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# Lin28B promotes melanoma growth by mediating a microRNA regulatory circuit

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## Abstract

It has been increasingly recognized that microRNAs (miRNAs) are often dysregulated in various human malignancies and can function as oncogenes or tumor-suppressors. However, the potential roles of miRNAs and components of the miRNA biogenesis pathway remain poorly defined in melanoma. Here, we systematically profiled miRNA expression in human melanocytes and melanoma cells, and identified a prominent function of miR-125a-5p in suppressing melanoma growth. Mechanistically, we discovered that Lin28B, a well-characterized inhibitor of let-7 miRNA biogenesis, was a direct target of miR-125a-5p in melanoma. We showed that the Lin28B was aberrantly expressed in a large proportion of melanoma patients and was functionally required for melanoma progression. We further demonstrated the involvement of let-7-dependent mechanism downstream of Lin28B, resulting in the activation of transforming growth factor-β signaling cascade. Collectively, our data implicate Lin28B as a novel oncogene in melanomagenesis by mediating a miRNA regulatory circuit.

## Introduction

Melanoma is the most aggressive skin cancer originating from neural crest-derived melanocytes (1). During progression, melanoma cells accumulate massive numbers of genetic alterations and consequently display a high degree of phenotypic plasticity (2,3). As a result, the discovery of tumor drivers is remarkably daunting in melanoma. Although a fraction of melanomas are driven by the activation of mitogen-activated protein kinase pathway and experience clinical benefit when treated with RAF/MEK inhibitors (4–6), almost all patients develop resistance to these agents due to the inherently dynamic behavior of signaling networks within tumor cells (7–9). Recently, microRNAs (miRNAs) have been shown to play a critical role in many physiological and pathological processes including tumorigenesis, and represent a less explored strategy to identify new oncogenic dependencies in melanoma (10–13).

miRNAs are a family of small non-coding RNAs, which can suppress gene expression by pairing to the 3′ untranslated regions (UTRs) of target mRNAs (14). Numerous studies have demonstrated that deregulation of miRNAs influences tumorigenicity in a variety of human cancers (15,16). One extensively documented example is let-7, a tumor suppressor miRNA family downregulated in multiple cancer types (17,18). The let-7 family of miRNAs negatively regulates the expression of a range of well-known oncogenes such as myc, kras and hmga2 (19–22). Not surprisingly, tumors have developed complex mechanisms to suppress let-7 in order to accommodate rapid cell proliferation.

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**Abbreviations**

| | |
|---|---|
| miRNAs | microRNAs |
| TGF | transforming growth factor |
| UTR | untranslated region |

As crucial posttranscriptional regulators of let-7 biogenesis, the oncofetal RNA-binding proteins Lin28A and Lin28B (collectively referred to as Lin28) are frequently overexpressed in a wide spectrum of human cancers, resulting in reduced levels of let-7 miRNAs (23–25). Importantly, Lin28 alone is sufficient to drive certain cancers in mice, e.g. lymphoma (26), neuroblastoma (27), liver (28) and kidney tumors (29). However, the role of Lin28 in melanoma has not been determined.

In this study, we performed a quantitative PCR-based miRNA profiling and identified that miR-125a-5p was differentially expressed in melanoma cells compared with melanocytes. Interestingly, we found that let-7 suppressor Lin28B was a direct target of miR-125a-5p. In this way, Lin28B was at the center of a miRNA regulatory axis to promote melanoma progression. We further demonstrated that Lin28B was involved in activating transforming growth factor (TGF)-β signaling pathway, thereby implicating the Lin28B/miRNA network as a novel oncogenic driver in melanomagenesis.

## Materials and methods

### Cell culture

Tumor cell lines were obtained from ATCC (authentication has been performed by ATCC based on morphology, karyotyping and PCR assays), and were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum (Invitrogen) for fewer than 6 months. Retroviral vector which contains Lin28B open reading frame (pBABE, Addgene) and lentiviral vector which contains Lin28B knockdown sequences (pLKO.1, Sigma) were transfected into HEK293T cells with packaging mixtures. Virus was collected, filtered and then incubated with target cells in growth medium containing 8 μg/ml polybrene (Millipore). Infected cells were selected with 5 μg/ml puromycin. For miRNA overexpression, oligos containing the miRNA sequences were cloned into the pLKO.1 lentiviral vector and virus was generated to infect target cells.

### Western blot

Tumor cells were lysed in RIPA buffer (Tris pH 7.4 50 mM, NaCl 150 mM, NP-40 1%, sodium dodecyl sulfate 0.1%, ethylenediaminetetraacetic acid 2 μM) containing proteinase inhibitors (Roche) and phosphatase inhibitors (Roche), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis. Antibodies against the following proteins were used: Lin28B, pSer-465/467 Smad2, Actin (Cell Signaling Technology).

### miRNA profiling and quantitative PCR

Total cell RNA was extracted with mirVana miRNA isolation kit (Ambion) following the manufacturer's protocol. miRNA in 500 ng total RNA was reverse transcribed using the Megaplex reverse transcriptase primer pools and the Taqman miRNA reverse transcription kit (Applied Biosystems), and loaded into the Taqman array miRNA cards. The real-time miRNA PCR array was performed on the Applied Biosystems ViiA7 machine. Relative expression levels of each miRNA were normalized to U6 snRNA as the endogenous control. Expression of individual miRNAs relative to U6 snRNA was quantified by TaqMan miRNA assays (Applied Biosystems). At least three biological replicates were included for each condition.

### Microarray analysis and quantitative PCR

Total messenger RNA was prepared with RNeasy plus mini kit (Qiagen) according to the manufacturer's protocol. The Affymetrix human genome U133 Plus 2.0 microarray was used for gene expression profiles. Three biological replicates per group were included for statistical analyses. Affymetrix microarray probe-level data were background-corrected, normalized and summarized using the robust multi-array average method. Differential gene expression was performed with linear models for microarray data (Limma) implemented in BioConductor. TaqMan gene expression assays (Applied Biosystems) were used for quantitative PCR analysis, with human beta-actin as the endogenous control. At least three biological replicates were included for each condition. TissueScan complementary DNA arrays were purchased from OriGene Technologies.

### Cell viability assays

Melanoma cells were seeded at 5000 cells per well in 96-well black-walled plates. The next day, cells were rinsed and fresh serum-free medium was added. Cells were incubated for 72 h. Cell viability was assayed using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's protocol.

### Dual luciferase reporter assay

For miRNA target validation, 3′ UTR segment of human Lin28B was cloned into a mammalian expression vector with dual luciferase reporter system (GeneCopoeia). COS-7 cells were transfected in 6-well plates using Lipofectamine 2000 (Invitrogen). Transfections were performed using 1 μg dual luciferase reporter plasmids and a final concentration of 10 nM synthetic miRNA mimics (Applied Biosystems). Forty-eight hours after transfection, dual luciferase assays were performed using Luc-Pair miR luciferase assay kit (GeneCopoeia) according to the manufacturer's instructions. Firefly luciferase activity was first normalized to Renilla luciferase expression control. For each reporter construct, the normalized value for miRNA transfection was then normalized to the value obtained from the same reporter construct co-transfected with control miRNA. Mean values, standard deviations and Student's *t*-test were calculated from seven independent transfections for each condition.

### Xenograft models

All animal protocols were reviewed and approved by the institute animal care and use committee. Tumor cells ($1 \times 10^6$) were mixed with Matrigel (BD Biosciences) and subcutaneously implanted into the right dorsal flanks of BALB/c Nude mice. When tumors reached ~150 $mm^3$, tumor volumes (10 animals per group) were measured twice weekly with digital caliper and calculated using the formula length × $width^2$ × 0.52.

### Statistical analysis

Statistical analysis was based on two-sided Student's *t*-test for two-group comparison and one-way analysis of variance, followed by Tukey's multiple comparisons test, was used to test for differences among more groups. *P*-values of $<0.05$ were considered statistically significant.

## Results

### Downregulation of miR-125a-5p accelerates melanoma growth

To identify miRNAs differentially expressed in melanoma cells relative to melanocytes, we performed a comprehensive miRNA profiling using Taqman micro fluidic cards (Supplementary Table 1, available at *Carcinogenesis* Online). We obtained four independent primary melanocyte lines from different commercial sources, and compared them with four patient-derived melanoma cell lines harboring diverse genetic variations. Hierarchical clustering analysis revealed that melanocytes and melanoma cells had distinct miRNA expression (Supplementary Figure 1, available at *Carcinogenesis* Online), with a handful of miRNAs most significantly differentially expressed (Figure 1A). Among these miRNAs, let-7b, miR-26a and miR-17 have been previously implicated in melanoma pathogenesis (30–32), thus validating the robustness of our approach.

To identify functionally important miRNAs, we individually modulated the expression of each candidate in melanoma cells and determined its impact on tumor growth *in vivo*. We found that

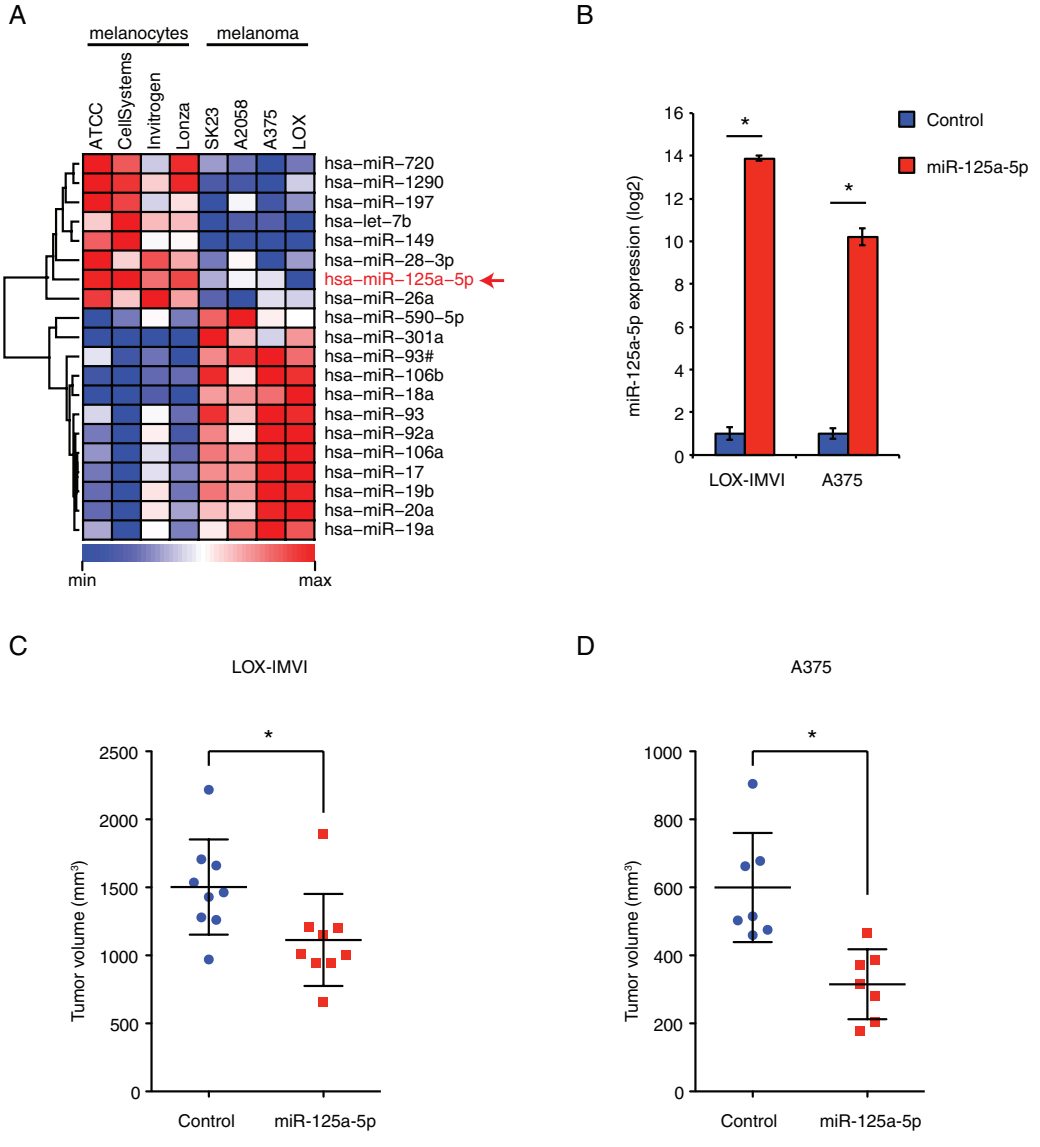


**Figure 1.** Downregulation of miR-125a-5p accelerates melanoma growth. (**A**) Heatmap of differentially expressed miRNAs in human melanoma cells compared with primary melanocytes. (**B**) Quantitative PCR of miR-125a-5p in LOX-IMVI and A375 cell lines infected with miR-125a-5p or control lentivirus. Each group had three biological replicates. *$P < 0.05$, Student's *t*-test. (**C**) Tumor volume of LOX-IMVI cells overexpressing miR-125a-5p or control sequence, ten mice per group. *$P < 0.05$, Student's *t*-test. (**D**) Tumor volume of A375 cells overexpressing miR-125a-5p or control sequence, ten mice per group. *$P < 0.05$, Student's *t*-test.

the majority of miRNAs had no obvious effects (Supplementary Figure 2, available at *Carcinogenesis* Online). However, when we re-expressed one downregulated miRNA, miR-125a-5p, in melanoma cells (Figure 1B), tumor xenograft growth was significantly suppressed. Similar results were observed in two cell lines LOX-IMVI (Figure 1C) and A375 (Figure 1D), suggesting that miR-125a-5p functions as a tumor suppressor in melanoma.

## Lin28B is a direct target and functional effector of miR-125a-5p in melanoma

We combined in silico analysis and experimental perturbation to uncover the molecular mechanisms through which miR-125a-5p inhibits melanoma growth. Initially, we predicted the gene targets of miR-125a-5p using TargetScan algorithm (33), which yielded a large number of putative target genes. Additionally, using microarray analysis, we thoroughly examined the expression levels of all genes in response to miR-125a-5p overexpression and identified 34 differentially expressed genes in two melanoma cell lines (Figure 2A). These complementary approaches nominated Lin28B as a potential target of miR-125a-5p. Indeed, the 3′ UTR of Lin28B contains a predicted binding site of miR-125a-5p, which is highly conserved across species (Figure 2B). Ectopic expression of miR-125a-5p significantly suppressed the activity of a luciferase reporter fused with wild-type Lin28B 3′ UTR, but not Lin28B 3′ UTR with mutated miR-125a-5p binding sites, suggesting that Lin28B gene is a direct target of miR-125a-5p (Figure 2C). Consistent with these findings, upon miR-125a-5p re-expression in LOX-IMVI and A375 cells, Lin28B was significantly downregulated at both RNA (Figure 2D) and protein levels (Figure 2E). To determine whether Lin28B is a functional mediator downstream of miR-125a-5p, we ectopically reintroduced Lin28B in the presence of miR-125a-5p overexpression (Figure 2F). The inhibition of miR-125a-5p on tumor

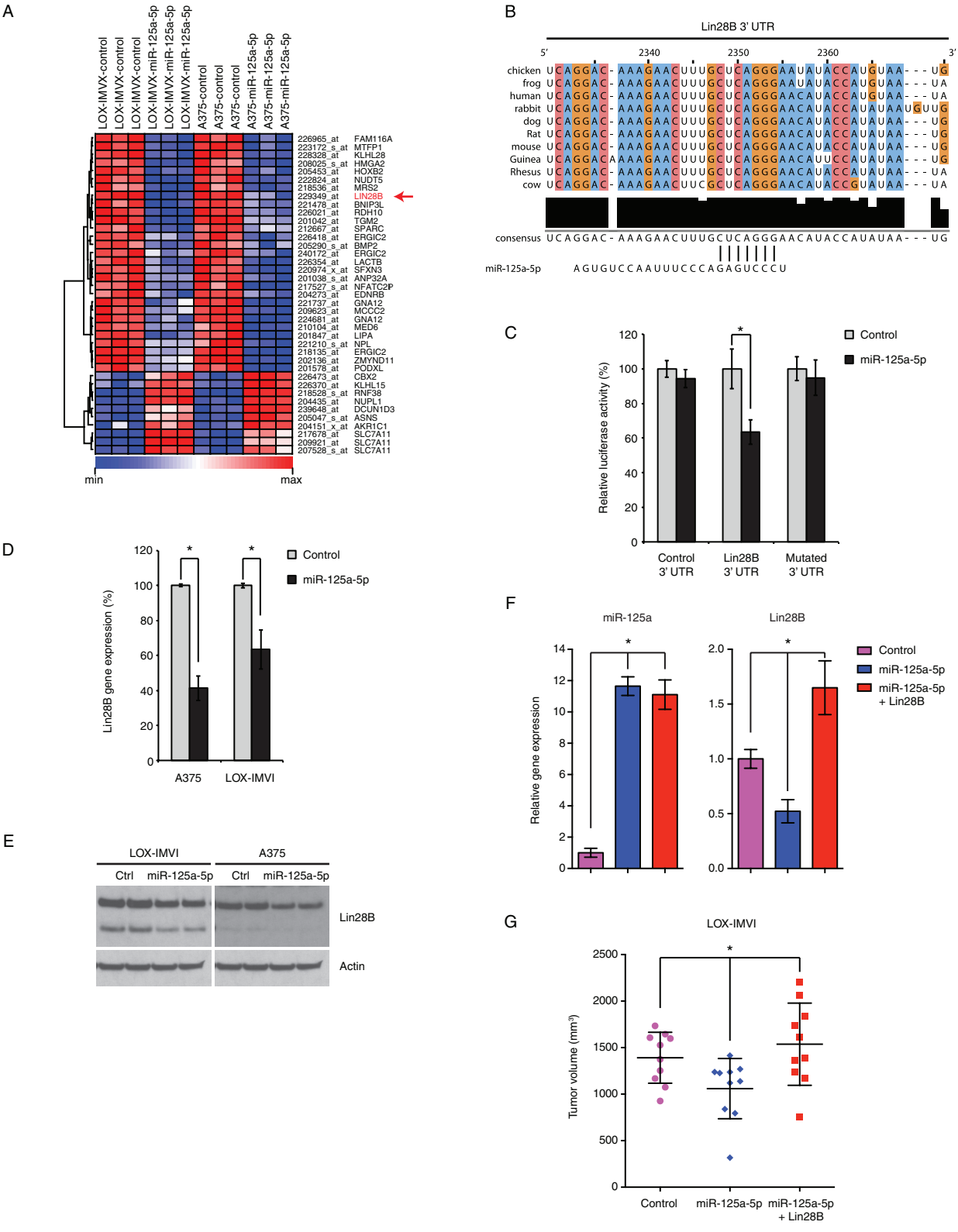


**Figure 2.** Lin28B is a direct target and functional effector of miR-125a-5p in melanoma. (**A**) Heatmap of differentially expressed genes in both melanoma cell lines overexpressing miR-125a-5p compared with control. (**B**) Alignment of the predicted miR-125a-5p binding site in Lin28B 3′ UTR. (**C**) Dual luciferase assay on wild-type or mutated Lin28B 3′ UTR in COS-7 cells transfected with control or miR-125a-5p. *$P < 0.05$, Student's *t*-test. (**D**) Quantitative PCR of Lin28B in LOX-IMVI and A375 cell lines infected with miR-125a-5p or control lentivirus. Each group had three biological replicates. *$P < 0.05$, Student's *t*-test. (**E**) Western blot analysis of Lin28B in LOX-IMVI and A375 cell lines infected with miR-125a-5p or control lentivirus. Two bands were detected, corresponding to the two Lin28B isoforms. (**F**) Quantitative PCR of miR-125a-5p and Lin28B in LOX-IMVI cells expressing control, miR-125a-5p or both miR-125a-5p and Lin28B. Each group had three biological replicates. *$P < 0.05$, analysis of variance followed by Tukey's post-test. (**G**) Tumor volume of LOX-IMVI cells expressing control, miR-125a-5p or both miR-125a-5p and Lin28B, ten mice per group. *$P < 0.05$, analysis of variance followed by Tukey's post-test.

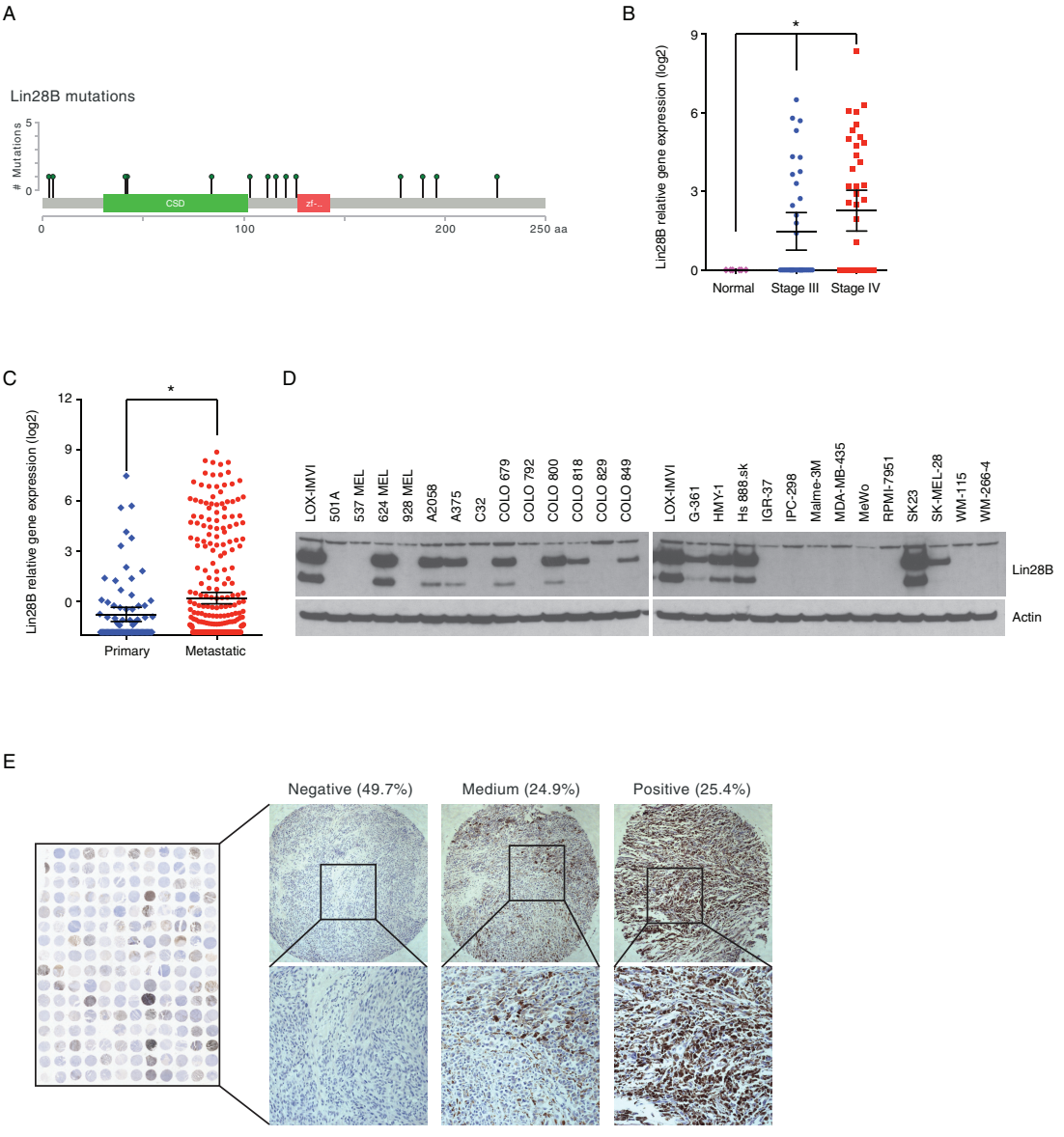


**Figure 3.** Lin28B is mutated and overexpressed in a subset of melanoma patients. (**A**) Mutation spectrum of Lin28B in melanoma patients. (**B**) Lin28B expression in normal skin tissues, early or late stages of melanoma, as measured by quantitative PCR. *$P < 0.05$, analysis of variance. (**C**) Lin28B expression in primary or metastatic melanoma, as measured by RNA-sequencing. *$P < 0.05$, Student's *t*-test. (**D**) Western blot analysis of Lin28B in a panel of melanoma cell lines. (**E**) Immunohistochemistry of Lin28B in a tissue microarray containing 191 melanoma samples.

growth was completely antagonized by enforced expression of Lin28B (Figure 2G). Taken together, we conclude that miR-125a-5p directly targets Lin28B to inhibit tumor growth in melanoma.

## Lin28B is mutated and overexpressed in a subset of melanoma patients

Emerging evidence suggests that Lin28B may contribute to the development of several types of cancer including chronic myeloid leukemia, hepatocellular carcinomas, Wilms' tumor and neuroblastoma (24,27,29,34,35). However, it remains unknown whether Lin28B plays a role in melanoma. We initially sought to determine whether Lin28B is aberrantly expressed in melanoma tissues. Unlike in neuroblastoma (27), Lin28B was rarely amplified in melanoma tumors as assessed by single nucleotide polymorphism array of 387 samples from the cancer genome atlas (TCGA) project (Supplementary Figure 3, available at *Carcinogenesis* Online). Next, we queried cBioPortal database which contains three independent melanoma projects (36,37). Interestingly, Lin28B was found to be somatically mutated in a subset of melanoma patients by all the three sequencing projects (Supplementary Figure 4, available at *Carcinogenesis* Online). These mutations are associated with amino acid substitutions scattered throughout the Lin28B protein (Figure 3A), and their biological impact remains to be investigated.

We resorted to pan-cancer RNA-seq analysis of 7660 tumors from the TCGA database to determine the relative Lin28B expression levels across a panel of 22 cancer types (38). Lin28B is most highly abundant in uterine and ovarian carcinomas, but is also expressed in melanoma patients at considerable levels (Supplementary Figure 5, available at *Carcinogenesis* Online). Next, using quantitative PCR of a skin tissue complementary DNA array, we analyzed Lin28B expression in a cohort of 77 clinical melanoma specimens representing late stages of tumor progression relative to normal skin. Lin28B expression was statistically significantly upregulated in melanoma

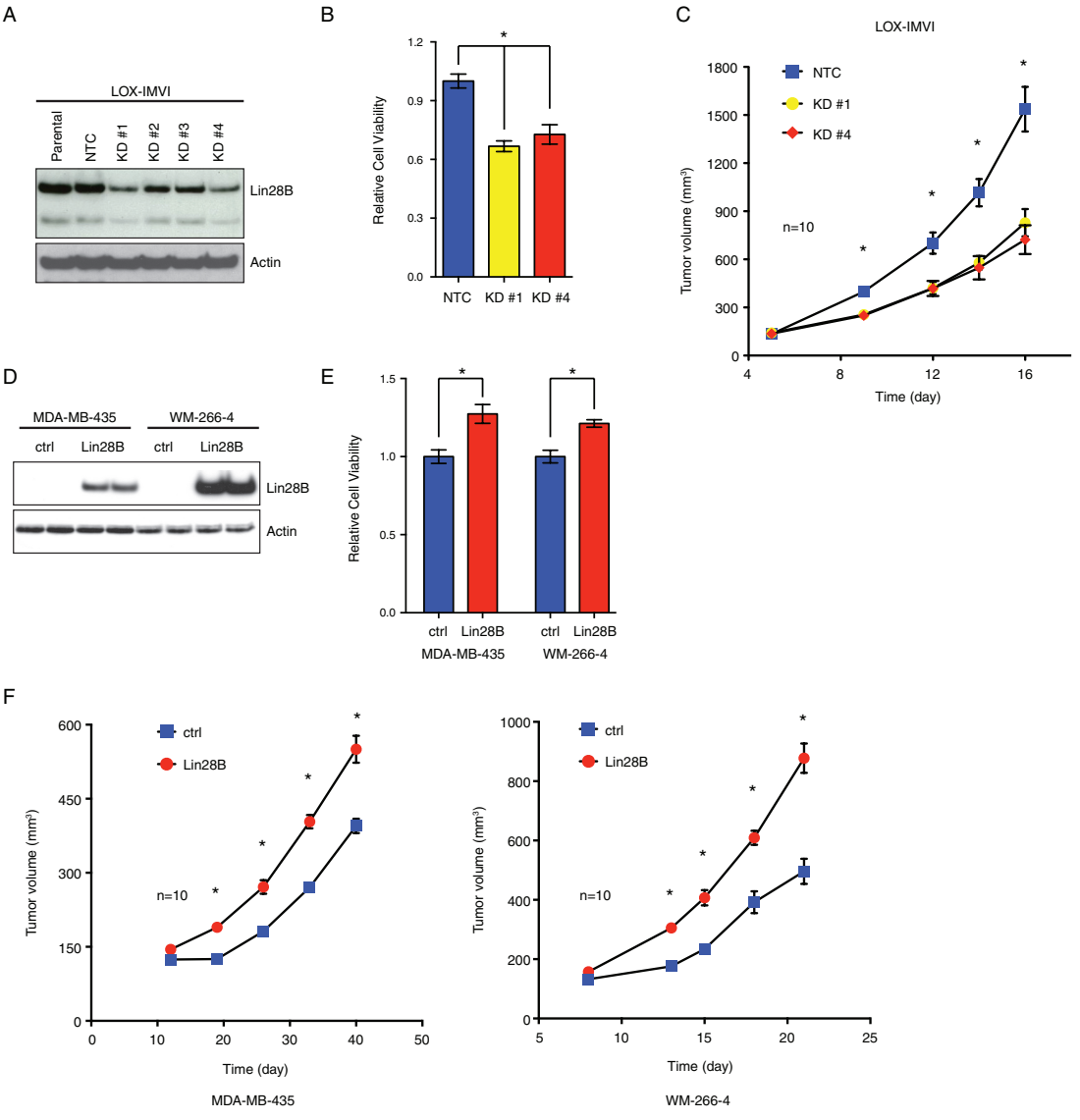


**Figure 4.** Lin28B promotes melanoma tumor growth *in vivo*. (**A**) Lin28B was knocked down in LOX-IMVI cells using four different short-hairpin RNAs. Knockdown efficiency was analyzed by western blot. (**B**) LOX-IMVI cell viability with Lin28B knockdown. *$P < 0.05$, analysis of variance followed by Tukey's post-test. (**C**) Tumor growth of LOX-IMVI cells with Lin28B knockdown, 10 mice per group. *$P < 0.05$, analysis of variance followed by Tukey's post-test. (**D**) Lin28B was overexpressed in MDA-MB-435 and WM-266-4 melanoma cell lines. Western blot demonstrated Lin28B overexpression. (**E**) Cell viability of MDA-MB-435 and WM-266-4 overexpressing Lin28B relative to control. *$P < 0.05$, Student's *t*-test. (**F**) Tumor growth of MDA-MB-435 and WM-266-4 cells overexpressing Lin28B relative to control, ten mice per group. *$P < 0.05$, Student's *t*-test.

tumors compared with non-malignant control tissues (Figure 3B). In addition, Lin28B level was further elevated in metastatic melanoma as compared with primary tumors (Figure 3C). Finally, Lin28B protein expression was assessed by western blotting in a panel of 27 melanoma cell lines (Figure 3D), and by immunohistochemistry in a melanoma tissue microarray of 191 cancer samples (Figure 3E). These analyses revealed that Lin28B expression was detectable in 13 of 27 (48.1%) cell lines and 96 of 191 (50.3%) tumor samples. Taken together, our data indicate that Lin28B is mutated and overexpressed in a subset of melanoma patients, suggesting that Lin28B may play an oncogenic role in melanoma.

## Lin28B promotes melanoma tumor growth *in vitro* and *in vivo*

Focusing on Lin28B, we conducted a series of experiments to gain further insight into its physiological function in melanoma. We stably knocked down Lin28B in LOX-IMVI cells using four different short-hairpin RNAs, two of which (KD #1 and KD #4) exhibited better efficiency to inhibit Lin28B expression (Figure 4A). Lin28B depletion significantly reduced cell viability *in vitro* (Figure 4B) and slowed down tumor formation of LOX-IMVI in immune-deficient mice (Figure 4C). Notably, the two short-hairpin RNAs led to similar results, suggesting that the phenotype is less likely due to unspecific effects. Conversely, we overexpressed Lin28B in MDA-MB-435 and WM-266-4 melanoma lines, both showing minimal endogenous expression of Lin28B protein (Figure 4D). As expected, we observed a statistically significant increase in cell viability (Figure 4E) and tumor growth (Figure 4F). Therefore, Lin28B is a bona fide oncogene in melanoma.

## Lin28B blocks the let-7 miRNA cluster and activates the TGF-β pathway in melanoma

Lin28B has been reported to inhibit the biogenesis of let-7 family miRNAs (39–42). To determine whether this molecular

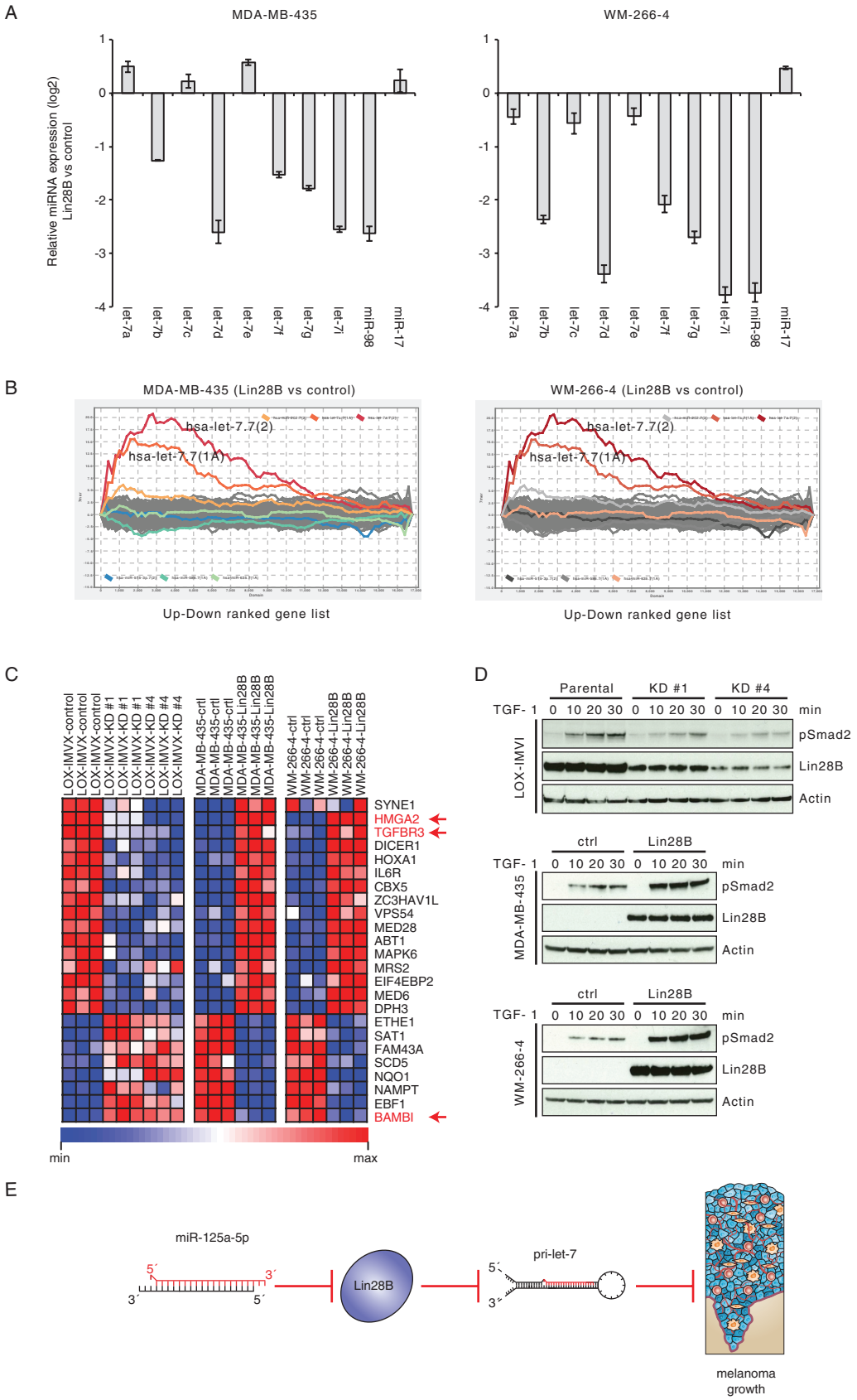


Figure 5. Lin28B blocks the let-7 miRNA cluster and activates the TGF-β pathway in melanoma. (**A**) Quantitative PCR of let-7 family miRNAs in melanoma cells over-expressing Lin28B relative to control. Each group had three biological replicates. *$P < 0.05$, Student's *t*-test. (**B**) Sylamer analysis of over-represented miRNA seed sites in melanoma cells overexpressing Lin28B. (**C**) Heatmap of differentially expressed genes regulated by Lin28B in all three models. (**D**) Western blot analysis of TGF-β1-induced pSmad2 upon Lin28B knockdown or overexpression. (**E**) A schematic model of Lin28B-regulated miRNA circuit in melanoma.

mechanism may account for the oncogenic role of Lin28B in melanoma, we investigated the effects of Lin28B overexpression in MDA-MB-435 and WM-266-4 cells. Among the let-7 miRNA genes examined, Lin28B decreased levels of let-7b, let-7d, let-7f, let-7g, let-7i and miR-98 in both cell lines (Figure 5A). As a result, let-7 sites were enriched among transcripts induced by Lin28B as revealed by microarray combined with Sylamer analysis (43), which detects over-represented miRNA seed sites within the 3′ UTRs of mRNAs (Figure 5B). On the contrary, targeted silencing of Lin28B in LOX-IMVI cells significantly increased expression levels of let-7 miRNAs (Supplementary Figure 6, available at *Carcinogenesis* Online). Therefore, these findings confirm an inverse relationship between Lin28B and let-7 miRNAs in melanoma.

To assess the relevant Lin28B/let-7 targets more specifically, we focused on a short list of genes that were consistently regulated by Lin28B in all our three cell models (Figure 5C). A strong correlation was observed between Lin28B and Hmga2, a putative target gene of Lin28B/let-7 (21,22). Hmga2 has been shown to serve as a competing endogenous RNA for the let-7 miRNA family, leading to the activation of TGF-β signaling pathway in non-small-cell lung cancer (44). Interestingly, we found that the TGF-β co-receptor Tgfbr3 was upregulated by Lin28B, and that a TGF-β pseudo-receptor Bambi (45) was downregulated by Lin28B (Figure 5C). These observations led us to speculate that Lin28B might regulate the activity of TGF-β pathway in melanoma. Indeed, knockdown of Lin28B inhibited the activation of TGF-β1-induced pSmad2, whereas overexpression of Lin28B promoted the activation of pSmad2 (Figure 5D). Thus, Lin28B functions, at least partially, through the TGF-β signaling axis in melanoma. Interestingly, exogenous TGF-β1 did not significantly promote melanoma cell growth (Supplementary Figure 7, available at *Carcinogenesis* Online), and we speculated that endogenous TGF-β1 produced by melanoma cells was sufficient to drive an autocrine pathway to affect cell physiology. It is also important to point out that our finding cannot rule out the possibility that other potential mechanisms downstream of Lin28B/let-7 may play a role in regulating melanomagenesis.

## Discussion

In this study, we have outlined a novel melanomagenic pathway in which the oncofetal RNA-binding protein Lin28B promotes melanoma progression by mediating a miRNA regulatory circuit. Our data demonstrated that Lin28B operates as a novel driver of melanoma tumorigenesis. Together with reports implicating Lin28B in other cancer types (24,27,29,34,35), our findings suggest that antagonizing Lin28B function in human malignancies may provide a means to reactivate the expression of let-7 family tumor suppressors, and may thus be therapeutically effective. Intriguingly, one rational target identified by our integrative analyses is the activation of TGF-β pathway downstream of Lin28B/let-7. This latter finding offers an alternative treatment approach and suggests a rationale for testing the therapeutic benefit of inhibiting TGF-β signaling in melanoma, particularly in tumors with Lin28B overexpression.

We showed that Lin28B is highly expressed in a considerable proportion of both melanoma cell lines and clinical specimens. However, our copy number analysis indicated that genetic amplification is unlikely the main mechanism for Lin28B upregulation. On the other hand, modulation of Lin28B levels could be achieved through epigenetic regulation such as miRNAs. Indeed, we identified miR-125a-5p, which directly targets Lin28B, to be downregulated in melanoma cells compared with normal melanocytes. Our finding was consistent with previous reports indicating Lin28 as a direct target of miR-125a/miR-125b (46,47), but specifically focused on its novel role in melanoma development. Considering other miRNAs reported to inhibit Lin28B in multiple cancers (30,35,48), it is tempting to propose a general model that Lin28B is aberrantly expressed in tumors largely due to the deregulation of tumor-suppressive miRNAs. In addition to overexpression, we found that Lin28B is somatically mutated in a subset of melanoma patients and further research is warranted to investigate the biological function of these mutations.

Although both Lin28A and Lin28B selectively repress the expression of let-7 miRNAs, we only observed Lin28B expression in melanoma whereas Lin28A appeared undetectable by western blot in all cell lines tested. Previous studies have illuminated the distinct molecular mechanisms by which Lin28A and Lin28B function to inhibit let-7 expression. Lin28A is predominantly cytoplasmic and recruits Zcchc11 to pre-let-7 to induce 3′-uridylation, thereby blocking Dicer cleavage and let-7 maturation. Unlike Lin28A, Lin28B localizes primarily to the nucleus and blocks let-7 maturation by binding to pri-let-7 and interfering with Drosha processing (49). Regarding to the upstream regulation of Lin28, we speculate that different mechanisms may exist to specifically regulate the expression of Lin28A and Lin28B. For example, Lin28A and Lin28B might exhibit lineage-specific expression. In addition, Lin28A was reported to contain two conserved miR-125 binding sites in its 3′UTR region (46), whereas we and others found that Lin28B only had one. Therefore, despite their high degree of homology, Lin28A and Lin28B may be involved in distinct oncogenic processes, such that in the case of melanoma, Lin28B plays a prominent role to promote tumor growth.

In conclusion, our results show that miR-125a-5p is underexpressed in melanoma and highlights the biological significance of miR-125a-5p in regulating Lin28B. Lin28B inhibits let-7 family miRNAs and activates TGF-β signaling pathway, thereby promoting melanoma tumor growth. Our data identify Lin28B as a novel oncogene in melanomagenesis and elucidate a previously unrecognized miRNA circuit underlying melanoma progression (a schematic model is shown in Figure 5E). These findings expand our understanding of molecular pathogenesis of human melanoma and indicate potential therapeutic avenues for the treatment of this aggressive disease.

## Supplementary material

Supplementary Figures 1–7 and Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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*Conflict of Interest Statement:* None declared.

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