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Growing oocyte specific transcription-dependent *de novo* DNA methylation at the imprinted *Zrsr1*-DMR

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Abstract

Zrsr1 is a paternally expressed imprinted gene located in the first intron of *Commd1*, and the *Zrsr1* promoter resides in a differentially methylated region (DMR) that is maternally methylated in the oocyte. However, a mechanism for the establishment of the methylation has remained obscure. *Commd1* is transcribed in the opposite direction to *Zrsr1* with predominant maternal expression, especially in the adult brain. In this study, we found *Commed1* transcribed through the DMR in the growing oocyte. *Zrsr1*-DMR methylation was abolished by the prevention of *Commd1* transcription. Furthermore, methylation did not occur at the artificially unmethylated maternal *Zrsr1*-DMR during embryonic development when transcription through the DMR was restored in the zygote. Loss of methylation at the maternal *Zrsr1*-DMR resulted in biallelic *Zrsr1* expression and reduced the extent of the predominant maternal expression of *Commd1.* These results indicate that the establishment of methylation at *Zrsr1*-DMR occurs in a transcription-dependent and oocyte-specific manner, and caused *Zrsr1* imprinting by repressing maternal expression. The predominant maternal expression of *Commd1* is likely caused by transcriptional interference by paternal *Zrsr1* expression.

Keywords DMR / DNA methylation / genomic imprinting

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Introduction

Genomic imprinting is an epigenetic phenomenon of parent-of-origin–dependent expression that is observed in a subset of mammalian genes. Imprinted genes are expressed exclusively or predominantly from one of the two parental alleles, and are frequently located in clusters known as imprinted domains. The expression of genes in an imprinted domain is regulated by a discrete element called an imprinting center (IC) or an imprinting control region (ICR). Imprinted genes or imprinted domains have been found to link to differentially methylated regions (DMRs) that exhibit parent-of-origin–specific DNA methylation. Two classes of DMRs have been identified as follows: germline DMRs (gDMRs), or primary DMRs; and somatic DMRs (sDMRs), or secondary DMRs. gDMR methylation is established during gametogenesis, and sDMRs acquire methylation after fertilization under the direction of gDMRs. The ICs of the imprinted genes are located in their corresponding gDMRs. More than 20 gDMRs have been identified in mice, of which only three are paternally methylated (Arnaud, 2010, Barlow & Bartolomei, 2014, Ferguson-Smith, 2011, Kobayashi, Sakurai et al., 2012). Recent studies have identified an additional 11 new putative maternally methylated gDMRs (Wang, Zhang et al., 2014).

DNA methylation at gDMRs is the primary determinant of the allelic expression of imprinted genes, and the mechanisms of methylation establishment have been extensively investigated. The specific recruitment of *de novo* methylation machineries to gDMR methylation sites via the recognition of sequence elements and/or chromatin structures has been considered as a potential mechanism of germline-specific gDMR methylation establishment (Arnaud, 2010, Bartolomei & Ferguson-Smith, 2011). However, efforts to identify sequence motifs for gDMR methylation have not been successful. Several trans-acting factors for maternally methylated

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gDMRs have been found to be essential for the establishment of germline methylation in mice. For example, Dnmt3a has been identified as the enzyme responsible for *de novo* methylation of many maternal gDMRs (Hata, Okano et al., 2002, Kaneda, Okano et al., 2004). Dnmt3l, a DNA methyltransferase (DNMT)-like protein without enzymatic activity, is the likely co-factor of DNMTs (Bourc'his, Xu et al., 2001). Ablation of Kdm1b, a histone demethylase of H3K4 di-and trimethylation, in oocytes resulted in the failure of methylation establishment at some maternal gDMRs (Ciccone, Su et al., 2009). In addition, the deletion of *Hira*, which encodes a histone H3.3 chaperon (Hira), led to global hypomethylation in oocytes (Nashun, Hill et al., 2015).

Kelsey et al. proposed a model for the establishment of methylation at the maternal gDMRs in the oocyte (Kelsey & Feil, 2013, Veselovska, Smallwood et al., 2015), which suggests that maternal methylation of gDMRs is regulated by the same mechanisms of general gene-body methylation reported for active genes (Ball, Li et al., 2009, Maunakea, Nagarajan et al., 2010). This was based on the findings that most maternal gDMRs are located in actively transcribed regions (Chotalia, Smallwood et al., 2009), that transcription is a prerequisite for the establishment of methylation at four maternal gDMRs (Chotalia et al., 2009, Singh, Sribenja et al., 2017, Smith, Futtner et al., 2011, Veselovska et al., 2015), and regarding the characteristics of the methylome and transcriptome in growing oocytes (Smallwood, Tomizawa et al., 2011, Veselovska et al., 2015). Analyses of the methylome revealed that most methylation in the oocyte genome occurs within actively transcribed regions, and that maternal gDMRs are not specifically targeted for methylation, but are instead methylated along with other parts of the transcribed regions where the gDMRs reside. Unlike the rest of the transcribed regions, gDMRs

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are likely protected against global demethylation during early embryonic development; only the gDMRs escape global demethylation and remain methylated for lifetime.

Methylation failed at the gDMRs in the *Gnas* locus and the KvDMR in the *Kcnq1* locus when a poly(A) signal-truncation cassette was inserted into these loci to prevent transcription from elongation through the gDMRs (Chotalia et al., 2009, Singh et al., 2017). Failure of methylation was also reported at PWS-IC and the *Zac1*-DMR when the promoter regions from which transcription originated and then proceeded through the maternal gDMRs were deleted (Smith et al., 2011, Veselovska et al., 2015). Furthermore, most of these maternal gDMRs were located within transcribed regions in the growing oocyte (Chotalia et al., 2009). On the other hand, the establishment of sDMR- and lineage-specific methylation during the post-implantation stage clearly indicates the *de novo* methylation potency of early embryonic cells, including naïve/primed pluripotent stem cells (Monk, 2015). It is unknown whether *de novo* methylation occurs at gDMRs during the post-implantation stage after failure to establish gDMR methylation in the oocyte.

Zrsr1 (*U2af1-rs1*) and *Commd1* (*Murr1*) are imprinted genes located in mouse proximal chromosome 11. *Zrsr1* is expressed ubiquitously in all adult tissues examined, and is expressed exclusively from the paternal allele. *Zrsr1* resides in the first intron of the *Commd1* gene and is transcribed in the opposite direction to the host gene. The *Zrsr1* promoter is located in a maternally methylated gDMR, i.e., the *Zrsr1*-DMR (Nabetani, Hatada et al., 1997). It is likely that maternal methylation at the *Zrsr1*-DMR causes imprinted expression by repressing maternal expression of the gene. *Commd1* is likewise expressed ubiquitously in adult mice, but is

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expressed from both parental alleles. However, *Commd1* expression from the maternal allele is stronger than expression from the paternal allele (i.e., predominant maternal expression), as exemplified in the adult brain. Although *Zrsr1*-DMR resides in the transcribed region of *Commd1*, the link between transcription and the establishment of methylation at this DMR has not been clarified.

In this study, we found that methylation at *Zrsr1*-DMR failed when transcription through the DMR was abolished by the insertion of a poly(A) signal cassette into the site between the *Commd1* promoter and the *Zrsr1* gene. Furthermore, upon deletion of the cassette in the zygote, *Zrsr1*-DMR transcription resumed, but methylation at the DMR during early development was not restored. These results indicate that transcription-dependent methylation at the DMR occurs specifically in the growing oocyte, but not during early development. We also found that interference with transcription likely caused predominant maternal expression of *Commd1* in the adult brain.

Results

Truncation of *Commd1* transcription results in methylation failure at *Zrsr1*-DMR in the growing oocyte

Methylation of maternal gDMRs of imprinted genes is established asynchronously during postnatal oocyte growth, typically between 5 and 25 days postpartum (dpp) (Lucifero, Mann et al., 2004). To verify that *de novo* methylation at *Zrsr1*-DMR is dependent on transcription resulting

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from *Commd1* expression in the oocyte, we analyzed *Commd1* expression and the methylation status of *Zrsr1*-DMR in growing oocytes from this period and in ovulated MII oocytes. *Commd1* was expressed in all periods of oocyte maturation analyzed (Fig 1B). *De novo* methylation at *Zrsr1*-DMR started after 10 dpp and was completed between 15 dpp and maturation (Fig 1C). Thus *Zrsr1*-DMR was transcribed before and during the establishment of methylation. *Zrsr1* expression was not detected by RT-PCR during oocyte maturation (Fig EV1).

To confirm that *de novo* methylation at *Zrsr1*-DMR was a prerequisite for *Commd1* expression, we inserted a truncation cassette containing three tandem copies of SV40 poly(A) signal into intron 1 of *Commd1* to generate the transcription-truncation allele $Commd1^{PA}$ (Fig 1A), and obtained $Commd1^{+/PA}$-heterozygous mice in a C57BL/6J background. No $Commd1^{PA/PA}$ mice were born from the intercrossing of heterozygous parents. $Commd1^{-/-}$ mice have been shown to be embryonically lethal at E9.5 to E10.5 (van de Sluis, Muller et al., 2007); the absence of homozygous pups for the truncation allele was thus likely attributable to embryonic lethality, which strongly suggests that a truncation occurred as expected and rendered the $Commd1^{PA}$ allele functionally null.

To assess the truncation of the $Commd1^{PA}$ allele and *Zrsr1*-DMR methylation in the MII oocyte, MII oocytes were obtained from adult F1 females generated from the cross between $Commd1^{+/PA}$ B6 females and WT PWK males. $Commd1^{PA(B6)/+(PWK)}$ mice, termed PA F1 mice, and $Commd1^{+(B)/+(PWK)}$ mice, termed WT F1 mice, were obtained from F1 littermates. The allelic expression of *Commd1* was analyzed by RFLP analysis of RT-PCR products with the primers Comm-F1 and Comm-R1, located at exon 1 and exon 2, respectively. Expression of the

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Commd1^{PA} allele was not detected in MII oocytes prepared from the PA F1 females, although expression of the PWK allele was detected. In contrast, both alleles were expressed in oocytes from WT F1 female littermates (Fig 2A). The *Zrsr1*-DMR was completely unmethylated on the truncated allele (B6) in the MII oocytes from the PA F1 females, in contrast to the WT PWK allele, which was completely methylated. As expected, in oocytes from the WT F1 females, *Zrsr1*-DMR was completely methylated on both the B6 and PWK alleles (Fig 2B). These results indicate that transcription termination occurred in intron 1, likely at the truncation cassette, and resulted in the loss of transcription through the DMR, which led to methylation failure at the DMR during oogenesis.

Maternal methylation at *Zrsr1*-DMR causes *Zrsr1* imprinting

To investigate the causative link between the maternal methylation at *Zrsr1*-DMR and the imprinted expression of *Zrsr1*, we analyzed *Zrsr1*-DMR methylation and *Zrsr1* allelic expression in the adult F1 mice described above. The truncation of *Commd1* transcription was also observed in the brain and liver of the PA F1 mice (Fig 3A). The *Zrsr1*-DMR on the maternal *Commd1*$^{PA(B6)}$ allele of the PA F1 mice was completely unmethylated, whereas normal maternal methylation was observed in the WT F1 mice (Fig 3B). These results indicate that the truncation cassette was also functional in the adult mice, and that the unmethylated status of maternal *Zrsr1*-DMR persisted throughout the embryo stage and postnatal growth to adulthood. *Zrsr1* was biallelically expressed in the PA F1 mice, although the gene exhibited paternal expression in the WT F1 mice (Fig 3C). Quantitative analysis of total *Zrsr1* mRNA levels indicated that the PA F1 mice expressed twice as much *Zrsr1* as the WT F1 mice (Fig 3D), which suggests that the

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maternal allele in the PA F1 mice was completely de-repressed and was expressed at the same level as the paternal allele. Thus it is evident that the *Zrsr1* gene is imprinted by suppression of maternal allele expression, and that maternal methylation at the DMR is the primary imprint mark.

Zygotic deletion of the truncation cassette does not result in the acquisition of methylation at *Zrsr1*-DMR during embryonic development and postnatal growth

We showed that *Zrsr1*-DMR acquires methylation in a transcription-dependent manner during oogenesis. However, Commd1 is expressed from both alleles and paternal Zrsr1-DMR is unmethylated in adult mice (Wang, Joh et al., 2004). This fact indicates that transcription-dependent methylation does not occur at paternal Zrsr1-DMR in differentiated somatic cells. However, it was not known that Commd1 is expressed in early embryo, in which genome-wide de novo methylation is about to occur (Howlett & Reik, 1991, Kafri, Ariel et al., 1992, Smallwood et al., 2011). *Commd1* was expressed from both alleles in the WT blastocysts described below (WT in Fig 4A and B), which indicated that the paternal *Zrsr1*-DMR was also unmethylated, even during transcription in pluripotent cells. To confirm this in the maternal allele, the floxed truncation cassette (refer to Fig 1A) was deleted from the maternal *Commd1*^{PA} allele via zygotic expression of Cre recombinase. Then, methylation at the maternal *Zrsr1*-DMR, which was inherited in an unmethylated state, was analyzed. The resulting deleted allele was denoted *Commd1*$^{\Delta PA}$.

The blastocysts were prepared via *in vitro* fertilization (IVF) with MII oocytes from the *Commd1*$^{+/PA}$ B6 females and sperm from CAG-Cre transgenic males in a BALB/c background.

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Deletion of the truncation cassette occurred in all blastocysts that inherited the *Commd1*^{PA} allele and the CAG-Cre transgene(s) (Fig EV2). Judging from the high incidence of deletion at the blastocyst stage, it is plausible that the deletion actually occurred much earlier. To determine whether deletion of the truncation cassette would restore expression of the maternal *Commd1* allele, we analyzed the allelic expression of *Commd1* in *Commd1*$^{+(B6)/+(BALB)}$ blastocysts (WT), *Commd1*$^{PA(B6)/+(BALB)}$ blastocysts (PA), and *Commd1*$^{\Delta PA(B6)/+(BALB)}$ blastocysts (ΔPA). Allelic expression was analyzed quantitatively by pyrosequencing of RT-PCR products with the primers CommExpre-F1-bio and CommExprePyro-R, which are located in exon 2. In the WT-blastocysts, *Commd1* was equally expressed from both parental alleles, but only the paternal allele was expressed in the PA-blastocysts. This indicates that the truncation cassette was also functional in the blastocysts, and suggests that *Commd1* was expressed from both alleles during the developmental period in which global *de novo* DNA methylation occurs. In ΔPA-blastocysts, in which the truncation cassette was deleted, the maternal ΔPA allele was expressed at levels comparable to the paternal allele (Fig 4A). Furthermore, the unmethylated status of maternal *Zrsr1*-DMR was maintained, despite transcription of the DMR (Fig 4B). To determine whether this status was maintained while under transcription from the early embryonic stage to adulthood, we analyzed the brain of ΔPA adult F1 mice from the cross between *Commd1*$^{+/PA}$ B6 females and CAG-Cre transgenic BALB/c males. The maternal *Zrsr1*-DMR in the *Commd1*$^{\Delta PA}$ allele was unmethylated, even though the maternal allele was expressed (Fig 4C and D). Thus, we concluded that maternal *Zrsr1*-DMR was not methylated after fertilization, irrespective of DMR transcription.

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Predominant maternal expression of *Commd1* is caused by the imprinted expression of *Zrsr1*

We reported predominant maternal expression of *Commd1*, especially in the brains of adult mice (Wang et al., 2004) (WT in Fig 4D and Fig EV3). Based on the two observations (Wang et al., 2004) discussed below, we hypothesized that the expression of *Zrsr1* decreased *Commd1* expression from the paternal allele by interfering with the transcription elongation of *Commd1* within the gene body and with transcription initiation in the *Commd1* promoter. First, in the adult liver, *Zrsr1* is expressed at extremely low levels (less than 10%) relative to *Commd1*, which exhibits less-pronounced predominant maternal expression (Fig EV3). However, in the adult brain, *Zrsr1* is expressed to the same extent as *Commd1*, which exhibits remarkable predominant maternal expression. Second, the antisense transcript of *Commd1* was observed in the promoter region of *Commd1* only in the paternal allele, and was observed at higher levels in the brain than in the liver (also refer to Fig 5B). The observed antisense *Commd1* transcript is presumably generated by read-through transcription of *Zrsr1*, which may interfere with the initiation of *Commd1* transcription.

According to this hypothesis, we expected that predominant maternal expression would not occur in the ΔPA mice, which express *Zrsr1* equally from both parental alleles. Indeed, quantitative analysis by pyrosequencing demonstrated that the ratio of maternal to paternal expression was significantly decreased in these mice relative to WT mice (Fig 4D). The total *Commd1* expression levels were then quantitatively analyzed in both lines using the TaqMan assay (Fig 4E). Expression of *Commd1* in the ΔPA mice was 57% of that in the WT mice, which

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is consistent with the notion of decreased maternal expression in the ΔPA mice. Moreover, the relative quantities of the overall expression were consistent with the quantitative allelic expression analysis shown in Fig 4D, in which the total expression levels were 5 (maternal = 4, paternal = 1) and 3 (maternal = 2, paternal = 1) in the WT mice and the ΔPA mice, respectively, given no change in the level of expression from the paternal allele. Collectively, these results indicate that expression of *Zrsr1* reduces the expression of *Commd1* from the paternal allele, and results in the predominant maternal expression of *Commd1*.

In the *Commd1* promoter region, we analyzed antisense transcription of *Commd1* in F1 mice generated from the cross between *Commd1*$^{+/PA}$ B6 females and WT PWK males. Antisense transcripts were expressed at higher levels in the brain than in the liver of the WT mice, as reported previously (Wang et al., 2004), and the same transcript was observed in the PA mice (Fig 5B). In addition, the antisense transcript was expressed only from the paternal allele in the WT mice, which is congruent with previous studies (Wang et al., 2004). However, it was expressed from both alleles in the PA mice (Fig 5C), which was consistent with the pattern of allelic expression of *Zrsr1* in these mice. This consistency of allelic expression between the opposite transcripts and *Zrsr1* strongly supports the idea that to some degree the transcription of *Zrsr1* extends to the *Commd1* promoter region beyond the poly(A) signal of the *Zrsr1* gene. Such antisense transcription might interfere with the initiation of *Commd1* transcription and thereby contribute to its predominant maternal expression, especially in the brain.

Discussion

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The results of this study indicate that transcription through the *Zrsr1*-DMR is essential for the establishment of methylation at the DMR in the growing oocyte, as reported for four other imprinted loci (Chotalia et al., 2009, Singh et al., 2017, Smith et al., 2011, Veselovska et al., 2015). Our results also indicate that transcription-dependent methylation in the *Zrsr1*-DMR does not occur after fertilization, which suggests that the mechanism of *de novo* methylation of the *Zrsr1*-DMR is oocyte-specific. We also found that paternally imprinted expression of *Zrsr1* resulted in predominant maternal expression of *Commd1*, which is likely caused by transcriptional interference between *Zrsr1* and *Commd1* on the paternal allele.

The mouse embryo undergoes genome-wide *de novo* DNA methylation during the post-implantation period, at a level comparable to the growing oocyte (Howlett & Reik, 1991, Kafri et al., 1992, Smallwood et al., 2011). In fact, the early embryo expresses factors essential for *de novo* DNA methylation, such as Dnmt3a, Dnmt3b, and Dnmt3l (Okano, Bell et al., 1999, Uysal, Akkoyunlu et al., 2015, Watanabe, Suetake et al., 2002). In this respect, the *de novo* DNA methylation activity in the pluripotent cells of the early embryo is comparable to that in the growing oocyte. However, our results indicated that methylation at *Zrsr1*-DMR does not occur on either parental allele in the early embryo, despite *Commd1* expression from both alleles (Fig 4A, B, C). This suggests some differences in the mechanisms of *de novo* methylation between the early embryo and the growing oocyte, at least in the gDMR. Two possible differences are considered: first, there may be factor(s) inhibiting *de novo* methylation at *Zrsr1*-DMR in the early embryo, e.g., histone modification(s) at the DMR, or a trans-acting factor(s) expressed in pluripotent cells; second, the early embryo may lack factor(s) required for *de novo* methylation at

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the DMR, which could include epigenetic modification(s) at the DMR that are erased after fertilization, or trans-acting oocyte-specific factor(s). In contrast to the methylation at *Zrsr1*-DMR, transcription-dependent methylation occurs during embryonic development at sDMRs located upstream of *Zdbf2*, which is an imprinted gene on the paternal allele, during the period around implantation (Greenberg, Glaser et al., 2017). This suggests variation in DNA methylation mechanisms among DMRs in the imprinted loci, and it will be interesting to examine whether transcription-dependent methylation is also oocyte-specific at the four previously reported loci.

Methylation of a gene promoter usually represses expression of the respective gene (Bird, 2002, Esteller, 2007). Thus, the paternal allele-specific expression of *Zrsr1* seems to be caused by maternal methylation at *Zrsr1*-DMR. This was confirmed by analyses of the expression and methylation of *Zrsr1* in the PA mice, in which complete unmethylation at the maternal *Zrsr1*-DMR resulted in equal levels of gene expression from both alleles (Fig 3C and D). A decrease in maternal expression of *Commd1* also occurred concomitantly with the activation of maternal *Zrsr1*, which strongly suggests that the predominant maternal expression of *Commd1* results from *Zrsr1*-mediated reduction in the expression of *Commd1* from the paternal allele. Studies on genetic organization in genomes have identified overlapping and antisense-oriented genes, and the mutual *cis*-acting effect on their expression, which is termed transcriptional interference (Bordoy & Chatterjee, 2015). The active transcription of *Zrsr1* within *Commd1* may cause collisions between the opposing *Zrsr1* and *Commd1* elongation complexes, which would result in the reduction in their expression. We observed an antisense-oriented transcript in the *Commd1* promoter region, which was hypothesized to be an overshoot of *Zrsr1* transcription (Fig 5) and

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suggests that *Zrsr1* transcription potentially interferes with the initiation of *Commd1* transcription. However, complete biallelic *Zrsr1* expression did not give rise to equal levels of *Commd1* expression from both parental alleles (Fig 4D). Therefore, unidentified differences between the parental *Commd1* alleles may exist, despite the fact that thus far we have found no differences in DNA methylation or some histone modifications in our examination of the *Commd1* promoter (Joh, Yatsuki et al., 2009).

Our results implicate the existence of unidentified growing-oocyte–specific factors for the establishment of methylation at maternal gDMRs. We have yet to determine whether these factors are proteins, RNAs, or chromatin structures. Future extensive proteomic, transcriptomic, and epigenetic analyses of the growing oocyte may elucidate the underlying mechanism for the primary establishment of methylation at the maternal gDMRs.

Materials and Methods

Generation of *Commd1-PA* mice

A truncation cassette was constructed by cloning three copies of the SV40 poly(A) signal from the expression vector pGFP-N1 (Clontech) into pT7Blue (Novagen). The truncation cassette was inserted at the genomic site 23 bp downstream of exon 1 using the gene-targeting method (Fig 1A). The targeting construct was generated by homologous recombination with a truncation cassette clone and a mouse *Commd1* BAC clone (BAC RP24-216A32, BACPAC Resources) in *E. coli* as previously described (Zhang, Muyrers et al., 2000). The targeting construct contained

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the following mouse *Commd1* genomic sequences: a 5.2-kb 5′ sequence containing exon 1, and a 5.1-kb 3′-sequence containing a part of intron 1. An embryonic stem cell (ES) clone with the truncation cassette inserted in the precise genomic position was identified by Southern blotting and PCR analyses of genomic structure, and was used to generate chimeric *Commd1-PA* mice. The *neo* gene in the targeting vector was flanked by FRTs and removed from the *Commd1*-truncated mice via Flippase expression. *Commd1-PA* mice in a C57BL/6J (denoted B6) genetic background was obtained by backcrossing with wild type (WT) C57BL/6J mice for five generations. This study was approved by the Ethical Committee for Animal Experiment of Saga University.

Preparation of primordial germ cells, metaphase II oocytes, and blastocysts

Mouse female primordial germ cells (PGC) were prepared from Day 5, Day 10, and Day 15 female C57BL/6 neonates as previously described (Kobayashi, Sakurai et al., 2013). The metaphase II (MII) oocytes were obtained from superovulated six- to eight-week-old female mice using the procedure described by Nakagata *et al*. (Nakagata, Takeo et al., 2013). Blastocysts were prepared by *in vitro* fertilization (IVF) using MII oocytes from *Commd1*$^{PA/+}$ B6 mice and sperm from 12- to 14-week-old CAG-Cre transgenic BALB/c mice. After cumulus-oocyte complexes had been coincubated with sperm for 3 h, fertilized oocytes were cultured to the blastocyst stage at 37 °C and 5% CO_2 in humidified air for 96–120 h, as previously described (24). CAG-Cre BALB/c mice were obtained from the RIKEN BioResource Center (RBRC No. RBRC06155). The CAG promoter drives ubiquitous expression of Cre recombinase in mice.

DNA preparation and methylation analysis

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Approximately 200 growing oocytes and MII oocytes were lysed in lysis buffer (0.5% SDS, 250 ng/µl proteinase K, 100 ng/µl yeast tRNA) at 37 °C for 60 min, and DNA in the lysate was bisulfite-converted by treating the lysate with the EpiTect Bisulfite Kit (QIAGEN, #59104). Recovered DNA was used for amplification of *Zrsr1*-DMR via nested PCR. Amplified DNA was cloned in pT7Blue T-vector (Novagen, #69820) and the resulting sequences were analyzed with BigDye Terminator v3.1 (Applied Biosystems, #4337455) on an ABI3130 sequencer (Applied Biosystems). Blastocyst DNA was prepared from the precipitate of ISOGEN II lysate during RNA preparation with ISOGENOME (NIPPON GENE, #318-08111). DNA from adult tissues was prepared using QIAamp DNA Mini Kit (QIAGEN, #51306). Blastocyst DNA was genotyped by genomic PCR, using primers on both sides of the truncation cassette (Fig EV2). DNA from blastocysts and tissues was also bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research Corp., #D5001). PCR, cloning, and sequencing were performed as described for oocyte DNA.

RNA preparation

RNA was prepared from approximately 100 growing and MII oocytes using the RNeasy Micro Kit (QIAGEN, #74004). For RNA preparation from blastocysts and adult tissues, samples were lysed with ISOGEN II (NIPPON GENE, #311-07361), and the cleared lysates were recovered by centrifugation according to the manufacturer's instructions. Three blastocysts were pooled for RNA preparation, and the lysate was loaded onto a spin column from the RNeasy Mini Kit (QIAGEN, #74104). DNase I treatment, column wash, and RNA elution were performed according to the manufacturer's instructions.

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Expression analysis

To analyze *Commd1* expression in oocyte and adult tissues, cDNA was synthesized with random primers (Takara, #3802) and reverse transcriptase (TOYOBO, TRT-101), and the *Commd1* cDNA was amplified by PCR with the primers Comm-F1 (exon 1) and Comm-R1 (exon 2). To analyze allelic expression, restriction fragment length polymorphism (RFLP) analysis was performed via NlaIII digestion of the amplified cDNA. Allelic expression of *Zrsr1* was analyzed by BigDye terminator sequencing of the cDNA amplified with the primers Zrsr-F1 and Zrsr-R1. No rs numbers were assigned to the single nucleotide polymorphisms (SNPs) that were used to discriminate between the C57BL/6J and PWK alleles. Quantitative allelic expression analysis of *Commd1* in blastocyst and adult tissues was performed in triplicate by pyrosequencing with PyroMark Q24 (QIAGEN) in the AQ assay mode. The rs26846230 SNP was used to discriminate between the C57BL/6J and BALBc alleles. Total expression levels were quantitated in triplicate using the StepOnePlus real-time PCR (RT-PCR) system with TaqMan Gene Expression Assay (Applied Biosystems) Mm00495837_s1 and Mm01239669_m1 for *Zrsr1* and *Commd1*, respectively. Actb (β-actin) mRNA was quantitated as an internal control using TaqMan Assay Mm00607939_s1.

Primers

All primers used in this study are listed in Table EV1.

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Acknowledgements

We thank Dr Tatsuya Kishino from the Gene Research Center, Center for Frontier Life Sciences, Nagasaki University, for providing the PWK mice used in this study.

Author contributions

KJ designed and carried out most of the experiments. KN and HK contributed the generation of PA-mice. FM, SK, and TK contributed materials. HY and KH contributed analysis tools. HS obtained funding, designed experiments, and oversaw the research. KJ and HS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Structure of the *Zrsr1*/*Commd1* locus and analysis of *Commd1* expression and *Zrsr1*-DMR methylation in the oocyte.

A *Zrsr1*, an approximately 2.8-kb intronless gene, and the first two exons of *Commd1* are represented by gray and white boxes, respectively. The schematic is not drawn to scale. Arrows above (maternal allele) and below (paternal allele) exon 1 and the *Zrsr1* gene represent the direction of transcription and the allelic expression status of the genes. The open and closed circles at the *Zrsr1* promoter indicate unmethylation and methylation, respectively. A schematic of the targeting vector is shown under the gene. The closed and hatched boxes represent the truncation cassette and the neo-selection marker gene, respectively. These elements are flanked by the 5.2-kb left arm containing exon 1, and the 5.1-kb right arm, which contains part of intron 1. The truncation cassette is flanked by loxP sites, represented by gray arrowheads enclosed in open rectangles. Expected transcription patterns of the WT and PA alleles are shown above the gene schematic.

B RT-PCR analysis of *Commd1* expression in growing oocytes prepared from B6 female neonates at Day 5 (D5), Day 10 (D10), and Day 15 (D15) postpartum, and fully grown MII oocytes (MII) from B6 adult females. PC: positive control for RT-PCR using adult brain cDNA. MW: molecular weight marker.

C Analysis of methylation at *Zrsr1*-DMR in growing and fully-grown oocytes used in (B). The 223-bp region in the DMR containing 14 CpGs was analyzed via bisulfite sequencing. Each

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row represents a dataset from one clone, and each circle represents one CpG site. Closed and open circles depict methylated and unmethylated CpGs, respectively.

Figure 2. Analysis of allelic *Commd1* expression and *Zrsr1*-DMR methylation in PA and WT MII oocytes.

A MII oocytes were prepared from *Commd1*$^{PA(B6)/+(PWK)}$ females (PA) and *Commd1*$^{+(B6)/+(PWK)}$ females (WT). RT-PCR was performed as in Figure 1B. Amplified cDNA (250 bp) was digested with NlaIII (CATG). There are two NlaIII restriction sites in the B6 amplicon, but one of these is absent in the PWK amplicon because of a single nucleotide polymorphism (SNP) between the two strains. Adult brain RNA from each of the strains was used as the control. MW: molecular weight marker.

B A 278-bp region in *Zrsr1*-DMR containing 15 CpG sites (B6) or 14 CpG sites (PWK) was analyzed via bisulfite sequencing. The alleles were discriminated between via an SNP (C in B6 and A in PWK) indicated as the leftmost CpG site in the B6 sequence.

Figure 3. Analysis of *Commd1* and *Zrsr1* expression and *Zrsr1*-DMR methylation in adult mice.

A Allelic expression of *Commd1* was analyzed as in Figure 2A in the brain (Br) and liver (Lv) of *Commd1*$^{PA(B6)/+(PWK)}$ (PA) and *Commd1*$^{+(B6)/+(PWK)}$ (WT) adult F1 mice. MW: molecular weight marker.

B Methylation at *Zrsr1*-DMR was analyzed as in Figure 2B in the brain of adult F1 mice.

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C Allelic expression of *Zrsr1* was analyzed in the F1 adult brain by direct RT-PCR sequencing of the cDNA, which contains two SNP sites between B6 and PWK.

D Total *Zrsr1* expression was analyzed in triplicate in the adult F1 brain and liver via TaqMan RT-PCR. Total expression is presented relative to the WT expression level in each tissue. The differences in levels of expression between WT and ΔPA F1 mice were statistically significant in the brain and liver (two-sided Student's *t* test, * $P < 0.01$).

Figure 4. Quantitative analysis of *Commd1* expression and *Zrsr1*-DMR methylation in ΔPA blastocysts and the ΔPA adult brain.

A Allelic expression of *Commd1* was analyzed in $Commd1^{+(B6)/+(BALB)}$ (WT), $Commd1^{PA(B6)/+(BALB)}$ (PA), and $Commd1^{\Delta PA(B6)/+(BALB)}$ (ΔPA) blastocysts in triplicate by pyrosequencing. Alleles were discriminated between using an SNP between B6 (Mat) and BALB/c (Pat) located in exon 2 (rs26846230; C in B6, T in BALB/c). The allelic expression ratios are presented relative to paternal expression in each blastocyst sample.

B Methylation at *Zrsr1*-DMR was analyzed in the blastocysts used in (A). The 343-bp region in the DMR containing 25 CpG sites (B6; maternal allele) or 24 CpG sites (BALB/c; paternal allele) was analyzed via bisulfite sequencing. The alleles were discriminated between using an SNP (rs26846192; C in B6, A in BALB/c) located in a CpG site in the B6 sequence.

C Methylation at *Zrsr1*-DMR was analyzed in the brains of ΔPA and WT adult F1 mice as described in (B).

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D Allelic expression of *Commd1* in the adult F1 brain was quantitatively analyzed in triplicate by pyrosequencing and is presented as in (A), showing the average values from four WT brains and five ΔPA brains.

E Total *Commd1* expression in an adult F1 brain of each genotype was analyzed in triplicate via TaqMan RT-PCR. Total expression is presented relative to the WT expression level. Asterisks (*) indicate statistical significance ($P < 0.01$) according to the two-sided Student's *t*-test.

Figure 5. The antisense transcript in the promoter region of *Commd1* in adult tissues.

A Strand-specific RT-PCR was performed to detect an antisense transcript to *Commd1* transcription in the promoter region. The arrows represent the primers CommUP-F1, for cDNA synthesis, and CommUP-F2 and CommUP-R2, for subsequent RT-PCR. The amplicon is 206 bp in size. CommUP-R2 is located approximately 100 bp upstream from the putative transcription start site of *Commd1*.

B Brain (Br) and liver (Lv) RNA were analyzed. Tissues were prepared from adult F1 mice generated from crossing PA-B6 females with WT-PWK males. cDNA synthesis was performed with (RTase +) or without (RTase −) reverse transcriptase. MW: molecular weight marker.

C Allelic expression of the antisense transcripts was analyzed via direct sequencing of the amplified brain cDNA from the WT and PA mice shown in (B).

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Expanded View Figure legends

Figure EV1. RT-PCR analysis of *Zrsr1* expression in growing oocytes

RT-PCR was done with primers Zrsr-F1 and Zrsr-R1 using cDNAs in Fig 1B. Growing oocytes were prepared from B6 female neonates at Day 5 (D5), Day 10 (D10), and Day 15 (D15) postpartum, and fully grown MII oocytes (MII) from B6 adult females. PC: positive control for RT-PCR using adult brain cDNA. MW: molecular weight marker. Two different cDNA batches were used for D5 RNA.

Figure EV2. Genotyping PCR of blastocysts carrying *Commd1*^{PA} and CAG-Cre transgene

A Schematic representation of PCR for three *Commd1* alleles, *Commd1*^{PA} (PA), *Commd1*^{DPA} (DPA) and *Commd1*$^{+}$ (WT).

B Electrophoresis of PCR products of nine blastocysts positive for *Commd1*^{PA} and CAG-Cre transgene among 24 blastocysts obtained from an IVF performed with oocytes from PA female mice and sperm from CAG-Cre male mice. Two blastocysts (#5, #9) contained small amount of undeleted truncation cassette. MW: molecular weight marker.

Figure EV3. Quantitative analysis of the allelic expression of *Commd1* in adult mice

The allelic expression was quantitatively analyzed by pyrosequencing. The levels of expression from the parental alleles are shown relative to the level of the paternal allele (1.0). Each sample was analyzed in triplicate.

A Brains and livers from two WT adult F1 mice between B6 females and PWK males.

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B Brains and livers from three WT adult F1 mice between B6 females and BALBc males.

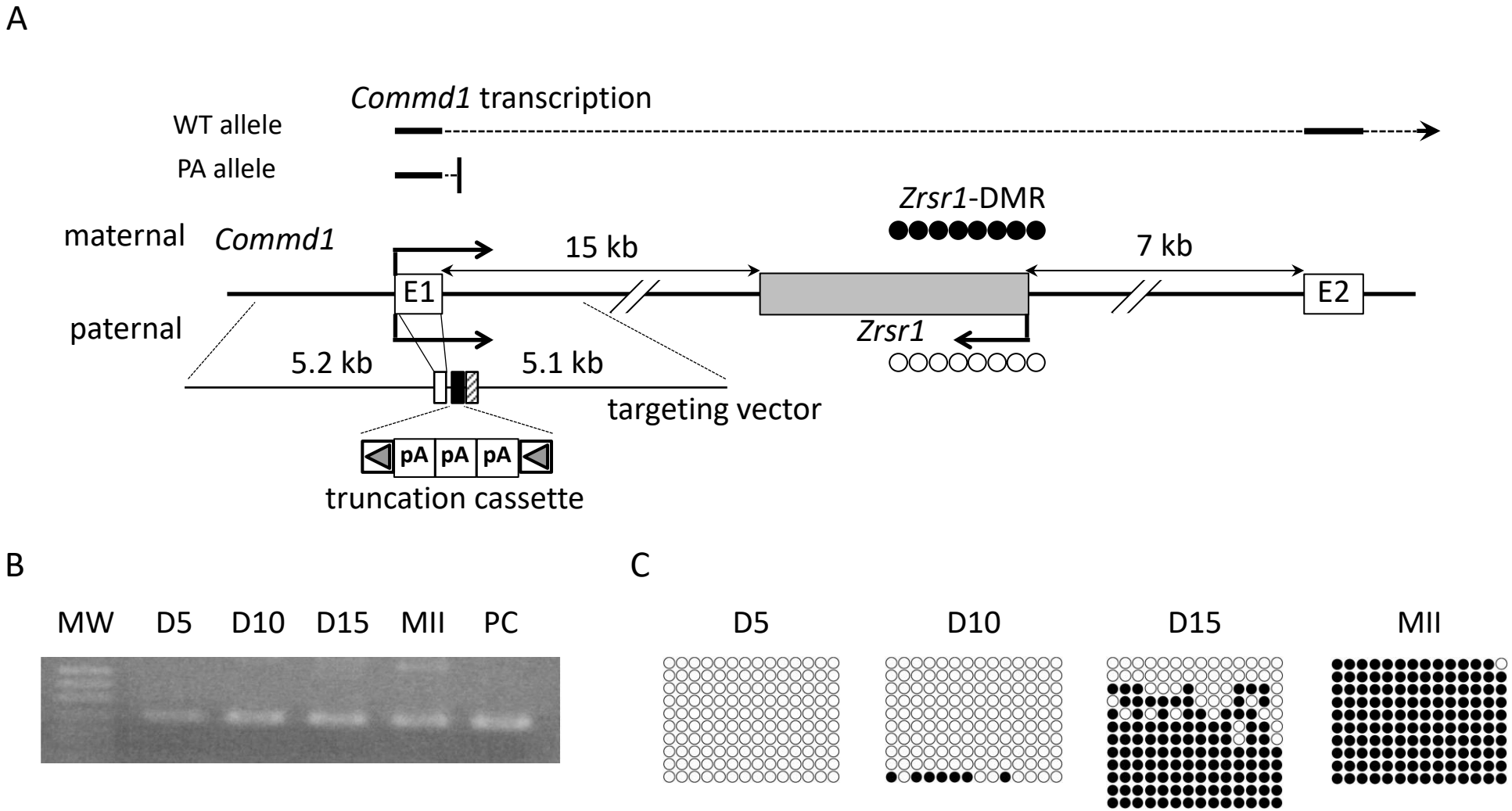


Figure 1. The structure of the *Zrsr1/Commd1* locus and the analysis of the *Commd1* expression and the *Zrsr1*-DMR methylation in oocyte.

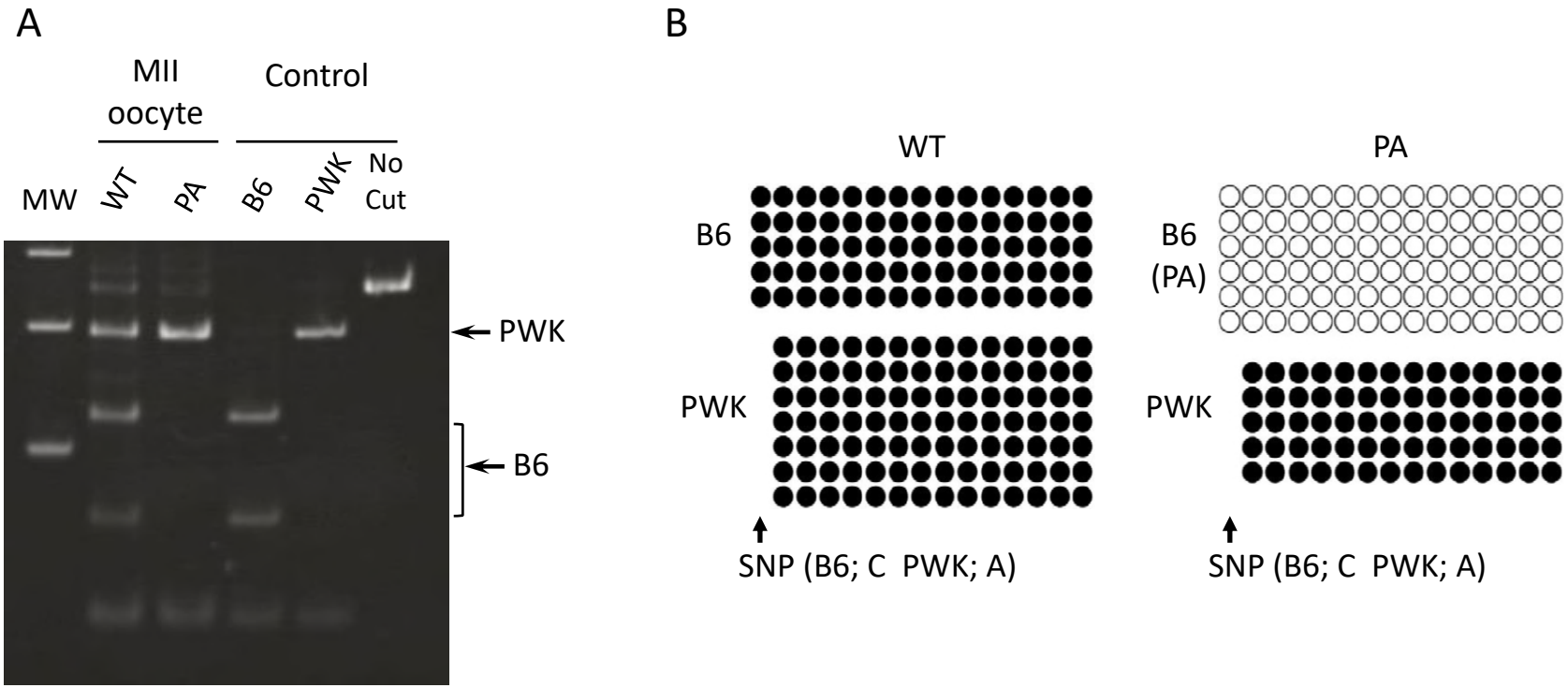


Figure 2. Analyses of the allelic expression of *Commd1* and the methylation status at the *Zrsr1*-DMR in PA and WT MII oocytes.

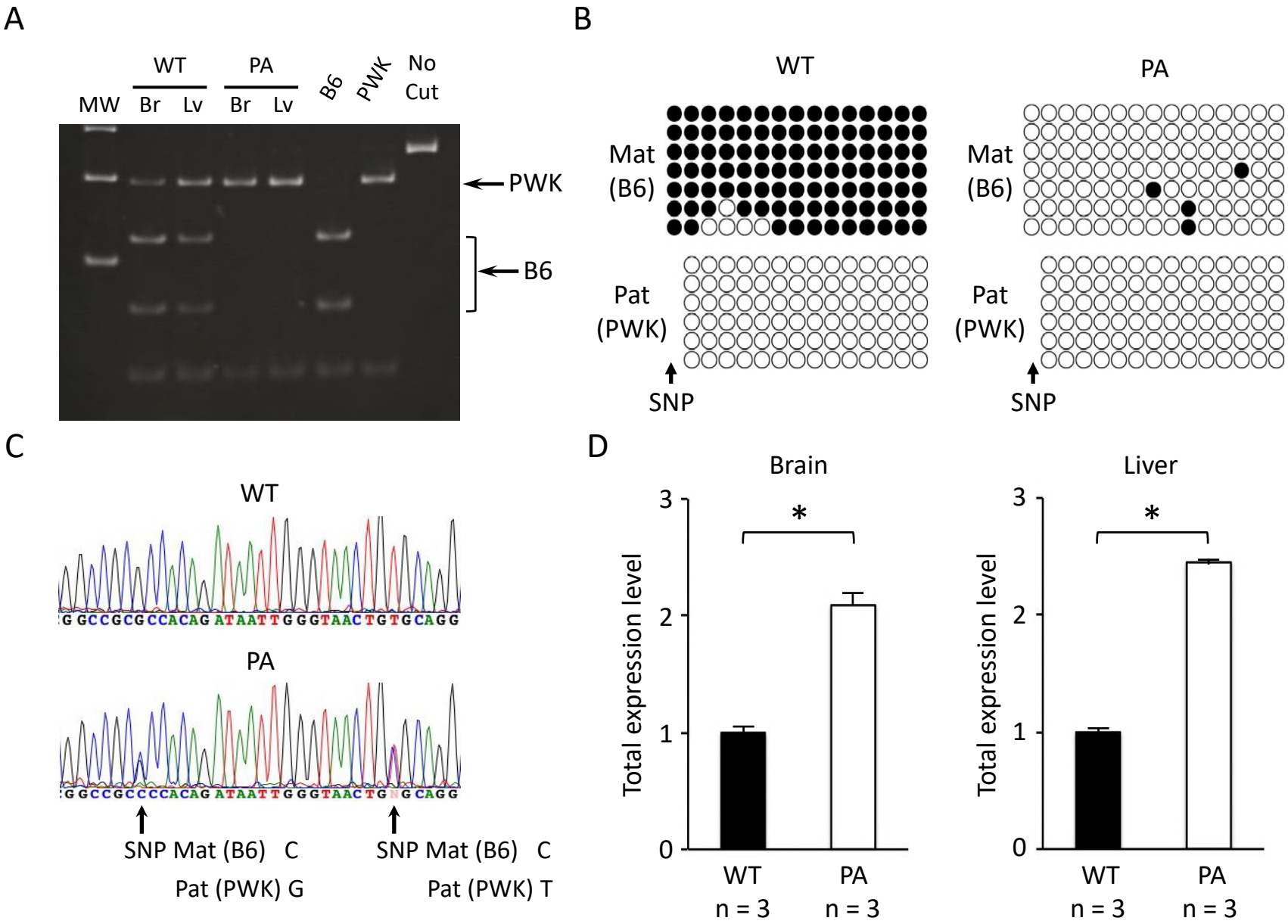


Figure 3. Analysis of the expression of *Commd1* and *Zrsr1* and of the methylation status of the *Zrsr1*-DMR in adult mice.

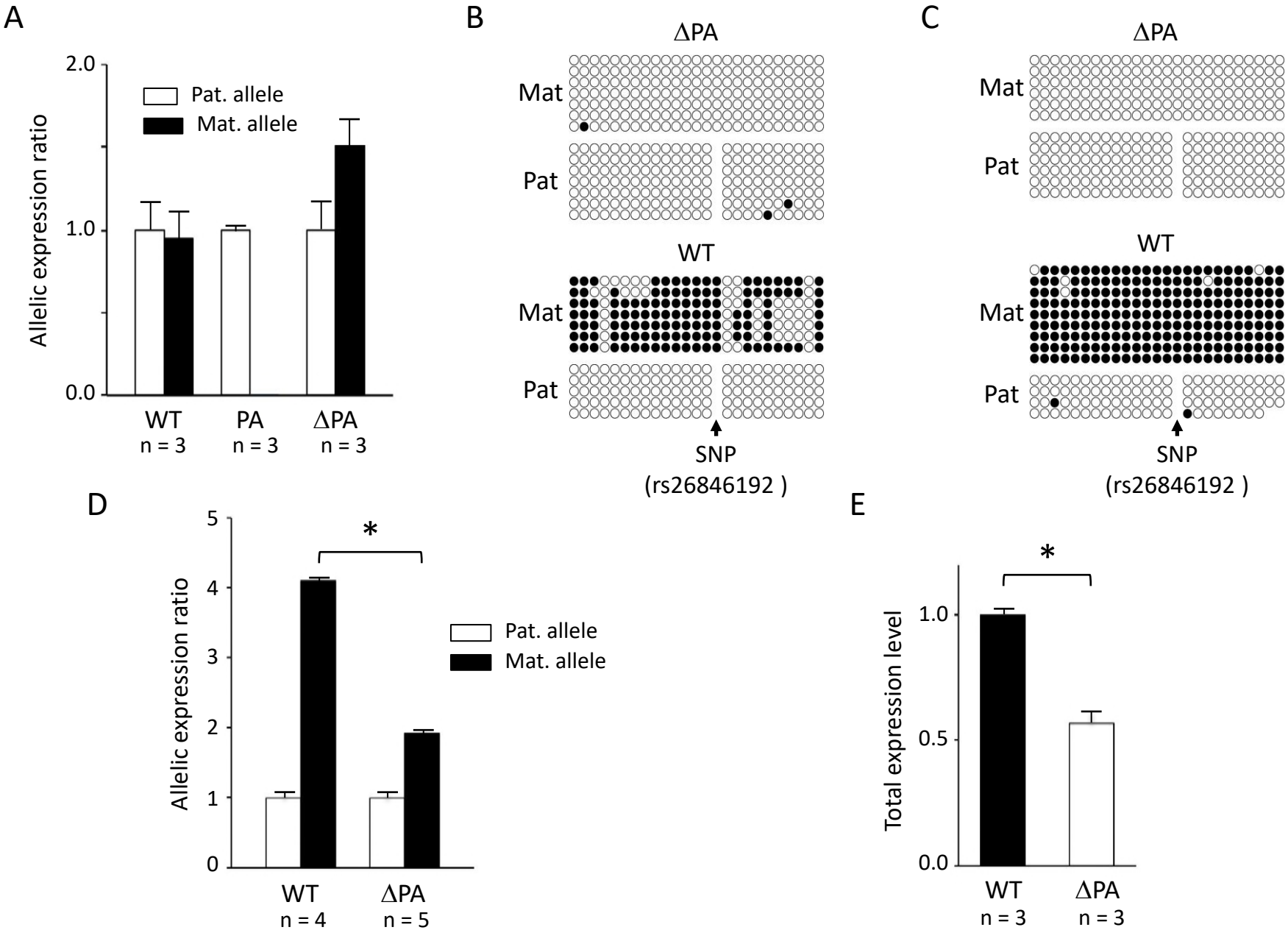
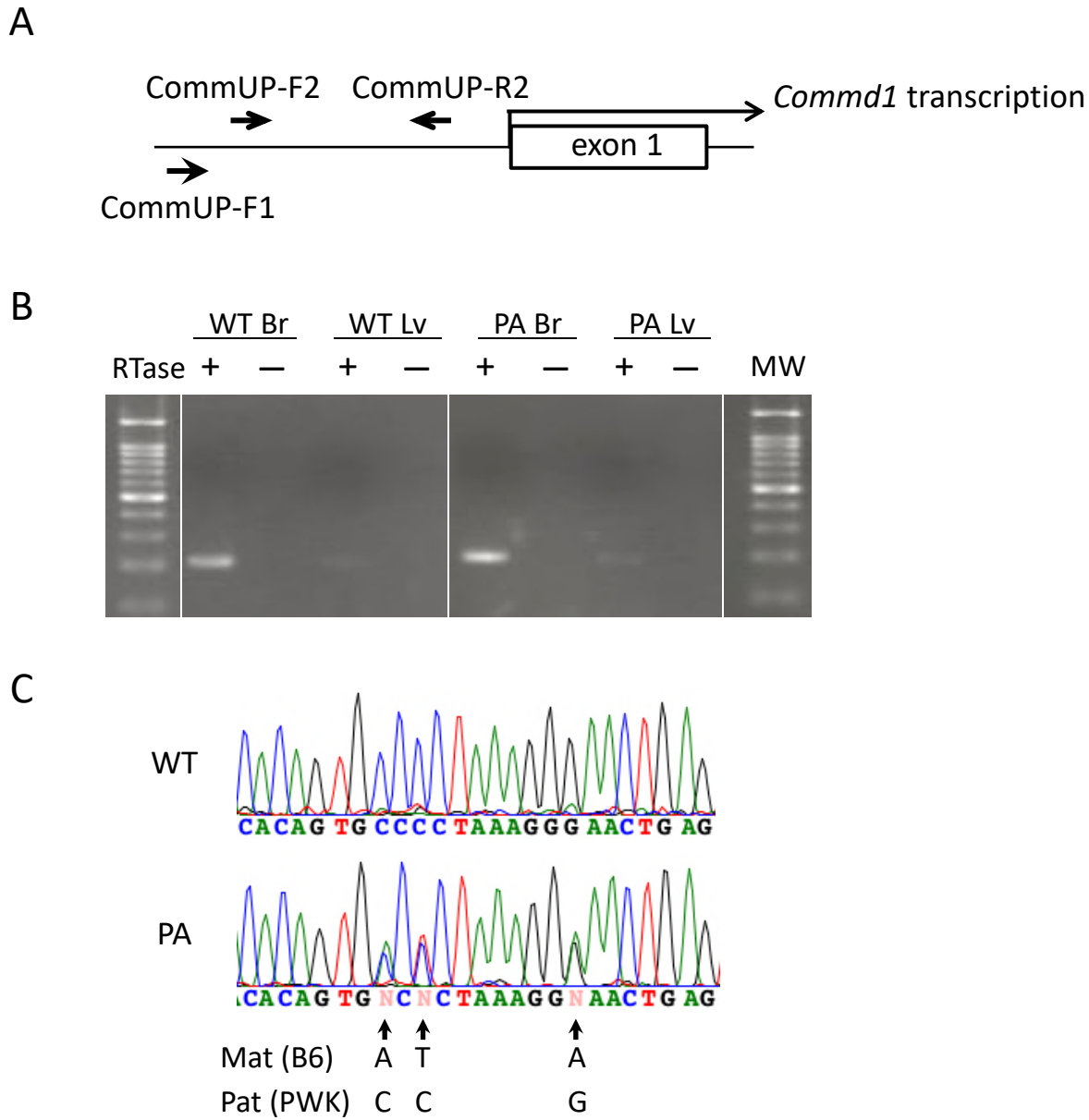


Figure 4. Quantitative expression analysis of *Commd1* and the methylation at the *Zrsr1*-DMR in ΔPA-blastocyst and ΔPA-adult brain.



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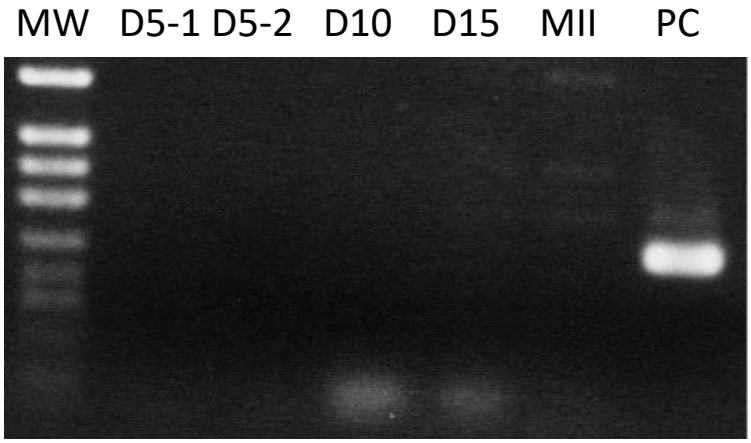

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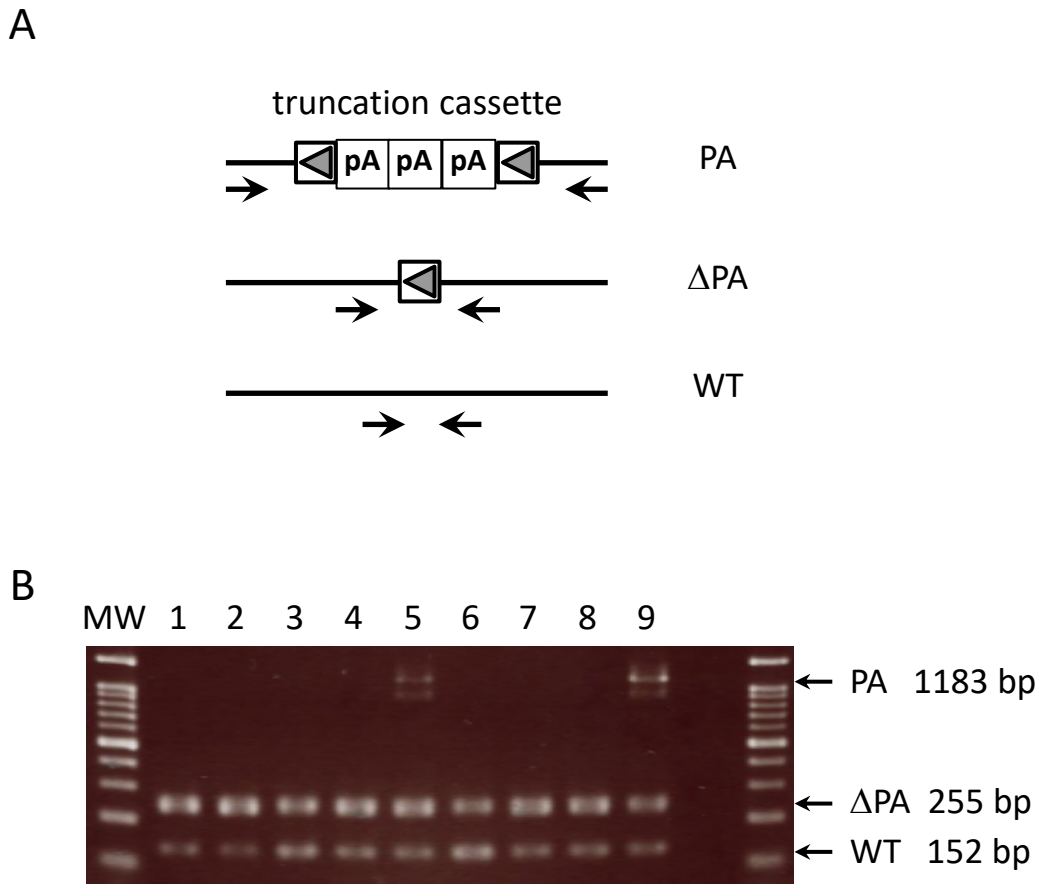

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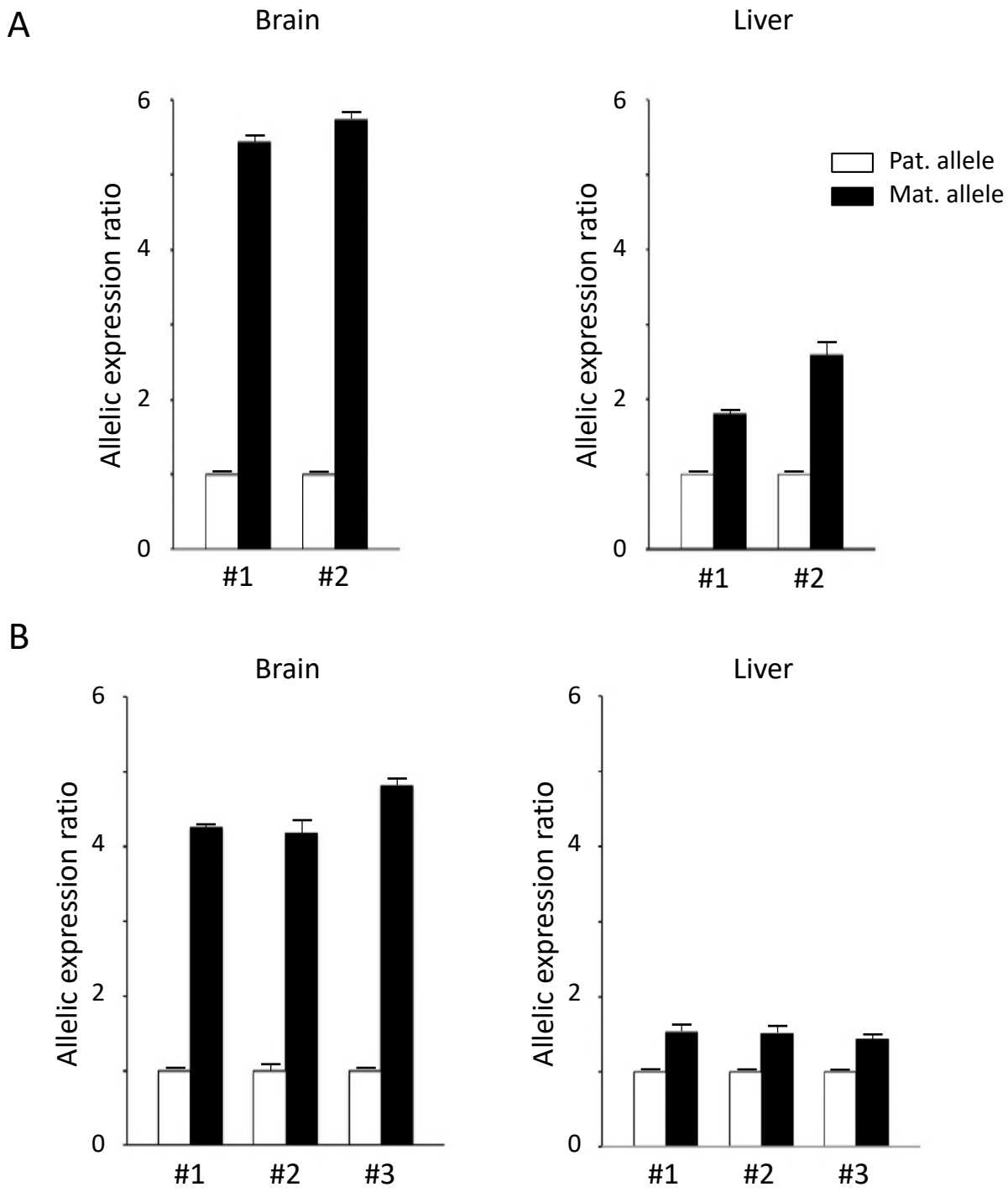

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B Brains and livers from three WT adult F1 mice between B6 females and BALBc males.