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Trends in Cancer

Review

Interplay between K-RAS and miRNAs

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K-RAS **is frequently mutated in cancers, and its overactivation can lead to oncogene-induced senescence (OIS), a barrier to cellular transformation. Feedback onto K-RAS limits its signaling to avoid senescence while achieving the appropriate level of activation that promotes proliferation and survival. Such regulation could be mediated by miRNAs, as aberrant RAS signaling and miRNA activity coexist in several cancers, with miRNAs acting both up- and downstream of K-RAS. Several miRNAs both regulate and are regulated by K-RAS, suggesting a noncoding RNA-based feedback mechanism. Functional interactions between K-RAS and the miRNA machinery have also begun to unfold. This review comprehensively surveys the state of knowledge connecting K-RAS to miRNA function and proposes a model for the regulation of K-RAS signaling by noncoding RNAs.**

Highlights

K-RAS is frequently mutated in human cancers. Its influence on downstream pathways is tightly regulated by feedback mechanisms that serve to circumvent oncogene-induced senescence (OIS).

miRNAs are noncoding RNAs that simultaneously repress mRNA translation of a diverse array of genes. Numerous miRNAs act up- and downstream of K-RAS, serving as mediators of K-RAS signaling to a wider range of targets.

Several miRNAs both directly target and are regulated by K-RAS, forming regulatory circuits such as positive feedback, negative feedback, and mutual suppression. miRNA-mediated feedback on K-RAS constitutes another layer of the complex regulatory network that governs appropriate K-RAS signaling.

Dysregulated K-RAS has a multifaceted effect on the activity of RNA-induced silencing complex (RISC) through modulation of AGO2 phosphorylation.

Principles of K-RAS signaling

K-RAS belongs to a family of membrane-associated monomeric GTPases that function as intracellular switches to control cellular responses to extracellular signals. In response to growth factor stimulation, K-RAS is activated through binding to GTP that is stimulated by guanine nucleotide exchange factors (GEFs). GTP-bound K-RAS assumes a structural conformation that allows for its interaction with effectors, triggering downstream signaling. Hydrolysis of GTP to GDP, potentiated by GTPase-activating proteins (GAPs), terminates signaling by returning K-RAS to its inactive conformation [1]. GEFs and GAPs, therefore, play a key role in regulating the activation state of K-RAS itself and the duration of signaling (Figure 1).

Oncogenic K-RAS mutations increase the steady-state level of K-RAS-GTP by promoting intrinsic nucleotide exchange and/or by decreasing GTP hydrolysis. This increase in K-RAS-GTP leads to enhanced downstream signaling through the RAF/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, and Ral guanine nucleotide dissociation stimulator (RalGDS) pathways, among others. Subsequently increased cell proliferation and cell survival jointly contribute to tumorigenesis [1]. Much attention has been paid to these downstream pathways as potential therapeutic targets for K-RAS-mutant cancers. Nevertheless, the preclinical efficacy of effector-based therapies has not translated into clinical efficacy, in part because of a lack of comprehensive understanding of the nuances of K-RAS signaling. Considerable work over the past decade has characterized various facets of miRNA-mediated regulation of K-RAS and the impact of dysregulated K-RAS on the miRNA landscape. In this review, we systematically explore the interplay between K-RAS signaling and miRNA, aiming to connect a key signaling regulator to the dominant post-transcriptional regulation machinery in the context of physiology and pathology.

Feedback mechanisms in K-RAS signaling

High levels of mitogenic signaling can be deleterious for cells. As a result, signaling pathways are tightly controlled by numerous regulatory mechanisms. For example, an intricate feedback network – emanating largely from ERK1/2 [2] – ensures that K-RAS/mitogen-activated protein kinase (MAPK) signaling oscillates in a range that supports normal cellular physiology. ERK

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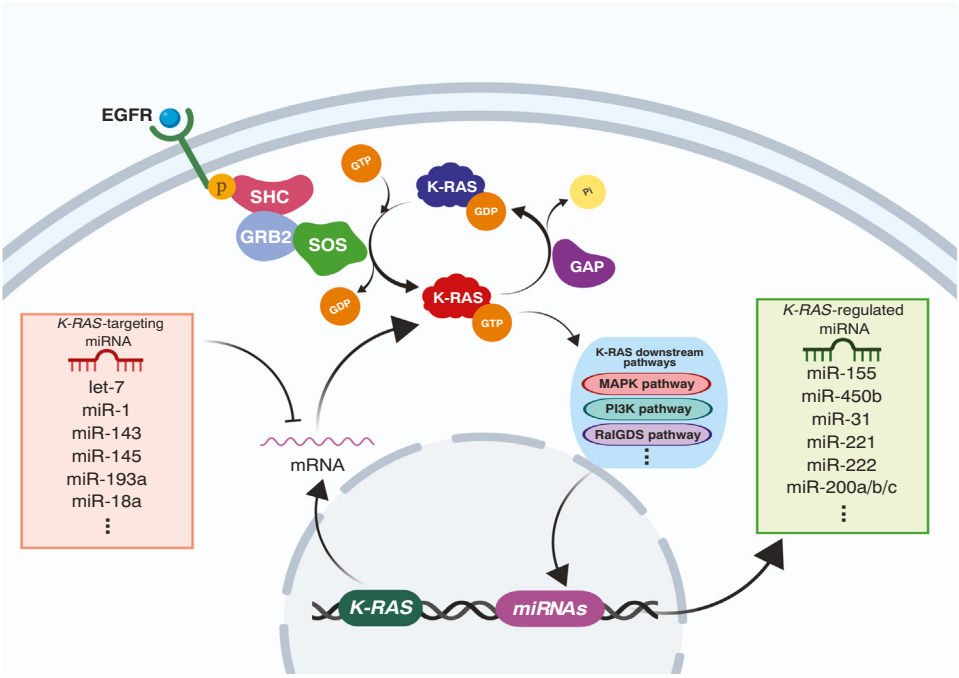

Figure 1. miRNAs up- and downstream of K-RAS signaling. K-RAS is a GTPase that is activated through guanine nucleotide exchange factor (GEF)-mediated binding to GTP. Its inactivation is through GTP hydrolysis facilitated by GTPase-activating proteins (GAPs). Proper functioning of GEFs, such as SOS, requires upstream signaling activation of EGFR and subsequent recruitment of SHC and GRB2 to the plasma membrane. An increase in the K-RAS-GTP level potentiates downstream signaling such as RAF/ERK, PI3K/AKT, and RalGDS pathways [1]. Various miRNAs act up- and downstream of the K-RAS signaling pathway. Several miRNAs have been identified to directly inhibit K-RAS translation, including *let-7, miR-143/145, miR-193a,* etc. [23–26]. Dysregulation of these miRNAs is frequently detected in cancers and potentially exhibits tumor-suppressive effects due to their targeted suppression of K-RAS. Indirect inhibition of K-RAS activity can also be achieved by miRNAs targeting key regulators such as GAPs. *miR-21*- and *miR-31*-mediated suppression of RASA1, a prominent GAP, enhances K-RAS activation by decreasing its hydrolysis potential [28–30]. Hyperactivation of K-RAS signaling also induces drastic changes in downstream miRNA levels, including upregulation of *miR-155, miR-450b, miR-31,* and *miR-21* [30,36,46,47]. These changes in the miRNA level and activity induced by K-RAS could contribute to shaping a protumorigenic environment or serve as a negative regulator that dampens the oncogenic signals. Abbreviations: EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; RalGDS, Ral guanine nucleotide dissociation stimulator; SHC, Src homology 2 domain-containing transforming protein; GRB2, growth factor receptor bound 2.

proteins efficiently limit their activation through negative feedback on upstream signaling factors via inhibitory phosphorylation of nearly all core members of the pathway: epidermal growth factor receptor (EGFR), son of sevenless homolog 1 (SOS1), RAF, and MEK1/2. For example, increased levels of K-RAS-GTP lead to ERK1/2 activation, which in turn phosphorylates Thr669 of EGFR. Thr669 phosphorylation downregulates EGFR activity by suppressing its C-terminal tyrosine phosphorylations (Tyr845/974/1045/1068/1173) that are required for EGFR activation in homo- and heterodimeric conformations [3]. Thus, activated ERK1/2 eventually dampens K-RAS signaling through suppression of its upstream regulator EGFR. ERK1/2 can also feedback onto SOS upstream of K-RAS. As a GEF, SOS1 promotes K-RAS activation by enhancing GDP-to-GTP exchange, which occurs at the plasma membrane through recruitment by growth factor receptor bound 2 (GRB2). ERK2-mediated phosphorylation of SOS1 on various sites (Ser1137/1167/1178/1193/1197) disrupts the interaction between SOS1 and GRB2, rendering SOS1 nonfunctional and unable to promote K-RAS activation [4,5]. In addition to upstream regulators of K-RAS, downstream effectors such as RAF and MEK are also targets of ERK1/2. RAF-

1 can be phosphorylated by ERK2 at Ser29/289/296/301/642. These phosphorylations prevent RAF-1 from engaging with activated K-RAS at the plasma membrane and result in dampening of MAPK pathway activation [6]. B-RAF can also be phosphorylated by ERK1/2 on Ser750, Thr753, Ser151, and Thr401 sites, which inhibit B-RAF function through an analogous mechanism [7,8]. Similar to RAF-1 and B-RAF, phosphorylation of Thr292 of MEK1 by ERK1/2 significantly reduces the kinase activity of the MEK1–MEK2 dimer by interfering with MEK2 binding to ERK1 [9].

In addition to phosphorylation-dependent feedback, ERK1/2 activation triggers the transcriptional upregulation of negative regulators of the K-RAS-MAPK signaling cascade such as dual-specificity phosphatases (DUSPs) and Sprouty proteins [2]. DUSPs, in particular, possess the unique capability of removing both threonine and tyrosine phosphorylations that are required for full ERK1/2 activation in the nucleus (DUSP1/2/4/5) and the cytoplasm (DUSP6/7/9) [2].

Feedback mechanisms that cells utilize to avoid aberrant activation of critical cellular signaling through transcriptional and post-translational mechanisms have been extensively elucidated. Yet, much less well understood is the role that miRNA-mediated post-transcriptional regulation plays in modulating K-RAS signaling under physiological and pathological conditions.

miRNAs in cancer

miRNAs are a class of small (~22 nt) noncoding RNAs that modulate gene expression [10]. Proper miRNA activity requires both functional biogenesis and gene suppression machinery. Its promiscuous targeting mechanism allows an individual miRNA to regulate a wide array of genes simultaneously (Box 1). Owing to their ability to fine-tune cellular signaling, miRNAs are frequently dysregulated in cancer. The connection between miRNA and cancer first became evident when *miR-15/16* was found to be frequently deleted in B cell chronic lymphocytic leukemia (CLL) [11]. Efforts in the following decade revealed important roles for the miRNA machinery and

Box 1. miRNA biogenesis and functions

During canonical miRNA biogenesis, miRNA genes are transcribed by RNA polymerase II, forming pri-miRNAs with stem-loop structures. In the nucleus, the microprocessor complex, composed of the RNase III Drosha and its RNA-binding partner DGCR8, cleaves the stem-loops off pri-miRNAs to form pre-miRNAs. Exportin 5, together with Ran-GTP, exports the pre-miRNA to the cytoplasm, wherein it undergoes further processing by the RNase III protein Dicer, generating a short double-stranded RNA (dsRNA) as the final product [10]. The dsRNA is then loaded onto one member of the Argonaute (AGO1–4) protein family, within which one RNA strand is degraded, while the other one remains as the functional mature miRNA. Serving as guides, AGO-bound miRNAs recruit a larger multiprotein complex known as the RISC to specific regions of their target mRNA transcripts, usually the 3′ UTR region, to halt translation and/or to induce mRNA degradation via recruitment of the decapping proteins and deadenylases [10].

A mammalian miRNA's targeting specificity is determined primarily by its 'seed sequence' (nucleotides 2–7 of the miRNA) [10]. Computational algorithms have been developed to identify putative mRNA targets for each miRNA. However, each miRNA is predicted to have several hundred targets due to its short seed sequence. Additionally, substantial discrepancies exist when comparing results from different algorithms (such as TargetScan, PicTar, PITA, miRanda) [36] and whether a specific miRNA binds to a specific target site is affected by a wide range of cell context-specific variables. Hence, experimental validation is critical when investigating downstream targets of specific miRNAs. In the past decade, several variations of nucleic acid/protein crosslinking immunoprecipitation (CLIP) followed by NGS [HITS-CLIP (high-throughput sequencing of RNA isolated by CLIP), eCLIP (enhanced CLIP), iCLIP (individual-nucleotide resolution CLIP), PAR-CLIP (photoactivatable-ribonucleoside-enhanced CLIP), CLASH (crosslinking, ligation, and sequencing of hybrids), HEAP (halo-enhanced Ago2 pull-down), etc.] have been developed to experimentally identify mRNA–miRNA interactions in a high-throughput manner [73–78]. These experimental approaches have offered great insights into the direct targets of miRNAs.

Due to their promiscuous targeting, miRNAs have been implicated in a diverse array of biological processes, spanning homeostatic physiology to disease pathogenesis. *lin-4* and *let-7*, the first two miRNAs identified in *Caenorhabditis elegans,* are both regulators of cellular differentiation and development [79–81]. Therefore, miRNAs were originally hypothesized to support terminal differentiation and suppress cell division. This notion was supported by the increase of miRNA expression during neutrophilic differentiation *in vitro* [12] and dysregulated differentiation in Dicer-deficient mouse embryonic stem cells with global miRNA KO [82].

individual miRNAs in the pathogenesis and development of multiple cancer types. miRNAs commonly dysregulated in cancers are often referred to as 'oncomiRs' or 'tumor suppressor miRNAs' based on whether their overall downstream effects facilitate or suppress tumor initiation and progression. However, due to the wide array of downstream targets for each miRNA, this categorization is rarely straightforward and some miRNAs can have both oncogenic and tumor-suppressive mechanisms, depending on the particular context.

With the development of microarray-based techniques and the identification of most miRNAs in the mammalian genome, comprehensive profiling of miRNA expression in tissues became possible. Early application of miRNA profiling in various cancers identified a general decrease of miRNA expression in tumor tissue compared with paired normal tissue, consistent with the notion that miRNA expression prevents cell division but promotes differentiation [12]. Since then, abnormal expression of miRNAs has been linked to the pathogenesis of different cancers and the oncogenic/tumor-suppressive effects of a few miRNAs have been validated *in vivo*. One example is the *miR-15/16* cluster, which is a putative tumor suppressor due to its frequent loss in B cell CLL. Genetic knockout (KO) of *miR-15/16* in mice induced B cell-autonomous, clonal lymphoproliferative disorders that recapitulated phenotypes of CLL patients. Several genes involved in cell cycle regulation, such as *CCNE, CCND2, CCND3, CDK4,* and *CDK6,* were identified as *miR-15/16* targets, and their perturbed expressions upon *miR-15/16* KO were associated with increased B cell proliferation [13]. Hence, *miR-15/16* is considered a tumor suppressor. *miR-155* and *miR-17~92* are considered oncomiRs, with enriched expressions in various cancers [14,15]. Overexpression of *miR-155* in murine B cells induced preleukemic pre-B cell proliferation with subsequent B cell malignancy, corroborating its oncogenic potential *in vivo* [16]. *miR-17~92* augmented Myc-driven B cell lymphoma development when overexpressed in the hematopoietic stem cells, while its forced expression in murine embryonic lung epithelium promoted proliferation and inhibited differentiation [14,17,18]. Furthermore, targeted deletion of *miR-17~92* hindered B cell development and induced lung hypoplasia in mice, suggesting that *miR-17~92* could play an essential role in both B cell/lung development as well as B cell lymphoma/lung cancer pathogenesis [19–22].

K-RAS regulation by miRNA

As a proto-oncogene that regulates several oncogenic pathways, K-RAS has always been considered a central signaling modulator. Thus, it is no surprise that much effort has been devoted to studying miRNAs that regulate K-RAS expression, with *let-7* being a prime example [23]. More recent efforts have used expression profile differences in tumor versus normal tissue to identify miRNA candidates that are likely to play a role in K-RAS regulation. For example, expression of *miR-143/145* negatively correlates with the K-RAS protein level in tissues, and forced expression of *miR-143/145 in vitro* significantly suppresses Ras signaling, decreasing proliferation and migration. These effects were shown to be mediated, at least in part, by direct binding of *miR-143/145* to the *K-RAS* 3′ untranslated region (UTR) [24,25]. Similarly, *miR-193a* was found to be upregulated in transformed cells relative to untransformed cells and to directly regulate *K-RAS*. Consistent with this model, ectopic expression of *miR-193a* decreased the K-Ras protein level and suppressed tumorigenicity [26]. Based on these results, *miR-193a* can be viewed as a tumor suppressor, providing a dampening effect on the activation of oncogenic pathways, although conclusive evidence for a true tumor suppressor role will require additional loss-of-function studies *in vivo*. Apart from dysregulated miRNA expression, SNPs in the *K-RAS* 3′ UTR also contribute to miRNA-mediated direct regulation of K-RAS via disruption of miRNA:*K-RAS* binding (Box 2).

miRNA-mediated regulation on *K-RAS* can also be indirect and multilayered. For example, *miR-18a* was shown to directly target *K-RAS,* but *miR-18a* biogenesis is itself controlled by

Box 2. SNPs affect miRNA binding to *K-RAS*

In addition to perturbations in the expression of *K-RAS*-targeting miRNA, SNPs in the *K-RAS* 3′ UTR also affect miRNA-mediated regulation of K-RAS activity. Since miRNA seed (nt 2–7) matching to the target site is necessary for miRNA-mediated gene suppression, it is reasonable that alterations, even SNPs, in miRNA-binding sites significantly influence miRNA functioning. rs61764370 was an SNP identified in the *let-7* target site in the 3′ UTR of *K-RAS* and decreased *let-7:K-Ras* binding. The presence of this SNP was significantly enriched in patients with NSCLC, with ~20% occurrence in patients versus ~5% in the whole population. Interestingly, the presence of this SNP strongly increased the risk for NSCLC in humans and resulted in upregulation of K-RAS *in vitro* [83].

Another SNP (rs712) was identified through sequencing of *the K-RAS* gene in oral squamous cell carcinoma (OSCC) samples. GT and TT genotypes of rs712 correlated with lower risk for OSCC [84]. rs712 showed predictive power for the risk of cancer in several other cases. However, TT genotype carriers, who had a lower risk for OSCC, showed an increased risk of gastric cancer and CRC, underlining potential context specificity of miRNA targeting and function [85,86]. Extensive sequencing of the *K-RAS* 3′ UTR in NSCLC cell lines found rs712 and rs9266 SNPs to be significantly enriched. Functionally, these SNPs potentially disrupted proper binding between *K-RAS* 3′ UTR and regulatory miRNAs such as *let-7* and *miR-181* [87].

an RNA-binding protein, hnRNPA1, which is in turn modulated by *miR-15a-5p* and *miR-35-3p* [27]. The level of active Ras (Ras-GTP) also depends on the balance between GTP hydrolysis and GDP-to-GTP exchange. Thus, miRNAs can exert indirect influences on K-RAS activity by regulating the levels of GAPs and GEFs. RASA1, a prominent Ras-GAP, serves as a convincing example, being targeted by both *miR-21* and *miR-31*. Forced expression of *miR-21* and *miR-31* *in vivo* enhanced tumor growth in xenografts and initiated murine lung adenocarcinomas in cooperation with K-Ras^{G12D}, respectively [28–30]. Furthermore, miRNAs can also indirectly influence K-RAS expression. Overexpression of *miR-222-3p* in several clear cell renal cell carcinoma (ccRCC) cell lines facilitates K-RAS expression and enhances proliferation, migration, and invasion through direct targeting of *SLC4A4*, an antagonist of K-RAS expression [31].

Given the promiscuous and context-specific nature of miRNA targeting, many interactions remain to be elucidated. The level of K-RAS protein and the activation of RAS signaling was shown to be regulated by miRNAs in many studies without a clear mechanistic link. For example, *miR-487b* expression has been reported to be significantly lower in liver metastatic lesions from primary colorectal cancer (CRC) patients compared with normal tissue, and *in vitro* studies using CRC cell lines indicated that *miR-487b* directly suppressed both K-RAS expression and activation of its downstream pathways. However, *K-RAS* was not identified as a direct target of *miR-487b*, and the exact mechanism of this interaction has not yet been demonstrated [32]. Another example is *miR-340*, which was shown to be downregulated in a panel of melanoma cell lines compared with normal human epidermal melanocytes. Its expression significantly suppressed the activation of the RAS/MEK/ERK axis, and computational prediction identified many targets of *miR-340* to be within the pathway [33]. Further experimental validation is required to illuminate the mechanism of this regulation.

miRNA-mediated negative feedback onto K-RAS

Feedback mechanisms regulating K-RAS and involving transcriptional and post-translational processes have been well documented. In principle, miRNAs can also contribute to the regulation of K-RAS activity through similar molecular circuits, including negative feedback loops, positive feedback loops, and mutual suppressions (Figure 2). *miR-181a* serves as an excellent example as the forced expression of oncogenic *K-RAS* results in upregulation of *miR-181a* [26]. The positive correlation between K-RAS activation and *miR-181a* expression was also reported in 3D cultures of CRC cells carrying oncogenic K-RAS compared with those carrying K-RAS^{WT} [34]. Intriguingly, *K-RAS* is itself a direct target of *miR-181a*, as the introduction of *miR-181a* *in vitro* decreases cellular proliferation, migration, and anchorage-independent growth [35]. These results

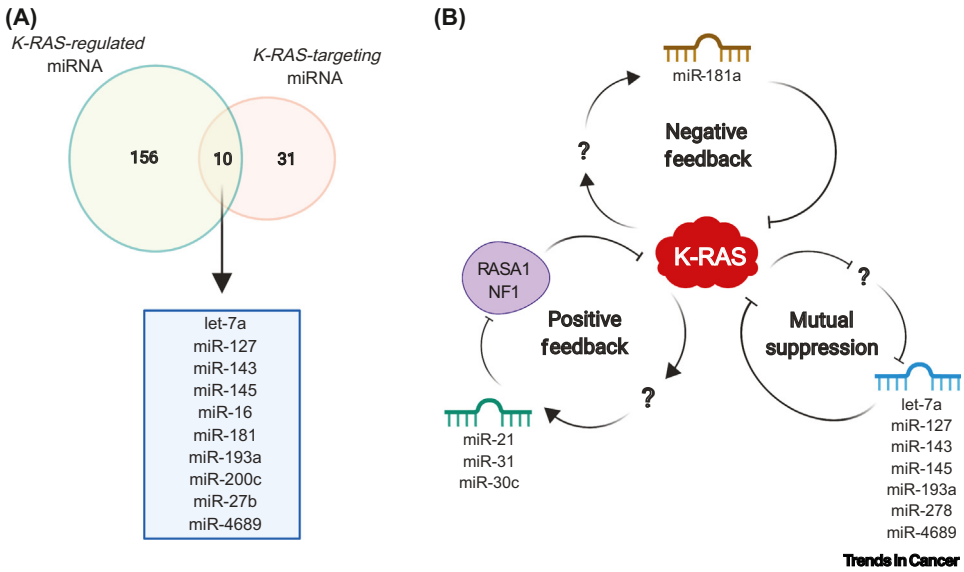

Figure 2. miRNA-mediated feedback regulation of K-RAS. (A) Extensive survey of all past literature yields 166 human miRNAs as downstream to K-RAS, as their expressions are reported to be dysregulated upon hyperactivation of K-RAS signaling at least once. A total of 39 miRNAs are classified as *K-RAS*-targeting miRNAs, with experimental evidence of direct miRNA targeting-induced K-RAS suppression in various cancer cell lines. Overlapping the two cohorts presents ten miRNAs that act both up- and downstream of K-RAS: *let-7a, miR-127, miR-143, miR-145, miR-16, miR-181, miR-193a, miR-200C, miR-27B,* and *miR-4689.* (B) Three distinct modes of feedback regulation emerge upon integration of K-RAS-induced effects on miRNAs with miRNA-mediated direct/indirect regulation of K-RAS. *miR-16* and *miR-200c* are not included here due to conflicting reports of their expression changes upon hyperactivation of K-RAS. Expression of K-RAS-targeting *miR-181a* is upregulated upon expression of oncogenic K-RAS, forming a negative feedback loop [26,34,35]. *miR-21, miR-31,* and *miR-30c* target RAS GAPs such as RASA1 and NF1 [28–30]. Their expressions are upregulated upon K-RAS hyperactivation [30,36], suggesting that these miRNAs can positively feedback onto K-RAS by suppressing its negative regulators to further enhance the signaling activation. Furthermore, several K-RAS-targeting miRNAs (*let-7a, miR-127, miR-143,* etc.) are downregulated by K-RAS, establishing a mutual suppressive relationship [26,37,38]. Activity of the miRNA and K-RAS signaling level likely form a delicate equilibrium during homeostasis, and its disruption in various pathologies could exacerbate the hyperactivation of oncogenic K-RAS. Abbreviation: GAP, GTPase-activating protein.

indicate the existence of a negative feedback loop involving K-RAS-induced *miR-181a* expression and *miR-181a*-mediated K-RAS suppression. Negative feedback mechanisms between K-RAS and miRNAs might have evolved as a cellular response to dysregulated signaling, aiming to dampen the surge of undesired cellular changes.

miRNA-mediated positive feedback onto K-RAS

Certain miRNAs regulated by K-RAS can suppress inhibitors of K-RAS to accomplish positive feedback. The previously discussed *RASA1*-targeting miRNAs, *miR-21* and *miR-31*, are both upregulated by activation of K-RAS signaling [30,36], achieving such regulation. Other miRNAs have been involved in similar feedback loops with K-RAS. For example, overexpression of K-RAS^{WT} and K-RAS^{G12D} in H1299 cells results in upregulation of *miR-30c*, which in turn represses negative regulators of K-RAS, such as RASA1 and neurofibromin 1 (NF1) [36].

Mutual suppression between K-RAS and miRNAs

In addition to using positive feedback loops to potentiate its activity, oncogenic K-RAS uses miRNAs to further accentuate the downstream effects. Previously, we discussed miRNAs that directly suppress K-RAS expression. Interestingly, several of these miRNAs can also be transcriptionally downregulated by K-RAS, thus forming mutually suppressive circuits with K-RAS. An example is provided by *miR-143/145*, the expression of which is inhibited upon K-Ras

hyperactivation in a Ras-responsive element-binding protein 1 (Rreb1)-dependent fashion and directly represses both K-Ras and Rreb1. The same regulatory mechanism has been established for several other miRNA suppressors of K-RAS expression, including *let-7a, miR-27b, miR-127, miR-193a,* and *miR-4686* [26,37,38]. Under physiological conditions, these mutually suppressive circuits between K-RAS and miRNAs form a delicate equilibrium that contributes to the homeostasis of cellular signaling. However, this balance is often disrupted in diseases with hyperactivated K-RAS. With one side of the seesaw significantly enhanced (K-RAS), the other side drops (miRNA). The decrease in expression of K-RAS-targeting miRNA further enhances K-RAS activation and potentially aids in the development of many K-RAS-related pathologies.

Uncertainties in the mutual interaction between miRNA and K-RAS

With the development of miRNA profiling techniques – from microarray to next-generation sequencing (NGS)-based methods – global miRNA expression has been analyzed across tissues under homeostatic conditions and in response to perturbations. However, a great deal of variability from these studies rendered a loose foundation for further investigations. Studies of miRNAs up- and downstream of K-RAS are no exception. For instance, changes in *miR-16* expression have been reported upon K-RAS perturbations, however with inconsistent results. In both 2D and 3D cultured CRC cells expressing oncogenic K-RAS, *miR-16* expression was significantly elevated relative to a K-RAS wild-type (WT) isogenic cell line. This change in *miR-16* diminished when examining miRNA expression in CRC patients without controlling for mutation background [34]. However, *miR-16* expression was reported to be significantly downregulated by overexpression of both WT and oncogenic K-RAS in immortalized human bronchial epithelial cells (NHBE-T) [38]. Similar controversial expression profiles induced by K-RAS activity were also demonstrated regarding *miR-200c*. Comparison between isogenic CRC cells with/without oncogenic K-RAS (DLD-1/DKO-4 and HCT116/HKe3) using qRT-PCR revealed K-RAS-induced *miR-200c* expression only when the cells were maintained in 3D cultures [34,39]. Yet, the opposite trend was discovered when overexpressing oncogenic K-RAS^{G12D} in both human lung fibroblasts (IMR90) and mammary gland epithelial cells (MCF10A) [40]. Another layer of complexity was added to the uncertainty of the interaction between K-RAS and *miR-16* or *miR-200c*, as both miRNAs were validated as direct regulators of K-RAS in other studies [41,42]. These inconsistencies complicate the identification of *bona fide* regulatory relationships.

K-RAS regulates miRNA expression

Early microarray studies uncovered specific miRNA signatures associated with K-RAS-mutant colorectal and lung cancers. These miRNA signatures could distinguish between *B-RAF*- and *K-RAS*-mutated CRCs and between lung cancers driven by *ALK* fusions, *EGFR* mutations, or *K-RAS* mutations [43–45]. These results suggested that the mutational status of *K-RAS* in tumor samples can have a significant impact on miRNA expression, despite being confounded by the noise from the sample's diverse genetic background. Due to their descriptive and correlative nature, however, these studies did not provide any mechanistic link between the presence of the *K-RAS*-mutant allele and the associated miRNA signature.

Changes in the expression of many miRNAs downstream of K-RAS contribute to sculpting a protumorigenic environment by regulating other cancer-related pathways (Figure 1). A good example is provided by *miR-155*, the expression of which is induced by K-RAS^{G12V} *in vitro* and directly represses the FOXO3a transcription factor. Reduced FOXO3a subsequent to *miR-155* upregulation was shown to cause a decrease in antioxidants such as superoxide dismutase 2 (SOD2) and catalase, contributing to increased reactive oxygen species (ROS) stress and promoting tumorigenicity [46]. Another approach for elucidating miRNAs downstream of K-RAS is through miRNA profiling in cancers with high *K-RAS* mutation burden, including CRC, non-

small cell lung cancer (NSCLC), and pancreatic ductal adenocarcinoma (PDAC). Using this approach, elevated levels of *miR-450b-5p* were discovered in microarray data of CRC tissues and shown to correlate with poor prognosis. *miR-450b-5p* directly targeted the ß-catenin regulators SFRP2 and SIAH1, enhancing WNT signaling, cell proliferation, and tumor growth. Further investigation *in vivo* suggested that *miR-450b-5p* expression was regulated by K-RAS/AP-1 signaling [47]. Thus, *miR-450b-5p* serves to bridge Ras and Wnt signaling, both of which are critical players in CRC pathogenesis. In addition, K-RAS can affect the levels of miRNAs by regulating their biogenesis. Hyperactivated K-RAS signaling was shown to regulate miRNA processing via an MYC-targeted long noncoding RNA (lncRNA), *KIMAT1*. Activated MAPK signaling downstream of K-RAS amplification in lung cancer upregulated *KIMAT1* expression, subsequently stabilizing miRNA processing components DHX9 and NPM1. This process was shown to promote the processing of oncogenic miRNAs and reduce tumor suppressor miRNAs, facilitating a positive feedback loop that further enhances *K-RAS* expression and contributes to the oncogenicity of hyperactivated K-RAS [48].

Though miRNA profiling studies have provided important insights into the roles of miRNAs in cancer, the aforementioned inconsistency of miRNA signatures from different studies of the same cancer significantly hinders the functional study of miRNAs in signaling. In a summary of miRNAs dysregulated in CRC, most miRNAs were only identified as differentially expressed once in the 23 studies compiled [49]. These discrepancies likely stem from differences in sample harvesting, miRNA extraction, profiling technique, and, most importantly, the variable genetic backgrounds of the samples. Additionally, the nature of the computational algorithms used to identify miRNA/mRNA regulatory networks might contribute to these interstudy differences. Several miRNA profiling studies constructed miRNA signaling networks by linking miRNA and mRNA expressions from tumor tissues using computationally predicted miRNA–target relationships [50,51]. Despite offering insights into central pathways regulated by miRNAs in cancer, these studies are confounded by the tissue context-dependent nature of miRNA targeting, which is commonly disregarded by computational algorithms [52]. Due to our lack of understanding of the principles governing tissue- and context-specific miRNA targeting, predictions from computational algorithms may yield many false positives/negatives when applied *in vivo* and in disease contexts. To further our knowledge regarding miRNA/mRNA targeting *in vivo,* future studies should use high-throughput experimental approaches such as CLIP-Seq under a defined genetic background to identify physiological miRNA targets and to offer insights into the rules regulating tissue- and context-specific functions of miRNA.

K-RAS regulates RNA-induced silencing complex activity

Until recently, it has been tacitly assumed that the RNA-induced silencing complex (RISC) is constitutively assembled and active in all cell types and conditions. Therefore, miRNAs have been thought to be universally active, and their activity depends solely on their expression levels. However, it is now becoming clear that RISC assembly, and consequentially the ability of miRNAs to repress gene expression, is a highly regulated process with extensive tissue specificity [52,53]. For example, in T cells, the activation of the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway led to more effective repression of a cognate target by the *miR-15/16* family without changing the expression of all members of this miRNA family [52,54]. Here, the enhancement of miRNA function can be attributed, at least in part, to PI3K/Akt/mTOR pathway-induced expression of Trinucleotide repeat-containing gene 6 (Tnrc6), a core component necessary for RISC assembly and target engagement. However, details on additional mechanisms that regulate RISC assembly/function have just begun to emerge. Recently, Bridge and colleagues identified LIM domain-containing protein 1 (LIMD1) as essential for securing the Argonaute 2 (AGO2)–TNRC6 interaction through AKT3-mediated mechanism [55]. Additionally, Golden *et al.* have shown that the antagonistic activities of the kinase Casein kinase I isoform alpha (CSNK1A1)

and the phosphatase serine/threonine-protein phosphatase 6 (ANKRD52-PPP6C) resulted in specific phosphorylations on AGO proteins, which dictated RISC engagements and repression of mRNAs [56]. Moreover, it has been shown that phosphorylation of AGO2 Y529 inhibits the loading of small RNA, while EGFR-dependent phosphorylation of AGO2 Tyr393 prevents processing of looped precursor RNAs into mature miRNAs [57]. Given that K-RAS regulates several major phosphorylation cascades, mounting evidence suggests that K-RAS also participates in the regulation of RISC function (Figure 3 and Box 3).

Direct interaction between K-RAS and AGO2

In addition to the complex regulatory network directly connecting K-RAS and miRNA expression, interactions between K-RAS and components of the miRISC have also been described. A recent report identified AGO2 as an interactor of K-RAS through coimmunoprecipitation of RAS [58]. The interaction between K-RAS and AGO2 was shown to be direct and mediated by the N terminus of AGO2 (49–139) and Y64 of the K-RAS switch 2 region. K-RAS–AGO2 complex colocalized to the endoplasmic reticulum. According to this study, expression of AGO2 appeared essential for the activation of K-RAS downstream signaling and K-RAS-mediated cell proliferation and transformation in mutant K-RAS-dependent cells. Additionally, mutant K-RAS was proposed to inhibit the unwinding of miRNA duplex and mature miRNA biogenesis through its interaction with AGO2. Further studies

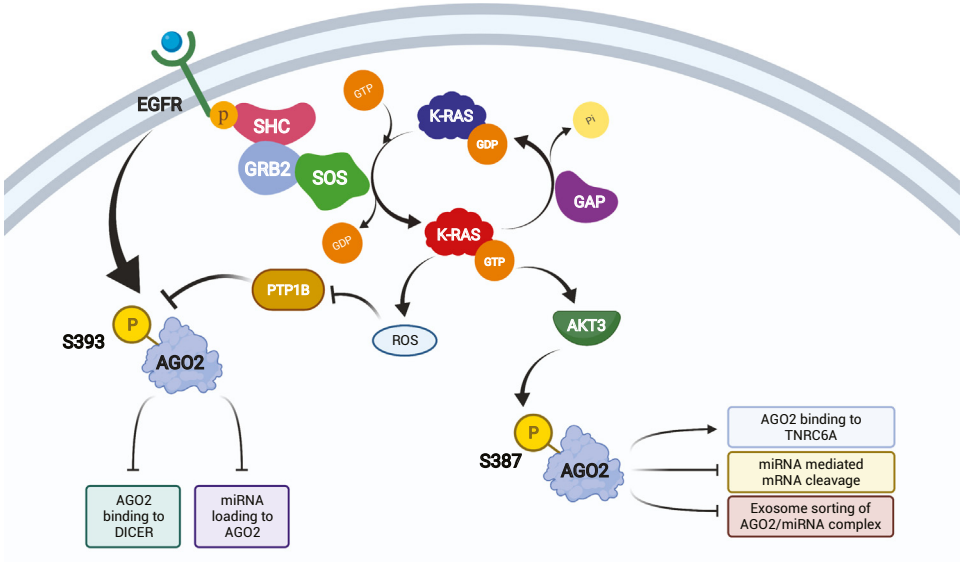

Figure 3. AGO2 phosphorylations by the K-RAS pathway. Proper phosphorylations of AGO2 are critical for its function. Several of these sites are regulated by members of the RAS pathway. AGO2 Ser387 phosphorylation is critical for its association with TNRC6 to form the RNA-induced silencing complex (RISC) at the processing body (P-body). Additionally, Ser387 phosphorylation inhibits both miRNA-mediated AGO2 cleavage of target mRNA transcripts and exosome sorting of AGO2–miRNA complexes. This critical phospho-site is regulated by AKT3, a kinase in the PI3K/AKT pathway downstream of K-RAS [88–90]. Therefore, hyperactivation of K-RAS could elevate AGO2 Ser387 phosphorylation to regulate its function. AGO2 Tyr393 represents another functional phospho-site that regulates AGO2: Dicer binding and miRNA loading to AGO2, both of which are required for proper AGO2 function. Its phosphorylation is regulated by EGFR, an upstream activator of K-RAS signaling that is susceptible to MAPK-induced negative feedback [3,57]. Thus, it is reasonable that the negative feedback suppression of EGFR triggered by oncogenic K-RAS signaling would undermine phosphorylation of AGO2 Tyr393, facilitating its function by enhancing AGO2 interaction with Dicer and miRNA. Additionally, PTP1B, the phosphatase governing AGO2 Tyr393, is also regulated by K-RAS. An increase of K-RAS signaling upregulates reactive oxygen species (ROS). This in turn inhibits PTP1B function and upregulates AGO2 Tyr393 phosphorylation [92], suggesting a multifaceted role of K-RAS in regulating AGO2 Tyr393. Abbreviations: EGFR, epidermal growth factor receptor; GAP, GTPase-activating protein; GRB2, growth factor receptor bound 2; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PTP1B, protein tyrosine phosphatase 1B.

Box 3. Phosphorylations of the miRNA machinery by the K-RAS pathway

Much of the impact from K-RAS hyperactivation stems from changes in downstream kinase and phosphatase activity (e.g., MAPK and PI3K–AKT pathways), and a flurry of recent studies have identified AGO2 as a substrate for several of these kinases and phosphatases (see Figure 3 in main text). Phosphorylation of Ser387 on AGO2 was shown to facilitate localization of AGO2 to processing bodies (P-bodies) for its activity. MAPK signaling was determined to be necessary for Ser387 phosphorylation. Yet, this process was independent of canonical MAPK pathway kinases JNK and MEK [88]. A subsequent study demonstrated that phosphorylation at Ser387 is directly mediated by AKT3. Phospho-Ser387 increased AGO2-dependent translational repression and its interaction with TNRC6A in the P-body. Interestingly, despite potentiating AGO2-mediated repression of gene expression, Ser387 phosphorylation reduced miRNA-targeted mRNA cleavage [89]. Furthermore, K-RAS activation-induced elevation of Ago2 Ser387 phosphorylation inhibited sorting of the AGO2–miRNA complex to exosomes [90]. In addition to affecting exosome sorting directly, hyperactivated K-RAS signaling also resulted in altered miRNA expression in exosomes, as certain miRNAs, such as *miR-100*, were significantly enriched in exosomes from cells carrying mutant K-RAS [91].

Besides Ser387, additional sites of phosphorylation were later identified, including Tyr393 and Tyr529 [57]. The phosphorylation of Tyr393 was also tightly regulated by factors up-/downstream of the RAS signaling cascade. EGFR, an upstream regulation of RAS, was identified as the kinase targeting the Tyr393 site of AGO2. In response to hypoxic stress, EGFR suppressed the maturation of tumor suppressor-like miRNAs by increasing the phospho-Tyr393 level and inhibiting AGO2 binding to Dicer. Together, the phosphorylation of Tyr393 of AGO2 by EGFR altered gene suppression in tumor cells undergoing hypoxia [57]. Dephosphorylation of the Tyr393 site was regulated by phosphatase protein tyrosine phosphatase 1B (PTP1B). H-RAS^{G12V}-induced ROS production inhibited PTP1B, which led to the accumulation of phospho-AGO^{Tyr393}. Corroborating previous studies, phosphorylation of the Tyr393 site inhibited miRNA-mediated gene silencing through disrupting proper miRNA loading into AGO2, ultimately contributing to H-RAS^{G12V}-induced senescence in primary cells [92].

demonstrated that Ago2 was essential for the development and progression of mouse models of K-Ras-driven pancreatic cancer and NSCLC [59,60]. However, the exact regulatory relationship between K-RAS and AGO2 remains unclear as both activation and suppression of K-RAS signaling have been reported upon knockdown/KO of AGO2.

miRNAs in K-RAS-driven cancer initiation and progression

Hyperactivation of K-RAS is a common initiating event in cancers of the pancreas, colon, lung, and hematopoietic system, as its gain-of-function mutations alone trigger dysplastic changes in these tissues [61–64]. Given the intricate network of miRNAs downstream of K-RAS, various aspects of the tumor initiation are likely regulated by miRNAs. *miR-34* is frequently downregulated in K-RAS-driven lung cancer and was postulated to function as a tumor suppressor. Exogenous delivery of *miR-34* via lentivirus before induction of K-Ras^{G12D} expression in mouse lung ameliorated tumor initiation, underlining the necessity of *miR-34* suppression during tumorigenesis [65]. Additionally, the deletion of *Dicer* from K-Ras-driven pancreatic cancers in mice accelerated the dedifferentiation of dysplastic cells while promoting apoptosis during tumor initiation, highlighting the complexity of phenotypes regulated by miRNAs [66]. However, functional impacts of the dysregulation of individual miRNAs demand detailed dissection in the context of tumor initiation utilizing animal models harboring an inducible deletion of specific miRNAs. Furthermore, tissue-specific contexts may also influence the K-RAS:miRNA dynamics during cancer progression as *Dicer* loss in mice does not accelerate overall pancreatic cancer development, while it promotes the cellular transformation and enhanced tumor burden in K-RAS-driven lung tumor [66,67].

K-RAS also plays a significant role in tumor progression, with its hyperactivation facilitating adenoma transformation to adenocarcinoma in CRC and other malignancies. Distinct tumor stages also exhibited unique miRNA profiles, with dysregulation of certain miRNAs sculpting stage-specific cellular environments that could promote or inhibit disease development [68]. Certain miRNAs, such as *miR-21* and *miR-135a*, are dysregulated in CRC in general, with the extent of their upregulation positively correlated with disease stages. More advanced diseases demonstrate a higher increase in their levels, suggesting that these miRNAs might contribute to the

progression of the disease [69]. Metastasis, a crucial step in the disease course, is associated with the dysregulation of certain miRNAs that contribute to cancers' metastatic potential. For example, suppression of the *miR-200* family during mouse pancreatic tumor metastasis derepresses Zeb1, which subsequently antagonizes E-cadherin and promotes epithelial–mesenchymal transition [68]. Many other miRNAs demonstrated similar capacity in regulating metastasis through direct targeting of K-RAS [70,71].

Given their layered roles in cancer initiation, progression, and metastasis, many miRNAs up- and downstream of K-RAS have been associated with various disease stages, prognosis, and therapeutic responses. In lung adenocarcinoma, an miRNA signature derived from *miR-769-5p* and *let-7d-5p* predicted patient survival, with higher expression of these miRNAs correlated with poor prognosis [45]. Furthermore, suppression of *miR-143,* a K-RAS-targeting miRNA, predicted both poor survival and lack of therapeutic response to EGFR inhibition in colon cancers expressing WT K-RAS [72]. Besides miRNA levels, SNPs can interfere with miRNA–target interactions and therefore contribute to patient prognosis (Box 2). Overall, epidemiological studies correlating miRNA profiles with disease status could help guide clinical decisions on diagnosis and treatment. Nevertheless, understanding the functional mechanisms underlying such correlations is critical for establishing validity. Therefore, rigorous experimental efforts are needed to fully uncover the interplay between miRNAs and the development of K-RAS-driven malignancies.

Concluding remarks

K-RAS is a master regulator of cell signaling, profoundly impacting cellular functions through a complex regulatory network rather than through a linear axis. After decades of investigation, there are still aspects of the K-RAS signaling cascade that remain elusive, however. The discovery of miRNA as a new class of translational regulators unveiled a new aspect of K-RAS signaling. miRNA-mediated feedback regulation on K-RAS, for example, represents an additional layer of signaling control that cells could use to fine-tune signaling for optimal output (Figure 2). Nevertheless, tissue and context-specific investigations of physiological miRNA activities are needed to fully extract the therapeutic potential of these interactions (see Outstanding questions).

Outstanding questions

While targeting *K-RAS,* miRNAs such as *let-7, miR-143, miR-145,* and *miR-193a* likely also suppress a diverse cohort of gene targets, not all of which are likely to exert similar effects on cell behavior. What is the overall physiological impact of the dysregulation of these miRNAs? Is the suppression of *K-RAS* their dominant signaling output?

miRNAs generally exhibit a modest impact on an individual gene's expression. In physiological conditions, are miRNA-mediated effects downstream of K-RAS governed through a linear axis of miRNA–target gene, or rather through an aggregated network of target genes emanating from the miRNAs?

Profiling by NGS presents a snapshot of the miRNA landscape upon K-RAS hyperactivation, yet lacks a mechanism that bridges oncogenic K-RAS with miRNA changes. What transcriptional programs governing miRNA levels are regulated by K-RAS signaling?

Investigations of functional miRNA targets have depended largely on computational predictions. How does K-RAS signaling influence the physiological miRNA target profile?

How does the context of specific tissues, physiological conditions, and pathologies affect the miRNA levels up- and downstream of K-RAS, the functional miRNA target repertoire, and the regulation of RISC status by K-RAS?

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Declaration of interests

The authors declare no competing interests.

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