Histone deacetylase 1 enhances microRNA processing via deacetylation of DGCR8

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Relatively little is known about the regulatory mechanisms of the Drosha/DGCR8 complex, which processes miRNAs at the initial step of biogenesis. We found that histone deacetylase 1 (HDAC1) increases the expression levels of mature miRNAs despite repressing the transcription of host genes. HDAC1 is an integral component of the Drosha/DGCR8 complex and enhances miRNA processing by increasing the affinity of DGCR8 to primary miRNA transcripts via deacetylation of critical lysine residues in the RNA-binding domains of DGCR8. This finding suggests that HDACs have two arms for gene silencing: transcriptional repression by promoter histone deacetylation and post-transcriptional inhibition by increasing miRNA abundance.

Keywords: acetylation; DGCR8; histone deacetylase; microRNA; miRNA processing

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INTRODUCTION

MicroRNAs (miRNAs) are a diverse class of short-length (21–23 nt in mammals) RNAs that regulate the cellular gene expression programmes in a negative manner by affecting the stability and translational efficiency of mRNAs. Accordingly, miRNAs are involved in multiple cellular functions that are essential for normal development and cell homeostasis in various cell types including embryonic stem (ES) cells (Marson *et al*, 2008; Ware *et al*, 2009). Hence, it is important to understand the regulatory mechanisms of miRNA biogenesis thoroughly.

Recent progress in miRNA research has unveiled the detailed process of miRNA biogenesis (Kim *et al*, 2009). MicroRNAs are first transcribed from the genome by RNA polymerase II (Pol II) as long primary transcripts (pri-miRNAs) concomitantly with host genes in which miRNAs are located in introns or even in exons (Lee *et al*, 2004). Pri-miRNAs are cropped into 60- to 70-nt hairpin-structured precursors (pre-miRNAs) by the Microprocessor complex composed of an RNase III enzyme Drosha, a double-stranded RNA-binding protein DGCR8 and a variety of cofactors

called Drosha-associated polypeptides (DAPs), including DEAD box helicases p68/DDX5 and p72/DDX17 (Denli *et al*, 2004; Gregory *et al*, 2004; Fukuda *et al*, 2007). DGCR8 binds to the junction between single- and double-stranded regions of pri-miRNAs through two double-stranded RNA-binding domains (dsRBDs), and directs Drosha to cleave the site ~11 bp away from the junction to generate pre-miRNAs (Han *et al*, 2006). Pre-miRNAs are then transported into the cytoplasm and further processed by another RNase III enzyme Dicer to become mature miRNAs. The final abundance of cellular miRNAs is controlled at multiple steps from the transcription of pri-miRNAs to the processing of pri- and pre-miRNAs in cell type- and developmental stage-specific manners. Despite increased knowledge of the pathway of miRNA biogenesis, relatively little is known about the mechanisms regulating the activity of the pathway components.

Histone deacetylases (HDACs) are a family of enzymes that catalyse the removal of acetyl groups from core histones and surrounding proteins in promoter regions, leading to chromatin compaction and transcriptional repression of Pol II-regulated genes (Yang & Seto, 2008). The effects of HDACs are counteracted by a group of enzymes known as histone acetyltransferases (HATs), and the balance between the two groups of enzymes determines transcriptional activity of genes. Because most miRNAs possess canonical core promoters and enhancers under the control of Pol II in their upstream regions (Saini *et al*, 2007; Marson *et al*, 2008), we postulated that HDACs negatively affect the abundance of miRNAs through transcriptional repression. In contrast to the initial assumption, however, our investigations revealed that HDAC1 functions as a positive regulator of miRNA abundance by enhancing Microprocessor-mediated processing.

RESULTS

HDAC1 increases the expression levels of mature miRNAs

To investigate whether HDACs have regulatory roles in miRNA biogenesis, we modified the expression levels of HDACs and evaluated the changes in mature miRNA abundance using miRNA arrays. For this purpose, we overexpressed HDAC1, which accounts for more than half of cellular HDAC activity and cannot be compensated by other HDACs (Dovey *et al*, 2010), in HEK293 cells, because intrinsic HDAC1 expression is relatively low in this cell line (Fig 1A, left lower panel). HDAC1 overexpression downregulated the expression of only 9.0% of mature miRNA

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Fig 1 | HDAC1 positively regulates the abundance of miRNAs. (A) We took advantage of the lentiviral expression system to achieve HDAC1 overexpression and knockdown in HEK293 and K562 cells, respectively. After 48 h, we sorted transduced cells using flow cytometry, isolated total cellular RNAs, including small RNAs, and subjected them to miRNA expression profiling using microarrays. Left panel: the y axis in the upper panel indicates the percentage of miRNAs affected by HDAC1 overexpression and knockdown. We defined >1.5-fold increase and >50% reduction as upregulation and downregulation, respectively. Representative results of the miRNA set with an expression cutoff value >100 are shown. We obtained the same tendency by analysing miRNA sets with different cutoff values (supplementary Fig S1A online). The efficiency of overexpression and knockdown was monitored by immunoblotting HDAC1 and GAPDH (lower panel). Right panel: we performed linear regression analysis of 78 miRNAs regulated by HDAC1 commonly in HEK293 (x axis) and K562 (y axis) cells. (B) RNA samples isolated as described above were subjected to real-time quantitative RT-PCR. The data were quantified with the $2^{-\Delta\Delta Ct}$ method using simultaneously amplified U6 snRNA, GAPDH and β -actin as references. The y axes indicate relative gene expression in HDAC1- and siHDAC1-transduced cells against corresponding Mock- and siControl-transduced cells with the expression levels of the latter being set at 1.0. The means $\pm$ s.d. (bars) of five independent experiments are shown (* $P < 0.05$ by Student's *t*-test). (C) We transfected HEK293 and K562 cells with KAT2B expression vector and lentiviral shRNA expression vectors for p300, respectively, and carried out RT-PCR analysis as described above. The means $\pm$ s.d. (bars) of three independent experiments are shown (* $P < 0.05$ by Student's *t*-test). (D) We cultured normal human CD34⁺ bone marrow mononuclear cells in serum-free liquid medium containing appropriate cytokines (Wada *et al*, 2009). After 24 h, total cellular RNA was isolated and subjected to real-time quantitative RT-PCR for miRNAs (upper panel) and semiquantitative RT-PCR for HDAC1 (lower panel). The means $\pm$ s.d. (bars) of three independent experiments are shown (* $P < 0.05$ by Student's *t*-test). BMMNC, bone marrow mononuclear cell; Ex, exon; HAT, histone acetyltransferase; HDAC, histone deacetylase; miRNA, micro RNA; pri-miR, primary transcript; pre-miR, precursor transcript; shRNA, short hairpin RNA.

species placed on an array, but rather upregulated the abundance greater than 1.5-fold in 46.1% (Fig 1A, left upper panel and supplementary Table S1 online). We carried out a reciprocal experiment in which HDAC1 expression was reduced by the aid of the short hairpin RNA/small interfering RNA system in HDAC1-overexpressing K562 cells (Kikuchi *et al*, 2010). HDAC1 knockdown resulted in a decrease of 67.2% of the miRNAs examined, and did not increase the abundance of any miRNA >1.5-fold in K562 cells (Fig 1A; supplementary Table S2 online). Moreover, linear regression analysis revealed a statistically significant inverse correlation among 78 miRNAs regulated by HDAC1 commonly in HEK293 and K562 cells (Fig 1A, right panel).

Next, we attempted to confirm the results of miRNA array analysis with real-time quantitative RT-PCR for three intronic miRNAs, miR-106b, miR-25 and miR-330, and examined the expression of the corresponding primary transcripts and host genes, MCM7 and EML2, simultaneously. Consistent with the array results, HDAC1 overexpression increased the expression levels of miR-106b, miR-25 and miR-330, whereas HDAC1 knockdown decreased them (Fig 1B). As anticipated, HDAC1 tended to repress the transcription of MCM7 and EML2, but did not significantly alter the expression levels of pri-miRNAs (Fig 1B). HDAC1 also marginally affected the abundance of primary transcripts of two intergenic miRNAs, pri-miR-29a and pri-miR-101, whose mature products were considerably upregulated in response to HDAC1 overexpression (supplementary Fig S1B online). In addition, we examined miR-939 and miR-921, whose expression was unaffected in microarray experiments (supplementary Table S1 online), to serve as negative controls for RT-PCR analysis. Neither HDAC1 overexpression nor knockdown significantly changed the expression of miR-939 and miR-921 in HEK293 and K562 cells (supplementary Fig S1C online).

To validate the results of HDAC1 modulation, we investigated whether HAT overexpression and knockdown affect the abundance of miRNAs in a reciprocal manner. As shown in Fig 1C, HAT (KAT2B) overexpression decreased the expression levels of miRNAs that were downregulated by HDAC1 overexpression. Similarly, knockdown of p300 histone acetyltransferase resulted in a marked increase in the expression of a set of miRNAs that were upregulated by HDAC1 knockdown.

Finally, we sought to verify the HDAC-mediated increase in miRNA expression in a natural context. Previously, we found that HDAC1 expression was relatively low in human CD34⁺ hematopoietic progenitor cells, and was upregulated during their commitment and differentiation (Wada *et al*, 2009). Using this system, we showed that the expression levels of miR-106b, miR-330 and miR-17-5b increased in parallel with the upregulation of HDAC1 (Fig 1D). Moreover, we examined the effects of enzymatic inhibition of HDACs on miRNA expression in K562 and other haematopoietic cell lines. Treatment with the HDAC inhibitor romidepsin reduced the expression of most but not all miRNA examined (supplementary Fig S1D online). Taken together, these data indicate that the expression of some types of miRNA and their host genes is differentially regulated by HDACs *in vivo*, despite the fact that they share common transcription units (Lee *et al*, 2004; Saini *et al*, 2007).

HDAC1 enhances miRNA processing *in vitro* and *in vivo*

Given that HDACs did not activate the transcription of miRNAs, we reasoned that the HDAC-mediated increase in miRNA abundance

occurs in post-transcriptional steps. To test this hypothesis, we performed *in vitro* miRNA processing assays. As shown in Fig 2A, pretreatment of HEK293 nuclear extracts with purified HDAC1 significantly enhanced the processing of *in vitro*-transcribed pri-miR-101 and pri-miR-106b to pre-miRNAs. In contrast, pretreatment with purified p300 led to a decrease in miRNA processing. To validate these findings in a more sophisticated system, we carried out the same experiments with the Microprocessor complex purified from HEK293 cells using anti-Drosha antibody. Again, HDAC1 significantly enhanced miRNA processing by a purified Microprocessor complex in a dose-dependent manner, whereas p300 inhibited it (Fig 2B; supplementary Fig S2B,C online).

In addition, we examined the expression of pre-miRNAs using real-time RT-PCR with hairpin-specific primers (supplementary Table S3 online). As shown in Fig 1B, HDAC1 induced the changes of pre-miRNA levels in accordance with those of mature miRNAs, implying that HDAC1 modulates the abundance of miRNAs at the processing step *in vivo*.

HDAC1 modifies the acetylation status of DGCR8

During the process of purification, we found that the Microprocessor complex contained class I HDACs (HDAC1, HDAC2 and HDAC3) in addition to Drosha, DGCR8 and two DEAD box helicases (Fig 3A). The members of class II and III HDACs, such as HDAC4, HDAC6 and SIRT6, were not detectable in Drosha immunoprecipitates (supplementary Fig S3A online). To identify the function of HDAC1 in the Microprocessor complex, we checked the acetylation status of each component of the complex. As shown in Fig 3B, lysine acetylation was readily detected in DGCR8, but not Drosha and p68/DDX5, in a native complex from HEK293 cells. The acetylation of DGCR8 was greatly enhanced by p300 and almost completely abrogated by HDAC1 (Fig 3B, right panel). In contrast, both p300 and HDAC1 marginally affected the acetylation status of Drosha and p68/DDX5. In addition, HDAC1 overexpression and knockdown caused DGCR8 deacetylation and hyperacetylation, respectively, *in vivo* (Fig 3B, left panel). These results strongly suggest that HDAC1 specifically modifies the acetylation levels of DGCR8 in the Microprocessor complex.

Next, we sought to determine the functional consequence of DGCR8 acetylation/deacetylation. Given that lysine acetylation generally influences the physical interactions of corresponding proteins with nucleic acids, we first examined the effects of HDAC1 on the association between the Microprocessor complex and pri-miRNA transcripts *in vivo* using native RNA immunoprecipitation. As shown in Fig 3C, HDAC1 overexpression augmented the association between the Microprocessor complex and primary transcripts of miR-106b, miR-101 and miR-29a, whose mature products were upregulated in response to HDAC1. In contrast, there was no detectable change in the association of the Microprocessor complex with pri-miR-921 and pri-miR-939, whose maturation was unaffected by HDAC1. Because miRNA processing proceeds co-transcriptionally at the site of active transcription (Morlando *et al*, 2008; Pawlicki & Steitz, 2008), we took the same approach to examine the association between pri-miRNA transcripts and core histones. As anticipated, HDAC1 overexpression liberated primary transcripts of miR-106b, miR-101 and miR-29a, all of which responded to HDAC overexpression, from histone H3 (Fig 3D). Again, there was no obvious change in the association of histone H3 with pri-miR-223

Fig 2 | HDAC1 enhances *in vitro* miRNA processing. (A) Primary miRNA transcripts were transcribed from linearized plasmids encoding pri-miR-106b and pri-miR-101 in the presence of [α - 32 P]CTP. *In vitro* processing was conducted in a reaction mixture containing pri-miRNA (20,000 c.p.m.) in the absence or presence of HEK293 nuclear extracts. Before the processing reaction, nuclear extracts were treated with either purified recombinant p300 (67 ng/ml) or HDAC1 (8 ng/ml), whose effects were monitored by histone H3 acetylation (supplementary Fig S2A online). (B) We carried out an *in vitro* miRNA processing assay in the absence (no Microprocessor) or presence of the Microprocessor complex purified from HEK293 cells using anti-Drosha antibody. The Microprocessor complex was treated with either purified recombinant HDAC1 (0, 0.8 and 8 ng/ml) or p300 (0, 6.7 and 67 ng/ml), and dialysed against reaction buffer before processing assays. The results of pri-miR-101 are shown; those of pri-miR-106b and pri-miR-939 are in supplementary Fig S2B,C online. The positions of RNA size marker are shown in the left. Lower panels: we quantified signal intensities of pre-miRNA and pri-miRNA bands using the Scion Image software, calculated processing efficiency according to the formula (pre-miRNA)/[(pri-miRNA) + (pre-miRNA)] and show the results as a ratio against untreated controls. The means $\pm$ s.d. (bars) of three independent experiments are shown (* P < 0.05 by one-way analysis of variance with the Bonferroni *post hoc* test). BPB, bromophenol blue; HAT, histone acetyltransferase; HDAC, histone deacetylase; miRNA, micro RNA; pri-miR, primary transcript; pre-miR, precursor transcript.

and pri-miR-939. It is noteworthy that the levels of mature miRNA upregulation correlated well with the association of primary transcripts to Drosha and the liberation from histone H3 in HDAC1-overexpressing cells. These results imply that HDACs facilitate the binding of nascent pri-miRNA transcripts to the Microprocessor complex through DGCR8.

HDAC1 increases DGCR8 affinity for pri-miRNAs

Recent crystallographic studies have demonstrated that human DGCR8 has two $\alpha\beta\beta\alpha$ -structured dsRBDs, and the second α -helices (designated as H2 and H2') in each dsRBD provide a binding platform for pri-miRNA substrates (Sohn *et al*, 2007). Importantly, the conserved lysine and arginine residues

◀ **Fig 3** | HDAC1 is an integral component of the Microprocessor complex and modifies its affinity to primary miRNA transcripts. (A) We prepared whole-cell lysates from HEK293 cells transfected with either empty (mock) or HDAC1 expression vector (HDAC1) and K562 cells transfected with siRNA vectors against either scrambled sequences (siControl) or HDAC1 mRNA (siHDAC1), and subjected them to immunoprecipitation with anti-Drosha antibody. The precipitated complexes were separated on SDS-PAGE, followed by immunoblot analysis for the presence of indicated proteins. Drosha expression serves as a control of equal efficiency of immunoprecipitation. The heavy chain of anti-Drosha antibody is shown as a loading control. (B) Left panel: we subjected the samples in A to immunoprecipitation with anti-DGCR8 antibody, followed by immunoblotting with anti-acetylated lysine and anti-DGCR8 antibodies. Right panel: purified Microprocessor complex was treated with either p300 (67 ng/ml) or HDAC1 (8 ng/ml) and was subjected to immunoprecipitation with antibodies against DGCR8, Drosha and p68/DDX5. The precipitated complexes were separated on SDS-PAGE, followed by immunoblotting with either an anti-acetylated lysine antibody or corresponding antibodies. (C) We isolated nuclear extracts from cells treated as described and carried out native RNA immunoprecipitation. Precipitated RNA was subjected to semiquantitative RT-PCR for primary miRNA transcripts and GAPDH (internal control). The results of linear amplification cycles are shown. Right panel: signal intensities were quantified using the Scion Image software and are shown as a fold increase against corresponding mock or siControl data after normalization with those of the input. (D) Nuclear extracts from HDAC1-overexpressing 293 cells were subjected to RNA immunoprecipitation with anti-histone H3 antibody (H3 bound). The remaining supernatants were handled exactly similar to the input chromatin (H3 unbound). Right panel: quantified signal intensities are shown as a ratio of unbound/bound after normalization with those of the input. The means $\pm$ s.d. (bars) of three independent experiments are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAT, histone acetyltransferase; HDAC, histone deacetylase; IgH, immunoglobulin heavy chain; miRNA, micro RNA; pri-miR, primary transcript; pre-miR, precursor transcript; siRNA, small interfering RNA.

in H2 and H2' (K561/K562/K565 and K669/R670/K673, respectively) were shown to be indispensable for the recognition of pri-miRNA, as the substitution of either K561/K562/K565 or K669/R670/K673 to AAA (designated as D1H2 and D2H2, respectively) resulted in over 50% reduction of DGCR8 binding to pri-miRNA probes in electrophoretic mobility shift assays (Fig 4A,B). It is therefore tempting to speculate that the acetylation status of these residues affects the affinity of DGCR8 to pri-miRNA transcripts. In fact, p300-mediated acetylation apparently inhibited the binding of recombinant DGCR8 to pri-miR-106b, and subsequent treatment with HDAC1 was able to reverse this inhibition in wild-type DGCR8 (Fig 4A; supplementary Fig S3C online). Importantly, p300 almost completely abrogated the binding of D2H2 mutant, which lost K669/R670/K673 of H2' but retained K561/K562/K565 of H2, but HDAC1 still restored the binding of this mutant to pri-miRNA probably through reversal of p300-mediated acetylation of K561/K562/K565 (Fig 4A; supplementary Fig S3C online).

Finally, we asked whether the affinity of DGCR8 to pri-miRNAs is dependent on its acetylation status *in vivo*. To this end, we transduced HEK293 cells with wild-type and mutant DGCR8, and examined the effects of HAT overexpression on the abundance of mature miRNAs. As shown in Fig 4C, miRNA expression was significantly downregulated by HAT overexpression when wild-type DGCR8 was introduced into HEK293 cells. In striking contrast, HAT failed to decrease the expression levels of miRNAs in the presence of D2H2 mutant. Taken together, these results strongly suggest that HDACs enhance miRNA processing by increasing the affinity of DGCR8 to primary miRNA transcripts via deacetylation of critical lysine residues in the dsRBDs of DGCR8.

DISCUSSION

In this study, we show that HDACs are involved in post-transcriptional gene silencing via increasing miRNA abundance. The large Microprocessor complex contains at least 17 cofactors called DAPs (Gregory *et al*, 2004); DAP60, 55 and 52 might correspond to HDAC1, 2 and 3, respectively, because of size similarities. The Drosha-associated HDACs might keep DGCR8

hypoacetylated in a steady state to maintain baseline expression of miRNAs. At the time of inducible transcription, DGCR8 might be coincidentally acetylated by HATs, resulting in temporal suppression of miRNA processing. This scenario is best exemplified by the recent finding that oestrogen receptor- α (ER α) inhibits the maturation of a subset of miRNAs via estrogen-dependent physical interaction with pri-miRNA transcripts (Yamagata *et al*, 2009). As ER α recruits HATs to facilitate the transcription of oestrogen-regulated genes, it is highly possible that ER-associated HATs are responsible for the inhibition of miRNA processing. However, DGCR8 is rapidly deacetylated by HDACs in the Microprocessor complex to facilitate transcription-coupled miRNA processing (Fig 4D).

Given the generality of the proposed mechanism of HDAC-mediated enhancement of miRNA processing, HDACs are supposed to affect the abundance of most, if not all, miRNA species. However, HDACs appeared to govern the maturation of many, but not all, miRNAs in our experiments. It has been reported that several auxiliary factors, such as p53, SMAD and KSRP, are involved in the processing of different subsets of miRNAs under the influence of various environmental cues. For example, ATM-activated p53 and KSRP enhance the maturation of specific miRNAs, such as miR-15, miR-16 and miR-206, as part of the DNA damage response (Suzuki *et al*, 2009; Zhang *et al*, 2011). HDAC1 might cooperate with these factors to promote the processing of individual miRNAs, underlying the selection of HDAC-responsive miRNAs (Fig 4D). This view is supported by our unexpected observation that pri-miR-939, which was unresponsive to HDAC1 in microarray and RT-PCR experiments, bound to recombinant DGCR8 in electrophoretic mobility shift assays (supplementary Fig S3D online) and responded to HDAC and HAT treatment in *in vitro* processing assays with purified Microprocessor (supplementary Fig S2C online) but not crude extracts (supplementary Fig S2D online).

Accumulating evidence indicates that the expression levels of mature miRNAs are generally low during early development because of insufficient processing of miRNAs (Viswanathan *et al*, 2008; Ware *et al*, 2009; Suh *et al*, 2010). Accordingly, miRNA abundance is also very low in ES cells, which might contribute to

Fig 4 | Acetylation status of critical lysine residues in dsRBD affects the binding of DGCR8 to primary miRNA transcripts. (A) We generated recombinant wild-type DGCR8M protein and D1H2 and D2H2 mutants, in which K561/K562/K565 and K669/R670/K673 were, respectively, substituted with Ala, in *Escherichia coli*. Purified DGCR8M proteins (0–4.0 μM) were incubated with radiolabelled pri-miR-106b transcript (0.25 nM) in EMSA buffer and subjected to SDS–PAGE to separate the DGCR8M/pri-miRNA complexes (B) and free probes (F). Before the binding reaction, DGCR8M proteins were either untreated or treated with p300 (67 ng/ml) with or without subsequent incubation with HDAC1 (8 ng/ml) in appropriate buffers. The effects of p300 and HDAC1 were monitored by immunoblotting with anti-acetylated lysine antibody (supplementary Fig S3B online). (B) Signal intensities of the DGCR8M/pri-miRNA complexes (bound) and free probes were quantified using the Scion Image software and are shown as a ratio of (bound)/[(bound) + (free)] on y axes. Means ± s.d. (bars) of three independent experiments are shown. (C) We overexpressed either wild-type or mutant (D2H2) DGCR8 in HEK293 cells, and examined the effects of KAT2B (HAT) overexpression on the expression levels of mature miRNAs using RT–PCR. Lower panel: the y axis indicates relative expression against untreated HEK293 cells. The means ± s.d. (bars) of three independent experiments of individual miRNAs are shown. Upper panel: the results of miR-106b, miR-25, miR-330, miR-29a and miR-101 were accumulated and evaluated by paired Student's *t*-test. Equal expression of the introduced genes was confirmed by immunoblotting (supplementary Fig S3E online). (D) Schematic representation of the mechanisms by which HDACs regulate the cellular gene expression programs. See Discussion for the detail. dsRBD, double-stranded RNA-binding domain; HAT, histone acetyltransferase; HDAC, histone deacetylase; EMSA, electrophoretic mobility shift assay; miRNA, micro RNA; Pol II, RNA polymerase II; pri-miR, primary transcript; pre-miR, precursor transcript.

their rich transcriptome to maintain stemness and pluripotency (Kosik, 2010). We and others have documented that HDAC expression is relatively low in multipotent stem/progenitor cells such as ES and hematopoietic progenitor cells (Wada et al, 2009; Lin et al, 2011). Low HDAC activity might be implicated in the insufficient maturation of miRNAs in these cells (Ware et al, 2009), and thus could be a target of therapeutic manipulation. Indeed, the efficiency of iPS cell generation was greatly improved by HDAC inhibitors and/or introduction of certain subsets of miRNAs (Anokye-Danson et al, 2011; Lin et al,

2011; Pfaff et al, 2011). Our present finding provides mechanistic insight into these facts and a rationale for HDAC inhibition strategies in regenerative medicine.

METHODS

Global analysis of miRNA expression. Cellular RNAs were hybridized with miRNA microarrays according to the manufacturer's instruction. A set of representative data is provided in supplementary Tables S1 and S2 online. The array results were verified by RT–PCR.

Native RNA immunoprecipitation. RNA immunoprecipitation was performed under native conditions according to the method described by Zhao *et al* (2008) with minor modifications. See supplementary information online for the detailed methods.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: T.W. and Y.F. designed research; T.W. conducted experiments; T.W., J.K. and Y.F. analysed data; and T.W. and Y.F. wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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