A Model for Epigenetic Inhibition via Transvection in the Mouse

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ABSTRACT Transvection is broadly defined as the ability of one locus to affect its homologous locus *in trans*. Although it was first discovered in the 1950s, there are only two known cases in mammals. Here, we report another instance of mammalian transvection induced by the *Cre/LoxP* system, which is widely used for conditional gene targeting in the mouse. We attempted to use the germline-expressed *Vasa-Cre* transgene to engineer a mouse mutation, but observe a dramatic reduction of *LoxP* recombination in mice that inherit an already deleted *LoxP* allele *in trans*. A similar phenomenon has previously been observed with another *Cre* that is expressed during meiosis: *Sycp-1-Cre*. This second example of *LoxP* inhibition *in trans* reinforces the conclusion that certain meiotically expressed *Cre* alleles can initiate transvection in mammals. However, unlike the previous example, we find that the inhibition of *LoxP* recombination is not due to DNA methylation. In addition, we demonstrate that *LoxP* inhibition is easily alleviated by adding an extra generation to our crossing scheme. This finding confirms that the *LoxP* sites are inhibited via an epigenetic mechanism, and provides a method for the use of other *Cre* transgenes associated with a similar *LoxP* inhibition event. Furthermore, the abrogation of *LoxP* inhibition by the simple addition of an extra generation in our crosses establishes a unique mouse system for future studies to uncover the mechanism of transvection in mammals.

KEYWORDS LoxP Cre; epigenetics; germline; mouse; transvection

TRANSVECTION is broadly defined as the ability of one locus to affect a homologous locus *in trans*. This phenomenon was first discovered in *Drosophila* at the *Bithorax* complex (Lewis 1954). Subsequent examples have been found in both plants and fungi (Coe 1966; Aramayo and Metzenberg 1996; Woodhouse *et al.* 2006). However, although the few known examples of transvection occur in a wide range of taxa, to our knowledge, only two cases have ever been observed in mammals (Rassoulzadegan *et al.* 2002; Sandhu *et al.* 2009).

One example of mammalian transvection was identified during an attempt to use the *Cre/LoxP* system to engineer a gene deletion in the mouse germline (Rassoulzadegan *et al.* 2002). *LoxP* sequences, originally identified in the P1 bacteriophage, recombine with nearly perfect efficiency in the presence of the CRE recombinase protein. As a result, the *Cre/LoxP* system has been successfully utilized for conditional gene targeting in virtually all mouse tissues (Sauer 1998). However, using a transgene with *Cre* driven by the *Sycp-1* male meiosis-specific promoter, the Cuzin group identified a notable exception. During the initial exposure to CRE in the germline of male mice, they found that *LoxP* recombination occurs with very high efficiency (Rassoulzadegan *et al.* 2002). However, the recombination efficiency declined sharply during the second passage through the germline (Rassoulzadegan *et al.* 2002). These results suggest that the initial meiotic recombination event can lead to the inhibition of the floxed (flanked by *LoxP* recombination sites) allele on the other homologous chromosome—a classic example of transvection. In addition, they observed that the methylation status of the *LoxP* sequences correlated with a failure to recombine, and methylating the *LoxP* sequences in a plasmid prior to transfection into mammalian cells inhibited recombination (Rassoulzadegan *et al.* 2002).

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Based on this evidence, they concluded that the *LoxP* sites are inhibited by DNA methylation, which they hypothesized blocks the CRE recombinase protein from recognizing its target sequence (Rassoulzadegan *et al.* 2002).

Recently, another example of transvection was identified in mammals. Imprinted loci are controlled by *cis*-acting sequences known as imprinting control regions (ICRs). Sandhu *et al.* (2009) found that ICRs from several imprinted loci physically interact. This allows the CTCF binding sites at the *H19* ICR to influence the replication timing of other ICRs *in trans* (Sandhu *et al.* 2009). However, despite this second example, the mechanism of transvection remains unknown, and the small number of mammalian examples precludes further investigation into this phenomenon.

Although the mechanism of mammalian transvection remains unknown, there are two prevailing models. One possibility is that transvection occurs when homologous chromosomes are paired during meiosis. The example of CRE-driven transvection in mice is consistent with this first model, since *Cre* is expressed during male meiosis, when chromosomes are maintained in close proximity (Rassoulzadegan *et al.* 2002). This model is also supported by the second example of mammalian transvection, where ICRs physically interact, though this interaction occurs in germ cells that are not undergoing meiosis (Sandhu *et al.* 2009).

Alternatively, it is possible that transvection does not require any physical interaction between homologous chromosomes. In this case, transvection could be mediated by a molecule that diffuses between the two chromosomes (Arteaga-Vazquez and Chandler 2010). This model is supported by evidence from maize. At the b1 locus, the weakly expressed B' allele can epigenetically repress the B–I allele, causing it to become as weakly expressed as B'. This effect, known as paramutation, can be stably propagated throughout generations even in the absence of the initiating B' allele (Chandler 2007). The mechanism of paramutation at the b1-locus is thought to be mediated by a small interfering RNA (siRNA) that diffuses between the two homologous chromosomes.

In this study, we attempted to use a germline expressed *Cre*, *Vasa-Cre*, to conditionally delete the histone demethylase *Kdm1a/Lsd1* (hereafter referred to as *Kdm1a*) (Gallardo *et al.* 2007; Wang *et al.* 2007). Similar to what was previously reported with *Sycp1-Cre*, we find that recombination of the *Kdm1a* floxed allele becomes inhibited *in trans*. This second example of *LoxP* inhibition reinforces the conclusion that certain *Cre* alleles expressed during meiosis can initiate transvection in mammals. However, unlike the prior report, our data show that DNA methylation does not inhibit *LoxP* recombination. Furthermore, we demonstrate that the addition of an extra generation between the initial recombination event and the inhibition event alleviates the inhibition of *LoxP* recombination. This result provides three critical insights. First, it suggests a useful strategy to overcome instances where other *Cre* transgenes lead to similar *LoxP* inhibition events. Second, our observation that two genotypically identical mice with differing parental history can exhibit dramatically different outcomes provides strong evidence that transvection in these mice is an epigenetic phenomenon. Finally, the juxtaposition of our original crosses, where we observe *LoxP* inhibition *in trans*, with our extra cross, where inhibition is eliminated, establishes an ideal system for future studies to elucidate the mechanism of transvection in mammals.

Materials and Methods

Animal husbandry and ethics statement

The following mouse strains were used: *Kdm1a/Lsd1* floxed allele (Wang *et al.* 2007), *Vasa-Cre* (Gallardo *et al.* 2007) and *Arl13b* floxed allele (Su *et al.* 2012). The strains were obtained directly from the Rosenfeld, Castrillon, and Caspary laboratories. All mice were of a mixed C57BL/6 strain background. Genotyping primers are listed in Supplemental Material, Table S1. All mouse work was performed under protocols approved by the Emory University Institutional Animal Care and Use Committee.

Crossing scheme

During the original cross, floxed P0 *Kdm1a* males were mated to *Vasa-Cre* females so that maternally provided *Cre* results in deletion in the F1 early embryo. This enabled us to genotype the tails of the resulting progeny. A total of 40 different litters resulting from 17 different crosses yielded 134/134 F1 male progeny with a properly deleted allele. Predominantly male animals were genotyped because only males can be used in the subsequent crosses (to avoid *Kdm1a* embryonic lethality in the resulting progeny). However, to be sure there was no sex-specific bias, we also genotyped nine females from three litters generated from three different crosses. All nine females had a properly deleted allele (data not included).

Of the 43 subsequent F2 animals assessed for proper recombination in the germline (Figure S1), 21 were females. The 21 assessed females were all mated to WT, and the tails of the resulting F3 progeny were genotyped for deletion. If recombination occurred properly, all F3 progeny from these crosses should be deleted over WT. Cases where multiple F3 animals were genotyped as floxed were scored as *LoxP* does not delete (14 animals); this included 29 genotyped litters. Cases where all resulting F3 animals had the proper deleted over WT genotype were scored as *LoxP* deletes (six animals); this included 20 litters. Of these assessed F2 animals, 22 were males. For the 22 F2 males, proper deletion of *Kdm1a* results in a complete loss of germ cells (eight animals) (Lambrot *et al.* 2015; Myrick *et al.* 2017)—a phenotype that is easily scored by dramatic reduction in the size of the adult testes. The lack of germ cells makes genotyping impossible. However, five of these F2 males were mated prior to dissection. All mated males that were sterile were subsequently verified as having smaller testes. In contrast, all fertile males improperly passed on the floxed allele (genotyped in F3 tails) and were verified as having normal sized testes (15 animals); this

included 30 litters. The extra cross animals were scored in an identical fashion, with 20 mated F3 females properly passing on the deleted allele 100% of the time. This included 67 genotyped litters. In addition, 15 F3 males all yielded the sterile phenotype with smaller testes. Finally, for the heterologous cross, recombination in the F2 germline was assessed by mating to WT and genotyping tails of the subsequent F3 progeny.

Testis immunostaining

Dissected testes were fixed for 210 min at 4° in 4% paraformaldehyde, washed in 1× PBS for 2 hr, then transferred to a 30% sucrose solution overnight at 4°. The tissue was then embedded in OCT compound (Tissue-Tek). Sections (10 μm) were incubated in a humidified chamber with anti-mouse CRE antibody (Sigma Aldrich C7988) diluted to 1:500 in 1× PBS, 1% heat-inactivated goat serum (Invitrogen 16210072), and 0.5% Triton X-100 overnight at 4°, and then in secondary goat anti-mouse antibody (Invitrogen A11001 used at 1:500) at room temperature for 2 hr. Slides were then washed in 1× PBS three times and mounted in ProLong antifade (Molecular Probes).

Bisulfite analysis

Bisulfite conversion was performed using 400 ng of tail DNA using the EZ Methylation Kit (Zymogen). Following bisulfite conversion, the samples were amplified using the primers listed in Table S1 and TA cloned (Invitrogen 450040) for sequencing. BiQ Analyzer was used to analyze bisulfite sequencing data (Bock *et al.* 2005). The methylation status of the *Kdm1a* P0 floxed allele prior to recombination was determined from the tail DNA of five P0 mice. The methylation status of the *Kdm1a* F1 deleted allele that was deleted throughout the mouse due to maternal CRE protein deposited into the very early F1 embryo, was determined using tail DNA from five original cross F1 mice. The methylation status of the original cross F2 floxed allele was determined from the tail DNA of 13 original cross F2 mice. This included nine mice where the *LoxP* sites were inhibited from recombination in the F2 germline, and four mice where the *LoxP* sites recombined normally in the F2 germline. The methylation status of the *Arl13b* P0 floxed allele was determined using tail DNA from three P0 mice.

Electrophoretic mobility shift assay (EMSA)

LoxP oligonucleotides, 34 bp in length, were ordered from Integrated DNA Technologies (either methylated or unmethylated at both CpG residues), labeled with T4 polynucleotide kinase (M0201S), and annealed. Probe sequences are listed in Table S1. All probes were purified in 5% acrylamide gels. Mobility shift reactions were carried out in 30 μl at room temperature for 30 min in CRE buffer (NEB) with 20,000 cpm radiolabeled DNA, 0.15 μg/μl poly (dI:dC) and 1 unit of CRE protein (NEB). Reaction mixtures were analyzed without loading dye on 1.5 mm thick 5% acrylamide gels in 1/4X TBE.

Next generation sequencing

Sperm from male mice was collected from the cauda epididymis from both original cross F1 males and extra cross F2 males. Four independent samples were collected (two for each cross). RNA was extracted from each sperm sample using Trizol, and the samples were treated as biological replicates. Between 250 and 1500 ng of RNA were used to prepare small RNA libraries using the TruSeq Small RNA Sample Prep kit according to the manufacturer's instructions (Illumina).

Small RNA libraries were clipped using FASTX Toolkit version 0.0.13, and reads longer than 18 nucleotides after clipping were mapped to the mouse genome (mm10) using Bowtie 1.1.2 (allowing up to one mismatch). Reads mapping to the genome were then mapped sequentially to a series of FASTA files containing rRNAs (Ensembl), miRNAs (hairpin from miRBase), tRNAs (GtRNA db), repeats (Repbase), piRNA clusters (Li *et al.* 2013), and Refseq (NCBI). To identify potential small RNAs targeting the LoxP site or its immediate surroundings, all reads >18 nt after clipping were mapped using Bowtie (one mismatch allowed) to the floxed *Kdm1a* allele. Coverage plots for the floxed *Kdm1a* and for tRNAs were then produced using BEDTools v2.24.0-33 (Quinlan and Hall 2010), and plotted using R (R Core Team 2013).

Data availability

All strains and reagents are available upon request. Gene expression data have been deposited into the Gene Expression Omnibus with accession number GSE97971. Figure S1 details the germ cell outcomes from the cross in Figure 1B. Figure S2 contains immunofluorescence images of *Vasa-Cre* expression in inhibited F2 testes. Figure S3 shows scatter plots comparing RNA-seq transcript levels in F1 extra cross and F1 original cross sperm. Figure S4 displays non-CpG methylation at the *LoxP* recognition site. Table S1 contains primer sequences used for EMSA and bisulfite analysis.

Results

LoxP recombination is inhibited during germline conditional deletion

In order to conditionally delete a floxed allele of *Kdm1a* (Figure 1A), we crossed floxed *Kdm1a* mice (Wang *et al.* 2007) to a transgenic *Cre* recombinase line in which *Cre* is expressed from the germline specific *Vasa/Ddx4* promoter (hereafter referred to as *Vasa-Cre*) (Figure 1B). The *Vasa-Cre* transgene is expressed exclusively in the germline of male and female mice beginning just prior to birth (Gallardo *et al.* 2007). Additionally, in mothers carrying the transgene, VASA-CRE protein is maintained in the mature oocyte, and can induce *LoxP* recombination in the early embryo. Therefore to avoid the maternal contribution, *Vasa-Cre* males were used during F1 crosses (Figure 1B).

Upon crossing floxed P0 *Kdm1a* males to *Vasa-Cre* females, we observed 100% recombination between *LoxP* sites (Figure 1, B and C). However, during subsequent F1 crosses to generate homozygous conditional mutants, we find that the efficiency of *LoxP* recombination is dramatically reduced, with a failure to recombine observed in 67% of progeny ($N = 43$) (Figure 1, B and C and Figure S1). Importantly, this reduction in *LoxP* recombination efficiency is specific to the *Vasa-Cre*

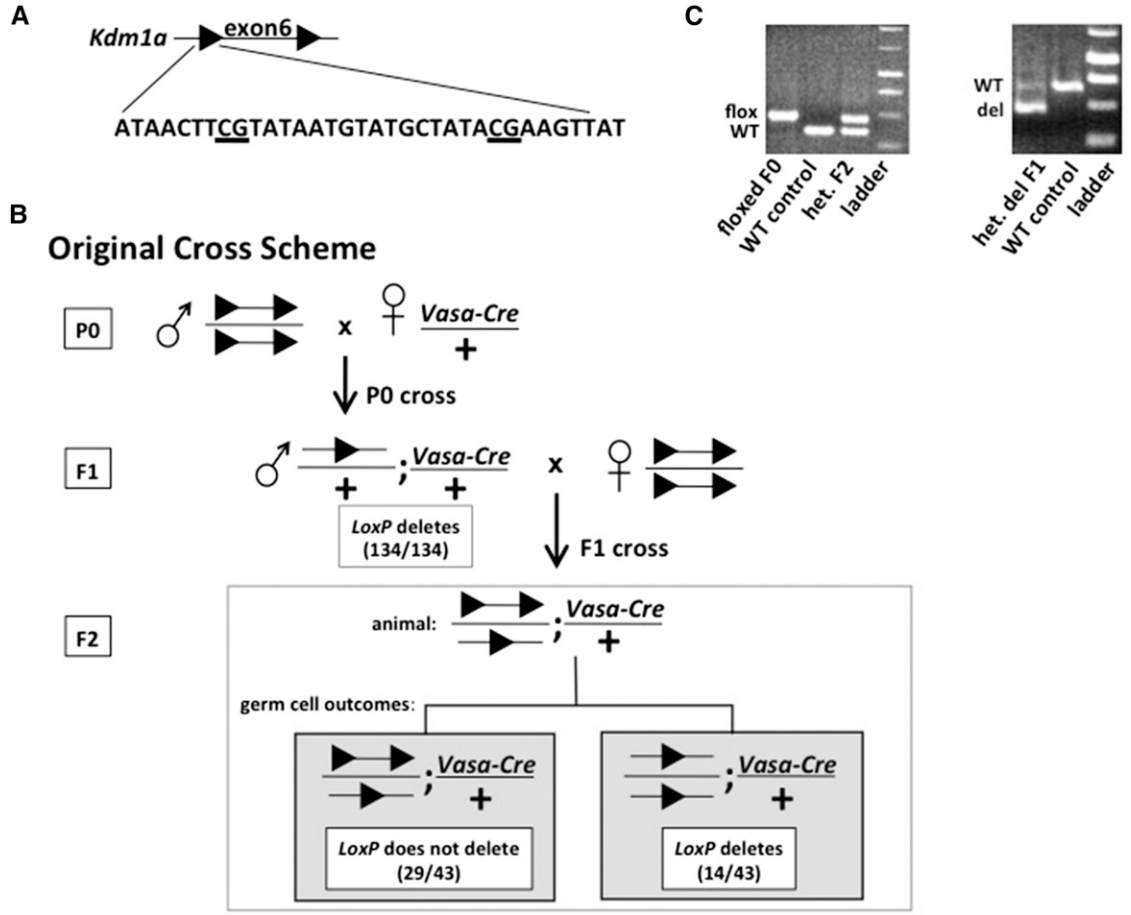

Figure 1 Inhibition of *LoxP* recombination during second passage through the germline. (A) Diagram of the floxed *Kdm1a* locus. The two CpG residues in the 34 bp *LoxP* recognition sequence are underlined. (B) Diagram of the original cross scheme used to generate germline *Kdm1a* conditional knockout mice. In F1 mice, the floxed *Kdm1a* locus deletes with 100% efficiency. However, in the germline of F2 mice, the *LoxP* sites are largely inhibited from recombining. This was determined by genotyping the resulting F3 progeny (see *Materials and Methods* and Figure S1 for details). (C) Sample genotyping showing the ability to distinguish all possible genotypes during the crosses.

transgene, as 100% recombination is achieved in both P0 and F1 crosses when *Kdm1a* is conditionally deleted with either of the oocyte-expressed *Cre* transgenes, *Zp3-Cre* and *Gdf9-Cre* (Ancelin *et al.* 2016; Wasson *et al.* 2016).

The Vasa-Cre transgene is still expressed during LoxP inhibition

It is possible that the dramatic reduction in the efficiency of *LoxP* recombination in F2 mice is caused by silencing of the *Vasa-Cre* transgene. To examine this possibility, we performed immunofluorescence on F2 testes in which the *LoxP* sites fail to recombine. In the F2 testes that largely fail to undergo recombination, we observe that CRE protein is expressed in meiotic SYCP3 positive leptotene, zygotene and pachytene spermatocytes, as well as spermatids (Figure 2 and Figure S2, A–C). In contrast, CRE protein is absent from spermatagonia, including all PLZF positive As (spermatogonial stem cells), Apr and Aal spermatogonia (Figure 2 and Figure S2, D–F). This expression pattern is consistent with what was originally reported for the *Vasa-Cre* transgene (Gallardo *et al.* 2007). This suggests that the inhibition of recombination is likely caused by inhibition of the *LoxP* sites themselves, rather than the lack of CRE expression from the *Vasa-Cre* transgene. This conclusion is identical to what has previously been suggested for *Sycp1-Cre* mediated transvection (Rassoulzadegan *et al.* 2002).

Adding an extra generation alleviates LoxP inhibition

Little is known about the mechanism of transvection in mammals. However, based on the example of paramutation at the b1 locus in maize, transvection might be facilitated by a molecule able to diffuse between the two homologous chromosomes. During our crosses to conditionally delete *Kdm1a* in the germline, a *LoxP* targeted diffusible molecule could be generated in the germline of F1 animals. This molecule could then be packaged into the F1 sperm, and direct the inhibition machinery in the early embryo to the *LoxP* sites on the maternally inherited floxed chromosome. If inhibition in our crosses occurred via this mechanism, then we could potentially alleviate it by crossing our F1 mice to wild type for a generation before backcrossing to the *Kdm1a* floxed mice. In this case, the sperm would no longer contain the diffusible molecule generated in the F1 germline. To test this hypothesis, we added an

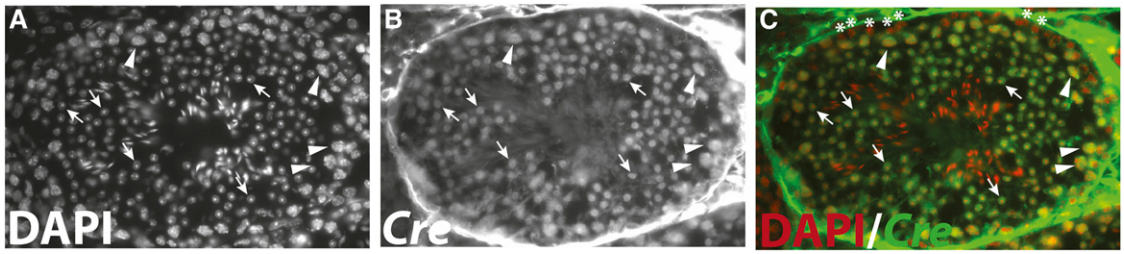

Figure 2 The expression of CRE in inhibited F2 testes. Representative DAPI (A), VASA-CRE (B), and merge (C) immunofluorescence ($N = 3$). VASA-CRE is still expressed in spermatocytes (arrowheads) and spermatids (arrows) of F2 testes that fail to recombine. VASA-CRE is not expressed in spermatagonia (asterisks).

additional cross to wild type after the F1 generation to generate F2 mice (hereafter referred to as extra cross F2 mice) (Figure 3A). These resulting extra cross F2 mice are genotypically identical to the F1 mice in our original cross (and to their F1 fathers), but differ in their parental history. Compared to the original cross F1 mice, which resulted in only 33% recombination, the addition of this extra cross restored *LoxP* recombination to 100% efficiency ($N = 35$) (Figure 3A). This result suggests that the *LoxP* sites in our original cross are being epigenetically inhibited. Importantly, based on our original cross, it was possible that paternal inheritance resulted in *LoxP* inhibition. However, in the extra cross, the *Cre* allele is paternally inherited identical to the original cross. This demonstrates that the paternal inheritance of the *Cre* transgene is competent for 100% *LoxP* recombination efficiency. In addition, it was possible that the *LoxP* inhibition in our original cross was influenced by strain background. However, the mixed C57BL/6 strain background in the extra cross is the same as the original cross, one generation removed. This suggests that the *LoxP* inhibition is not simply due to strain background.

The alleviation of inhibition with the extra cross is consistent with a model where a freely diffusible molecule can inhibit the recombination of *LoxP* sequences *in trans*. Based on this model, crossing to a different floxed gene might result in *LoxP* inhibition at this additional locus. To test this possibility, we crossed F1 mice, containing one deleted allele and one wild-type allele of *Kdm1a*, to mice that are homozygous for the floxed allele of *Arl13b* (Figure 3, B and C). In this cross (hereafter referred to as the heterologous cross), we find that in the resulting F2 progeny, the floxed allele of *Arl13b* recombines 100% of the time (Figure 3, B and C), suggesting that inhibition in our crosses is confined to the *Kdm1a* locus. This indicates that the *LoxP* recognition sequence is not sufficient to initiate inhibition, and that sequences flanking the *LoxP* may also be involved.

Sperm RNA does not target LoxP sites for inactivation

The successful recombination in the F1 extra cross mice supports a transvection model involving a freely diffusible molecule. Based on the example of paramutation at the b1 locus in maize, we considered the possibility that this molecule could be an RNA. For example, if small RNAs targeting the *LoxP* site were generated in the germline as a response to the first recombination event, they could be deposited in F1 sperm, and initiate inhibition of the *LoxP* sites on the homologous chromosome in the early original cross F2 embryo. The plausibility of such a model has been demonstrated at the *Kit* locus in mice, where sperm RNA mediates a *trans*-generational inhibition event (Rassoulzadegan *et al.* 2006).

If the observed inhibition were due to small RNAs targeting the floxed *Kdm1a* allele, we reasoned they should only be present, or at least be more abundant, in F1 sperm from the original cross. To examine this possibility, we produced small RNA libraries from multiple sperm samples derived either from original cross F1 mice or extra cross F2 mice. However, we did not detect any RNA reads specifically overlapping with the *LoxP* recognition site in any of our libraries. We also failed to detect any enrichment for original cross F1 small RNAs mapping to the regions flanking the *LoxP* sites (Figure 4A). This lack of small RNA enrichment at the *Kdm1* floxed locus was not due to a deficiency in our small RNA libraries, as we detect the normal high number of reads mapping to the 5′ half of *tRNA-Gly-GCC-5-1* (Figure 4B). Furthermore, we also did not detect any consistent differences in micro RNAs (miRNA), piwi-RNAs (piRNA), repeats, ribosomal RNAs (rRNA), or transfer RNAs (tRNAs) between replicates (Figure S3). Taken together, these results seem to exclude the possibility that *LoxP* targeted small RNA molecules deposited in sperm are responsible for inhibition of the *LoxP* site *in trans*.

LoxP sites are DNA methylated prior to LoxP inhibition

Previously, it was suggested that inhibition of *LoxP* recombination occurs via DNA methylation of two CpG dinucleotides within the 34 bp *LoxP* site (Figure 1A) (Rassoulzadegan *et al.* 2002). This methylation was hypothesized to block the binding of the CRE recombinase to the *LoxP* recognition site (Rassoulzadegan *et al.* 2002). To determine if DNA methylation might also account for the decreased recombination efficiency in our original cross F2 mice, we used bisulfite DNA methylation analysis to determine the methylation status of the *LoxP* sites in our crosses. Consistent with the observations of Rassoulzadegan and colleagues, we found DNA methylation at two CpG dinucleotides within the LoxP recognition sites that were inhibited from recombining (Figure 5C). We also detect methylation at CpG dinucleotides immediately

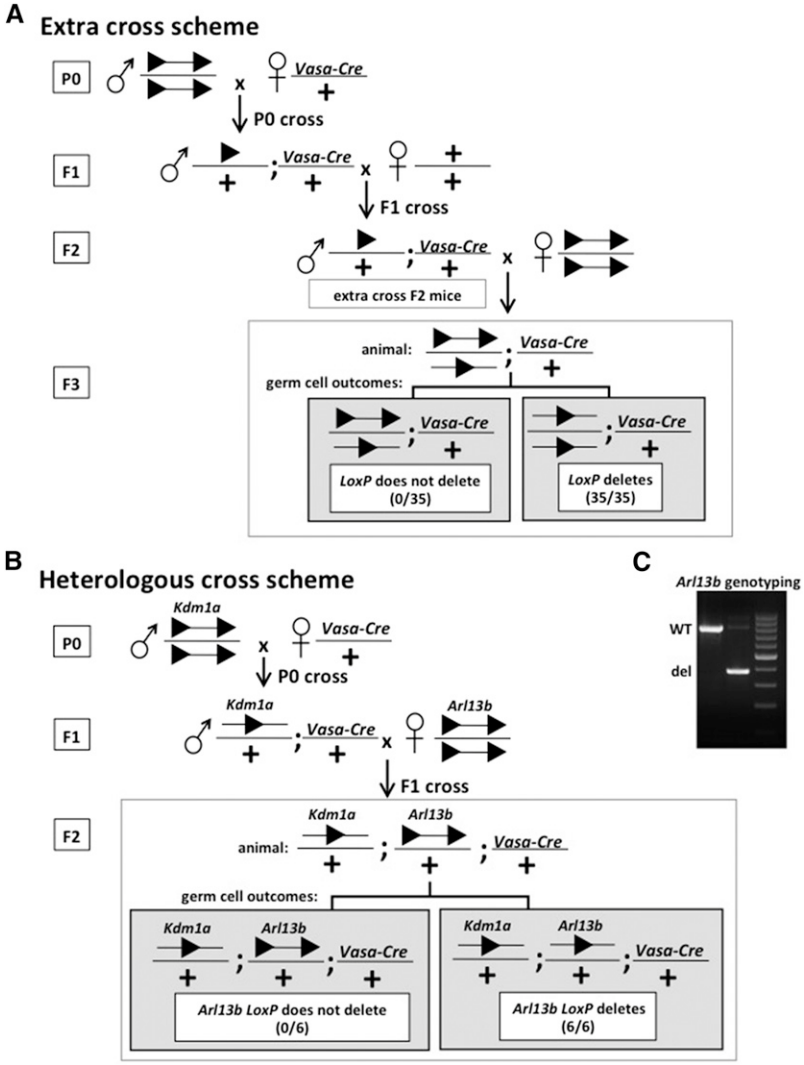

Figure 3 Diagram of the extra cross (A) and heterologous cross (B) mating scheme. As in the original cross (Figure 1), floxed *Kdm1a* mice are initially crossed to *Vasa-Cre* to generate F1 heterozygotes. However, unlike in the original cross, the F1 heterozygotes are subsequently crossed to WT to generate extra cross F2 mice. These mice are genotypically identical to the original cross F1 mice (and their fathers), but differ in their parental history. Upon backcrossing to the floxed mice, the floxed allele in the germline of the extra cross F3 progeny now recombines with 100% efficiency. This was determined by genotyping the F4 progeny (see *Materials and Methods* and Figure S1 for details). (B) Alternatively, F1 heterozygotes are crossed to floxed *Arl13b* mice (heterologous cross). In the heterologous cross F2 mice, the *Arl13b LoxP* sequences recombine with 100% efficiency (see *Materials and Methods* and Figure S1 for details on how recombination was determined). (C) Sample genotyping showing the ability to distinguish *Arl13b* genotypes.

adjacent (one CpG 11 bp 5′ and two CpGs 9 bp 3′ and 28 bp 3′) to the *LoxP* site (Figure 5C). However, we observed a similar methylation profile in original cross F1 alleles that recombined with perfect efficiency (Figure 5B). Furthermore, the P0 floxed *Kdm1a* allele used to initiate these crosses was also fully methylated at these CpG dinucleotides, in the complete absence of any *Cre* transgene (Figure 5A). These data strongly suggest that DNA methylation is not sufficient to inhibit *LoxP* recombination in these mice.

In addition to CpG DNA methylation, we also observe non-CpG methylation in the *LoxP* recognition site (Figure S4, A–C). This methylation occurs at one of the cytosine residues that was also previously reported to be methylated in *Sycp1-Cre* crosses (Rassoulzadegan *et al.* 2002). The observation of this non-CpG methylation in original cross F1 mice, where recombination occurs with full efficiency, indicates that this non-CpG methylation also does not inhibit *LoxP* recombination (Figure S4B).

We were surprised to find that *Kdm1a LoxP* sites were already methylated at CpG residues prior to the introduction of the *Cre* transgene. This suggests that *LoxP* sites may be stochastically targeted by DNA methylation. To determine if this is the case, we assayed the DNA methylation status of the *LoxP* recognition sequence in another floxed allele at the *Arl13b* locus (Su *et al.* 2012). These mice have been shown to undergo normal *LoxP* recombination with several different *Cre* transgenic lines (Su *et al.* 2012; Higginbotham *et al.* 2013). Similar to what we observed in *Kdm1a* mice, we find that the two CpG dinucleotides in the *Arl13b LoxP* recognition sequence are also largely methylated (Figure 5D). This result is consistent with our conclusion that DNA methylation is not sufficient to inhibit *LoxP* recombination.

CpG methylation does not inhibit CRE binding to LoxP sites

Our observations that *LoxP* sites can recombine normally even when they are fully methylated suggests that the CRE recombinase must be able to bind to methylated *LoxP* recognition sequences. To test the ability of CRE to bind methylated *LoxP* sites, we performed EMSAs with radiolabeled 34 bp *LoxP* probes that were either methylated or unmethylated at both CpG dinucleotides (Figure 1A). Recombinant

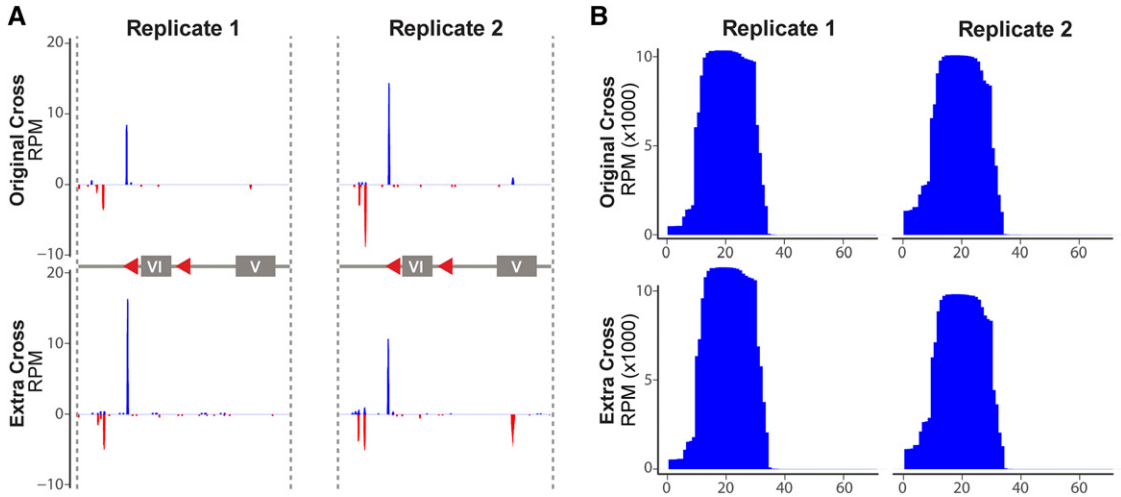

Figure 4 Sperm small RNAs do not target *LoxP* sites. (A) Coverage plots of *LoxP* sites (red arrows) flanking exon 6 in *Kdm1a*, showing very few reads mapping to the *Kdm1a* floxed locus and no difference between original cross F1 sperm (top) and extra cross F2 sperm (bottom) in two biological replicates. The *LoxP* sites are intended to show the relative position, and are not drawn to scale. Plus strand is shown in blue and minus strand is shown in red. (B) For comparison, coverage plots of the plus strand (blue) of *tRNA-Gly-GCC-5-1* are shown for the same libraries.

CRE protein produced a strong retarded mobility complex with the unmethylated *LoxP* recognition sequence, indicating that CRE binds with high affinity to the *LoxP* site (Figure 6, lanes 1 and 2). This binding was specific, as *LoxP* sequences, but not an unrelated promoter sequence, could compete for binding at 200-fold excess (Figure 6, lanes 3 and 4). Most importantly, CRE binding was also independent of methylation status, as the fully methylated *LoxP* probe produced an identical pattern of strongly retarded mobility (Figure 6, lanes 5–8). This result, *i.e.*, that CRE binds to the *LoxP* recognition sequence regardless of DNA methylation status, is consistent with our *in vivo* observations that DNA methylation does not inhibit *LoxP* recombination.

Discussion

To generate mice with *Kdm1a* deleted in the germline, we crossed floxed *Kdm1a* mice to *Vasa-Cre* transgenic mice (Gallardo *et al.* 2007; Wang *et al.* 2007). In the initial F1 cross, *LoxP* sites recombined with high efficiency. However, upon backcrossing to generate germline homozygous deleted mice, we found that the efficiency of *LoxP* recombination on the remaining floxed allele was dramatically reduced. This reduction was not caused by silencing of the *Vasa-Cre* transgene, as CRE remained robustly expressed in mice where the *LoxP* sites failed to recombine. This suggests that the *LoxP* sites themselves are becoming inhibited. In addition, the decrease in recombination efficiency is specific to *Vasa-Cre*, as normal recombination is observed with both *Zp3-Cre* and *Gdf9-Cre* (Ancelin *et al.* 2016; Wasson *et al.* 2016). Since the inhibition of *LoxP* recombination in the original cross F2 germline only occurs after initial exposure to VASA-CRE, we conclude that it is dependent upon germline exposure to CRE in the F1 animals. Thus, initial exposure to CRE must trigger an alteration, either in the chromatin itself or in the associated environment, which can be propagated through sperm to the next generation. In the subsequent F2 animals, this alteration can then inhibit the floxed *Kdm1a* allele on the homologous chromosome from efficient recombination. Therefore, we propose, as was previously proposed for *Sycp1-Cre*, that the inhibition of *LoxP* recombination occurs via a transvection event. This is the second example of *LoxP* inhibition from a *Cre* allele expressed during meiosis, which reinforces the idea that transvection in mammals may be triggered by meiotically expressed *Cre*.

Since transvection can be initiated by two different *Cre* transgenes that are expressed during meiosis, it is tempting to hypothesize that the phenomenon is triggered by *LoxP* recombination specifically during meiosis. However, in our crosses, recombination first occurs in the early F1 embryo, due to maternally inherited CRE from the P0 mother, well before meiotic onset. Therefore, we propose that inhibition in our crosses may be triggered by CRE protein binding to the already deleted *LoxP* site during meiosis, rather than by meiotic *LoxP* recombination. It is possible that binding of ectopic CRE recombinase to DNA during meiosis, and/or the recombinase trying repeatedly to initiate a double stranded break, could trigger a surveillance mechanism targeted to DNA damage or foreign elements.

There are two prevailing models for the mechanism of transvection. The first theorizes that transvection occurs during via a physical interaction between the homologous chromosomes when they are paired during meiosis. The second invokes a molecule able to freely diffuse between the homologous chromosomes. Thus far, the only two examples of *Cre*-initiated transvection occur in animals expressing *Cre* during meiosis. These observations seem to be consistent with a model where transvection requires a close physical interaction between chromosomes. However, the floxed *Kdm1a* allele in original cross F2 mice is blocked from recombining via

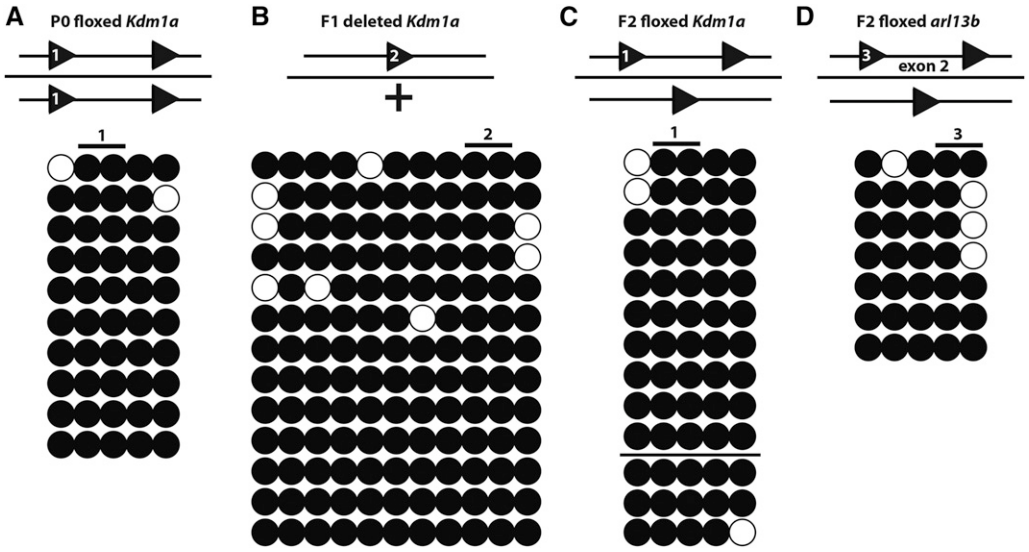

Figure 5 Bisulfite DNA methylation analysis of the *LoxP* recognition site. *In vivo* bisulfite analysis of the two CpG dinucleotides (Figure 1A) in the *LoxP* recognition sequence (diagrammed above), as well as CpG residues from the flanking region, in the *Kdm1a* P0 floxed allele (marked with 1) prior to recombination (A) (*N* = 5 mice), the *Kdm1a* F1 deleted allele (marked with 2) after recombination (B) (*N* = 5 mice), *Kdm1a* F2 floxed alleles (marked with 1) that do not recombine (nine lollipop rows above the horizontal black line), and *Kdm1a* F2 floxed alleles recombine normally (four lollipop rows below the horizontal black line) (C) (*N* = 13 total mice), and the *Arl13b* P0 floxed allele (marked with 3) (D) (*N* = 3 mice). Open circles indicate unmethylated CpG residues. Closed circles indicate methylated CpG residues.

an event that must occur prior to the onset of *Vasa-Cre* expression. Otherwise, the floxed *Kdm1a* allele would presumably recombine with full efficiency. In male mice, expression of *Vasa-Cre* begins at embryonic day 18 with full deletion obtained before birth (Gallardo *et al.* 2007). Surprisingly, this expression is much earlier than the onset of meiosis in males, which occurs after birth (Bowles and Koopman 2007). This suggests that the *Kdm1a* inhibition occurs in germ cells before birth, arguing against a simple model where it occurs via physical interaction during meiosis.

If *Cre*-mediated transvection does not require the physical association of the floxed and deleted alleles, it is possible that a freely diffusible molecule allows for communication between chromosomes *in trans*. If such a molecule is generated during the initial *LoxP* recombination in the F1 germline, it could be packaged into sperm and target the remaining floxed allele on the homologous chromosome for inhibition in the embryo following fertilization. To test this possibility, we performed an extra cross after the F1 generation. Remarkably, this extra cross completely restored recombination efficiency, raising the possibility that a diffusible molecule could be propagated through sperm in our crosses. Based on previous evidence from the *Kit* locus in mice, we wondered whether this diffusible molecule could be a specific RNA (Rassoulzadegan *et al.* 2006). However, we fail to detect any RNAs selectively present in the original cross sperm that could specifically target the *LoxP* sites. Thus, our data fail to support a model in which inhibition is initiated by an RNA corresponding to the *LoxP* recognition site.

Although we still do not know the mechanism of mammalian transvection, our data nevertheless provide significant insight. We propose that the binding of CRE recombinase protein to the *LoxP* recognition site during meiosis triggers an alteration that can be propagated through sperm to initiate transvection in the resulting embryo. It is possible that this alteration could be a type of histone modification (Hammoud *et al.* 2009; Brykczynska *et al.* 2010), or an unknown molecule. Regardless, the alteration must then be maintained throughout embryonic development into the germline, where it initiates the event that blocks the recombination of the *LoxP* sites on the homologous chromosome. Furthermore, this inhibition of the *LoxP* sites must occur in germ cells prior to the expression of *Vasa-Cre* at birth, well before the onset of meiosis. Intriguingly, the ICRs of imprinted loci have recently been shown to initiate a transvection event in premeiotic germ cells (Sandhu *et al.* 2009). Based on this example, we speculate that CRE initiated transvection may also occur in premeiotic germ cells, either via a physical interaction or a freely diffusible molecule. However, we find that adding an extra cross eliminates the *Kdm1a LoxP* inhibition. This suggests that any alteration propagated through sperm to initiate transvection is not stable enough to be maintained through a subsequent generation. Thus, the alteration may be reprogrammed in the germline, to prevent it from being passed on to future generations.

Our data also provide mechanistic insight into the mechanism of *LoxP* inhibition. In the previous case of meiotic *Cre*-initiated transvection, it was concluded that DNA methylation inhibits *LoxP* recombination (Rassoulzadegan *et al.* 2002). Our results, both *in vivo* and *in vitro*, directly contradict this conclusion. It is possible that these two observations are mechanistically unrelated. However, since both cases involve the inhibition of *LoxP* recombination via a transvection event when *Cre* is expressed during meiosis, we favor the idea that these phenomena are related. If this is the case, the observed acquisition of DNA methylation in the case of

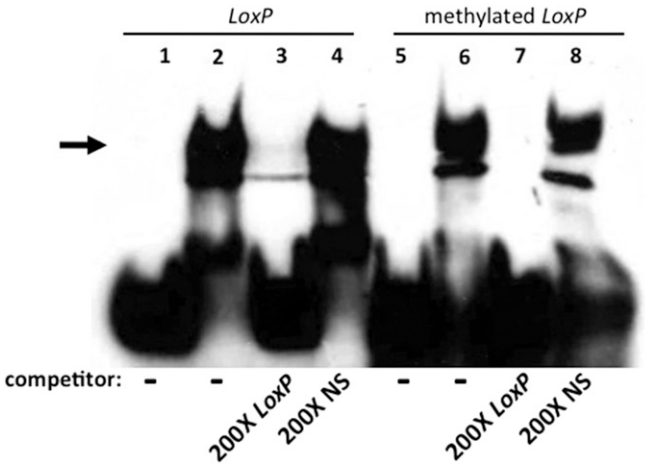

Figure 6 Binding of CRE to the *LoxP* recognition sequence. EMSAs with the unmethylated *LoxP* recognition sequence (lanes 1–4) and the fully methylated *LoxP* site (lanes 5–8) bound to recombinant CRE protein (lanes 2–4 and 6–8) or with probe alone (lanes 1 and 5). The retarded mobility shift complex is indicated by the black arrow to the left. In lanes 3 and 7, the retarded mobility shift complex has been competed with 200-fold molar excess of specific *LoxP* competitor probe, while in lanes 4 and 8, the retarded mobility shift complex has been competed with nonspecific (NS) competitor.

Sycp-1 may have been correlative rather than causative (Rassoulzadegan *et al.* 2002).

Importantly, we find that adding an extra generation after the F1 generation completely eliminates *LoxP* inhibition, despite the fact that both crosses were performed with animals that are genetically identical at the floxed locus and have the same strain background. This finding leads to three new insights. First, the fact that parental history, rather than genotype, determines the outcome strongly suggests that inhibition of *LoxP* recombination is an epigenetic phenomenon. Second, our results provide a roadmap for the use of other meiotic *Cre* transgenes that may be associated with similar *LoxP* inhibition. Although we are aware of only one additional published example of meiotic *Cre* initiated transvection (Rassoulzadegan *et al.* 2002), personal communications indicate other likely examples of meiotic *Cre*-initiated *LoxP* inhibition. The data presented here suggest that the simple addition of an extra cross could enable efficient use of these meiotically expressed *Cre* transgenes. Finally, by uncovering a system where the simple addition of an extra cross completely eliminates transvection, we have established an important *in vivo* mouse model that can be used to elucidate the mechanism of transvection in mammals.

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