

# MRG15 is required for pre-mRNA splicing and spermatogenesis

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Splicing can be epigenetically regulated and involved in cellular differentiation in somatic cells, but the interplay of epigenetic factors and the splicing machinery during spermatogenesis remains unclear. To study these interactions in vivo, we generated a germline deletion of MORF-related gene on chromosome 15 (MRG15), a multifunctional chromatin organizer that binds to methylated histone H3 lysine 36 (H3K36) in introns of transcriptionally active genes and has been implicated in regulation of histone acetylation, homology-directed DNA repair, and alternative splicing in somatic cells. Conditional KO (cKO) males lacking MRG15 in the germline are sterile secondary to spermatogenic arrest at the round spermatid stage. There were no significant alterations in meiotic division and histone acetylation. Specific mRNA sequences disappeared from 66 germ cell-expressed genes in the absence of MRG15, and specific intronic sequences were retained in mRNAs of 4 genes in the MRG15 cKO testes. In particular, introns were retained in mRNAs encoding the transition proteins that replace histones during sperm chromatin condensation. In round spermatids, MRG15 localizes with splicing factors PTBP1 and PTBP2 at H3K36me3 sites between the exons and single intron of transition nuclear protein 2 (*Tnp2*). Thus, our results reveal that MRG15 is essential for pre-mRNA splicing during spermatogenesis and that epigenetic regulation of pre-mRNA splicing by histone modification could be useful to understand not only spermatogenesis but also, epigenetic disorders underlying male infertile patients.

infertility | fertility defects | splicing defects | epigenetics | spermiogenesis

Spermatogenesis is a complex process involving several biological events and dramatic changes of chromatin structure. Male germ cells undergo stem cell self-renewal, mitotic divisions in spermatogonial proliferation, genomic rearrangement by meiotic homologous recombination at the spermatocyte stage, and morphological changes of round spermatids into elongated spermatids to form mature spermatozoa (1–3). During spermiogenesis, nucleosomal histone proteins are replaced with transition nuclear proteins (TNPs) and subsequently, protamines, the major nucleosomal proteins in spermatozoa. Moreover, epigenetic modifications, such as histone methylation, dramatically change throughout spermatogenesis (3, 4).

Pre-mRNA splicing generates protein diversity and is involved in the regulation of cellular differentiation (5, 6). Recent advances have shown that histone modifications regulate alternative splicing through recruitment of splicing regulators via chromatin binding proteins, such as MORF-related gene on chromosome 15 (MRG15) (7). Histone H3 lysine 36 (H3K36) is methylated proximal to tissue-specific splicing regions (8–12). MRG15 specifically recognizes the methylated H3K36 and recruits polypyrimidine tract binding protein (PTB) at intronic splicing silencer elements near an exon to suppress

exon insertions into mRNA (7). MRG15 is also a component of histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes and regulates transcription by balancing histone acetylation (7, 13–21). During spermatogenesis, before histones are replaced with transition proteins and protamines, histone H4 is highly acetylated, and the acetylation of histones is required for histone removal. In addition to regulation of pre-mRNA splicing and histone acetylation, MRG15 contributes to homology-directed DNA repair. Although MRG15 functions in multiple biological processes, the roles of MRG15 during spermatogenesis are unknown, because *Mrg15* null mice are embryonic lethal (22).

In this report, we have deleted MRG15 specifically in post-natal male germ cells and analyzed the role of MRG15 during spermatogenesis. Spermatogenesis in the *Mrg15* null testis arrests at the round spermatid stage without affecting meiotic division and histone acetylation. MRG15 contributes to epigenetic regulation of pre-mRNA splicing of *Tnp2*, one of the reasons for the arrest of spermatogenesis in the *Mrg15* null germ cells.

## Results

**MRG15 Is Required for Postmeiotic Spermatogenesis.** MRG15 mRNA levels are extremely high in testis, and expression initiates at 20 d after birth, when late-stage spermatocytes differentiate into round

### Significance

Pre-mRNA splicing generates protein diversity, is involved in the regulation of cellular differentiation, and can be epigenetically regulated by histone modifications. Chromatin binding proteins, which recognize histone modifications, recruit splicing regulators to methylated histones around tissue-specific splicing regions and regulate pre-mRNA splicing. However, the interplay of epigenetic factors and the splicing machinery during spermatogenesis remains unclear. Here, we show that epigenetic regulation of pre-mRNA splicing is required for spermatogenesis and male fertility. Thus, novel splicing diversity is important for spermatogenesis, and defects in this system may trigger disease.

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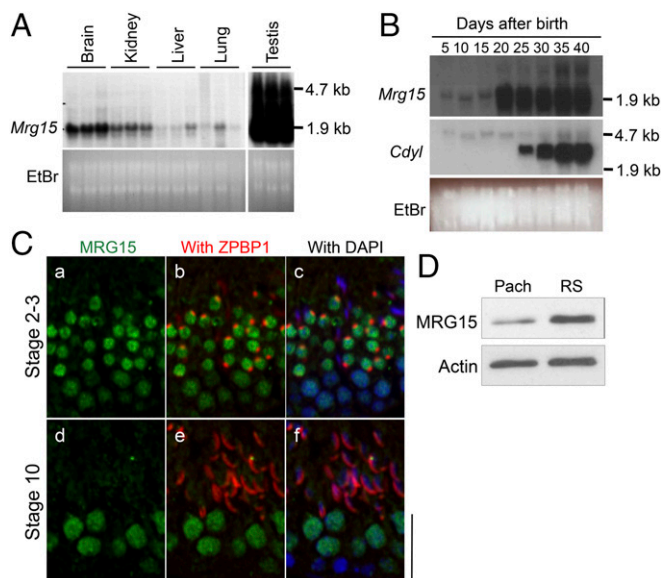
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The authors declare no conflict of interest.

Data deposition: The RNA sequencing and ChIP sequencing data have been deposited in the DDBJ Sequence Read Archive (DRA; accession nos. [DRA004783](https://www.ncbi.nlm.nih.gov/sra/DRA004783) and [DRA004778](https://www.ncbi.nlm.nih.gov/sra/DRA004778), respectively).

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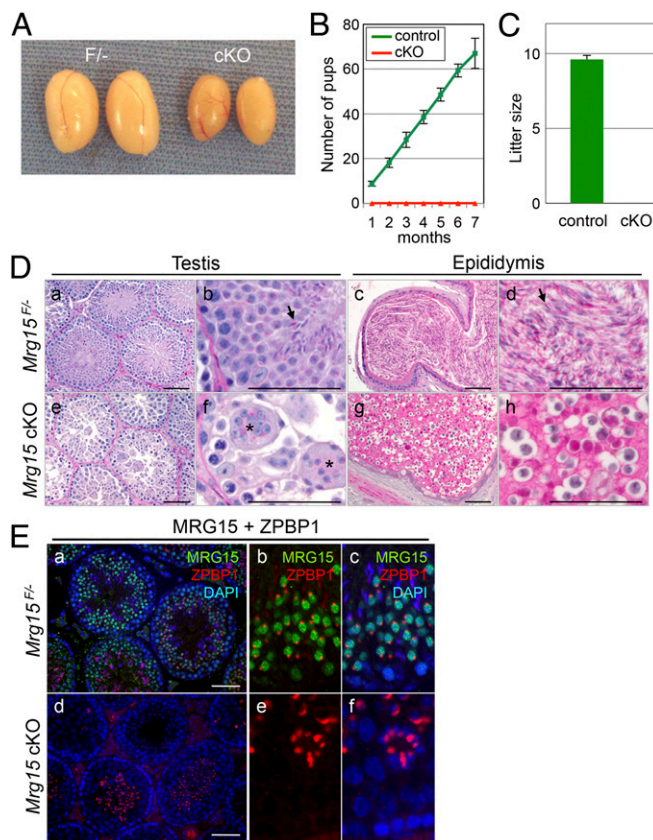
**Fig. 1.** Spatiotemporal expression of MRG15 in the testis. (A and B) Transcripts of *Mrg15* were examined by Northern blot analysis (A) in multiple tissues and (B) during postnatal development of the testis. Expression of chromodomain protein, Y chromosome-like (*Cdy1*) and/or ethidium bromide (EtBr) staining were performed as controls. The testis lanes in A were separately exposed to avoid strong radioactive signal from the testis lanes from invading adjacent lanes. (C) Immunolocalization of MRG15 in the testes. MRG15 (green), ZBPB1 (red), and DAPI (blue) localization in (a–c) stages 2 and 3 and (d–f) stage 10 seminiferous epithelium is shown. Seminiferous stages were determined by patterns of an acrosome staining of ZBPB1. (Scale bar: 10  $\mu$ m.) (D) Western blot analysis of MRG15 in purified pachytene spermatocytes (Pach) and round spermatids (RS). Expression analysis of actin was performed as a control.

spermatids (Fig. 1 A and B). MRG15 protein localizes in spermatocytes, and its expression is highest in round spermatids during spermatogenesis (Fig. 1 C and D). To address the *in vivo* postnatal roles of MRG15 during spermatogenesis, an MRG15-floxed allele was generated in ES cells, and mice carrying this allele were crossed with *Stra8-Cre* mice to achieve germline-specific deletion (SI Appendix, Fig. S1) (23). Germline-specific *Mrg15* null males have smaller testes and are sterile compared with control males (Fig. 2 A–C). In the *Mrg15* null testes, spermatogenesis is arrested at the round spermatid stage, abnormal multinucleated cells are abundant, and there are no detectable mature sperm with condensed nuclei (Fig. 2D). Whereas spermatogonia and spermatocytes are histologically normal in *Mrg15* null testes, spermatogenesis is arrested before round spermatid step 7, with significant apoptotic cell death (Fig. 2E and SI Appendix, Fig. S2). These findings suggest that MRG15 functions in round spermatids or late-stage spermatocytes for progression of spermatogenesis.

**MRG15 Is Not Required for Meiotic Progression.** Because MRG15 plays an essential role during homology-directed DNA damage repair, SCP1,  $\gamma$ H2AX, and ubiquitylated H2A were analyzed in spermatocytes to examine whether MRG15 is required for meiotic homologous recombination (20, 21, 24). However, there were no significant differences in localizations of meiosis regulating proteins between control and *Mrg15* null testes (SI Appendix, Figs. S3 and S4A). H3K36me3 and H4K20me3, both of which are recognized by MRG15, were not changed by lack of MRG15, indicating that MRG15 is not essential for meiotic homologous recombination (SI Appendix, Fig. S3) (25, 26).

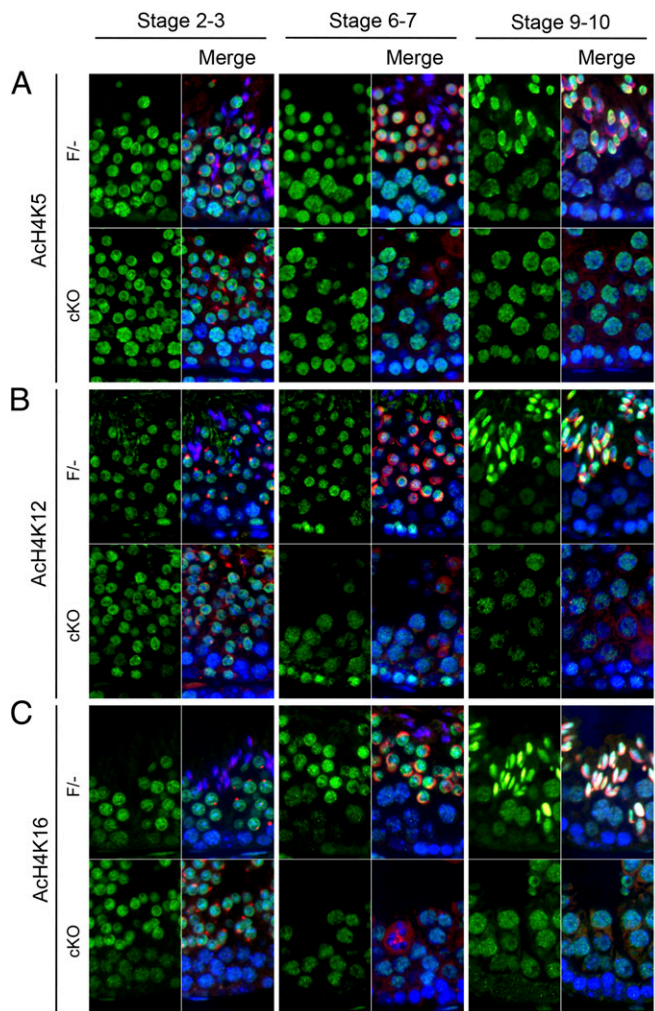
**Spermatogenesis in *Mrg15* Null Testes Arrests Before Hyperacetylation of Histone H4.** During spermatogenesis, histone H4 is highly acetylated before replacement of nucleosomal proteins from

histones to TNPs to protamines (27). Because MRG15 is found in both HAT and HDAC complexes (13–21), MRG15 may be essential for postmeiotic events. To investigate whether MRG15 is involved in histone acetylation in postmeiotic spermatogenesis, acetylated histone H3 levels in elongated spermatids in control testes, acetylated histone H3 levels in round spermatids are not significantly different between control and MRG15 conditional KO (cKO) testes (SI Appendix, Fig. S4B). CDYL, a histone H4 acetyltransferase that recognizes methylated H3K9 and is expressed in the testes after postnatal day 25 (Fig. 1B), is detectable in elongating spermatids in control testes but not in MRG15 cKO testes, probably because spermatogenesis is arrested before the elongating spermatid stage (SI Appendix, Fig. S4C) (28). When acetylation of lysines 5, 8, 12, and 16 of histone H4 was analyzed, these acetylated forms were reduced in the *Mrg15* null testes (Fig. 3 and SI Appendix, Fig. S5). However, high accumulation of acetylation on each residue was observed in elongating spermatids of control testes, and these germ cells are no longer present in *Mrg15* null testes (Fig. 3 and SI Appendix, Fig. S5A). When the amounts of histone H4 acetylation on each residue were compared in spermatid fractions between 22-d-old WT testes, in which spermatogenesis has developed to the round spermatid stage, and adult *Mrg15* null testes, there were no significant differences (SI Appendix, Fig. S5B). Because



**Fig. 2.** Infertile phenotype of germ cell-specific MRG15 null mice. (A) Gross images of *Mrg15*<sup>F/-</sup> (F/-) and *Mrg15*<sup>F/-:Stra8-Cre+</sup> (cKO) testes at 10 wk of age. (B) Average number of pups produced by F/- (control) and cKO males over 6 mo of breeding (*n* = 10 per genotype). (C) Average litter size produced by control and cKO males. (D) Histological analyses of (a, b, e, and f) testes and (c, d, g, and h) epididymis of (a–d) control and (e–h) cKO at 10 wk of age. Arrows and asterisks indicate mature sperm and abnormal multinucleated cells, respectively. (Scale bar: 50  $\mu$ m.) (E) Validation of seminiferous stage of spermatogenetic arrest by lack of MRG15. MRG15 (green), ZBPB1 (red), and DAPI (blue) in (a–c) control and (d–f) cKO are shown. (Scale bar: 50  $\mu$ m.)





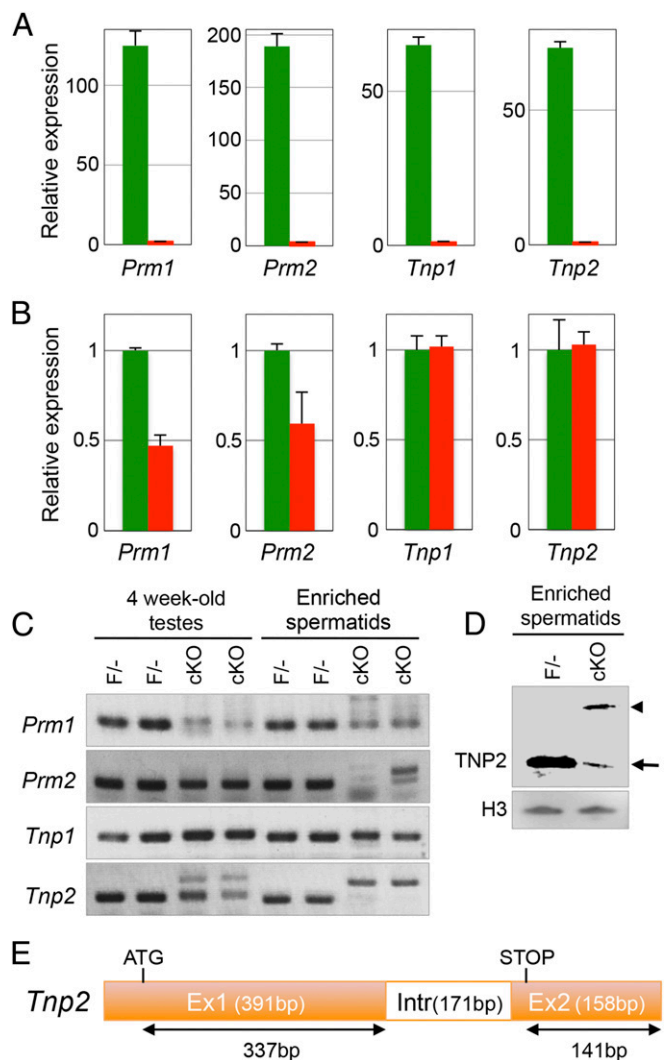
**Fig. 3.** Analyses of histone acetylation in MRG15 null spermatogenesis. Immunolocalization of acetylated histones in seminiferous cycle. Immunolocalization of (A) AcH4K5, (B) AcH4K12, and (C) AcH4K16 in stages 2 and 3, 6 and 7, and 9 and 10 of seminiferous cycles of both *F/−* and *cKO* are shown. Blue, DAPI; green, acetylated histones; red, ZBP1. (Scale bar: 50  $\mu$ m.)

spermatogenesis in *Mrg15* null testes arrests at the round spermatid stage before histone H4 is hyperacetylated, it is difficult to determine if MRG15 is necessary for replacement of nucleosomal proteins.

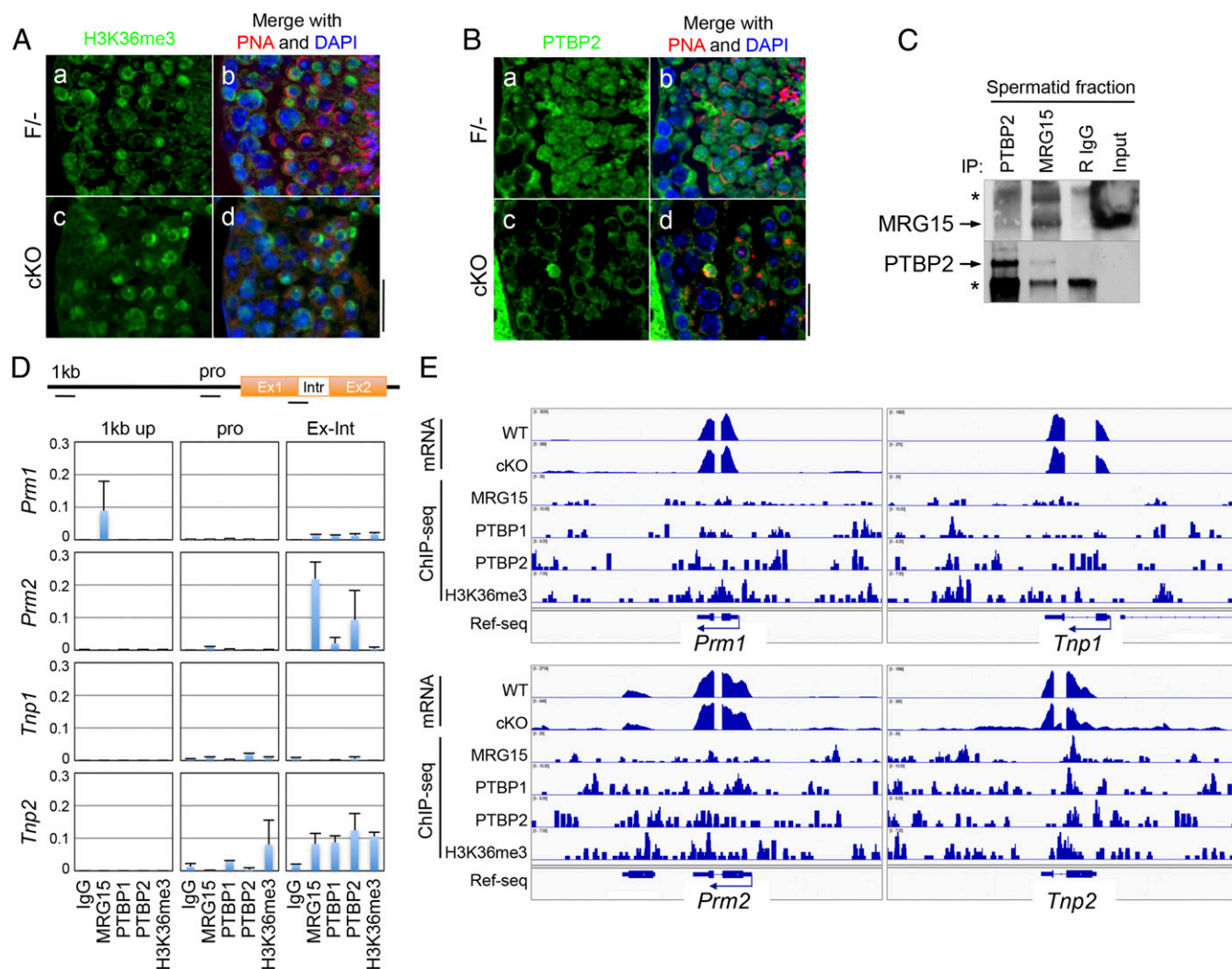
**Histone Modification Regulates Splicing of *Tnp2* by MRG15.** To study these processes in more molecular detail, expression of protamines (*Prm1* and *Prm2*) and Tnps (*Tnp1* and *Tnp2*) in testes and spermatids was analyzed in WT and *Mrg15* null spermatid fraction. Expression levels of both *Prm* and *Tnp* genes were significantly reduced in the *Mrg15* null adult testes, possibly because the expression of these proteins is the highest in latter stages of spermiogenesis, which are absent in the *Mrg15* null testes (Fig. 4A). To further interpret our data, expression of the *Prm* and *Tnp* genes was compared between spermatids fractions isolated from 22-d-old WT and *Mrg15* null testes, in which spermatogenesis has progressed to the early stage of round spermatids. Whereas expression levels of *Prm1* and *Prm2* are decreased in the absence of MRG15, *Tnp1* and *Tnp2* expression levels are not changed by *Mrg15* deletion (Fig. 4B). However, the *Tnp2* PCR products were larger in size in the absence of MRG15, and the shifts in size of the larger bands were exactly the size of the *Tnp2* intron sequences (Fig. 4C). Larger bands in the absence of MRG15 were also detected in the *Prm2* amplification in the

spermatid-enriched fraction, although the larger bands were not observed in 4-wk-old testes. When expression of TNP2 protein was analyzed in the spermatid fraction, the amount of TNP2 (16 kDa) was extremely low in the absence of MRG15 compared with control testis, and a larger TNP2 protein was detected specifically in *Mrg15* null spermatids (Fig. 4D), suggesting that splicing of the *Tnp2* gene (Fig. 4E) could be regulated by MRG15.

Because MRG15 regulates alternative splicing with recognition of methylated H3K36 and cooperation of PTB proteins, localization of methylated H3K36 and interaction between MRG15 and PTB proteins were analyzed. Methylated H3K36 is detected in a variety of male germ cells and observed at high



**Fig. 4.** Splicing defects observed in the absence of MRG15. (A and B) Quantitative expressions of *Prm1*, *Prm2*, *Tnp1*, and *Tnp2* were examined by quantitative RT-PCR in (A) testes and (B) spermatid fractions of 22-d-old WT (green) and MRG15 *cKO* (red) mice. *Hprt* was used as an internal control. (C) Validation of the size of the PCR products of *Prm1*, *Prm2*, *Tnp1*, and *Tnp2* in 4-wk-old testes and spermatid fractions of *F/−* and *cKO* males by electrophoresis. (D) Detection of TNP2 protein in *F/−* and *cKO* spermatids. Whole-protein extracts of crude spermatid fractions of *F/−* and *cKO* were separated by electrophoresis and probed with anti-TNP2 antibody. Arrow and arrowhead indicate a predicted size of TNP2 and a larger size of intron retained TNP2, respectively. Histone H3 was performed as control. (E) Structure of *Tnp2* gene. *Tnp2* mRNA consists of two exons (391 and 158 bp) and one intron (171 bp). The ORF of *Tnp2* is 354 bp. When the intron is retained in the *Tnp2* mRNA, the predicted length is 525 bp.



**Fig. 5.** MRG15 and PTBP2 cooperatively regulate splicing of *Tnp2* gene. (A) Accumulation of H3K36me3 in the testes. (a and c) H3K36me3 (green) and (b and d) peanut agglutinin (PNA; red) were visualized in the (a and b) F<sup>-/-</sup> and (c and d) cKO testes. (B) Immunolocalization of PTBP2. (a and c) PTBP2 (green) and (b and d) PNA (red) were stained in the (a and b) F<sup>-/-</sup> and (c and d) cKO testes. (Scale bar: 50  $\mu$ m.) (C) Coimmunoprecipitation of MRG15 and PTBP2 in WT spermatid fraction. Arrows indicate the bands of each protein. IP, immunoprecipitation; R, rabbit; \*, IgG. (D) ChIP analyses of the protamine and transition protein genes. Quantitative PCR using primers located on 1-kb upstream, promoter, and exon–intron junction of *Prm1*, *Prm2*, *Tnp1*, and *Tnp2* (each location is shown in Upper) was performed in ChIPs with indicated antibodies. Results are shown as relative amplification against input genome. (E) Colocalization of retained intron and MRG15 complexes. Genome browser views show the alignment of the read sequences of mRNA and ChIP with MRG15, PTBP1, PTBP2, and H3K36me3 on the mouse genomic regions of *Prm1*, *Prm2*, *Tnp1*, and *Tnp2* of WT spermatids. Ref-seq, reference sequence.

levels in the nucleus of both control and *Mrg15* null round spermatids (Fig. 5A), suggesting that H3K36 methylation is not affected by lack of MRG15. MRG15 interacts with PTBP2 in the WT spermatid fractions, and PTBP2 localizes to round spermatids similar to MRG15 and methylated H3K36 (Figs. 5A–C). In the absence of MRG15, PTBP2 is excluded from the nuclei of round spermatids, suggesting that recruitment of PTBP2 to the splicing machinery is defective in *Mrg15* null round spermatids (Fig. 5B). To further verify the recruitment of MRG15 and PTBP proteins as well as H3K36me3 was analyzed by ChIP. We discovered specific colocalization of MRG15 and PTBP proteins between exons and introns of the *Tnp2* gene but did not discover specific colocalization on either the 1-kb upstream region or the promoter region of the *Tnp2* gene or the *Prm1*, *Prm2*, and *Tnp1* genes (Fig. 5D).

To investigate a more widespread link between splicing defects and the lack of MRG15 and MRG15-containing protein complexes, we performed whole-mRNA sequencing and ChIP

sequencing (ChIP-seq) against H3K36me3, MRG15, PTBP1, and PTBP2 using enriched spermatids collected from 22- to 25-d-old testes. We found that specific mRNA sequences disappeared from 66 germ cell-expressed genes in the absence of MRG15 and that specific intronic sequences were retained in mRNAs of 4 genes in the MRG15 cKO testes (Tables 1 and 2). In the *Mrg15* null spermatids, colocalization of MRG15 complexes and H3K36me3 near splicing-defective regions was observed in 61 genes that had skipped sequences and all 4 genes that had retained sequences (Tables 1 and 2). Strong colocalization of MRG15, PTBP proteins, and H3K36me3 at the junction between exon and intron of the *Tnp2* gene was confirmed by the ChIP-seq experiments (Fig. 5E). Although weak colocalization of the MRG15 complex and H3K36me3 was detected at the junction between exon and intron of *Prm2*, there was only a slight intron retention of the *Prm2* transcript detected in deep sequencing results (Fig. 5E). Because the intron of *Tnp2* was retained in its precursor mRNA, other genes, in which an intron sequence remained in their mRNAs in the absence of MRG15, were

**Table 1. Genes that have skipped sequences in the absence of MRG15**

Gene	Chromosome	Start	Stop	Gene ontology term (biological process)	Gene ontology term (cellular)	Ref.
<i>Ankrd44</i>	Chr1	54,784,415	54,786,577	—	—	
<i>Ccdc93</i>	Chr1	123,383,243	123,387,698	Golgi to plasma membrane transport	Endosome	
<i>Mtap2</i>	Chr1	66,448,096	66,466,708	Microtubule bundle formation	Cytoskeleton	29
<i>Plekha6</i>	Chr1	135,142,720	135,155,585	—	—	
<i>Ptpn4</i>	Chr1	121,680,167	121,698,899	Protein dephosphorylation	Cytoskeleton	
<i>Spata17</i>	Chr1	188,964,376	188,998,253	—	Cytoplasm	35
<i>Ccdc34</i>	Chr2	109,872,597	109,880,730	—	—	
<i>Frdm5</i>	Chr2	121,417,499	121,632,465	—	Cytoskeleton	
<i>Pfkfb3</i>	Chr2	11,401,999	11,403,497	Fructose 2,6-bisphosphate metabolism	Nucleoplasm	46
<i>Zhx3</i>	Chr2	160,658,155	160,698,526	Transcription, DNA templated	Nucleus	
<i>Nbea</i>	Chr3	55,514,662	55,521,698	Protein targeting	Trans-Golgi network	
<i>Pdzk1</i>	Chr3	96,634,080	96,654,150	Calnitine transport	Plasma membrane	
<i>Postn</i>	Chr3	54,181,529	54,187,309	Cell adhesion	Extracellular region	
<i>Sgms2</i>	Chr3	131,045,379	131,047,507	Lipid metabolic process	Golgi apparatus	
<i>Aof2</i>	Chr4	136,110,316	136,111,087	Transcription, DNA templated	Nuclear chromatin	
<i>Eif4g3</i>	Chr4	137,702,289	137,707,496	Translation	—	30
<i>Map3k7</i>	Chr4	32,097,397	32,102,913	MAPK cascade	Plasma membrane	
<i>Pef1</i>	Chr4	129,798,686	129,802,306	Proteolysis	Cytoplasm	
<i>Slc9a1</i>	Chr4	132,972,414	132,973,814	Ion transport	Plasma membrane	
<i>Ssbp3</i>	Chr4	106,703,697	106,709,540	Transcription, DNA templated	Nucleus	
<i>Tssk3</i>	Chr4	129,166,977	129,167,705	Spermatogenesis	Intracellular	
<i>1700023E05Rik</i>	Chr5	77,445,664	77,471,120	—	—	
<i>4931409K22Rik*</i>	Chr5	24,056,624	24,057,778	—	—	
<i>Prom1</i>	Chr5	44,454,424	44,485,633	Retina-layer formation	Extracellular space	
<i>Cpa5</i>	Chr6	30,561,444	30,562,506	Proteolysis	Extracellular region	
<i>Vgll4</i>	Chr6	114,814,098	114,840,634	Transcription, DNA templated	Nucleus	
<i>Vhlh</i>	Chr6	113,574,373	113,578,085	Transcription, DNA templated	Nucleus	
<i>Eftud1</i>	Chr7	89,846,528	89,899,301	Translation	Intracellular	
<i>Igf2*</i>	Chr7	149,841,722	149,844,268	Protein phosphorylation	Extracellular region	
<i>Vasp</i>	Chr7	19,843,269	19,844,151	Actin cytoskeleton organization	Cytoskeleton	
<i>Dynlrb2</i>	Chr8	119,038,866	119,039,579	Microtubule-based movement	Cytoskeleton	
<i>Psd3</i>	Chr8	70,314,841	70,341,846	ARF protein signal transduction	Plasma membrane	
<i>Stox2</i>	Chr8	48,288,628	48,437,384	—	—	
<i>Abhd14b</i>	Chr9	106,352,563	106,353,723	Transcription, DNA templated	Nucleus	
<i>Ccdc33*</i>	Chr9	57,878,228	57,879,421	Spermatogenesis	Peroxisome	
<i>Dixdc1</i>	Chr9	50,519,078	50,535,890	Wnt signaling pathway	Cytoplasm	
<i>Ncam1</i>	Chr9	49,365,344	49,372,973	Cell adhesion	Plasma membrane	
<i>Nptn</i>	Chr9	58,491,610	58,498,303	Cell adhesion	Plasma membrane	
<i>Osbp110</i>	Chr9	115,085,201	115,116,631	Lipid metabolic process	Cytoskeleton	
<i>Pkm2</i>	Chr9	59,519,881	59,523,374	Glycolytic process	Mitochondrion	
<i>Usp2</i>	Chr9	43,875,304	43,883,448	Protein deubiquitination	Nucleus	31
<i>Hbs1l</i>	Chr10	21,056,882	21,061,519	Translation	Intracellular	
<i>Mtap7</i>	Chr10	19,868,887	19,950,589	Microtubule cytoskeleton organization	Cytoskeleton	32
<i>4933404M19Rik</i>	Chr11	78,017,700	78,018,341	—	—	
<i>Ace</i>	Chr11	105,832,798	105,833,330	Proteolysis	Extracellular region	33
<i>Akap1</i>	Chr11	88,707,180	88,725,710	Regulation of PKA signaling	Mitochondrion	
<i>Ccdc46</i>	Chr11	108,392,780	108,431,601	Receptor localization to synapse	Cytoskeleton	
<i>Tex14</i>	Chr11	87,356,880	87,362,933	Mitotic nuclear division	Kinetochore	34
<i>4930579E17Rik</i>	Chr12	37,274,548	37,414,835	Axon guidance	—	
<i>Ankrd9*</i>	Chr12	112,215,759	112,216,157	Hydrolase activity	—	
<i>Spata7</i>	Chr12	99,870,248	99,872,433	Response to stimulus	Cytoskeleton	
<i>2010111101Rik</i>	Chr13	63,383,567	63,398,165	Proteolysis	Cytoplasm	
<i>Bmp6</i>	Chr13	38,438,457	38,561,482	BMP signaling pathway	Extracellular region	
<i>Cdc14b</i>	Chr13	64,297,984	64,306,578	DNA repair	Nucleus	38
<i>4933401F05Rik</i>	Chr14	64,700,689	64,704,147	Proteolysis	Membrane	
<i>Diap3</i>	Chr14	87,172,510	87,210,142	Actin cytoskeleton organization	Nucleus	
<i>Apo17b</i>	Chr15	77,258,240	77,277,809	—	—	
<i>Dab2</i>	Chr15	6,249,900	6,366,805	Endocytosis	Plasma membrane	
<i>Gm628</i>	Chr15	73,623,216	73,629,309	—	—	
<i>Lpp</i>	Chr16	24,681,958	24,761,678	Cell adhesion	Cytoplasm	
<i>AW554918</i>	Chr18	25,362,478	25,448,439	—	—	
<i>Sncaip</i>	Chr18	53,067,129	53,074,960	Cell death	Cytoplasm	
<i>Tcf4</i>	Chr18	69,802,670	69,810,875	Transcription, DNA templated	Nucleus	
<i>6030443O07Rik</i>	Chr19	45,046,176	45,047,623	DNA repair	Nucleus	
<i>Sorbs1</i>	Chr19	40,374,012	40,386,158	Actin filament organization	Cytoskeleton	
<i>Rpgr*</i>	ChrX	9,743,403	9,755,396	Cilium assembly	Cytoskeleton	36

\*These genes have skipped sequences but do not show colocalization of H3K36me3 and MRG15 complexes.



**Table 2. Genes with retained sequences in *Mrg15* cKO but not in WT spermatids**

Gene	Chromosome	Start	Stop	Intron no.	Full name-location
<i>Mfap5</i>	Chr6	122,471,947	122,474,504	6	Mfap5_chr6_122471947_122474504_6
<i>Nmnat3</i>	Chr9	98,304,033	98,310,495	4	Nmnat3_chr9_98304033_98310495_4
<i>Tpm1</i>	Chr9	66,895,719	66,896,825	1	Tpm1_chr9_66895719_66896825_1
<i>Tnp2</i>	Chr16	10,788,187	10,788,357	1	Tnp2_chr16_10788187_10788357_1

further investigated. Among four genes that had retained sequences, only *Tnp2* retained the whole intron sequence, whereas *Mfap5* and *Nmnat3* retained a part of the intronic sequences, and *Tpm1* retained an exon that was not expressed in MRG15-positive spermatids. We confirmed splicing defects of three genes in the absence of MRG15 and also, weak colocalization of MRG15, PTBPs, and H3K36me3 around each defective locus using mRNA sequencing and ChIP-seq (SI Appendix, Fig. S6). The results at least indicate that MRG15 recognizes H3K36me3 and recruits PTBP2 to the splicing machinery of the *Tnp2* gene, and this protein complex regulates pre-mRNA splicing of *Tnp2* cooperatively (Fig. 6).

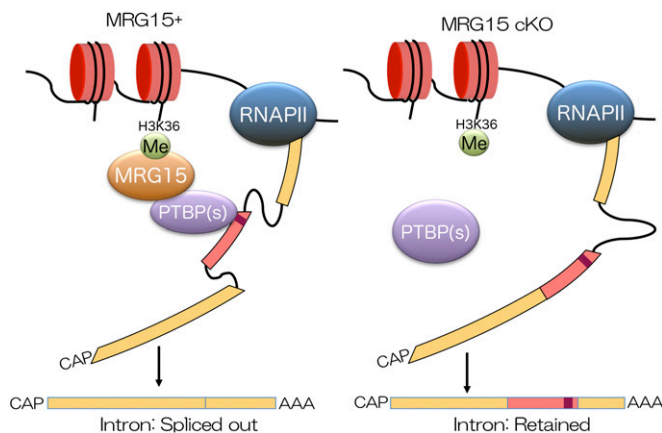
In addition, at least *Mtap2*, *Eif4g3*, *Usp2*, *Mtap7*, *Ace*, and *Tex14* are known to play a role in male fertility (29–34), and overexpression of *Spata17* and *Rpgr* leads to male infertility (35, 36). Therefore, the infertility in the *Mrg15* cKO mice could be secondary to alterations in the levels of these proteins. For example, our group has described the essential roles of TEX14 in male fertility and formation of intercellular bridges (34). The lack of TEX14 leads to the loss of intercellular bridges and the failure of proliferation and differentiation of spermatogonia (34, 37). Although the roles of TEX14 and intercellular bridges during postmeiotic process still remain to be elucidated, abnormal TEX14 might be a cause of the defects in spermatogenesis in the absence of *Mrg15*. Furthermore, *Mtap2*, *Spata17*, *Frmf5*, *Vasp*, *Dynlrb2*, *Oshpl10*, *Mtap7*, *Ccdc46*, *Spata7*, *Cdc14b*, *Sorbs1*, and *Rpgr* could be important for postmeiotic spermatogenic progression and might also be involved in the defects in the *Mrg15* null germ cells (Table 1) (38); these proteins are associated with microtubules and cytoskeleton, and suppression of their levels could be involved in the formation of multinuclear round spermatids and the failure to form mature sperm.

## Discussion

One of the biological events in which MRG15 is involved is homology-directed DNA repair after DNA damage (21). Many proteins that control homology-directed DNA repair, such as RAD51, BRCA1, BRCA2, and PALB2, are also involved in the regulation of meiotic homologous recombination (39–42). When MRG15 is down-regulated in somatic cells, localization of RAD51, BRCA2, and PALB2 to DNA damage sites is suppressed (21). However, the formation of RAD51 foci was not affected by the deletion of MRG15. Although localization of PALB2 to DNA damage sites can be regulated by BRCA1 independently with MRG15 and depletion of PALB2 leads to loss of RAD51 focus formation in the DNA damage sites of somatic cells, inhibition of the interaction between PALB2 and BRCA1 does not affect formation of RAD51 foci in meiotic double-strand breaks, even if it results in male sterility, likely secondary to defect in sex chromosome synapsis (42). Because there were no significant defects in meiosis *Mrg15* null testes, MRG15 is apparently not required for meiotic homologous recombination.

Herein, we found splicing failures in multiple genes in the absence of MRG15 and showed that histone modification regulates splicing via chromatin binding proteins, such as MRG15 (7) (Fig. 6). These splicing forms might produce different products and have different functions from the typical annotated sequences. The results suggest that absence of a spliced form of a gene could be important for progression of spermatogenesis. When an abnormal splicing form is expressed in WT spermatids,

the gene product could have different functions compared with the original product. In contrast, the gene product could lose its own function or exert an antagonistic effect on the original product when it is expressed in the *Mrg15* null spermatids. In this study, we focused on the abnormally spliced mRNA, in which intronic sequences remained in the absence of MRG15. Among four genes with retained sequences, strong interaction between MRG15 complexes and abnormal splicing was found in the *Tnp2* gene, whereas weak interaction was found in the other three genes: *Mfap5*, *Nmnat3*, and *Tpm1*. Of note, the retained sequence of *Tpm1* in the absence of MRG15 was derived from an exon but not derived from an intron, indicating that MRG15 complexes could regulate alternatively splicing of *Tpm1*. In addition, skipped exon 3 of *Tpm1* is a known target of PTBP (43). Interestingly, there was slight and weak colocalization of MRG15–PTBPs complexes around introns of *Prm1* and *Prm2*, respectively, despite that H3K36me3 was strongly accumulated at the junction between exon and intron of *Prm1* and *Prm2*. Some other proteins or modifications might participate in the regulation of splicing events of *Tpm1*, *Prm1*, and *Prm2* mRNAs. Because MRG15 can interact with HAT, HDAC complexes, and other histone modifications, such as methylated H4K20, localization of MRG15 on the genome may be regulated by not only H3K36me3 but also, other histone modifications and/or protein complexes (13–21, 26). PTBPs are known to antagonize exon definition; therefore, those retained sequences could be recognized as an exon in the absence of MRG15 (44). Although detailed molecular mechanisms underlying epigenetic regulation of specific sequence retention still remain to be elucidated, amounts of MRG15–PTBPs complexes localized in the regulatory region seem to be correlated with ratios of splicing defects in the lack of MRG15 (Figs. 4C and 5E). However, many abnormally spliced mRNAs, in which exon sequences were skipped by the lack of MRG15, were identified. MRG15–PTBPs complexes could repress splicing of those skipped sequences in the absence of MRG15. Because *Tnp2* null mice are not sterile, defects in other



**Fig. 6.** Proposed model for how MRG15 regulates pre-mRNA splicing of germ cell genes, such as *Tnp2*. AAA, poly A tail of mRNA; CAP, 5' cap structure of mRNA; Me, methylation; RNAPII, RNA polymerase II.

genes must be involved in the phenotype of the *Mrg15* cKO mice (45). The most apparent defect in *Mrg15* null spermatogenesis was spermatogenic arrest at the round spermatid stage. The epididymides of the *Mrg15* cKO mice were filled with round spermatids, suggesting that the absence of MRG15 might induce splicing defects in the genes, which were essential for round spermatids to proceed to the elongation step. Moreover, multinuclear round spermatids were frequently detected in testes lacking MRG15, indicating that some genes, which were essential for cell division, are altered by the lack of MRG15. Among 61 genes with MRG15-regulated intron skipped sequences, 9 genes are categorized as cytoskeleton-associated genes, and 9 genes are categorized as microtubule, actin, or cytoskeletal protein binding genes (Table 1). Particularly, *Mtap7* is localized in the spermatid manchette, and *Mtap7* mutant mice are sterile because of deformation of spermatid nuclei. Another cytoskeletal-associated gene, *Mtap2*, is required for spermatocytes to exit meiotic prophase I via the G2/MI transition. Although the role of MTAP2 in round spermatids is not known, abnormal MTAP2 could be a cause of the formation of multinuclear round spermatids. CDC14B, which is a dual-specificity phosphatase, could also be involved in the formation of multinuclear round spermatids, because it can bind, bundle, and stabilize microtubules independent of its phosphatase activity (38). However, PFKFB3 could be involved in formation of mature sperm; PFKFB3 is present only in immature sperm and replaced with PFKFB4 during maturation of sperm (46). Thus, splicing defects of multiple genes regulated by MRG15–PTBPs complexes could cause the defects in spermatogenesis in the absence of *Mrg15*. Additional characterization as well as biological analyses of each abnormal splicing form and ChIP-seq using pure spermatids may uncover additional spermatogenic phenotypes in the *Mrg15* null testes.

Germline-specific deletion of *Ptbp2* results in male sterility secondary to defects in alternative splicing (47). In the *Ptbp2* null mice, spermatogenesis also arrests at the round spermatid stage, and there are multinucleated cells as observed in the *Mrg15* null germ cells. These results suggest that MRG15 and PTBP2 share target genes and regulate pre-mRNA splicing cooperatively. However, splicing defects that were found in the absence of PTBP2 were not altered in the *Mrg15* null spermatids, suggesting that MRG15 and PTBP2 share some but not all splicing events (SI Appendix, Fig. S6) (47).

A large part of splicing forms could be regulated by other histone modifications and/or chromatin binding proteins but could not be regulated by H3K36me3 and MRG15. In fact, there are several complexes that include chromatin binding proteins (which recognize other histone modifications) and a splicing factor, such as H3K9me3/HP1a/hnRNPs, H3K4me3/CHD1/U2 snRNP, or acetylated H3/GCN5/U2 snRNP (5, 48–51). Splicing regulation via an MRG15–PTBP2 complex may also be physiologically relevant to the brain, because PTBP2 expression is restricted to testis and brain, and MRG15 is ubiquitously expressed (52–54). Moreover, regulation of splicing might be detectable in the hematopoietic lineage, because PTBP3 expression is restricted in hematopoietic cells (55). Thus, splicing regulation may be involved in other tissues, and tissue specificity might be regulated by not only the combination of chromatin binding proteins and splicing regulators present but also, regulation of histone modification. Alternative splicing forms and splicing regulation generate protein diversity; therefore, defects in this system may trigger disease.

## Materials and Methods

**Generation of Germ Cell-Specific *Mrg15* Null Mice and Fertility Analysis.** Details regarding the targeting construct and generation of conditional null allele of MRG15 are in SI Appendix, SI Materials and Methods. A germline-specific deletion of *Mrg15* was produced by mating with *Stra8-Cre* mice provided by Robert E. Braun, Jackson Laboratory, Bar Harbor, ME (23). For fertility analysis, 6-wk-old control (*Mrg15<sup>f/f</sup>;Stra8-Cre<sup>-</sup>*) and *Mrg15* cKO (*Mrg15<sup>f/f</sup>;Stra8-Cre<sup>+</sup>*) male littermates were individually bred to WT females. The numbers of litters and pups born per litter were monitored over a 6-mo period. All mouse experiments were performed on a C57BL/6J:129S5 hybrid background in accordance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and the Experimental Animal Care Committee of Kyushu University.

**Northern Blot Analysis and Semiquantitative and Quantitative RT-PCR.** Total RNA from mouse adult tissue samples and developing testes samples and cultured and isolated cell samples were extracted and subjected to Northern blot, semiquantitative RT-PCR, and quantitative PCR. Detailed methods and the primer sequences used in the experiments can be found in SI Appendix, SI Materials and Methods and Table S1, respectively.

**Histology, Immunofluorescence, and TUNEL Staining.** For testis histology, 5- $\mu$ m sections were stained with periodic acid-Schiff reagent and counterstained with hematoxylin. For immunofluorescence of tissue sections, paraformaldehyde-fixed sections were retrieved by microwave and then, incubated with primary antibodies overnight at 4 °C followed by Alexa 488- and Alexa 594-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Fluorescent sections were mounted with VECTASHIELD containing DAPI (VECTOR Laboratories). To detect apoptotic cells, DNA fragmentation was analyzed by the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instruction. Details regarding antibodies are included in SI Appendix, SI Materials and Methods.

**Chromosome Analysis.** Chromosome spread analysis was performed as previously described (56). Antibody information is included in SI Appendix, SI Materials and Methods.

**ChIP.** Approximately 10 million spermatids were cross-linked and sonicated until DNA was fragmented to an average length 200–500 bp. Control immunoprecipitation was performed with normal rabbit IgG. Immunoprecipitated DNA and input DNA were analyzed by PCR using the primers listed in SI Appendix, Table S1 or sequenced by Illumina HiSeq1500. Details regarding ChIP and antibodies used can be found in SI Appendix, SI Materials and Methods.

**RNA Deep Sequencing and ChIP-Seq Analyses.** Details regarding RNA deep sequencing, ChIP sequencing, and their analyses are in SI Appendix, SI Materials and Methods. RNA sequencing and ChIP-seq data have been deposited in the DDBJ (DNA Data Bank of Japan) Sequence Read Archive under accession numbers DRA004783 and DRA004778, respectively.

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