Development 140, 4407-4416 (2013) doi:10.1242/dev.099390
© 2013. Published by The Company of Biologists Ltd

Evf2 (*Dlx6as*) lncRNA regulates ultraconserved enhancer methylation and the differential transcriptional control of adjacent genes

Emily G. Berghoff, Mary F. Clark, Sean Chen, Ivelisse Cajigas, David E. Leib and Jhumku D. Kohtz*

SUMMARY

Several lines of evidence suggest that long non-coding RNA (lncRNA)-dependent mechanisms regulate transcription and CpG DNA methylation. Whereas CpG island methylation has been studied in detail, the significance of enhancer DNA methylation and its relationship with lncRNAs is relatively unexplored. Previous experiments proposed that the ultraconserved lncRNA *Evf2* represses transcription through *Dlx6* antisense (*Dlx6as*) transcription and methyl-CpG binding protein (MECP2) recruitment to the Dlx5/6 ultraconserved DNA regulatory enhancer (Dlx5/6ei) in embryonic day 13.5 medial ganglionic eminence (E13.5 MGE). Here, genetic epistasis experiments show that MECP2 transcriptional repression of *Evf2* and *Dlx5*, but not *Dlx6*, occurs through antagonism of DLX1/2 in E13.5 MGE. Analysis of E13.5 MGE from mice lacking *Evf2* and of partially rescued *Evf2* transgenic mice shows that *Evf2* prevents site-specific CpG DNA methylation of Dlx5/6ei in *trans*, without altering *Dlx5/6* expression. *Dlx1/2* loss increases CpG DNA methylation, whereas *Mecp2* loss does not affect Dlx5/6ei methylation. Based on these studies, we propose a model in which *Evf2* inhibits enhancer DNA methylation, effectively modulating competition between the DLX1/2 activator and MECP2 repressor. *Evf2* antisense transcription and *Evf2*-dependent balanced recruitment of activator and repressor proteins enables differential transcriptional control of adjacent genes with shared DNA regulatory elements.

KEY WORDS: Forebrain, Ultraconserved enhancer methylation, MECP2, Mouse

INTRODUCTION

DNA methylation is an epigenetic modification associated with transcriptional changes in plants and animals (Feng et al., 2010; Smith and Meissner, 2013). Although factors involved in DNA methylation have been studied in depth, it is still not known how specific sites are selected. Multiple lines of evidence demonstrate the involvement of long non-coding RNAs (lncRNAs), specifically those known to regulate dosage compensation and imprinting through control of DNA methylation in *cis* (Barlow, 2011; Lee and Bartolomei, 2013). In addition to their *cis* regulation, lncRNAs that control imprinting also act in *trans*, as shown by studies with the *H19* lncRNA (Forné et al., 1997). One possible mechanism for lncRNA control of DNA methylation has been proposed for *Kcnq1ot1* (Mohammad et al., 2010), an lncRNA that forms a complex with DNA methyltransferase 1 (DNMT1), recruiting DNMT1 to chromatin and increasing paternal-specific CpG island methylation. The RNA-binding properties of DNMTs and methyl-CpG binding protein 2 (MECP2) (Nan et al., 1997) raised the possibility that large subgroups of non-coding RNAs might be directly involved in DNA methylation (Jeffery and Nakielny, 2004).

Besides imprinting and gene dosage compensation, lncRNA control of DNA methylation is less well understood. Genome-wide roles for antisense lncRNAs and other lncRNAs in gene regulation have been proposed (Lee, 2012), increasing their potential for controlling DNA methylation. In embryonic stem cells, the majority of lncRNAs are close to, or overlapping with, protein-coding genes (Sigova et al., 2013), adding further to this potential.

Evidence that antisense lncRNAs can differentially control DNA methylation in *trans* was provided by work on two different lncRNAs: *Khps1a* (Imamura et al., 2004) and *p15AS* (Yu et al., 2008). Whereas *Khps1a* facilitates CpG island demethylation in the promoter of its sense partner *Sphk1*, *p15AS* increases methylation in the *p15* (*Cdkn2b*) promoter. In a screen for functional relationships between sense/antisense pairs in the genome, both positive and negative regulatory effects were found (Katayama et al., 2005), a result supported by the differential effects of *Khps1a* and *p15AS* on methylation. Therefore, lncRNAs can mediate changes in methylation, possibly depending on DNMT and methyl-binding protein recruitment.

Work from our laboratory showed that *Evf2* (*Dlx6as*; also known as *Dlx6os1*) (Feng et al., 2006) is an ultraconserved, developmentally regulated *Dlx6* antisense lncRNA, that regulates activity of *Dlx5/6* DNA regulatory sequences ei and eii (Zerucha et al., 2000). *Evf2* recruits both known activator (DLX) (Panganiban and Rubenstein, 2002) and repressor (MECP2) proteins (Bond et al., 2009). It is unknown whether this recruitment of a methylation binding protein (MECP2) has an effect on methylation in this region. In addition, the precise mechanism of action of the DLX activator and MECP2 repressor proteins on the enhancer is unknown.

The biological significance of *Evf2*-Dlx5/6ei interactions is supported by altered adult hippocampal GABA circuitry in mice lacking *Evf2* (Bond et al., 2009), transcriptional effects of a single nucleotide polymorphism (SNP) in Dlx5/6ei linked to autism (Poitras et al., 2010), and the established role of MECP2 in autism (Guy et al., 2007; Guy et al., 2001). Loss of *Evf2* results in increased *Dlx5* and *Dlx6* expression in E13.5 MGE, a major site of sonic hedgehog-activated *Dlx* and *Evf2* gene regulatory events crucial for GABAergic interneuron development (Anderson et al., 1997a; Feng et al., 2006; Kohtz et al., 1998).

Developmental Biology and Department of Pediatrics, Lurie Children's Research Center, and Feinberg School of Medicine, Northwestern University, Box 204, 2430 N. Halsted, Chicago, IL 60614, USA.

*Author for correspondence (j-kohtz@northwestern.edu)

Accepted 20 August 2013

The ability of *Evf2* to recruit MECP2 to Dlx5/6ei and eii (Bond et al., 2009), and the role of MECP2 in chromatin organization in the *Dlx5/6* region (Horike et al., 2005) raised several questions regarding the relationship between transcriptional effects of MECP2 recruitment and Dlx5/6ei DNA methylation. In this article, we show that loss of *Mecp2* in E13.5 MGE results in increased expression of *Evf2* and *Dlx5*, with no change in *Dlx6* expression. *Evf2* prevents CpG methylation in Dlx5/6ei, indicating that methylated CpG sites are not responsible for MECP2 recruitment. Using a transgenic mouse model with reduced *Evf2* expression (~0.38× wild-type expression levels), we show that *Evf2 trans* activity inhibits methylation of the *Dlx5/6* ultraconserved enhancer in mice lacking *Evf2* (*Evf2*$^{TS/TS}$), without altering *Dlx5/6* gene expression. Based on both genetic epistasis and rescue experiments, we propose a model in which *Evf2* lncRNA inhibits *Dlx5/6* ultraconserved DNA methylation, facilitating antagonistic interactions between repressive and activating transcription factors MECP2 and DLXs. These interactions allow differential control of adjacent genes by shared DNA regulatory elements.

MATERIALS AND METHODS

PCR

Total RNA was extracted from pairs of mouse embryonic day (E) 13.5 medial ganglionic eminence (MGE) tissue using the EZNA MicroElute Total RNA Kit (Omega). E13.5 MGE dissections were as previously described (Feng et al., 2006). cDNA was synthesized using the qScript cDNA Synthesis Kit (Quanta Biosystems). The resulting cDNA served as a template for the amplification of the genes of interest normalized to the constitutive gene (*Actb*, encoding β-actin) by quantitative real-time PCR, using TaqMan or SYBR Green reagents. For TaqMan PCR, we used the following: *Dlx5* (Mm00438430_m1), *Dlx6* (Mm01166201_m1) and *Actb* (Mm00607939_s1). Rat-specific *Evf2* primers were previously described (Feng et al., 2006). For SYBR Green PCR, we used the following primers: *mEvf2*-F (0.2 μM, 5′-CTCCCTCCGCTCAGTATAGATTTC-3′); *mEvf2*-R (0.2 μM, 5′-CCTCCCCGGTGAATATCTCTT-3′); *β-actin*-F (0.3 μM, 5′-GCGAGCACAGCTTCTTTGC-3′); and *β-actin*-R (0.3 μM, 5′-TCGTCATCCATGGCGAACT-3′).

For *Dlx5* imprinting analysis, primers spanning a SNP in *Dlx5* were used to amplify transcripts from E13.5 MGE cDNA as previously described (Horike et al., 2005), cut with *Hin*dIII and fragments analyzed by agarose gel electrophoresis. Ratios of resulting products were quantified by ImageJ software. Values were averaged from three embryos for each genotype, as indicated.

Mice

Evf2 rescue (*Evf2R*) mice were generated using the *Dlx1/2* enhancer (Ghanem et al., 2007), floxed TS (transcription stop) sequence (Soriano, 1999), and full-length rat *Evf2* cDNA (Feng et al., 2006). The TS sequence was removed by crossing *Evf2R*^{floxedTS} to EIIAcre mice [Jackson, FVB/N-TgN (EIIa-Cre) C5379Lmgd] to allow expression of rat *Evf2* in *Dlx1/2*-expressing cells. *Evf2R*^{EIIAcre} was crossed to *Evf2*$^{TS/TS}$, to generate *Evf2*$^{TS/TS}$;*R*$^{+/-}$ and *Evf2*$^{TS/TS}$;*R*$^{-/-}$ embryos for the gene expression and methylation analysis shown in Fig. 4. *Evf2*$^{TS/TS}$ mice were generated as previously described (Bond et al., 2009). B6.129P2(C)-Mecp2$^{tm1.1Bird}$/J mice (Guy et al., 2001) were obtained from the Jackson laboratory. As *Mecp2* is X-linked, *Mecp2* null mice were generated by crossing *Mecp2*$^{+/-}$ females to Bl6 males, and analyzing male E13.5 embryos (*Mecp2*$^{-/y}$). *Dlx1/2*$^{+/-}$ mice (Anderson et al., 1997a) were generously given by Dr Kenneth Campbell (Children's Hospital Research Foundation, Cincinnati, OH, USA).

Methylation analysis

Genomic DNA from mouse E13.5 MGE tissue was modified with the methylSEQr Bisulfite Conversion Kit (Applied Biosystems). The genomic region spanning the *Dlx5/6* ultraconserved enhancer (ei) was amplified by using nested PCR, using the following primers: External primers: F: 5′-GATTTGGGTATTTTTAAATTATG-3′ and R: 5′-AAAATAAATACAAAAACATCAACC-3′; Internal primers: F: 5′-GTTATTTATTAGAAGTTAATAGAG-3′ and R: 5′-TAAACATTTTCTAATTTCAAAATTC-3′. The PCR products were cloned into pGEM T-easy vector (Promega) and individual clones were sequenced. A minimum of 45 clones was sequenced from each genotype.

Global methylation analysis of mouse B1 line elements (1-4) from *Evf2*$^{+/+}$ and *Evf2*$^{TS/TS}$ E13.5 MGE was performed by EpigenDx by pyrosequencing PCR products from bisulfite-treated DNA (Uhlmann et al., 2002). E13.5 MGE tissue was digested with proteinase K (40 μg) at 50°C for 30 minutes in a volume of 40 μl, and cell debris pelleted by centrifugation at 14,000 ***g*** for 10 minutes. Digested tissue (20 μl) was used in the bisulfite conversion reaction; E13.5 MGE DNA (500 ng) was bisulfite treated by EpigenDx using a proprietary bisulfite salt solution. DNA was diluted to 45 μl, and 5 μl of 3 N NaOH for 30 minutes at 42°C to denature the DNA. Bisulfite salt solution (100 μl) was added to the DNA and incubated for 14 hours at 50°C. Bisulfite-treated DNA was purified using Zymogen DNA columns and eluted in 20 μl TE (10 mM Tris pH 7.5, 0.1 mM EDTA); 1 μl was used for each PCR.

The PCR was performed with 0.2 μM of each mouse B element primer (1-4). One of the PCR primers was biotinylated to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences), and the Sepharose beads containing the immobilized PCR product were purified, washed and denatured in 0.2 M NaOH solution, and rewashed using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen). Pyrosequencing primer (0.2 μM) was annealed to the purified single-stranded PCR product. PCR products (10 μl) were sequenced by Pyrosequencing PSQ96 HS System (Biotage), following the manufacturer's instructions (Pyrosequencing, Qiagen). The methylation status of mouse B1 line elements was analyzed individually using QCpG software (Pyrosequencing, Qiagen).

RESULTS

Antagonism between MECP2 and DLX1/2

Although it has been shown that MECP2 is a transcriptional repressor of *Dlx5* and *Dlx6* in postnatal brain (Horike et al., 2005), the role of *Mecp2* in embryonic brain development has not been defined. In addition, MECP2 exhibits both activator and repressor roles *in vivo*, raising the question of whether MECP2 represses or activates *Dlx5/6* expression in E13.5 MGE (Chahrour et al., 2008). Previous results showed that loss of *Evf2* in E13.5 MGE decreases binding of MECP2 to Dlx5/6ei and eii, with decreased histone deacetylase (HDAC1) binding to Dlx5/6eii (Bond et al., 2009). Thus, decreased HDAC1 binding to Dlx5/6eii is one of the possible explanations for increased expression of *Dlx5* and *Dlx6*.

In order to determine the effect of MECP2 on *Dlx5/6* enhancer activity *in vivo*, we compared *Evf2*, *Dlx5* and *Dlx6* expression in E13.5 MGE of *Mecp2* null mice [Fig. 1, mutant (gray bars) and wild-type littermates (black bars)]. Loss of *Mecp2* [*Mecp2* null (*Mecp2*$^{-/y}$)] increases *Evf2* (~2-fold) and *Dlx5* (~2.5-fold) transcripts, with no detectable changes in *Dlx6*. Horike et al. (Horike et al., 2005) reported ~2-fold increase in *Dlx5* and ~1.8-fold increase in *Dlx6* in *Mecp2* null postnatal cortex. Therefore, E13.5 MGE and postnatal cortex are similar in that MECP2 moderately inhibits *Dlx5* expression (~2- to 2.5-fold), but these regions differ in control of *Dlx6*.

In E13.5 MGE, removal of one copy of *Dlx1/2* from *Mecp2* null mice (*Mecp2null; Dlx1/2*$^{+/-}$) prevents activation of *Evf2* and *Dlx5*. This result suggests that increased *Evf2* and *Dlx5*, in the absence of MECP2, is mediated by DLX1/2. Removal of one copy of *Dlx1/2* (*Dlx1/2*$^{+/-}$) inhibits *Evf2* and *Dlx5* expression, indicating dose-dependent *Dlx1/2* regulation of *Evf2* and *Dlx5*. Complete loss of *Dlx1/2* (*Dlx1/2*$^{-/-}$) results in loss of *Evf2*, *Dlx5* and *Dlx6*, in agreement with previous demonstrations that *Dlx1/2* is crucial for

Development

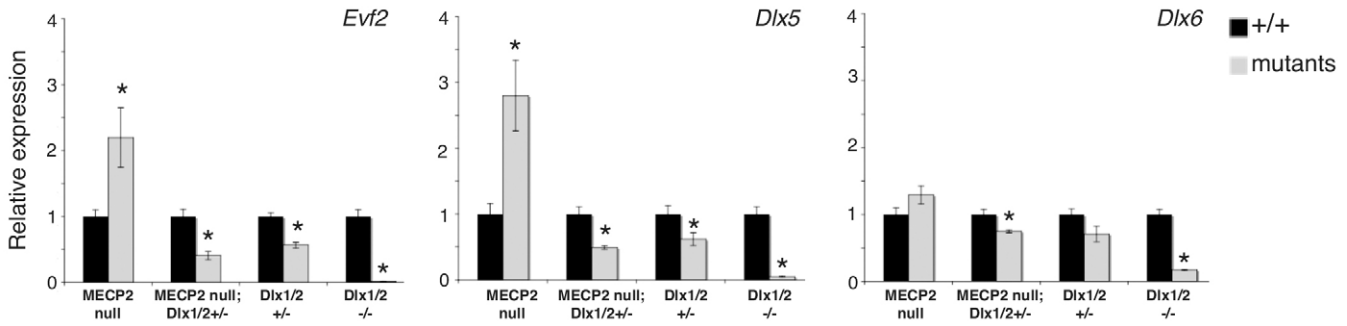


Fig. 1. MECP2 represses *Evf2* and *Dlx5* expression through antagonism of DLX1/2. Quantitative PCR on cDNA isolated from E13.5 medial ganglionic eminence was performed to determine expression levels of *Evf2*, *Dlx5* and *Dlx6*. Values are normalized to *Actb*, and expression compared from different genotypes with respective wild-type littermates (+/+, black). The following mutants (gray) were used: *Mecp2null* (*Mecp2*$^{-/y}$), *Mecp2null; Dlx1/2*$^{+/-}$ [*Mecp2*$^{-/y}$ with one copy of *Dlx1/2* (Anderson et al., 1997b)], *Dlx1/2*$^{+/-}$ (heterozygote with one copy of *Dlx1/2*) and *Dlx1/2*$^{-/-}$ (*Dlx1/2* null lacking *Dlx1* and *Dlx2*). *Mecp2* represses *Evf2* and *Dlx5*, but not *Dlx6*. *Dlx1/2* are activators of *Evf2*, *Dlx5* and *Dlx6*. *Dlx1/2* control of *Evf2* and *Dlx5* is dose dependent, as removal of one copy (*Dlx1/2*$^{+/-}$) reduces *Evf2* and *Dlx5* expression. n=3 each genotype. *P<0.01 (Student's t-test). Error bars represent s.e.m.

Dlx5/6 gene activation (Anderson et al., 1997b; Zerucha et al., 2000). Loss of *Mecp2* does not change levels of *Dlx1* or *Dlx2* transcripts (supplementary material Fig. S1). Therefore, increased expression of *Evf2* and *Dlx5* in *Mecp2* null mice does not result from increased *Dlx1/2* expression.

These genetic epistasis experiments suggest that DLX1/2 and MECP2 interactions are antagonistic rather than cooperative. Antagonistic interactions suggest that MECP2 and DLX1/2 compete for binding on the same allele, arguing against parent of origin-specific *Dlx5* regulation. Although *Dlx5* imprinting has been demonstrated in humans (Okita et al., 2003), there has been controversy in the literature regarding *Dlx5* imprinting in mice (Horike et al., 2005; Kimura et al., 2004; Schüle et al., 2007).

In order to address whether *Dlx5* is imprinted in E13.5 MGE, and whether *Evf2* regulation of *Dlx5* is parentally controlled, we used a previously identified *Dlx5* SNP in JF1 mice (Horike et al., 2005) to distinguish between parental alleles. We find that *Dlx5* is not imprinted in E13.5 MGE, as the ratio between maternal *Dlx5* and paternal *Dlx5* transcripts is either 1.18 or 1.10 (Fig. 2A, lanes 1 and 2, corresponding to genotypes in schematics 1 and 2). When *Evf2* is truncated on the paternal (Fig. 2A, lane 3) or maternal (Fig. 2A, lane 4) alleles, *Dlx5* increases (Fig. 2A, green arrows). These data suggest that *Evf2* repression of *Dlx5* is equal on maternal and paternal alleles (Fig. 2A, ratio between maternal *Dlx5* and paternal *Dlx5* transcripts is ~2.3 for genotypes in schematics 3 and 4). Fig. 2B shows E13.5 MGE qRT-PCR analysis of *Dlx5* expression in *Evf2TS*^{pat}/+^{mat} (pink bars), *Evf2TS*^{mat}/+^{pat} (blue bars) and *Evf2*$^{+/+}$ littermates (black bars). *Dlx5* increases to 1.5× wild-type levels upon maternal or paternal *Evf2* loss. Together, these data show that *Dlx5* is not imprinted at this time in development, and that *Evf2* represses *Dlx5* on both maternal and paternal alleles, arguing against MECP2 allele-specific or parentally controlled *Dlx5* expression in E13.5 MGE.

Evf2 controls site-specific CpG DNA methylation in the *Dlx5/6* ultraconserved enhancer ei

Given that MECP2 is a methyl-CpG binding protein, we next asked whether *Evf2* recruits MECP2 by increasing CpG methylation within Dlx5/6ei. We used previously described mice lacking *Evf2* (*Evf2*$^{TS/TS}$) (Bond et al., 2009), in which a triple poly(A) transcription stop site is inserted into the 5′ end of *Evf2*. Bisulfite sequencing analysis of 890 bp spanning Dlx5/6ei (13 potential CpG methylation sites) on E13.5 MGE DNA was performed on *Evf2* wild-type littermates (*Evf2*$^{+/+}$) and compared with *Evf2*$^{TS/TS}$ mutants (Fig. 3A,B). Increased methylation is observed in *Evf2*$^{TS/TS}$ E13.5 MGE DNA at two specific CpG sites, ^{576}CpG and ^{757}CpG (Fig. 3B), showing that *Evf2* inhibits site-specific CpG DNA methylation in the Dlx5/6 ultraconserved enhancer ei. Global methylation analysis of mouse B1 line elements at four sites (1-4) shows that *Evf2* loss does not increase global methylation (Fig. 3C), indicating that methylation increase at Dlx5/6ei does not result from global effects.

We next asked whether *Evf2* controls Dlx5/6ei CpG methylation through *trans* or *cis* mechanisms. In order to answer this question, we developed an *Evf2* rescue transgenic model, in which rat *Evf2* cDNA (Feng et al., 2006) is driven by a *Dlx1/2* enhancer (Ghanem et al., 2007). *Evf2* rescue transgenic mice (*Evf2R*) were crossed to *Evf2*$^{TS/TS}$ mice to generate mice that only expressed *Evf2* from the transgene (*Evf2*$^{TS/TS}$*;R*). Fig. 4A,B shows a schematic of the *Evf2* transgene and genotyping results of cre-mediated removal of the triple poly(A) stop site (TS) placed at the 5′ end of the rat *Evf2* transgene (*Evf2*$^{TS/TS}$*;R*). Although we have not ruled out the possibility that transgene integration is in chromosome 6, transgene inheritance does not appear to be linked to *Dlx5/6*, as *Evf2*$^{TS/+}$*;R* offspring from crosses of *Evf2*$^{TS/+}$ × *Evf2*$^{+/+}$*;R* are obtained at expected frequencies.

Quantitative RT-PCR analysis of E13.5 MGE from *Evf2*$^{TS/TS}$*;R* mice shows that rat-specific *Evf2* is expressed from the transgene (Fig. 4C). Comparison of *Evf2* transcript levels expressed by the transgene and wild-type *Evf2* levels is possible using primers that recognize both rat and mouse transcripts (Fig. 4D, *Evf2*); the transgene is expressed at 0.38× wild-type levels (Fig. 4D). *Dlx5* and *Dlx6* expression does not differ between *Evf2*$^{TS/TS}$*;R* and *Evf2*$^{TS/TS}$ E13.5 MGE (Fig. 4E). However, Dlx5/6ei ^{576}CpG and ^{757}CpG methylation is significantly decreased in E13.5 MGE DNA of *Evf2*$^{TS/TS}$*;R* transgenic rescue mice compared with *Evf2*$^{TS/TS}$ (Fig. 4F). There is a slight (<15%), but statistically significant, increase in ^{626}CpG methylation, raising the possibility of opposing methylation effects of *Evf2* on sites other than ^{576}CpG and ^{757}CpG. The ability of the *Evf2* transgene, even at 0.38× wild-type levels, to significantly decrease ^{576}CpG and ^{757}CpG methylation of Dlx5/6ei, suggests that *Evf2* inhibits methylation in *trans*.

Loss of *Dlx1/2* but not *Mecp2* results in ^{576}CpG and ^{757}CpG Dlx5/6ei methylation

The proximity of ^{576}CpG to previously defined DLX1/2 binding sites (Fig. 3A, D1 and D2, green) in Dlx5/6ei (Zerucha et al., 2000)

Development

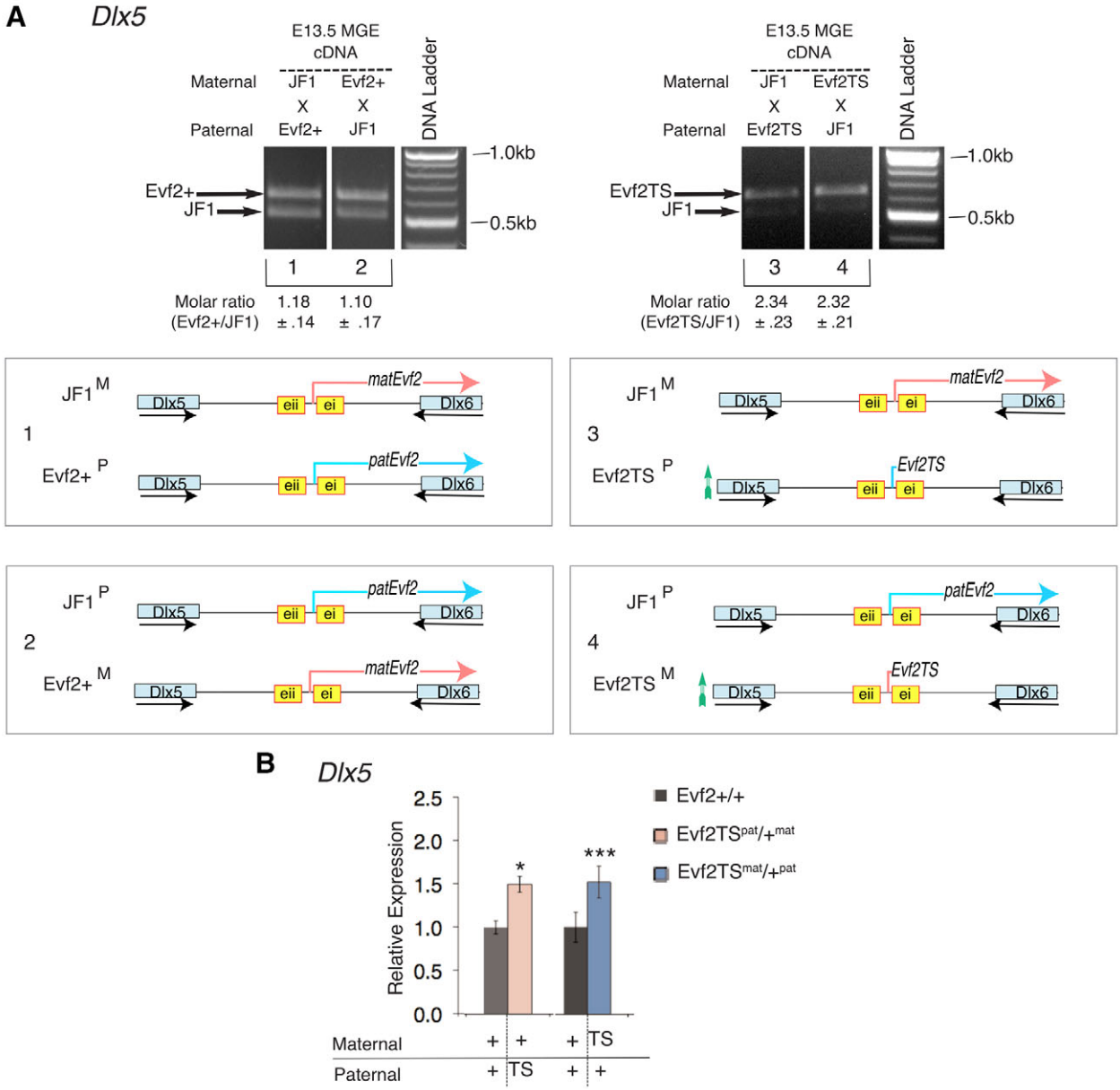


Fig. 2. *Evf2* represses *Dlx5* equally on maternal and paternal alleles. (**A**) Imprinting analysis of *Dlx5* RNA in E13.5 MGE. A SNP within *Dlx5* generates a *Hin*dIII site in JF1 (Horike et al., 2005), distinguishing parental origin of *Dlx5* transcripts in crosses of JF1 and *Evf2*+ (mixed 129/Bl6) mice. $Evf2^{TS/+}$ mice are on a mixed 129/Bl6 background. Wild types are referred to as *Evf2*+ or *Evf2*+/+ to indicate littermate controls. Crosses of (1) $JF1^{mat} \times Evf2+^{pat}$ or (2) $JF1^{pat} \times Evf2+^{mat}$ indicate equal expression of *Dlx5* from maternal and paternal alleles, showing that *Dlx5* is not imprinted [ratios (1) *Evf2*+/*JF1*=1.18±0.14, (2) *Evf2*+/JF1=1.10±0.17; *n*=4, *P*>0.05]. Analysis of *Dlx5* expression in (3) $JF1^{mat} \times Evf2TS^{pat}$ and (4) $JF1^{pat} \times Evf2TS^{mat}$ shows that *Dlx5* expressed adjacent to transcription stop site insertion is increased (~2.3-fold) for both maternal and paternal alleles; *n*=3 for each genotype, *P*>0.05. Schematics of the genotypes of crosses (1-4) corresponding to gel lanes are shown. M, maternal; P, paternal; pink, maternal *Evf2* transcript; blue, paternal *Evf2* transcript; TS, transcription stop; *Evf2TS*, truncated transcript from TS insertion; green arrow, increased *Dlx5* expression adjacent to TS insertion. (**B**) *Dlx5* expression increases to the same level upon maternal *Evf2* or paternal *Evf2* loss. Crosses of *Evf2TS*/+ with *Evf2*+/+ generate $Evf2TS^{pat}/+^{mat}$ (pink) and $Evf2TS^{pat}/+^{mat}$ (blue), depending on *Evf2TS* parental origin, as well as *Evf2*+/+ littermates (black). *n*=5 for $Evf2TS^{pat}/+^{mat}$, and *n*=5 *Evf2*+/+ littermates (**P*=0.03), *n*=6 $Evf2TS^{mat}/+^{pat}$, *n*=6 *Evf2*+/+ littermates (****P*=3.4×10^{-4}). *P* values are generated by Student's two-tailed *t*-test.

raises the possibility that *Evf2* recruitment of DLX1/2 plays a role in ^{576}CpG and ^{757}CpG methylation. However, loss of DLX1/2 loss also results in *Evf2* loss (Fig. 1), making it difficult to distinguish between the role of DLX1/2 binding to Dlx5/6ei and the role of *Evf2* in methylation. Analysis of $Dlx1/2^{-/-}$ E13.5 MGE (Fig. 5A) shows that ^{576}CpG and ^{757}CpG Dlx5/6ei methylation increases in the absence of *Dlx1/2*. This result is consistent with increased ^{576}CpG and ^{757}CpG Dlx5/6ei methylation observed in $Evf2^{TS/TS}$ mice, and further supports the idea that *Evf2* prevents site-specific ^{576}CpG and ^{757}CpG Dlx5/6ei methylation.

Given that *Evf2* also recruits MECP2 to Dlx5/6ei (Bond et al., 2009), we next asked whether MECP2 recruitment plays a role in Dlx5/6ei methylation. Analysis of Dlx5/6ei methylation in $Mecp2null;Dlx1/2^{+/-}$ mice (Fig. 5B) shows no significant difference in CpG methylation profile across Dlx5/6ei, compared with wild-type littermates. Although there appears to be slightly increased

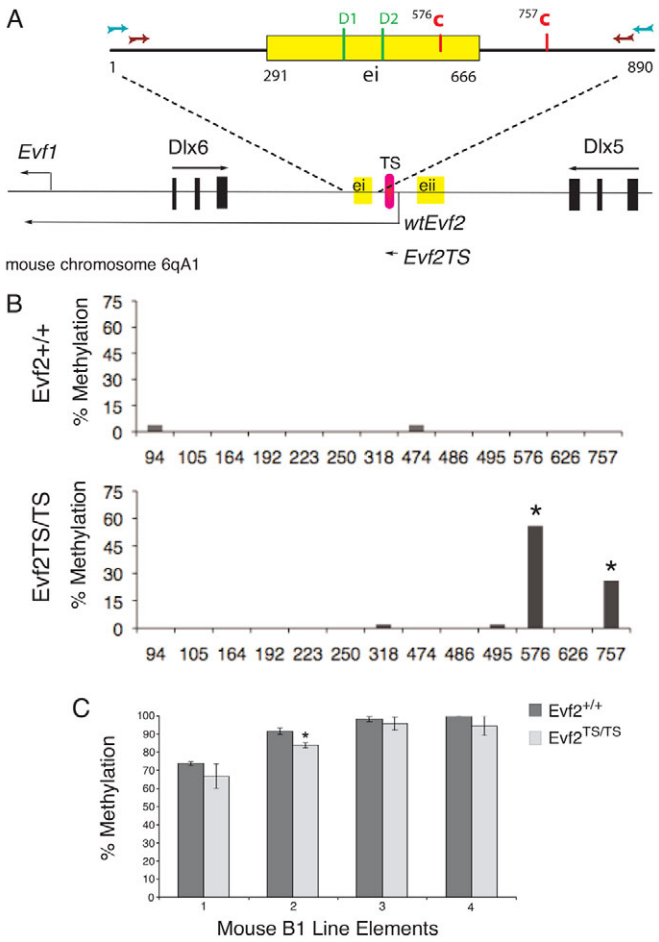


Fig. 3. *Evf2* lncRNA prevents site-specific CpG DNA methylation within the Dlx5/6 ultraconserved enhancer ei. E13.5 MGE DNA isolated from three $Evf2^{+/+}$ and three $Evf2^{TS/TS}$ mutants was bisulfite treated, PCR amplified, subcloned, and individual clones sequenced. (**A**) Schematic of the Dlx5/6 intergenic region, containing the intergenic enhancers ei (ultraconserved) and eii, with expansion of the 890 PCR region spanning ei (blue and brown arrows indicate nested primers, where blue arrows indicate external primers, and brown arrows indicate internal primers). There are 13 possible CpG DNA methylation sites within this 890-nucleotide (nt) region. ^{576}CpG and ^{757}CpG are each marked by a red C. Pink oval represents the location of the triple poly(A) transcription stop (TS) insertion site at the 5′ end of *Evf2* in $Evf2^{TS/TS}$ mice. The wild-type *Evf2* transcript is ~3.7 kb, whereas *Evf2TS* generates a predicted truncated transcript (80 nt) before transcription termination. DLX1/2 binding sites, as previously identified (Zerucha et al., 2000), within ei are in green (D1 and D2). (**B**) Graph of percentage methylation comparing $Evf2^{+/+}$ and $Evf2^{TS/TS}$ E13.5 MGE at 13 possible CpG sites within the 890 PCR region shown in A. Data are obtained from 52 $Evf2^{+/+}$ and 56 $Evf2^{TS/TS}$ individual clones. *n*=3 for each genotype. Loss of *Evf2* results in increased methylation at sites ^{576}CpG and ^{757}CpG, **P*<0.01. (**C**) Global methylation analysis of four different B1 line elements (1-4) in $Evf2^{+/+}$ and $Evf2^{TS/TS}$ E13.5 MGE DNA shows that *Evf2* loss does not increase global methylation. Error bars represent s.e.m.

methylation at sites ^{626}CpG and ^{757}CpG, these differences are not statistically significant. It is interesting to note that *Evf2* expression in *Mecp2null;Dlx1/2*$^{+/-}$ and $Evf2^{TS/TS}$*;R* mice (Fig. 1) are similar, and that Dlx5/6ei methylation profiles are also similar [*Mecp2null;Dlx1/2*$^{+/-}$ (Fig. 5B) and $Evf2^{TS/TS}$*;R* (Fig. 4F)]. Although we did not determine Dlx5/6ei methylation profiles in *Dlx1/2*$^{+/-}$ mice, it would be expected that *Mecp2null;Dlx1/2*$^{+/-}$ and *Dlx1/2*$^{+/-}$ methylation profiles would also be similar, as *Evf2* levels are similar between *Mecp2null;Dlx1/2*$^{+/-}$ and *Dlx1/2*$^{+/-}$ mice. Together, these data support the idea that *Evf2*, but not MECP2, controls Dlx5/6ei methylation.

DISCUSSION

Six key results presented in this paper are listed below, followed by their implications.

(1) MECP2 represses *Dlx5* and *Evf2*, but not *Dlx6*. *Evf2* recruitment of MECP2 to Dlx5/6 intergenic enhancers inhibits *Dlx5* expression, whereas *Evf2* antisense transcriptional regulation inhibits *Dlx6* expression. Therefore, the *Evf2* lncRNA enables differential regulation of genes with shared regulatory elements.

(2) Loss of one copy of *Dlx1/2* in *Mecp2* null E13.5 MGE rescues increased expression of *Dlx5* and *Evf2*. MECP2 inhibits *Dlx5* and *Evf2* expression by antagonizing DLX1/2 transcriptional activation.

(3) *Dlx5* is not imprinted in E13.5 MGE tissue. *Evf2* represses *Dlx5* equally on maternal and paternal alleles. *Evf2* recruitment of MECP2 is unlikely to be involved in *Dlx5* imprinting, and therefore unlikely to be allele specific, at this time in development.

(4) *Evf2* prevents methylation at two specific sites in the Dlx5/6ei ultraconserved enhancer. *Evf2* transgene expression at 0.38× wild-type levels reduces site-specific methylation of the Dlx5/6ei ultraconserved enhancer. An lncRNA can determine site-specific methylation pattern across an enhancer, at least in part, through *trans*-acting mechanisms.

(5) Despite reduced site-specific enhancer methylation, *Evf2* transgene expression at 0.38× wild-type levels does not change *Dlx5* and *Dlx6* expression. The lncRNA-dependent methylation pattern across an enhancer might not reflect the activity state of the enhancer, but rather the presence of the lncRNA, which can have activator or repressor activity.

(6) Loss of *Dlx1/2*, but not *Mecp2*, increases site-specific methylation of the Dlx5/6ei ultraconserved enhancer. *Evf2* recruitment of MECP2 is not involved in regulating site-specific methylation of the Dlx5/6ei ultraconserved enhancer.

These results are based on analysis of gene expression and methylation patterns in mouse mutants either lacking or expressing different doses of *Evf2*, *Dlx1/2* or *Mecp2*. This information, combined with previously published chromatin immunoprecipitation, leads to models (Fig. 6A,B) that describe how *Evf2* methylation control and transcription factor recruitment contribute to Dlx5/6ei enhancer-dependent gene regulation. These models are described in detail below.

Relationship between *Mecp2*, *Dlx1/2* and *Dlx5/6* enhancer activity

Based on genetic manipulation of *Mecp2* and *Dlx1/2 in vivo* (Fig. 1), the schematics in Fig. 6A describe possibilities for MECP2 and DLX1/2 occupancy of Dlx5/6ei/eii. Fig. 1 shows that loss of MECP2 activates *Evf2* (~2-fold) and *Dlx5* (~2.5-fold) expression. When one copy of *Dlx1/2* is also lost (double mutant, *Mecp2null;Dlx1/2*$^{+/-}$), increased *Evf2* and *Dlx5* expression is lost. This supports the idea that MECP2 inhibits *Evf2* and *Dlx5* expression by preventing DLX1/2 activation of Dlx5/6ei/eii, suggesting antagonism between MECP2 and DLX1/2. There is an additional effect resulting from removal of one copy of *Dlx1/2*, whereby both *Evf2* and *Dlx5* decrease further compared with wild type (~0.5-fold). One possible explanation of why *Evf2* and *Dlx5* do not return to wild-type levels, but decrease even further is their dose-dependent regulation by DLX1/2. Dose-dependent regulation of *Evf2* and *Dlx5* by DLX1/2 is supported by removing one copy of

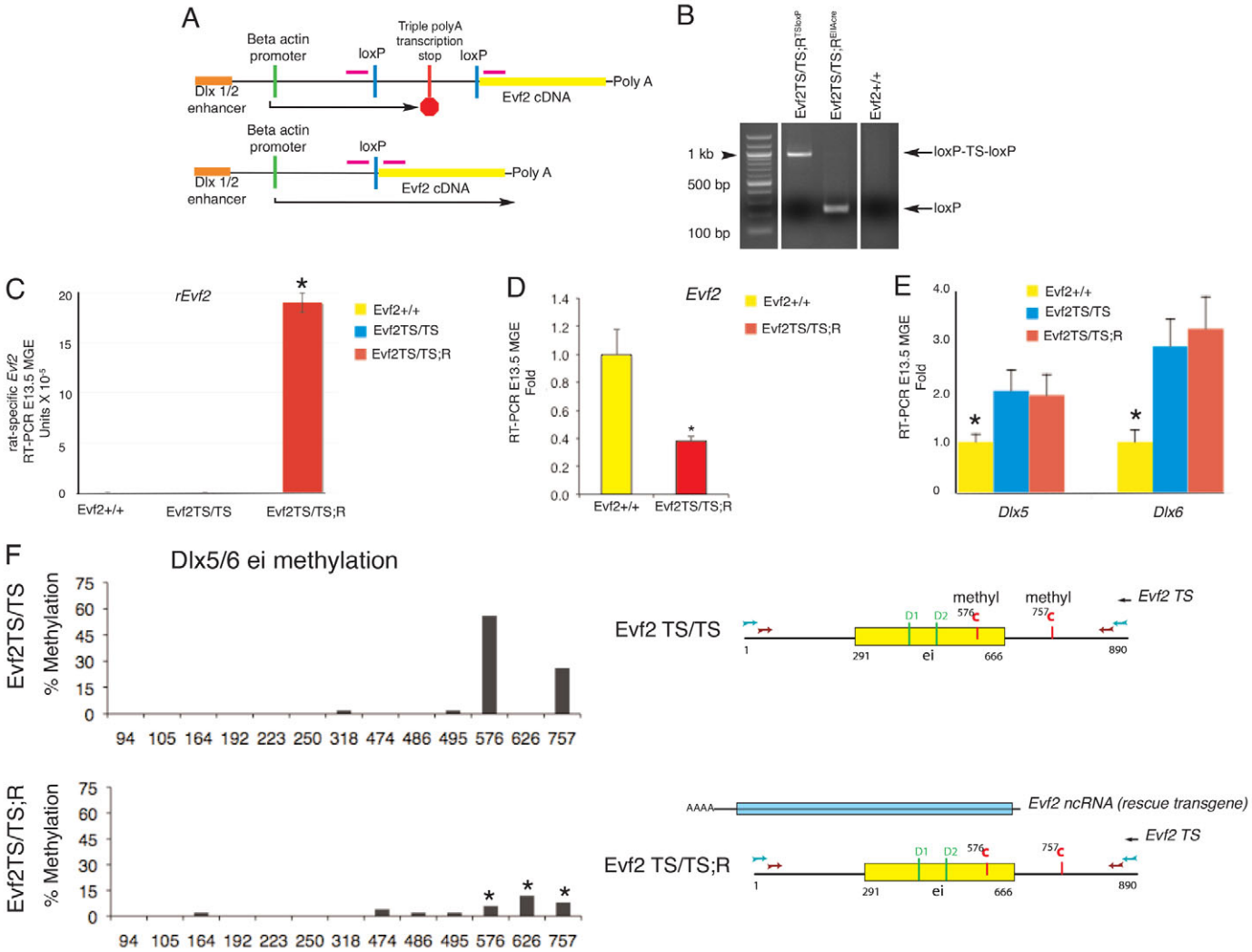


Fig. 4. *Evf2* lncRNA *trans* activity controls Dlx5/6 ultraconserved enhancer methylation. *Evf2* rescue mice (*Evf2R*) were generated using a transgene expressing full-length rat *Evf2* (3.7 kb) driven by Dlx1/2 enhancer 1b (Ghanem et al., 2007) and *Actb* promoter. (**A**) Schematic of the construct used to express rat *Evf2*; a floxed TS sequence precedes the 5′ end of *Evf2*, stopping transcription, and allowing transcription after cre-mediated removal. Pink bars show where genotyping primers are placed to distinguish loxP-TS-loxP from a single loxP remaining site after cre removal. (**B**) Genotyping results of $Evf2^{TS/TS}$;$R^{loxP\text{-}TS\text{-}loxP}$ (longer fragment) and $Evf2^{TS/TS}$;$R^{EIIAcre,\ loxP}$ (shorter fragment). (**C-E**) Quantitative RT-PCR of E13.5 MGE from $Evf2^{+/+}$ (yellow), $Evf2^{TS/TS}$ (blue) and $Evf2^{TS/TS}$;*R* (red, $Evf2^{TS/TS}$;$R^{EIIAcre,\ loxP}$). (C) Detection of rat-specific *Evf2* transcripts, only expressed in $Evf2^{TS/TS}$;*R* tissue (red bar). (D) *Evf2* expressed from the transgene (red bar) is expressed at ~0.38× wild-type levels (yellow bar). (E) Transgenic expression of *Evf2* does not significantly change *Dlx5* or *Dlx6* expression in $Evf2^{TS/TS}$ mice. $Evf2^{+/+}$ is significantly different from $Evf2^{TS/TS}$ and $Evf2^{TS/TS}$;*R* (P<0.01, two-way ANOVA), n=3 for each genotype. Error bars represent s.e.m. (**F**) *Evf2* transgene ($Evf2^{TS/TS}$;*R*) reduces methylation at ^{576}CpG and ^{757}CpG in Dlx5/6ei, compared with $Evf2^{TS/TS}$. Bisulfite sequencing of the same 890-bp region spanning Dlx5/6ei on E13.5 MGE DNA as in Fig. 3. Significant differences are detected at sites ^{576}CpG and ^{757}CpG, where $Evf2^{TS/TS}$;*R* E13.5 MGE has decreased methylation compared with $Evf2^{TS/TS}$. There is a slight increase in methylation at ^{626}CpG (<15%). n=3 embryos for each genotype, *P<0.01 (Student's t-test). A minimum of 45 clones were sequenced for each genotype.

Dlx1/2 ($Dlx1/2^{+/-}$), which also results in *Evf2* and *Dlx5* expression at ~0.5× wild-type levels. The fact that levels of *Evf2* and *Dlx5* are equal in $Dlx1/2^{+/-}$ and *Mecp2null;*$Dlx1/2^{+/-}$ shows that loss of MECP2 has no effect when DLX1/2 levels are limiting.

The schematics in Fig. 6A illustrate a possible explanation of why *Evf2* and *Dlx5* levels are the same in $Dlx1/2^{+/-}$ and *Mecp2null;*$Dlx1/2^{+/-}$ mice. In the wild-type situation, there are three possible states of Dlx5/6ei/eii occupancy: inactive (both copies are bound by MECP2; Fig. 6Aa); low activity (one copy is bound by MECP2 and the other by DLX1/2; Fig. 6Ab); high activity (both copies are bound by DLX1/2; Fig. 6Ac).

In *Mecp2null;*$Dlx1/2^{+/+}$ mice (Fig. 6Ae), MECP2 is absent, but DLX1/2 is at wild-type levels; DLX1/2 is expected to bind both copies, generating a high activity state (Fig. 6Ac,e). In *Mecp2null;*$Dlx1/2^{+/-}$ mice (Fig. 6Ad), MECP2 is still absent, but DLX1/2 levels are limiting; DLX1/2 only binds one copy of Dlx5/6ei/eii, generating a low activity state (Fig. 6Ab,d*). In $Dlx1/2^{-/-}$ mice (Fig. 6Af), DLX1/2 is absent, generating an inactive state (Fig. 6Aa,f). MECP2 is shown bound to Dlx5/6ei/eii in (Fig. 6Af); however, given previous chromatin immunoprecipitation (ChiP) data showing that *Evf2* recruits MECP2 to Dlx5/6ei and eii (Bond et al. 2009), it is also possible that MECP2 does not bind Dlx5/6ei and eii in the absence of *Evf2* and DLX1/2. In $Dlx1/2^{+/-}$ mice, DLX1/2 levels are limiting (Fig. 6Ag); DLX1/2 binds one copy of Dlx5/6ei/eii, whereas MECP2 binds the other copy, generating a low activity state (Fig. 6Ab,d*,g*). The fact that genotypes d* and

Development

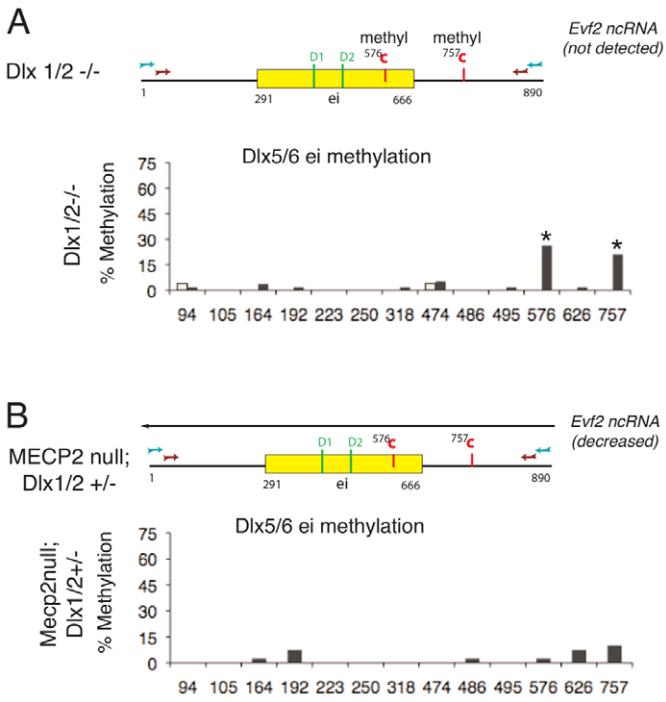


Fig. 5. DLX1/2 but not MECP2 represses ^{576}CpG and ^{757}CpG methylation of Dlx5/6 ultraconserved enhancer. (**A**) Bisulfite sequencing of the same 890-bp region spanning Dlx5/6ei as described in Figs 3 and 4, was performed on E13.5 MGE DNA isolated from *Dlx1/2*$^{-/-}$ (black bars) compared with wild-type littermates (white bars). Loss of *Dlx1/2* increases methylation at ^{576}CpG and ^{757}CpG; *P<0.01, n=3 for each genotype, minimum of 45 clones sequences for each genotype. (**B**) *Mecp2null;Dlx1/2*$^{+/-}$ (black bars) and wild-type littermates (shown in white bars in A) do not differ from each other. n=2 embryos/genotype and a minimum of 45 clones sequenced/genotype. P>0.05.

g* generate similar states of low Dlx5/6ei/eii activity supports the idea that the major role of MECP2 is to antagonize DLX1/2. Although this model is an over simplification of complex interactions that influence Dlx5/6ei/eii activity, they are consistent with both present genetic data (Fig. 1) and previous ChIP data showing binding of MECP2 and DLX1/2 to Dlx5/6ei and eii (Bond et al., 2009).

Although it would be interesting to perform an analysis of double mutants lacking both *Mecp2* and *Dlx1/2* (*Mecp2null; Dlx1/2*$^{-/-}$), *Mecp2null; Dlx1/2*$^{+/-}$ females proved to be very poor breeders. Therefore, analysis of the double mutants proved unfeasible. However, future experiments to determine the state of MECP2 binding to Dlx5/6ei/eii in *Dlx1/2*$^{-/-}$ E13.5 MGE might be informative. As *Evf2* is lost in *Dlx1/2*$^{-/-}$ E13.5 MGE, lack of MECP2 binding to Dlx5/6ei/eii would further support *Evf2*-dependent recruitment of MECP2. However, if MECP2 remains associated to Dlx5/6ei/eii in the absence of both DLX1/2 and *Evf2*, this would support an alternate state in which *Evf2* is not required for MECP2 recruitment in the absence of DLX1/2.

Trans-acting mechanisms involving *Evf2*

In previous experiments, ectopic assays using cultured neural cell lines (Feng et al., 2006) or E13.5 MGE explants (Bond et al., 2009), suggested that *Evf2* transcriptional activity occurs in *trans*. Co-transfection of *Evf2* and *Dlx2* cDNAs into C17 neural cells demonstrated increased activity of Dlx5/6ei and eii in luciferase reporter assays (Feng et al., 2006). Electroporation of *Evf2* cDNA into E13.5 MGE increased expression of *Dlx5* and *Dlx6*, also supporting the observation that *Evf2* increases Dlx5/6ei and eii enhancer activity in *trans* (Bond et al., 2009). However, the levels of *Evf2* expressed by electroporation could not be determined. Evidence in this paper supports the involvement of *trans*-acting mechanisms during *Evf2* interactions with the Dlx5/6ei enhancer. Using *Evf2*$^{TS/TS}$*;R* transgenic mice, we show that *Evf2* transgene expression at these reduced levels does not change *Dlx5* or *Dlx6* expression, but does prevent methylation of the Dlx5/6ei enhancer. Multiple factors, including culture-induced conditions, could contribute to the differences found between the two assays. The finding that *Evf2* expressed by the transgene in *Evf2*$^{TS/TS}$*;R* mice is reduced (~0.38×) compared with wild type raises the possibility that low levels of *Evf2* can inhibit Dlx5/6ei methylation, but higher levels may be necessary to elicit transcriptional changes. Therefore, one possibility is that inhibition of Dlx5/6ei methylation is necessary, but not sufficient during *Evf2* transcriptional regulation. By lowering the level of *Evf2 in vivo*, transgenic expression might distinguish between methylation and transcriptional effects of *Evf2* on Dlx5/6ei. Another possibility is that complete methylation inhibition is not achieved by the transgene, as *Evf2*$^{TS/TS}$*;R* methylation (Fig. 4F) profiles are slightly different compared with those of *Evf2*$^{+/+}$ (Fig. 3B).

Enhancer methylation and transcriptional control

DNA methylation analyses have focused on CpG islands and the role of site-specific CpG methylation across enhancers is not established. Our results on the relationship between *Evf2* lncRNA, *Dlx1/2*, *Mecp2*, Dlx5/6ei enhancer CpG DNA methylation and transcriptional activity suggest a complex relationship in which *Evf2* is a key regulator of enhancer methylation profile.

Based on the results in this article combined with previous ChIP experiments showing *Evf2*-dependent recruitment of DLX and MECP2 proteins to Dlx5/6ei (Bond et al., 2009), a model for interactions at Dlx5/6ei is proposed (Fig. 6B). Evidence from this article and others demonstrates that *Dlx1/2* is required for *Evf2*, *Dlx5* and *Dlx6* expression (Fig. 6B, green arrows). Support that *Evf2* represses *Dlx6* expression through antisense inhibition (Fig. 6B, red arrow), is supported by Bond et al. (Bond et al., 2009), who showed that *Evf2* inhibition of *Dlx6* is greater than *Dlx5* inhibition, and that electroporated *Evf2* is unable to rescue *Dlx6* expression. The transgenic model in this article further supports the involvement of *Evf2 cis* regulatory activity (*Evf2*$^{TS/TS}$*;R*), i.e. *Dlx6* remains unaltered by *Evf2* transgene expression (Fig. 4), and *Dlx5* adjacent to *Evf2TS* increases (Fig. 2A, lanes 3 and 4). *Evf2ncRNA-trans* inhibits Dlx5/6ei methylation at two specific sites: ^{576}CpG and ^{757}CpG (Fig. 3A, red). This is supported by the analysis provided in Fig. 3B (^{576}CpG and ^{757}CpG methylation increases in *Evf2*$^{TS/TS}$ mutants compared with wild types), and Fig. 4F (^{576}CpG and ^{757}CpG methylation decreases in *Evf2*$^{TS/TS}$*;R* compared with *Evf2*$^{TS/TS}$).

Previous experiments (Bond et al., 2009) suggested that one possible explanation for *Evf2* repression of Dlx5/6eii is that *Evf2* recruits MECP2, recruiting HDAC1 to Dlx5/6eii, and inhibiting *Evf2* and *Dlx5* expression (red). However, in this article, we show that *Dlx6* levels do not change upon loss of MECP2 (Fig. 1). Therefore, it is possible that either MECP2 does not inhibit Dlx6 expression, or, by inhibiting *Evf2* expression, decreased *Dlx6* antisense inhibition balances MECP2 repressive effects. Whereas *Evf2*, *Dlx5* and *Dlx6* expression requires *Dlx1/2* (Fig. 1), loss of *Evf2* results in decreased DLX1/2 binding to Dlx5/6ei and eii (Bond et al., 2009). This leads us to propose that *Evf2* stabilizes, but is not

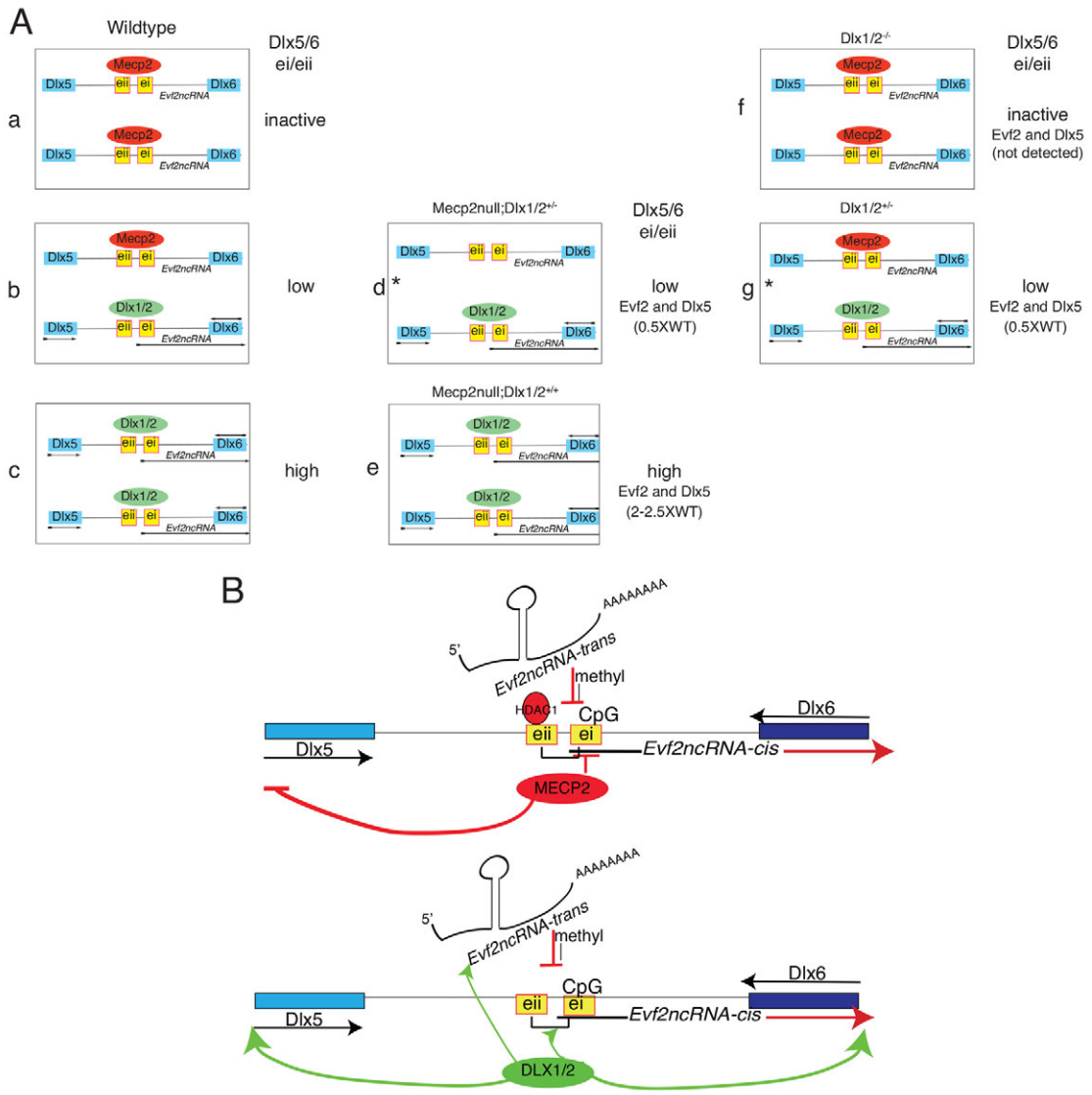


Fig. 6. Models describing *Mecp2*, *Dlx1/2* and *Dlx5/6* enhancer interactions. (**A**) Model describing the relationship between *Mecp2* and *Dlx1/2* occupancy of Dlx5/6ei and eii enhancers and transcriptional activity (see Discussion for details). (**B**) Model describing how the *Evf2* lncRNA facilitates differential dosage control of adjacent genes regulated by common enhancer elements. *Evf2* lncRNA inhibits enhancer methylation and mediates recruitment of transcriptional repressor and activator. Schematic summarizes the relationship between enhancer methylation, *Evf2* lncRNA *trans*- and *cis*-effects, and antagonism between recruited transcription factors DLX1/2 and MECP2. Genetic epistasis experiments support the hypothesis that binding of MECP2 occurs in competition with DLX1/2 at Dlx5/6ei and eii, rather than cooperatively. Removal of one copy of DLX1/2 from MECP2 null mice decreases levels of *Evf2* and *Dlx5*, supporting antagonism between MECP2 and DLX1/2. Whereas MECP2 represses *Dlx5* and *Evf2*, DLX1/2 activates *Dlx5*, *Dlx6* and *Evf2* expression. DLX1/2 increases *Evf2* expression, which inhibits ^{576}CpG and ^{757}CpG site-specific methylation of Dlx5/6ei in *trans*.

required for DLX1/2 binding, counteracting MECP2 repressive effects. In this model, *Evf2* lncRNA facilitates differential dosage of adjacent genes that are under the control of the same DNA regulatory enhancers through balanced recruitment of both activator and repressor, and antisense inhibition of one of the genes in a bi-gene cluster. A major question for future studies will be to determine how the levels of *Evf2* activator and repressor recruitment are determined.

Methylation studies of Dlx5/6ei in this article suggest that the methylation profile across the enhancer is determined by the *Evf2* lncRNA. However, the functional state of the Dlx5/6ei enhancer cannot be assessed by the CpG methylation profile. Dlx5/6ei methylation increases when the enhancer is inactive (*Dlx1/2*$^{-/-}$) and also when enhancer activity increases (*Evf2*$^{TS/TS}$). This is consistent with the idea that methylation reflects the presence of an RNA regulator that recruits both positive and negatively acting transcription factors. The model is complicated by the fact that when the *Evf2* transgene is expressed at 0.38× wild-type levels, Dlx5/6ei methylation significantly decreases, but *Dlx5* or *Dlx6* expression levels are not altered (Fig. 4D,E). There are many possible explanations. For instance, it could be argued that, although statistically reduced, the transgene does not completely inhibit methylation to the levels seen in wild type (compare Dlx5/6ei methylation profiles obtained in Fig. 3B and Fig. 4F). Therefore, the *Evf2* dose might need to be higher. Alternatively, *Evf2* *cis*-regulation might play a role in methylation inhibition.

An alternative possibility is that Dlx5/6ei enhancer methylation does not play a role in transcriptional regulation, reflecting only the

Development

presence of *Evf2* RNA. However, an argument against this is that *Evf2* prevents site-specific ^{576}CpG and ^{757}CpG Dlx5/6ei methylation, rather than random methylation over the 13 possible CpG sites tested. If methylation status is just a consequence of the presence of the RNA and does not play a functional role, site-specific methylation effects would not be expected. At this point, nothing is known about additional factors necessary for methylation of these two specific sites.

Taken together, the model described in Fig. 6B raises an intriguing possibility that the relationship between *Evf2* and enhancer methylation is dynamic, reflecting the dynamic nature of positive and negative transcription factor occupancy of Dlx5/6ei. If so, such dynamic changes might make it difficult to correlate on/off enhancer activity states with DNA methylation profiles *in vivo*.

Future directions

Given the identification of diverse classes of lncRNAs at the genome-wide level, this work raises many questions regarding lncRNA-dependent site-specific DNA methylation. (1) Do different classes of lncRNAs, in addition to ultraconserved lncRNAs, regulate site-specific methylation patterns in enhancer and non-enhancer regions? Or, is this mechanism unique to *Evf2* and Dlx5/6 ei ultraconserved enhancer? (2) How do site-specific enhancer methylation, lncRNA presence, enhancer activity and histone modifications correlate? Is there a histone modification signature that is associated with RNA-dependent enhancer methylation? (3) Are DNMTs and additional factors involved in RNA-mediated site-specific enhancer methylation? Future experiments will be necessary to define the significance of these relationships to gene regulation.

Acknowledgements

We thank Dr Marc Ekker (University of Ottawa) for Dlx1/2 enhancer plasmid; Dr Kenneth Campbell (University of Cincinnati, Children's Hospital) for sending *Dlx1/2*$^{+/-}$ mice; the Northwestern transgenic facility for pronuclear injections generating conditional *Evf2;R*$^{floxed\ TS}$ mice; Fabricio Costa and Bento Soares (Lurie Children's Research Center, Northwestern University) for advice on bisulfite sequencing; and Liying Yang (EpigenDx) for global methylation analysis of mouse B1-4 elements.

Funding

This work was funded by the American Reinvestment and Recovery Act award from the National Institute of Child Health and Human Development (NICHD) [R01HD056504]; the National Institute of Mental Health (NIMH) [R01MH094653]; Illinois Excellence in Academic Medicine; and Lurie Children's Research Center [all to J.D.K.]. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Author contributions

E.G.B. performed experiments shown in Figs 1 and 3-5, provided critical comments on manuscript and helped with manuscript preparation. M.F.C. developed and characterized *Evf2* rescue transgenic line (*Evf2*$^{TS/TS}$*;R*), contributing to the analysis shown in Fig. 4. S.C. performed experiments shown in Figs 2 and 4, and provided critical comments on manuscript. I.C. performed experiments shown in Figs 1, 4 and Fig. S1 and provided critical comments. D.E.L. contributed to experiments shown in Figs 1, 4 and 5. J.D.K. conceived of and directed experiments, and wrote the manuscript.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099390/-/DC1

References

Anderson, S. A., Eisenstat, D. D., Shi, L. and Rubenstein, J. L. (1997a). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* **278**, 474-476.

Anderson, S. A., Qiu, M., Bulfone, A., Eisenstat, D. D., Meneses, J., Pedersen, R. and Rubenstein, J. L. (1997b). Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron* **19**, 27-37.

Barlow, D. P. (2011). Genomic imprinting: a mammalian epigenetic discovery model. *Annu. Rev. Genet.* **45**, 379-403.

Bond, A. M., Vangompel, M. J., Sametsky, E. A., Clark, M. F., Savage, J. C., Disterhoft, J. F. and Kohtz, J. D. (2009). Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. *Nat. Neurosci.* **12**, 1020-1027.

Chahrour, M., Jung, S. Y., Shaw, C., Zhou, X., Wong, S. T., Qin, J. and Zoghbi, H. Y. (2008). MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* **320**, 1224-1229.

Feng, J., Bi, C., Clark, B. S., Mady, R., Shah, P. and Kohtz, J. D. (2006). The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. *Genes Dev.* **20**, 1470-1484.

Feng, S., Jacobsen, S. E. and Reik, W. (2010). Epigenetic reprogramming in plant and animal development. *Science* **330**, 622-627.

Forné, T., Oswald, J., Dean, W., Saam, J. R., Bailleul, B., Dandolo, L., Tilghman, S. M., Walter, J. and Reik, W. (1997). Loss of the maternal H19 gene induces changes in Igf2 methylation in both cis and trans. *Proc. Natl. Acad. Sci. USA* **94**, 10243-10248.

Ghanem, N., Yu, M., Long, J., Hatch, G., Rubenstein, J. L. and Ekker, M. (2007). Distinct cis-regulatory elements from the Dlx1/Dlx2 locus mark different progenitor cell populations in the ganglionic eminences and different subtypes of adult cortical interneurons. *J. Neurosci.* **27**, 5012-5022.

Guy, J., Hendrich, B., Holmes, M., Martin, J. E. and Bird, A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat. Genet.* **27**, 322-326.

Guy, J., Gan, J., Selfridge, J., Cobb, S. and Bird, A. (2007). Reversal of neurological defects in a mouse model of Rett syndrome. *Science* **315**, 1143-1147.

Horike, S., Cai, S., Miyano, M., Cheng, J. F. and Kohwi-Shigematsu, T. (2005). Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nat. Genet.* **37**, 31-40.

Imamura, T., Yamamoto, S., Ohgane, J., Hattori, N., Tanaka, S. and Shiota, K. (2004). Non-coding RNA directed DNA demethylation of Sphk1 CpG island. *Biochem. Biophys. Res. Commun.* **322**, 593-600.

Jeffery, L. and Nakielny, S. (2004). Components of the DNA methylation system of chromatin control are RNA-binding proteins. *J. Biol. Chem.* **279**, 49479-49487.

Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., Nishida, H., Yap, C. C., Suzuki, M., Kawai, J. et al.; RIKEN Genome Exploration Research Group; Genome Science Group (Genome Network Project Core Group); FANTOM Consortium (2005). Antisense transcription in the mammalian transcriptome. *Science* **309**, 1564-1566.

Kimura, M. I., Kazuki, Y., Kashiwagi, A., Kai, Y., Abe, S., Barbieri, O., Levi, G. and Oshimura, M. (2004). Dlx5, the mouse homologue of the human-imprinted DLX5 gene, is biallelically expressed in the mouse brain. *J. Hum. Genet.* **49**, 273-277.

Kohtz, J. D., Baker, D. P., Corte, G. and Fishell, G. (1998). Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. *Development* **125**, 5079-5089.

Lee, J. T. (2012). Epigenetic regulation by long noncoding RNAs. *Science* **338**, 1435-1439.

Lee, J. T. and Bartolomei, M. S. (2013). X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell* **152**, 1308-1323.

Mohammad, F., Mondal, T., Guseva, N., Pandey, G. K. and Kanduri, C. (2010). Kcnq1ot1 noncoding RNA mediates transcriptional gene silencing by interacting with Dnmt1. *Development* **137**, 2493-2499.

Nan, X., Campoy, F. J. and Bird, A. (1997). MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* **88**, 471-481.

Okita, C., Meguro, M., Hoshiya, H., Haruta, M., Sakamoto, Y. K. and Oshimura, M. (2003). A new imprinted cluster on the human chromosome 7q21-q31, identified by human-mouse monochromosomal hybrids. *Genomics* **81**, 556-559.

Panganiban, G. and Rubenstein, J. L. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. *Development* **129**, 4371-4386.

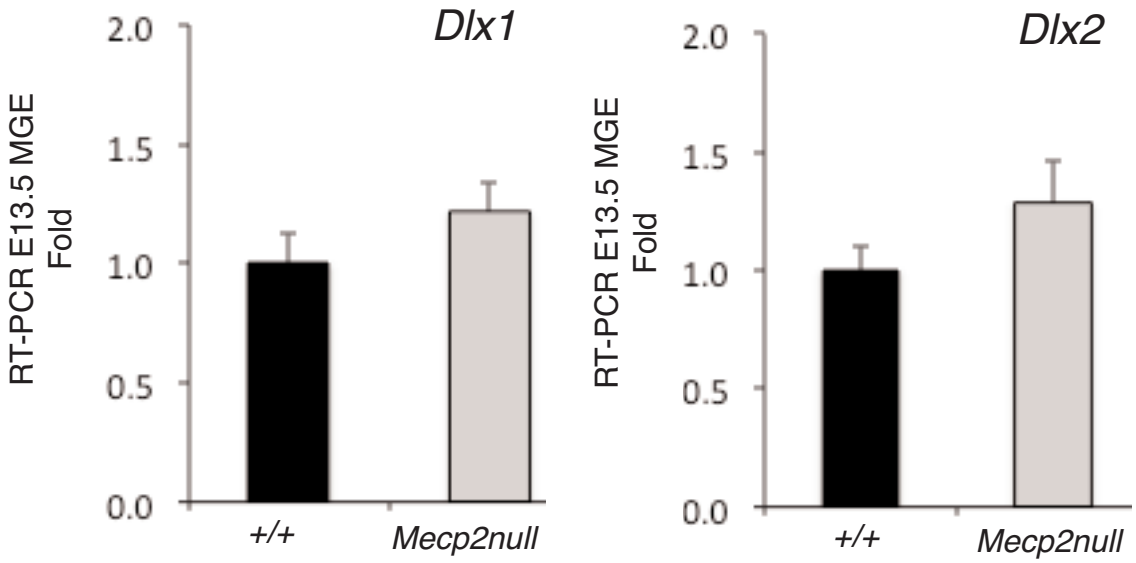
Poitras, L., Yu, M., Lesage-Pelletier, C., Macdonald, R. B., Gagné, J. P., Hatch, G., Kelly, I., Hamilton, S. P., Rubenstein, J. L., Poirier, G. G. et al. (2010). An SNP in an ultraconserved regulatory element affects Dlx5/Dlx6 regulation in the forebrain. *Development* **137**, 3089-3097.

Schüle, B., Li, H. H., Fisch-Kohl, C., Purmann, C. and Francke, U. (2007). DLX5 and DLX6 expression is biallelic and not modulated by MeCP2 deficiency. *Am. J. Hum. Genet.* **81**, 492-506.

Sigova, A. A., Mullen, A. C., Molinie, B., Gupta, S., Orlando, D. A., Guenther, M. G., Almada, A. E., Lin, C., Sharp, P. A., Giallourakis, C. C. et al. (2013). Divergent transcription of long noncoding RNA/mRNA gene pairs in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **110**, 2876-2881.

Smith, Z. D. and Meissner, A. (2013). DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14**, 204-220.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Uhlmann, K., Brinckmann, A., Toliat, M. R., Ritter, H. and Nürnberg, P. (2002). Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. *Electrophoresis* **23**, 4072-4079.
Yu, W., Gius, D., Onyango, P., Muldoon-Jacobs, K., Karp, J., Feinberg, A. P. and Cui, H. (2008). Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA. *Nature* **451**, 202-206.
Zerucha, T., Stühmer, T., Hatch, G., Park, B. K., Long, Q., Yu, G., Gambarotta, A., Schultz, J. R., Rubenstein, J. L. and Ekker, M. (2000). A highly conserved enhancer in the Dlx5/Dlx6 intergenic region is the site of cross-regulatory interactions between Dlx genes in the embryonic forebrain. *J. Neurosci.* **20**, 709-721.

Development



Supplemental Figure 1. *Mecp2* does not repress *Dlx1* or *Dlx2*. E13.5 MGE qRT-PCR from *+/+* and *Mecp2null* littermates compare levels of *Dlx1* and *Dlx2* transcripts. *+/+* (Black bars) *MECP2null* (gray bars). n= 3 for each genotype. $p>0.05$ for *Dlx1* and *Dlx2*. No significant differences are detected in *Dlx1* or *Dlx2* mRNA levels upon loss of *Mecp2*.