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

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 and acute myeloid leukemia.1 MLL is a histone H3 lysine 4 (H3K4) methyltransferase that can execute methylation on a subset of target genes through its evolutionarily conserved SET domain, an activity that is essential for normal MLL function.2 MLL is proteolytically cleaved into two distinct subunits: MLL(1-280) and MLL(281-530), which non-covalently interact to assemble an intramolecular complex involved in epigenetic transcriptional regulation.3

MLL is routinely regarded as a nuclear protein. Interestingly, however, our recent research revealed that the MLL(1-280) subunit alone can localize to cytoplasmic processing bodies (P-bodies).3,4 Where microRNA (miRNA)-mediated gene silencing takes place,5 and affect the function of a subset of miRNA, as exemplified by the let-7a family.3,4 The dysregulated function of let-7a resulting from the reduced expression of MLL was very important for maintaining a high level of MYC in MLL leukemia.7 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 participates in miRNA-mediated translational repression. In this study, we demonstrated that MLL was required to recruit let-7a and miR-10a to the miRNA-induced silencing complex (miRISC), partly through its binding partner RAN. The methods and datasets are available as Online Supplementary Information files.

Most miRNA are loaded onto Argonaute (AGO) proteins in the miRISC and act as post-transcriptional regulators of their target mRNA.6 Unfortunately, how these miRNA 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 and B, Online Supplementary Figure S1A-C), suggesting an interaction between AGO1 and MLL. Using a specific P-body marker DCP1A, we further confirmed that MLL and AGO1 co-localized in the cytoplasmic P-bodies (Online Supplementary Figure S1D). Previous studies showed that Argonaute proteins could accumulate in stress granules 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 stress granules, as indicated by the specific stress granule marker GRP78 (Online Supplementary Figure S1E). These results are consistent with those of our previous study showing that MLL was present not only in P-bodies but also in stress granules.9 Co-immunoprecipitation experiments showed that MLL but not MLL interacts with AGO1 (Figure 1C). Additionally, we demonstrated that the interaction between MLL and AGO1 preferentially occurs in the cytoplasm, and not in the nucleus (Figure 1D). Interestingly, the interaction between MLL and AGO1 decreased dramatically after RNase A treatment, as revealed by co-immunoprecipitation assays, indicating that this interaction was an RNA-dependent indirect interaction, rather than a direct protein-protein interac-
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, which recognizes MLL C180 (aa2829-2883), was used to detect MLL. Scale bar, 5 μm. (B) Mll wild-type (Mll+/+) and Mll knockout (Mll-/-) MEF cells were transfected with GFP-AGO1. 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), MLLC320, MLLC180 or empty vector. Cell lysates were prepared and subjected to 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 co-immunoprecipitation using anti-MLL antibodies. Co-purified proteins were examined by immunoblotting using the indicated antibodies. (E) 293T cell lysates were treated with RNase A followed by anti-MLL immunoprecipitation. Western blots were performed using the 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 immunoblotting with indicated antibodies. (G) Extracts of 293T-shScr and 293T-shMLL cells were subjected to RNA immunoprecipitation (RIP) analysis using anti-AGO1 antibody, and pulled down RNA were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using specific primers for let-7a. (H) Mll+/+ and Mll-/- MEF cellular lysates were subjected to a biotinylated-let-7a RNA pull-down assay. Then let-7a-immunoprecipitated AGO1 proteins were subjected to western blot analysis. Scrambled miRNA was used as a negative control. (I, K) 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 h post-transfection. Total RNAs were isolated to analyze the MYC mRNA level by qRT-PCR. (J) MLLFL rescue experiments with exogenous shRNA-resistant MLL N320, MLL C180 or MLLFL were performed with anti-AGO1 RIP experiments at 24 h after transfection. Total RNA were isolated to analyze the let-7a (J) and MYC (K) levels by qRT-PCR using specific primers. NS, no significant difference. *P<0.05, **P<0.01, ***P<0.001. Data represent mean and standard error of mean of three independent experiments.
Figure 2. Legend on following page.
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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 the indicated antibodies. (C) The interaction between MLL andS180 was examined. Left panels: western blots showing the inputs of purified GST-RAN and MLL-S180. Right panels: the pull-down immunoblots were shown with GST-RAN as the bait and the pulled MLL-S180 detected by an anti-Myc antibody. (D) 293T cells were untreated (upper panels) or treated with arsenite (0.5 mM, 45 min) (lower panels), then fixed and stained with the indicated antibodies. Note that eif3 is specific for stress granules. Arrowheads show the localization of MLL with RAN and eif3. Scale bar, 5 µm. (E) The RIPSeq tool was used to predict the interactions between RAN and let-7a or pre-let-7a. The random forest (RF) classifier and support vector machine (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. Pulled down RNA were isolated, pre-let-7a and mature let-7a were analyzed by qRT-PCR using specific primers (G). These results were consistent with a previous finding 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 Online Supplementary Figure S2D,E, luciferase activity in RAN-depleted cells was 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 was decreased in RAN-depleted cells, an effect that could be recovered by the reintroduction of RAN (Figure 2I, Online Supplementary Figure S2F-I). Our previous studies demonstrated that MLL has a causal role in the RNAi functional efficiency, so we investigated the role of RAN-binding in MLL-regulated miRNA function. We found that MLL fails to rescue the miRNA activity when RAN was depleted, an effect that could be recovered by MLL together with reintroduction of RAN, suggesting that RAN was required for the MLL-mediated miRNA regulation (Online Supplementary Figure S2J-K). Given the fact that RAN is a small GTPase involved in nucleocytoplasmic transport, we determined whether the GTPase activity of RAN is required for the functional interaction of the MLL-miRISC complex. As revealed in Online Supplementary Figure S2L, both wild-type RAN (RANwt) and GTPase-deficient mutant (RANQ69L) could partially reverse the defects 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 RANwt or RANQ69L re-expression (Online Supplementary Figure S2M), suggesting that the GTPase activity of RAN was not required for the function of the MLL-miRISC complex. Additionally, the binding of AGO1 to let-7a or mir-10a was decreased in RAN-depleted cells as revealed by a pull-down assay using biotinylated let-7a or mir-10a (Figure 2F, Online Supplementary Figure S2N). These results indicated that RAN, beyond pre-miRNA export, was required for miRNA-mediated gene silencing.

To decipher the function 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-immunoprecipitation 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 2I), indicating that MLL is accountable for these interactions. To further strengthen our findings, we explored how RAN behaves in a leukemia context. Co-immunoprecipitation assays performed in three leukemia cell lines, JM1, REH and U937, showed that MLL interacts with RAN (Online Supplementary Figure S2O). In REH and U937 cells, MLL together with RAN co-localized to stress granules following arsenite treatment, as illustrated by immunofluorescence assay (Online Supplementary Figure S2P). Additionally, we found that the binding of let-7a to AGO1 was decreased in RAN-depleted REH and U937 cells, an effect that could be restored by the reintroduction of RAN (Online Supplementary Figure S2Q-R). Consistently with the results obtained from 293T cells, we observed that AGO1 had an RNA-dependent indirect interaction with RAN in REH cells (Online Supplementary Figure S2S). Moreover, the interaction between AGO1 and RAN was impaired in MLL-depleted REH cells (Online Supplementary Figure S2T-V). As expected, the binding of RAN to AGO1 was reduced in MLL leukemic cells due to the downregulation of MLL (Online Supplementary Figure S2W).

Collectively, in the present study, we demonstrated that MLL was required for recruiting let-7a and its target miRNA 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 provide 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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According to the RIPSeq online tool, RAN could bind the pre-miRNA and the mature miRNA (Figure 2E, Online Supplementary Figure S2A). Next, our RIP experiments confirmed that RAN could bind not only the pre-miRNA but also the mature miRNA (Figure 2F, Online Supplementary Figure S2B). Moreover, RNA pull-down results showed a higher RAN expression in the let-7a or miR-10a-biotinylated group compared to that in the control group (Figure 2G, Online Supplementary Figure S2C). These results were consistent with a previous finding that RAN was an RNA-binding protein, 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 Online Supplementary Figure S2D,E, luciferase activity in RAN-depleted cells was 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 was decreased in RAN-depleted cells, an effect that could be recovered by the reintroduction of RAN (Figure 2I, Online Supplementary Figure S2F-I). Our previous studies demonstrated that MLL has a causal role in the RNAi functional efficiency, so we investigated the role of RAN-binding in MLL-regulated miRNA function. We found that MLL failed to rescue the miRNA activity when RAN was depleted, an effect that could be recovered by MLL together with reintroduction of RAN, suggesting that RAN was required for the MLL-mediated miRNA regulation (Online Supplementary Figure S2J-K). Given the fact that RAN is a small GTPase involved in nucleocytoplasmic transport, we determined whether the GTPase activity of RAN is required for the functional interaction of the MLL-miRISC complex. As revealed in Online Supplementary Figure S2L, both wild-type RAN (RANwt) and GTPase-deficient mutant (RANQ69L) could partially reverse the defects 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 RANwt or RANQ69L re-expression (Online Supplementary Figure S2M), suggesting that the GTPase activity of RAN was not required for the function of the MLL-miRISC complex. Additionally, the binding of AGO1 to let-7a or mir-10a was decreased in RAN-depleted cells as revealed by a pull-down assay using biotinylated let-7a or mir-10a (Figure 2F, Online Supplementary Figure S2N). These results indicated that RAN, beyond pre-miRNA export, was required for miRNA-mediated gene silencing.

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Received: July 29, 2020.

Accepted: December 11, 2020.


Disclosures: no conflicts of interest to disclose.

Contributions: SHZ, ZHC and DJ designed and performed most of the experiments, analyzed the data and wrote the draft manuscript; RHW, DL, QYX, FZ, GJG and WK provided technical assistance for the immunofluorescence experiments and data analyses; CJZ performed some experiments and provided expertise and extensively edited the manuscript; HL and ZKL contributed grant support, designed the entire project, wrote the manuscript and supervised the project. All authors discussed the results and commented on the manuscript.

Acknowledgments: we would like to thank all the members of Liu’s laboratory for their technical assistance. We also thank the Core Facility and Technical Service Center (Shanghai Institute of Hematology) for generous support with cell imaging. We apologize for not citing all the relevant references due to space limitations.

Funding: this work was supported by the National Key Research and Development Program of China (2018YFA0107802), the National Natural Science Foundation of China (81973996, 81900107 and 81572119), the Program of Shanghai Academic/Technology Research Leader (19XD1402500), the Shanghai Municipal Education Commission Guangfeng Clinical Medicine grant (2016304), the Shanghai Municipal Health Commission (2019ZXJQ01), the Shu Guang project (14SG15), the Collaborative Innovation Center of Hematology, and the Samuel Waxman Cancer Research Foundation.

References