

# A Novel Target of microRNA-29, Ring1 and YY1 Binding Protein (Rybp), Negatively Regulates Skeletal Myogenesis

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Running title: Rybp is a novel suppressor of myogenesis

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**Background:** microRNA-29-YY1 regulatory circuitry functions during skeletal myogenesis.

**Results:** a genome-wide search revealed Rybp as a novel target of miR-29 which silences myogenic loci together with YY1.

**Conclusion:** Rybp functions as a repressor of myogenesis.

**Significance:** this study identifies a novel regulatory circuitry underlying muscle formation and highlights the intimate interplay among transcription factors, epigenetic regulators and microRNAs.

## SUMMARY

Skeletal muscle cell differentiation (myogenesis) is a process orchestrated by a complex network involving transcription factors, epigenetic regulators, and microRNAs. Previous studies identified miR-29 as a pro-myogenic factor that interacts with components of Polycomb repressive complex (PRC), YY1 and Ezh2. In a genome-wide survey of miR-29 mediated

transcriptome changes in C2C12 myoblasts, many epigenetic factors were found to be down-regulated by miR-29. Among them, Rybp was shown to be a direct target of miR-29 through binding to its 3' UTR region. Functional studies demonstrated that Rybp is down-regulated during myogenesis and acts as a negative regulator of skeletal myogenesis both *in vitro* during C2C12 differentiation and *in vivo* in injury-induced muscle regeneration. Furthermore, we found that Rybp and YY1 co-occupy several myogenic loci, including miR-29 itself, to silence their expression, thus forming a Rybp-miR-29 feedback loop. Rybp over-expression was found to enhance the enrichment of Ezh2 and tri-methylation of H3K27 at target loci, suggesting it may facilitate the recruitment or stabilization of PRC. Collectively, our results identify Rybp as a novel regulator of myogenesis that co-acts with YY1 to silence miR-29 and other myogenic loci.

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Skeletal muscle growth and regeneration are attributed to satellite cells (muscle stem cells), which are characterized by the expression of paired-box transcription factor 7 (Pax7) and when activated, become immature muscle cells or myoblasts which will proliferate and differentiate. The formation of mature muscle proceeds with the exit of myoblasts from the cell cycle, the expression of muscle-specific genes and the suppression of genes that are specific to other cell lineages and tissues (1). A major portion of our understanding of myogenic differentiation is focused at the level of transcription, orchestrated by a complex network of muscle-specific transcription factors (TFs) including MyoD, Myogenic Factor 5 (Myf5), Myogenin, Myogenic Regulatory Factor 4 (MRF4), and myocyte enhancer factor 2 (Mef2). These factors activate muscle specific genes to coordinate myoblasts to terminally withdraw from the cell cycle and subsequently fuse into multinucleated myotubes (2). In addition to TFs, epigenetic regulators exert an important layer of transcriptional control (3). The interplay between TFs and these regulators on muscle loci constitutes an important part of the signaling pathways.

Studies from recent years have also incorporated microRNAs (miRNAs) into the complex network of myogenic regulation. As non-coding single-stranded RNAs of 21 to 25 nucleotides and a novel class of gene regulators, miRNAs negatively regulate their targets at the post-transcriptional level through binding to their 3' UTR regions leading to either mRNA cleavage or translational repression (4). A subset of these miRNAs, miR-1, miR-133, and miR-206, play central regulatory roles in muscle biology and are called muscle miRs. In addition to the above muscle miRs, emerging evidences also support the involvement of non-muscle miRs in myogenesis (5). In an effort to elucidate the transcriptional and post-transcriptional regulation during skeletal myogenesis, we

identified an NF-KappaB (NF- $\kappa$ B)-Yin Yang1 (YY1)-miR-29 signaling axis (6). YY1, a ubiquitously expressed transcription factor, upon activation by NF- $\kappa$ B signaling, functions to repress muscle differentiation by epigenetically silencing the transcription of miR-29 through recruiting histone methyltransferase Ezh2 as well as histone deacetylase 1 (HDAC1)(6-7). At the onset of myogenesis, YY1 is down-regulated by decreased NF- $\kappa$ B signaling, which results in the replacement of YY1/Ezh2/HDAC1 repressive complex by an activator complex containing MyoD, serum response factor (SRF) along with associated acetyltransferases CBP and p300/CBP-associated factor (PCAF), leading to the activation of miR-29. miR-29 in turns targets YY1 resulting in its further decrease. These findings highlight the intimate interaction of TF, miRNA and chromatin modulator.

In addition to YY1, miR-29 directly targets DNMT3a and DNMT3b in lung cancer and acute myeloid leukemia (AML) (8-9). miR-29b can also indirectly silence DNMT1 by targeting SP1, a transactivator of DNMT1 (9). These findings together with ours support the ability of miR-29 in regulating epigenetic machinery. We thus speculated that in addition to YY1, miR-29 may regulate many other epigenetic factors whose unveiling will be facilitated by a genome-wide search.

As members of Polycomb group proteins (PcGs), YY1 and Ezh2 together regulate a number of muscle loci including both muscle structure genes and muscle relevant miRNAs(3). The PcG genes encode a structurally diverse group of proteins, which form large complexes arising from their mutual interactions. Despite their well characterized functions in embryonic stem cells (ESCs) (10-11), it is unknown if other PcG proteins are involved in myogenesis and whether they are part of the YY1/Ezh2 containing PRC.

In this work, a genome-wide transcriptome survey was employed to uncover that a number of epigenetic factors were down-regulated in miR-29 expressing C2C12 cells, with Rybp, an YY1 interacting PcG protein, being one of them. miR-29 was able to target Rybp directly through a conserved binding site on its 3'UTR region. Both *in vitro* and *in vivo* functional studies showed that Rybp is a negative regulator of skeletal myogenesis which functions as a co-repressor of YY1 to silence the myogenic loci including miR-29 itself. Our study thus identified a novel functional Rybp-miR-29 regulatory circuitry in skeletal muscle formation.

## EXPERIMENTAL PROCEDURES

*Cells* - Mouse C2C12 myoblasts (CRL-1772) were obtained from ATCC and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg of Streptomycin at 37°C in 5% CO<sub>2</sub>. For myogenic differentiation, cells were seeded in 60 mm or 100 mm plates and shifted to DMEM containing 2% horse serum when 90% confluence. Primary myoblasts were isolated from approximately one week old mice muscles by the described procedures (12-13). Briefly, total hind limb muscles (3 to 6 mice per group) were digested with type IV collagenase (Invitrogen, 5 mg/ml) and dispase II (Invitrogen, 1.4 mg/ml) for 0.5 hr, and cell suspensions were further homogenized by pipetting before being filtered through 70 µm and 40 µm filters. The obtained cells were pre-plated on uncoated cell culture plates in F10 media (Invitrogen) to selectively enrich for myoblasts. After two rounds of pre-plating, the cell suspension was plated on Gelatin-coated plates (Iwaki) in F10 medium (Invitrogen) supplemented with 20% FBS and Basic Fibroblast Growth Factor (Invitrogen, 25 ng/ml). Primary myoblasts were used at passage 3-5 after isolation.

*Transfection and infection* - Transient transfection with miRNA precursor oligos, siRNA oligos or DNA plasmids was performed on 60mm or 100mm dishes with Lipofectamine 2000 reagent as suggested by the manufacturer (Invitrogen). For luciferase experiments, C2C12 and primary myoblasts were transfected in 12-well plates. Cell extracts were prepared and luciferase activity was monitored as previously described (14) or using dual-luciferase kit (Promega). To generate C2C12 cells stably expressing Rybp, a Rybp expressing plasmid (4µg) was transfected into C2C12 cells using Lipofectamin 2000 (Invitrogen). 36 hours after transfection, cells were placed in 400µg/ml G418 (Invitrogen) for stable selection. Stable clones were pooled together after ~ 2 week selection.

*Oligonucleotides* - Precursor miRNA oligos were obtained from Ambion. Mercury LNA microRNA or control oligos were obtained from Exiqon. The 19-nucleotide siRNA duplexes against mouse Rybp coding region (siRNA, 5'-CGACATGTCAGCAGTGAAT -3') or scrambled oligos were obtained from RiboBio. In each case 100nM oligos were used for transient transfection into cells.

*DNA constructs* - To construct a Rybp-3'UTR reporter plasmid, a 401bp fragment encompassing miR-29 binding site was amplified by primers (Forward primer: 5'-AGTTCTAGAAGACTCCATCCTGTCAGCATATC-3'; Reverse primer: 5'-AGTTCTAGAGGAAAAAGGCCGGAATT CGA-3') and cloned into pGL3 vector (Promega) at Xba I site. To construct a mutant reporter plasmid, the seed region was mutated from TGGTGCTT to TCATAAGT. To generate a Rybp expression plasmid, the full-length coding region of Rybp was amplified by primers (Forward primer: 5'-AGTGCTAGCATGACCATGGGCGACAA GAAG-3'; Reverse primer: 5'-AGTGGTACCTCAGAAAGATTCATCATT

CACTGC-3'), digested and cloned into pcDNA3.1(+) vector (Invitrogen) at NheI and KpnI sites. An YY1 expression plasmid was a gift from Y. Shi (Harvard University) and used as described (15). A miR-29-promoter luciferase reporter was created and used as described (6). Renilla luciferase reporter was obtained from Promega and used according to manufactory.

*RT-PCR and Real-time RT-PCR* - Total RNAs were extracted using TRIzol reagent (Invitrogen). Expression of mature miRNAs was determined using the miRNA-specific Taqman microRNA assay kit (Applied Biosystem) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystem). U6 was used for normalization. Expression of mRNA analysis was performed with SYBR Green Master Mix (Bio-Rad Laboratories) as described using GAPDH for normalization (15). Primers used are listed in Supplemental Table S1.

*Immunoblotting, Immunostaining and Immunohistochemistry* - For Western blotting analyses, total cell extracts were prepared and used as previously described (16). The following dilutions were used for each antibody: Myogenin (Santa Cruz Biotechnology; 1:2000), YY1 (Santa Cruz Biotechnology; 1:2000), Rybp (Millipore; 1: 2000), Troponin (Sigma; 1:2000), MyHC (Sigma; 2,000),  $\alpha$ -Tubulin (Sigma; 1:5000), Pax 7 (Developmental Studies Hybridoma Bank; 1:2000), eMyHC (Leica, 1:2000), and GAPDH (Santa Cruz Biotechnology; 1:5000). Densimetric quantification of the Western bands was performed using the Quantity One software (Bio-Rad). Immunofluorescence on cultured cells was performed using the following antibodies: Rybp (Millipore; 1:400), MyHC (Sigma; 1:350), YY1 (Santa Cruz Biotechnology; 1:400). Frozen muscle sections were prepared and stained as previously described (17). Immunofluorescence staining on frozen muscle sections was performed using the following antibodies: MyoD (Santa Cruz,

1:100); Myogenin (Santa Cruz, 1:100), Pax7 (DSHB, 1:100). H&E staining on frozen muscle sections was performed as previously described (35). Quantification of number of fibers with centrally located nuclei (CLN) and IF positively stained cells was performed from a minimum of 20 randomly chosen fields, from 5-6 sections throughout the length of the muscle in 4-6 per group. All fluorescent images were captured with a confocal laser scanning microscope (FV1000, Olympus, Japan). All samples were imaged with the 20 $\times$  or 40 $\times$  objective lens. Pictures were captured in Kahlman frame giving an average of two scans using the Olympus microscope FV1000 and the accompanying software FV10-ASW (version 01.07.02.02, Olympus).

*Coimmunoprecipitation assays*- Co-IP was performed as described before (18). Briefly, 10  $\mu$ g of Normal IgG (Santa Cruz Biotechnology), antibodies against YY1 (Santa Cruz Biotechnology) or Rybp (Millipore) was cross-linked to 50  $\mu$ l (bed volume) of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) by Dimethyl pimelimidate (DMP, Sigma). C2C12 cells were first cross-linked with 200  $\mu$ g/ml of 3,3'-Dithiodipropionic acid di(N-hydroxysuccinimide ester) (Sigma) for 20 min and then harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM HEPES, pH 7.4, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1  $\times$  Protease Inhibitor Cocktail). The antibody-conjugated beads were incubated with 500  $\mu$ g of the above cell lysate overnight at 4 $^{\circ}$ C with rotation. After extensive washing with RIPA buffer, the bound proteins were eluted by boiling in 20  $\mu$ l of 2  $\times$  sample buffer (125 mM Tris-HCl, pH 6.8, with 4% SDS, 20% (v/v) glycerol) and subjected to Western blot analysis.

*ChIP assay* - ChIP assays were performed as recommended by the manufacturer (Upstate) using 5  $\mu$ g of antibodies against Rybp (Millipore), YY1 (Santa Cruz Biotechnology),

Ezh2 (Cell signaling), trimethyl-histone H3-K27 (Millipore), or isotype IgG (Santa Cruz Biotechnology) used as a negative control. Genomic DNA pellets were resuspended in 20  $\mu$ l of water. qRT-PCR was performed with 1  $\mu$ l of immunoprecipitated material with SYBR Green Master Mix (Bio-Rad Laboratories). Relative enrichment is calculated as the amount of amplified DNA normalized to input and relative to values obtained after normal IgG immunoprecipitation, which were set as 1. Primers used are listed in Supplemental Table S1.

*Animal studies* - C57B/L mice were housed in the animal facility of the Chinese University of Hong Kong (CUHK) under conventional conditions with constant temperature and humidity and fed a standard diet. Animal experimentation was approved by the CUHK Animal Experimentation Ethics Committee (Ref No. 10/027/MIS). For Cardiotoxin (CTX) injection, approximately five week old mice were injected with 60  $\mu$ l of CTX at 10  $\mu$ g/ml into the tibialis anterior muscles. Oligos were prepared by pre-incubating 10  $\mu$ M of siRNA oligos with Lipofectamine 2000 for 15 minutes and injections were made in a final volume of 60  $\mu$ l in OPTI-EM (GIBCO) at day 1/4, 2 and 4. **Mice were sacrificed and TA muscles were harvested at day 1, 2, 4 and 6, and total RNAs and proteins were extracted for real-time RT-PCR and Western analyses. For IF staining of MyoD, Myogenin and Pax7, muscle sections were collected at day 3.**

*Statistical analysis* - Statistical significance was assessed by the Student's t-test. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

## RESULTS

*Rybp is down-regulated in miR-29 expressing C2C12 cells* - In order to gain insights into the miR-29 mediated events in muscle cells, we previously performed a

genome-wide transcriptome analysis by high throughput RNA-sequencing (19). 739 genes were found to be down-regulated by miR-29 over-expression. Interestingly, many epigenetic factors were decreased, including previously identified targets of miR-29, *Hdac4* and *Dnmt1* (Fig. 1A-B), suggesting miR-29 is an important epigenetic regulator. When compared to vector control cells, these genes were suppressed to a different extent with *Cbx4* 38% while *Suz12* 16%. YY1 RNAs, on the other hand, was not found to be significantly down-regulated. This is in line with our previous finding showing miR-29 represses YY1 translation but not transcription (6). Among the down-regulated genes, *Rybp* caught our attention because it was consistently predicted to be a direct target of miR-29 by three algorithms, TargetScan (20), miRanda (21), PicTar (22). Moreover, as a known modifier of chromatin and a member of PcG genes, its role in myogenesis has never been explored. As revealed by RNA-seq, it was suppressed about 21% by miR-29 over-expression (Fig. 1A and 1C).

*miR-29 directly targets Rybp through binding to its 3'UTR* - To investigate whether the down-regulation of *Rybp* is due to direct targeting, A search for miR-29 binding sites within *Rybp* 3'UTR revealed that all three miR-29 family members, miR-29a, -29b, -29c, were predicted to hybridize to an evolutionarily conserved site among vertebrate species (Fig. 2A and Supplemental Fig. S1). A perfect match exists between the seed region of miR-29 and the 3'UTR of *Rybp*, suggesting that miR-29 can directly repress *Rybp* expression. This was tested by cloning a 400bp fragment of the *Rybp* 3'UTR encompassing the target site downstream of the firefly luciferase gene. Co-transfection of this reporter (WT) with each of the miR-29 family members caused similar repression of luciferase reporter (Fig. 2B). This was specific to miR-29 binding since the reporter activity was less affected when transfections were repeated with

an irrelevant miRNA, miR-194, or with a mutant miR-29 binding site in the Rybp 3'UTR. At the functional level, we predicted that miR-29 binding to the Rybp 3'UTR would lead to Rybp repression. Indeed, ectopic expression of miR-29 by transfection of miR-29 oligos or transfection of a pMIF-miR-29 plasmid caused decreased expression of Rybp at both RNA and protein levels (Fig. 2C-D and Supplemental Fig. S2A-D), and knock-down miR-29 by anti-miR oligos led to increased expression of Rybp (Fig. 2C-D and Supplemental Fig. S2E). Interestingly, in addition to miR-29, Rybp is also predicted to be targeted by muscle miRNAs, miR-1 and miR-206 (Supplemental Fig. S3A). Indeed, results from expression and luciferase reporter assays performed as above demonstrated that miR-1 and miR-206 can also directly target Rybp (Supplemental Fig. S3B-F). This is consistent with the common idea that an mRNA can be targeted by multiple miRNAs. It also further indicates the relevancy of Rybp to myogenesis.

*Rybp is down-regulated in C2C12 myogenesis concomitant with YY1* - To probe into the function of Rybp in skeletal myogenesis, we examined the expression levels of Rybp during C2C12 myoblast differentiation. As shown in Fig. 3A, its expression gradually decreased during a time course of day 0, 2, 4 and 6 whereas the myogenic marker, Myosin Heavy Chain (MyHC) increased. This was concomitant with gradually decreased YY1 expression as well as a gradual increase of miR-29, miR-1 and miR-206 (Supplemental Fig. S4), suggesting a functional connection between Rybp and YY1. Consistently, in primary myoblasts isolated from mouse limb muscles, expression of Rybp decreased gradually along with YY1 during differentiation in contrast to the up-regulated expressions of myogenic markers, alpha Skeletal Actin ( $\alpha$ -Actin), Troponin and MyHC (Fig. 3B). Immuno-fluorescence (IF) staining also revealed that Rybp was co-localized with YY1 in the

nuclei of C2C12 myoblasts (Fig. 3C). Furthermore, a physical interaction of Rybp and YY1 was detected in C2C12 myoblasts by Co-Immunoprecipitation (Co-IP) (Fig. 3D). Together, the above findings suggest that Rybp may be an anti-myogenic factor that acts in concert with YY1. Additional evidence for their functional relationship came from the expression patterns of their transcripts across tissues. Expressions of Rybp and YY1 were found to be highly coordinated with the highest expression levels in lung and the lowest in skeletal muscle (Fig. 3E-F). Although Rybp is low in mature skeletal muscle, it was found to be highly enriched in freshly isolated myoblasts together with YY1 (Fig. 3G), suggesting its role in regulating myoblast growth or differentiation.

*Rybp functions as a negative regulator of skeletal myogenesis* - To test the functional relevance of Rybp during myogenesis, we knocked-down Rybp in C2C12 cells with a siRNA oligo (siRybp). The decreased expression of Rybp was found to enhance C2C12 myogenic differentiation as evidenced by the up-regulated expressions of MyHC, Troponin,  $\alpha$ -Actin and Myogenin at both protein and RNA levels (Fig. 4A-B). Specifically, miR-29 and miR-1, two other direct transcriptional targets of YY1, were up-regulated by Rybp decrease (Fig. 4C). IF staining also revealed an increased number of MyHC-positive myotubes upon decrease of Rybp (Fig. 4D). Reporter assay with luciferase reporter plasmids containing Troponin, MyHC, miR-29 promoter/enhancer upstream of luciferase gene, demonstrated an increase of luciferase activity in siRybp cells as compared to cells transfected with negative control oligos (siNC) (Fig. 4E). Collectively, the above findings suggest that Rybp inhibits C2C12 myogenic differentiation. To strengthen the above findings, over-expressing Rybp stably in C2C12 was found to delay differentiation compared to cells expressing vector control as revealed by expression assay, IF and reporter



assay performed as the above (Fig. 4F-J).

*Rybp functions as a co-repressor of YY1 to silence myogenic loci in myoblasts* - To gain insights into the molecular mechanism on how Rybp repressed myogenesis, we speculated that Rybp may function in the same repressive complex containing YY1 and Ezh2 to epigenetically silence muscle genes and muscle relevant miRNA targets **in myoblasts**. To test this notion, ChIP assays were performed against Rybp and YY1 using chromatin isolated from C2C12 cells stably expressing vector or Rybp. Both proliferating myoblasts (MBs) and differentiated myotubes (MTs) were included in the study. Promoters/enhancers of three known YY1 targets, miR-29, miR-1 and Tnni2, were examined for their association with Rybp and YY1. As expected, in vector MBs, strong binding of Rybp was detected on all three target regions along with YY1, and the association dramatically reduced in MTs (Fig. 5A-B), suggesting that Rybp functions together with YY1 to silence target expression **in proliferating myoblasts and removed together upon myogenic differentiation**. Rybp over-expression led to an increase of Rybp binding on these loci but not YY1 enrichment (Fig. 5A-B), implying that Rybp may not have impact on YY1 targeting. Knowing that Ezh2 is a co-repressor of YY1 on these loci, we further examined Ezh2 binding. Interestingly, Rybp over-expression was found to enhance Ezh2 association (Fig. 5C). Consistently, levels of H3K27me3 displayed the same pattern as Ezh2 (Fig. 5D). Collectively, these data suggest that Rybp functions to suppress muscle genes and miRNAs transcription together with YY1 and Ezh2. This notion was further strengthened by transfecting YY1 or/and Rybp expression plasmids along with a miR-29 promoter reporter in C2C12 cells. Rybp or YY1 alone was found to inhibit the reporter activity to a similar extent whereas Rybp expression increased YY1 inhibition at a dose dependent manner (Fig. 5E).

*Knock-down of Rybp in vivo enhances injury-induced muscle regeneration* - Lastly, to extend our *in vitro* findings to *in vivo* muscle formation, we explored the function of Rybp in a widely-used muscle regeneration model in which the injection of cardiotoxin (CTX) results in muscle injury and in turn induces muscle regeneration. After injection with CTX, tibialis anterior (TA) muscle displays the typical degeneration-regeneration process: fiber degeneration and immune cell infiltration were immediately evident within 1 to 2 days, meanwhile satellite cells start proliferating followed by activation and myogenic differentiation 3 to 4 days afterwards, newly formed fibers with centrally located nuclei (CLN) are evident within 5 to 6 days, and muscle architecture is largely restored within 10 days (12).

The expression profile of Rybp mRNAs was examined at day 1, 2, 3, and 6 days post-injection. A sharp increase of more than 10 folds in the first two days post-injury was observed, after which its expression level gradually fell down until day 6 (Fig. 6A, left panel). This was temporally correlated with YY1 expression (middle panel), suggesting that a dynamic and coordinated change of Rybp and YY1 expressions occurred during *in vivo* muscle degeneration-regeneration. On the contrary, miR-29 expression was inversely correlated with Rybp and YY1 (right panel): it sharply decreased in degeneration stage, and then gradually increased in regeneration stage. This was in keeping with the *in vitro* finding that Rybp and YY1 together inhibit miR-29 transcription.

To ascertain the functional significance of Rybp regulation, siNC or siRybp oligos were injected into CTX-injured TA muscles following the administration scheme outlined in Fig. 6B. Six hours after CTX injection into TA muscles, siNC or siRybp oligos were administrated into left and right hind limbs, respectively. The injections were made every two days for a total

of three times. The treated muscles were collected at day 1, 2, 4 and 6. Results from qRT-PCR and Western analyses revealed that the expressions of Rybp were decreased at all the time points (Fig. 6C-D), suggesting a successful knock-down of Rybp by this approach. Expression levels of MyoD, Myogenin and embryonic-MyHC (e-MyHC, a marker for regenerating fibers) were found to be up-regulated by siRybp injection (Fig. 6C-D), suggesting an acceleration of myogenic program as discovered *in vitro*. Consistently, miR-29 expression was found to be up-regulated at day 1 and 2 during the regeneration peak (Fig. 6C, day 1 and 2). Interestingly, the expression of Pax7 was found to be down-regulated (Fig. 6C-D). Further evidence came from IF staining of the above markers on frozen muscle sections. MyoD and Myogenin positively stained cells were significantly increased by siRybp treatment (Fig. 6E-F). Pax7 positively stained cells, on the other hand, were decreased (Fig. 6G). Additionally, siRybp treatment led to increased number of newly formed fibers characterized by centrally located nuclei (CLN, Fig. 6H). Together, these findings imply that Rybp is a functional anti-myogenic factor during muscle regeneration *in vivo*.

## DISCUSSION

This study was initiated to investigate the genome-wide impact of miR-29 on epigenetic regulators in skeletal muscle cells. Unexpectedly, Rybp, a PcG member was discovered to be a likely target of miR-29. Subsequent study validated that Rybp is a novel target of miR-29. Further investigation identified Rybp as a negative regulator of skeletal myogenesis which acts in concert with YY1. Rybp and YY1 were found to co-occupy several target promoters/enhancers of YY1 including miR-29 itself to silence their expression in myoblasts, thus forming a miR-29-Rybp feedback

regulatory loop. Increased Rybp led to an enhancement of Ezh2 association on the target loci, suggesting that Rybp may facilitate PRC recruitment or stabilization. Moreover, we demonstrated that knock-down of Rybp accelerated injury-induced muscle regeneration *in vivo*, strengthening the argument that Rybp is a relevant regulator of skeletal muscle formation (Fig. 7).

*Rybp is a novel PcG repressor of skeletal myogenesis* - PcG repressor proteins play key roles in establishing and maintaining gene expression patterns during cellular differentiation and development. There are two major biochemical complexes, PRC1 and PRC2, which have inherent histone-modifying activity critical for their function in gene repression. PcG target genes are often co-regulated by PRC1 and PRC2 which are hierarchically recruited to target loci (23). It is commonly accepted that Ezh2-containing PRC2 promotes the tri-methylation of H3K27 which then leads to recruitment of PRC1. Being able to interact with both YY1 and PRC1 protein, Ring1, Rybp and its homolog YAF2 is proposed to recruit PRC1 through interacting with RING1(24). Previous studies have identified Rybp as a transcriptional silencer in cancer (25-26), embryogenesis (27) and central nervous system development (28). Our current study is the first to demonstrate a role of Rybp in skeletal myogenesis. In line with the other studies, our results suggested Rybp as a transcriptional repressor and a repressor of myogenesis. Knock-down of Rybp was sufficient to accelerate C2C12 myogenesis, suggesting it as an indispensable factor. This is in contrast to a recent report showing Rybp is dispensable for gene repression in ESCs (29). Moreover, *in vivo* injection of siRybp oligos accelerated CTX induced muscle regeneration, demonstrating that the role of Rybp is relevant in muscle formation. In contrast to increasing the number of cells stained with MyoD, Myogenin and eMyHC, siRybp injection down-regulated of



the number of cells with Pax7 staining, suggesting it may positively regulate satellite cell proliferation. This will be an interesting direction for future investigation. Previously, we showed that YY1 is relevant in DMD pathogenesis and treatment of dystrophic muscles with miR-29 can increase muscle regeneration through down-regulating YY1 levels (17). Considering their intimate interaction, it will be interestingly to find out whether Rybp is also involved in this type of muscle disease.

*Rybp functions as a co-repressor of YY1* - Majority of the studies on Rybp characterized it as a recruiter of PRC1. Rybp can bind both Ring1B and its substrate, ubiquitinated histone H2A thus participate in the long-term stable repression established by PRC1 complex (30). A very latest finding unveiled a H3K27me3 independent pathway of PRC1 recruitment, which is mediated directly through Rybp (31). However, it was not know how Rybp is recruited to target loci. Even though the physical interaction of YY1 and Rybp has been discovered long time ago in an *in vitro* study (27), the biological significance of this interaction was hardly explored. **In one study, Rybp and YY1 were found to form a transcriptional complex with E2F2/E2F3 controlling Cdc6 promoter activation (32).** It was also proposed that YY1/Rybp act as recruiter of PcG complexes to their targets. However, despite some evidence for such an activity, chromatin association studies in ESCs failed to show YY1 co-localization with PcG targets (33). Our results, on the other hand, provided clear evidence for an *in vivo* association of YY1/Rybp on target loci. Co-occupancy of YY1, Rybp, Ezh2 and H3K27me3 at the same genomic region was observed on three known YY1 target loci, miR-29 and Tnni2 promoter (6-7) as well as miR-1 enhancer (12). This is distinct to the recent observation showing Rybp binds to genomic regions with no or very low H3K27me3

in ESCs (29), suggesting a distinct function of Rybp in skeletal muscle cells versus ESCs. Furthermore, the fact that our ChIP assays detected co-occupancy of Ezh2, H3K27me3 and Rybp on the same genomic location suggest that Rybp may be somehow involved in recruitment or stabilization of PRC2. This notion is in line with a recent study of PcG regulation on HOXD cluster in hESC differentiation (34). Knock-down of RYBP resulted in a loss of both BMI1 (PRC1) and SUZ12 (PRC2) from the promoter and a complete loss of H3K27me3. Thus, YY1/Rybp interaction may be involved in PRC2 recruitment/stabilization in addition to PRC1 recruitment. Nevertheless, we cannot exclude the possibility that such mechanism only occurs on the limited genomic loci. Rybp may exert diverse functions in a target and cell-type dependent manner. The coordinated function of YY1 and Rybp was further evidenced from their correlated expression patterns in both *in vitro* C2C12 cell differentiation and *in vivo* CTX-induced muscle regeneration. Interestingly, this expression pattern was also observed in multiple tissues, suggesting that the interaction of YY1 and Rybp may not be limited to muscle only.

*miR-29 functions as an epigenetic modulator* - Our results reinforced the idea that miR-29 is a critical regulator of epigenetic machinery. In addition to Rybp and known targets, Hdac4 (35), Dnmt1 (9), a dozen of epigenetic modulators were down-regulated in miR-29 expressing cells. Among them, Bmi1, Cbx4, Cbx7, Suz12 are all PcG genes, suggesting miR-29 has strong impact on PcG-mediated chromatin modification. A number of Hdac genes, Hdac4, Hdac5, Hdac7, and Hdac8, were also down-regulated, suggesting a likely regulation of miR-29 on HDAC mediated chromatin remodeling. Not only it directly targets Rybp, its own expression is under transcriptional suppression by Rybp, thus forming a negative feedback loop. This regulation ensures that in myoblasts miR-29 is

silenced; once differentiation ensued, NF- $\kappa$ B decreased YY1 relieves miR-29 suppression. Increasing level of miR-29 in turn down-regulates both YY1 and Rybp thereby promoting myogenic program. Although both YY1 and Rybp are directly targeted by miR-29, the former is at translational level only (Fig. 1 RNA-seq data and (6)) but the latter is at both transcriptional and translational levels. Our studies thus demonstrate the prevalence of miRNA-involved regulatory loops.

Other than miR-29, miR-1 was found to form a direct regulatory circuitry with YY1 (12) and Rybp; miR-26 (36) and miR-214 (37) regulate Ezh2. These miRs thus constitute a growing list of epi-miRs in skeletal muscle. Our current study highlights the fact that skeletal

myogenesis is a sophisticated process involving interactions of epi-miRs, epigenetic modulators and transcription factors. Understanding these interactions will not only deepen our knowledge in the field of transcriptional/post-transcriptional regulation of myogenesis but also offer novel insights into the mechanisms behind various muscle diseases. For example, disruption of NF- $\kappa$ B-YY1-miR-29 circuitry leads to the formation of Rhabdomyosarcoma (6) and also contributes to muscle fibrogenesis in Duchenne muscular dystrophy (17). Our current study showed that knock-down of Rybp *in vivo* up-regulates miR-29 and accelerates injury-induced muscle regeneration (Fig. 6), suggesting this regulatory axis could be relevant in muscle diseases.

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## FOOTNOTES

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## FIGURE LEGENDS

**FIGURE 1. Over-expression of miR-29 in C2C12 cells down-regulates Rybp.** (A) Down-regulated epigenetic factors in C2C12 cells stably expressing miR-29 as revealed by RNA-seq. The abundances were reported in FPKM (fragments per kilobase of transcript per million fragments mapped). Suppression ratio was obtained by calculating percentage of the expression difference between vector and miR-29 cells relative to vector. (B) Coverage plot showing ~200 kb genomic region encompassing *Hdac4* gene on chromosome 1; the gene structure is shown below the graph. (C) Coverage plot showing ~60 kb region encompassing *Rybp* gene on chromosome (Chr) 6; the gene structure is shown below the graph.

**FIGURE 2. miR-29 suppresses Rybp expression through direct binding to its 3'UTR.** (A) A schematic illustration of base pairing between mmu-miR-29c with 3'UTR region of mouse *Rybp* gene. (B) A wild type (WT) or mutant *Rybp* 3'UTR luciferase reporter was transfected into C2C12 cells along with indicated precursor miRNA oligos. Luciferase activities were determined at 48 hr post-transfection and normalized to Renilla luciferase activity. Data represent the average of three independent experiments  $\pm$  SD. (C) *Rybp* mRNA expression was measured in NC, miR-29, Anti-NC or Anti-miR-29 transfected C2C12 cells by qRT-PCR and normalized with GAPDH. Expression folds are shown with respect to NC or Anti-NC cells where normalized copy numbers were set to 1. (D) *Rybp* protein expression was measured in the above transfected C2C12 cells by Western blotting using  $\alpha$ -Tubulin as a loading control. Numbers below indicate densitometric quantification of the Western bands.

**FIGURE 3. Rybp is down-regulated during C2C12 myogenesis concomitant with YY1.** (A) C2C12 myoblasts were induced to differentiate in differentiation medium (DM) for 0, 2, 4 or 6 days. Total proteins were extracted and used for Western blotting for *Rybp*, YY1 and MyHC. GAPDH was used as a loading control. (B) Primary myoblasts were induced to differentiate in DM for 0, 2, 4 days. Total proteins were extracted and used for Western blotting analysis for *Rybp*, YY1,  $\alpha$ -Actin, Troponin and MyHC.  $\alpha$ -Tubulin was used as a loading control. (C) C2C12 cells were stained for *Rybp* (red) and YY1 (green) by immunofluorescence (IF). The merged signals of *Rybp* and YY1 are yellow. DAPI staining indicates the nuclei. Bars=50 $\mu$ m. (D) Protein lysates were extracted from C2C12 myoblasts and subjected to Co-IP assay using antibodies against YY1 or *Rybp*. (E-F) Total RNAs were extracted from the indicated mouse tissues, and the expression levels of *Rybp* and YY1 mRNAs were measured by qRT-PCR and normalized with GAPDH. Data are represented as mean  $\pm$  SD. (G) Expression levels of *Rybp* or YY1 in muscle tissue or primary myoblasts were measured by qRT-PCR.

**FIGURE 4. Rybp suppresses myogenic differentiation.** (A) C2C12 cells were transfected with siNC or siRybp oligos, and differentiated for 2 days. Total proteins were isolated and Western blotting was performed to probe for Rybp, Troponin,  $\alpha$ -Actin, Myogenin expressions using  $\alpha$ -Tubulin as a loading control. Numbers below indicate densitometric quantification of the Western bands. (B) Total RNAs were extracted from the above transfected cells and used for qRT-PCR analysis of Rybp, MyHC, Troponin,  $\alpha$ -Actin, and Myogenin normalized with GAPDH. Expression folds are shown with respect to siNC cells where normalized copy numbers were set to 1. Data are plotted as mean  $\pm$  S.D. (C) qRT-PCR was performed in the above transfected cells to detect the expressions of miR-29 and miR-1 using U6 as normalization control. (D) The above transfected cells were stained for MyHC by IF (left). Bars=50 $\mu$ m. Positively stained cells were quantified by counting 10 randomly chosen fields and are represented as mean  $\pm$  S.D. (right). (E) C2C12 cells were transfected with Tnni2-Luc, MyHC-Luc or miR-29-Luc reporters along with pCMV-LacZ and siNC or siRybp oligos. Cells were differentiated for 48 hr, and luciferase activities were determined and normalized to activities of  $\beta$ -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. (F) C2C12 cells stably expressing Rybp or vector control were differentiated and harvested at the indicated time points. Total proteins were isolated and Western blotting was performed to probe for Rybp, MyHC, Troponin and  $\alpha$ -Actin expressions using  $\alpha$ -Tubulin as loading control. (G) C2C12 cells were transfected with Rybp expressing plasmid or vector control, and then differentiated for 2 days. Total RNAs were extracted from the above transfected cells and used for qRT-PCR analysis of Rybp, MyHC, Troponin,  $\alpha$ -Actin, and Myogenin as in (B). (H) qRT-PCR was performed in the above transfected cells to detect the expression of miR-29 and miR-1 using U6 as normalization control. (I) The above transfected cells were stained for MyHC by IF (left). Bars=50 $\mu$ m. Positively stained cells were quantified by counting 10 randomly chosen fields and are represented as mean  $\pm$  S.D. (right). (J) C2C12 cells were transfected with Tnni2-Luc, MyHC-Luc or miR-29-Luc reporters along with pCMV-LacZ and vector or Rybp expressing plasmids. Cells were differentiated for 48 hr, and luciferase activities were determined as in (E).

**FIGURE 5. Rybp functions as a co-repressor of YY1 to silence myogenic loci.** (A-D) C2C12 cells expressing vector control or Rybp were cultured as Myoblasts (MBs) or differentiated into Myotubes (MTs), at which stages chromatin was collected for ChIP-PCR assays using antibodies against Rybp, YY1, Ezh2, H3K27me3 or IgG as controls. Primers for amplifying promoters/enhancers of miR-29, miR-1 and Tnni2 were used for qRT-PCR analysis. Enrichment folds are shown with respect to IgG control where normalized PCR values were set to 1. Data are plotted as mean  $\pm$  S.D. (E) C2C12 cells were transfected with 0.25  $\mu$ g of miR-29-promoter-luc reporter plasmid along with 0.5 $\mu$ g Rybp, or 0.5  $\mu$ g YY1 plasmid combined with Rybp plasmids (0, 0.2, 0.3, 0.4, or 0.5  $\mu$ g). Relative activities were shown with respect to control cells, where normalized luciferase values were set to 1. Data represent the average of three independent experiments  $\pm$  SD.

**FIGURE 6. Knock-down of Rybp improves muscle regeneration *in vivo*.** (A) Tibialis anterior (TA) muscles from six-week old C57/BL6 background mice were injected with cardiotoxin (CTX). RNAs were then extracted from injected muscles at the indicated days post-injection, and qRT-PCR was performed to measure the expressions of Rybp and YY1 normalized with GAPDH or miR-29



normalized with U6. Expression folds are shown with respect to day 1 where Rybp, YY1 and miR-29 levels were set to a value of 1. Quantitative values are represented as means  $\pm$  S.D. (B) TA muscles were injected with CTX at day 0, followed by injection with siNC (left leg) and siRybp1 oligos (right leg) 6 hours later. And re-injection of siRNA oligos was performed every other day for two more times. The injected muscles were harvested at the indicated days. N=4 for each group. (C) Total RNAs were extracted from the above injected muscles. Expression levels of Rybp, MyoD, Myogenin, eMyHC, miR-29 and Pax7 RNAs were detected by qRT-PCR. Expression folds are shown with respect to siNC where expression levels were set to a value of 1. Data are represented as mean  $\pm$  S.D. (D) Proteins were extracted from the above injected muscles and probed for Rybp, MyoD, Myogenin, eMyHC, or Pax7 using  $\alpha$ -tubulin as a loading control. (E-G) Frozen muscle sections were prepared from the above injected muscles on day 3 post-injection and IF staining was performed for MyoD, Myogenin or Pax7. (H). H&E staining was used to visualize fibers with centrally located nuclei (CLN) on day 6 post CTX injection. Positively stained cells or fibers with CLN were quantified. \* $p$ <0.05; \*\* $p$ <0.01.

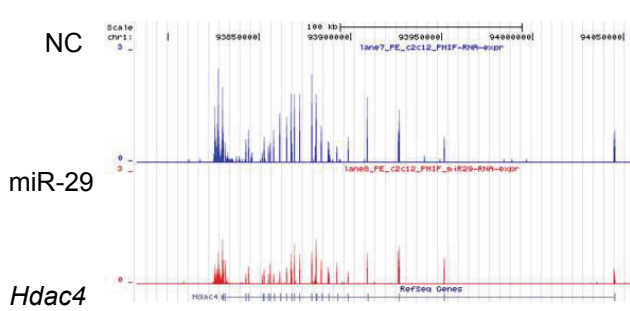
**FIGURE 7. A model of Rybp/YY1-miR-29 regulatory circuitry in skeletal myogenesis.** The model depicts the role of the Rybp/YY1-miR-29 regulatory circuitry in myogenic differentiation. In myoblast, a repressive complex containing YY1/Rybp/Ezh2/HDAC4 binds to the promoter region of miR-29, leading to the trimethylation of histone lysine 27 and subsequent silencing of miR-29 transcription. Upon myogenesis starts, the recruitment of MyoD/SRF activating complex and the displacement of the repressive complex lead to the activation of miR-29, which in turns targets both Rybp and YY1 to ensure the progress of myogenic differentiation. Straight line, promoter/enhancer region of mmu-miR-29b/c with arrow denotes TSS; CCAT, YY1 binding element; E-box, MyoD binding site; Me, methylation of histone lysine 27; Ac, acetylation of histones.

Figure 1 Zhou et al

A

Gene ID	Chrosomal location	Vector (FPKM)	miR-29 (FPKM)	Suppression % (Vector-miR-29/Vector)
<i>Bmi1</i>	2:18598644-18608256	14.0093	10.0326	28.3861
<i>Cbx4</i>	11:118938886-118947535	14.9745	9.22796	38.3755
<i>Cbx7</i>	15:79746236-79801549	23.4262	14.6354	37.5255
<i>Dnmt1</i>	9:20711649-20757317	25.1897	20.2815	19.4849
<i>Hdac4</i>	1:93829333-94044970	9.22078	5.89644	36.0527
<i>Hdac5</i>	11:102055745-102091480	160.129	86.5175	45.9701
<i>Hdac7</i>	15:97614785-97674933	29.9493	19.4492	35.0596
<i>Hdac8</i>	X:99447860-99700698	5.90241	3.18482	46.0420
<i>Hdac10</i>	15:88953732-88959130	11.9574	9.13218	23.6274
<i>Hdac11</i>	6:91106658-91124686	9.26428	6.10939	34.0543
<i>Rybp</i>	6:100178559-100237435	7.31249	5.78492	20.8899
<i>Suz12</i>	11:79806607-79847625	5.34988	4.49326	16.0119
<i>Yy1</i>	12:110031182-110054842	23.0989	22.2637	3.6158

B



C

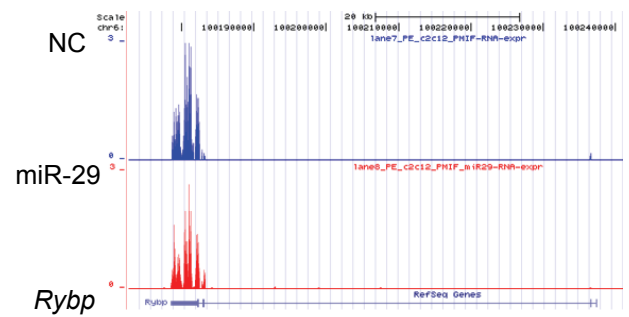


Figure 2 Zhou et al

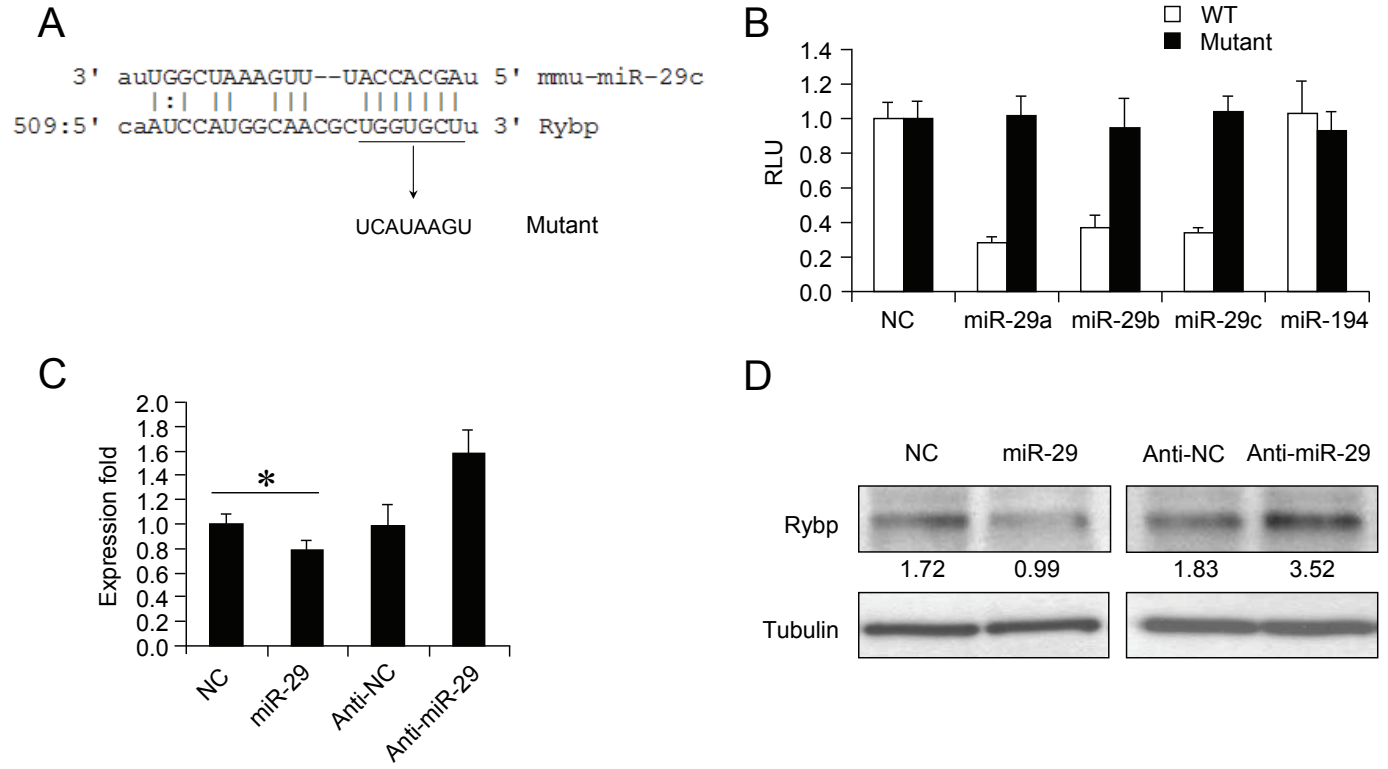
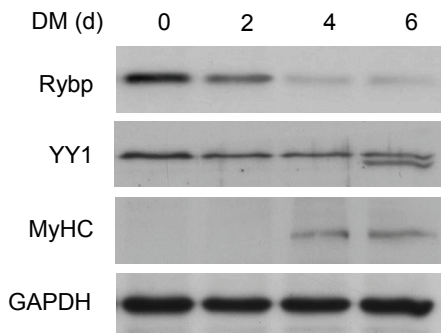
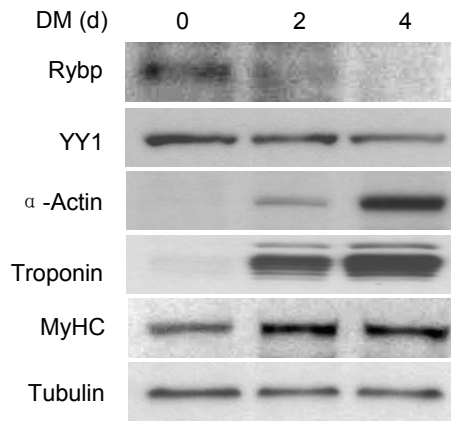


Figure 3 Zhou et al

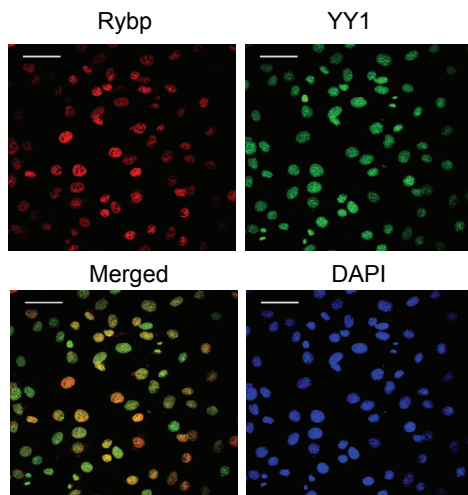
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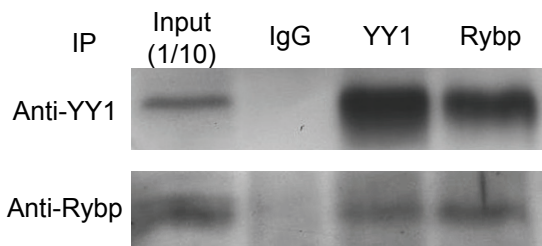
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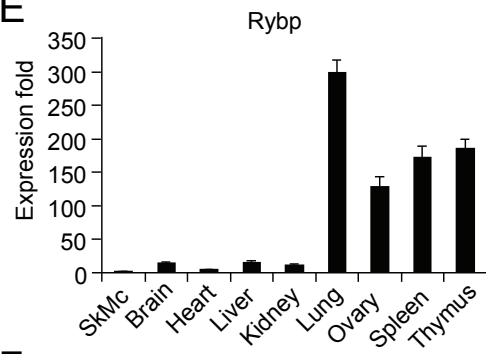
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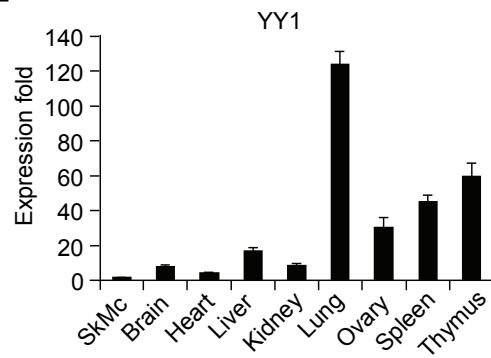
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**E**



**F**



**G**

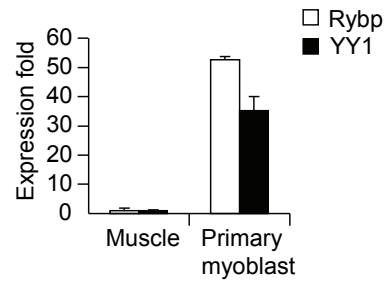


Figure 4 Zhou et al

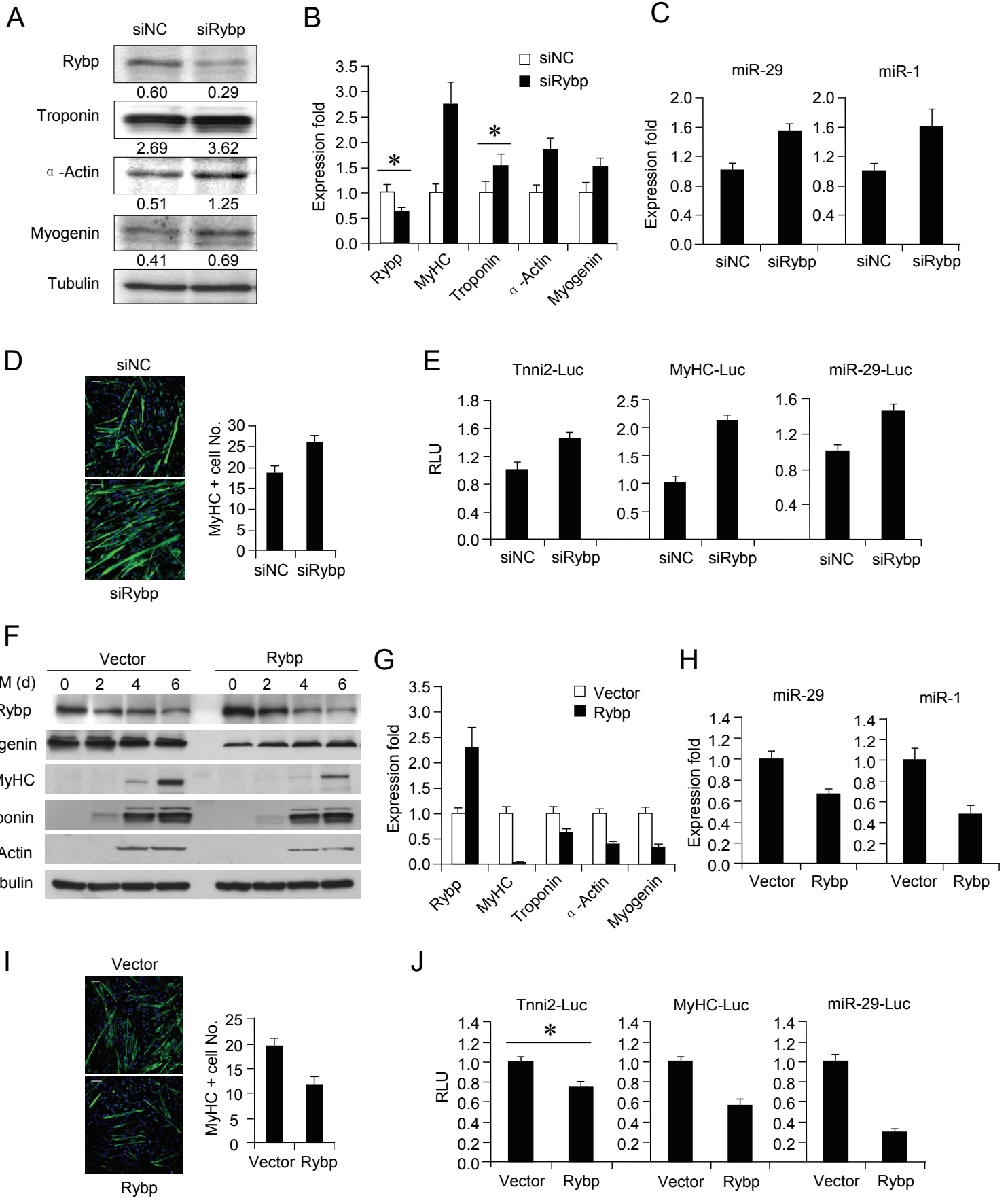


Figure 5 Zhou et al

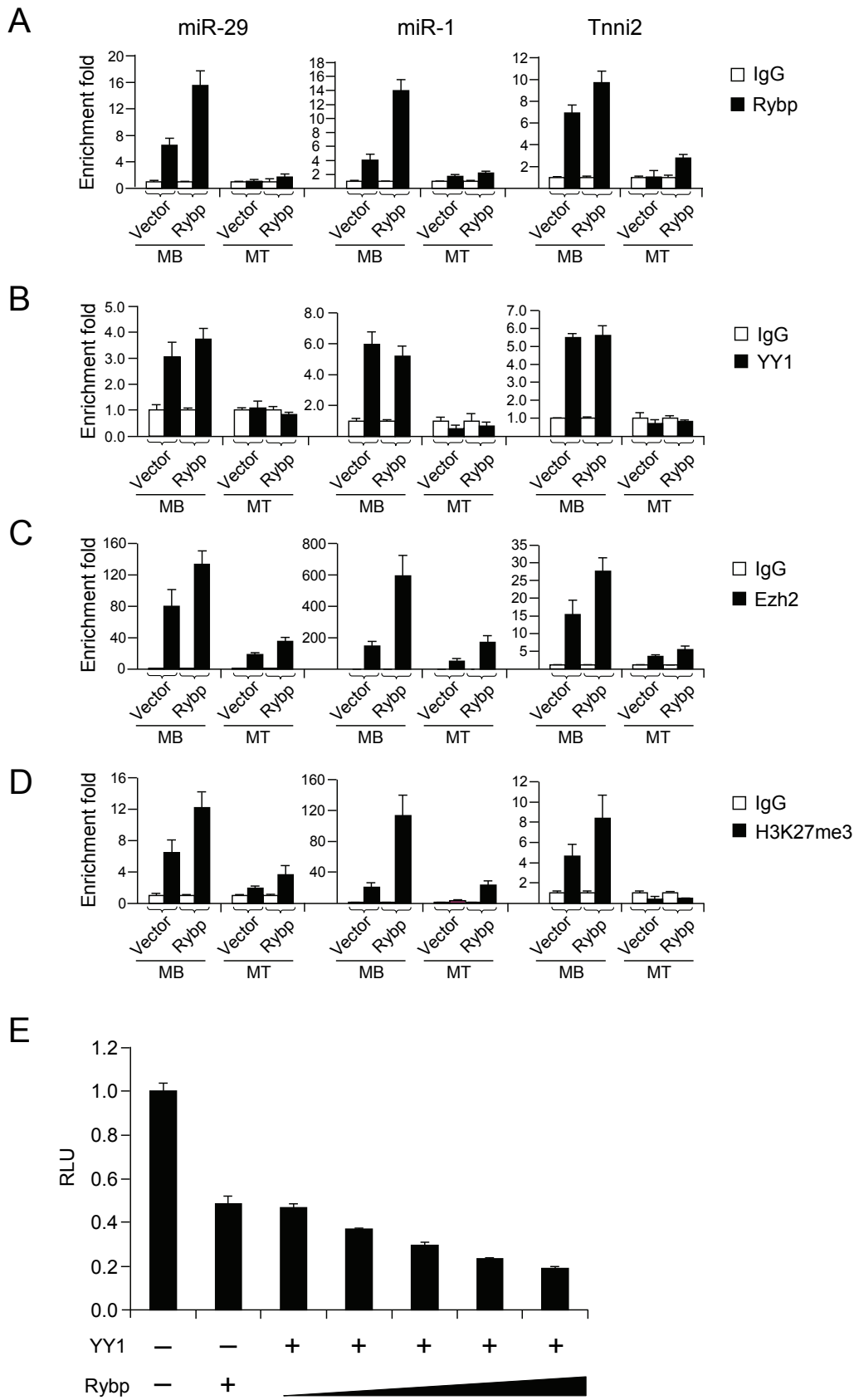




Figure 6 Zhou et al

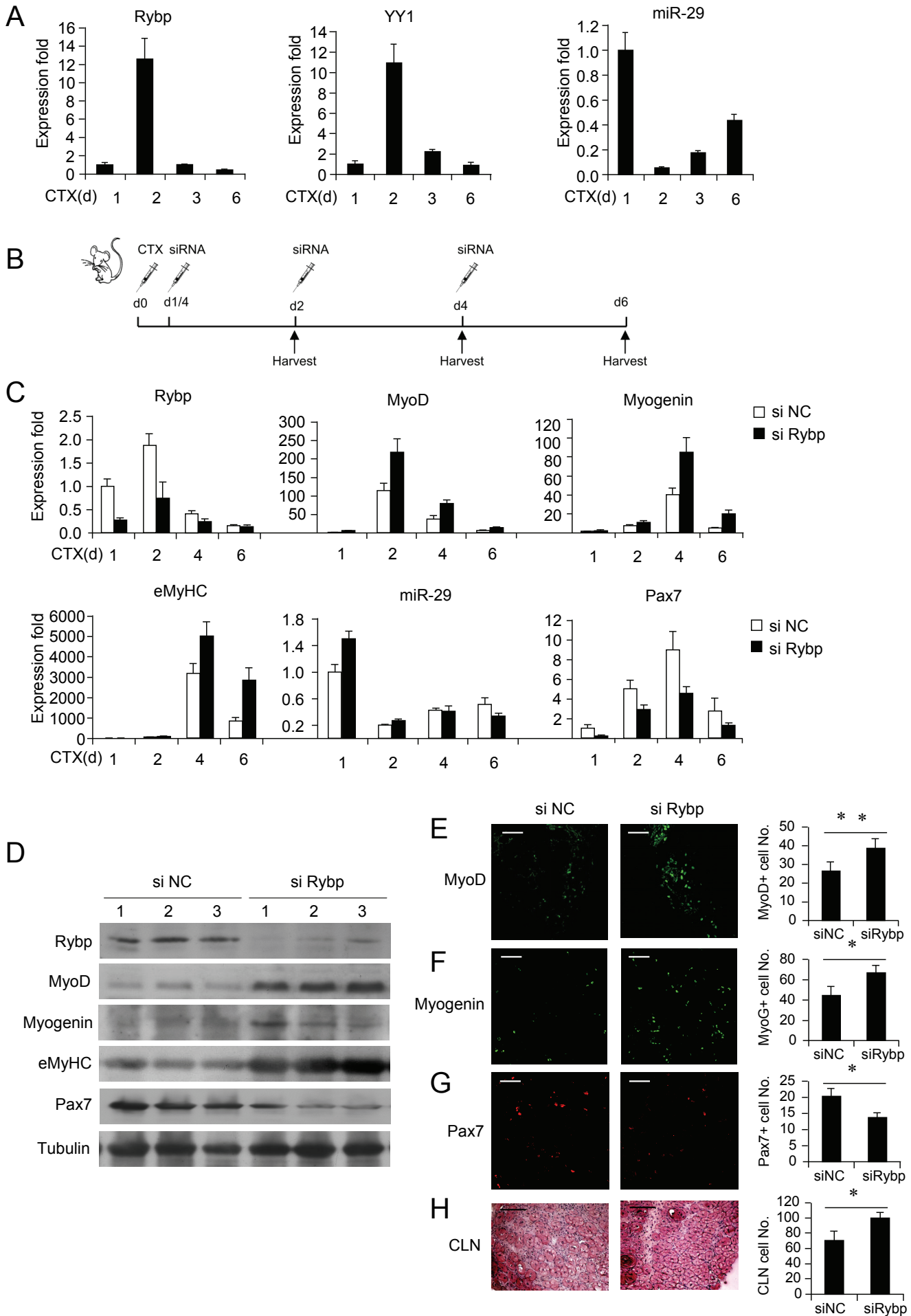


Figure 7 Zhou et al

