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Mixed-lineage leukemia protein modulates the loading of let-7a onto AGO1 by recruiting RAN

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Running Title: MLL contributes to miRNA loading onto AGO1

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Dear Editor,

The mixed-lineage leukemia (MLL) proto-oncogenic protein, as the founding member of human TrxG proteins, was originally identified through its association with both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)\(^1\). MLL is a histone H3 Lysine4 (H3K4) methyltransferase that can execute methylation on a subset of target genes through its evolutionarily conserved SET domain, and this activity is very essential for normal MLL function\(^2\). MLL is proteolytically cleaved into two distinct subunits: MLL\(^{C180}\) and MLL\(^{N320}\), which non-covalently interact to assemble an intramolecular complex involved in epigenetic transcriptional regulation\(^2\).

MLL is routinely regarded as a nuclear protein. Interestingly enough, our recent findings revealed that MLL C180 subunit alone could localize to the cytoplasmic processing bodies (P-bodies)\(^3, 4\), where miRNA-mediated gene silencing takes place\(^5\), and affect the function of a subset of miRNAs as exemplified by the *let-7a* family\(^3, 4\). The dysregulated function of *let-7a* resulted from the reduced expression of MLL\(^{C180}\) was very important for maintaining high level of MYC in MLL leukemia\(^4\). Thus, our work uncovered an unexpected role for MLL in miRNA-mediated translational repression. However, how MLL participates in the regulation of miRNA function remains elusive. We therefore sought to uncover the underlying mechanisms of how MLL participated in the miRNA-mediated translational repression. In this study, we demonstrated that MLL was required for recruiting *let-7a* and *miR-10a* to the miRNA-induced silencing complex (miRISC), partly through its binding partner RAN.

Most miRNAs are loaded onto Argonaute (AGO) proteins in the miRISC and act as post-transcriptional regulators of their target mRNAs\(^6\). Unfortunately, how these miRNAs are selectively loaded onto AGO proteins still remains poorly understood\(^6\). Among miRISC-associated factors, AGO1 plays a predominant and specific role in miRNA-mediated translational repression\(^7\). Our immunofluorescence results demonstrated that AGO1 and MLL were localized in the same cytoplasmic foci, which was disrupted upon MLL depletion (Figure 1A-B and Supplementary Figure S1A-C), suggesting an interaction between AGO1 and MLL. Using specific P-body marker DCP1A, we further confirmed that MLL and AGO1 co-localized in the cytoplasmic P-bodies (Supplementary Figure S1D). Previous studies have shown that Argonaute proteins could accumulate to stress granules (SGs) in addition to P-bodies when cells were subjected to stress\(^8\). We observed that upon arsenite treatment MLL together with AGO1 could co-localize to SGs as indicated by the specific SG marker G3BP1 (Supplementary Figure S1E). These results were consistent with our previous report showing MLL was present not only in P-bodies but also in the SGs\(^3\). Co-IP experiments showed that MLL\(^{C180}\) but not MLL\(^{N320}\) interacts with AGO1 (Figure 1C). Additionally, we demonstrated that the interaction between MLL\(^{C180}\) and AGO1 preferentially occurs in cytoplasm, but not in nucleus (Figure 1D). Interestingly, the interaction between MLL and
AGO1 decreased dramatically after RNase A treatment as revealed by Co-IP assays, indicating that this interaction was an RNA-dependent indirect interaction, rather than a direct protein-protein interaction (Figure 1E and Supplementary Figure S1F). Indeed, the interaction between MLL and AGO1 was enhanced by co-transfected let-7a (Figure 1F and Supplementary Figure S1G), indicating that miRNAs might play a critical role in MLL and AGO1 axis.

miRNA-mediated gene silencing requires miRNAs to associate with AGO proteins and other silencing factors to form a functional miRISC to repress target mRNAs. Given that miRNAs may fully function in mediating gene silencing even without the existence of microscopically visible P-bodies, functional miRISCs may still be formed upon MLL depletion. We thus further examined whether the depletion of MLL would impair the recruitment of miRNA to form the functional miRISC. We focused on let-7a and miR-10a, which were two MLL-binding miRNAs reported in our previous studies. We performed anti-AGO1 RNA immunoprecipitation (RIP) experiments and the results showed that MLL depletion resulted in the loss binding of let-7a and miR-10a to AGO1 (Figure 1G and Supplementary Figure S1H-K). Pull-down assay using biotinylated let-7a further validated that the binding of AGO1 to let-7a was reduced in Mll knockout (Mll−/−) MEF cells (Figure 1H). In addition, the recruitment of let-7a and miR-10a target mRNAs, MYC, HRAS and HOXA1, to AGO1 were also largely impaired in the MLL-depleted cells (Figure 1I and Supplementary Figure S1L-M). Notably, AGO1 expression was not affected by the knockdown of MLL (Supplementary Figure S1H), suggesting this impaired recruitment of miRNAs and its target mRNAs to AGO1 was not likely caused by the reduced AGO1 protein levels. Together with the above-mentioned data showing the interaction between MLL and AGO1 was RNA-dependent, these results indicated that MLL and miRNA may require each other in order to be efficiently recruited by AGO1 and form a functional miRISC.

To further investigate the role for MLL in the recruitment of miRNA to miRISC, we reintroduced shRNA-resistant MLLN320, MLLC180 or full-length MLL (MLLFL) into MLL knockdown 293T cells or Mll knockout (Mll−/−) MEF cells and found that the recruitment of let-7a and miR-10a to miRISC were rescued by exogenous MLLC180 (Figure 1J-K and Supplementary Figure S1P-S). Altogether, these results indicated that MLL plays a causal role in targeting miRNAs and their target mRNAs to AGO1 to form a translationally repressed miRISC complex, highlighting the importance for MLL in the control of miRNA-mediated expression.

MLLC180 itself does not possess any predictable RNA recognition motif, so we reasoned that MLL might recruit RNA components indirectly through its binding partners. Our proteomics data showed that RAN, a small GTPase involved in the import of cargo through nuclear pore complexes, was one of the proteins displaying strong interactions with MLL in cytoplasm (Figure 2A). In line with a previous study, we identified that RAN interacted with MLL in an RNA-independent manner (Figure 2B). We further confirmed that RAN could...
pull down MLL$^{C180}$, indicating a direct interaction between MLL$^{C180}$ and RAN (Figure 2C). Moreover, immunofluorescence data showed that upon arsenite treatment, MLL together with RAN co-localized to SGs as revealed by SG marker elf3 (Figure 2D), suggesting a potential role of RAN in regulating mRNA translation besides involving in the import of cargo. RAN and XPO5 can form a complex which plays a critical role in nucleocytoplasmic transport of pre-miRNA molecules$^{10}$. Unlike XPO5, which dissociates from pre-miRNA in the cytoplasm, RAN could still associate with pre-miRNA in the cytoplasm$^{12}$. According to the RPISeq online tool$^{13}$, RAN could bind the pre-miRNA and the mature miRNA (Figure 2E and Supplementary Figure S2A). Next, our RIP assay confirmed that RAN could bind not only the pre-miRNA but also the mature miRNA (Figure 2F and Supplementary Figure S2B). Moreover, RNA pull-down results showed a higher RAN expression in the let-7a or miR-10a biotinylated group compared to control group (Figure 2G and Supplementary Figure S2C). These results were consistent with a previous finding reporting that RAN was an RNA-binding protein$^{14}$, suggesting that RAN may be involved in the later steps of miRNA processing and function.

We next probed whether RAN is required to mediate gene silencing of miRNA targets. As shown in Figure 2H and Supplementary Figure S2D-E, luciferase activities in RAN-depleted cells were increased compared with that in control cells, indicating that the loss of RAN impaired the let-7a and miR-10a silencing functions. RIP experiments showed that the binding of both let-7a, miR-10a and MYC, HOXA1 to AGO1 were decreased in RAN-depleted cells, which could be recovered by RAN reintroduction (Figure 2I and Supplementary Figure S2F-I). Our previous studies have demonstrated that MLL$^{C180}$ plays a causal role in the miRNA functional deficiency$^{3, 4}$, we then investigated the role of RAN-binding in the MLL$^{C180}$-regulated miRNA function. Our results showed that MLL$^{C180}$ failed to rescue the miRNA activity when Ran was depleted, which could be recovered by MLL$^{C180}$ together with RAN reintroduction, suggesting that RAN was required for the MLL$^{C180}$-mediated miRNA regulation (Supplementary Figure S2J-K). Given the fact that RAN was a small GTPase involved in nucleocytoplasmic transport$^{10}$, we determined whether the GTPase activity of RAN is required for the functional interaction of the MLL-miRISC complex. As revealed in Supplementary Figure S2L, both wild-type RAN (RAN$^{WT}$) and GTPase-deficient mutant (RAN$^{Q69L}$) could partially reverse the deficits in the binding of let-7a to AGO1 caused by loss of endogenous RAN. We also observed that depletion of RAN significantly impaired the interaction between MLL and AGO1, which could be recovered by RAN$^{WT}$ or RAN$^{Q69L}$ re-expression (Supplementary Figure S2M), suggesting that the GTPase activity of RAN was not required for the MLL-miRISC complex function. Additionally, the binding of AGO1 to let-7a or miR-10a were decreased in RAN-depleted cells as revealed by pull-down assay using biotinylated let-7a or miR-10a (Figure 2J and Supplementary Figure S2N). These results indicated that RAN beyond the pre-miRNA export was required
for miRNA-mediated gene silencing. To decipher the role of RAN in the function of miRISC, we tested the interaction between RAN and AGO1. We observed that AGO1 had an RNA-dependent indirect interaction with RAN (Figure 2K). Importantly, Co-IP experiments revealed that besides AGO1, DDX6 a key P-body protein specifically involved in miRNA-mediated translational repression, interacts with RAN, but these interactions decreased significantly upon MLL depletion (Figure 2L), indicating MLL is accountable for these interactions.

To further strengthen our findings, we explored how RAN is behaving in a leukemic context. Co-IP assays performed in three leukemia cell lines JM1, REH and U937 showed that MLL interacts with RAN (Supplementary Figure S2O). In REH and U937 cells, MLL together with RAN co-localized to SGs following arsenite treatment as illustrated by immunofluorescence assay (Supplementary Figure S2P). Additionally, we found that the binding of let-7a to AGO1 was decreased in RAN-depleted REH and U937 cells, which could be restored by RAN reintroduction (Supplementary Figure S2Q-R). Consistent with the results obtained from 293T cells, we observed that AGO1 had an RNA-dependent indirect interaction with RAN in REH cells (Supplementary Figure S2S). Moreover, the interaction between AGO1 and RAN was impaired in MLL-depleted REH cells (Supplementary Figure S2T-V). As expected, the binding of RAN to AGO1 was reduced in MLL leukemic cells due to the downregulation of MLL (Supplementary Fig. S2W).

Collectively, in the present study, we demonstrated that MLL was required for recruiting let-7a and its target mRNAs to the miRISC, partly through its direct binding partner RAN (Figure 2M), unraveling an unexpected role for RAN in the loading of miRNA onto AGO1. Our findings provided an alternate mechanism and expanded the functional scope of RAN in the miRNA processing pathway. Thus, the discovery of interplay between MLL and miRNA represents a new regulatory layer, and an additional level of complexity, in the control of gene expression.

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**Author Contributions**

S.H.Z., Z.H.C. and D.J. designed and performed most of experiments, analyzed the data and wrote the draft manuscript; R.H.W, D.L., Q.Y.X., F.Z., G.J.G and W.K. provided technical assistance for the immunofluorescence experiments and data analyses; C.J.Z. performed some experiments and provided expertise and extensively edited the manuscript; H.L. and Z.K.L. contributed grant support, designed the entire project, wrote the manuscript and supervised the project. All authors discussed the results and commented on the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Additional Information**

Methods and data sets are available as Supplementary Information files.

**References**


Figure Legends

**Figure 1.** MLL is required for the loading of *let-7a* onto AGO1. (A) 293T cells were transfected with GFP-AGO1. Immunofluorescence experiments were performed to visualize the localization of GFP-AGO1 and MLL. MLL-CT antibody that recognizes MLL\textsuperscript{C180} (aa2829-2883) was used to detect MLL. Scale bar, 5 um. (B) *Mll* wild-type (*Mll*\textsuperscript{+/+}) and *Mll* knockout (*Mll*\textsuperscript{-/-}) MEF cells were transfected with GFP-AGO1, respectively. Immunofluorescence experiments were performed to visualize the localization of GFP-AGO1 and MLL. Arrowheads show the localization of MLL with the GFP-AGO1. Scale bar,
5 μm. (C) 293T cells were transfected with FLAG-tagged full-length MLL (MLLFL), MLLN320, MLLC180 or empty vector, respectively. Cell lysates were prepared and subjected to the anti-FLAG immunoprecipitation assays. The interaction between MLL and AGO1 was analyzed by Western blot assays using indicated antibodies. (D) The cytosolic and nuclear fractions of 293T cells were separated and subjected to immunoprecipitation using anti-MLL antibodies. Co-purified proteins were examined by immunoblots using the indicated antibodies. (E) 293T cell lysates were treated with RNase A followed by anti-MLL immunoprecipitation. Western blots were performed using indicated antibodies. (F) The interaction between MLL and AGO1 was assessed after let-7a transfection. Anti-MLL immunoprecipitation assays were performed, results were analyzed by immunoblots with indicated antibodies. (G) Extracts of 293T-shScr and 293T-shMLL cells were subjected to RIP analysis using anti-AGO1 antibody, and pull-downed RNAs were analyzed by qRT-PCR using specific primers for let-7a. (H) Mll wild-type (Mll+/+) and Mll knockout (Mll−/−) MEF cellular lysates were subjected to Biotinylated-let-7a RNA pull down assay. Then let-7a-immunoprecipitated AGO1 proteins were subjected to the Western blot analysis. Scrambled miRNAs were used as negative control. (I) 293T-shScr and 293T-shMLL cells were transfected with Agomir-negative control (NC) and Agomir-let-7a mimic (let-7a) followed by anti-AGO1 RIP experiments at 24 hours post-transfection. Total RNAs were isolated to analyze the MYC mRNA level by qRT-PCR. (J-K) 293T-shScr and 293T-shMLL cells with the latter being rescued by exogenous shRNA-resistant MLLN320, MLLC180 or full-length MLL (MLLFL) were performed with anti-AGO1 RIP experiments at 24 hours post-transfection. Total RNAs were isolated to analyze the let-7a (J) and MYC (K) levels by qRT-PCR using specific primers. NS, no significant difference. * for P<0.05, ** for P<0.01, *** for P<0.001. Data represent mean and s.e.m of three independent experiments.

Figure 2. MLL contributes to the loading of let-7a onto AGO1 through interacting with RAN. (A) List of MLL-associated proteins identified by mass spectrometric analysis. 293T cells transfected with MLL were harvested and subjected to the nuclear-cytoplasmic fractionation. The cytoplasmic fractions were prepared for the immunoprecipitation assays followed by mass spectrometric analysis. (B) 293T cell lysates were treated with RNase A followed by anti-MLL immunoprecipitation. Western blots were performed using indicated antibodies. (C) Direct interaction between MLLC180 and GST-RAN was examined. Left panels: Western blots shown the inputs of purified GST-RAN and Myc-MLLC180. Right panels: the pull down immunoblots were shown with GST-RAN as the bait and the pulled MLLC180 detected by an anti-Myc antibody. (D) 293T cells were untreated (upper panels) or arsenite treated (0.5 mM, 45 min) (lower panels), then fixed and stained with indicated antibodies. Note that eIF3 is specific for the stress granules. Arrowheads show the localization of MLL with the RAN and eIF3. Scale bar, 5 μm. (E) The
RPISeq tool was used to predict the interactions between RAN and *let-7a* or *pre-let-7a*. The RF classifier and SVM classifier represent the confidence of the prediction. In performance evaluation experiments, predictions with probabilities > 0.5 were considered “positive”. (F) 293T cellular lysates were prepared and anti-RAN RIP experiments were performed. Pull-downed RNAs were isolated, *pre-let-7a* and mature *let-7a* were analyzed by qRT-PCR using specific primers. (G) 293T cellular lysates were subjected to Biotinylated-*let-7a* RNA pull down assay. Then *let-7a*-immunoprecipitated RAN proteins were subjected to the Western blot analysis. Scrambled miRNAs were used as negative control. (H) 293T-shScr and shRAN cells transfected with Agomir-negative control (NC) or Agomir-*let-7a* mimic (*let-7a*) were subjected to dual luciferase reporter assays. The ratio of luciferase activity was measured and normalized to the value of the cells transfected with the control reporter and NC. (I) Extracts of 293T-shScr and shRAN cells with the latter being rescued by shRNA-resistant RAN were subjected to anti-AGO1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for *let-7a*. (J) 293T-shScr and 293T-shRAN cellular lysates were subjected to Biotinylated-*let-7a* RNA pull down assay. Then *let-7a*-immunoprecipitated AGO1 proteins were subjected to the Western blot analysis. Scrambled miRNAs were used as negative control. (K) 293T cell lysates were treated with RNase A followed by anti-AGO1 immunoprecipitation. Western blots were performed using indicated antibodies. (L) Extracts of 293T-shScr and 293T-shMLL cells were collected and Co-immunoprecipitation assays were performed and analyzed using the indicated antibodies. (M) The proposed mechanism through which MLL and RAN are involved in the loading of *let-7a* onto AGO1. MLL is required for the loading of *let-7a* onto AGO1 via the direct interaction with RAN. Thus, RAN serves as a molecular adaptor for the assembly of MLL-associated miRISC. NS, no significant difference. * for *P*<0.05, ** for *P*<0.01, *** for *P*<0.001. Data represent mean and s.e.m of three independent experiments.
Supplementary Figure S1. MLL plays a causal role in the recruitment of let-7a and miR-10a to miRISC.

(A) 293T cells were transfected with Myc-AGO1. Immunofluorescence experiments were performed to visualize the localization of Myc-AGO1 and MLL. anti-Myc antibody was used to detect Myc-AGO1. MLL-CT antibody that recognizes MLLC180 (aa2829-2883) was used to detect MLL. Scale bar, 5 um.

(B) 293T cells were transfected with GFP-AGO1. Immunofluorescence experiments were performed to visualize the localization of GFP-AGO1 and MLL. A300-374A antibody recognizing aa2725-2775 of MLL was used to detect MLL. Scale bar, 5 um.

(C) The localization of AGO1 after MLL depletion. 293T-shScr and 293T-shMLL cells were transfected with GFP-AGO1 and visualized...
by confocal microscopy at 24 hours post-transfection. Scale bar, 5 um. (D) 293T cells were transfected with GFP-AGO1. Endogenous MLL and P-body proteins DCP1A were probed using indicated antibodies. The localization of GFP-AGO1, MLL and P-bodies were visualized by immunofluorescence assays. Scale bar, 5 um. (E) 293T cells were untreated (upper panels) or arsenite treated (0.5 mM, 45 min) (lower panels), then fixed and stained with indicated antibodies. Note that G3BP1 is specific for the stress granule. Scale bar, 5 um. (F) MEF cell lysates were untreated or treated with RNase A followed by anti-MLL immunoprecipitation. Immunopurified complexes were analyzed by Western blot with indicated antibodies. (G) The interaction between MII and Ago1 in MEF cells was assessed after let-7a transfection. Anti-MII immunoprecipitation assays were performed and analyzed by immunoblots using indicated antibodies. (H) AGO1 expression level upon MLL depletion were confirmed by Western blot assays using indicated antibodies. (I) 293T-shScr and 293T-shMLL cells were prepared for the RIP analysis using anti-AGO1 antibody, then the bound miRNAs were subjected to the Northern blot assays against let-7a. U6 snRNA was used as loading control. (J) Extracts of 293T-shScr and 293T-shMLL cells were subjected to RIP analysis using anti-AGO1 antibody, and pull-downed RNAs were analyzed by qRT-PCR using specific primers for miR-10a. (K) 293T-shScr and 293T-shMLL cells were prepared for the RIP analysis using anti-AGO1 antibody, then the bound miRNAs were subjected to the Northern blot assays against miR-10a. U6 snRNA was used as loading control. (L) 293T-shScr and 293T-shMLL cells were transfected with negative control (NC) and let-7a followed by anti-AG01 RIP experiments at 24h post-transfection. Total RNAs were isolated to analyze the HRAS mRNA level by qRT-PCR. (M) 293T-shScr and 293T-shMLL cells were transfected with negative control (NC) and miR-10a followed by anti-AG01 RIP experiments at 24h post-transfection. Total RNAs were isolated to analyze the HOXA1 mRNA level by qRT-PCR. (N-O) Extracts of Myl wild-type (Myl+/+) and Myl knockout (Myl−/−) MEF cells with the latter being rescued by MLLC180 were subjected to anti-Ago1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for let-7a (N) and Myc (O). (P-Q) Extracts of Myl wild-type (Myl+/+) and Myl knockout (Myl−/−) MEF cells with the latter being rescued by MLLC180 were subjected to anti-Ago1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for miR-10a (P) and Hoxa1 (Q). (R-S) 293T-shScr and 293T-shMLL cells with the latter being rescued by exogenous shRNA-resistant MLLN320, MLLC180 or full-length MLL (MLLF) were performed with anti-AGO1 RIP experiments at 24 hours post-transfection. Total RNAs were isolated to analyze the miR-10a (R) and HOXA1 (S) levels by qRT-PCR using specific primers. NS, no significant difference. * for P<0.05, ** for P<0.01, *** for P<0.001. Data represent mean and s.e.m of three independent experiments.
Supplementary Figure S2. MLL-RAN interaction contributes to the loading of let-7a and miR-10a onto AGO1.

(A) The RPISeq tool was used to predict the interactions between RAN and miR-10a or pre-miR-10a. The RF classifier and SVM classifier represent the confidence of the prediction. In performance evaluation experiments, predictions with probabilities > 0.5 were considered “positive”. (B) 293T cells lysate were prepared and anti-RAN RIP experiments were performed. Pull-downed RNAs were isolated, pre-miR-10a and mature miR-10a were analyzed by qRT-PCR using specific primers. (C) 293T cellular lysates were subjected to Biotinylated-miR-10a RNA pull down assay. Then miR-10a-immunoprecipitated RAN proteins were subjected to the Western blot analysis. Scrambled miRNAs were used as negative control. (D) 293T cells stably expressing control or RAN shRNAs were harvested for the Western blot assays using the indicated antibodies. (E) 293T-shScr and shRAN cells transfected with Agomir-negative
control (NC) or Agomir-miR-10a mimic (miR-10a) were subjected to dual luciferase reporter assays. The ratio of luciferase activity was measured and normalized to the value of the cells transfected with the control reporter and NC.

(F) 293T-shScr and 293T-shRAN cells with the latter being rescued by exogenous shRNA-resistant RAN were prepared for the RIP analysis using anti-AGO1 antibody, then the bound miRNAs were subjected to the Northern blot assays against let-7a. U6 snRNA was used as loading control. (G) Extracts of 293T-shScr and shRAN cells with the latter being rescued by shRNA-resistant RAN were subjected to anti-AGO1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for MYC.

(H) 293T-shScr and 293T-shRAN cells with the latter being rescued by exogenous shRNA-resistant RAN were prepared for the RIP analysis using anti-AGO1 antibody, then the bound miRNAs were subjected to the Northern blot assays against miR-10a. U6 snRNA was used as loading control. (I) Extracts of 293T-shScr and shRAN cells with the latter being rescued by shRNA-resistant RAN were subjected to anti-AGO1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for miR-10a and HOXA1.

(J) Mll knockout (Mll−/−) MEF cells were transduced with lentivirus specifically targeting control or the 3'UTR region of Ran. Cells were harvested for the Western blot assays using the indicated antibodies. (K) Mll knockout (Mll−/−) MEF cells transfected with Agomir-negative control (NC) or Agomir-let-7a mimic (let-7a) were subjected to dual luciferase reporter assays. shRNA-resistant MLLC180 with or without RAN were reintroduced into the Ran-depleted Mll−/− MEF cells to determine the linking role of RAN in the MLLC180-mediated miRNA function. The ratio of luciferase activity was measured and normalized to the value of the cells transfected with the control reporter and NC.

(L) 293T-shScr and 293T-shRAN cells with the latter being rescued by shRNA-resistant RANWT or RANQ69L were subjected to anti-AGO1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for let-7a.

(M) Transfections of RAN knockdown 293T cells with shRNA-resistant wild-type RAN (RANWT) or GTPase-deficient mutant RANQ69L (RANQ69L) were confirmed by Western blot assays. Co-IP assays were performed to check the interaction between MLL and AGO1. Antibodies were used as indicated. (N) 293T-shScr and 293T-shRAN cellular lysates were subjected to Biotinylated-miR-10a RNA pull down assay. Then miR-10a-immunoprecipitated AGO1 proteins were subjected to the Western blot analysis. Scrambled miRNAs were used as negative control.

(O) JM1, REH and U937 cells were collected followed by anti-MLL immunoprecipitation. Western blots were performed using indicated antibodies. (P) REH (upper panels) and U937 (lower panels) cells were untreated or arsenite treated (0.1 mM, 30 min), then fixed and stained with indicated antibodies. Note that eIF3 is specific for the stress granules. Arrowheads show the localization of MLL with the RAN and eIF3. Scale bar, 2 um.
using the indicated antibodies. (R) Extracts of scramble control and shRAN cells with the latter being rescued by shRNA-resistant RAN were subjected to anti-AGO1 RIP assays. Pull-downed RNAs were analyzed by qRT-PCR using specific primers for let-7a. Left: REH cells; Right: U937 cells. (S) REH cell lysates were treated with RNase A followed by anti-AGO1 immunoprecipitation. Western blots were performed using indicated antibodies. (T) REH cells stably expressing control or MLL shRNAs were harvested for the Western blot assays using the indicated antibodies. (U-V) Extracts of REH-shScr and REH-shMLL cells were collected and Co-immunoprecipitation assays were performed and analyzed using the indicated antibodies. (W) JM1, REH, RS4;11, SEM and KOPN8 cells were collected followed by anti-AGO1 immunoprecipitation. Western blots were performed using indicated antibodies. JM1 and REH were wild-type MLL harboring cell lines, RS4;11 and SEM were MLL-AF4 harboring cell lines, KOPN8 was the MLL-ENL harboring cell line. * for P<0.05, ** for P<0.01. Data represent mean and s.e.m of three independent experiments.
Supplementary Figure S3. Unprocessed original Western blot images of this manuscript.