Ribonuclease activity of MARF1 controls oocyte RNA homeostasis and genome integrity in mice

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Producing normal eggs for fertilization and species propagation requires completion of meiosis and protection of the genome from the ravages of retrotransposons. Mutation of Marf1 (meiosis regulator and mRNA stability factor 1) results in defects in both these key processes in mouse oocytes and thus in infertility. MARF1 was predicted to have ribonuclease activity, but the structural basis for the function of MARF1 and the contribution of its putative ribonuclease domain to the mutant oocyte phenotype was unknown. Therefore, we resolved the crystal structures of key domains of MARF1 and demonstrated by biochemical and mutagenic analyses that the ribonuclease activity of MARF1 controls oocyte meiotic progression and retrotransposon surveillance. The N-terminal NYN domain of MARF1 resembles the nuclease domains of Vpa0982, T4 RNase H, and MCPiPi1 and contains four conserved aspartate residues, D178, D215, D246, and D272. The C-terminal LOTUS domain of MARF1 adopts a winged helix-turn-helix fold and binds ssRNA and dsRNA. Purified MARF1 cleaved ssRNAs in vitro, but this cleavage activity was abolished by mutations of conserved aspartates in its NYN domain and truncation of the LOTUS domain. Furthermore, a point mutation in the D272 residue in vivo caused a female-only infertile phenotype in mice, with failure of meiotic resumption and elongation of Linel and lap retrotransposon transcripts and DNA double-strand breaks in oocytes. Therefore, the ribonuclease activity of MARF1 controls oocyte meiosis and genome integrity. This activity depends upon conserved aspartic residues in the catalytic NYN domain and the RNA-binding activity of the LOTUS domain.

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Significance

Although MARF1 (meiosis regulator and mRNA stability factor 1) is an ancient protein, identification of its function in mammalian female germ cell development and fertility is recent. It is crucial for the progression of oocyte meiosis and defense against the ravages of retrotransposons, which can cause damage to the oocyte's genome. These processes are dependent upon the ability of MARF1 to act alone both to bind RNA and to function as a ribonuclease during oogenesis. Here we reveal the molecular structure and functional mechanisms that enable MARF1 activity and provide insight into the complex posttranscriptional processes that shape the oocyte transcriptome.
and Tudor domain-containing proteins (TDRD) 5 and 7 owing to its presence in these proteins (36, 39). This LOTUS domain is predicted to bind to dsRNA or to stems of folded structures in RNAs, particularly those formed by small noncoding RNAs (snRNAs) after hybridizing with their RNA targets (36, 39). This RNA-binding property of the LOTUS domain equips it to be an “adapter” able to recruit targets for the effectors. Based on the copresence of the NYN RNA-like domain and the LOTUS RNA-binding domains in MARF1 protein, it has been hypothesized that MARF1 in oocytes may function as both an adapter, like the TDRD5/7 in male spermatocytes, to recruit specific RNA targets, including those for retrotransposons Line 1 and Iap, and an effector, similar to the PIWI domain in PIWI proteins to catalyze the specific cleavage of target RNA (2). According to this hypothesis, the MARF1 protein alone in oocytes may be capable of performing the regulatory role played by multiple factors in the PIWI-piRNA pathway of spermatocytes. This model could explain the sexual dimorphic control of mammalian germ cell meiosis and retrotransposon surveillance. However, this notion has remained conjecture because the molecular properties and the function of the NYN and LOTUS domain in MARF1 were unclear. Moreover, there was no direct experimental evidence showing that the oocyte-expressed MARF1 has RNase activity or that the absence of this activity contributes to the described phenotype of mutant Marf1 oocytes. Here, we resolved the crystal structures of the NYN and LOTUS domains of MARF1 and demonstrated through biochemical and mutagenic analyses that MARF1 is indeed an RNase that controls oocyte meiotic progression and retrotransposon surveillance.

Results and Discussion

Mouse MARF1 Cleaves ssRNA in Vitro. Phylogenetic analysis indicates that MARF1 is highly conserved in mammals (SI Appendix, Fig. S1). To test whether mammalian MARF1 exhibits nuclease activity, we purified residues 158–1,381 of the recombinant Mus musculus MARF1 protein containing all the functional domains (Fig. 1A) and incubated it with various RNA substrates labeled with $^{32}$P at the 5′ end. MARF1 efficiently cleaved ssRNAs but had only weak cleavage activity toward dsRNAs of the same size (Fig. 1B). Moreover, this activity of MARF1 was dose- and time-dependent (Fig. 1B and C), required the presence of specific divalent metal ions, i.e., Mg$^{2+}$ or Mn$^{2+}$, and was inhibited by EDTA (Fig. 1D). MARF1 cleaved circular RNA, although with weaker activity than toward the linear RNA substrate (Fig. 1E), indicating that it has exo- and endo-ribonuclease activities.

The RNA-Cleavage Activity of Mouse MARF1 Depends upon the RNA-Binding Ability of Its LOTUS Domains. The RNA-cleaving activity of MARF1 was severely compromised when it was truncated to delete the C-terminal repeat of LOTUS domains (Fig. 1F). This suggests that the presence of LOTUS domains is critical for MARF1 to elicit the RNase function. Given that the originally defined function of the LOTUS domain was RNA binding, it is tempting to speculate that the LOTUS domain could facilitate the cleavage activity of MARF1 by recognizing and binding the RNA substrates of MARF1. To determine whether, and to what extent, the M. musculus MARF1-LOTUS domain binds RNA, EMSAs were performed on different versions of MARF1 proteins. The C-terminal repeat of LOTUS domains itself binds ssRNA and dsRNA substrates with affinities comparable to MARF1 (residues 158–1,381) (SI Appendix, Fig. S2). However, recent studies in Drosophila suggested that the LOTUS domain of Oskar, a maternal effect gene product essential for germ cell formation, does not bind RNAs. Instead, it functions as a conserved regulator for the DEAD-box RNA helicase VASA in a protein–protein interaction module (40, 41). To determine the structural basis for the RNA-binding ability of M. musculus MARF1-LOTUS domains, we resolved the crystal structure of the first repeat of MARF1’s C-terminal LOTUS domains (LOTUS1) (Fig. 1G and SI Appendix, Table S1). Sequence alignment and structural superimposition of the LOTUS domains from M. musculus MARF1 and other proteins revealed that the M. musculus MARF1-LOTUS domains are different from Oskar and lack the C-terminal extended helix required for protein interaction (SI Appendix, Fig. S3). This result is consistent with the recent observation made by Jeske et al. (40) in Drosophila and provides more structural basis for dividing the LOTUS domain into two subclasses—the extended (e) and minimal (m) LOTUS depending on the presence (eLOTUS) or absence (mLOTUS) of the C-terminal extension. Therefore, the data presented here indicate that LOTUS domains are implicated in substrate recognition and binding by MARF1 and are therefore indispensable for the RNase function of MARF1.
The RNase Activity of Mouse MARF1 Is Determined by the Conserved Aspartic Residues Present in Its Catalytic NYN Domains. To gain mechanistic insight into the RNase activity of MARF1, we determined the crystal structure of the NYN domain (residues 158–230) at 1.75-Å resolution (SI Appendix, Table S1). The structure adopts an α/β sandwich fold, with β-strands 1–6 forming a parallel β-sheet surrounded by sets of α helices on both sides (Fig. 2). Dali server analysis showed that MARF1-NYN resembled the nuclease domains of T4 RNase H [Protein Data Bank (PDB) ID code ITRF], Vpa0982 (PDB ID code 2QIP), and HsMCPIP1 (PDB ID code 3V32) (SI Appendix, Fig. S4). However, unlike these three proteins that contain an additional helix, MARF1-NYN has a loop formed after α1. This additional loop orthogonally faces the core β-sheet and α1-helix (Fig. 2). Unlike the other two RNase H and HsMCPIP1 PIN-domain nucleases that have a helix insertion after β2, a structure tending to form a cap over the active site, the MARF1-NYN domain and Vpa0982 lack such a helix insertion after β2 (Fig. 2A and SI Appendix, Fig. S4), making the MARF1-NYN domain a relatively exposed active site.

Sequence alignment and structure superimposition of the typical NYN domains with other PIN proteins revealed that the MARF1-NYN domain contains four conserved aspartates, D178, D215, D246, and D272 (Fig. 2B and SI Appendix, Fig. S5A). Three of them, D178, D215, and D272, are located at the C terminus of strands β1, β2, and β4, respectively, and one acidic residue, D246, is at the N terminus of helix α3. Analysis of the electrostatic surface potential of the NYN domain revealed that these four aspartates are in a negatively charged pocket (Fig. 2C). These structural features and their conservation among the key nucleases suggest that these four aspartate residues could be critical for the nuclease activity of MARF1. We therefore tested this possibility by mutating these aspartate residues into alanine and assessing the RNA-cleavage activity of the resultant MARF1-mutant proteins. We first expressed and purified the MARF1-NYN domain and assessed its potential targets of MARF1 RNase activity. We hypothesized that supplementing the MARF1 WT oocytes with exogenous WT, but not mutated, MARF1 would reduce some, or all, of the transcripts that are expressed at higher than WT levels. A transcriptomic analysis demonstrated that supplementation with WT but not D272A-MARF1 caused profound changes in the transcriptome of the MARF1 mutant oocytes (3). Stable-state levels of 925 transcripts were uniquely down-regulated by WT MARF1, while only 113 transcripts were uniquely reduced by D272A-MARF1 (Fig. 3B and Datasets S1 and S2). Nearly half (46.7%) of the 925 down-regulated transcripts were among the transcripts identified as up-regulated in the GV-stage oocytes of MARF1-D272A mice (Fig. 3B). These included Ppyp2, whose up-regulation causes meiotic arrest in the MARF1 mutant oocytes (3), and others that were validated by real-time qRT-PCR (Fig. 3C). Since transcription in oocytes isolated from large antral follicles is normally silent (42), the decrease in the levels of these 925 transcripts in MARF1-D272A oocytes after overexpression of WT MARF1 is most likely attributed to their direct degradation by MARF1.

![Fig. 2. Crystal structure and mutagenic analysis of the NYN domain of MARF1.](image)

**Fig. 2.** Crystal structure and mutagenic analysis of the NYN domain of MARF1. (A) Cartoon diagram of the overall structure of the NYN domain. The secondary structure elements are labeled. (B) Superimposition of the conserved acidic residues of the MARF1-NYN structure (magenta) on the PIN domain of T4 RNase H (cyan) (Left), Vpa0982 (green) (Center), and MCPIP1 (slate) (Right). (C) Electrostatic surface potential analysis of the NYN structure. Conserved residues D178, D215, D246, and D272 are in the negatively charged pocket. (D) In vitro RNase activity assay of WT and mutant MARF1 in which the conserved aspartate residues (D178, D215, D246, and D272) were mutated individually into alanine.

Point-Mutation of the Conserved D272 Residue in the NYN Domain of MARF1 Causes a Female-Only Infertile Phenotype in Mice. What is the physiological role of the RNase activity of MARF1? To address this question, we generated knockin mice carrying the D272A mutation (hereafter referred to as “Marf1<sup>GT/GT</sup>”) by CRISPER/CAS9-mediated genome editing (SI Appendix, Fig. S6 A and B) and assessed the phenotype in the resultant mutant mice. No reduction in the expression of Marf1 mRNA (Fig. 4A) was detected in Marf1<sup>GT/GT</sup> oocytes. However, a dramatic increase in the levels of MARF1 protein was unexpectedly observed in Marf1<sup>GT/GT</sup> oocytes (Fig. 4B). The underlying cause for this increase is not known, but it could be a feedback response of the maternal oocytes to the loss of MARF1-NYN function. Marf1<sup>GT/GT</sup> females, but not males, are completely infertile (Fig. 4C). This indicates that the RNase activity of MARF1 is indispensable for female fertility. Furthermore, mice carrying both the Marf1<sup>GT</sup>-knockin allele and the Marf1<sup>GT</sup> allele (referred to hereafter as “Marf1<sup>GT/GT</sup>”) display the same phenotype as Marf1<sup>GT/GT</sup> mice (Fig. 4C), thus indicating that infertility in Marf1<sup>GT/GT</sup> females is not caused by potential off-target effects.

Meiotic Arrest and Activation of Retrotransposons in MARF1-D272A Point-Mutant Oocytes. There was no overt abnormality in folliculogenesis in either Marf1<sup>GT/GT</sup> or Marf1<sup>GT/GT</sup> females (SI Appendix, Fig. S7), and ovulation occurred normally as determined by the number of eggs recovered and the morphology of the expanded cumuli oophorus (Fig. 4 D–G and SI Appendix, Fig.
MARF1 Is an Executor of RNA Degradation in Mouse Oocytes. RNA-seq analysis revealed that the integrity of oocyte transcriptome was also profoundly impaired in Marf1\textsuperscript{D272A/D272A} and Marf1\textsuperscript{GT/D272A} females were immature and arrested at the GV stage (Fig. 4 F and G and SI Appendix, Fig. S6C). Therefore the inability of the oocyte to resume the first meiosis is the immediate cause of the infertility in Marf1\textsuperscript{D272A/D272A} females. Moreover, we found that levels of Linc1 and lap retrotransposon mRNAs were elevated in Marf1\textsuperscript{D272A/D272A} fully grown GV-stage oocytes isolated from large antral follicles (Fig. 5A), and the frequency of DNA double-stranded breaks, as demonstrated by γH2AX staining, was also increased in these oocytes (Fig. 5B). These data collectively indicate that the RNase activity of NYN domains mediates the function of MARF1 in controlling oocyte meiosis and retrotransposon silencing.

Interestingly, mRNA 3′ terminal uridylation catalyzed by the uridylytransferases TUT4 and -7 mediates the degradation of a cohort transcripts in the oocyte transcriptome, which is crucial for oocyte maturation and female fertility (43). Given the indispensable role of both TUT4/7- and MARF1-dependent pathways in sculpting the maternal transcriptome, we hypothesized that the two pathways may share some of the same mRNA targets in oocytes for degradation. By comparing our transcriptomic data with those published by Morgan et al. (43), we found that the expression levels of nearly half (46.1%) of the transcripts that are up-regulated in Tutt4-7-deleted oocytes are also elevated in Marf1\textsuperscript{D272A/D272A} (Fig. 5E). These data suggest that an intersection exists between these two pathways and that MARF1 could function as a downstream executor of the TUT4/7-mediated mRNA degradation pathway in oocytes. Interestingly, MARF1 and TUT4, along with some other proteins involved in mRNA decay pathways, are components that constitute the proteome of the process (P) bodies of human epithelial cells (44). Thus, MARF1 may coordinate with TUT4/7 in the same functional module in oocytes to specifically shape the maternal resolution of the NYN domains reveals that the degradation of these transcripts is a consequence of a specific RNase activity.

Fig. 3. (A) Schematic illustration of the experimental design for the analysis of the RNase activity of MARF1 in oocytes by mutating the conserved D272 residue in the NYN domain to alanine. IVT, in vitro transcription. (B) Venn diagram illustrating the relationship of the transcripts down-regulated by supplementation with either WT MARF1 (+WT-MARF1) or D272A-mutant MARF1 (+D272A-MARF1) in Marf1\textsuperscript{GT/GT} oocytes (+Marf1\textsuperscript{GT/GT} Up) compared with WT mouse oocytes. (C) Real-time RT-PCR validation of representative transcripts down-regulated by supplementation with WT but not D272A-mutant MARF1 in Marf1\textsuperscript{GT/GT} oocytes. Fold changes relative to the Marf1\textsuperscript{GT/GT} group are shown as mean ± SEM (n = 3). Different letters indicate a significant difference, P < 0.05, by one-way ANOVA and Tukey’s HSD test.
transcriptome for supporting oocyte maturation and preimplan-
tation development. LOTUS domains in MARF1 may play an
important role in selecting substrate for degradation by binding
specific RNA sequences. Nevertheless, although the data pre-
sented here demonstrate that MARF1 is a bona fide RNase, they
do not imply that MARF1 functions in the same way as PIWI
endonucleases to specifically slice the target into smaller frag-
ments (45, 46). Also, whether MARF1 catalyzes the production
of small RNAs in oocytes, particularly piRNAs and endo-siRNAs,
remains to be determined, as do the direct RNA targets of
MARF1.

Both the NYN and LOTUS domains are well conserved across
eukaryotic species and bacteria. Although the NYN and LOTUS domains
in eukaryotic proteins are usually fused with diverse types of
RNA-binding or protein–protein interaction domains, the bacte-
rial versions of these two domains almost always combine together
in one protein, with an N-terminal NYN domain fused to a single
or duplicated LOTUS domain (36). Therefore, MARF1 is an
ancient protein that probably originated in bacteria, and the NYN
domain fused with the LOTUS domain is probably the first ver-
sion that entered the eukaryotes and retained the original function
of its bacterial cognates (36). Remarkably, despite this fascinating
quality of being an ancient protein, the molecular properties of
MARF1, as well as its biological function in species other than
mammals, remain surprisingly unknown. Here, following up on
our initial identification of MARF1 as a key oogenic regulator
essential for mouse oocyte meiotic progression and silencing of
retrotransposon, we resolved the crystal structure of the NYN and
LOTUS domains in mouse MARF1 and demonstrated both
in vivo and in vitro that MARF1 is indeed an RNase that controls
these essential oogenic processes. These findings not only shed
light on the molecular properties of MARF1 but also provide
insight into the molecular mechanisms by which MARF1 controls
oocyte meiosis and genome integrity.

The quality of oocytes is well recognized as the key to repro-
ductive success and the creation of healthy individuals (47, 48).
Oocyte quality is largely determined by the autonomous gene
expression program built within the oocytes (42, 49). Key play-
ers in pathways involved in RNA degradation-mediated post-
transcriptional control have recently emerged as regulators of
oocyte gene expression; these are indispensable for oocyte ac-
quisation of meiotic and developmental competence (42, 43, 50–
54). The observations put forward here not only clarify the
structural basis for MARF1 function as a mammalian female-
specific regulator of germ cell meiosis and retrotransposon sur-
veillance but also provide insight into the posttranscriptional
control of oocyte gene expression. By functioning as an executor
of RNA-degradation processes in oocytes, MARF1 controls the
mRNA homeostasis and genome integrity of mammalian oocytes.

Materials and Methods

Unless otherwise specified, all reagents and chemicals were purchased from
Sigma-Aldrich Co.

Mice. Marf1<sup>GT/GT</sup> mice were imported from the Jackson Laboratory. D272A-
MARF1 knockin mice were generated by CRISPER-Cas9 technology as de-
tailed in SI Appendix, Materials and Methods. All mouse procedures and
protocols were approved by the Institutional Animal Care and Use Com-
mittee of Nanjing Medical University and were conducted in accordance
with the institutional guidelines for the care and use of laboratory animals.

Ovarian Histology and Oocyte Isolation, Microinjection, and Staining. Ovarian
sections were stained with periodic acid–Schiff reagent and Lillie–Mayer
hematoxylin. Fully grown GV-stage oocytes were isolated from mice that
were primed with equine chorionic gonadotropin (eCG). Microinjection and
immunofluorescent staining of the oocytes were carried out as described in
SI Appendix, Materials and Methods.

Western Blot and qRT-PCR Analyses. The same number of WT and Marf1–
mutant fully grown GV-stage oocytes were collected and subjected to
Western blot and qRT-PCR analyses.

RNA-Seq and Gene-Enrichment Analyses. Four sets of oocyte samples of each
genotype or treatment were collected and subjected to RNA-seq analysis as
described in SI Appendix, Materials and Methods. RNA-seq data have been
deposited in the Gene Expression Omnibus (accession no. GSE109213). Gene-
enrichment analysis of differentially expressed transcripts was carried out
using Metascape (metascape.org).

Cloning, Expression, Amino Acid Substitution, and Purification of Recombinant
MARF1. The coding sequence of Marf1 (residues 158–320, 158–407, 158–
690, 158–1381, and 687–1381) were cloned into the modified pET28a vector
with an N-terminal His<sub>6</sub>SUMO tag (residues 158–320, 158–407, 158–690;
Merck), the pfastBacHTe vector (residues 158–1381; Thermo Fisher Scientific,
Inc.), and the pET28a vector with a C-terminal His<sub>6</sub> tag (residues 687–1381;
Materials and Methods

The EMSA was carried out by incubating the recombinant proteins

Nucleic Acid Preparation and Nucleic Activity Assay. The dsRNA and circular ssRNA substrates were prepared using the labeled ssRNA H49 and H49AS that were obtained by in vitro transcription and were 5’ radiolabeled with 32P-γ-ATP (Perkin-Elmer Health Sciences). These 5’ 32P-labeled substrates were then incubated at 37 °C with the recombinant proteins for the nucleic activity assay.

EMSA. The EMSA was carried out by incubating the recombinant proteins with 5’ 32P-labeled substrates at 25 °C for 30 min.

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