NORE1A is a Ras senescence effector that controls the apoptotic/senescent balance of p53 via HIPK2

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The Ras oncoprotein is a key driver of cancer. However, Ras also provokes senescence, which serves as a major barrier to Ras-driven transformation. Ras senescence pathways remain poorly characterized. NORE1A is a novel Ras effector that serves as a tumor suppressor. It is frequently inactivated in tumors. We show that NORE1A is a powerful Ras senescence effector and that down-regulation of NORE1A suppresses senescence induction by Ras and enhances Ras transformation. We show that Ras induces the formation of a complex between NORE1A and the kinase HIPK2, enhancing HIPK2 association with p53. HIPK2 is a tumor suppressor that can induce either proapoptotic or prosenescent posttranslational modifications of p53. NORE1A acts to suppress its proapoptotic phosphorylation of p53 but enhance its prosenescent acetylation of p53. Thus, we identify a major new Ras signaling pathway that links Ras to the control of specific protein acetylation and show how NORE1A allows Ras to qualitatively modify p53 function to promote senescence.

Introduction

Activation of Ras oncoproteins is a driving event in many human cancers (Malumbres and Barbacid, 2003; Campbell and Der, 2004; Schubbert et al., 2011). Activated forms of Ras stimulate multiple mitogenic signaling pathways, including the Raf–MAPK and PI3 kinase pathways to promote transformation (Plyayeva-Gupta et al., 2011). However, activated forms of Ras also promote oncogene induced senescence (Serrano et al., 1997; Cox and Der, 2003; Lowe et al., 2004). The detection of Ras-induced senescence in multiple cell culture systems (Serrano et al., 1997; Ferbeyre et al., 2002), in vivo mouse models (Collado et al., 2005; Morton et al., 2010; Kennedy et al., 2011), human rasopathies (Courtois-Cox et al., 2006), and premalignant activated Ras-containing human pancreatic tumors (Caldwell et al., 2012) confirms that the process is physiological (Dimmuro and David, 2010; Kuijman et al., 2010). However, the mechanisms by which Ras drives senescence and how these mechanisms are subverted during the development of malignancy (Chen et al., 2005; Collado et al., 2005; Kuijman et al., 2010) remains poorly understood (Bianchi-Smiraglia and Nikiforov, 2012). One factor that is clearly important is the activation of the p53 tumor suppressor by Ras (Serrano et al., 1997).

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Abbreviations: MEF, mouse embryonic fibroblast; shRNA, short hairpin RNA.
NORE1A binds directly to Ras oncoproteins with all the characteristics of an effector that promotes apoptosis (Khokhlatchev et al., 2002; Vos et al., 2003) or cell cycle arrest (Aoyama et al., 2004; Calvisi et al., 2009). NORE1A lacks enzymatic activity and is thought to act as a scaffolding molecule. Like other RASSF family proteins, it can bind the proapoptotic kinases MST1 and MST2 and feeds into the Hippo signaling pathway (Khokhlatchev et al., 2002). However, NORE1A tumor suppressor activity does not require the interaction with MST kinases (Aoyama et al., 2004), suggesting other effectors are the key to its function. NORE1A is frequently down-regulated during tumor development (Donninger et al., 2007), and its dysregulation is implicated in a rare familial cancer syndrome (Chen et al., 2003). In primary tumors, inactivation of NORE1A expression often correlates with up-regulation of Ras activity (Calvisi et al., 2008) and exogenous expression of NORE1A suppresses the tumorigenic phenotype (Vos et al., 2003). Moreover, NORE1A+/− mouse embryo fibroblasts (MEFs) are susceptible to one-step transformation by activated Ras, which wild-type MEFs are not (Park et al., 2010). Thus, NORE1A serves as a Ras effector/tumor suppressor that likely plays a key role in restraining the transforming effects of mutant Ras.

Our recent studies have suggested that although NORE1A can induce apoptosis, a more physiological role may be in the regulation of the cell cycle by inducing a p53-dependent activation of the CDK inhibitor p21CIP1 (Calvisi et al., 2009). Moreover, in primary human tumors, NORE1A down-regulation and p53 inactivation are usually mutually exclusive, suggesting they lie in the same pathway (Calvisi et al., 2009). As Ras senescence pathways are known to operate, in part, via p53 (Serrano et al., 1997), we examined the role of NORE1A in Ras-mediated senescence and p53 activation.

We found that NORE1A is a potent inducer of p53-dependent senescence in multiple cell systems. Moreover, knockdown of NORE1A impaired Ras-induced senescence and enhanced the transforming activity of activated Ras. However, deletion mutagenesis of NORE1A showed that these biological effects were independent of canonical Hippo pathway signaling.

Further studies revealed that NORE1A forms an endogenous, Ras-regulated complex with the kinase HIPK2 and that this interaction is essential for full Ras/NORE1A-induced senescence. HIPK2 can positively regulate total p53 levels by down-regulating the p53-negative regulator mdr2 (Di Stefano et al., 2005a). It can also modulate the apoptotic or the senescence-promoting activity of p53 by promoting discrete posttranslational modifications (Puca et al., 2010). It stimulates the proapoptotic activity of p53 by direct phosphorylation of p53 residue S46 (D’Orazi et al., 2002; Di Stefano et al., 2004). However, HIPK2 can also indirectly promote the acetylation of p53 by recruiting the acetyl transferases PCAF and CBP/p300 into a complex (Hoffmann et al., 2002; Di Stefano et al., 2005b). Acetylation of p53 can enhance its affinity for the promoters of prosenescence genes such as p21CIP1 and is associated with senescence (Pearson et al., 2000; Di Stefano et al., 2005b; Brooks and Gu, 2011). Our studies show that NORE1A inhibits the proapoptotic phosphorylation of p53 on S46 and inhibits p53 proapoptotic signaling. However, NORE1A stimulates the acetylation of p53 and activates prosenescence signaling in a HIPK2-dependent manner. This appears to be due to a Ras-regulated scaffolding of p53 and HIPK2 by NORE1A.

Thus, we identify a novel NORE1A–HIPK2–p53 pathway that allows Ras to qualitatively control the posttranslational modification code of p53 to favor senescence over apoptosis. This pathway explains mechanistically the previous observations that Ras can promote p53 acetylation (Pearson et al., 2000), p21CIP1 activation (Di Stefano et al., 2005b), and senescence (Serrano et al., 1997). Inactivation of this pathway is frequent in primary tumors and its loss may play a key role in the ability of Ras-driven tumor cells to breach the senescence barrier and progress to malignancy.

Results

NORE1A is a Ras senescence effector

NORE1A is a Ras effector/tumor suppressor with a poorly characterized mode of action. We have recently shown that NORE1A activates p21CIP1 via p53 (Calvisi et al., 2009). As p53 activation of p21CIP1 is associated with the induction of senescence, we hypothesized that NORE1A might be part of the elusive senescence signaling machinery of Ras. To determine if this is the case, we examined the ability of NORE1A to induce senescence. A549 cells are a human lung tumor cell line that contains an activated Ras gene and is wild-type for p53 but does not express NORE1A (Aoyama et al., 2004). A549 cells were transiently transfected with a NORE1A expression vector and senescence assayed by β-galactosidase activity. NORE1A was found to be a potent inducer of senescence in A549 cells (Fig. 1a).

To confirm that NORE1A was acting via p53, we used siRNA knockdown of p53 in the cells to show that the senescence-inducing activity of NORE1A is p53 dependent (Fig. 1a). Additional studies showed NORE1A also induces senescence in primary MEFs (Fig. 1b). This ability was impaired in a NORE1A mutant that is defective for binding to Ras (NORE1A*; Aoyama et al., 2004). Moreover, knockdown of NORE1A using two previously validated short hairpin RNAs (shRNAs) against mouse NORE1A (Calvisi et al., 2009) inhibited the ability of Ras to induce senescence in MEFs (Fig. 1b). Thus, NORE1A appears to serve as a key component of a Ras–p53 senescence pathway.

To confirm the importance of NORE1A to Ras-induced senescence and to examine the role of the pathway in suppressing Ras-driven transformation, we used HBEC-3KT cells. These cells are a human lung epithelial cell line that has been immortalized in the absence of viral oncoproteins by telomerase and cdk4 (Ramirez et al., 2004). The cells retain NORE1A expression and exhibit a high background of senescence, as measured by β-galactosidase activity, under normal growth conditions (Fig. 1c). However, after stable transduction of NORE1A shRNA constructs and knockdown of NORE1A expression, the senescence is repressed (Fig. 1c). Fig. 1c (left) shows quantification of senescence in the cell system. Fig. 1c (right) shows a representative image of the cells and the degree of NORE1A protein knockdown. To substantiate that the high level of β-galactosidase staining observed in these cells was
Suppression of NORE1A enhances Ras-mediated transformation

To determine the effects of NORE1A knockdown on Ras-mediated senescence and transformation in the HBEC-3KT cells, we stably transfected a doxycycline-inducible expression vector for H-Ras12v (Ellis et al., 2002) into a matched pair of HBEC-3KT cells containing either the scrambled shRNA control or two different NORE1A shRNAs. Doxycycline was added to the cells and, after 72 h, the cells were assayed for senescence. The control cells showed a 30% increase over the high background, whereas the NORE1A knockdown cells showed only an ~5 and ~10% increase, respectively (Fig. 2 a). The levels of Ras protein induced in all three cell systems were approximately the same (Fig. 2 b). We then plated the cells in soft agar in the presence or absence of doxycycline and assayed for the growth of colonies after 3 wk. Colony formation was enhanced in the NORE1A knockdown cells (Fig. 2 c).

NORE1A senescence signaling does not require the Hippo pathway

NORE1A can modulate apoptosis by binding to the MST kinases that regulate the Hippo pathway using its C-terminal SARAH motif (Khokhlatchev et al., 2002). Yet it can modulate cell cycle arrest in an MST kinase-independent manner (Aoyama et al., 2004). To determine if the senescence-activating effects of NORE1A were dependent on the Hippo signaling pathway, we generated a deletion mutant of NORE1A (NORE1A-delSARAH) that lacks the region containing the SARAH motif essential for its interaction with MST kinases (Khokhlatchev et al., 2002). We then repeated the senescence assays in A549 cells

indeed senescence related, we measured two additional well-established markers of senescence, senescence-associated heterochromatin foci (SAHF) and Dec1 (Collado and Serrano, 2010). We observed that loss of NORE1A reduced both the levels of SAHF (Fig. 1 d) and Dec1 expression (Fig. 1 e), confirming that NORE1A is an important effector of senescence. Trypan blue staining showed no difference in the levels of cell viability (unpublished data).
HIPK2, a known regulator of p53 (Puca et al., 2010; Fig. 4 b, bottom). Further analysis confirmed that endogenous NORE1A could be co-immunoprecipitated with endogenous HIPK2 from HuH6 and HepG2 liver cancer cell lines (Fig. 4 c).

To determine the effects of Ras on the interaction of NORE1A and HIPK2, we performed co-transfection experiments in HEK-293T cells and measured the degree of complex formation. In the presence of activated Ras, the degree of complex formation between NORE1A and HIPK2 was dramatically increased (Fig. 4 d). To confirm the effects of Ras on the endogenous interaction between NORE1A and HIPK2, we used the HBEC-3KT cells stably transfected with an inducible Ras construct (Fig. 2). We found that after doxycycline treatment to induce Ras expression, there was a strong increase in the complex formation between endogenous NORE1A and endogenous HIPK2 (Fig. 4 e).

Ras/NORE1A act to stabilize HIPK2

During these experiments, we often observed an apparent increase in the levels of HIPK2 in the lysates of cells transfected with Ras and NORE1A (Fig. 4 d). This was particularly apparent when we examined the effects of activated Ras induction on endogenous HIPK2 levels (Fig. 4 e). HIPK2 is known to be an unstable protein (Calzado et al., 2009). By using cyclohexamide treatment to block translation after transient transfection of HEK-293T cells, we were able to analyze the effects of Ras and NORE1A on HIPK2 protein stability. We found that Ras acts to stabilize NORE1A and that both NORE1A and Ras stabilize HIPK2. The most effective stabilization was a combination of Ras and NORE1A. A representative experiment is shown in Fig. 5 a.

Quantification of three experiments is shown below as relative percentage decay (Fig. 5 b).

Interaction with HIPK2 is required for NORE1A-mediated senescence

HIPK2 is a tumor suppressor that binds p53 and modulates its activity both directly and indirectly (Puca et al., 2010). Thus, including the mutant NORE1A. The deletion mutant retained almost WT levels of senescence induction in A549 cells (Fig. 3).

NORE1A forms an endogenous, Ras-regulated complex with the p53 regulator HIPK2

The aforementioned experiments implicate NORE1A as a key Ras senescence effector that acts via p53 but does not use the canonical Hippo pathway. While attempting to determine the mechanism behind the effect, we examined the subcellular localization of endogenous NORE1A and found that it is primarily localized to the nucleus (Fig. 4 a). Transient transfections of NORE1A tagged with GFP confirmed a mainly nuclear localization but also revealed that some cells demonstrated NORE1A in nuclear speckles (Fig. 4 b, top). Systematic analysis of a series of fluorescently tagged known nuclear speckle proteins showed that NORE1A specifically co-localized with the kinase NORE1A suppression impairs Ras-mediated senescence induction and enhances Ras-mediated transformation. (A) An inducible form of activated H-Ras was transfected into the scrambled shRNA or the two NORE1A shRNA transduced HBEC-3KT stable cell lines described in Fig. 1. Ras was induced with doxycycline and, after 72 h, β-galactosidase activity was quantified. The percent increase over the uninduced control cells was measured. Data represent the mean ± SD of triplicate experiments. *, P < 0.05 compared with scrambled control. (B) Duplicate dishes were induced, lysed, and equal amounts of protein were loaded on a gel and immunoblotted for the expression of HRas to confirm equivalent expression. (B) The Ras-inducible HBEC-3KT cell lines were then plated in soft agar in the presence of doxycycline. Frequency of colony formation was quantified. Experiments were done in duplicate and the error bars show SD.
NORE1A and HIPK2, we used deletion mutagenesis followed by point mutagenesis to identify the minimal domain required for the interaction of NORE1A with HIPK2. Ultimately, Arginines 92–94 of NORE1A were mutated to Alanines. The resultant NORE1AR3A mutant was defective for the interaction with HIPK2 and defective for the induction of senescence in A549 cells (Fig. 6 c) and showed a reduced ability to stabilize HIPK2 (Fig. S1 a). However, it retained WT binding to MST1 and retained WT apoptosis capacity (Fig. 6 d).

HIPK2 is a good candidate for a downstream component of NORE1A senescence signaling. To determine the importance of HIPK2 for NORE1A-mediated senescence, we generated stable clones of A549 cells transfected with shRNAs against HIPK2. Compared with the scrambled shRNA control, the HIPK2 knockdown cells were resistant to NORE1A induced senescence (Fig. 6 a). Suppression of HIPK2 similarly reduced baseline senescence in HBEC-3KT cells (Fig. 6 b). To further confirm the biological importance of the interaction between NORE1A and HIPK2, we used deletion mutagenesis followed by point mutagenesis to identify the minimal domain required for the interaction of NORE1A with HIPK2. Ultimately, Arginines 92–94 of NORE1A were mutated to Alanines. The resultant NORE1AB92-94A mutant (NORE1A(B92-94A)) was defective for the interaction with HIPK2 and defective for the induction of senescence in A549 cells (Fig. 6 c) and showed a reduced ability to stabilize HIPK2 (Fig. S1 a). However, it retained WT binding to MST1 and retained WT apoptosis capacity (Fig. 6 d).
action of p53 by enhancing its affinity for the promoters of pro-apoptotic genes such as Bax (Hofmann et al., 2002). However, HIPK2 also has indirect effects on p53, as it can recruit the acetyltransferases PCAF and CBP/p300 to acetylate p53 on specific lysine residues (Puca et al., 2010). This acetylation can switch the activity of p53 from apoptosis to senescence by enhancing its affinity for specific promoters such as p21 CIP1 (Di Stefano et al., 2005b). Ras has previously been shown to promote the acetylation of p53 on residue K382 by an unknown mechanism (Pearson et al., 2000).

As we had observed induction of senescence by NORE1A, we examined the effects of NORE1A on p53 acetylation at residue K382 using a specific antibody. In transient transfections of A549 cells, we observed that NORE1A, but not the HIPK2 binding defective mutant of NORE1A, induced p53 acetylation at residue 382 (Fig. 8 a). We then examined the relationship between NORE1A and acetylated p53 in a panel of WT p53 primary human tumor samples. We found that the expression levels of NORE1A closely correlated with the acetylation of p53 (correlation coefficient \( r = 0.811; r^2 = 0.6578; P < 0.0001 \); Fig. 8 b).

Further studies using transient transfections in HEK-293 with the validated NORE1A shRNAs showed that Ras requires...
NORE1A to acetylate p53 (Fig. 8 c) and that NORE1A requires HIPK2 to acetylate p53 (Fig. 8 d). Thus, NORE1A/HIPK2 appears to be the link between Ras and p53 acetylation.

We then examined the effects of NORE1A on the HIPK2-dependent proapoptotic posttranslational modification of p53. Using transient transfections in HEK-293 cells and a phospho S46-specific antibody, we found that NORE1A does not activate phosphorylation of p53 on S46 (Fig. 8 e, left). Indeed, it appears to suppress it. This contrasts with the effects on p53 K382 acetylation which is up-regulated by WT NORE1A in the HEK-293 cells (Fig. 8 e, right). Thus, in the same cells, NORE1A is modulating the posttranslational modifications of p53, activating it toward a prosenescent function. Interestingly, the NORE1A point mutant R3A that is defective for HIPK2 binding, and does not enhance p53 K382 acetylation, also failed to activate p53 S46 phosphorylation (Fig. 8 e). Co-transfection experiments with activated Ras also showed that it fails to stimulate phosphorylation of p53 S46. Indeed, S46 phosphorylation is inhibited by Ras/NORE1A (Fig. 8 f).

Upon phosphorylation by HIPK2, p53 preferentially binds and activates the promoters of proapoptotic genes (Hofmann et al., 2002). As we observed the inhibition of p53 phosphorylation by NORE1A we examined the effects of NORE1A on a Bax luciferase reporter. Fig. 9 a shows that NORE1A suppresses the activation of Bax. Because NORE1A enhances senescence, we also examined its effects on p21, a p53-responsive gene that is associated with senescence (Pearson et al., 2000; Di Stefano et al., 2005b; Brooks and Gu, 2011) and found that NORE1A strongly increases p21 expression (Fig. 9 b). Thus, NORE1A appears to repress p53-responsive genes associated...
Ras-driven senescence in tumor systems (Chen et al., 2009; Collado and Serrano, 2010; Dimauro and David, 2010; Morton et al., 2010).

The mechanisms underlying Ras induced senescence pathways and how they are subverted to facilitate human tumor development remain poorly understood. One component that has been shown to play a clear role in Ras-mediated senescence is the p53 tumor suppressor (Serrano et al., 1997). Ras has been shown to induce senescence-associated posttranslational modifications of p53 (Pearson et al., 2000), but the connection between Ras and p53 remains unclear.

NORE1A is a member of the RASSF family of proteins (RASSF5). RASSF family members are tumor suppressors that can serve as Ras death effectors (Donninger et al., 2007). Both RASSF1A and NORE1A (RASSF5) can serve as proapoptotic Ras effectors connecting Ras to the Hippo pathway (Khokhlatchev et al., 2002; Matallanas et al., 2011). Thus, the frequent loss of RASSF1A and NORE1A expression in human tumors due to epigenetic inactivation may be a major permissive factor for Ras transformation by uncoupling Ras from apoptosis.

Although initial studies identified an apoptotic function for NORE1A (Khokhlatchev et al., 2002; Vos et al., 2003), we previously identified a more likely physiological role in the regulation of cell cycle arrest by p53 (Calvisi et al., 2009). In following through on these studies, we have identified NORE1A as a key component of Ras-driven senescence signaling to p53. Thus, the frequent loss of NORE1A in human tumors will partially uncouple p53-dependent senescence from upstream regulating events such as Ras. This may explain why MEFs derived from NORE1A knockout mice can be transformed in a single step by oncogenic Ras (Park et al., 2010), instead of undergoing senescence (Serrano et al., 1997).

The link between Ras/NORE1A and p53 appears to be primarily the HIPK2 protein. NORE1A forms an endogenous, Ras-regulated complex with HIPK2 that stabilizes the protein. As NORE1A has been shown to directly bind the related HIPK1 (Lee et al., 2012), it seems likely that the interaction with HIPK2 is also direct. HIPK2 is itself a tumor suppressor (D’Orazi et al., 2012) with a broad range of functions. It has been shown to regulate apoptosis, in large part by phosphorylating p53 on residue S46 (D’Orazi et al., 2002; Hofmann et al., 2002). This enhances the affinity for p53 for the promoters of proapoptotic genes (Puca et al., 2010). However, HIPK2 can also recruit the acetyltransferases CBP/p300 (Hofmann et al., 2002) and PCAF (Di Stefano et al., 2005b) to acetylate p53. The former induces acetylation of p53 at residue K382, whereas the latter acetylates K320. Acetylation at K382 in combination with S46 phosphorylation appears to promote p53 apoptotic functions (Puca et al., 2010). Acetylation of K382 alone has been associated with senescence (Pearson et al., 2000). Moreover, acetylation at K320 has been reported to enhance p53 association with the p21CIP1 promoter, enhancing expression, which can lead to senescence (Taira and Yoshida, 2012). Here, we show that in the presence of Ras/NORE1A signaling, HIPK2 promotes the acetylation of p53 at K382 but suppresses the phosphorylation of p53 at S46.

We have also detected NORE1A-induced, prosenescent acetylation of p53 at K320 (Fig. S1 b). Thus, NORE1A is specifically with apoptosis and enhance expression of p53-responsive genes associated with senescence, suggesting that NORE1A is a key part of the signaling machinery that determines if p53 drives apoptosis or senescence.

**Discussion**

Ras oncoproteins are powerfully transforming, but aberrant Ras signaling can also activate potent cell death pathways, including both apoptosis and senescence (Serrano et al., 1997; Feig and Buchsbaum, 2002; Cox and Der, 2003; Lowe et al., 2004). It has been hypothesized that Ras-induced senescence pathways serve to impede the development of cancer in cells that acquire activation of Ras (Prieur and Peiper, 2008; Collado and Serrano, 2010; Overmeyer and Maltese, 2011). Their loss should facilitate Ras-driven transformation. Although Ras-induced senescence under physiological conditions now appears a more complex process than originally thought, depending on both signal intensity and cell type (Studebaker et al., 2008; Kuilman et al., 2010; Bianchi-Smiraglia and Nikiforov, 2012), in vivo studies have now confirmed the occurrence and importance of Ras-driven senescence in tumor systems (Chen et al., 2009; Collado and Serrano, 2010; Dimauro and David, 2010; Morton et al., 2010).

Figure 7. NORE1A scaffolds p53 and HIPK2. (A) NORE1A forms a Ras-regulated complex with endogenous p53. HEK-293 cells were transfected with NORE1A in the presence or absence of activated Ras. Cells were lysed, immunoprecipitated (IP) for NORE1A, and immunoblotted (IB) for p53. (B) NORE1A promotes the formation of a p53–HIPK2 complex. HEK-293 cells were transfected with HIPK2, NORE1A, and activated Ras. Cells were lysed and immunoprecipitated (IP) for GFP-HIPK2. The IP was then examined for the presence of endogenous p53.
Figure 8. **NORE1A mediates specific posttranslational modifications of p53 via HIPK2.** (A) NORE1A promotes acetylation of p53 on residue K382. A549 cells were transfected with NORE1A WT or the HIPK2-defective NORE1A<sup>RA</sup> mutant. The cells were lysed and immunoblotted for p53 acetylated at K382 using a K382-specific antibody. (B) NORE1A expression correlates with acetylated p53 expression in primary human tumor samples. The relationship between the protein levels (as assessed by Western blotting and quantification of the chemiluminescence signal) of NORE1A and those of acetylated p53 (K382) was measured in a collection of human HCC (n = 40) harboring WT p53. Axes are shown as relative luminescent units from samples with equal total protein loading. A significant, direct correlation was found between NORE1A and acetylated p53 levels. GraphPad Prism 5.01 was used to evaluate statistical significance by Tukey–Kramer and linear regression analyses (correlation coefficient [r] = 0.811, r<sup>2</sup> = 0.6578, P < 0.0001). (C) Ras requires NORE1A to modulate p53 acetylation. HEK-293 cells were transiently transfected with the NORE1A shRNAs and activated H-Ras. Cells were lysed and assayed for endogenous p53 K382 acetylation after 24 h. Immunoreactive bands were quantified by densitometry and the results of 3 experiments plotted as a bar graph with data normalized to the scrambled control, *P < 0.05 compared with cells transfected with the scrambled control. (D) NORE1A requires HIPK2 to modulate p53 acetylation. HEK-293 cells were transfected with NORE1A in the presence or absence of the HIPK2 shRNAs. After 24 h cells were lysed and assayed for endogenous p53 K382 acetylation. Immunoreactive bands were quantified by densitometry and the results of three experiments plotted as a bar graph, with data normalized to control cells transfected with the scrambled shRNA. *P < 0.05 compared with cell transfected with the scrambled shRNA. (E) NORE1A suppresses S46 phosphorylation of p53. HEK-293 cells were transfected with expression constructs for NORE1A or the NORE1A<sup>RA</sup> mutant. The cells were lysed and immunoblotted for endogenous p53 phosphorylated at S46 (left) or acetylated at K382 (right). (F) Ras suppresses p53 S46 phosphorylation. HEK-293 cells were transfected with expression constructs for NORE1A, activated H-Ras, or both. The level of S46 phosphorylation of endogenous p53 was then assayed by Western blot using a phospho S46–specific antibody. The Western blot shown is representative of three experiments. Total levels of endogenous p53 and transfected NORE1A/Ras are shown in the lower panels.
As we have previously shown that NORE1A stabilizes p53 in (Lee et al., 2012). Both p53 and HIPK2 regulate and are regulated by mdm2 (Di Stefano et al., 2005a; Rinaldo et al., 2009). As we have previously shown that NORE1A stabilizes p53 in the nucleus (Calvisi et al., 2009), it may be interesting to examine the action of NORE1A on mdm2, which could allow both quantitative and qualitative (via HIPK2) regulation of p53.

Currently, the main RASSF family activated anti-growth pathway that has been described is the Hippo pathway (Fausti et al., 2012). In its simplest form the Hippo pathway consists of an MST kinase, which phosphorylates and activates a LATS kinase that then phosphorylates the related transcriptional co-activators YAP and TAZ to modulate their activity. RASSF proteins such as NORE1A can bind and activate MST kinases to contribute to the Hippo pathway (Avruch et al., 2012). In these studies, we included a NORE1A mutant that does not bind MST kinases to determine that the p53 pathway/senescent-inducing effects of NORE1A appear to be largely independent of the canonical Hippo pathway. However, recent studies have identified a potential role for HIPK2 in modulating the Hippo pathway by interacting with YAP (Poon et al., 2012), and YAP has now been implicated in p53-mediated senescence (Xie et al., 2013). Therefore, NORE1A-induced senescence may be more complex, and there may be a role for NORE1A and other RASSF proteins in the induction of the Hippo pathway by non-canonical activity at the level of YAP/TAZ, independently of MST kinases.

Thus, we identify a novel Ras–NORE1A–HIPK2–p53 signaling pathway that is critical and specific for Ras-mediated senescence. The pathway promotes the induction of specific prosenescent posttranslational modifications of p53. Its inactivation uncouples Ras from p53, allowing Ras to drive transformation without the restriction of senescence, even in the presence of WT p53. NORE1A is down-regulated in many human tumors and we found that hepatocellular carcinomas that lose NORE1A expression usually demonstrate reduced p53 acetylation. This observation supports the concept that senescence pathways must be inactivated for malignant progression of cancer and that NORE1A is a critical component of such a pathway. This work also identifies a novel mechanism of action for a RASSF family protein: the regulation of protein acetylation. We suspect this function may be common to other RASSF family members, and may involve targets in addition to p53.

Materials and methods

Plasmids

Human NORE1A cDNA was obtained from Origene and confirmed by sequencing. HA, KATE, and GFP-NORE1A expression plasmids were generated by cloning the full-length NORE1A cDNA as a BglII–EcoRI fragment into a pCDNA vector with an HA epitope tag, pmKATE2 (Evrogen) and pEGFP-C1 [Takara Bio]. pmKATE2 encodes the far-red fluorescent protein mKATE2 as a codon-shifted variant of WT GFP. All three fusion proteins were under the control of a CMV promoter. pCGNHA-HRas12v was created by cloning the full-length HRas12v cDNA as a BamHI fragment into pCGN vector under the control of a CMV promoter (Ellis et al., 2002) and pLRT-HRas12v by cloning the full-length HRas12v cDNA as a XhoI–NotI fragment into the pLRT retroviral vector (Watsuji et al., 1997). The GFP tagged HIPK2 expression plasmid was generated by cloning the HIPK2 cDNA into the pEGFP-C2 expression vector and was a gift of Y. Kim (National Institutes of Health, Bethesda, MD). shRNAs for human NORE1A (#1: 5'-TATATATAGCTATATGCCT-3'; #2: 5'-AGCTTGGCCTAAGGAGA-3'; Scrambled: 5'-ATCTCGCTTGGGCGAGAGTAAG-3'; RHS4531-EG83593) were obtained from Open Biosystems and HIPK2 shRNAs (#1: 5'-CTCGCCAGCCTCCACCCTTACACTGGAAT-3'; #2: 5'-GCCTTGGAGACTGGAACACCGACGGAG-3'; TR304106) were obtained from Origene. p53 and control siRNAs were from Applied Biosystems [ID 2714] and...
mouse NOR1A shRNAAs (#1: 5’-GGGTGCCTCAGAAGCTCATTCGGTGTTG-GAC-3’; #2: 5’-CCGACGTTAGAAGCTCCTTGCAGCCCG-3’) from Origene and have been described previously [Calvisi et al., 2009]. The NOR1AΔ74 mutant (92/94: RRR:AAA) was generated using a QuikChange kit (Stratagene) as described by the manufacturer. The NOR1A RA mutant was also generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) in a similar manner based on the mutant described in Aird et al. [2004]. The NOR1A-sarah-del mutant was generated by PCR and lacks the C-terminal 168 bases pairs. All mutants were sequenced to confirm fidelity before use. MST1 constructs were a generous gift (J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA).

**Tissue culture and transfections**

HEK-293, A549, NCH1792, HepG2, and COS-7 cells were obtained from the American Type Culture Collection. MEFs were a gift from D. Dean (University of Louisville, Louisville, KY). Cells were grown in DMEM/10% FBS. HBE-C3KT cells were provided courtesy of Jerry Shay (UT Southwestern, Dallas, TX) and cultured in keratinocyte-serum-free medium containing bovine pituitary extract and recombinant epidermal growth factor (Invitrogen). A summary of the cell lines used in this study with respect to their Ras and p53 status is provided in **Tables S1 and S2**. Transient transfections were performed using JetPrime (Polyplus) as described by the manufacturer. GFP and RFP proteins were visualized using an Olympus IX50 fluorescent microscope at room temperature in growth medium with a 100x/1.30 Olympus UPlanFl objective. β-galactosidase assays were performed using a BioVision kit (BioVision). Samples were imaged using an IX50 inverted system microscope (Olympus) with a UPlanFl 4x/0.13 Ph or LCPlanFl 20x/0.40 Ph1 objective (Olympus) and a SPOT camera (Diagnostic Instruments Inc.). Senescence-associated heterochromatin foci (SAHF) were detected essentially as previously described (Aird and Zhang, 2013). In brief, cells were plated in glass-bottom microwell dishes (MatTek Corp). The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then washed with PBS. They were then permeabilized with 0.2% (vol/vol) Triton X-100 for 5 min at room temperature, blocked with 3% BSA in PBS for 5 min, and stained with 0.15 µg/ml DAPI diluted in 3% BSA in PBS for 3 min at room temperature. SAHF were observed using an Olympus IX50 fluorescent microscope with a 100x/1.30 Olympus UPlanFl oil immersion objective. APOPTOSIS ASSAYS WERE PERFORMED USING THE CASPASE-3 SENSOR INDICATOR PLASMID, AS DESCRIBED PREVIOUSLY (Vos et al., 2003). In brief, COS-7 cells were co-transfected with either red fluorescent expression vector pmKATE-NOR1A or NOR1AΔ74 and yellow fluorescent protein-sensor caspase activity reporter plasmid (Takara Bio). Localization of the pSensor indicator protein in cotransfected cells was determined 24 h later by fluorescent microscopy. Cycloheximide (Sigma-Aldrich) treatments were performed 24 h after transfection at a concentration of 20 µg/ml. Soft agar assays were performed as described previously (Ellis et al., 2002). Cells (104) were resuspended in 0.35% agar and plated on a prehardened 0.7% agar base and incubated at 37°C for 14 d before scoring by microscopic evaluation. pRTH-Ras12v transfected cells were selected in 5 µg/ml Blasticidin (Invitrogen) and induced with 1 µg/ml doxycycline (Sigma-Aldrich).

**Human tissue samples**

A collection of 40 p53 WT HCCs were used. Liver tissues were provided by S.S. Torgerison (Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD). Institutional Review Board approval was obtained at participating hospitals in the Liver Tissue Procurement and Distribution System [University of Minnesota, Minneapolis, MN], and University of Pittsburgh, Pittsburgh, PA and the National Institutes of Health. The clinical-pathological features of HCC patient samples are summarized in Table S2.

**Western blots and immunoprecipitation**

Monoclonal NOR1A antibodies were a gift from A. Kholkhatchev (University of Virginia, Richmond, VA), and were raised against human NOR1A amino acid residues 119–416. Rabbit polyclonal NOR1A antibodies were raised against the synthetic human NOR1A peptide: KYDKFRQKLEELRELSQGKPQ by ProSci (Poway, CA). Rabbit monoclonal HIPK2 antibodies were from Epitomics (Burlingame, CA). Rabbit polyclonal Dec1 was from Ingexen (Littleton, CO), and rabbit polyclonal RFP and mouse monoclonal GFP were from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-H-Ras antibody 146 was from the NCI antibody repository. Mouse monoclonal FLAG and β-actin antibodies were obtained from Sigma-Aldrich and mouse monoclonal HA antibodies were from Covance. Rabbit polyclonal p53 Ac382 and Phospho-S46 and p38 were obtained from Cell Signaling Technology. Immunoprecipitations were performed using loma anti-GFP, Allele Biotech, or mouse monoclonal HA beads (Sigma-Aldrich). Goat anti-mouse and anti–rabbit secondary antibodies conjugated to peroxidase were from Thermo Fisher Scientific. Western blots were developed using a West-Pico kit (Thermo Fisher Scientific). Protein acetylation was detected by immunoprecipitation with rabbit anti–acetyl-Hisylseye beads (ImmuneChem) followed by Western blot with the appropriate antibody. Alternatively, the protein was immunoprecipitated and then Western blotted with rabbit anti-acetyl lysine (Cell Signaling Technology).

**Image acquisition and processing**

Images were scanned and quantified using a Phoros FX plus Molecular Imager (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories). Images were compiled using Photoshop (Adobe).

**Online supplemental material**

Fig. S1 shows that the Ras binding defective mutant of NOR1A (NOR1AΔ74) fails to stabilize HIPK2 protein and that NOR1A also promotes acetylation of p53 at K320. Table S1 provides a description of the cell lines used. Table S2 provides a description of the clinicopathological features of HCC patient samples. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201408087/DC1.

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**References**


NOR1A is a Ras senescence effector  Donninger et al. 787

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**Figure S1. NORE1A<sup>R3A</sup> fails to stabilize HIPK2.** (a) HEK-293 cells were transfected with HIPK2, NORE1A and activated H-Ras expression constructs. The cells were treated with cyclohexamide and lysed at the indicated time points. Levels of protein were determined by Western blot analysis. (b) HEK293 cells were transiently transfected with vector, NORE1A or p53 expression plasmids. The cell lysates were then Western blotted for the total levels of each protein and the degree of p53 acetylation at residue K320 using a specific antibody (Cell Signaling Technology). In the presence of NORE1A, endogenous p53 exhibited enhanced K320 acetylation.
### Table S1. Description of cell lines used

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>RAS Status</th>
<th>P53 status</th>
<th>HIPK2 status</th>
<th>NORE1A Status</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 human lung tumor cell line</td>
<td>mutant</td>
<td>WT</td>
<td>+</td>
<td>-</td>
<td>Can be induced to undergo senescence by NORE1A transfection.</td>
</tr>
<tr>
<td>MEFs</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>Primary murine cells with senescence machinery intact</td>
</tr>
<tr>
<td>HBEC-3KT Human lung</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>Human lung epithelial cells immortalized without the use of viral oncogenes.</td>
</tr>
<tr>
<td>HEK-293 cells Human kidney</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>Human embryonic kidney cells. Excellent cell line for transient transfection experiments due to ease of transfection and relatively high levels of expression.</td>
</tr>
<tr>
<td>HEK-293T Human kidney</td>
<td>WT</td>
<td>inhibited</td>
<td>+</td>
<td>+</td>
<td>As above but expressing SV40 LT which compromises p53. Higher levels of expression from transiently transfected plasmids.</td>
</tr>
<tr>
<td>COS-7 Monkey kidney</td>
<td>WT</td>
<td>WT</td>
<td>?</td>
<td>?</td>
<td>Express high levels of protein from transiently transfected plasmids. Possess large, flat nuclei, ideal for visualizing fluorescent nuclear proteins.</td>
</tr>
<tr>
<td>HuH6 Human hepatocellular carcinoma</td>
<td>WT (MET*)</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>Express both NORE1A and HIPK2. Constitutively phosphorylated MET receptor may activate the WT Ras pathway.</td>
</tr>
<tr>
<td>HepG2 Human hepatocellular carcinoma</td>
<td>mutant</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>Contain mutant Ras, express both NORE1A and HIPK2. Can be used for endogenous co-IP.</td>
</tr>
</tbody>
</table>

### Table S2. The clinicopathological features of HCC patient samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCCB</th>
<th>HCCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>71.8 ± 8.9</td>
<td>73.4 ± 10.0</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>HCV</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>–</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>&lt;5 cm</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Edmondson and Steiner grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>IV</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Alpha-fetoprotein secretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;300 ng/ml of serum</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>&lt;300 ng/ml of serum</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

*HCCB: HCC with better outcome/longer survival (survival >3 yr after partial liver resection)

*HCCP: HCC with poorer outcome/shorter survival (survival <3 yr after partial liver resection)*