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The oncogenic RNA-binding protein Musashi1 is regulated by tumor suppressor miRNAs

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Abbreviations: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GFP, green fluorescent protein; hg18, human genome build 18; mRNA, messenger RNA; Msi1, musashi1; MTS, (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PEST, proline-glutamic acid-serine-threonine peptide sequence; RBP, RNA-binding protein; RT-PCR, reverse transcription-polymerase chain reaction; shRNA, short hairpin RNA; UTR, untranslated region

Musashi1 (Msi1) is an evolutionarily conserved RNA-binding protein that has been implicated in processes like stem cell fate, nervous system development and tumorigenesis via its activities as a specific regulator of translation. While Msi1 is barely detected in normal adult tissue, it has been observed to be highly expressed in numerous tumor types (e.g., breast, colon, medulloblastoma, glioblastoma, et cetera). Unfortunately, the molecular cues that are responsible for Msi1 upregulation in cancer cells are largely unknown. Tumor suppressor microRNAs (miRNAs) are known for targeting genes with oncogenic properties like Msi1 and for being either downregulated or deleted in tumor tissue. We observed that Msi1 long 3'UTR region is potentially targeted by several tumor suppressor miRNAs (miR-34a, -101, -128, -137 and -138). Western blotting of endogenous Msi1 protein as well as luciferase assays confirmed Msi1 regulation by these tumor suppressor miRNAs. Furthermore, we observed when examining different cellular states that these miRNAs and Msi1 have opposite expression profiles. Cell proliferation inhibition induced by the tumor suppressor miRNAs was partially rescued by Msi1 transgenic expression. We conclude that tumor suppressor miRNAs are direct and influential regulators of Msi1, affecting its expression pattern during tumorigenesis of malignant nervous system tumors.

Introduction

RNA-binding proteins (RBPs) recognize specific motifs in pre-mRNAs and mRNAs and by doing so, regulate gene expression via splicing, transport, stability, localization and translation. RBPs can contribute or lead to tumor formation when aberrantly expressed as they can interfere with the expression of specific gene subsets in cell cycle control, proliferation, apoptosis and differentiation.[1,2]

Musashi1 (Msi1) is an RBP that has been connected to the development of multiple tumor types.[3-9] Msi1 is evolutionarily conserved, being initially identified in *Drosophila melanogaster* where it is required for development of adult external sensory organs (sensilla) and for maintenance of germ-line stem cell identity.[10,11] In vertebrates, Msi1 has been equally implicated in nervous system development. Its expression in adults is mainly restricted to stem and precursors cells.[12] Msi1 defines a population of multipotent stem cells in the brain, intestinal crypt cells, breast and hair follicles.[13-20] Msi1 has been determined to be highly expressed in several malignancies, such as glioblastoma multiforme, medulloblastoma, cervical carcinoma, lung and colon cancers.[3-9] A connection between Msi1 expression and prognosis was established in the case of gliomas.[5] A study done with HCT116 colon cancer cells revealed that knockdown of Msi1 produces tumor growth arrest in xenografts, reduces cancer cell proliferation and increases apoptosis alone and in combination with radiation injury.[21] Similar results were obtained with breast cancer cells where Msi1 was determine to be a negative prognostic indicator of breast cancer patient survival, and an indicator of tumor cells with stem cell-like characteristics. A recent study illustrates that Msi1 can function as a proto-oncogene. Intestinal epithelium progenitor cells overexpressing Msi1 showed an increase in proliferation via the activation of Wnt and Notch pathways. Importantly, these cells acquired tumorigenic properties as observed in xenograft experiments.[22]

A very important question is which regulatory elements trigger Msi1 high expression in cancer cells. Its long 3' untranslated region (UTR) suggests that post-transcriptional regulation might play a major role; this would include the action of microRNAs (miRNAs). In fact, several miRNAs have been determined

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Table 1. Identified Msi1-targeting tumor suppressor miRNAs

microRNA ID	Chromosomal Coordinates	Deregulation in Cancer	miRNA Targets	Biological/Cellular Processes	References
hsa-miR-34a	1:9211727-9211836 [-]	↓ in retinoblastoma, neuroblastoma, uveal melanoma, hepatocellular carcinoma, hematological malignancies, glioblastoma, lung cancer, cervical carcinoma	*SIRT1, NOTCH1, JAG1, FOXP1,* E2F pathway	Cell proliferation, transcriptional regulation, apoptosis, histone modification, apoptosis, DNA repair	30–32, 50, 57–68
hsa-miR-101	1:65524117-65524191 [-]	↓ in anaplastic large cell lymphoma, gastric cancer, lung cancer, prostate cancer, hepatocellular carcinoma, colon cancer, endometrial serous carcinoma, transitional cell carcinoma, glioblastoma	*PTGS2, EZH2, MAGI2, APP, MCL1, FOS*	Cyclooxygenase production, apoptosis, cell proliferation, TGFβ production, chromatin organization, histone modification, transcriptional regulation	33, 69–80
hsa-miR-128	2:136422967-136423048 [+]	↓ in endometrial carcinoma, prostate cancer, neuroblastoma, glioblastoma	*FOXO1, RELN/DCX, BMI1, E2F3a, BAX*	Transcriptional regulation, cell cycle regulation, cell proliferation, apoptosis	34–36, 81–84
hsa-miR-137	1:98511626-98511727 [-]	↓ in colon cancer, head/neck squamous cell carcinoma, glioblastoma, oral squamous cell carcinoma, melanoma	*MIB1, CDC42, MITF*	Cell cycle regulation, cell proliferation, neural differentiation	33, 85–89
hsa-miR-138	3:44155704-44155802 [+]	↓ in anaplastic thyroid carcinoma, head/neck squamous cell carcinoma, glioblastoma	*TERT, EID1, EZH2*	Telomere maintenance, cell differentiation	90–93

The chromosome, chromosomal coordinates, and strandedness are listed for each miRNA. Each of the studied miRNA have been shown to be downregulated and implicated in the pathogenesis of many different cancers targeting many different mRNAs implicated in various cancer-related cellular and biological processes.

to function as tumor suppressors; when downregulated or deleted in tumor cells, this event can lead to increased expression of oncogenic proteins such as Musashi1.[23] We have identified several miRNAs (mir-34a, miR-101, miR-128, miR-137 and mir-138) that affect Msi1 expression. They have been shown to function as tumor suppressors and to be downregulated in malignant tumors of the nervous system. We suggest then that the downregulation of these miRNAs in cancer cells could contribute to an increase in Msi1 expression and subsequently to tumor formation.

Results and Discussion

Musashi1 expression is regulated by microRNAs. One of the recent developments in the study of malignant tumors of the nervous system is the participation of microRNAs (miRNAs). Each microRNA can target hundreds of mRNAs, and many mRNAs may be targeted by multiple microRNAs, thus creating a miRNA-mediated genetic regulatory network with complex topological features. In recent studies, microRNA profiling of malignant tumors of the nervous system had identified a specific subset of miRNAs being deregulated, most likely due to genomic rearrangements or miRNA promoter methylation.[24] Several of these miRNAs have been later shown to function as tumor suppressor miRNAs as their downregulation can lead to activation of oncogenic pathways.[23] Interestingly, some of these microRNAs have also been implicated in neural stem cells and neural development, thus intertwining stem cell biology and tumorigenesis.[25] Since Musashi1 is equally implicated in both processes, we believe that miRNAs, particularly tumor suppressor miRNAs, might play a role in its regulation. Moreover, the Msi1 mRNA has a long 3' untranslated region (1,797 nucleotides in length), making it an excellent candidate for miRNA regulation.[26-28]

For the identification of putative Msi1-targeted miRNAs, we conducted an in silico search using the TargetScanHuman webform.[29] To narrow down the list, we specifically searched for tumor suppressing miRNAs that have been implicated in malignant tumors of the nervous system. **Table 1** lists the miRNAs that have been evaluated in our studies. Putative tumor suppressor miRNAs selected as candidates for Msi1 regulation includes miR-34a, miR-101, miR-128, miR-137 and miR-138.[30-38] A synopsis of their profiling studies, mRNA targets and impact on biological functions is summarized in **Table 1**. These miRNAs have decreased or no expression in brain tumors, as compared to normal brain tissue.[25] When their expression is restored in cancer cells these miRNAs can slow down cellular proliferation and promote neural differentiation; additionally, these miRNAs have also been linked to stem cell self-renewal.

We first studied the regulation of Msi1 by these tumor suppressor miRNAs by functional analysis. The interaction between the miRNA and the Msi1 3' UTR is shown in **Figure 1A**. We expressed the miRNA mimics in U251 glioblastoma and Daoy medulloblastoma cells and evaluated its impact on Msi1 expression by RT-qPCR and western blot. Transfection efficiency was monitored by quantitative PCR (**Fig. S1**). With all five selected miRNAs, repression of Msi1 is seen at the protein level (**Fig. 1B and C**). Additionally, all five miRNAs were able to partially decrease Msi1 mRNA levels by 20 to 60% in U251 glioblastoma cells and 25 to 60% in Daoy medulloblastoma cells (**Fig. 1D and E**), suggesting that the regulation takes place both at translation and mRNA decay steps.[39,40] This combined regulation could explain the strong effect observed at the protein level. However, further mechanistic studies of the miRNA action on Msi1 mRNA are needed to complement the end point data presented here. We then utilized a reporter assay to confirm that the selected miRNA targets the Msi1 3' UTR region.[41] The Msi1 3' UTR region was

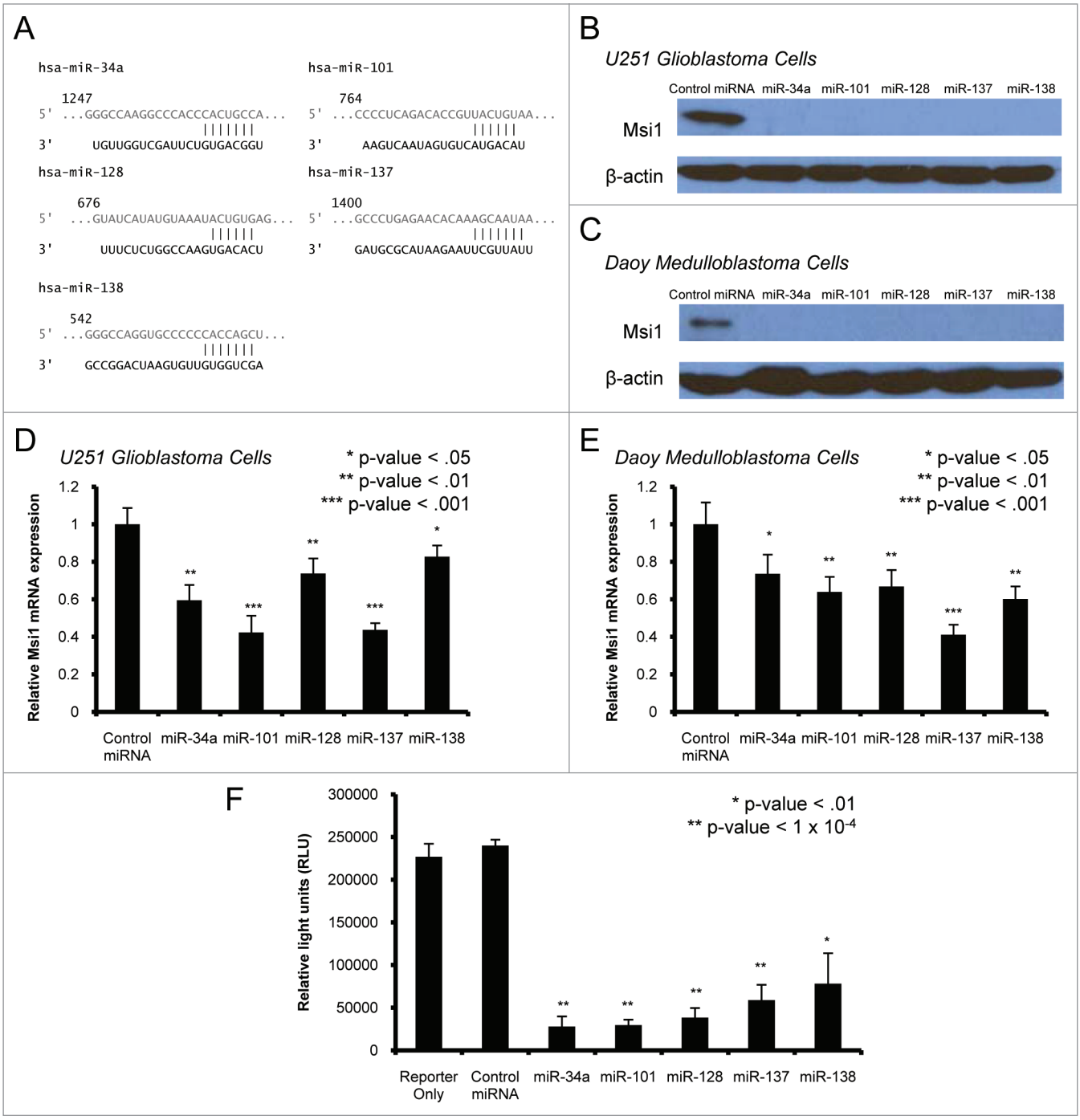

Figure 1. Putative tumor suppressor microRNAs repress Musashi1 at the 3' untranslated region. (A) Predicted Msi1 mRNA:miRNA binding interactions. The region of the Msi1 3' UTR (light gray) that corresponds to the miRNA binding site is shown, with the position of the first shown nucleotide numbered above (5' to 3' direction). The miRNA (black) is shown in the 3' to 5' direction. Watson Crick base pairing interaction is defined between the mRNA and the miRNA seed region with vertical lines. U251 glioblastoma cells (B) and Daoy medulloblastoma cells (C) were transfected with miRNA mimics and Musashi1 protein expression was assessed by western blotting. A scrambled negative control miRNA (Control miRNA) was also transfected as a negative control for miRNA function. β-actin was included as an endogenous loading control. U251 glioblastoma cells (D) and Daoy medulloblastoma cells (E) were transfected with miRNA mimics and Musashi1 messenger RNA levels was assessed by RT-qPCR. A scrambled negative control miRNA (Control miRNA) was also transfected as a negative control for miRNA function. Data was normalized using the $2^{-\Delta\Delta Ct}$ methodology and normalized to *ACTB* mRNA levels. (F) HeLa cells were cotransfected with a Msi1 3' UTR luciferase reporter vector with putative miRNAs mimics and luminescence was measured. The reporter by itself (Reporter Only) and the reporter cotransfected with a scrambled negative control miRNA (Control miRNA) were included as a negative control. Experiments were performed in triplicate. Data was analyzed with Student t-test, comparing mRNA levels of the miRNA mimic transfection to that of the control miRNA expression level. The mean and standard error of measurement is displayed.

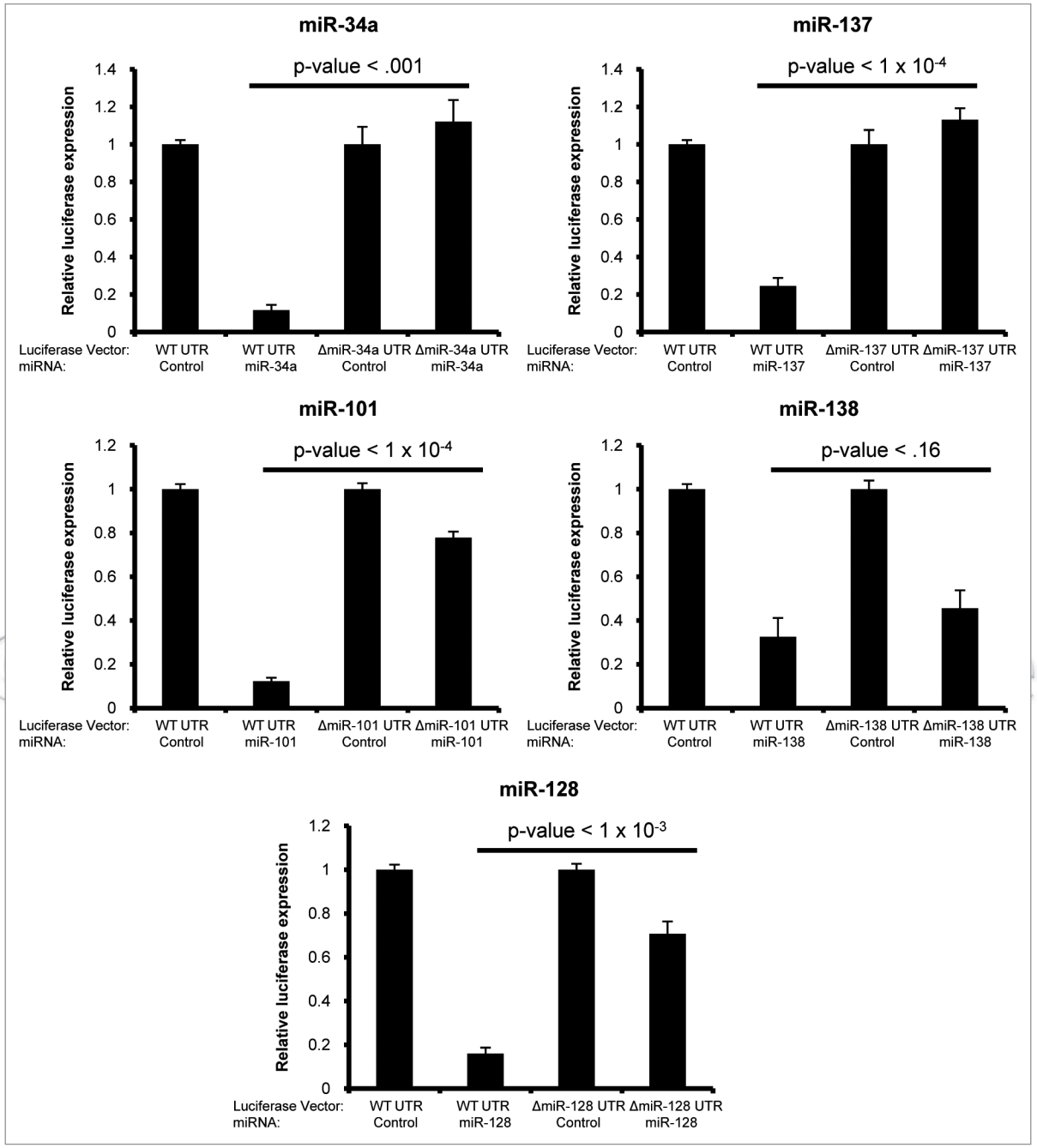

Figure 2. Putative Msi1-targeted tumor suppressor miRNAs act on the Msi1 3' UTR at predicted miRNA binding sites. Deletions of Target Scan Human predicted sites on the luciferase Msi1 3' untranslated region construct were performed using a high-fidelity PCR-based procedure. Cotransfections of luciferase:Msi1 3' UTR construct and miRNA mimics were performed in HeLa cells using Thermo Scientific Dharmafect Duo transfection reagent. A control miRNA was used as a negative control. Deletion of predicted miRNA sites abolished the miRNA-mediated regulation as reflected by luciferase results. The only exception was miR-138, which was still of capable of decreasing the expression of its cognate 3' UTR deletion construct. Experiments were performed in triplicate. Data was analyzed with Student t-test, comparing the mutated UTR luciferase expression to that of the wild type UTR. The mean and standard error of measurement is displayed.

cloned downstream of a destabilized luciferase gene containing a PEST sequence.[42] The reporter assay indicated that all the miRNAs were able to repress the expression of luciferase (**Fig. 1F**).

Furthermore, deletion of the putative miRNA binding sites in the luciferase:UTR vector rendered the UTR refractory to repression by the miRNA mimic, further confirming the action of the selected tumor suppressor miRNAs via the predicted targets sites (**Fig. 2**). The only exception was miR-138. The deletion of the predicted site caused only a partial effect on miRNA-mediated regulation. We later observed that the luciferase gene (*luc2*) contains putative binding sites for miR-138 that may mask the results of our deletion analysis. Two 6-mer miR-138 binding

sites were identified in the open reading frame for the *luc2* gene. To determine that miR-138 is able to target the luciferase open reading frame, its mimic was cotransfected with the pGL4 vector, which contains a constitutively-driven *luc2* gene and lacks a 3'UTR region. As predicted, repression of luciferase expression was observed (**Fig. S2**). Regardless the result of the luciferase experiments, we are confident that Msi1 is a target for miR-138 as evident from the profound impact of miR-138 on the endogenous Msi1 mRNA and protein levels (**Fig. 1B–E**).

To evaluate if the different deletion constructs were not deleterious to the function of the 3' UTR, each deletion construct was cotransfected with two noncognate miRNAs; in all cases, the noncognate miRNAs were able to repress the luciferase deletion construct, signifying that the deletion did not affect the features of the 3' UTR (**Fig. S3**).

Evolutionary conservation of miRNA sites in the Msi1 3' UTR region. We used the MULTIZ 44-way vertebrate genome alignment referenced on human hg18 to analyze conservation of the miRNA target sites in the 3' UTR of Msi1.[43] Although the Msi1 coding sequence shows conservation through all vertebrates, portions of the human 3' UTR are absent outside mammals. Conservation within aligned sequences of the 3' UTR is quite high. A portion at the 3' terminus of the annotated RefSeq transcript for Msi1, covering approximately 580 bases, is conserved through mammals, birds and reptiles, while only very small parts of this segment appear to be conserved in some fish. Among the 5 target sites we examined, only miR-34a and miR-137 sites are within these 580 bases. The seed segment of the miR-34a target site is only conserved through primates (**Fig. 3**). The miR-137 seed site is conserved perfectly in all species but zebrafish (**Fig. 3**). The miR-138, miR-128 and miR-101 sites appear not to exist outside mammals. The nucleotide-level conservation in these sites shows very high conservation, with very few substitutions among mammals.

miRNAs and Msi1 have opposing patterns of expression. In neural stem cells, Musashi1 is highly expressed while it is barely detected in mature neurons.[13] These findings suggest that Musashi1 plays important roles in maintaining neural stem cell identity and that its expression needs to be downregulated to allow for neuronal differentiation.[44] However, it is unclear how Msi1 is regulated during this process. Recent studies in miRNAs and nervous system development suggest that miRNAs play vital roles in maintaining neural stem cell identity and lineage specification.[45] Therefore, we suggest that the miRNAs in this study can contribute to a downregulation of Msi1 during cell differentiation. The dual roles of these tumor suppressor miRNAs implies an overlap between development and cancer.[25]

We utilized a neuroblastic differentiation assay to monitor changes in both Msi1 and miRNA expression. In this assay, SK-N-BE neuroblastoma cells, which display high Msi1 expression, can be differentiated along the neuronal lineage using all-trans retinoic acid for a period of one week.[46] On gross visualization, cell morphology changes are evident by the development of neuritic processes that extends from the cell body when induced with all-trans retinoic acid (**Fig. 4A**). The decrease in Msi1 expression after all-trans retinoic acid-induced differentiation is seen at the mRNA (**Fig. 4B**) and protein level (**Fig. 4C**). Consequently, we hypothesize that the miRNAs we selected to analyze should have a higher expression after differentiation to suppress protein output of Msi1 after differentiation. Using quantitative real-time PCR, we observe an increase in miR-34a, miR-101, miR-137 and miR-138 but not miR-128 (**Fig. 4D**) ranging from a 2- to 5-fold over undifferentiated cells.

Breast cancer and medulloblastoma cells maintained under conditions that favor spheroid formation and consequently, the expansion of cells with stem-like characteristics, show higher Msi1 expression when compared to cells grown as monolayers.[3,47] Similarly, in two glioblastoma multiforme lines established from patient samples,[48] we observed a 2- to 3-fold increase in Msi1 mRNA levels when cells were grown as tumorspheroids (**Fig. 5A**). Interestingly, we observed that miR-34a, miR-101, miR-128 and miR-137 but not miR-138 have an opposite pattern of expression, having a higher expression in monolayers than in the tumorspheres (**Fig. 5B**). We then suggest that a decrease in expression of miRNAs targeting Msi1 in cancer cells with stem-like characteristics allows for a higher expression of Msi1.

To determine if Msi1 is required for the proliferation of glioblastoma tumorspheroids, we created a stable cell line expressing a short hairpin RNA directed against Msi1. A control line was created with a non-silencing shRNA. Forty-eight hours after plating, cells were counted. Msi1 knockdown cells showed ~50% reduction in growth when compared to control, suggesting that Msi1 is a major contributor of "cancer stem cell" proliferation in glioblastoma (**Fig. 5C**). We then explored the effects of the miRNAs on glioblastoma tumorspheroid proliferation. In all cases, transfection of miRNA mimics decreased cell proliferation; miR-34a had the most profound effect, reducing proliferation by approximately 60%, while miR-101 only reduced cell proliferation by approximately 27% (**Fig. 5D**).

Msi1 transgenic expression partially suppresses miRNA-induced inhibition of cell proliferation. To ascertain that the tumor suppressing miRNAs act, in part, through Musashi1 regulation, we performed a rescue experiment in U251 glioblastoma cells. A stable U251 glioblastoma cell line containing a Musashi1 transgene was established. The vector utilized to create the stable cell line contains a constitutive elongation factor 1α promoter and lacks any Msi1-specific regulatory elements such as the 3' UTR, allowing the ectopic expression of Msi1 to be "immune" to any miRNA-mediated regulation. A complementary U251 cell line overexpressing green fluorescent protein was used as negative control. Quantitative RT-PCR confirms that the cell line express the Msi1 transgene (**Fig. S4**). When miRNA mimics were expressed, cell proliferation was reduced in the control GFP cell line (**Fig. 6**). Although, Msi1 transgenic expression did not fully reinstate the wild type levels of proliferation, the impact of miRNA mimics on cell proliferation was definitely less pronounced (**Fig. 6**). The partial recovery is explained based on the repression of other putative miRNA targets, some of which have been previously studied (**Table 1**).

While many studies have identified putative tumor suppressing and oncogenic miRNAs in malignant nervous system tumors, fewer studies have identified and characterized targets of these

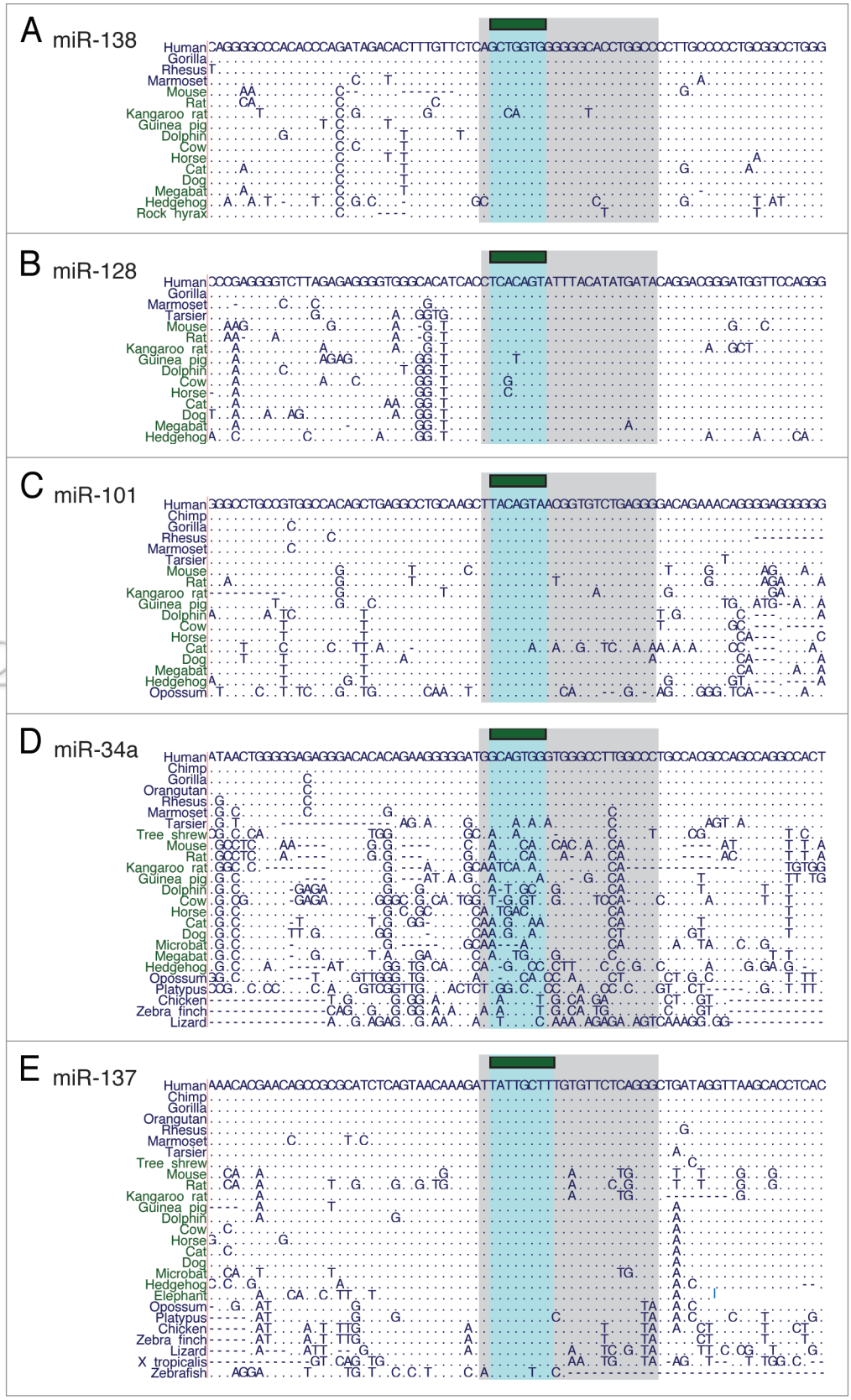

Figure 3. Multiple-species alignments of miRNA target sites in the Msi1 3' UTR, including flanking sequence. Alignments include only species for which orthologous sequence exists at corresponding sites.

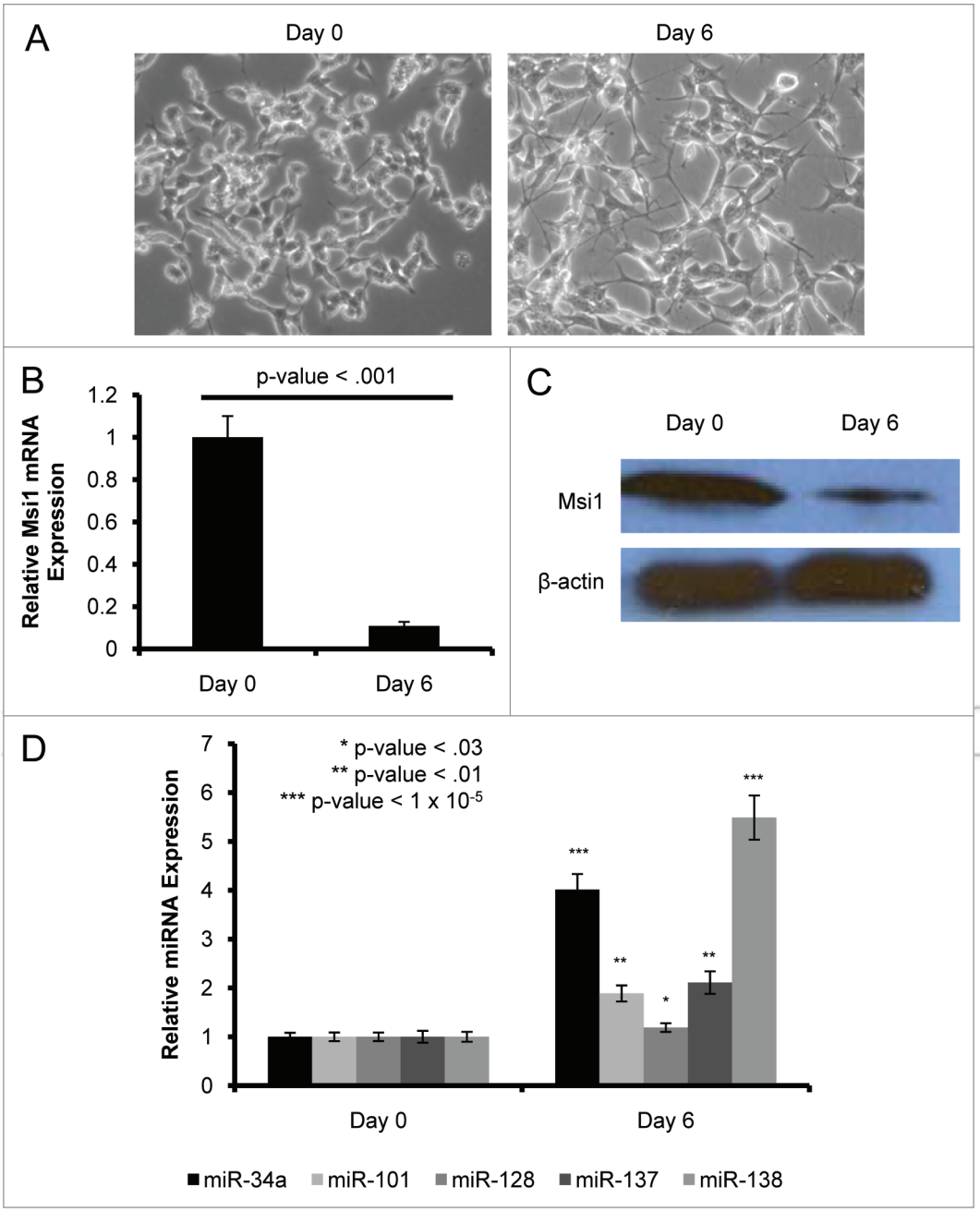

Figure 4. All-trans retinoic acid-mediated induction of neuronal differentiation of SK-N-BE neuroblastoma cells causes downregulation of Msi1 expression and concomitant increase in miRNA expression. (A) SK-N-BE cells were differentiatied with 10 uM of all-trans retinoic acid for a period of 6 days. On visual inspection, potent formation of neuritic processes are seen after six days of differentiation induction with all-trans retinoic acid. After 6 days of differentiation with all-trans retinoic acid, a downregulation of Msi1 expression is observed at the mRNA level (B) and at the protein level (C). The *ACTB* mRNA was used for normalization when using the $2^{-\Delta\Delta Ct}$ method for mRNA relative quantification and β-actin was included as an endogenous protein loading control. (D) After differentiation, miRNA expression increased when measured with miRNA relative quantification ($2^{-\Delta\Delta Ct}$ method) using the *RNU48* C/D box snoRNA as an endogenous control. Experiments were performed in triplicate. Data was analyzed with Student t-test, comparing the expression levels of either Msi1 or the miRNAs at time zero to that after 6 days of differentiation. The mean and standard error of measurement is displayed.

miRNAs. In our study, we suggest that the oncogenic RNA-binding protein Musashi1 is highly regulated by a combinatorial group of tumor suppressor miRNAs in malignant nervous system tumors, a connection of multiple miRNAs:single target phenomenon that is only beginning to be appreciated.[49] Regulation of RNA-binding proteins by miRNAs is a rational mechanism by which miRNAs can indirectly regulate the expression of downstream target mRNAs in addition to its direct mRNA targets, thus broadly influencing the biology, behavior and physiology of a cancer cell. Moreover, the important role of miRNAs in genetic

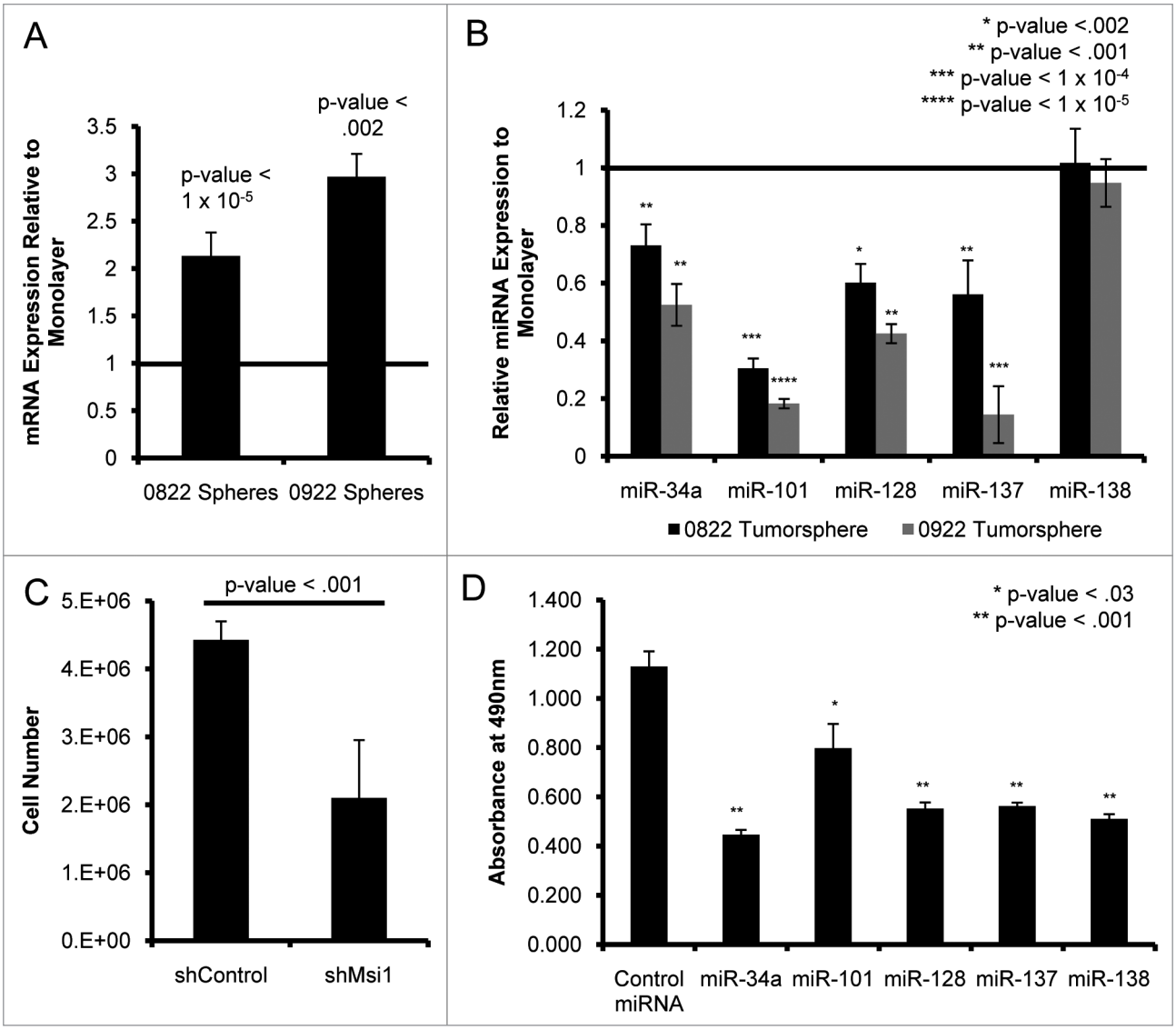

Figure 5. Primary glioblastoma cell proliferation is regulated by Msi1 through a reduction of miRNA expression. (A) When primary glioblastoma cells are grown in a stem cell-enriching, free-floating spheroid formation in serum-less Neurobasal media supplemented with basic fibroblast growth factor and epidermal growth factor, Msi1 mRNA expression levels increase relative to levels in primary glioblastoma cells growth in a serum-supplemented, adherent, monolayer condition. Expression was assessed in two primary glioblastoma samples. The *ACTB* mRNA was used for normalization when using the $2^{-\Delta\Delta Ct}$ method for mRNA relative quantification. A horizontal line crossing the ordinate at a value of 1 indicates the relative expression level in the monolayer culture. (B) Putative Msi1-targeting tumor suppressor miRNAs expression decrease when primary glioblastoma cells are grown in a neurophere condition as compared to a monolayer culture with the exception of miR-138. The *RNU48* C/D box small nucleolar RNA was used as an endogenous control when using the $2^{-\Delta\Delta Ct}$ method for miRNA relative quantification. Experiments were performed in triplicate. Data was analyzed with Student t-test, comparing expression levels in the spheroid conditions to that of the monolayer. The mean and standard error of measurement is displayed. A horizontal line crossing the ordinate at a value of 1 indicates the relative expression level in the monolayer culture. (C) Stable silencing of Msi1 was prepared in primary glioblastoma cells after transduction of a lentivirus carrying a short hairpin RNA expression construct and selection using 3 ug/mL of puromycin. A control cell line was prepared with a non-silencing shRNA. 5 x 10^5 cells were plated in a 24-well plate and counted 48 hours later. A decrease of cell proliferation was observed in the silenced Msi1 cell line, as determined by cell counting. (D) miRNA mimics were transfected into the primary glioblastoma cell line and cell proliferation was monitored 48 hours later. With all five miRNA mimics, a decrease in cell proliferation was observed in the primary glioblastoma cell line, as measured by MTS assay. Experiments were performed in triplicate. Data was analyzed with Student t-test, comparing proliferation in the miRNA mimic experiment to that of the control miRNA. The mean and standard error of measurement is displayed.

regulatory networks has been underscored by the realization of miRNA-mediated networks in managing noise and robustness in an ever-changing environment, organizing developmental signals and potentiating oncogenic transformation.

For instance, in our study, we demonstrate that Msi1 is a target of miR-34a. Knowing that Notch-1 is also a target of miR-34a[50] and that Msi1 is involved in the Notch-1 and Hes-1 expression via its repressive function on Numb, we could speculate that

miR-34a may act in a coherent type I feed forward regulatory network (**Fig. 7A**) in the Notch signaling pathway via Msi1 and Notch-1 (**Fig. 7B**).[3,19,21,22,47,51-54] Finally, in addition to target identification, our study offered insight into potential microRNA therapeutic replacement modalities for the interventional treatment of malignant nervous system tumors through the suppression of the Musashi1 RNA-binding protein.[55]

Material and Methods

Musashi1 untranslated region conservation analysis. Cross-species conservation was taken from full-genome alignments of 44 vertebrates, as available from the UCSC Genome Bioinformatics group; the alignment was constructed using the MULTIZ algorithm.[43]

Cell culture. SK-N-BE neuroblastoma, Daoy medulloblastoma, U251 glioblastoma and HeLa cervical adenocarcinoma cell lines were obtained from American Type Culture Collection (American Type Culture Collection, Manassas, VA). SK-N-BE, U251 and Daoy cell lines were propagated in Dulbecco's Modified Essential Medium (Thermo Scientific, Rockford, IL), which contained 10% fetal bovine serum, penicillin and streptomycin. HeLa cells were maintained in culture in Minimum Essential Medium (Thermo Scientific) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Primary glioblastoma tumorspheres were obtained from patient samples at the Cancer Therapy & Research Center at the University of Texas Health Science Center at San Antonio and propagated in neurobasal media containing L-glutamine, N2 supplement, B27 supplement, heparin, epidermal growth factor (EGF) (Peprotech, Inc., Rocky Hill, NJ) and basic fibroblast growth factor (bFGF) (Peprotech, Inc.). For growth of primary glioblastoma cells as monolayers, the cells were cultured in the presence of Dulbecco's Modified Essential Medium with 10% FBS and pen/strep.

For the establishment of Msi1 ectopic expression U251 cell line, the Msi1 coding region was cloned into pEF1/Myc-His A mammalian expression vector (Invitrogen, Carlsbad, CA). GFP was cloned as a negative control vector. pEF1/Myc-His-Msi1 and pEF1/Myc-His-GFP were transfected into U251 cells using the Lipofectamine 2000 transfection reagent (Invitrogen). G418 (800 ug/mL) was used as a selection agent. The cells were propagated for 2 weeks and quantitative RT-PCR used to evaluate transgenic expression.

Stable primary glioblastoma cell lines expressing the Msi1 short hairpin RNA (shRNA) were created utilizing lentiviral transduction. A control non-silencing shRNA directed is used as a negative control. Lentiviruses harboring a shRNA expression cassette was obtained from Open Biosystems (Open Biosystems, Huntsville, AL) and lentiviral particles were prepared according to manufacturers' protocol. After transduction, stable transformants were selected using 3 ug/mL of puromycin (Invitrogen) in neurobasal media supplemented with L-glutamine, N2 supplement, B27 supplement, heparin, epidermal growth factor (EGF) (Peprotech, Inc.) and basic fibroblast growth factor (bFGF) (Peprotech, Inc.).

Microscopy. Microscopic images of cell morphology were acquired using a Zeiss Axiovert 200 M epifluorescence microscope (Carl Zeiss Microimaging, LLC, Thornwood, NY) equipped with a 63x oil immersion objective.

Transfection. U251 glioblastoma and Daoy medulloblastoma cells were reverse transfected with miRNA mimics with Lipofectamine RNAiMAX transfection reagent (Invitrogen). For luciferase assays, HeLa cells were co-transfected with the pSGG-Msi1 reporter vector and miRNA mimic using DharmaFECT

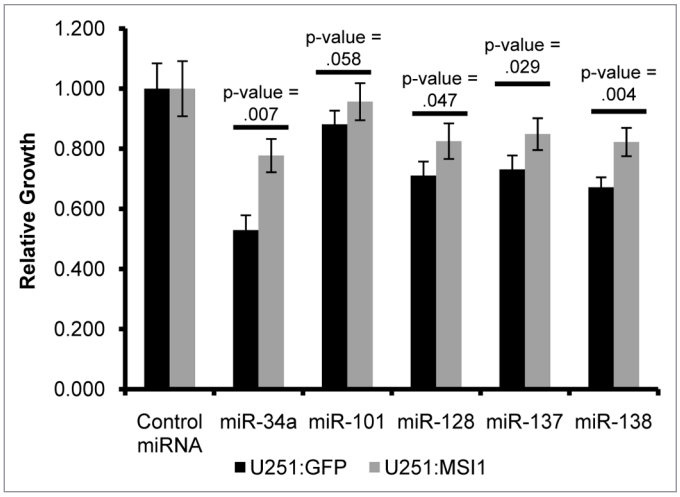

Figure 6. Complementation by ectopically expressed Msi1 partially reverses cell proliferation suppression induced by miRNA mimics. miRNA mimics were transfected in the GFP and Msi1 overexpressing cell lines. Cell growth was measured by the MTS assay 48 hours post-transfection. The effect on growth suppression was partially reversed in the Msi1 overexpressing stable U251 cell line. A control miRNA was included as a negative control for miRNA transfection. Student t-test was used to perform the statistical analysis. Experiments were performed in triplicate. The mean and standard error of measurement is displayed.

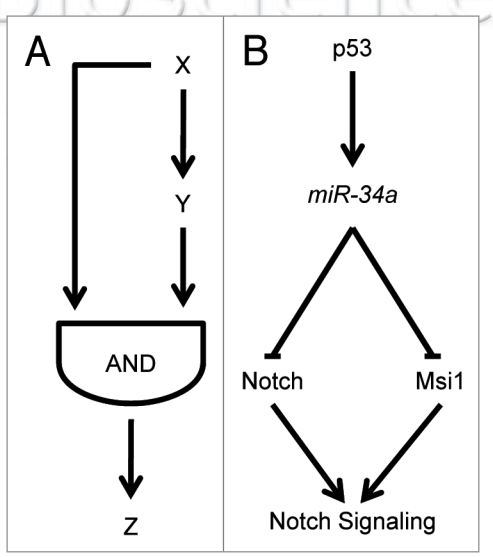

Figure 7. miR-34a may act in a coherent type I feed forward module in the Notch signaling pathway. (A) Schematic of a coherent type I feed forward network motif. (B) Hypothetical coherent type I feed forward network motif involving miR-34a, Msi1 and the Notch signaling pathway.

Duo transfection reagent (Thermo Fisher Scientific, Dharmacon Products, Lafayette, CO).

Cell differentiation. SK-N-BE cells were plated at a density of 1 x 10^5 cells per 100 mm culture dish. Of all-trans retinoic acid, ten uM solubilized in 200-proof ethanol, were added to each culture dish for the specified amount of time. The media and all-trans retinoic acid were changed every 24 h.

Plasmids and mutagenesis. pSGG-Msi1 3' untranslated region chimeric reporter vector were obtained from Switchgear Genomics (Menlo Park, CA). Deletion of putative miRNA binding sites was performed using a optimized high fidelity PCR-based procedure described by L.O.F. Penalva and J. Valcárcel [Technical Tips Online (tto.trends.com)].[56]

Luciferase assays. Luciferase assays were performed according to Boutz et al.,[41] 7.5 x 10^3 cells were plated 24 hours priors to transfection in each well in a 96-well cluster plate. 100 ng of pSGG-Msi1 3' UTR reporter vector was mixed with 10 pmol of the miRNA mimic in OptiMEM (Invitrogen). 0.15 uL of DharmaFECT Duo transfection reagent (Thermo Fisher Scientific, Dharmacon Products) was added. After formation of the nucleic acid:lipid complex, the transfection solution was overlaid onto the previously plated HeLa cells. After incubation for 24 hours, the HeLa cell extract was prepared using the Reporter Lysis Buffer from Promega (Madison, WI). The 100 uL of Luciferase Assay Reagent was added to 20 uL of cell lysate, and the luminescence was read on a Berthold Technologies AutoLumat LB 953 Multi-Tube luminometer (Berthold Technologies, Oak Ridge, TN). As a control procedure, potential variation in the luciferase assay was measured by 6 separate transfection preparations of the reporter vector and a control miRNA; we did not observe any remarkable variation in luciferase expression (**Fig. S5**).

RNA preparation and qRT-PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen). Briefly, TRIzol was added to the cells for lysis and dissociation of any RNA:protein complexes. Chloroform was added for phase separation. Total RNA, located in the aqueous phase, was precipitated using isopropyl alcohol. After centrifugation, the RNA pellet was washed in 75% ethanol and resuspended in nuclease-free water.

Reverse transcription of miRNAs was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and the miRNA gene-specific reverse primer from the TaqMan MicroRNA Assay (Applied Biosystems). Reverse transcription of messenger RNAs was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random priming. After reverse transcription, quantitative PCR was performed using the TaqMan primer/probe set in the TaqMan MicroRNA Assay for miRNA analysis or Gene Expression Assay (Applied Biosystems) in TaqMan Gene Expression Master Mix (Applied Biosystems) for mRNA analysis. Real-time PCRs were performed on a 7500 Real Time PCR System (Applied Biosystems). Data was acquired using the SDS 2.0.1 software package (Applied Biosystems) and analyzed using the $2^{-\Delta\Delta Ct}$ method using the *RNU48* C/D box small nucleolar RNA gene as an endogenous control for miRNAs or β-actin as an endogenous control for Msi1 mRNA.

Western blotting. U251 glioblastoma and Daoy medulloblastoma cells were transfected in 100 mm culture plates for Musashi1 protein analysis. After 48 h of incubation post-transfection, cells were scraped from the plate and pelleted. After centrifugation, cells were resuspended and sonicated in 2x SDS Laemmli sample buffer. Cell lysates were ran on a Tris-glycine-SDS PAGE gel that has a 4% stacking gel and 10% resolving gel. After electrophoresis, a semi-dry transfer procedure was carried out onto a nitrocellulose membrane. After transfer, the membrane was blocked in Tris-buffered saline with Tween 20 and 5% skim milk. The membrane was probed with either a rabbit monoclonal anti-Musashi1 antibody (Abcam, Cambridge, MA) or mouse monoclonal anti-β-actin antibody (Abcam). HRP-conjugated goat anti-rabbit antibody (Santa Cruz Biotechology, Santa Cruz, CA) was used as a secondary antibody for Musashi1 or HRP-conjugated goat anti-mouse antibody (Zymed Laboratories, Carlsbad, CA) was used as a secondary antibody for β-actin. Electrochemiluminescence was used to detect the Musashi1 or β-actin protein.

MTS cellular proliferation assay. Stable transgenic Msi1 expressing (or transgenic GFP expressing negative control) U251 glioblastoma cells were transfected with miRNA mimics using RNAiMAX transfection reagent (Invitrogen) in a 96 well plate. After 2 days incubation, cell proliferation was measured with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS assay) (Promega). After incubation with the assay reagent for 1 hour, absorbance at 490 nm was measured on a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT).

Statistical analysis. Student's t-test was performed on all analysis, comparing the control miRNA or siRNA experiment to that of the miRNA mimic or Msi1 siRNA data. p-values less than 0.05 were considered statistically significant. All data was performed in triplicate and the data is presented as the mean ± standard error of measurement.

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Note

Supplemental materials can be found at:
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