

# NF- $\kappa$ B–YY1–miR-29 Regulatory Circuitry in Skeletal Myogenesis and Rhabdomyosarcoma

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## SUMMARY

Studies support the importance of microRNAs in physiological and pathological processes. Here we describe the regulation and function of miR-29 in myogenesis and rhabdomyosarcoma (RMS). Results demonstrate that in myoblasts, miR-29 is repressed by NF- $\kappa$ B acting through YY1 and the Polycomb group. During myogenesis, NF- $\kappa$ B and YY1 downregulation causes derepression of miR-29, which in turn accelerates differentiation by targeting its repressor YY1. However, in RMS cells and primary tumors that possess impaired differentiation, miR-29 is epigenetically silenced by an activated NF- $\kappa$ B–YY1 pathway. Reconstitution of miR-29 in RMS in mice inhibits tumor growth and stimulates differentiation, suggesting that miR-29 acts as a tumor suppressor through its promyogenic function. Together, these results identify a NF- $\kappa$ B–YY1–miR-29 regulatory circuit whose disruption may contribute to RMS.

## INTRODUCTION

MicroRNAs (miRNAs) are small noncoding single-stranded RNAs that constitute a novel class of gene regulators. These RNAs are initially transcribed by RNA polymerase II as primary transcripts (Lee et al., 2004; Zeng et al., 2005) that are then processed in the nucleus by Drosha into an ~70 nucleotide precursor miRNA that forms hairpin structures (Gregory et al., 2005). Precursor miRNAs are then exported into the cytoplasm and serve as substrates to generate mature miRNAs (He and Hanon, 2004). Approximately 50% of miRNAs are found in clusters, transcribed as polycistronic primary transcripts (Mourelatos et al., 2002).

miRNAs negatively regulate gene expression at the posttranscriptional level by base pairing with the 3' untranslated region (UTR) of their target mRNAs. It is believed that if this pairing is perfect or nearly perfect, as seen predominantly in plants (Gregory

et al., 2005), the mRNA becomes cleaved and degraded (Pattanayak et al., 2005). However, with most mammalian miRNAs, the pairing is imperfect, resulting in translational repression (Hutvagner, 2005). Since their initial discovery, more than 3000 miRNAs have been identified in animals, plants, and viruses. With over 500 miRNAs in the human genome and a plethora of predicted mRNA targets, it is believed that these small RNAs have an enormous regulatory potential in gene expression programs (Bartel, 2004). Indeed, the functions of miRNAs have been found to extend to both physiological and pathological conditions, including cell proliferation, cell death, differentiation, development, metabolism, viral infection, and cancer (Miska, 2005; Shivdasani, 2006).

One cellular process under miRNA control is skeletal muscle differentiation. This process is orchestrated by the transcription factors MyoD, Myf5, myogenin, MRF4, and Mef2. These factors activate muscle genes to coordinate myoblasts to terminally

## SIGNIFICANCE

MicroRNAs regulate skeletal myogenesis, but their impact in muscle diseases is not well understood. Here we describe miR-29 as an enhancer of myogenic differentiation and a suppressor of rhabdomyosarcoma (RMS). We find that miR-29 exists in a regulatory circuit involving NF- $\kappa$ B and YY1. In myoblasts, NF- $\kappa$ B acts through YY1 to epigenetically suppress miR-29, while during differentiation, miR-29 is induced to facilitate myogenesis by a negative feedback on YY1. Significantly, RMS tumors lose miR-29 due to an elevation in NF- $\kappa$ B and YY1, and readjustment of miR-29 levels in RMS stimulates differentiation. Thus, myogenesis is dependent on NF- $\kappa$ B–YY1–miR-29 circuitry whose dysfunction may contribute to RMS pathogenesis. Such findings offer potential avenues for the diagnosis and treatment of muscle-relevant cancers.

withdraw from the cell cycle and subsequently fuse into multinucleated myotubes (Sabourin and Rudnicki, 2000). Several muscle miRNAs are regulated by these myogenic transcription factors and are required for skeletal muscle formation. For instance, MyoD and Mef2 regulate expression of miR-1 that suppresses HDAC4, resulting in augmented Mef2 activity (Rao et al., 2006; Zhao et al., 2005). MyoD induces miR-206, which targets DNA polymerase to facilitate cell-cycle exit, as well as follistatin to enhance myogenesis (Anderson et al., 2006; Kim et al., 2006; Rosenberg et al., 2006). In addition, miR-133 synthesis is controlled by Mef2, but functionally this miRNA inhibits myogenesis by downregulating serum response factor (SRF), which maintains myoblast proliferation (Chen et al., 2006; Liu et al., 2007).

Analogous to muscle miRNAs, signal transduction pathways function within a hierarchical network to positively and negatively regulate myogenesis. Among these is NF- $\kappa$ B, which is active in myoblasts and functions to block differentiation *in vitro* and *in vivo* (Acharyya et al., 2007; Guttridge et al., 2000; Wang et al., 2007). NF- $\kappa$ B exists as a dimer, with the p50/p65 heterodimer form being the most common (Hayden and Ghosh, 2004). In resting cells, NF- $\kappa$ B is retained in the cytoplasm through binding of its I $\kappa$ B inhibitor. Classical activation of NF- $\kappa$ B occurs by factors that stimulate the I $\kappa$ B kinase complex to phosphorylate and degrade I $\kappa$ B, leading to NF- $\kappa$ B nuclear translocation and subsequent target gene expression (Karin and Ben-Neriah, 2000).

Recent work has demonstrated that NF- $\kappa$ B's ability to repress myogenesis occurs through multiple mechanisms that depend on components of the classical NF- $\kappa$ B pathway (Bakkar et al., 2008). One such mechanism involves the target gene Yin Yang 1 (YY1), which itself is capable of repressing muscle differentiation (Caretti et al., 2004; Lee et al., 1994; Wang et al., 2007). YY1 is also a member of the Polycomb group (PcG), which functions to silence transcription of a selected set of genes by chromatin modification (Sparmann and van Lohuizen, 2006). In myoblasts, NF- $\kappa$ B regulates YY1, which in turn binds and inhibits myofibrillar promoters by recruiting the PcG member Ezh2 as well as the histone deacetylase protein HDAC1 (Caretti et al., 2004; Wang et al., 2007). Thus, activation of NF- $\kappa$ B ensures that YY1 levels and Polycomb activity are maintained in undifferentiated cells.

In addition to their underlying etiology in a variety of muscle-related diseases including dystrophies and cachexia, a skeletal muscle lineage is also considered to be the origin of rhabdomyosarcoma (RMS), an aggressive, malignant pediatric soft-tissue sarcoma (Breitfeld and Meyer, 2005; Mackall and Helman, 2001). These tumors are thought to arise as a consequence of a dysfunctional balance of the growth and terminal differentiation of muscle progenitor cells. Despite current modes of intensive chemotherapy, radiation, and surgery, survival for children with advanced disease is dismal, and prognosis has remained unchanged for decades. RMS is divided into two major histological subtypes, embryonal and alveolar. Whereas embryonal RMS (ERMS) recapitulates the phenotypical and biological features of embryonic muscle, alveolar RMS (ARMS) is a more loosely organized tumor displaying poor muscle differentiation (Qualman et al., 1998). Current knowledge of the molecular mechanisms that contribute to the failure in differentiation of RMS tumors is limited. Although miRNAs are considered to act as tumor suppressors or oncogenes in an assortment of cancers, notably,

their potential role in RMS has not been explored. In this study, we demonstrate the regulation of miR-29 by the NF- $\kappa$ B-YY1 regulatory pathway and elucidate its significance in normal skeletal muscle differentiation and RMS.

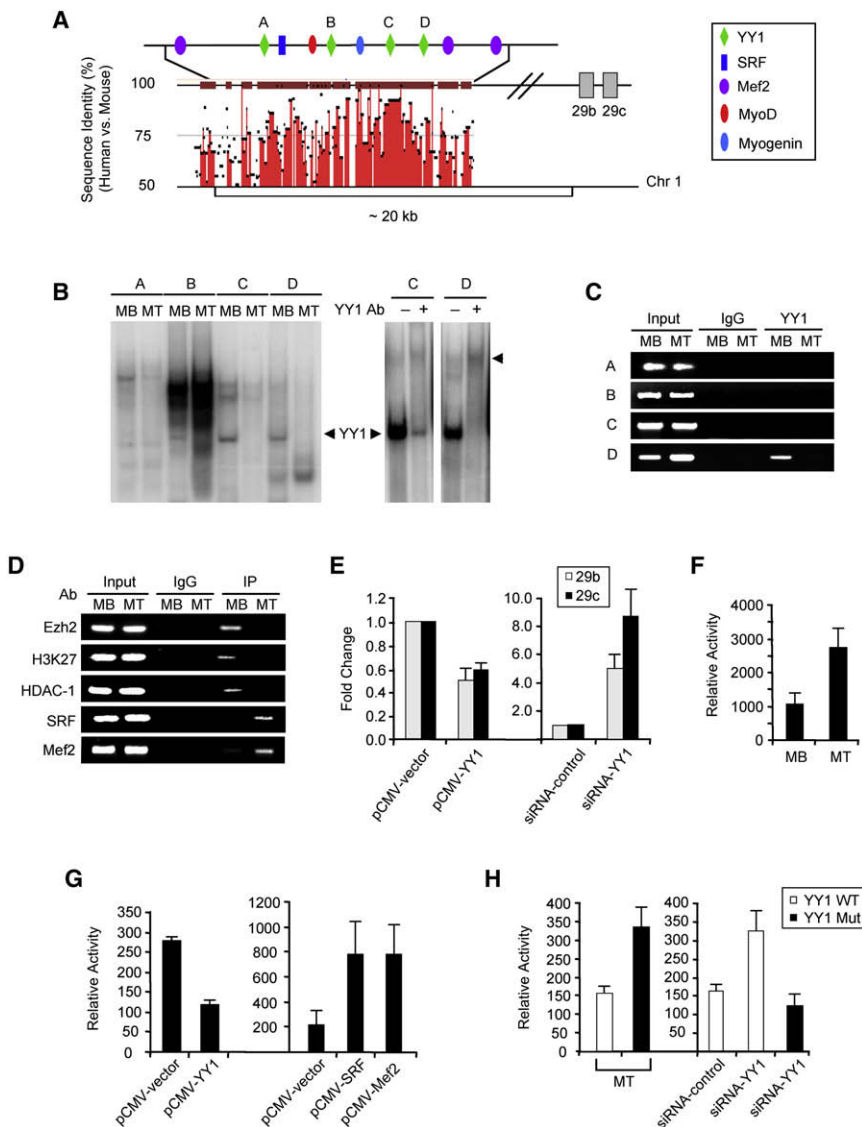
## RESULTS

### NF- $\kappa$ B and YY1 Negatively Regulate miR-29b/c in C2C12 Myoblasts

Although miRNAs are involved in the complex network of skeletal myogenesis, their interactions with other myogenic regulatory factors are only beginning to be revealed. Of particular interest is the regulation of miRNA by transcription factors. Since we recently found that NF- $\kappa$ B functions to inhibit myogenesis through YY1 (Wang et al., 2007), we initiated a screen for miRNAs potentially regulated by these two transcription factors. The use of positional weight matrix from TRANSFAC (Wingender et al., 1997) identified numerous YY1 binding sites upstream of pre-miRNAs. To narrow our search, we utilized the UCSC genome browser (Kent et al., 2002) and rVISTA (Loots and Ovcharenko, 2004) to find YY1 sites conserved among species. This gave rise to a handful of miRNAs that represented potential targets of YY1 (see Table S1 available online). Among these, we singled out miR-29b2 and miR-29c, which lie in close proximity to each other on mouse and human chromosome 1 (Figure S1A). Four YY1 binding sites (referred to as A, B, C, and D) were identified in a highly conserved region ~20 kb upstream of miR-29b2 and miR-29c (Figure 1A; Figure S1B). This same region also contains several conserved *cis*-elements for the myogenic transcription factors MyoD, myogenin, Mef2, and SRF. In comparison, no conserved YY1 sites were identified upstream of miR-29a and miR-29b1, clustered on mouse chromosome 6 and human chromosome 7 (Figure S1A).

To test whether sites A through D upstream of the miR-29b2 (hereafter referred to simply as miR-29b) and miR-29c locus were competent for YY1 binding, electrophoretic mobility shift assays (EMSA) were performed with extracts prepared from C2C12 myoblasts and myotubes. Results showed that all four sites produced binding complexes in myoblasts (MB) (Figure 1B), but only complexes C and D were supershifted with YY1 antisera. Furthermore, in line with previous findings that YY1 activity decreases in myogenesis (Caretti et al., 2004; Wang et al., 2007), complex C and D were also reduced in myotubes (MT). However, by chromatin immunoprecipitation (ChIP), only site D was bound by YY1 (Figure 1C). Consistent with EMSA, binding was only detected in MB, suggesting that YY1 binds to a putative regulatory element of miR-29b/c.

Since YY1 represses myogenic genes through recruitment of the PcG and HDAC1 (Caretti et al., 2004), we asked whether similar regulation occurred on the miR-29b/c locus. Indeed, in addition to YY1, binding for Ezh2 and its trimethylated activity on histone H3 lysine 27 (H3K27) was also detected, along with HDAC1 (Figure 1D). In MT, these repressors were replaced with SRF and Mef2, whose presence is associated with activation of muscle-specific genes. To further investigate the regulation of miR-29b/c by YY1, C2C12 myoblasts were transfected with a YY1 expression plasmid. By quantitative RT-PCR, miR-29b and miR-29c levels decreased 2-fold in the presence of YY1 (Figure 1E). In contrast, siRNA knockdown of YY1 significantly



**Figure 1. YY1 Represses miR-29b/c through Binding to a Conserved Regulatory Region**

(A) An rVISTA schematic showing the degree of sequence conservation between human and mouse chromosome (Chr) 1 in a region upstream of the miR-29b/c cluster. Predicted YY1, MyoD, myogenin, SRF, and Mef2 sites are displayed.

(B) EMSA performed with C2C12 myoblasts (MB) or myotubes (MT) using probes corresponding to YY1 sites A–D. With MB extracts, a supershift EMSA was performed using YY1 antisera. Arrowheads denote YY1/DNA-bound complexes.

(C) ChIP assays for YY1 were performed with chromatin from C2C12 MB or MT. Precipitated DNA was amplified with oligonucleotides spanning regions A–D. Total inputs are indicated.

(D) ChIP assays as in (C) were repeated for Ezh2, H3K27, HDAC1, SRF, and Mef2.

(E) MB were transfected with either a YY1 expression plasmid (pCMV-YY1) or YY1 siRNA oligos and then induced to differentiate for 24 hr, at which time miR-29b and miR-29c were measured by qRT-PCR and normalized to U6. Fold changes are shown with respect to control siRNA-transfected cells, where miR-29 levels were set to a value of 1. (F) MB were transfected with a miR-29b/c-enhancer-Luc reporter and maintained as MB or differentiated in MT for 48 hr, at which time luciferase activity was determined.

(G) MB were transfected with a miR-29b/c-enhancer-Luc reporter along with plasmids for YY1, SRF, or Mef2. Cells were then switched to differentiation conditions and subsequently measured for luciferase activity.

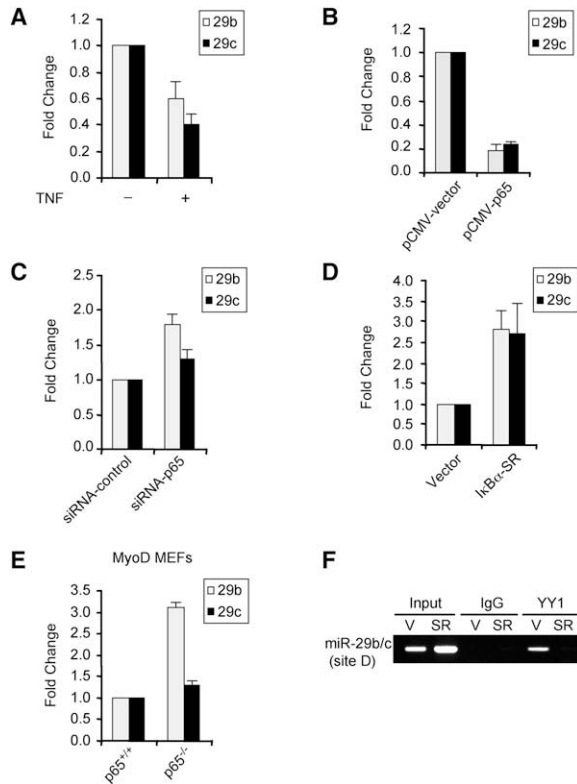
(H) MB were transfected with the miR-29b/c-enhancer-Luc reporter (YY1 WT) or with an enhancer reporter containing a deletion mutation in the YY1 D site (YY1 Mut) and subsequently differentiated for 48 hr (MT), at which time luciferase activity was determined (left graph). Separate transfections were performed with YY1 WT and YY1 Mut reporters along with a YY1 expression plasmid (pCMV-YY1) or YY1 siRNA (right graph). Cells were subsequently differentiated for 48 hr, at which time luciferase activity was determined. Vector siRNA oligo-transfected cells were used as a control.

All luciferase data were normalized to  $\beta$ -galactosidase protein and represent the average of three independent experiments  $\pm$  SD.

enhanced expression of both miRNAs (Figure 1E; Figure S2A), suggesting that YY1 is a repressor of miR-29b/c. Next we tested the activity of the miR-29b/c upstream regulatory region by cloning a 4.5 kb fragment spanning YY1, SRF, and Mef2 binding sites into a luciferase reporter plasmid. Myogenesis caused an increase in reporter activity (Figure 1F). Furthermore, this activity was repressed by addition of YY1 but stimulated by either YY1 knockdown or addition of SRF or Mef2 (Figures 1G and 1H), suggesting that this region upstream of miR-29b/c functions as an enhancer. To examine the requirement of YY1 binding for enhancer activity, the D site was mutated and compared to its wild-type counterpart. Loss of the YY1 site led to higher reporter activity, and, compared to the wild-type enhancer, the mutant site was unresponsive to YY1 knockdown (Figure 1H). Collec-

tively, these data demonstrate that the miR-29b/c locus contains a regulatory region that is under negative control by YY1 and the PcG in MB and is positively regulated by myogenic transcription factors in MT.

Since YY1 is a transcriptional target of NF- $\kappa$ B (Wang et al., 2007), we reasoned that miR-29b/c should also come under negative control of NF- $\kappa$ B. Consistent with this thinking, treatment of C2C12 myoblasts with TNF $\alpha$  as an activator of NF- $\kappa$ B reduced miR-29b/c expression (Figure 2A). Similar reduction of miR-29 was observed in C2C12 cells stably expressing the p65 subunit of NF- $\kappa$ B (Figure 2B). Conversely, MB containing siRNA against p65 or expressing the I $\kappa$ B $\alpha$ -SR inhibitor of NF- $\kappa$ B led to higher levels of miR-29b/c as compared to control cells (Figures 2C and 2D; Figure S2B). Similar enhancement of miR-29



**Figure 2. NF-κB Negatively Regulates miR-29b/c**

(A) C2C12 cells were treated with TNF $\alpha$ , and miR-29 expression was measured by qRT-PCR normalized to U6. Fold changes are shown with respect to vector cells, where miR-29 levels were set to a value of 1.  
 (B) MB were transfected with either vector or p65 plasmid, and miR-29b/c levels were measured 48 hr posttransfection.  
 (C) MB were transfected with either vector or p65 siRNA oligos, and miR-29b/c expression was then measured as in (B).  
 (D) miR-29b and miR-29c were measured in MB stably expressing vector or I $\kappa$ B $\alpha$ -SR. Fold changes are shown with respect to vector cells, which were set to a value of 1.  
 (E) MyoD was stably expressed in p65<sup>+/+</sup> or p65<sup>-/-</sup> mouse embryonic fibroblasts (Bakkar et al., 2008), and qRT-PCR was performed for miR-29b and miR-29c.  
 (F) ChIP assays with YY1 or control IgG were performed on chromatin derived from either vector control (V)- or I $\kappa$ B $\alpha$ -SR (SR)-expressing MB. Primers specific to site D were used for PCR amplification. Total inputs are indicated. All quantitative data are represented as mean  $\pm$  SD.

was observed in p65<sup>-/-</sup> fibroblasts expressing MyoD (Figure 2E). In addition, ChIP analysis showed that YY1 was bound to the miR-29b/c locus in vector MB but absent in cells devoid of NF-κB (Figure 2F). These findings strongly suggest that NF-κB negatively regulates miR-29b/c via YY1.

**miR-29 Functions as a Positive Regulator of Myogenesis**

Results above led us to speculate that miR-29b/c might itself be regulated during muscle differentiation. To test this, we examined miR-29 in both in vitro and in vivo models of myogenesis. The results showed that levels of miR-29b/c steadily increased in differentiating C2C12 cells, correlating with induction of known differentiation markers and repression of its inhibitors NF-κB and YY1 (Figures 3A–3C). Interestingly, miR-29a was also induced

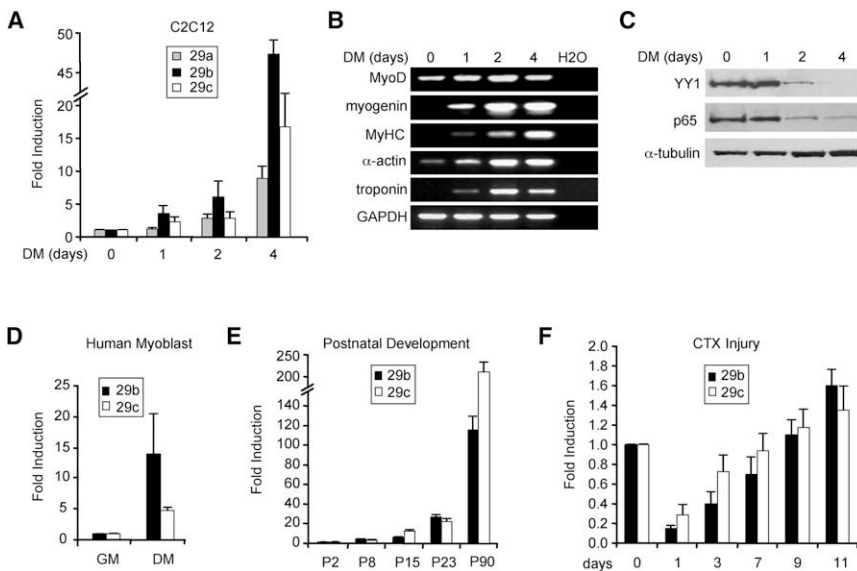
during myogenesis, albeit to lower levels, indicating that this miRNA might be subject to similar transcriptional silencing in MB. Regulation of miR-29b was not species specific since similar induction was seen in differentiating human muscle cells (Figure 3D). We also found that miR-29b/c was stimulated in developing postnatal muscle (Figure 3E), suggesting that this miRNA is relevant in skeletal muscle in vivo, and similar to cultured cells, there was an inverse relationship between miR-29b/c and NF-κB/YY1 expression (Figure S3A). Furthermore, in a cardiotoxin (CTX) model of muscle injury, miR-29b/c levels initially dropped and then steadily increased in accordance with the regenerative program. This regulation again correlated strongly with changes in YY1 (Figure 3F; Figure S3B). Altogether, these results support that miR-29 is positively regulated during muscle differentiation.

To test the functional relevance of miR-29 regulation during myogenesis, we employed a gain-of-function approach by ectopically expressing miR-29 in C2C12 MB with precursor miRNA oligos (Pre-29). Expression of miR-29 by this technique was verified by qRT-PCR (Figure S4A). Compared to control (Pre-NC), Pre-29 stimulated promoter activities of myofibrillar genes (Figure 4A) and increased endogenous MyHC and tropinin protein (Figure 4B) and RNA (Figure S4B). Although Pre-29c was used in this analysis, we observed that all three miR-29 members were capable of strongly enhancing muscle differentiation (Figure 4C; Figure S4C). To verify miR-29 specificity, transfections were repeated with a mutant form of Pre-29 lacking its seed region (Pre-29-mut). Results showed that MT formation was virtually identical in cells expressing Pre-29-mut compared to control (Figure S4D). To further confirm specificity, we carried out reciprocal loss-of-function experiments in which C2C12 MB were administered antisense RNA oligos (Anti-29), which were seen to successfully decrease endogenous miR-29 (Figure S4E). Compared to the control (Anti-NC), addition of Anti-29 caused a significant reduction in myogenic activity (Figure 4D) that correlated tightly with decreases in MyHC, tropinin, and MT formation (Figures 4E and 4F; Figure S4F). Taken together, these results suggest that miR-29 functions in muscle cells as an enhancer of differentiation.

**miR-29 Regulates Myogenesis through Feedback Inhibition of YY1**

We subsequently investigated the mechanism through which miR-29 regulates differentiation by searching for targets that might mediate its effect. Remarkably, one of the protein targets consistently predicted by three algorithms, TargetScan (Lewis et al., 2003), miRanda (Enright et al., 2003), and PicTar (Krek et al., 2005), was YY1. Based on this information and our results showing an inverse relationship in expression between miR-29 and YY1 during myogenesis (Figure 3), we speculated that miR-29 acts in a feedback loop to suppress YY1 and its antimyogenic activity.

A search for miR-29 binding sites within the YY1 3'UTR revealed that all miR-29 family members were predicted to hybridize to an evolutionarily conserved site among vertebrate species (Figure 5A; Figure S5A). Secondary structure analysis also showed a favorable minimum free energy in the formation of the miR-29:YY1 3'UTR duplex stem-loop (Figure 5B). Furthermore, a perfect match exists between the seed region of miR-29



**Figure 3. miR-29 Is Induced During Muscle Differentiation In Vitro and In Vivo**

(A) C2C12 MB were induced to differentiate up to 4 days in differentiation medium (DM), and at the indicated times, miR-29a, miR-29b, and miR-29c were measured by qRT-PCR.

(B) RT-PCR analysis of the myogenic markers MyoD, myogenin, MyHC IIb, skeletal actin ( $\alpha$ -actin), and troponin T performed at times similar to those in (A).

(C) Western blots probing for YY1 and nuclear p65 in differentiating C2C12 cells.

(D) Expression of miR-29b and miR-29c in primary human myoblasts (GM) and myotubes (DM).

(E) Measurement of miR-29 from either lower limb muscles at P15, P23, and P90 in C57BL/6 mice. (F) Same measurement as in (E) from cardiotoxin (CTX)-injected TA muscles. Quantitative values for miR-29 are represented as mean  $\pm$  SD.

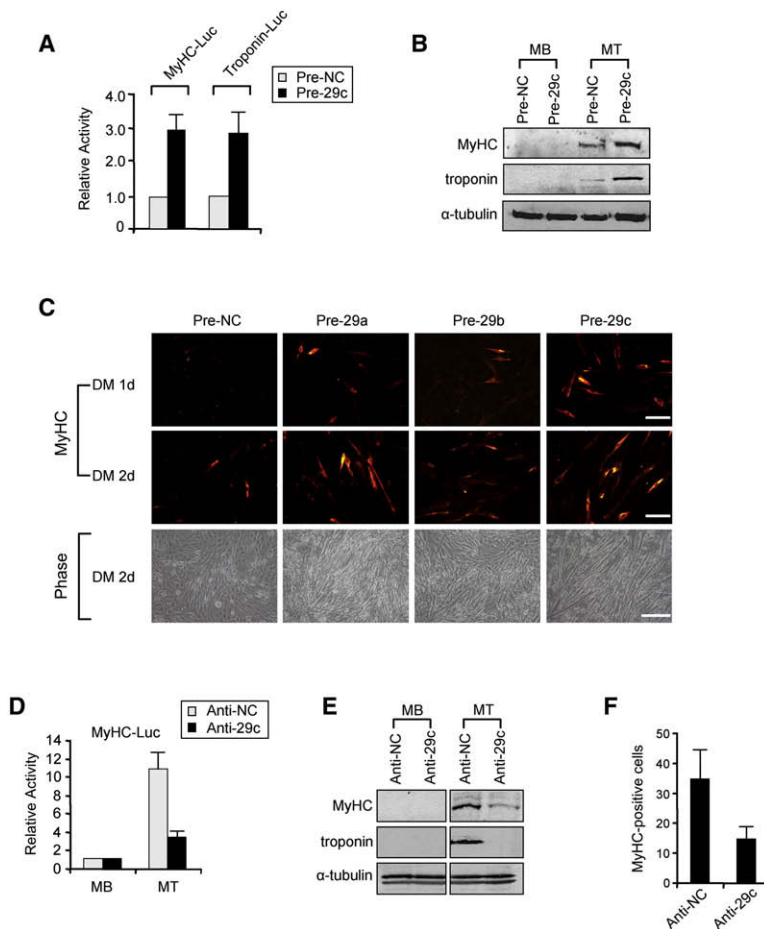
and the 3'UTR of YY1, suggesting that miR-29 is involved in translational repression of YY1. This was tested by cloning a 500 bp fragment of the YY1 3'UTR downstream of the firefly luciferase (*Luc*) gene. Cotransfections of this reporter (WT) with each of the miR-29 family members caused similar repressions of luciferase (Figure 5C). This was specific to miR-29 binding since luciferase was less affected when transfections were repeated with an irrelevant miR or with a mutant lacking the miR-29 binding site in the YY1 3'UTR. We also observed similar miR-29-mediated downregulation of luciferase when the full-length YY1 3'UTR was inserted downstream of the reporter (Figure S5B). At the functional level, we predicted that miR-29 binding to the YY1 3'UTR would lead to YY1 translational repression. Indeed, introduction of Pre-29 caused YY1 protein, but not RNA, to be reduced, while Anti-29 resulted in the opposite effect (Figure 5D; Figure S5C). To address whether YY1 targeting by miR-29 was relevant in vivo, we administered Anti-29 oligos in hindlimb muscles of neonatal mice and subsequently probed for YY1. Although some variability was observed with control oligos, addition of Anti-29 led to reproducible increases in YY1 (Figure 5E; Figure S5D). To ascertain the significance of these changes in YY1, we used siRNA to knockdown YY1 in differentiating C2C12 cells. Consistent with the role of YY1 as a negative regulator of myogenesis, depletion of YY1 caused a 2.3-fold increase in myotube formation (Figure 5F). Given that a similar phenotype was seen upon miR-29 overexpression in differentiating MB, this suggests that miR-29 action in skeletal muscle is largely mediated through negative feedback on YY1.

### miR-29 Functions as a Tumor Suppressor in Rhabdomyosarcoma

RMS cells are prohibited from terminal differentiation despite their commitment to a myogenic pathway. Our result showing that miR-29 promotes myogenesis led us to investigate whether miR-29 might be a contributing factor in RMS. Functionally, miR-29 acts as a tumor suppressor through its proapoptotic activity and targeting of the prosurvival products TCL (Pekarsky et al., 2006) and Mcl-1 (Mott et al., 2007). To address the role of

miR-29 in RMS, we examined its expression in cell lines derived from alveolar (ARMS) and embryonal (ERMS) RMS tumors. Significantly, miR-29b expression was strongly reduced in each of the RMS cell lines (Figure 6A), indicating that regulation of miR-29 does not discriminate among RMS subtypes. Consistent with these results, miR-29b was also highly suppressed in primary RMS tumors, and similar to the cell lines, this reduction did not discriminate between ARMS and ERMS (Figure 6B). To assess the specificity of this regulation with respect to miR-29, expression of two other muscle-associated microRNAs, miR-1 and miR-206, was examined in RMS. Whereas miR-1 was also reduced in most tumors, no uniform pattern of regulation occurred with miR-206 (Figures S6A and S6B), suggesting that RMS is not associated with a general downregulation of muscle-associated miRNAs.

To further investigate the tumor suppressor role of miR-29 in RMS, we tested the effects of ectopic expression of miR-29 in the RH30 ARMS cell line. Addition of Pre-29b oligos in these cells caused a 2-fold reduction in cell growth compared to Pre-NC control (Figure S6C). This difference in cell number was further enhanced when miR-29b was stably expressed using lentiviral (pMIF) infection (Figure 6C). Decreases in proliferation correlated with regulation of cell-cycle proteins, the cyclin-dependent kinase inhibitor p21<sup>CIP/WAF1</sup>, and cyclin D1 (Figure 6D). This regulation was also not specific to ARMS since a similar defect in growth was observed when Pre-29b was added to RD2 ERMS cells (data not shown). In addition, miR-29 caused only a mild effect on apoptosis and was not seen to affect expression of the prosurvival protein Mcl-1 (Figure S6D). Significantly, although RH30 tumor cells fail to undergo terminal differentiation, stable expression of miR-29b resulted in increased levels of the differentiation markers MyHC,  $\alpha$ -actin, and troponin (Figure 6E). Morphologically, the cell shape changed from a round to an elongated appearance, suggestive of an increased tendency toward differentiation (Figure 6F). Indeed, upon addition of differentiation medium, multinucleated myotube-like cells readily formed in miR-29b-expressing RH30 cells at levels approximately ten times that of vector (Figure 6G; Figure S6E). This effect was



**Figure 4. miR-29 Accelerates Muscle Differentiation**

(A) C2C12 cells were transfected with 0.2  $\mu$ g of MyHC-Luc or troponin-Luc reporters along with 0.2  $\mu$ g pCMV-LacZ and 50  $\mu$ M precursor control (Pre-NC) or miR-29c (Pre-29c) oligos. Cells were differentiated for 48 hr, and luciferase was determined and normalized to  $\beta$ -galactosidase. Relative activity is shown with respect to control cells, where normalized luciferase values were set to 1. Data represent the average of three independent experiments  $\pm$  SD.

(B) C2C12 MB were transfected with Pre-NC or Pre-29c oligos. Cells were then maintained as MB or differentiated into MT. Lysates were prepared and probed for MyHC IIb and troponin T.

(C) MB were transfected with Pre-NC or Pre-29a, b, or c members and differentiated (DM) for 1 or 2 days (d), at which time cells were immunostained for MyHC. Cell morphology was visualized by phase-contrast microscopy. Scale bars = 100  $\mu$ m for the top two rows and 200  $\mu$ m for the bottom row. (D) C2C12 cells were transfected with MyHC-Luc along with Anti-miR control (Anti-NC) or Anti-miR-29c (Anti-29c). Cells were then maintained as MB or differentiated into MT for 48 hr, at which time luciferase activity was determined. Reporter data represent the average of three independent experiments  $\pm$  SD.

(E) MB were administered Anti-NC or Anti-29c, and MyHC and troponin were then probed in cells maintained as MB or differentiated into MT.

(F) MB were transfected with Anti-NC or Anti-29c oligos, and cells were subsequently differentiated for 3 days, at which time cultures were stained for MyHC. Positively stained cells were counted from a minimum of ten randomly chosen fields from three individual plates and are represented as mean  $\pm$  SD.

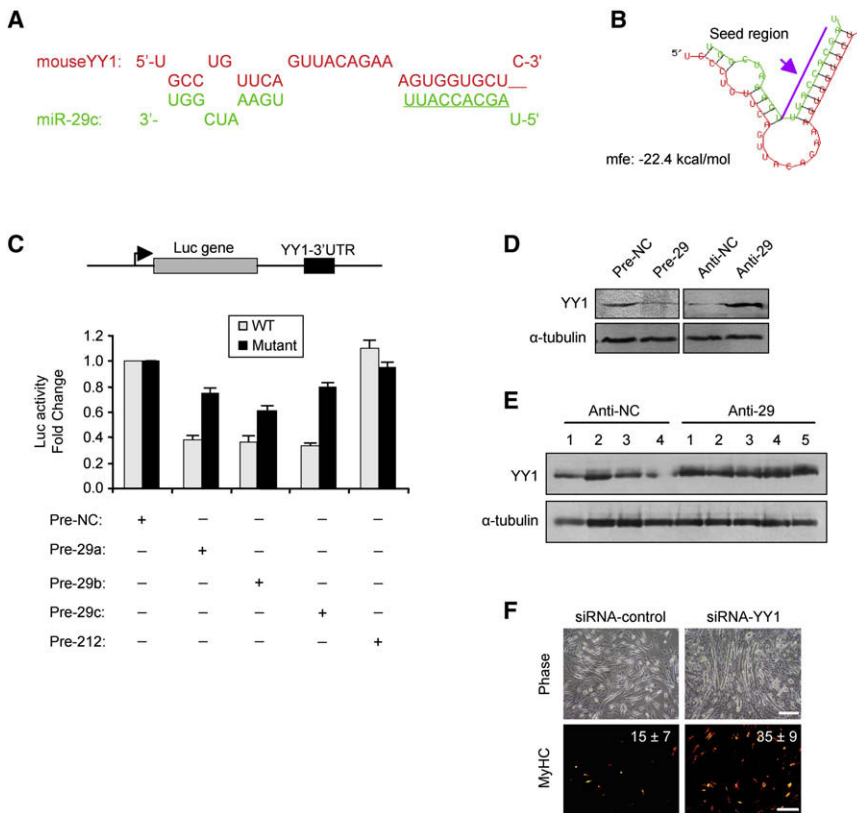
not specific to ARMS since similar differentiation-accelerating effects by miR-29b were observed in RD2 ERMS cells (Figures S6F and S6G). These results indicate that miR-29 limits RMS cell growth through its promotion of myogenic differentiation.

Next, we evaluated the antitumor effect of miR-29 in vivo by establishing RH30 tumors in immunocompromised mice. Tumors were allowed to reach a size of at least 0.1  $\text{cm}^3$ , at which time  $1 \times 10^9$  plaque-forming units (pfu) of pMIF-vector or pMIF-miR-29b-expressing lentivirus were administered intratumorally, and tumor size was monitored daily for a period of 3 weeks. Whereas tumors injected with vector control virus grew rapidly, with a doubling time of 2 days, tumors injected with miR-29b showed visibly slower growth, with cell doubling occurring at around 6 days (Figure 7A). Differences in tumor volumes became more apparent after 8 days postinjection and persisted until the experimental endpoint, when control tumors were on average 1.9 times larger than those injected with miR-29b ( $n = 6$ ;  $p = 0.0029$ ). In some animals, tumors were resected to evaluate the mechanism of miR-29 action. Hematoxylin and eosin (H&E) staining from both groups revealed densely packed, small, round cells with scant cytoplasm, consistent with the ARMS origin of the RH30 cell line (Figure 7B). Significantly, intratumoral addition of miR-29 stimulated myogenic differentiation, as evidenced by the increased expression of MyHC,  $\alpha$ -actin, and troponin (Figure 7C). This was associated with reduced cellular proliferation since Ki67 and phosphohistone H3 (p-H3) staining were

2.3- and 1.9-fold lower, respectively, in miR-29-injected tumors versus control (Figures 7B and 7D;  $p < 0.05$ ). Similar to in vitro analysis, we again found no clear evidence of apoptosis by TUNEL staining in miR-29-expressing tumors (Figure 7D), suggesting that in comparison to other malignancies, the proapoptotic activity of miR-29 may not be a primary tumor suppressor mechanism in RMS. To verify that our results were not a consequence of lentivirus delivery, RMS tumors were reestablished in nude mice and subsequently injected with Pre-29b versus control Pre-NC oligos. Analogous to lentivirus delivery, tumors administered with Pre-29b were on average 2.2 times smaller than controls (Figure 7E;  $n = 4$ ,  $p = 0.0047$ ). Collectively, these data support that miR-29 is a tumor suppressor in RMS functioning through its prodifferentiation activity.

#### Evidence of Dysregulation of NF- $\kappa$ B-YY1-miR-29 Circuitry in RMS

To further address the mechanism of miR-29 tumor suppressor activity in RMS, we considered YY1 as a likely downstream effector since this was a miR-29 target identified in differentiating muscle cells. Dysregulated expression of YY1 has also been associated with multiple cancers (Baritaki et al., 2007; de Nigris et al., 2006; Seligson et al., 2005). In line with our hypothesis, higher levels of YY1 were detected in RMS cell lines (Figure 8A) as well as in patient tumors (Figure S7A) compared to normal adjacent tissues (Figure 8B). Significantly, these results coincided



**Figure 5. miR-29 Suppresses YY1 through Binding to Its 3'UTR**

(A) Predicted target site of miR-29c (green) in the 3'UTR of mouse YY1 (red), with the seed region underlined.

(B) Predicted folding structure from mFOLD between miR-29c (green) and the YY1 3'UTR (red). The minimal free energy (mfe) is indicated, and the seed region is shown by the purple line and arrow.

(C) A wild-type (WT) luciferase reporter was generated by fusing an ~500 bp fragment of the YY1 3'UTR encompassing the miR-29 binding site downstream of the luciferase reporter gene. The mutant plasmid was generated by deleting the miR-29 binding site. WT or mutant reporter constructs were then transfected into MB with indicated precursor miRNA oligos. Luciferase was determined at 48 hr posttransfection and normalized to  $\beta$ -galactosidase. Data represent the average of three independent experiments  $\pm$  SD.

(D) MB were transfected with precursor (Pre-NC) or Anti-miR control (Anti-NC) or precursor miR-29c (Pre-29) or anti-miR-29c (Anti-29) oligos. YY1 protein was then probed in extracts from cells differentiated for 48 hr. Blots were stripped and reprobed for  $\alpha$ -tubulin.

(E) P5 neonatal mice were injected with Anti-miR control (Anti-NC) or Anti-miR-29c (Anti-29) oligos in lower limb muscles. Forty-eight hours post-injection, lysates were probed for YY1.

(F) MB were transfected with siRNA control or siRNA against YY1. Cells were then differentiated for 24 hr, at which time they were photographed under phase contrast or immunostained for MyHC. Numbers indicate averages of MyHC-positive cells counted from a minimum of ten randomly chosen fields, represented as mean  $\pm$  SD. Scale bar = 200  $\mu$ m at top and 100  $\mu$ m at bottom.

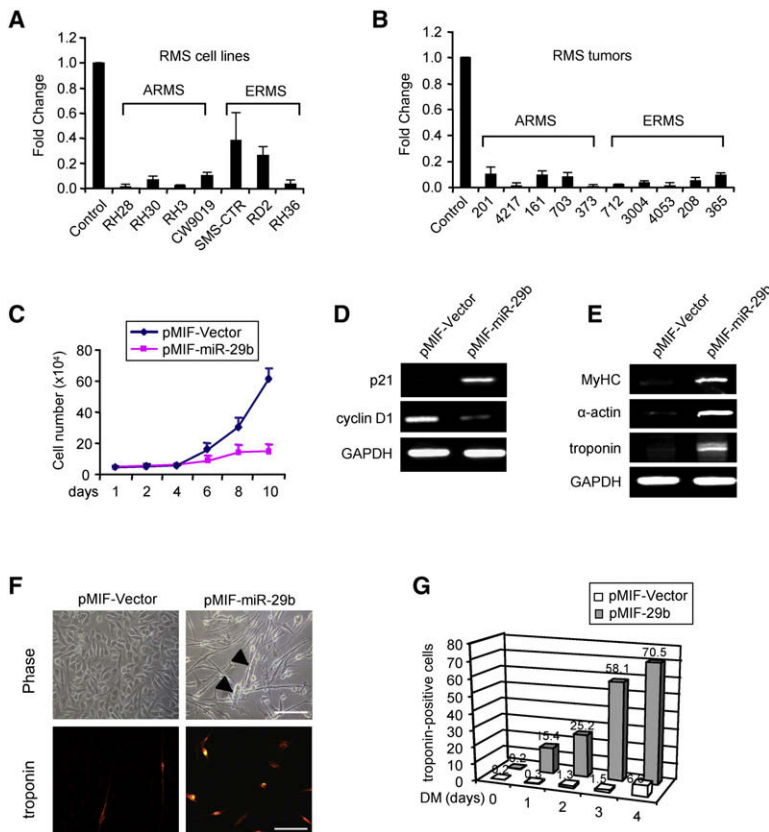
strongly with lower levels of miR-29 (Figure 6), implying that tumor suppressor activity of miR-29 occurred by targeting YY1. In support of this notion, expression of miR-29b in RH30 cells caused a concomitant decrease in YY1 (Figure S7B), and similar to what was observed in C2C12 MB, downregulation of YY1 in RH30 cells occurred through the conserved binding site in its 3'UTR, as ectopic expression of miR-29 repressed activity of luciferase reporter containing the wild-type, but not mutant, miR-29 binding site (Figure S7C). Furthermore, depletion of YY1 with siRNA in RH30 cells and xenograft tumors induced differentiation markers (Figure 8C and Figure 8D, respectively) comparable to what was observed with reconstituted miR-29. These data indicate that loss of miR-29 in RMS leads to dysregulated expression of YY1, thus favoring an undifferentiated phenotype.

In probing into the mechanism underlying the downregulation of miR-29 in RMS, we found that similar to YY1, Ezh2 was also pronouncedly elevated in RMS cell lines and patient tumors (Figures 8A and 8B). This suggested that epigenetic silencing by the PcG complex might account for loss of miR-29. Consistent with this thinking, YY1 and Ezh2 binding was detected on the miR-29b/c locus in RH30 cells, but interestingly, this binding occurred on site B (Figure 8E) rather than on the YY1 D binding site that was identified in C2C12 MB (Figure 1). The reason for this difference in binding site occupancy is not known. It is possible that such selectivity is dependent on the dosage of the PcG complex

or the presence of a tumor factor that preferentially directs YY1 binding. YY1 knockdown by siRNA oligos in RH30 cells led to an increase in miR-29b and to a lesser extent miR-29c (Figures S7D and S7E), supporting data above that YY1 is required for miR-29 suppression in RMS. Furthermore, this suppression was also regulated by NF- $\kappa$ B since addition of I $\kappa$ B $\alpha$ -SR lowered YY1 while concurrently increasing levels of miR-29b (Figure 8F). This was consistent with the increase in p65 detected in RMS cell lines and patient tumors (Figures 8A and 8B). Finally, we also observed that inactivation of NF- $\kappa$ B by stable expression of I $\kappa$ B $\alpha$ -SR in RH30 cells stimulated their myogenic potential as assessed by measurement of differentiation markers and quantitation of troponin-positive cells (Figures 8G and 8H; Figures S8A–S8C), which mirrored the phenotype caused by miR-29 overexpression (Figure 6). Together, these data support that dysregulation of NF- $\kappa$ B–YY1–miR-29 circuitry is a contributing factor for RMS tumorigenesis by interfering with the muscle differentiation program.

## DISCUSSION

This study was undertaken to investigate the potential interplay between the classical transcription factors NF- $\kappa$ B/YY1 and novel gene regulator miRNAs in both normal and abnormal myogenesis. Results led to the identification of miR-29 as a target of the



**Figure 6. miR-29 Functions as a Tumor Suppressor in Rhabdomyosarcoma**

(A) Normal human skeletal muscle cells were cultured along with alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS) cell lines, and qRT-PCR was performed to measure miR-29b.

(B) Total RNAs were obtained from ten RMS patient tumors (numbers represent patient identification), and expression of miR-29b was measured by qRT-PCR. Total RNAs from human skeletal muscle were used as the control. For (A) and (B), quantitative data for miR-29 values are represented as mean  $\pm$  SD.

(C) RH30 cells were transduced with vector or miR-29b-expressing lentiviruses, and stable cell lines were generated. Cell numbers were determined in triplicate by trypan blue staining over the course of 10 days; values are expressed as mean  $\pm$  SD.

(D) Total RNAs were extracted from vector or miR-29b-expressing RH30 cells, and semiquantitative RT-PCR was performed probing for p21<sup>CIP/WAF1</sup> and cyclin D1. GAPDH was used as a control.

(E) Analysis as in (D) was performed for the differentiation markers MyHC,  $\alpha$ -actin, and troponin T.

(F) Phase-contrast images of control and miR-29-expressing RH30 cells. Arrowheads indicate myotube-like structures. Cells were treated with differentiation medium for 2 days and immunostained for troponin. Scale bars = 200  $\mu$ m.

(G) Vector control or miR-29-expressing RH30 cells were differentiated (DM) for 0, 1, 2, 3, or 4 days and subsequently immunostained for troponin. The graph represents the average number of troponin-positive cells that were counted from a minimum of ten randomly chosen fields from three culture plates.

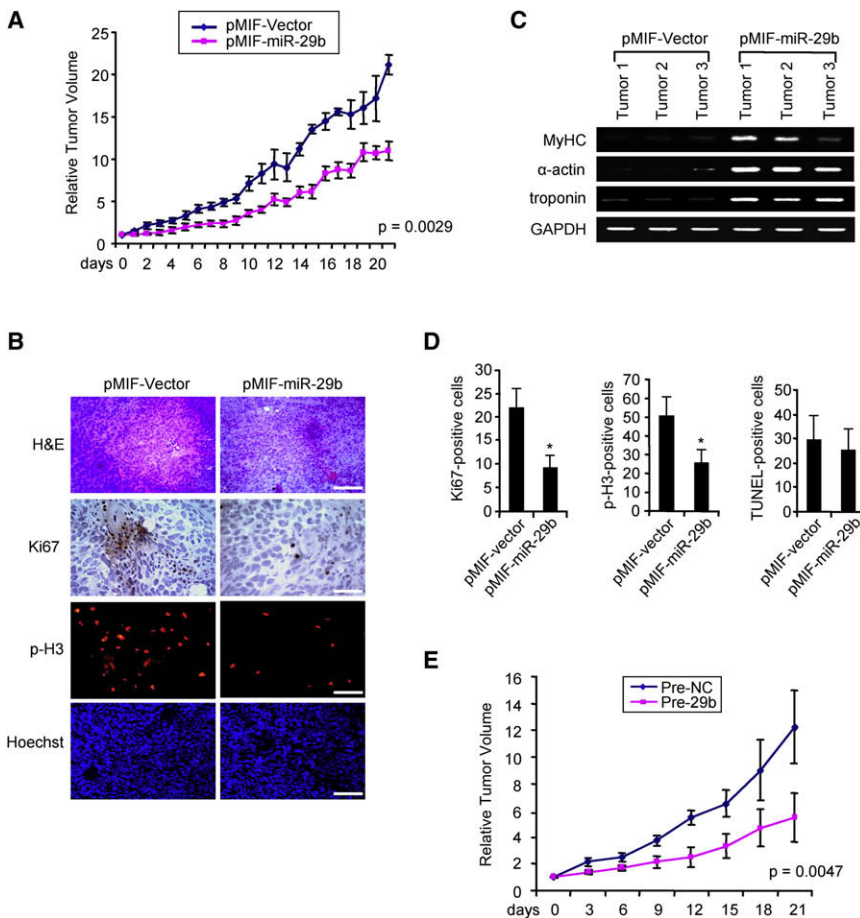
NF- $\kappa$ B-YY1 pathway. Although miRNA biology has greatly advanced in the last few years, much of the effort has focused on describing the targets of miRNAs rather than understanding the regulation of miRNA genes themselves. The nature of miRNA promoter/regulator elements remains one of the most interesting and open questions in the study of miRNA regulation, and their identification as recently described for embryonic stem cells (Marson et al., 2008) is likely to help unravel the regulatory networks involved by these miRNAs. Our current study identified a regulatory element located  $\sim$ 20 kb upstream from the miR-29b/c cluster on chromosome 1. This element contains a YY1 binding site that also associates with the transcriptional repressor factors Ezh2 and HDAC1. We showed that this repressive complex binds specifically in myoblasts when miR-29b/c expression was at its lowest and that loss of YY1 and Ezh2 binding during myogenesis leads to induction of miR-29. This suggests that YY1 and the PcG complex function through this regulatory element to epigenetically repress miR-29b/c transcription in progenitor muscle cells. Recent work has shown that a similar PcG complex under NF- $\kappa$ B control is responsible for the silencing of multiple myofibrillar genes in myoblasts (Wang et al., 2007). Likewise, we found that miR-29 expression was also negatively regulated by NF- $\kappa$ B, suggesting that miR-29 regulation is controlled by a mechanism analogous to that described for protein-coding genes.

The difficulty of accurately identifying the transcriptional start site (TSS) of miRNAs has hindered studies of intergenic miRNA regulation. However, recently Chang and colleagues (2008) used the rapid amplification of cDNA ends (RACE) technique to

characterize the TSS of miR-29b/c. Impressively, their results placed the TSS within the conserved regulatory region described in this report. The four YY1 sites that we identified are located in close proximity to the TSS. Site D, which is occupied by YY1 in myoblasts, lies 2 kb downstream of the pre-miR-29b/c start site in the first intron, whereas the functional YY1 binding site B in RMS lies only 200 bp upstream of the TSS. This is consistent with previous findings that map YY1 regulatory sites within the proximal promoter regions of myofibrillar genes (Wang et al., 2007), again supporting the argument that miR-29 can be regulated in a fashion similar to that of protein-coding genes.

The decrease in NF- $\kappa$ B and YY1 activities, in conjunction with an upregulation of miR-29 during in vitro and in vivo differentiation of skeletal muscle cells, argues that this family of miRNAs plays a role in normal myogenesis. Our results from combined gain-of-function and loss-of-function experiments support this notion. We further showed that the mechanism by which the miR-29 family functions in this capacity is by targeting its own repressor, YY1. Reports have shown that YY1 mRNA and protein are down-regulated during muscle differentiation (Caretti et al., 2004; Wang et al., 2007), in part through the concomitant reduction of p65 and the classical signaling pathway of NF- $\kappa$ B (Bakkar et al., 2008). Our current findings suggest that miR-29 is an additional factor ensuring YY1 downregulation during skeletal myogenesis. Therefore, similar to multiple myogenic transcription factors, cell-cycle regulators, and signaling pathways, miR-29 represents a group of noncoding RNAs including the muscle-specific miRs 1, 133, and 206 that participates in fine tuning the fidelity of skeletal muscle cell differentiation.





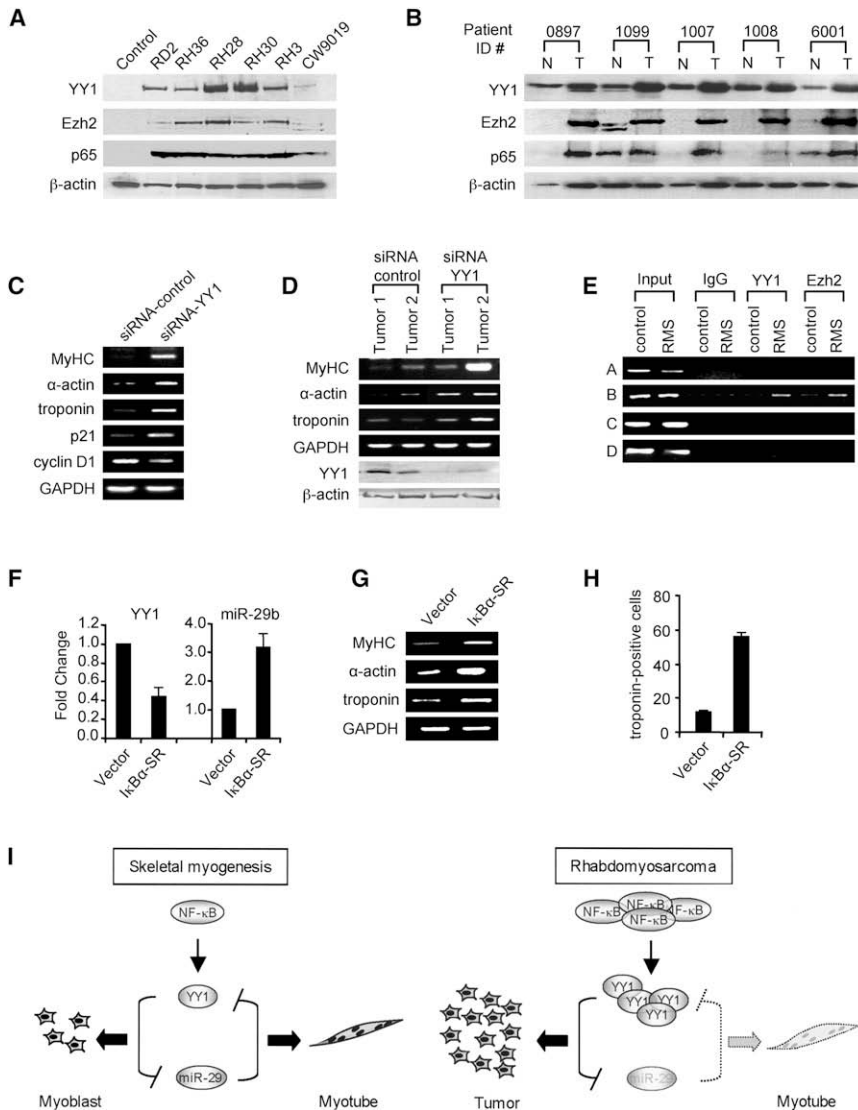
**Figure 7. miR-29 Inhibits RMS Tumor Growth In Vivo**

(A) RH30 tumors were established in mice and then injected with vector or miR-29b-expressing lentiviruses. Tumor volume was recorded daily for 21 days; values are expressed as mean  $\pm$  SD. (B) Tumors were sectioned for histological staining with hematoxylin and eosin (H&E) or the proliferation markers Ki67 and phosphohistone H3 (p-H3). Apoptotic cells were stained by a standard TUNEL assay. Scale bars = 200  $\mu$ m for top two rows and 100  $\mu$ m for bottom two rows. (C) During the course of miRNA treatment, three tumors from vector control- and miR-29b-injected groups were resected. Total RNAs were prepared, and semiquantitative RT-PCR was performed probing for differentiation markers. (D) Ki67-, p-H3-, and TUNEL-positive cells were quantitated by counting positively stained cells from ten randomly chosen fields from six sections per tumor, represented as mean  $\pm$  SD. \* $p$  < 0.05. (E) RH30 xenograft tumors were established as in (A) and then injected with precursor control miR (Pre-NC) or miR-29b (Pre-29b) oligos. Tumor volume was recorded over 21 days; data are represented as in (A).

The ability of miR-29 to stimulate normal myogenesis prompted us to ask whether it could also contribute to the aberrant myogenic differentiation and tumor development in RMS. Indeed, our results support that miR-29 acts as a tumor suppressor in skeletal muscle cells. This conclusion was derived from findings showing that miR-29 expression was dramatically reduced in RMS cancer cell lines and patient tumor samples. These data are consistent with a recently performed miRNA profiling study that identified the downregulation of miR-29 in four patients with ARMS (Subramanian et al., 2007). Further support for tumor suppressor actions of miR-29 in RMS came from our results showing that forced expression of miR-29 restored the potential to reengage a muscle differentiation program and led to a reduction in RH30 proliferation. Significantly, the tumor suppressor activities of miR-29 were also confirmed in an RMS xenograft model. Similar to cultured cells, miR-29-expressing tumors displayed increased differentiation and reduced cellular proliferation. Thus, loss of miR-29 in RMS likely facilitates tumor development through an inhibition of myogenic differentiation. Previous studies have suggested that miR-29 is involved in B cell chronic lymphocytic leukemia and cholangiocarcinoma by regulating an apoptotic pathway. However, our results in RMS did not reveal a significant effect on apoptosis, suggesting that miR-29 exerts its tumor suppressor function in RMS through a mechanism distinct from those previously described.

mediated by a translational repression mechanism similar to the one that we observed in C2C12 MB. These data further suggest that YY1 is a downstream effector of miR-29. YY1 is thought to function as an oncogene through the modulation of key genes involved in cell-cycle regulation and apoptosis (Gordon et al., 2006). However, our results demonstrated that in RMS, the oncogenic activity of YY1 arises from its antidifferentiation property in myogenesis. Furthermore, YY1 has been shown to regulate p53 (Sui et al., 2004). Findings demonstrate that p53 is required for normal skeletal myogenesis and that its inactivation correlates with RMS (Cam et al., 2006). Therefore, it is possible that deregulated expression of YY1 in RMS leads to p53 inactivation in muscle cells that contributes to their transformed phenotype predominantly by a block in myogenic differentiation. In addition to YY1, we have identified several sarcoma-associated oncogenes that qualify as miR-29 targets (Table S2), suggesting that the tumor suppressor activity of miR-29 in muscle cells may occur through different mechanisms or that miR-29 may function as a tumor suppressor in multiple sarcomas other than RMS.

In contrast to the majority of tumor suppressor miRNAs, which reside in fragile sites and exhibit loss of heterozygosity (Calin et al., 2004), miR-29 genes reside in chromosomal regions not typically described to undergo rearrangements (Barr, 2001; Douglass et al., 1985). This suggests that mechanisms other than chromosomal alterations exist to suppress miR-29. Our current



**Figure 8. NF- $\kappa$ B-YY1-miR-29 Circuitry Is Dysregulated in RMS**

(A) Extracts were prepared from normal human muscle and RMS cell lines, and immunoblots were performed probing for YY1, Ezh2, and p65. (B) Lysates were prepared from five RMS patient tumors (T) and adjacent normal muscle tissue (N) and probed for YY1, Ezh2, and p65 proteins.

(C) RH30 cells were transfected with control siRNA oligos or siRNA-YY1 oligos. Cells were differentiated, and semiquantitative RT-PCR was performed probing for differentiation markers. GAPDH was used as a control.

(D) RH30 tumors were established in nude mice and then injected with siRNA oligos every 3 days for 1 week. RNA and protein lysates were prepared from tumors and subsequently probed by RT-PCR for MyHC,  $\alpha$ -actin, and troponin and by western blot for YY1.

(E) ChIP assays with either a YY1 antibody or control IgG were performed on chromatin isolated from human skeletal muscle cells (control) or RH30 cells (RMS). Precipitated DNA fragments were amplified with oligonucleotides spanning regions A–D of the human miR-29b/c regulatory region. Total inputs are indicated.

(F) RH30 cells were infected with adenoviruses expressing vector control or I $\kappa$ B $\alpha$ -SR. YY1 and miR-29b levels were measured at 48 hr postinfection by qRT-PCR and are represented as mean  $\pm$  SD.

(G) RH30 cells were infected with control or I $\kappa$ B $\alpha$ -SR adenovirus, and differentiation markers were probed by qRT-PCR.

(H) RH30 cells were transduced with vector or I $\kappa$ B $\alpha$ -SR-expressing retroviruses to generate stable cell lines. Cells were then treated with differentiation medium for 2 days and immunostained for troponin and quantified for myofibrillar expression, represented as mean  $\pm$  SD.

(I) Model depicting the role of the NF- $\kappa$ B-YY1-miR-29 regulatory circuit in both normal myogenic differentiation and RMS. In myogenesis, this circuit involves constitutive activity of NF- $\kappa$ B in myoblasts regulating YY1, which subsequently

epigenetically suppresses miR-29 and maintains cells in an undifferentiated state. As differentiation ensues, downregulation of the NF- $\kappa$ B-YY1 pathway leads to upregulation of miR-29 that in turns further decreases YY1 levels to ensure proper differentiation into myotubes. In RMS, this circuit becomes dysregulated due to an increase in the NF- $\kappa$ B-YY1 pathway that constitutively represses miR-29. In the absence of miR-29 tumor suppressor activity, YY1 is left uncontrolled, thereby impairing differentiation and leading to rhabdomyosarcomagenesis.

findings support that such suppression results from a dysregulation of YY1 in RMS. YY1 is bound to the regulatory region of miR-29b/c in RMS cells, suggesting that suppression of miR-29b/c occurs by epigenetic silencing. Our finding that Ezh2 levels are also elevated in RMS is consistent with findings that PcG proteins contribute to oncogenesis (Sparrmann and van Lohuizen, 2006). Interestingly, recent work has elucidated that miR-29 can target methyltransferases in lung tumor cells and that forced expression of miR-29 leads to reduced global DNA methylation (Fabbri et al., 2007). Thus, miR-29 might represent a unique class of miRNAs involved in cancer epigenomics with respect to their regulation and function.

Taken together, these results identify the involvement of miR-29 in a regulatory circuit relevant in both skeletal myogenesis and RMS. As modeled in Figure 8I, we propose that miR-29 and

YY1 are interlinked by a mutual negative feedback loop whereby miR-29 blocks YY1 translation and YY1 inhibits miR-29 transcription. This loop is further regulated by NF- $\kappa$ B, which indirectly controls miR-29 through YY1. In normal proliferating myoblasts, constitutive NF- $\kappa$ B activity functions in part by maintaining levels of YY1 that repress miR-29 by an epigenetic mechanism. Once differentiation ensues, NF- $\kappa$ B activity is decreased, leading to a transcriptional reduction in YY1 and subsequent derepression of miR-29. Increasing levels of miR-29 in turn inhibit YY1 translation, thereby promoting myofibrillar gene expression. However, in RMS this regulatory circuit is compromised by constitutive activation of NF- $\kappa$ B-YY1 that, in combination with elevated PcG activity, promotes sustained silencing of miR-29. In the absence of miR-29, YY1 levels are further elevated to inhibit differentiation, thereby facilitating tumor development. These findings not only

provide insights into the interplay between transcription factors and miRNAs during muscle differentiation but also have implications for the diagnosis and treatment of RMS.

## EXPERIMENTAL PROCEDURES

### Cell Lines

C2C12 myoblasts (MB) were cultured and differentiated as described previously (Guttridge et al., 1999). Human MB were obtained from Clonetics and maintained in F12 medium supplemented with 20% FBS and 4% chicken embryo extract. Human RMS cell lines were maintained in RPMI medium supplemented with 10% FBS.

### Transfection and Infections

Transfections with miRNA precursor oligos and siRNA oligos were performed with Lipofectamine 2000 as suggested by the manufacturer (Invitrogen). For luciferase assays, cells were transfected in 12-well plates and luciferase activity was monitored as described previously (Guttridge et al., 1999). For lentivirus production, pMIF-cGFP-Zeo vector or pMIF-cGFP-Zeo-miR-29b plasmids along with pPACK packaging plasmid mix (System Biosciences) were transfected into 293TN cells maintained in 10% FBS. Forty-eight hours after transfection, supernatant was harvested from these cells and viral titers were estimated by fluorescence-activated cell sorting analysis. Approximately  $1 \times 10^9$  virus particles were used to transduce RH30 or RD2 cells, which were subsequently placed in 200  $\mu\text{g}/\text{ml}$  Zeocin for stable selection. Adenovirus particles expressing I $\kappa$ B $\alpha$ -SR or GFP vector control (University of North Carolina Vector Core Facility) were used to infect RH30 cells. RNAs were prepared at 48 hr postinfection and used for subsequent analysis. pBabe vector and pBabe-I $\kappa$ B $\alpha$ -SR retroviruses were produced as described previously (Hertlein et al., 2005).

### Oligonucleotides and Plasmids

Precursor miRNA oligos were obtained from Ambion. 2'-O'-methyl- and 3'-amino-C6-modified Anti-miR oligos were obtained from Fidelity Systems. siRNA oligos against human or mouse YY1 were obtained from Santa Cruz Biotechnology, and siRNA oligos against p65 were obtained from Dharmacon. In each case, 50  $\mu\text{M}$  oligos were used for transient transfections. Details on the construction and use of plasmids can be found in Supplemental Experimental Procedures.

### RT-PCR and Real-Time RT-PCR

Total RNAs from cells were extracted using TRIzol reagent (Invitrogen). Expression of mature miRNAs was determined using a miRNA-specific TaqMan microRNA assay kit (Applied Biosystems) in an iCycler (Bio-Rad Laboratories). U6 was used for normalization.

### Immunoblotting and Immunostaining

Cell extracts were prepared and used for immunoblotting as described previously (Guttridge et al., 1999). Source and dilution for each antibody were as follows: YY1 (Santa Cruz, 1:1000), troponin T (Sigma, 1:1000), MyHC (Sigma, 1:1000), Ezh2 (Zymed, 1:1000), p65 (Rockland, 1:10000),  $\alpha$ -tubulin (Sigma, 1:1000), and  $\beta$ -actin (Santa Cruz, 1:1000). For immunofluorescence of C2C12 cells, antibodies used were MyHC or troponin T (Sigma, 1:500). For tumor sections, antibodies used were anti-phosphohistone H3 (Cell Signaling, 1:200) and Ki67 (Dako, 1:50).

### EMSAs and ChIP

EMSAs and ChIP were performed as described previously (Wang et al., 2007). For ChIP assays, 2  $\mu\text{g}$  of antibodies against YY1 (Santa Cruz), Ezh2 (Zymed), HDAC1 (Santa Cruz), trimethyl histone H3K27 (Upstate), SRF (Santa Cruz), Mef2 (Santa Cruz), or isotype IgG (Sigma) were used. Genomic DNA pellets were resuspended in 20  $\mu\text{l}$  of water. PCR was performed with 2  $\mu\text{l}$  of immunoprecipitated material, and products were analyzed on an agarose gel visualized by a gel documentation system.

### Tumor Samples

Specimens were obtained from the Cooperative Human Tissue Network in accordance with the institutional review boards of The Ohio State University and Nationwide Children's Hospital. Details on the characterization of each tumor specimen can be found in Supplemental Experimental Procedures.

### Animal Studies

Mice were housed in the animal facilities of the Arthur G. James Comprehensive Cancer Center under conventional conditions with constant temperature and humidity and fed a standard diet. Treatment of mice was in accordance with the institutional guidelines of The Ohio State University Animal Care and Use Program. For Anti-miR oligo injection, P5 C57BL/6 mice were injected with 5 nM Anti-NC or Anti-miR-29 oligos in the lower limb. Muscles were collected 48 hr postinjection and used for western blotting. For cardiotoxin (CTX) studies, 4-week-old mice were injected with 50  $\mu\text{l}$  of CTX at 10  $\mu\text{g}/\text{ml}$  in the tibialis anterior. Muscles were harvested and RNAs extracted for real-time RT-PCR analysis. For xenograft studies, 3- to 4-week-old athymic *nu/nu* female mice (Charles River Laboratories, NCI) were injected subcutaneously with  $3 \times 10^6$  RH30 cells. Tumor diameter was measured two-dimensionally daily with electronic calipers. Tumor volume was calculated using the following formula:  $(\pi/6) \times D_1 \times D_2 \times D_3$ , where  $D_1$  is the largest diameter and  $D_2$  is the smallest diameter. Pre-miR oligos or siRNA oligos (5  $\mu\text{M}$ ) were preincubated with Lipofectamine (Invitrogen) for 15 min prior to injection into tumors in a final volume of 60  $\mu\text{l}$  of Opti-MEM (Invitrogen).

### Bioinformatic and Statistical Analysis

Promoter sequences were retrieved from UCSC Genome Bioinformatics (<http://genome.ucsc.edu/>). Prediction of the transcription factor binding sites was performed using the rVISTA sequence analysis tool (<http://rlista.dcode.org/>). Prediction of miRNA targets was performed using three publicly available algorithms: TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/>), and PicTar (New York University Center for Comparative Functional Genomics). Statistical significance of tumor growth was assessed using a paired Student's t test.

## SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, eight figures, and two tables and can be found with this article online at [http://www.cancer-cell.org/supplemental/S1535-6108\(08\)00330-9](http://www.cancer-cell.org/supplemental/S1535-6108(08)00330-9).

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