damage and tumor cell death is through Nrf2 up-regulation and low ROS levels and therefore low levels of Ser-15 phosphorylation leading to p53 degradation.

We found the requirement of KRas for the maintenance of low p53 levels is Ras isoform-
specific as depletion of HRas and NRas resulted in little up-regulation of p53. This is consistent with previous reports that showed that over-expressing mt KRas, but not HRas or NRas, reduced p53 levels (31). Furthermore, we have shown that downstream of KRas, RalB, but not Akt1/2 and c-Raf, is required for maintaining low levels of p53 (11). However, whether RalB regulates p53 stability through the antioxidant defense system is not known. In this study, we found that depletion of RalB leads to down-regulation of Nrf2, decreased GSH/GSSG ratio, and increased ROS and that the resulting increased p53 Ser-15 phosphorylation and p53 up-regulation require increased intracellular levels of ROS.

RalB is a member of an IκB kinase-related TBK1 complex that contributes to cancer cell survival (18), which led us to investigate whether TBK1 also regulates p53 stability. Our results show that depletion of TBK1 increases p53 Ser-15 phosphorylation and up-regulates p53 and that this is mediated by ROS. Further support for the regulation of p53 by the TBK1/NFκB pathway was provided by the demonstration that the NFκB pathway inhibitors, BAY 11-7085 and the dominant-negative IκBαM, increased p53 phosphorylation and p53 levels in a ROS-dependent manner. Although the involvement of the TBK1/NFκB pathway in the regulation of p53 and its dependence on the anti-oxidant program is novel, the decrease in Nrf2 levels by BAY 11-7085 and IκBαM is consistent with reports that showed that NFκB binds κB binding sites on the Nrf2 promoter and increases its mRNA expression in leukemia cells (32). Furthermore, the same study showed that NFκB inhibitors decreased Nrf2 levels and significantly reduced clonogenicity of leukemia patient cells and improved their chemotherapeutic responsiveness (32). Although, our studies do not demonstrate a causal link between Nrf2 and the NF-kappaB pathway-dependent regulation of p53, together with these previous results (32), they suggest that one potential way that tumors maintain low levels of ROS and p53 is through TBK1/NFκB-mediated Nrf2 redox detoxification.

Although our results show that, downstream of KRas, the Ral/TBK1/NFκB pathway contributes to the tumor’s ability to maintain low ROS levels, other pathways may also be involved in KRas maintaining low ROS levels. For example, in MEFs expressing G12D KRas the MEK inhibitor AZD6244 decreased Nrf2 and its target genes and increased ROS levels whereas endogenous expression of activated B-Raf increased P-ERK and Nrf2 and decreased ROS (30). The demonstration that depletion of KRas, RalB, and TBK1, ectopic expression of IkBaM, and treatment with BAY 11-7085 all lead to decreased Nrf2 and increased ROS opens up new therapeutic avenues of targeting these pathways to up-regulate WT p53, which in turn can induce tumor cell death. This is significant as tumor cells and cancer stem cells must maintain low levels of intracellular ROS for their survival and to preserve self-renewal capacities (24, 26, 33, 34).

Finally, although studies in this manuscript do not demonstrate a tumorigenic potential of Nrf2-mediated alterations of the p53 pathway, our previous studies showed that depletion of K-Ras and RalB activates p53 and inhibits anchorage-independent growth and invasion and interferes with cell cycle progression in a p53-dependent manner (11).

Taken together, our results have provided mechanistic insights to the central question of how mt KRas regulates WT p53 by uncovering the critical role of the Nrf2 antioxidant defense system in KRas regulation of p53 phosphorylation and stability. This is a novel mechanism that is relevant to KRas-driven cancer development. Nrf2, a key regulator for the maintenance of redox homeostasis, has been shown to contribute to several hallmarks of cancer, including uncontrolled proliferation, self-renewal, metabolic reprogramming, drug resistance, invasion, and metastasis (14-16). In the present study, we provided evidence for a new oncogenic role of Nrf2 in cancer development as a mediator of oncogenic KRas suppression of p53.

**EXPERIMENTAL PROCEDURES**

**Cell culture and reagents**

The human lung cancer cell lines A549, H460, and H1944 were obtained from ATCC and grown as described by us (11). NAC (A9166), 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, D6883), and Erastin (E7781) were from Sigma-Aldrich. BAY 11-7085 (sc-202490) was from Santa Cruz.

**siRNAs and antibodies**

Non-targeting siRNAs with sequence GUCGACGUUAUUUGCCGGUCGUU (custom,
KRas suppresses p53 by Nrf2 activation

synthesis oligo identification no. YHKIK-000001), KRas (M-005069-00-0005), HRas (M-004142-00), NRas (M-003919-00), and RalB (M-008403-00-0005) were from Dharmacon/GE Healthcare. TBK1 siRNA (sc-39058) was from Santa Cruz. Anti-IκBα (4812S), p21Waf1/cip1 (DSC60) (2946S), and TBK1 (D1B4) (3540S) antibodies were from Cell Signaling. Antibodies against p53 (DO-1) (sc-126), HRas (C-20) (sc-520), and NRas (C-20) (sc-519) were from Santa Cruz. Anti-vinculin (V9131-2ML) and β-actin (A5441) antibodies were from Sigma. KRas antibody (Ab-1; OP24) was from Calbiochem. RalB antibody (04-037) was from EMD Millipore. Nrf2 antibody (EP1080Y) was from Abcam.

siRNA transfection
Cancer cells (0.2 × 10⁶/well) were plated in six-well plates and transiently transfected the next day with 20 µM of siRNA using Lipofectamine RNAiMAX transfection reagent (13778-150, Invitrogen) according to the manufacturer’s instructions. Transfected cells were collected 48 hours after transfection for ROS and GSH assays, and RT-PCR and Western blot analyses.

DNA transfection
Cells were plated overnight to reach 70% to 80% confluence and were then transfected with plasmid DNA of pCMX IκBα mt (IκBαM, Addgene, 12329) using Lipofectamine 2000 (11668-027, Invitrogen) transfection reagent according to the manufacturer’s instructions. Transfected cells were harvested 48 hours after transfection for ROS, luciferase reporter assay, and assessment of mRNA and protein expression levels.

Western blotting
Cells were harvested, washed, and processed for Western blotting as described by us (11).

Measurement of intracellular ROS levels
Intracellular ROS levels were estimated using the cell-permeable oxidation-sensitive probe H2DCF-DA. Cells were seeded into dark, clear-bottom 96-well plates at 1,000 cells/well. Cells were transfected the next day with siRNA; 48 hours after transfection, cells were washed with Hanks’ balanced salt solution (HBSS; 14025-076, Invitrogen). The cells were then incubated with 20 µM of H2DCF-DA in HBSS in the dark at 37°C for 45 minutes. After incubation, H2DCF-DA was removed and cells were washed with HBSS. The ROS levels were determined by measurement of fluorescence of dichlorofluorescein (excitation at 480 nm and emission at 530 nm) and normalized to cellular protein content, which was determined by the sulforhodamine B (SRB, 230162, Sigma) assay (35).

Microtiter plate assay for the measurement of GSH/GSSG ratio
Cells were harvested using 0.05%Trypsin-EDTA (1X) and washed 3 times with ice-cold PBS. GSH and GSSG levels were measured by enzymatic assays using the EnzyChrom GSH/GSSG assay kit (EGTT-100; BioAssay System, Hayward, CA), following the manufacturer’s instructions. Detection of reaction products was monitored at 0 and 10 min using a microplate reader at 412 nm. GSH and GSSG levels were calculated from standard curves of GSH and GSSG.

RNA purification and quantitative RT-PCR
Total cellular RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using ProtoScript first-strand cDNA synthesis kit (E6300S, New England Biolabs), according to the manufacturer’s instructions. Quantitative RT-PCR was carried out using PowerUp SYBR green PCR Master Mix (A25742, Applied Biosystems). Primer sequences used included the following: 5′-GGTTTCTTTCGGCTACGTTT-3′ (forward) and 5′-TAACTCAGGAATGGATAATAGCTC-3′ (reverse) for human Nrf2; 5′-GGCAAGTCCATCCCAACTG-3′ (forward) and 5′-GCA AGT CAG GGA AGC CTG GA-3′ (reverse) for human NQO1; 5′-ATGCAGTGCCAGATCTTTATATGTC-3′ (reverse) for human SLC7A11; and 5′-GTATTGGGCGCCTGGTCACC-3′ (forward) and 5′-CGGGAAGATGG TGATGG-3′ (reverse) for GAPDH. All primers were synthesized by Integrated DNA Technologies. The RT-PCR reactions were performed in 20-µL volumes that included 2 µL RT products (1:100 diluted) as template, 10 µL of SYBR green PCR master mix, and 20 pmol of individual primer. The PCR amplifications (40 cycles at 95°C for 15 seconds and at 55°C for 60 seconds) were performed using
the QuantStudio 3 RT-PCR System (Applied Biosystems). For each target gene, the average Ct values calculated from triplicate PCR reactions were normalized to the average Ct values for GAPDH. These normalized values were then used to calculate a value expressing the extent of knockdown relative to the non-specific control siRNA according to the formula $2^{-\Delta\Delta Ct}$.

**Luciferase reporter assay**

Cells (3000/well) were seeded in 96-well plates and co-transfected the next day with DNA: 300 ng of pNFκB MetLuc reporter (Clontech) and 20 ng of β-galactosidase according to the manufacturer’s instructions.

**Statistical analyses**

Graphics were prepared using GraphPad Prism software. In this study, $t$ tests were used to determine the statistical significance of the results. $P$ values < 0.05 were chosen as a threshold for statistical significance.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

H.Y., S.X., and A.K. performed experiments, collected data, and prepared the figures. S.M.S. designed experiments, supervised the work, and wrote the manuscript.

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Figure legends

Figure 1. Depletion of KRas and RalB activates p53 by increasing the levels phospho-p53, p53, and p21Waf1/cip1 in a ROS-dependent manner. Depletion of KRas and RalB (A) promotes ROS generation as determined by dichlorofluorescein (DCF) and (B) reduces the ratio of GSH/GSSG, and (C) increases phospho-p53 on Ser-15 (P-p53), p53, and p21Waf1/cip1 levels in A549 cells. (D) The ROS scavenger NAC prevents p53 activation following KRas and RalB depletion in A549 cells. (E, F) Erastin, the inhibitor of the cystine-glutamate antiporter SLC7A11, decreases ROS levels and activates p53 in a concentration-dependent manner.

Figure 2: Depletion of KRas and RalB downregulates Nrf2 and its transcriptional targets. Depletion of KRas and RalB in A549 cells (A) decreases Nrf2 protein levels and induces p53 phosphorylation (P-p53), p53, and p21Waf1/cip1 as determined by Western blotting and (B-D) decreases mRNA levels of Nrf2 (B), NQO1 (C), and SLC7A11 (D) in A549 cells as determined by quantitative RT-PCR.

Figure 3: TBK1 depletion downregulates Nrf2 and its targets and activates p53 in a ROS-dependent manner. Depletion of TBK1 (A) decreases Nrf2 protein levels and induces p53 phosphorylation (P-p53), p53, and p21Waf1/cip1 as determined by Western blotting and (B-D) decreases mRNA levels of Nrf2 (B), NQO1 (C), and SLC7A11 (D) in A549 cells as determined by quantitative RT-PCR. (E) Increases intracellular ROS levels in A549 cells. (F) The ROS scavenger NAC prevents p53 activation following TBK1 depletion in A549 cells.

Figure 4. Blockade of the NFkB pathway activates p53 in a ROS-dependent manner. Overexpression of dominant-negative mutant of IκBα (IκBαM) in A549 cells (A) inhibits NFkB activity, (B) decreases Nrf2, Nqo1 and SLC7A11 mRNA levels, (C) increases the level of intracellular ROS, and (D) increases the levels of p53 phosphorylation (P-p53), p53, and p21Waf1/cip1 in a ROS-dependent manner. Treatment with NAC rescues from the effects of IκBαM. The IKK inhibitor BAY 11-7085 (20 µM) (E) inhibits NFkB activity, (F) decreases Nrf2, Nqo1 and SLC7A11 mRNA levels, (G) increases the level of intracellular ROS, and (H) increases the levels of P-p53, p53, and p21Waf1/cip1 in a ROS-dependent manner. Treatment with NAC rescues from the effects of BAY 11-7085.

Figure 5. Model: KRas suppresses p53 by activating the Nrf2 antioxidant defense system
**Figure 1**

A. ROS (DCF fluorescence) levels in control, NT, KRas, and RalB conditions.

B. GSH/GSSG ratio comparison among control, NT, KRas, and RalB.

C. Western blot analysis of KRas, RalB, p-p53, p53, p21, and Actin in different conditions.

D. Effects of NAC on ROS (DCF fluorescence) in NT, KRas, and RalB conditions.

E. Comparison of ROS (DCF fluorescence) levels with Erastin at 0, 1, 3, and 10 µM.

F. Western blot analysis of p-p53, p53, p21, and Actin with different Erastin concentrations.
Figure 2

A

Kras
RalB
Nrf2
p-p53
P53
P21
Actin

B

Nrf2

Relative mRNA expression of Nrf2

C

NQO-1

Relative mRNA expression of NQO-1

D

SLC7A11

Relative mRNA expression of SLC7A11
Figure 4

A

B

C

D

E

F

G

H
Figure 5

Cell membrane

KRas

Ral-GDS

Ral

TBK1

ROS

ATM

NAC

NRF2

Anti-oxidant defense system

NQO1

SLC7A11

Erastin

Stable P53

p21

CDDO-ME

NFSB

Stable P53

P

S15

Bay 11-7085

IkBαM
The GTPase KRAS suppresses the P53 tumor suppressor by activating the NRF2-regulated antioxidant defense system in cancer cells
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