Meiosis arrest female 1 (MARF1) has nuage-like function in mammalian oocytes

You-Qiang Su,a,b Fengyun Suna, Mary Ann Handela, John C. Schimenti,c and John J. Eppiga,d*,1

*The Jackson Laboratory, Bar Harbor, ME 04609; bState Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, People’s Republic of China; and cCollege of Veterinary Medicine, Cornell University, Ithaca, NY 14853

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Ordered regulation of meiosis and protection of germline genomic integrity from transposable elements are essential for male and female gamete development. In the male germ line, these processes are ensured by proteins associated with cytoplasmic nuage, but morphologically similar germ granules or nuage have not been identified in mammalian female germ cells. Indeed, many mutations affecting nuage-associated proteins such as PIWI and tudor domain containing proteins 5 and 7 (TDRD5/7) can result in failure of meiosis, up-regulation of retrotransposons, and infertility only in males and not in females. We recently identified MARF1 (meiosis arrest female 1) as a protein essential for controlling meiosis and retrotransposon surveillance in oocytes; and in contrast to PIWI-pathway mutations, Marf1 mutant females are infertile, whereas mutant males are fertile. Here we put forward the hypothesis that MARF1 in mouse oocytes is a functional counterpart of the nuage-associated components of spermatocytes. We describe the developmental pattern of Marf1 expression and its roles in retrotransposon silencing and protection from DNA double-strand breaks. Analysis of MARF1 protein domains compared with PIWI and TDRD5/7 revealed that these functional similarities are reflected in remarkable structural analogies. Thus, functions that in the male germ line require protein interactions and cooperative scaffolding are combined in MARF1, allowing a single molecule to execute crucial activities of meiotic regulation and protection of germline genomic integrity.

Gamete development encompasses key cellular and molecular processes common to gametes of both sexes as well as sex-specific processes crucial for fertilization and embryogenesis. Common gametogenic pathways include meiosis, to carry out recombination and segregation of homologous chromosomes, and silencing of retrotransposons, to maintain genomic integrity. Precise control of these processes is essential for transmission of parental genetic materials to subsequent generations and creation of genetic diversity. Errors can cause infertility, miscarriage, or birth defects and pose genetic threats to the offspring and subsequent generations. Here we put forward the hypothesis that meiosis arrest female 1 (MARF1) expressed in mouse oocytes is a functional counterpart of the nuage-associated components of spermatocytes that carry out these crucial facets of regulation of meiosis and control of retrotransposon expression.

MARF1 Controls Meiosis and Retrotransposon Silencing in Female but Not Male Germ Cells in Mice

We previously used a forward genetics approach to identify genes controlling oogenesis and female fertility in mice, revealing a gene encoding a master regulator of oogenic processes (1). This gene, originally referred to as 4921513D23Rik, is now named meiosis arrest female 1 (Marf1). Mutations of Marf1 cause infertility only in females, a phenotype attributed to failure in oocyte maturation to progress beyond the meiotic vesicle (GV)-stage and ovulation of immature GV stage oocytes. Up-regulation of protein phosphatase 2 catalytic subunit (PPP2CB) is a key feature of the meiotic arrest phenotype of Marf1 mutant oocytes. This overexpression in Marf1 mutant oocytes results in the failure to activate a maturation promoting factor that is essential for driving meiosis beyond prophase I (Fig. S1 shows a diagram illustrating the normal meiotic function of MARF1). Further studies on the mechanisms defining this phenotype led to uncovering significant defects in oogenic processes in Marf1 mutant oocytes, including up-regulation of Line1 and Iap retrotransposon mRNAs, which correlates with an increase in the number of nuclear DNA double-strand breaks. A cohort of other transcripts is also significantly up-regulated in the Marf1 mutant oocytes, whereas very few transcripts are down-regulated, suggesting that the Marf1 mutant phenotype results from aberrant RNA homeostasis and the absence of normal putative MARF1 RNase activity encoded by the NYN domain (Fig. 1). Thus, MARF1 controls meiosis and retrotransposon silencing in mammalian oocytes. Moreover, other oocyte developmental processes essential for preparation for fertilization and embryogenesis are also affected by Marf1 mutation (1). A common thread uniting these seemingly disconnected oogenic processes is the disruption of oocyte RNA homeostatic mechanisms. Such homeostatic functions are not limited to MARF1 in oocytes but are complemented by the activities of endogenous small interfering RNAs (endo-siRNAs) (2, 3) as discussed later in more detail.

In contrast to females, male Marf1 mutants are fully fertile, indicating apparently normal spermatogenesis. Thus, MARF1 function is required only during oogenesis. However, key gametogenic pathways, such as those controlling meiosis and suppression of retrotransposon expression, have counterparts in both the female and male germ line, and in the context of sexually dimorphic mechanisms of gametogenesis it is instructive to examine both similarities and differences.

PIWI-Interacting (pi)RNA Pathway Controls Meiosis and Retrotransposon Silencing in Male but Not Female Germ Cells in Mice

The functions of MARF1 in female germ cells are reminiscent of a mechanism regulating meiosis and retrotransposon silencing in male germ cells. These functions involve the PIWI subfamily of the argonaute proteins and their interacting partners, such as certain members of the tudor-domain-containing protein (TDRD) family and PIWI-interacting RNAs, known as piRNAs (4–6). These proteins and RNAs are localized to nuage or germ granules, which are cell-unique nonmembranous cytoplasmic microstructures aggregated with electron-dense ribonucleoprotein complexes (7, 8). Central to the control of spermatogenic processes by nuage components are the biogenesis of piRNA (9–11), piRNA-guided posttranscriptional cleavage of specific transposon...
RNA by PIWI proteins (12), and piRNA-PIWI directed transcriptional suppression of transposon expression (12–14).

The piRNAs, generally 26–31 nucleotides in length, are a class of small noncoding RNAs processed from long single-stranded precursors in a DICER-independent mechanism. A significant number of piRNAs are derived from repetitive sequences, including retrotansposons, and are enriched in germline cells (11, 15–18). PIWI proteins are key components of nuage and play a central role in silencing transposable elements in germ cells (14, 19, 20). PIWI proteins have two major domains: the C-terminal PIWI domain and a central PAZ domain (Fig. 1) (21, 22). The RNase H-like endonuclease (slicer) activity of the PIWI domain is central to the function of some PIWI proteins and requires the binding of PIWI Argonaut and Zwille (PAZ) domains to piRNAs (23–25). The piRNAs bound by PAZ domains function as a guide for PIWI proteins by base-pairing to the cRNA targets (26). The function of PIWI proteins also requires interaction and coordination of PIWI proteins with other proteins, as well as residing in nuage. The partners most critical for the function of PIWI proteins are TDRDs, which are enriched in germ cell nuage (5, 27). The TDRDs contain various numbers of Tudor domains, which bind to symmetrically dimethylated arginines residues located in the N termini of PIWI proteins (11, 15–18).

Regulation of the mRNAs encoding retrotransposons Line1 and/or Iap (9, 10, 19, 36–46). The male-specific infertility phenotypes exhibited by these mutants suggests that the PIWI-piRNA pathway is not necessary for the control of meiosis and retrotransposon silencing in mammalian oocytes.

This unique facet of the sex dimorphism in the control of mammalian meiosis may be attributable to the difference in the timing of genome-wide DNA methylation in mammalian germ cells. Meiosis in mammals is indeed sexually dimorphic; both in terms of the biological processes and the genetic control mechanisms (47, 48). Meiosis in female mammals initiates soon after sex determination in fetal gonads, whereas it does not commence in males until puberty. Unlike the continuous process of meiosis in males, meiosis in females is subject to regulated starts and stops, with meiosis arrested at diplote stage of the first meiotic prophase shortly after birth and throughout follicular development. Meiosis resumes in oocytes of nonatretic Graafian follicles only after a preovulatory surge of luteinizing hormone and is arrested again at metaphase II until fertilization. Mice bearing mutations in genes encoding several regulators of the earlier meiotic events often exhibit differences in the control of prophase I events in males and females (48–51). Interestingly, the timing of crucial reprogramming of DNA methylation also differs between males and females. De novo DNA methylation takes place in both female and male germ cells after genome-wide DNA demethylation in primordial germ cells, which allows reestablishment of the maternal and paternal specific imprints (52). The demethylation starts at approximately embryonic day (E) 7.5 when primordial germ cells (PGCs) migrate to and colonize at the genital ridge and is completed by E13.5 (53, 54). The genome-wide DNA methylation takes place in males in prospermatogonia before birth, at approximately E13.5 and is completed 3 d after birth [postnatal day (P) 3] (55, 56). However, in females, DNA demethylation does not begin until after birth in growing oocytes that have already completed the earlier meiotic events and have progressed to the diplote stage of prophase I (57). Thus, in males, demethylation is earlier in the development of the germline and premeiotic, whereas in females it is later in the development of the germline and postmeiotic prophase. Moreover, no prominent nuage or germ granules, similar to the intermitochondrial cement or chromatoid bodies present in male mouse germ cells, have been found in growing oocytes (7). Although Balbiani bodies consisting of endoplasmic reticulum and mitochondria interspersed with nuage surrounding Golgi stacks have been reported in nongrowing mouse oocytes, they disappear soon after the oocytes commence their extensive growth phase (58). However, the global DNA demethylation reprogramming in primordial germ cells creates a window of vulnerability to possible escape and activation of transposable elements in both male and female germ cells. In the face of demethylation, mammalian germ cells of both sexes require mechanisms that defend against activation of retrotransposons. Thus, do female germ cells use mechanisms similar to the PIWI-piRNA pathway in male germ cells, or are there completely different female-specific mechanisms that control meiosis and retrotransposon silencing in mammalian oocytes?

**Structural and Functional Analogies Between Oocyte MARF1 and Spermatocyte PIWI-piRNA Pathway**

To address this question, we first asked whether there are any protein structure or molecular function similarities between MARF1 and the components of mouse male germ cell nuage involved in the PIWI-piRNA pathway.

The MARF1 protein has three major domain motifs: an N-terminal “LK-Nuc” domain, two central “RRM” domains, and C-terminal “OST-HTH/LOTUS” domains (Fig. 1) (59, 60). The “LK-Nuc” domain is now officially termed “NYN” (Nedd4-BP1, YacP Nucleases, NYN) domain, because of its original identification in the eukaryotic proteins Nedd4-binding protein 1 and the
bacterial YacP-like proteins. It is described as an RNase domain belonging to the superfamily that includes the 5′→3′ nuclease, PIN, NYN, and phage T4-type viral RNase H domains (61). The OST-HTH/LOTUS domain is a recently identified unique RNA-binding domain, which has a winged helix-turn-helix fold and is predicted to bind specifically to dsRNAs or stems of folded structures in RNAs (59, 60). This domain is also present in Drosophila Oskar and mammalian TDRD5/7 proteins (Fig. 1). Oskar is essential for the assembly of germ plasm in Drosophila, whereas TDRD5/7 proteins are required for formation of normal chromatoid bodies in mouse spermatids (39, 40, 62). Mutations in these genes cause failure of germ cell specification in Drosophila and interruption of normal spermatogenesis and retrotransposon repression in mice, respectively (39, 40, 63). Because dsRNAs formed by micro(mi)RNAs, repeat associated siRNAs, and piRNAs hybridized to their targets are readily found in nuage, Oskar formed by micro(mi)RNAs, repeat associated siRNAs, and piRNAs is suggested to recruit specific RNA targets including those for retrotransposons Line1 and Iap, and an effector, like the PIWI domain in PIWI protein to catalyze the specific cleavages of target RNAs (Fig. 1). Specificity of MARF1 function may be reinforced by the RRM (RNA recognition motif) domain, which is known to bind single-stranded RNAs. With these multiple functional domains, only one key protein, MARF1, could elicit the function carried out by multiple proteins in male germ cell nuage. Nevertheless, the function of MARF1 could well require interacting proteins, and the presence other adaptor molecules in oocytes is certainly possible; however, they apparently do not form visible ribonucleoprotein aggregates similar to male germ cell nuage.

MARF1 protein contains the phylogenetically conserved PIH/LOTUS/CHROMO domain and NYN domains (12). The OST-HTH/LOTUS domain and NYN domains indicate that they both probably emerged in bacteria (59). With the exception of MARF1, in most eu- karyotic OST-HTH/LOTUS domain-containing proteins the OST-HTH/LOTUS domains are fused with different RNA-binding or protein–protein interaction domains. However, almost all of the bacterial versions of OST-HTH/LOTUS domain-containing proteins, similar to MARF1, have one or more OST-HTH/LOTUS domains fused to a N-terminal NYN domain (59). It has been suggested that the OST-HTH/LOTUS domain-containing protein having OST-HTH/LOTUS domains fused with an N-terminal NYN domain is the first version appearing in euukaryotes (59). In this view, MARF1 is probably an ancient protein retaining the role of RNA degradation found in its bacterial cognates. Furthermore, Oskar is also formed by fusion of an OST-HTH/LOTUS domain with a C-terminal “SONFI” hydrolase of bacterial species (Fig. 1) (59, 64). Taken together, these structural considerations suggest that genes for Oskar, TDRD5/7, and MARF1 might have originated from similar ancestors during evolution through gene duplication events and could play conserved roles in germline development.

We assessed similarities in expression of MARF1 and nuage components. In male mice, PIWI proteins and TDRDs are both enriched in germ cells. The expression of PIW1L (MIWI) begins in midmeiotic prophase (the pachytene stage) spermatocytes at approximately P14 and persists to the stage of haploid round spermatids (37, 65). PIW1L2 (MIWI2) is expressed in prospermatogonia of fetal testes beginning at E12.5 and is continuously expressed in germ cells after birth until the stage when haploid round spermatids are formed (12, 36, 65, 66). PIW1L4 (MIWI2) expression also begins in prospermatogonia of fetal testes and persists until shortly after birth (P3) (12). TDRD5 and -7, as most of the other TDRDs including TDRD1, -2, and -7, are expressed in both fetal and adult germ cells (5, 31, 39, 67).

Consistent with the expression patterns of PIW1L and TDRDs in male germ cells, MARF1 is highly expressed in fully grown oocytes, but it is barely detectable in the somatic cell compartments of large antral follicles in mouse ovaries (1). Moreover, oocytes express a splice variant of Marf1, which is different from the annotated form expressed in somatic cells, and lacks the 3′-537-bp nucleotides in exon 3. Lack of this part of exon 3 does not change the composition of the major domains in MARF1 protein (1). Although the biological function difference between the oocyte-expressed MARF1 isoform and the somatic cell-expressed MARF1 is not clear, the ovarian function of MARF1 is clearly restricted to oocytes. To further refine the functional window for MARF1 in the control of female fertility, we determined temporal expression of Marf1 in mouse oocytes and how expression changes during oocyte and preimplantation development. We collected oocytes and preimplantation embryos at different developmental stages and examined the expression of Marf1 at the levels of both mRNA and protein. As seen in Fig. 2, both Marf1 mRNA and protein were detected in germ cells as early as the smallest sized oocytes isolated from newborn P0 mouse ovaries. These oocytes are quiescent and have not yet commenced the growth phase. The levels of Marf1 mRNA and protein increased significantly after oocytes initiated growth, and follicular development reached the primary follicle stage at P6. Thereafter, Marf1 mRNA and protein remained at the similar levels, both in growing oocytes isolated from early secondary follicles at P12 and in fully grown oocytes isolated from Graafian follicles of equine choriandronadropin-activated mouse oocytes (ECG-stimulated P22 mice. This expression pattern is consistent with that of the Marf1 β-galactosidase (GAL) reporter, where low X-GAL staining was found in quiescent oocytes of primordial follicles, with extent and intensity of staining increasing in growing and fully grown oocytes (1). Interestingly, this expression pattern of MARF1 in mouse oocytes falls into the window when genome-wide de novo DNA methylation takes place (57). The lower levels of MARF1 expression in non-growing oocytes of Marf1 may not play a significant role during early stages of oocyte development.

The steady-state level of Marf1 mRNA was reduced by approximately half during maturation to the metaphase II (MII) stage, and this level persisted after fertilization and one-cell-stage zygote formation (Fig. 2). Thereafter, the levels of Marf1 mRNA decreased dramatically, by >95%, at two-cell stage embryos and remained constantly low during the following stages of embryo development up to the blastocyst stage. In contrast, levels of Marf1 protein were unchanged during oocyte maturation. This suggests continual translation or stability of MARF1 protein to maintain a constant level, and that MARF1 may play an important role during the processes of oocyte meiotic maturation. At the one-cell stage, even though Marf1 mRNA levels remained at the same level as in MII oocytes, the protein declined to almost undetectable levels, suggesting posttranscriptional degradation after fertilization. MARF1 protein remained at barely detectable levels thereafter in two-cell to blastocyst stage embryos, suggesting it does not function during these stages.

As shown previously (1), MARF1 is implicated in retrotransposon suppression in oocytes. Indeed, one key role of the PIWI-piRNA nuage components is to suppress the activation of retrotransposons, particularly Line1 and/or Iap, thereby serving to defend genomic integrity. For example, mutations of Piwil1, Ttdr5, and Ttdr7 cause the up-regulation of Line1 mRNA (25, 39, 40), whereas mutations of Piwil2 and Piwil4 result in up-regulation of both Line1 and Iap (13). Similar to these phenotypes, Marf1
mutations cause up-regulation of Line1 and Iap mRNA in fully grown oocytes from large antral follicles (1). However, the temporal onset of this change of Iap and Line1 expression was not known. The presence of MARF1 protein in oocytes of earlier developmental stages could enable the regulation of Iap and Line1 mRNA during these early oogenic stages. However, as shown in Fig. 3, no significant up-regulation of Iap mRNA was detected in nongrowing oocytes isolated from P0, P6, or P12 Marf1 mutant mouse ovaries. Nonetheless, Line 1 mRNA was found to be up-regulated in growing oocytes of P12 Marf1 mutant ovaries. Therefore, stage-dependent MARF1 regulation of Iap and Line1 retrotransposon expression occurs only after oocyte midgrowth stage, when follicular development advances to the secondary (late preantral) stage. Possibly retrotransposon silencing at earlier developmental stages is by a MARF1-independent mechanism. Notably, DNA methylation of Iap and meiotic progression in
oocytes at earlier developmental stages is regulated by HELLS (formerly known as LSH), a member of the SNF2 family of chromatin remodeling ATPases, which controls accessibility of DNA to de novo DNA methyltransferases DNMT3A and DNMT3B (68). Hells knockout oocytes display severe defects in homologous chromosome synapsis, repair of DNA double-stranded breaks, and up-regulation of Iap mRNA, which causes meiotic arrest at pachytene stage and subsequent loss of oocytes and failure of ovarian follicle formation (68). This meiotic function of HELLS is apparently not sexually dimorphic, because it also controls early meiotic events in spermatocytes (69); and moreover, the function of HELLS is apparently not germ cell-specific, because HELLS is crucial for normal development, and Hells knockout causes early postnatal lethality (70).

The insertion of mobile elements into the host genome after retrotransposon activation causes nuclear DNA double-strand breaks, and indeed we previously observed an increase in the number of DNA double-strand breaks in fully-grown Marf1 mutant oocytes coincident with up-regulation of Iap and Line 1 mRNA (1). Therefore, we examined the temporal correlation of nuclear DNA double-strand breaks with Iap and Line 1 mRNA expression in Marf1 mutant oocytes. As shown in Fig. S2, oocytes at various stages (leptonema, zygonema, and pachynema) of early meiotic prophase I were found to be present in P0 ovaries, and comparable numbers of positively stained γH2AX foci were found in both wild-type and mutant Marf1 oocytes at each corresponding meiotic stage, as expected because of the low expression of Marf1 in wild-type oocytes. This indicates no increase in the number of nuclear DNA double-strand breaks in mutant nongrowing oocytes. Interestingly, unlike male spermatocytes, in which almost all naturally occurring double-strand breaks were already repaired at pachytene stage, a significant number of breaks were still present in both wild-type and mutant pachytene stage oocytes. This hitherto unappreciated sexual dimorphism in meiotic DNA double-strand break repair may help explain observed differences in effects of mutations in this pathway. By the diplo-tene stage in growing oocytes isolated from both wild-type and mutant P12 ovaries, significantly more γH2AX foci were found in mutant Marf1 oocytes compared with wild type (Fig. 4), indicating increased DNA double-strand breaks in P12 mutant oocytes.

PIWI proteins and their interacting partners in male germ cell nuage control the activation of retrotransposons at both transcriptional and posttranscriptional levels. Cytosine methylation of the Cpg dinucleotides in the promoter region of repetitive elements is a key mechanism for suppressing retrotransposon activation at transcriptional levels in germ cells. In Tdrd5-knockout testis, up-regulation of Line1 mRNA is correlated with demethylation of their promoter elements (39). Similarly, in Piwil2 and Piwil4 knockout testis, DNA methylation at Line1 and Iap elements is also significantly reduced (13). These findings prompted us to determine whether the up-regulation of Line1 and Iap mRNAs in Marf1 fully grown oocytes is accompanied by changes in DNA methylation of their promoter regions. The DNA methylation patterns of Line1 and Iap promoters in Marf1 wild-type and mutant fully grown oocytes were determined by bisulfite sequencing. As indicated in Fig. 5, comparable numbers of methylated CpGs were identified in the 5′ LTR of Iap and 5′ UTRs of two subtypes (type A and type G1) of Line1 DNAs, respectively, in both Marf1 wild-type and mutant oocytes, with no significant difference in the patterns of DNA methylation between wild-type and mutant oocytes at the sites examined. Therefore, up-regulation of Iap and Line 1 mRNAs in Marf1 mutant oocytes is not caused by suppression of DNA methylation. Although defects in other mechanisms that suppress repetitive elements at transcriptional levels, such as histone modification and heterochromatin formation, could potentially contribute to the up-regulation of Iap and Line 1 mRNAs in Marf1 mutant oocytes, a loss of posttranscriptional control of Iap and Line 1 mRNAs seems to be a likely cause. This conclusion is buttressed by our previous observation that, along with up-regulation of Iap and Line 1 mRNAs, a significant cohort (377) of transcripts was markedly elevated (>fourfold in Marf1 mutant oocytes, whereas only a small number (27) of transcripts was down-regulated to the same extent (1). Furthermore, posttranscriptional regulation by MARF1 protein was also implicated by the observation that, despite dramatic increases in mRNAs of a few representative transcripts from this up-regulated cohort, the levels of their cognate unprocessed heterogeneous nuclear RNA were not changed in mutant oocytes (1). Posttranscriptional regulation of retrotransposon expression also exists in male germ cells. The production of secondary piRNAs through piRNA-guided catalysis of the target retrotransposons by PIWIL2 and PIWIL4 is one major mechanism for posttranscriptional regulation of retrotransposon expression (71). In addition to this piRNA biogenesis-dependent mechanism of silencing retrotransposons, a posttranscriptional mechanism of direct degradation of retrotransposon mRNAs by PIWIL1 occurs in male germ cells. This PIWIL1 “slicer”-dependent mechanism functions without the amplification of piRNAs (25). Interestingly, TDRD7 regulates retrotransposon silencing through a mechanism independent of piRNA biogenesis, one at the level of translational control (40).

The production of piRNA is essential for the PIWI protein-piRNA pathway to function in mouse male germ cells (71). The predicted preference of the OST-HTH/LOTUS domain to bind dsRNAs, particularly those formed by small noncoding RNAs (snRNAs) after hybridizing with their RNA targets, implies that
the function of MARF1 in mouse oocytes may also require the participation of snRNAs (59). MicroRNAs are abundant in mouse oocytes. However, interruption of miRNA production caused by oocyte-specific knockout of Dicer8 does not affect oogenesis and female fertility (72), indicating that miRNAs are not essential for MARF1 function. Endo-siRNAs are also present in mouse oocytes and probably function in the control of oocyte development and repression of retrotransposon expression (2, 3). However, the phenotypes of the oocyte-specific knockout of Dicer1, a gene encoding the critical enzyme for miRNA and siRNA production, are different from those of Marf1 mutants. The Dicer1-cKO oocytes can undergo maturation but have severe defects in spindle formation and chromosome alignments (73, 74), and up-regulation of miRNAs for retrotransposons Mt and Sine, but not Line 1 and Iap (73). This suggests that mouse oocytes use different mechanisms to control different retrotransposon elements. Although a large number of piRNA are detected in mouse oocytes, the role of piRNAs in mouse oocytes remains unknown (2, 3). Whether the functions of MARF1 require the presence of a specific set of piRNAs, or whether MARF1 is actually involved in the production of piRNA in mouse oocytes, awaits investigation.

Concluding Remarks

Sexually dimorphic mechanisms control gametogenic processes in mammals. In males, the RNA “slicer” activity of some PIWI proteins is probably essential for the control of meiosis and silencing of retrotransposons. The function of PIWI proteins requires facilitation by their specific interacting partners—proteins and RNAs tethered together in nuage or germ granules. Tudor domain-containing proteins, such as TDRD5 and TDRD7, play key roles in facilitating the function of PIWIs by acting as scaffolds. In females, no prominent nuage structure is obvious in oocytes, and PIWI proteins and their interacting partners are dispensable for the control of meiosis and retrotransposon silencing. Instead, a female-specific mechanism using MARF1 is used to control these key oogenic processes. Given that the RNase-like NYN domain present in MARF1 protein may resemble the RNA slice activity of PIWI domains in PIWI proteins, and that the OST-HTH/LOTUS that is also present in TDRD5 and TDRD7 may provide specific RNA binding properties, MARF1 alone may be capable of performing regulatory functions similar to those carried out by multiple components in male germ cells. Nevertheless, coordination with other proteins and RNAs may be required for MARF1 function. Identifying these potential partners of MARF1 and resolving their specific interactions will be key to deciphering the molecular mechanisms by which this complex and fascinating protein uniquely orchestrates events essential to oogenesis.

Materials and Methods

Animals. The production and genotyping of Marf1 wild-type and mutant (Marf1<sup><i>enu</i></sup>) mice were carried out as described previously (1). Both the Marf1<sup><i>enu</i></sup> and the C57BL6/6XSLF1 F1 mice were raised under the standard conditions at the investigators’ colonies at The Jackson Laboratory. All mouse procedures and protocols were in accordance with National Institutes of Health guide for animal care and use and were approved by the Animal Care and Use Committee at The Jackson Laboratory.
Oocytes and Embryo Isolation. Nongrowing oocytes, growing oocytes from primary and early secondary follicles, GV-stage fully grown oocytes from large antral follicles, and ovulated mature MII oocytes were isolated from newborn mice at P0, P6, and P12, from P22 mice stimulated with equine chorionic gonadotropin (eCG) for 44 h, or from P24 mice first stimulated with eCG for 44 h then human chorionic gonadotropin (hCG) for 14 h, respectively, as described previously (75–77). To obtain preimplantation embryos at various developmental stages, P22 mice were first stimulated with eCG for 44 h and then injected with 5 IU hCG and mated with adult B6SJL F1 male mice. Embryos at one-cell, two-cell, four-cell, eight-cell, morula, and blastocyst stages were then collected from females with visible vaginal plugs 18 h, 44 h, 56 h, 68 h, 78 h, and 96 h after receiving hCG injection, respectively.

Real-Time RT-PCR and Western Blot Analyses. These were carried out as described previously (1). To compare the steady-state levels of mRNA in GV-stage fully grown oocytes, MII stage mature oocytes, and preimplantation embryos at various stages of development (P6), the number (60) of oocytes and preimplantation embryos were used. To normalize potential variation among samples originating from pipetting and RNA isolation processes, rabbit β-globin mRNA was added into each sample at the beginning of RNA extraction. The concentration of DNA 125 ng per oocyte to serve as an external control. In the other real-time RT-PCR experiments, Rpf19 was used as internal control for normalization of the variation among samples. For Western Blot analysis of MARF1 and ACTB proteins in oocytes at various developmental stages, 60- and 480-P6 growing oocytes, and P0 oocytes with the total amount of protein equal to 480-P6 growing oocytes were loaded onto the same gel. For Western blot analysis of MARF1 and ACTB proteins in oocytes and preimplantation embryos at various developmental stages, the same number (60) of oocytes (GV and MII stages) and preimplantation embryos (one-cell, two-cell, four-cell, eight-cell, morula, and blastocyst stages) were loaded onto the same gel.

Oocyte Spread Preparation and Staining. For preparation of spreads of non-growing oocytes, oocytes from P0 Marf1 mutant mice and wild-type littermate controls were processed for immunofluorescence staining. For preparation of spreads of growing oocytes, oocytes from P12 Marf1 mutant mice and wild-type littermate controls were collected in cold PBS. The oocytes were digested with 0.5 mg/mL collagenase for 30 min in hypotonic extraction buffer (pH 8.2) [15 mM Tris, 50 mM sucrose, 2 mM tritium citrate, 5 mM EDTA, and a protease inhibitor mixture] and then transferred into 100 mM sucrose solution (pH 8.2) and disrupted by pipetting until a single-cell suspension was formed. This ovarian single-cell suspension was subsequently dispensed onto glass slides covered with 1% paraformaldehyde (PFA) containing 0.1% Triton X-100 (pH 9.2) and stayed in a humidified chamber for 3–5 h until the PFA solution was almost dried out. These ovarian cell spreads were washed with 0.4% Photo-Flo (Electron Microscopy Sciences), air dried at room temperature, and stored at −20°C until being processed for immunofluorescence staining. For preparation of spreads of growing oocytes, oocytes from P12 Marf1 mutant mice and wild-type littermate controls were collected in cold PBS containing 0.3% BSA, and oocytes were isolated by digesting with 0.1% collagenase as described previously (76). The isolated oocytes were then transferred into 50 μL hypotonic extraction buffer (15 mM Tris-HCl, 50 mM sucrose, 20 mM citrate, 5 mM EDTA, 0.5 mM DTT, and 0.09 mg/mL PMSF, pH 8.2) and incubated for 30 min at room temperature. After the incubation, 20 μL hypotonic extraction buffer was replaced with 20 μL 0.1 M sucrose, and the oocytes were incubated for another 30 min. Thereafter, the oocyte spreads were prepared following the same procedure for preparing P0 oocyte spreads as described above. Both the P0 and P12 oocyte spreads were then washed in 10% antibody dilution buffer [3% (v/v) BSA, 10% (v/v) goat serum, 0.05% Triton X-100] in PBS. The cells were labeled with rabbit anti-phospho-H2AX (rH2AX) (Abcam, catalog no. 07-164) at 1:200 human anti-centromere antibody (Antibodies Incorporated, catalog no. 15-234), rat anti-SYCP3 (see ref. 78 for reference) at 1:1,000. After three washes in PBS, the cells were incubated with secondary antibodies: Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen, catalog no. A21245), Alexa Fluor 594 donkey anti-rat IgG (Invitrogen, catalog no. A21209), Alexa Fluor 488 goat anti-human IgG (Invitrogen, catalog no. A11013), at 1:1,000 at room temperature for 1 h, washed, and mounted onto slides with Vectashield containing DAPI (Vector, catalog no. H-1200) to visualize DNA. The staining was evaluated using a Zeiss Axio Observer Z1 microscope equipped with suitable filters, and images were captured using the attached Zeiss AxioCam camera.

Bisulfite Sequencing. Fifty to 100 Marf1 wild-type and mutant fully grown oocytes were collected in 10 μL PBS and then subjected to sample lysis and bisulfite conversion using the EpioTec Plus LysExA Bisulfite Kit (catalog no. 59164, Qiagen) according to the manufacturer’s instructions. The resulting DNA was then used for amplification of the selected 5’ LTR region of lap DNA that contains 11 CpGs, and the 5’ UTRs of Line-Gf and Line-A DNAs that have 9 and 14 CpGs, respectively, using the following primers: lap forward TTGGTTTAGATGGTTAATAATTAG, lap reverse AAAAAACACAAAAACCAAAACCTTACCTAT; Line-1 forward TGGATTAGGTGTGGTTTTAATAT, Line-1 reverse TATATCCTACCTCAAAATTCT; Line-1Gf forward GTTAAAGATTGATTTGTTGAAGGT, Line-1Gf reverse CACAAAAACATTTCTCAAAACTATAT. The PCR products were purified using the QiAquick Gel Extraction Kit (catalog no. 28706, Qiagen) and cloned into pCRII-TOPO vector using TOPO TA Cloning Dual Promoter kit (catalog no. K46001, Invitrogen). Positive clones were picked and amplified and plasmid DNA of each clone was purified and sequenced from both ends.

Statistical Analysis. All experiments were performed at least three times, and data are presented as mean ± SEM. In experiments that have only two groups, differences between two groups were analyzed by t tests. In experiments having more than two groups, differences between groups were compared by one-way ANOVA followed by Tukey’s honestly significant difference test using JMP software (SAS Institute). In the experiment of h2HAX immunostaining, z2 was used for analysis of difference between Marf1 wild-type and mutant groups. Statistical significance was defined as P < 0.05.

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Supporting Information
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Fig. S1. Meiotic resumption in mouse oocytes is induced by active maturation promoting factor (MPF), which is promoted by CDC25B, and inhibited by protein kinase A (PKA). PKA is activated by high levels of cAMP, which in turn inhibits CDC25B. The function of MARF1 in oocytes is downstream of PKA and upstream of MPF. Mutations of MARF1 cause the up-regulation of Ppp2cb mRNA, whereas down-regulation of the expression of Ppp2cb or protein in Marf1 mutant oocytes reverses the meiotic arrest, suggesting that MARF1 promotes oocyte meiotic progression by suppressing expression of Ppp2cb. Inhibition of PPP2CB in Marf1 mutant oocytes causes activation of MPF and resumption of meiosis. Overexpression of CDC25B in Marf1 mutant oocytes also reverses the meiotic arrest, indicating that the function of MARF1 is upstream of CDC25B. Red lines and molecules indicate negative regulation of meiotic resumption, whereas those marked in green indicate positive regulators of meiotic resumption in mouse oocytes. Dotted lines indicated possible indirect actions. Arrows indicate stimulatory effect, whereas T-shaped connectors indicate inhibitory effect.
No significant changes in the number of DNA double-stranded breaks in developmentally quiescent Marf1 mutant oocytes isolated from day 0 newborn mouse ovaries. Confocal microscopy of Marf1 WT (A) and mutant (B) oocyte chromatin spread at different stages of first meiotic prophase, immunolabeled with antibodies to γH2AX (red), CREST (green), and SYCP3 (purple), and counterstained with DAPI (blue). (Scale bars, 5 μm.)