A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression

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As bona fide p53 transcriptional targets, miR-34 microRNAs (miRNAs) exhibit frequent alterations in many human tumor types and elicit multiple p53 downstream effects upon overexpression. Unexpectedly, miR-34 deletion alone fails to impair multiple miR-34-mediated tumor suppressor effects in mice, possibly due to the considerable redundancy in the p53 pathway. Here, we demonstrate that miR-34a represses HDM4, a potent negative regulator of p53, creating a positive feedback loop acting on p53. In a Kras-induced mouse lung cancer model, miR-34a deficiency alone does not exhibit a strong oncogenic effect. However, miR-34a deficiency strongly promotes tumorigenesis when p53 is haploinsufficient, suggesting that the defective p53–miR-34 feedback loop can enhance oncogenesis in a specific context. The importance of the p53/miR-34/HDM4 feedback loop is further confirmed by an inverse correlation between miR-34 and full-length HDM4 in human lung adenocarcinomas. In addition, human lung adenocarcinomas generate an elevated level of a short HDM4 isoform through alternative polyadenylation. This short HDM4 isoform lacks miR-34-binding sites in the 3′ untranslated region (UTR), thereby evading miR-34 regulation to disable the p53-miR-34 positive feedback. Taken together, our results elucidated the intricate cross-talk between p53 and miR-34 miRNAs and revealed an important tumor suppressor effect generated by this positive feedback loop.

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p53, the “guardian of the genome,” functions at the center of a complex molecular network to mediate tumor suppression [Riley et al. 2008; Vousden and Prives 2009; Lane and Levine 2010]. In response to various oncogene and cellular stresses, p53 activates or represses a large number of downstream targets in a context- and stimulus-dependent manner, promoting multiple cellular processes to collectively repress tumor formation [Riley et al. 2008]. In addition to the numerous protein-coding p53 targets, noncoding RNAs—in particular, microRNAs (miRNAs)—are increasingly recognized as essential components of the p53 pathway to mediate post-transcriptional gene repression downstream from p53 [Hermeking 2007; Lin et al. 2012].

miRNAs are a class of small, regulatory noncoding RNAs that mediate post-transcriptional gene silencing of specific mRNAs [Ambros 2004; Zamore and Haley 2005; Filipowicz et al. 2008; Bartel 2009]. Among the best-studied p53-regulated miRNAs are miR-34 miRNAs, including miR-34a, miR-34b, and miR-34c, which are encoded at two distinct loci: mir-34a and mir-34b/34c [Chang et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Tarasov et al. 2007]. Acute miR-34 overexpression elicits various p53 downstream effects in a context-dependent manner, including cell cycle arrest, apoptosis, senescence, and suppression of the epithelial–mesenchymal transition (EMT) [Chang et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Kim et al. 2011]. Such miR-34 effects are mediated at least in part by post-transcriptional repression of targets, including cyclin D1, cyclin E2, Cdk4,
c-Met, c-Myc, Bcl2, Snail1, and Sirt1, which collectively promote multiple oncogenic processes [Hermeking 2009]. More importantly, acute miR-34 expression also leads to tumor repression in vivo in both a genetically engineered lung adenocarcinoma model and multiple xenograft models [Wiggins et al. 2010; Kasinski and Slack 2012]. Consistently, a low-level miR-34 expression has been detected in a variety of human cancer types due to aberrant transcriptional regulation, genomic deletions, and promoter hypermethylation [Kaghad et al. 1997; Lodygin et al. 2008; Hermeking 2009; Wang et al. 2011b; Tanaka et al. 2012]. These observations imply the functional significance of miR-34 deficiency in tumorigenesis and suggest that miR-34 miRNAs could act as important components of the tumor suppressor network.

Unexpectedly, the initial phenotypic characterization of miR-34 knockout mice has yielded a confusing picture. miR-34-deficient mouse embryonic fibroblasts [MEFs] exhibited no defects in p53-dependent cell cycle arrest, apoptosis, or replicative senescence [Choi et al. 2011; Concepcion et al. 2012]. In addition, the miR-34a−/−; mir-34b/34c−/− mouse showed no increase in p53-dependent spontaneous tumorigenesis, irradiation-induced lymphomagenesis, or oncogenic cooperation in the Eμ-myc B-cell lymphoma model [Concepcion et al. 2012]. In these contexts, it is conceivable that the redundant pathways downstream from p53 could compensate for miR-34 loss in vivo. Nevertheless, deficiency of miR-34a or mir-34b/34c partially phenocopies p53 deficiency in promoting somatic reprogramming [Choi et al. 2011], implicating that miR-34 miRNAs can mediate the p53 downstream effects in a context-dependent manner [Jain and Barton 2012]. Due to the redundant nature of the p53 pathway, we hypothesize that the function of the p53–miR-34 axis in tumor suppression could be best revealed in systems where the robustness of the p53 response is compromised.

In this study, we demonstrated a positive feedback loop between p53 and miR-34 that is at least in part due to the miR-34a-mediated repression of a negative p53 regulator, Mdm4. Using a Kras-induced mouse lung adenocarcinoma model, we demonstrated the functional importance of this p53–miR-34a positive feedback in tumor suppression. Given the redundancy of the p53 network, the p53–miR-34 positive feedback confers an important tumor suppressor effect that is only evident when p53 is haploinsufficient in vivo. Consistently, human lung adenocarcinomas, when compared with normal lung tissues, not only exhibit an inverse correlation between miR-34 and full-length HDM4 but also have an elevated level of a shorter HDM4 isoform that evades miR-34-dependent regulation. Altogether, our studies elucidate an intricate cross-talk between p53 and miR-34 miRNAs and reveal the oncogenic consequences when this feedback loop is disrupted in vivo.

Results

miR-34a deficiency promotes KrasG12D-induced lung adenocarcinomas when p53 is haploinsufficient

Emerging evidence implicates the p53-regulated miR-34 miRNAs as integral components of the tumor suppressor pathway in the development of lung adenocarcinomas [Wiggins et al. 2010; Kasinski and Slack 2012]. miR-34 loci were frequently hypermethylated and down-regulated in human non-small-cell lung cancers (NSCLCs) [Lodygin et al. 2008; Wang et al. 2011b; Tanaka et al. 2012]. Similarly, the expression of miR-34 was readily detected in both neonatal and adult mouse lungs but was significantly decreased at the terminal lung adenocarcinomas induced by activating Kras mutation [KrasG12D] and p53 deletion [Fig. 1A,B, Supplemental Fig. S1A,B]. Consistently, enforced exogenous miR-34 expression prevented the initiation and progression of lung cancer development in mice in both a xenograft model and a genetically engineered lung adenocarcinoma model induced by KrasG12D and a dominant-negative p53R172H mutation [Wiggins et al. 2010; Kasinski and Slack 2012]. These findings led us to hypothesize that miR-34 miRNAs, as integral components of the p53 pathway, could play an important role in suppressing lung cancer development.

Activating mutations in Kras, which occur in ~30% of human NSCLCs, act as a strong initiation lesion to promote the development of lung adenomas and adenocarcinomas in mice [Slebos et al. 1990; Rodenhuis and Slebos 1992]. A genetically engineered KrasLSL-G12D mouse strain has been widely employed to study the development of lung cancer [Jackson et al. 2001; Winslow et al. 2011]. In this mouse model, the expression of the oncogenic KrasG12D allele from its endogenous promoter can be specifically induced in lung by Adeno-Cre (AdCre) virus-mediated removal of a transcription termination element flanked with LoxP sites [designated as LSL cassette] [Jackson et al. 2001].

Aberrant Ras signaling, triggered by either Hras overexpression or the activating KrasG12D mutation, induces miR-34 expression in a p53-dependent manner [Supplemental Fig. S1C; He et al. 2007]. Using the KrasLSL-G12D lung cancer model, we investigated the tumor suppressor functions of miR-34a in vivo. The loss of miR-34a alone exhibited no significant oncogenic cooperation with KrasG12D in the lung [Fig. 1C; Supplemental Fig. S1D]. This finding is similar to a number of canonical p53 targets whose knockout in mice fail to promote tumorigenesis in vivo to the extent of p53 deficiency [Martin-Caballero et al. 2001; Choudhury et al. 2007; Michalak et al. 2008]. These findings imply the complex nature of functional redundancy downstream from p53 and suggest that the in vivo effects of miR-34a alone might be more apparent in a sensitized genetic background where this p53 redundancy is compromised.

Using this rationale, we disrupted the integrity of the p53 pathway in the KrasLSL-G12D model by introducing a deletion of one p53 allele. Although p53 acts as a haploinsufficient tumor suppressor in many cell types, p53 hemizygosity in KrasLSL-G12D mice did not significantly promote lung adenocarcinoma development [Supplemental Fig. S1E]. The resulting KrasLSL-G12D/+; p53−/− mice phenocopied the KrasLSL-G12D/+; p53−/− mice in tumor latency, tumor size, and tumor grade, indicating that p53 hemizygosity did not preclude the p53 tumor suppressor effects in response to Kras-induced tumorigenesis. Con-
sistently, we did not observe any p53 loss of heterozygosity (LOH) in high-grade lung tumors derived from the Kras\textsuperscript{LSL-G12D}; p53\textsuperscript{−/−} mice (Supplemental Fig. S1F). These findings suggest that in the Kras\textsuperscript{G12D}-driven lung adenocarcinoma model, one allele of wild-type p53 is sufficient to sustain p53-dependent tumor suppressor response.

Figure 1. miR-34a deficiency and p53 haploinsufficiency significantly promote Kras\textsuperscript{G12D}-induced oncogenesis in lung adenocarcinomas. (A) miR-34a expression levels were measured by real-time PCR analyses in various organs of neonatal (postnatal day 15 [P15]; top) and adult (5 wk, bottom) mice. (B) miR-34a expression was readily detected in normal adult lungs but significantly reduced in Kras\textsuperscript{G12D}; p53\textsuperscript{−/−} lung adenocarcinomas. (C) The representative lung images are shown from Kras\textsuperscript{LSL-G12D+/+} and Kras\textsuperscript{LSL-G12D−/−}; mir-34a\textsuperscript{−/−} mice 22 wk after AdCre infection [top] and Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−} and Kras\textsuperscript{LSL-G12D−/−}; p53\textsuperscript{−/−}; mir-34a\textsuperscript{−/−} mice 19 wk after AdCre infection [bottom]. White arrowheads indicate the lung adenomas or adenocarcinomas on the lung surface. (D) Hematoxylin and eosin (H&E) staining of lung sections indicated a significant increase in tumor burden of Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−}; mir-34a\textsuperscript{−/−} mice when compared with Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−} control mice. (E) Quantification of 779 Kras\textsuperscript{G12D+/+}; p53\textsuperscript{−/−} and 684 Kras\textsuperscript{G12D−/−}; p53\textsuperscript{−/−}; mir-34a\textsuperscript{−/−} lung tumors demonstrated that a significant increase in tumor size was caused by miR-34a deficiency. (F) A significant increase in tumor burden was observed in Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−}; mir-34a\textsuperscript{−/−} mice when compared with Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−} mice. The tumor burden was measured as the percentage of the tumor area versus the total lung area in six pairs of animals. (G) The tumor numbers in Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−} and Kras\textsuperscript{LSL-G12D−/−}; p53\textsuperscript{−/−}; mir-34a\textsuperscript{−/−} mice were similar (n = 6). (H) The percentage of tumor area versus the total lung area was measured for low-grade (grades 1 and 2) and high-grade (grades 3–5) tumors in Kras\textsuperscript{LSL-G12D+/+}; p53\textsuperscript{−/−} and Kras\textsuperscript{LSL-G12D−/−}; p53\textsuperscript{−/−}; mir-34a\textsuperscript{−/−} mice (n = 7). While the tumor burden for low-grade tumors was similar for both genotypes, the tumor burden for high-grade tumors was significantly increased as a result of miR-34a deficiency. All analyses described in D–H were performed using mice at 19 wk after AdCre infection. All error bars represent SEM; (n.s.) not significant; (*) P < 0.05; (**) P < 0.01; (***) P < 0.001.
Despite the similarities between Kras<sup>LSL-G12D</sup>/Kras<sup>LSL-G12D</sup>/p53<sup>+/+</sup> and Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> mice and MEFs [Supplemental Fig. S1E,G], complete p53 deficiency significantly promotes Kras<sup>G12D</sup>-driven lung tumors [Jackson et al. 2005], suggesting that a strong oncogenic effect could occur in this model when the p53 activity is below a critical threshold. Thus, compared with Kras<sup>G12D</sup>/+ mice that have intact p53 activity, Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> animals are likely more susceptible to malignant transformation upon further disruption of the p53 pathway, such as deficiency of miR-34 miRNAs.

Interestingly, miR-34a deficiency strongly promoted the development of Kras<sup>G12D</sup>-induced lung adenocarcinoma in combination with p53 hemizygosity, giving rise to mice with considerable respiratory difficulty or respiratory failure at 19 wk after AdCre exposure [Fig. 1C,D]. At 19 wk, the lungs of Kras<sup>LSL-G12D</sup>/p53<sup>+/+</sup> control mice had a cobblestone appearance, with multiple small lesions on the lung surface, the lungs of Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> mice displayed multiple large tumors on the lung surface (Fig. 1C). Further histological analyses comparing Kras<sup>LSL-G12D</sup>/p53<sup>+/+</sup> miR-34a<sup>−/−</sup> and control Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> mice revealed increased tumor area and tumor size [Fig. 1D–F], yet the overall tumor numbers remained the same (Fig. 1G). Interestingly, Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice exhibited enhanced tumor area and increased tumor number of high-grade tumors but had little effect on the tumor area or number of low-grade tumors in the lung (Fig. 1H; Supplemental Fig. S1H). The high-grade tumors identified in Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice were often characterized by their large tumor size, large and pleomorphic nuclei, and high degree of stromal desmoplasia [Supplemental Fig. S1I]. These findings suggest that when the robustness of the p53 response is compromised, miR-34a deficiency leads to a significant acceleration in tumor progression of Kras<sup>G12D</sup>-driven lung adenocarcinomas.

We carried out a detailed histological analysis to determine the cell type of transformed cells in Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice. The adenomas and adenocarcinomas developed in Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> and Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice were largely positive for surfactant apoprotein-C (SP-C), a marker for alveolar type II pneumocytes, and negative for CC10, a marker for Clara cells [Fig. 2A]. Thus, these tumors arose from alveolar type II pneumocytes or their precursors. Since acute miR-34a overexpression reduced cell proliferation in the Kras<sup>G12D</sup>/p53<sup>R172H</sup> lung tumors [Kasinski and Slack 2012], we examined the effect of miR-34a deficiency on cell proliferation. Lung tumors in the Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice harbored increased numbers of Ki67-positive cells compared with those from the Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> mice [Fig. 2B]. This observation was consistent with a moderately increased cell proliferation seen in miR-34a-deficient MEFs after serial passages [Lal et al. 2011; Concepcion et al. 2012; data not shown]. This enhanced cell proliferative phenotype associated with miR-34a deficiency likely contributed to the increased tumor progression for the high-grade tumors.

High-grade lung adenocarcinomas are often associated with enhanced MAPK signaling [Feldser et al. 2010]. Compared with the control Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> mice, the Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice developed more tumors that exhibited positive staining for phospho-Erk [p-Erk] (Fig. 2C). Previous studies suggested that the enhanced p-Erk signaling in high-grade Kras<sup>G12D</sup>-induced tumors invariably enhanced Arf expression, thus triggering potent p53-dependent tumor suppression to repress tumor progression [Feldser et al. 2010; Junttila et al. 2010]. This enhanced p53 signaling in high-grade Kras<sup>G12D</sup>-driven lung adenocarcinomas could, at least in part, counteract the enhanced MAPK signaling, thus delaying tumor progression [Feldser et al. 2010; Junttila et al. 2010]. Since miR-34a is an integral component of the p53 pathway, the specific increase of high-grade tumors in Kras<sup>LSL-G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> mice may reflect a compromised p53 activity in response to the aberrant MAPK signaling. Consistent with this hypothesis, Kras<sup>G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> lungs tumors, when compared with Kras<sup>G12D</sup>/p53<sup>−/−</sup> controls, exhibited a significant decrease in the induction of canonical p53 targets, such as p21 (Fig. 2D,E). Not only was the percentage of tumors with high p21 expression significantly decreased, the average percentage of p21-positive cells within each tumor was also decreased (Fig. 2D,E). Similarly, we also observed a decrease in p53 staining in Kras<sup>G12D</sup>/p53<sup>−/−</sup> miR-34a<sup>−/−</sup> high-grade lung tumors, but not in low-grade ones, compared with Kras<sup>G12D</sup>/p53<sup>−/−</sup> controls [Supplemental Fig. S1J]. Taken together, in the context of p53 hemizygosity, the loss of miR-34a yielded a compromised p53 response that failed to effectively antagonize the aberrant MAPK signaling in high-grade tumors, thus accelerating the tumor progression specifically in the high-grade lung tumors.

p53 and miR-34 miRNAs form a positive feedback loop

As bona fide p53 targets, miR-34 miRNAs also form a positive feedback loop with p53, which in turn regulates the degree of p53 activation [Yamakuchi et al. 2008; Hermeking 2009]. In both tumor cells and primary MEFs, the extent of cell cycle arrest induced by miR-34 miRNAs was heavily dependent on the p53 status. Enforced expression of miR-34 miRNAs significantly induced the G1 arrest in HCT116 cells harboring wild-type p53; however, this anti-proliferative effect of miR-34 was greatly dampened in p53<sup>−/−</sup> HCT116 cells [Fig. 3A]. Similarly, overexpression of pri-miR-34a and pri-miR-34b/34c in wild-type MEFs induced a robust G1 arrest and a strong decrease in the S-phase population [Fig. 3B], yet only a small effect was observed when p53<sup>−/−</sup> MEFs were subjected to the same treatment [Fig. 3B]. Notably, miR-34 overexpression can still induce a mild G1 arrest in the p53-deficient cells [Fig. 3A,B], presumably due to the inhibition of cell cycle regulators such as CCND1, CDK4, and CDK6 [Supplemental Fig. S2C]. Since p53 is crucial for the cell cycle arrest effect induced by miR-34, we examined the effects of miR-34 on p53 activity. Forced expression of miR-34 miRNAs increased the accumula-
Figure 2. Histopathological analysis of lung tumors of KrasLSL-G12D/+; p53+/-; mir-34a−/− mice. (A) The lung adenomas and adenocarcinomas from the KrasLSL-G12D/+; p53+/- and KrasLSL-G12D/+; p53+/-; mir-34a−/− mice were derived from the alveolar type II pneumocytes. Lung sections from both genotypes were stained with SP-C (a marker for alveolar type II cells; panels i–iv), CC10 (a marker for Clara cells; panels v and vi), and H&E (panels vii–x). Bars: 5× images, panels i, ii, vii, viii, 400 μm; 20× images, panels iii, iv, v, vi, 100 μm; 40× images, panels ix, x, 50 μm. (B) KrasG12D/+; p53+/−; mir-34a−/− lung tumors contained a greater percentage of proliferating cells compared with KrasG12D/+; p53+/− tumors. Representative images [left] and quantitation [right] of Ki67 staining are shown for KrasG12D/+; p53+/− (n = 18) and KrasG12D/+; p53+/−; mir-34a−/− (n = 24) tumors stained with Ki67, a cell proliferation marker. Bars, 50 μm. (C) KrasLSL-G12D/+; p53+/-; mir-34a−/− mice carried a greater percentage of p-Erk-positive lung tumors compared with KrasLSL-G12D/+; p53+/- mice. Representative images [left] and quantitation [right] of p-Erk staining are shown. Bars: 5× images, 400 μm; 20× images, 100 μm. (D) KrasG12D/+; p53+/−; mir-34a−/− lung tumors exhibited a decreased p21 staining compared with KrasG12D/+; p53+/− tumors. Representative images of p21 staining are shown for both genotypes [left], and the classification of lung tumors was performed for both genotypes by the degree of p21 expression [right]. Bars, 50 μm. (E) The percentage of p21-positive cells per tumor was calculated for 59 KrasG12D/+; p53+/− and 55 KrasG12D/+; p53+/−; mir-34a−/− lung tumors. All analyses were performed using mice 19 wk after AdCre infection. Error bars represent SEM; (**) P < 0.01; (***) P < 0.001.
miR-34 enhances p53 activity by post-transcriptional repression of HDM4. (A) The ability of miR-34 to induce G1 cell cycle arrest greatly depends on an intact p53. p53+/+ or p53−/− HCT116 cells were transfected with control siRNA (siGL3), miR-34a, miR-34b, or miR-34c and then treated with 100 ng/mL nocodazole to arrest cells in M phase. The percentage of G1 population in each transfection experiment was determined by flow cytometry. miR-34 enhanced G1 cell cycle arrest in p53+/+ HCT116 cells, but this effect was significantly reduced in p53−/− HCT116 cells. (B) miR-34 overexpression reduced the S-phase population in wild-type MEFs (left panel), but this effect was significantly reduced in p53−/− MEFs (right panel). Error bars are SD. (C) miR-34a and siHDM4 overexpression in A549, p53+/+, and p53−/− HCT116 cells significantly repressed the protein level of HDM4. miR-34a and siHDM4 overexpression in tumor cells with wild-type p53 caused a strong increase in p21 and a moderate increase in p53 protein level. (D) A schematic illustration of the full-length HDM4 3’ UTR and the predicted miR-34a-binding sites. (Bottom) Luciferase reporters were constructed to test the predicted miR-34a-binding sites. Two fragments of HDM4 3’ UTR (HDM4-3UTR-F1 and HDM4-3UTR-F2) were each cloned downstream from a luciferase reporter [Luc]. All predicted miR-34-binding sites in HDM4-3UTR-F1 or HDM4-3UTR-F2 shown in red were mutated in the luciferase reporter constructs [designated with asterisks]. The miR-34a-binding sites validated in luciferase assays are labeled in red, while those that failed to have an effect are labeled in orange. (E) miR-34 overexpression in A549 cells significantly reduced the mRNA level for HDM4. (F) In HCT116 dicer−/− cells, miR-34a specifically repressed luciferase reporters that carried HDM4 3’ UTR fragments with intact miR-34-binding sites. This miR-34a-dependent repression was abolished when the predicted miR-34-binding sites in the HDM4 3’ UTR fragments were mutated. (G,H) In human lung adenocarcinomas from the TCGA collection, expression of HDM4 (G) and P21 (H) exhibited inverse and positive correlation with the level of miR-34 miRNAs, respectively. Expression of miR-34 in the “miR-34 low” group was >16-fold greater than that in the “miR-34 high” group. In a separate cohort of 25 lung adenocarcinoma samples, real-time PCR analyses confirmed the inverse correlation between HDM4 and miR-34a [I] and the positive correlation between P21 and miR-34a [J]. Error bars represent SEM unless indicated otherwise. (*) P < 0.05; (**) P < 0.01; (***) P < 0.001.
tion of p53 protein in several tumor cell lines and in primary MEFs carrying the wild-type p53 [Fig. 3C; Supplemental Figs. S1G, S2A]; increased p53-mediated transcription was confirmed by the induction of several canonical p53 targets, such as p21 [Fig. 3C; Supplemental Fig. S2A,B; data not shown]. The induction of p21 by miR-34a depended on an intact p53, as no p21 induction was observed in p53−/− cells overexpressing miR-34a [Fig. 3C]. Our results were consistent with a previous report indicating that the ability of miR-34 miRNAs to induce apoptosis in HCT116 cells depended on the presence of an intact p53 [Raver-Shapira et al. 2007; Yamakuchi et al. 2008]. These findings suggest that p53 and miR-34 form a positive feedback loop to strengthen the downstream effects of p53. Thus, disruption of this positive feedback loop could significantly compromise the p53 tumor suppressor response.

This p53-miR-34 positive feedback could at least in part explain why the oncogenic effect of miR-34a deficiency in vivo was dependent on the p53 status. Deficiency of miR-34a disrupts the p53-miR-34 positive feedback, which could be compensated for by the redundancy of an intact p53 pathway in vivo. However, when p53 was haploinsufficient, miR-34a deficiency in KrasLSL-G12D/+; p53+/−; mir-34a−/− mice further disrupted the robustness of the p53 response, possibly creating a p53 activity that was below a critical threshold for maintaining adequate tumor suppression in vivo. Similarly, miR-34a deficiency in KrasLSL-G12D/+; p53+/− MEFs compromised the extent of p53 activation in response to the activating Kras mutation [Supplemental Fig. S2D]. Thus, the oncogenic effect conferred by miR-34a deficiency is dependent on the p53 status.

miR-34 represses Mdm4 to activate p53 and establish a positive feedback loop

A previous report indicated that miR-34 miRNAs promoted p53-mediated apoptosis in vitro by positively regulating p53 acetylation [Yamakuchi et al. 2008]. This is at least in part attributed to the ability of miR-34 miRNAs to directly repress SIRT1, which deacetylates p53 to impede p53-mediated transactivation [Yamakuchi et al. 2008]. Although we confirmed the direct inhibition of SIRT1 by miR-34 overexpression in multiple tumor cell lines [Supplemental Fig. S2E,F], we did not observe any inverse correlation between miR-34 and SIRT1 in human lung adenocarcinomas using RNA sequencing [RNA-seq] data from The Cancer Genome Atlas project [TCGA] [data not shown]. Thus, at least in the context of lung tumor development, additional mechanisms could mediate the positive feedback between p53 and miR-34.

To identify additional miR-34 targets that mediate its positive regulation of p53 activity, we focused on negative regulators of p53 that contain predicted miR-34 target sites in their 3′ untranslated regions (UTRs). Our analysis employed both computational predictions [Targetscape and RNA22] [Lewis et al. 2003; 2005; Miranda et al. 2006; Grimson et al. 2007] and data mining in published miR-34 pull-down data [Lal et al. 2011] and microarray data [Chang et al. 2007]. Among many reported putative miR-34 targets, the Mdm4 gene [also referred to as HDM4 in humans] emerged as a prominent candidate due to the presence of multiple predicted miR-34 target sites in the 3′ UTR [Fig. 3D; Supplemental Fig. S2H] and its important functions as one major negative regulator of p53 [Marine et al. 2006; Markey and Berberich 2008; Mandke et al. 2012; Wade et al. 2013]. mdm4/HDM4 and its closely related homolog, mdm2/HD2M2, both encode RING domain proteins that promote oncogenesis by inhibiting p53 [Marine et al. 2006; Wade et al. 2013]. The negative regulation of p53 by Mdm4 is best demonstrated by the aberrant p53 activation in Mdm4-deficient mice, which causes embryonic lethality at embryonic day 10.5 [E10.5] [Parant et al. 2001; Migliorini et al. 2002]. While Mdm2 acts as an ubiquitin E3 ligase that promotes p53 protein degradation via polyubiquitination [Eischen and Lozano 2009; Kruse and Gu 2009], Mdm4 does not possess E3 ubiquitin ligase activity by itself. In addition to repressing p53-dependent transactivation [Wade et al. 2013], Mdm4 positively regulates Mdm2 to promote p53 polyubiquitination, which leads to proteasomal degradation of p53 [Wang et al. 2011a].

Real-time PCR and Western analyses in a variety of mouse and human cell lines both indicated that endogenous Mdm4/HDM4 expression was subjected to the miR-34-dependent repression, with effects seen on both the transcript and protein levels [Fig. 3C,E, Supplemental Fig. S2I,J]. The repression of HDM4 by miR-34a in p53 wild-type tumor cells was correlated with a moderate increase in p53 protein level and a strong induction of canonical p53 targets, such as p21 [Fig. 3C; Supplemental Fig. S2A]. The moderate increase in p53 protein level was at least in part due to reduced ubiquitin-mediated proteasomal degradation [Supplemental Fig. S2G]. Interestingly, the extent of p21 induction by miR-34 was stronger than that of p53 protein increase [Fig. 3C; Supplemental Fig. S2A,E], suggesting that HDM4 modulates p53 activity in both a proteasome-dependent and proteasome-independent manner.

Using Targetscape and RNA22, we predicted five miR-34-binding sites in the 3′ UTR of the full-length HDM4 transcript [Fig. 3D]. Our luciferase reporter assays validated the functional importance of these predicted sites [Fig. 3F; Supplemental Fig. S2K]. Luciferase reporters carrying the wild-type 3′ UTR exhibited significant repression upon miR-34 overexpression; mutation of selected miR-34-binding sites abolished this miR-34-dependent regulation [Fig. 3F; Supplemental Fig. S2K]. In miR-34 overexpression studies, the extent of HDM4 repression was comparable with or stronger than that seen for other well-characterized miR-34 targets, including CCNDC1, CDK6, and SIRT1 [Fig. 3C; Supplemental Fig. S2C; Sun et al. 2008; Yamakuchi et al. 2008]. In mice, mdm4 could be transcribed into multiple isoforms in vivo, each of which was validated by 3′-RACE [data not shown]. miR-34 represses a long isoform of mdm4 in mice, which contains four predicted miR-34 targets sites in its 3′ UTR [Supplemental Fig. S2H,I; data not shown].
Human lung adenocarcinomas express a short HDM4 isoform that evades miR-34-mediated repression

Given the direct repression of HDM4 by miR-34 in vitro, we examined the correlation between miR-34 miRNAs and HDM4 in human lung adenocarcinomas. Using the RNA-seq data from TCGA, we observed an inverse correlation between the levels of full-length HDM4 mRNAs and those of miR-34 miRNAs in 327 lung adenocarcinomas (Fig. 3G). Not surprisingly, the HDM4 level inversely correlated with the levels of canonical p53 targets, such as P21 (Supplemental Fig. S3A). These findings were consistent with a positive correlation between the expression of miR-34 miRNAs and that of the canonical p53 target, such as P21, in these samples (Fig. 3H). We observed a similarly strong correlation when we examined the expression of miR-34 miRNAs and the expression of a cohort of validated p53 targets (Supplemental Fig. S3B). To validate our findings from TCGA data sets, we also collected 26 paired human lung adenocarcinomas and their normal adjacent controls and performed real-time PCR analyses to measure the expression levels of HDM4, mir-34 miRNAs, and P21. Using this cohort of lung adenocarcinoma samples, we confirmed the existence of an inverse correlation between full-length HDM4 and miR-34 miRNAs [Fig. 3I; Supplemental Fig. S3C] as well as a positive correlation between miR-34 miRNAs and P21 [Fig. 3J; Supplemental Fig. S3D]. Since miR-34 miRNAs are p53 targets, the correlation between miR-34 miRNAs and other p53 targets in human lung adenocarcinomas could also reflect the transcriptional activity of p53. Nevertheless, the inverse correlation between miR-34 and HDM4 and the ability of miR-34 to directly repress HDM4 and activate p53 strongly argue that the p53/miR-34/HDM4 positive feedback could, at least in part, contribute to this correlation. Collectively, our findings support the importance of the p53–miR-34–HDM4 positive feedback during the development of lung adenocarcinomas.

Interestingly, HDM4 encodes several isoforms in lung adenocarcinomas due to alternative polyadenylation. The full-length HDM4 isoform is produced with an intact 3′ UTR, while several HDM4 isoforms are generated with a shorter 3′ UTR that is devoid of all miR-34-binding sites (Fig. 4A). Although expression from the full-length HDM4 mRNA is inhibited by miR-34, expression from the short HDM4 isoforms largely evades miR-34-dependent regulation, as confirmed using luciferase reporter assays [Fig. 4B]. In both TCGA data sets and our own cohort of lung adenocarcinoma samples, the shorter HDM4 RNA isoforms accumulated to higher levels in lung adenocarcinoma tissues compared with their adjacent normal tissues. Hence, increased ratios of short versus full-length HDM4 RNA isoforms were seen in lung adenocarcinoma tissues (Fig. 4C, 4D). It is unclear how lung adenocarcinomas promote the alternative polyadenylation of the HDM4 transcript. It is also unclear how the expression levels of the short HDM4 isoforms compare with that of the full-length HDM4, yet the tumor-specific increase in the short HDM4 RNA isoforms is expected to permit bypassing of miR-34-dependent regulation of a subset of total HDM4 transcripts. Thus, deregulation of miR-34 or alternative HDM4 polyadenylation can evade miR-34-mediated HDM4 repression, thus dampening p53 response by disrupting the p53/miR-34/HDM4 feedback (Fig. 4E). Taken together, our results elucidated the intricate cross-talk between p53 and miR-34 miRNAs and revealed an important tumor suppressor effect generated by this positive feedback loop.

Discussion

The pathways downstream from p53 from p53 exhibit extensive redundancy. The deficiency of canonical p53 targets rarely recapitulated the spectrum and the extent of the oncogenic defects caused by the deficiency of p53 itself. Although the p53-regulated miR-34 miRNAs have been implicated as potent tumor suppressors, subsequent loss-of-function studies yielded a more complicated picture due to this p53 redundancy (Concepcion et al. 2012). Specifically, deletion of mir-34a and mir-34b/34c in MEFs caused little or no change in p53-mediated cell cycle arrest or apoptosis in vitro and caused no increase in tumorigenesis in vivo (Concepcion et al. 2012). The seemingly intact p53 response in miR-34-deficient cells and animals is not entirely surprising. It is conceivable that miR-34 miRNAs are integral components of a robust yet highly redundant p53 tumor suppressor pathway. The loss of mir-34a and mir-34b/34c could initiate a compensatory pathway mediated by either redundant effectors downstream from p53 or homologous miRNAs that share the same/similar target specificity. It is also possible that the functional importance of the p53–miR-34 axis is cell type-dependent and context-dependent, and the tumor suppressor effects of miR-34 miRNAs might be more apparent in a specific biological context.

While p53 induces the transcription of miR-34 miRNAs, these miRNAs in turn repress Mdm4 and/or Sirt1 post-transcriptionally to enhance p53 transcription activity and decrease p53 protein turnover. This positive feedback loop between p53 and mir-34 further strengthens the p53 activity upon p53 activation, conferring a robust tumor suppression response. In the Kras-induced lung adenocarcinoma model, the highly redundant and robust p53 pathway could tolerate a moderate disruption, such as that caused by miR-34a deficiency alone or by p53 hemizygosity. However, miR-34a deficiency combined with p53 hemizygosity further disrupts the p53 pathway, causing the strength of the p53 activity to be below a critical threshold required for sufficient tumor suppression in vivo. Our results may seem paradoxical to a previous study, where miR-34 deficiency in MEFs does not impact the p53-mediated cell cycle arrest and apoptosis in response to genotoxic stress (Concepcion et al. 2012). However, it is possible that the p53–miR-34 positive feedback loop has little impact on the acute p53 response but does affect the p53 activation that is required for tumor suppression under sustained oncogenic stresses.

The tumor suppressor effects of miR-34 in vivo could also be mediated through both p53-dependent and p53-
independent mechanisms. Several studies have demonstrated that the miR-34a targets are enriched for components in the growth factor signaling and cell cycle progression (Lal et al. 2011). In particular, multiple candidate miR-34a-regulated genes, including c-Met and Axl, participate in the RAS–RAF–MAPK signaling (Lal et al. 2011). This is consistent with the decreased Erk phosphorylation in KrasG12D/+; p53−/− tumor cell lines in response to miR-34a overexpression [data not shown]. This is also consistent with the enhanced Erk phosphorylation in KrasG12D/+; p53+/−; miR-34a−/− tumors when compared with the KrasG12D/+; p53+/+ controls. Increased MAPK signal-

Figure 4. Human lung adenocarcinomas express a short HDM4 isoform that evades miR-34-mediated repression. [A] A schematic illustration of the 3′ UTRs of a short HDM4 isoform and a full-length HDM4. The short HDM4 isoform does not contain any predicted miR-34-binding sites in the 3′ UTR. [B] The short HDM4 isoform carries a 3′ UTR that escapes miR-34 regulation. In HCT116 dicer−/− cells, miR-34a overexpression mediated a strong repression of the luciferase reporters that carried the 3′ UTR fragments from the full-length HDM4. However, miR-34a failed to alter the expression of the luciferase reporter that carried the 3′ UTR from the short HDM4 isoform. [C,D] Human lung adenocarcinomas exhibit a greater level of HDM4 short isoforms (shown at left) and a greater ratio between short and full-length HDM4 mRNAs. [C] The expression of HDM4 short isoforms and full-length HDM4 mRNAs was calculated as log2 counts in normal and tumor samples using the level III RNA-seq data from TCGA. [D] A similar comparison was performed using real-time PCR quantitation from a cohort of 24 paired lung adenocarcinoma and normal control samples. The average expression of the HDM4 short isoform in normal samples was normalized to 1, and the average ratio between short and full-length HDM4 mRNAs in normal samples was also normalized to 1. [E] A diagram describes our proposed model to explain the positive feedback between p53 and miR-34 miRNAs and how alternative polyadenylation of HDM4 could escape this feedback regulation. Error bars represent SEM. (**) P < 0.01; (***) P < 0.001.
ing in high-grade Kras	extsuperscript{G12D}-driven lung tumors often triggers p53 activation to repress tumor progression. Thus, in the Kras	extsuperscript{LSL-G12D/+}, p53	extsuperscript{+/-}; mir-34a	extsuperscript{-/-} mice, an increased MAPK signaling resulting from the activated Kras	extsuperscript{G12D} and mir-34a deficiency combined with a dampened p53 response due to p53 hemizygosity and miR-34a deficiency could generate a strong oncogenic effect that we observed in vivo. Consistently, when comparing Kras	extsuperscript{LSL-G12D/+}, p53	extsuperscript{+/-}; mir-34a	extsuperscript{-/-} tumors with Kras	extsuperscript{LSL-G12D/+}, p53	extsuperscript{+/-} tumors, a reduced p53 staining is evident in the high-grade tumors but not the low-grade tumors.

Our analysis of clinical samples implicates the functional significance of miR-34-dependent HDM4 repression. It is likely that disrupting the p53/miR-34/HDM4 feedback by miR-34 deficiency or by alternative polyadenylation of HDM4 could contribute to an increased oncogenesis in human lung adenocarcinomas. In two separate cohorts of lung adenocarcinomas, a strong inverse correlation exists between miR-34 miRNAs and full-length HDM4, and a considerable positive correlation occurs between miR-34 and canonical p53 targets. These results suggest that the disruption of the p53/miR-34/HDM4 feedback could represent an important mechanism to dampen the p53-mediated tumor suppression. In human lung adenocarcinomas, multiple mechanisms can act to disrupt this p53/miR-34/HDM4 feedback regulation. In addition to p53 mutations, deregulation of miR-34 miRNAs through either genomic deletion or promoter hypermethylation occurs frequently in lung cancers [Tanaka et al. 2012]. Furthermore, tumors exhibit an increased level of short HDM4 isoforms that are devoid of all miR-34-binding sites to bypass miR-34-mediated repression. The tumor-specific increase in the short HDM4 transcripts in human lung adenocarcinomas could constitute yet another mechanism to disrupt the p53–miR-34 feedback regulation and, ultimately, contribute to tumor development.

In the p53 pathway, there are many positive and negative feedback loops whose biological effects are cell type-dependent and context-dependent. Here, we demonstrated that the p53–miR-34 positive feedback constitutes an important regulation of the extent of tumor suppression during lung cancer development. This p53–miR-34 positive feedback regulation fine-tunes the degree of p53 activation and connects the p53 pathway with other signaling pathways, conferring both redundancy and robustness to the p53 tumor suppressor network.

Materials and methods

Animals

Mice of Kras	extsuperscript{LSL-G12D/+} genotype were crossed with p53	extsuperscript{+/-} or p53	extsuperscript{+/-}; mir-34a	extsuperscript{-/-} mice to generate Kras	extsuperscript{LSL-G12D/+}, p53	extsuperscript{+/-} and Kras	extsuperscript{LSL-G12D/+}, p53	extsuperscript{+/-}; mir-34a	extsuperscript{-/-} mice. Lung tumorigenesis was induced by intranasal incubation of 2.5 × 10⁷ plaque-forming units of recombinant AdCre virus (University of Iowa Gene Transfer Vector Core) in 8-wk-old mice.

Isolation of mouse tumor cells

Cell lines were isolated from individual lung tumors recovered at necropsy. Following harvest, tumors were cut into small pieces and digested for 30 min at 37°C in 2 mL of Hanks’ buffered salt solution [HBSS] containing dispase [Invitrogen, no. 17105-041] and collagenase type IV [Invitrogen, no. 17104-019]. Following digestion, ice-cold DNase (Roche Applied Science, no. 10104159001) was added, and the mixture was incubated for 10 min on ice. Digested tumor samples then were pressed through 40-µm cell strainers. Finally, samples were centrifuged at 1000 rpm for 5 min at room temperature, resuspended in culture medium [DMEM, Invitrogen, no. 11995-073] supplemented with 10% fetal bovine serum [FBS] and 1% penicillin and streptomycin [Invitrogen, no. 15140-163], and plated in 12-well plates.

Cell culture and transfection

MEFs and Phoenix-E cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Primary Kras	extsuperscript{LSL-G12D/+} MEFs with various genotypes were infected with AdCre virus by incubation for 12 h in DMEM supplemented with 2% FBS and 1% penicillin and streptomycin. A549 cells and primary mouse lung tumor-derived cells were maintained in RPMI1640 medium [Invitrogen, no. 11875-119] supplemented with 10% FBS and 1% penicillin and streptomycin. HCT116 cells were maintained McCoy’s 5A medium [Invitrogen, no. 16600-082] supplemented with 10% FBS and 1% penicillin and streptomycin. Transient transfection of HCT116 and A549 cells was performed using either Oligofectamine [Invitrogen, no. 12525-011] or Lipofectamine 2000 [Invitrogen, no. 11668-019] according to the manufacturer’s instruction.

Histopathology and immunohistochemistry (IHC)

For determining tumor incidence, area, and grade, whole lungs were fixed overnight in 10% buffered formalin [Fisher Scientific, no. SF100-4], dehydrated in a graded series of ethanol solutions, embedded in Paraplast X-TRA paraffin [Fisher Scientific, no. 23-021-401], sectioned at 6-µm thicknesses, mounted on glass slides, and stained with hematoxylin and eosin (H&E) using standard procedures. Lung and tumor areas were determined using Image Viewer (Ventana Medical Systems/Roche). Tumors were analyzed on H&E sections. Each tumor was given a score of grade 1–5 [Jackson et al. 2005]. Grades 1 and 2 were classified as low-grade tumors, and grades 3–5 were classified as high-grade tumors.

For IHC, paraffin sections were deparaffinized, dehydrated, and subjected to heat-induced antigen retrieval in a pressure cooker using 10 mM sodium citrate buffer (pH 6.0). Slides were incubated for 10 min with 3% H₂O₂, blocked for 3 h with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.3% Triton X-100, and incubated with primary antibody overnight in 1% BSA in PBS-0.3% Triton X-100. Horseradish peroxidase (HRP)-conjugated secondary antibodies [Invitrogen, no. G-21234; Santa Cruz Biotechnology, no. sc-2768] were incubated for 2 h at room temperature, with a 1:400 dilution in PBS containing 5% BSA and 0.3% Triton X-100. Peroxidase was then visualized by DAB staining [Invitrogen, no. 00-2014] and counterstaining with Mayer’s hematoxylin [Electron Microscopy Sciences, no. 26503-04].

Real-time PCR analyses

Total RNA was extracted from cells or tissues using Trizol [Invitrogen, no. 15596] according to the manufacturer’s instruc-
tion. For miRNA real-time PCR assays, cDNA was synthesized from total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, no. 4368814). Subsequently, quantitative PCR was carried out using TaqMan miRNA assays (Applied Biosystems) to detect mature miR-34a miRNA levels. For mRNA real-time PCR, cDNA was synthesized from total RNA using the iScript advanced cDNA synthesis kit (Bio-Rad, no. 170-8843), and mRNA expression levels were determined by real-time PCR with SYBR (Kapa Biosystems, no. KK4604). The primers used here are shown in Supplemental Table S1.

3′-end real-time PCR
Total RNA was extracted from cells or tissues using Trizol according to the manufacturer’s instruction. RNA was heat-fragmented for 5 min at 70°C with the RNA fragmentation reagent (Ambion, no. AM8740). The fragmented RNA was precipitated and then used to synthesize cDNA by RT–PCR with SuperScript III reverse transcriptase (Invitrogen, no. 18080044). mRNA expression levels were determined by real-time PCR with SYBR. The primers used here are shown in Supplemental Table S1.

3′-RACE
The 3′-RACE was performed using mouse testis marathon-ready cDNA (Clontech, no. 639405) according to the manufacturer’s protocol. We cloned multiple mdm4 isoforms with different lengths of 3′ UTRs. The longest mdm4 isoform, validated by RT–PCR, contains a 3′ UTR of 5435 nucleotides, which harbors four predicted miR-34 target sites.

Western blot analysis
Total cellular extracts resolved by SDS-PAGE were transferred to nitrocellulose filters. Western blotting was performed in TBST (100 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk or 5% BSA. Immunoreactive protein bands were visualized by using Pierce ECL Western blotting substrate (Thermo Scientific, no. 32106).

Antibodies
The following monoclonal (mAb) and polyclonal (pAb) primary antibodies were used for immunoblotting and IHC: SIRT1 pAb (1:1000; Abcam, no. ab13749), HDM4 pAb (1:1000; Bethyl Laboratories, no. A300-287A), p53 mAb (1:1000; Cell Signaling, no. 2522), pErk mAb (1:1000 [Cell Signaling, no. 4370] for immunoblotting, 1:75 [Cell Signaling no. 4376] for IHC), HDM4 mAb (1:1000; Millipore, no. OP43), surfactant protein C pAb (1:200; Millipore, no. AB3786), CC10 pAb (1:400; Santa Cruz, no. sc-25555), p21 mAb or pAb (1:100 [Santa Cruz, no. sc-6246]) for immunoblotting, 1:100 [Santa Cruz, sc-471] for IHC), α-tubulin mAb (1:5000; Sigma, no. T5168), and Ki67 mAb (1:200; Thermo Scientific, no. RM-9106-S1).

Ubiquitination assay
For the ubiquitination assay, HCT116 cells on one 10-cm plate were cotransfected with 25 nM RNA oligos (siGFP or siR-34a mimic), 4 μg of HA-ubiquitin plasmid, and 1 μg of pcDNA-p53 plasmid using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Cells were treated with 20 μM MG132 [Sigma, no. C2211] 4 h before harvesting. Forty-eight hours after transfection, cells were lysed in standard RIPA buffer (10 mM Tris at pH 8.0, 1% NP-40, 2 mM EDTA, 150 mM NaCl with the Roche protease inhibitor, no. 11697498001), and the whole-cell lysates were subjected to immunoprecipitation with 2 μg of anti-p53 [FL-993-G, Santa Cruz Biotechnology] antibody and protein A/G agarose [Pierce]. The immunoprecipitates were immunoblotted with anti-HA antibody [1:1000, Santa Cruz Biotechnology, no. 12CA5] to detect the ubiquitinated p53.

Plasmid DNA constructs
Three fragments corresponding to the HDM4 3′ UTR were amplified by PCR using human genomic DNA as a template with the primers shown in Supplemental Table S1. Fragments 1 and 2 (F1 and F2) were cloned downstream from a luciferase reporter in a pGL3 vector [Promega, no. E1751]. Seed regions were mutated to remove all complementarity to miR-34 miRNAs by QuickChange mutagenesis kit (Strategene) with the primers shown in Supplemental Table S1. The short form of the HDM4 3′ UTRs was similarly cloned into the same luciferase reporter vector.

Luciferase assay
HCT116 Dicer−/− cells were cotransfected with 100 ng of reporter constructs and 50 nM miRNAs (miR-34a or miR-124a) in the form of siRNA using LT1 (Mirus, no. MIR2300) and TKO [Mirus, no. MIR2150]. Ten nanograms of pRLTK [Promega, no. E2241] also was cotransfected as a normalization control. Cells were lysed 48 h after transfection, and ratios between firefly and Renilla luciferase activity were measured with the dual-luciferase reporter assay system [Promega, no. E1910].

Gene expression analysis of the TCGA data set
For the TCGA data set, miRNA-seq and RNA-seq data were generated using an Illumina HiSeq 2000 sequencing platform from lung adenocarcinoma samples and normal adjacent controls. The read counts were already summarized for each miRNA or mRNA isoform, and the expression level of each gene can be retrieved from the TCGA level 3 data (archives bcgsc.ca_LUAD.IlluminaHiSeq_miRNASeq.Level_3.1.4.0 and unc.edu_LUAD.IlluminaHiSeq_RNASeqV2.Level_3.1.8.0).

For miRNA-seq data, we summarized the miRNA-seq data at the level of the MIMATIs [miRNA IDs] and selected 631 miRNAs that have at least five read counts in at least 5% of the samples. The raw counts were normalized using upper-quantile. The plate 1757, which contained seven normal samples, constituted a batch different from the other normal samples and was not included in the analysis of the miRNA-seq data.

For RNA-seq data, the read counts already summarized at the gene and transcript levels were retrieved from the TCGA level 3 data (archive unc.edu_LUAD.IlluminaHiSeq_RNASeqV2.Level_3.1.8.0). RSEM was used to estimate gene and transcript abundance. Eighteen-thousand-three-hundred-eighteen genes and 59,847 transcripts with at least five counts in at least 5% of the samples were selected. The raw counts were then normalized using upper-quantile. Three HDM4 transcripts carried an intact 3′ UTR [uc0011bha.2, uc0011bhb.2, and uc0011bhc.2], and two short HDM4 transcripts carried a short 3′ UTR devoid of all miR-34-binding sites [uc010pqw.1 and uc010pqx.1]. Their counts were summed up to get one expression value per sample for the HDM4 long and short forms, respectively. The RNA-seq data included 395 tumor samples and 51 adjacent normal controls, of which 327 tumor and 12 adjacent normal samples were also available in the miRNA-seq data.
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