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The Role of the NORE1A Tumor Suppressor in Oncogene-Induced Senescence

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Abstract

The Ras genes are the most frequently mutated oncogenes in human cancer. However, Ras biology is quite complex. While Ras promotes tumorigenesis by regulating numerous growth promoting pathways, activated Ras can paradoxically also lead to cell cycle arrest, death, and Oncogene-Induced Senescence (OIS). OIS is thought to be a critical pathway that serves to protect cells against aberrant Ras signaling. Multiple reports have highlighted the importance of the p53 and Rb tumor suppressors in Ras mediated OIS. However, until recently, the molecular mechanisms connecting Ras to these proteins remained unknown. The RASSF family of tumor suppressors has recently been identified as direct effectors of Ras. One of these members, NORE1A (RASSF5), may be the missing link between Ras-induced senescence and the regulation of p53 and Rb. This occurs both quantitatively, by promoting protein stability, as well as qualitatively via promoting critical pro-senescent post-translational modifications. Here we review the mechanisms by which NORE1A can activate OIS as a barrier against Ras-mediated transformation, and how this could lead to improved therapeutic strategies against cancers having lost NORE1A expression.

Keywords

RASSF; HIPK2; PP1A; Retinoblastoma; p53; Ras

Introduction

Ras proteins make up a small family of three closely related proteins. They are small GTPases that act as molecular switches to regulate multiple pathways involved in

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proliferation and cell survival (1-3). Gain of function mutations in K-Ras, H-Ras, or N-Ras were the first oncogenic alterations identified in human cancer over 30 years ago (4), and the available data suggest that Ras may be distinguished as the most frequently mutated oncogene in human cancer (5). Ras is a key driver of transformation due to its ability to regulate multiple signaling pathways including growth, cell-cycle progression, cytoskeletal changes, and migration (4). Though Ras is mutated in approximately 30% of all human tumors (6), additional cancers contain genetic alterations in Ras regulatory components, such as the Epidermal Growth Factor Receptor (EGFR), Raf, and negative regulators of Ras, such as NF1 (Neurofibromin 1) and DAB2IP (DAB2 Interacting Protein) (7-9). Thus, tumors can be commonly driven by aberrant Ras activation even in the absence of *ras* gene mutations. While Ras proteins remain one of the cornerstones of cancer biology, Ras biology remains extremely complex and is not fully understood.

Although Ras acts as a powerful driver of growth and transformation, it has the ability to regulate several growth inhibitory pathways (10-12). Reports in the 1980s showed that in primary cells, mutant (activated) Ras inhibits cell growth rather than promotes transformation (13). Multiple groups have since shown that mutation or over-expression of Ras leads to cell death and cell cycle arrest. The ability of oncogenes like Ras to promote a state of irreversible cell-cycle arrest has been coined Oncogene-Induced Senescence (OIS) (14), and multiple reports have now implicated H-Ras, N-Ras, and even K-Ras in OIS (15-18). OIS is believed to be a critical barrier against the development of cancer, which must be overcome in order for oncogenes like Ras to promote tumorigenesis (16,19) (Figure 1). Interestingly, as Ras-driven tumors progress toward a more malignant phenotype, they lose multiple markers associated with senescence (20). The detection of Ras-mediated senescence in cell culture systems, primary human tumors, and animal models confirms OIS as a physiologically relevant tumor suppression process (16,20-24). However, the signaling pathways mediated by Ras that promote senescence are not fully defined.

Pro-mitogenic pathways controlled by Ras are regulated by numerous effector proteins, such as the Raf kinases and PI3-kinase (25,26). However, not all Ras effectors are mediators of survival signaling (10). Members of the family of proteins known as the RASSF family of proteins can serve as key effectors of Ras-mediated growth inhibition (27-29). The six core members have Ras Association (RA) domains and several have been shown to bind or complex with activated Ras (30). These proteins lack any apparent intrinsic enzymatic activity, and are thought to function as scaffolding and localization molecules, in turn regulating the function of downstream tumor suppressor proteins (29,31). Notably, most RASSF family members are down-regulated in human cancers, in part by epigenetic mechanisms (32). The best characterized member, RASSF1A, is now thought to be the most frequently silenced tumor suppressor in human cancers, highlighting the significance of this family in tumor suppression (31,33,34).

The second best characterized member of the RASSF family is NORE1A (RASSF5). NORE1A binds directly to Ras, acting as a *bona fide* Ras death effector/tumor suppressor (35-40). Initial evidence clearly pointed to NORE1A as a Ras effector and tumor suppressor. For example, over-expression of NORE1A inhibited growth in human cancer cells harboring mutant K-Ras (A549 lung adenocarcinoma) or mutant N-Ras (H1299 non-small cell lung

carcinoma), and inhibition of NORE1A expression enhanced cell proliferation (38,41). However, the underlying mechanisms of Ras/NORE1A action were not clear.

NORE1A shares about 50% homology with RASSF1A (35), but the proteins diverge significantly in their N-termini, suggesting overlapping, but distinct functions. As with RASSF1A, NORE1A is frequently inactivated in many human cancers by promoter methylation. It is also subject to post-transcriptional inactivation via calpain-mediated proteolysis (42). In addition, inactivation of NORE1A through a translocation event is linked to the development of clear cell renal cell carcinoma, a rare form of familial cancer (43). Thus, “knockout humans” are predisposed to cancer. Moreover, in primary Ras-driven tumors, loss of NORE1A is associated with a more malignant phenotype, suggesting an important link between Ras and NORE1A *in vivo* (44,45).

NORE1A was first thought to play a role in regulation of the HIPPO pathway due to its ability to bind to MST kinases (37). However, NORE1A does not seem to activate MST kinases, and the interaction between NORE1A and MST kinases is not required for NORE1A to inhibit growth (46,47). Collectively, the data suggest that NORE1A acts as a tumor suppressor independently of canonical HIPPO signaling. Additional work in this area, using more physiological systems, suggested that NORE1A had an impact on cell-cycle arrest rather than apoptosis (41). Several reports have now shown that NORE1A is a critical driver of Oncogene-Induced Senescence (48-52). In support of this premise, loss of NORE1A impairs the ability of Ras to induce senescence, providing strong evidence that NORE1A is an important component of Ras-induced senescence (48).

Ras-induced senescence appears to act as a critical barrier against the development of cancer, and OIS must be overcome in order for Ras to promote transformation (16,53,54). For over 25 years, both the p53 and Rb pathways have been implicated in OIS; however, the exact mechanisms by which Ras regulates these proteins was not defined. NORE1A has now been identified as the missing link between Ras and these critical tumor suppressors. Specifically, NORE1A can regulate both the p53 and Rb pathways quantitatively and qualitatively (48,49). This review describes our current understanding of how NORE1A regulates critical tumor suppressor pathways to promote OIS in order to protect against Ras-mediated transformation.

NORE1A Interacts with PP1A to Dephosphorylate and Activate Rb, Inducing OIS

The *Rb* gene was the first tumor suppressor gene identified and was initially linked to the formation of rare cases of pediatric tumors of the retina called retinoblastoma (55-57). Further studies have identified alterations in the *Rb* gene or inactivation of the Rb protein in a variety of human cancers, and it is now widely accepted that the inactivation of Rb may be one of the most frequent events in cancer (58-60). In addition to its important function in regulating the cell cycle, Rb has critical functions in other biological processes, including chromosomal stability, regulation of apoptosis, and control of OIS (16,61,62). In addition, inactivation of the *Rb* gene suppresses Ras-induced senescence (16,63). This implies that

Ras induces OIS, at least in part, via Rb. However, how Ras specifically regulates Rb to drive senescence was unknown until recently.

The regulation of Rb function involves both inhibitory phosphorylation and activating dephosphorylation events. Although the mechanisms of Rb phosphorylation by cyclin-dependent kinases are well characterized, the processes that activate Rb by dephosphorylation remain less so (64). Recent reports have shown that the phosphatases PP1 and PP2A play important roles in the mammalian cell cycle (65-67). Moreover, it has been shown that PP1 phosphatases can act on Rb to promote the formation of the active, hypophosphorylated form of the protein (68,69). Intriguingly, PP1A enzymatic activity can be regulated by Ras (70). Therefore, PP1A might act as a tumor suppressor and serve as the link between Ras and Rb, and Ras may promote senescence in part by activating PP1A, in turn promoting the dephosphorylation and activation of Rb (71). Recently, how Ras stimulates the activity of PP1A toward Rb was identified (49).

PP1A had been detected in complex with NORE1A in a yeast two-hybrid screen (72), and based on evidence suggesting that NORE1A was involved in OIS, it was plausible that NORE1A could be modulating senescence by regulating Rb function. Our group showed that NORE1A forms an endogenous complex with PP1A, and that this interaction is enhanced in the presence of either K- or H-Ras (49). In addition, we found that Ras/NORE1A cooperated to stabilize PP1A, as well as to scaffold PP1A to Rb, in turn promoting Rb dephosphorylation, a pro-senescent event (49). The enhanced PP1A association with Rb in the NORE1A complex may also have a second effect, as Hirschi and colleagues showed that overlapping kinase and phosphatase docking sites exist that regulate the activity of Rb (73). Therefore, NORE1A may also serve to hinder Rb phosphorylation by inhibiting the binding of Cdk.

While we found NORE1A in complex with Rb, this interaction is likely indirect, possibly via PP1A. However, this provides a mechanistic insight as to how Ras can regulate the post-translational modification code of Rb in order to activate Rb and promote senescence. Interestingly, Rb is regulated by other post-translational modifications (PTMs) in addition to phosphorylation, including acetylation, which is thought to also be important for senescence, and SUMOylation (74). The ability of NORE1A to regulate these PTMs on Rb is currently under investigation. In addition, we found that Ras/NORE1A stabilizes Rb (unpublished data), suggesting a quantitative mechanism of action in addition to activation via dephosphorylation.

NORE1A Promotes OIS by Activating p53 via HIPK2

Although Rb clearly plays an important role in NORE1A induced senescence, the suppression of Rb does not completely inhibit the process (49). This suggests multiple mechanisms of NORE1A induced senescence. Human cells require the loss of both the p53 and Rb pathways to fully bypass senescence. The p53 tumor suppressor is a powerful transcription factor and plays a central role in the regulation of the cell cycle, apoptosis, DNA repair, and senescence (75-77). Work by Calvisi and colleagues in 2009 showed that NORE1A induces p53-dependent activation of p21^{CIP1}, a cyclin dependent kinase inhibitor

associated with p53-dependent senescence (41). Later studies by Donninger et al. showed that Ras (K- as well as H-Ras) promotes the formation of a complex between NORE1A and HIPK2; HIPK2 is a kinase that can regulate the apoptosis/senescence balance of p53 by regulating critical PTMs on p53 (48). Mechanistically, NORE1A was found to scaffold HIPK2 to p53 in a Ras-regulated manner, in turn promoting the acetylation of p53 at lysines 320 and 382, two PTMs associated with p53 senescence activity, while simultaneously reducing the phosphorylation of p53 at serine 46 (48); the latter modification is known to favor p53-mediated apoptosis (78). As a result, NORE1A expression results in the up-regulation of p53-regulated senescence markers such as p21^{CIP1} and down-regulation of p53 apoptotic mediators, such as Bax (48).

In sum, NORE1A appears to restrain the transforming properties of Ras by simultaneously acting on both p53 and Rb, thus making NORE1A a powerful Ras senescence effector (50). This involves scaffolding of a kinase and a phosphatase to p53 and Rb, respectively (48,49). These collective findings have novel and powerful ramifications on Ras biology, providing mechanistic examples by which Ras uses its effectors to regulate the activity of downstream proteins by qualitatively controlling their PTMs. Additionally, loss of NORE1A uncouples Ras from both p53 and Rb, thus allowing Ras to bypass senescence, and in turn facilitating transformation by allowing the Ras-mediated pro-growth signals to predominate.

NORE1A Forms a Ras-Regulated Complex with β -TrCP and Suppresses the Expression of MDM2

The canonical Wnt signaling pathway is a highly conserved pathway important for tissue development and renewal, and has been shown to be frequently activated in human cancers (79). Interestingly, repression of Wnt signaling has been implicated as an early-activated trigger of the senescence program (80). Furthermore, it has been reported that activated β -Catenin, a critical transcription factor in Wnt signaling, suppresses senescence in melanocytes harboring mutant Ras (81). This strongly suggests that downregulation of β -Catenin may be critical for senescence induction. One of the ways to antagonize the Wnt pathway is by ubiquitin-mediated proteolysis of β -Catenin, which can be accomplished by the E3 ubiquitin ligase β -TrCP (82).

Work published by Schmidt and colleagues identified a direct interaction between NORE1A and β -TrCP, the substrate recognition subunit of the SCF β -TrCP E3 ubiquitin ligase, which is a critical regulator of the Wnt/ β -Catenin pathway (83). This raised the possibility that Ras may also be able to regulate the proteasome and protein turnover via NORE1A. Indeed, these studies showed that an H-Ras-regulated complex between NORE1A and β -TrCP promotes the degradation of β -Catenin, providing a mechanism by which Ras can negatively regulate Wnt signaling (83). While fascinating, the works begs an interesting question: is the effect of NORE1A on β -TrCP specific to β -Catenin, or are there additional targets that are affected by NORE1A binding to β -TrCP? One of these additional targets, which is known to indirectly play a role in OIS, is the Mouse Double Minute 2 Homolog, MDM2 (84).

MDM2 is an E3 ubiquitin ligase and a key negative regulator of p53 (85). MDM2 can negatively regulate p53 protein levels by binding and ubiquitinating p53, leading to its

nuclear export and degradation by the proteasome (86). MDM2 acts in an oncogenic fashion (87), and the *MDM2* gene is frequently found to be over-expressed in human cancers, in particular in tumors that contain wild type p53 (88,89). Recent work by Lee and colleagues reported that NORE1A forms a complex with MDM2 (90), and this interaction seems to play a role in regulating protein turnover. Because NORE1A also interacts with β -TrCP, a protein known to promote the degradation of MDM2 (91), it is possible that in addition to modulating the PTMs of p53, and thereby its activity, NORE1A may be stabilizing p53 by regulating MDM2. Further work by Schmidt et al. confirmed this hypothesis by showing that NORE1A does indeed down-regulate MDM2 protein expression. By using an MDM2 mutant that cannot auto-ubiquitylate itself, as well as a dominant-negative form of β -TrCP, they were able to confirm that the ability of NORE1A to down-regulate MDM2 occurs in a β -TrCP dependent manner (52). In addition, this group showed that MDM2 suppresses the ability of NORE1A to induce senescence in tumor cell lines harboring mutant Ras. These findings provide an explanation as to how NORE1A may enhance nuclear expression of p53 (41).

MDM2 is able to regulate other important proteins involved in multiple biological processes, including OIS. Other studies have shown that MDM2 is a negative regulator of Rb, and can contribute to tumorigenesis in part by destabilizing Rb (92). Our unpublished data indicate that Ras/NORE1A cooperate to stabilize Rb; however, the mechanism by which this occurs remains unknown, but it is possible that this occurs due to the ability of NORE1A to down-regulate MDM2. Furthermore, MDM2 can be found in an endogenous complex with PP1A (93), one of the key phosphatases required for Rb activation and induction of OIS. While the effects of MDM2 on PP1A stability have not been elucidated, we have shown that Ras/NORE1A can stabilize PP1A through a currently unknown mechanism (49). These data suggest that NORE1A could be regulating PP1A stability through its interaction with MDM2, in part by antagonizing the ubiquitination/degradation properties of MDM2.

RASSF1A, arguably the most frequently silenced tumor suppressor in human cancer (31,32,34), is another member of the RASSF family that plays an important role in β -Catenin signaling (32). Studies by Ram and colleagues showed that RASSF1A interacts with β -TrCP, and that the down-regulation of RASSF1A led to the stability of β -catenin (94), as is the case with NORE1A (83). Furthermore, loss of RASSF1A in *Apc^{+/Min}* mice (a model of intestinal cancer) resulted in accumulation of β -catenin and accelerated tumor development (95). Taken together, these findings highlight the importance of RASSF1A in regulation of β -catenin. RASSF1A has also been shown to promote MDM2 auto-ubiquitylation, in turn preventing p53 degradation (96). Collectively, this suggests that while RASSF1A is primarily considered as pro-apoptotic in nature, there is the possibility that it may also be involved in senescence. Indeed, results from Thaler et al. showed that RASSF1A can induce a senescence response both *in vitro* and *in vivo* (97). However, the precise role of Ras in the modulation of these effects remain unclear.

NORE1A and the Senescence-Associated Secretory Phenotype

Cells undergoing senescence undergo multiple morphological and biochemical changes, including the acquisition of a senescence-associated secretory phenotype (SASP) (98-100).

Senescent cells secrete specific inflammatory cytokines that can affect surrounding cells (101). Perhaps the most prominent cytokine of the SASP is interleukin-6 (IL-6). IL-6 has been reported to be associated with not only DNA-damaged induced senescence, but also oncogenic stress-induced senescence, such as activated Ras, and seems to occur independently of both the p53 and the Rb pathways (99).

IL-6 and IL-8 are secreted by multiple cell types in the tumor microenvironment and contribute to the SASP (102); however, SASP is quite complex, as SASP factors may act in both autocrine and paracrine manners to either promote tumorigenesis or to reinforce the senescent phenotype (103). IL-6 is one of the factors that is thought to reinforce the senescent phenotype (104). We found that NORE1A enhanced IL-6 promoter activity in human tumor cell lines harboring mutant K-Ras, although in contrast to previous reports (99), this appeared to be Rb dependent (49).

A role for NORE1A activity in SASP could explain our observation that although we find increasing loss of NORE1A expression in primary tumor tissue as they progress to malignancy, we have observed an elevated level of NORE1A expression in the stroma surrounding the tumors (41). Therefore, it is possible that the upregulation of NORE1A may promote the SASP in stromal cells while the downregulation of NORE1A in tumor cells inhibits OIS. However, whether NORE1A modulates senescence in stromal cells has not been determined, and further studies will be required to confirm a role for NORE1A in the SASP process.

Therapeutic Strategies to Target NORE1A-Negative Cancers

For over 30 years, countless therapeutic efforts have aimed at targeting Ras-driven tumors, accounting for over a third of all human cancers. Unfortunately, many of these efforts have failed, leading some to consider Ras as “undruggable.” However, new strategies are now aimed at targeting Ras-mediated pathways rather than Ras directly. As loss of NORE1A shows strong correlation with enhanced malignancy, NORE1A can serve as a prognostic tool (41). Since loss of NORE1A allows Ras to bypass senescence, it would seem logical to target such tumors with standard therapies that invoke a senescence response as part of their therapeutic effect, termed “therapy-induced senescence.” Indeed, the concept of pro-senescence therapy has emerged over recent years as a novel therapeutic approach to treat cancers, and it is now believed that the inclusion of deliberate pro-senescence therapy may be critical as part of cancer treatment regimens (105). Such approaches include drugs that enhance p53 activity and function, as well as drugs with the ability to modulate Rb activity through targeting Cdk and CDKIs (105). The collective work in this review indicates that the status of NORE1A in a tumor may play a role in dictating the sensitivity of tumor cells to any pro-senescence type therapy.

As loss of NORE1A expression primarily occurs via promoter methylation, it is a candidate for epigenetic therapy. It has already been shown that restoring NORE1A expression in several NORE1A-negative cancer cell lines using the DNA methyl-transferase inhibitor 5-Azacytidine restores NORE1A signaling pathways (41). Unfortunately, treatment with 5-Aza-C results in non-specific overall DNA demethylation and this process may affect

multiple regulatory pathways (106). This can likely reactivate expression of multiple silenced genes, including oncogenes and tumor suppressors in different cell types and in different cancers (107). However, the continued development of more specific epigenetic therapy agents, such as Nanaomycin A, which preferentially inhibits the key DNMT3B enzyme, may enhance the practicality of this approach. Recent evidence has shown that Nanaomycin A reduced global demethylation levels while reactivating transcription of several RASSF family members (108,109), though its effects on NORE1A expression have not yet been studied. Thus, a novel approach in NORE1A negative cancers might be to combine epigenetic therapy with senescence therapy to induce tumor regression.

Concluding Remarks

Ras is arguably one of the most important oncogenes in human cancer. However, Ras can paradoxically inhibit cell proliferation, and it is thought that loss of these growth inhibitory pathways drives Ras towards transformation. Given the overwhelming number of reports, using both *in vitro* and *in vivo* studies, it is clear that Oncogene-Induced Senescence is a critical protective mechanism against Ras-mediated transformation. For the most part, Ras can activate senescence through the p53 and Rb tumor suppressor pathways, though it was unclear until now as to how mechanistically Ras is able to regulate these proteins. Ras, being a critical node in signal transduction, must act through effector proteins in order to promote OIS. The RASSF family of tumor suppressors is a relatively new family of proteins known to act as “death” effectors of Ras, and one member, NORE1A, has now been identified as the link between Ras and the induction of OIS.

NORE1A is able to promote OIS via multiple mechanisms, such as stabilizing Rb and nuclear p53, scaffolding regulatory enzymes to promote key pro-senescent PTMs, regulating the expression of MDM2 and β -catenin, and potentially inducing the SASP (Figure 2). While these collective findings provide great insight as to how Ras is able to specifically activate OIS, these studies have even broader ramifications on Ras biology. First, they provide compelling evidence that Ras can qualitatively regulate downstream proteins by affecting their post-translational code via phosphorylation, acetylation, and potentially other modifications such as SUMOylation. In support of this premise, recent work by Kubota and colleagues has shown that Ras can regulate the MAPK cascade by SUMOylation (110). However, this new mode of regulation remains to be clearly defined, and deciphering the mechanisms and downstream targets Ras regulates by PTMs will be critical to gain a true understanding of Ras biology, especially as we attempt to identify new and improved therapeutic strategies to treat Ras-driven cancers.

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Abbreviations

OIS	Oncogene-Induced Senescence
NORE1A	Novel Ras Effector 1A
Rb	Retinoblastoma Protein
PTM	Post-Translational Modification
EGFR	Epidermal Growth Factor Receptor
NF1	Neurofibromin 1
DAB2IP	DAB2 Interacting Protein
RA	Ras Association
RASSF1A	Ras Association Domain Family 1 Isoform A
PP1A	Protein Phosphatase 1A
HIPK2	Homeodomain Interacting Protein Kinase 2
MDM2	Mouse Double Minute 2 Homolog
SASP	Senescence-Associated Secretory Phenotype
IL-6	Interleukin-6

Highlights

- Oncogene-Induced Senescence is a protective mechanism against transformation
- Ras can paradoxically inhibit growth by promoting Oncogene-Induced Senescence
- NORE1A is a tumor suppressor that is silenced in a variety of human cancers
- NORE1A links Ras to p53 and Rb to drive senescence
- Loss of NORE1A allows Ras to bypass senescence induction and promote transformation

Figure 1. The role of Oncogene-Induced Senescence as a critical barrier against transformation and tumor development

Normal growing cells can undergo mutations leading to activation of oncogenes, such as Ras. Aberrant Ras signaling can activate a failsafe mechanism known as Oncogene-Induced Senescence (OIS), and as long as the senescence machinery remains intact, it will suppress tumor progression. However, mutations or loss of tumor suppressor genes involved in OIS, such as NORE1A, p53, and p16^{INK4a}, allows Ras to bypass the senescence program and drive tumor progression towards malignancy.

Figure 2. The molecular mechanisms by which NORE1A promotes Oncogene-Induced Senescence (OIS)

In cells expressing NORE1A, aberrant Ras signaling leads to the activation of OIS, whereby NORE1A can activate multiple signaling pathways. NORE1A can stabilize Rb and nuclear p53, as well as affect pro-senescent post-translational modifications of both proteins. NORE1A scaffolds PP1A to Rb to activate Rb by dephosphorylation, and recruits HIPK2 to p53 to enhance pro-senescent p53 acetylation and suppress pro-apoptotic phosphorylation. In addition, NORE1A interacts with β-TrCP to suppress the expression of MDM2, a negative regulator of both p53 and Rb. Lastly, NORE1A may be regulating the senescence-associated secretory phenotype (SASP). Ac, acetylated lysine; P, phosphorylation; Ub, Ubiquitin.