EGR1 recruits TET1 to shape the brain methylome during development and upon neuronal activity

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Life experience can leave lasting marks, such as epigenetic changes, in the brain. How life experience is translated into storable epigenetic information remains largely unknown. With unbiased data-driven approaches, we predicted that Egr1, a transcription factor important for memory formation, plays an essential role in brain epigenetic programming. We performed EGR1 ChIP-seq and validated thousands of EGR1 binding sites with methylation patterns established during postnatal brain development. More specifically, these EGR1 binding sites become hypomethylated in mature neurons but remain heavily methylated in glia. We further demonstrated that EGR1 recruits a DNA demethylase TET1 to remove the methylation marks and activate downstream genes. The frontal cortices from the knockout mice lacking Egr1 or Tet1 share strikingly similar profiles in both gene expression and DNA methylation. In summary, our study reveals EGR1 programs the brain methylome together with TET1 providing new insight into how life experience may shape the brain methylome.
t has been well acknowledged that early postnatal experience is critical for brain development and may induce long-lasting epigenetic changes in postmitotic neurons. Growing evidence indicates that learning and memory are highly dependent on the function of epigenetic machinery such as DNA methyltransferases (DNMTs) and DNA demethylases, the Ten-Eleven Translocation (Tet) proteins including TET1, TET2, and TET3. Double knockout of Dnmt1 and Dnmt3a leads to abnormal gene expression contributing to synaptic plasticity and learning and memory deficits. Genetic deletion or knock-down of each TET enzyme results in a unique set of phenotypes. Tet1 is involved in neural progenitor cell proliferation and neuronal activity-induced active DNA demethylation in the dentate gyrus of the adult mouse brain. Tet knockout mice exhibited impaired hippocampal neurogenesis, significant deficiency in short-term memory retention, abnormal long-term depression and impaired memory extinction. The deletion of Tet3 leads to neonatal lethality and neural progenitor cells induced from Tet3 knockout ES cells undergo apoptosis rapidly with reduced terminal differentiation of neurons. Significant impairment in fear extinction memory was observed in mice with Tet3 knockdown via shRNA. Although little is known about the role of Tet2 in neuronal differentiation or function, Tet2 knockout mice show abnormal hyper-methylation in the frontal cortex.

Despite the known needs of DNMTs and TETs for learning and memory, how these enzymes are directed to specific genomic loci in neurons remain elusive.

Neuronal activity-induced DNA methylation changes may occur within hours after electroconvulsive stimulation. This suggests that neurons can react to environmental stimuli and guide the epigenetic machinery to desired genomic loci swiftly. As an immediate early gene, Egr1 in mice, EGR1 in humans, also known as Zif268, Krox-24, and NGFI-A, can be rapidly and transiently induced by neuronal activity. Egr1 is a critical transcriptional regulator involved in brain development, learning, and long-term neuronal plasticity. With a rapid increase in expression during the first few weeks after birth, Egr1 controls the selection, maturation and functional integration of newborn neurons. A seminal study has established a link between maternal care and methylation programming during early postnatal brain development, and Egr1 was proposed to be an epigenetic regulator of glucocorticoid receptor. More interestingly, EGR1 has a binding motif containing CpG dinucleotides (5′-CGTGGGGGCGG-3′) and the binding of EGR1 to target DNA is insensitive to methylation. However, whether EGR1 can direct epigenetic machinery to its target sites upon neuronal activation is unknown.

Recently, we have implemented a nonparametric Bayesian clustering approach to identify genomic loci with bipolar DNA methylation patterns: the presence of both hypo-methylated and hyper-methylated patterns within a mixed cell population. In other words, for sequence reads mapped to a bipolar methylated locus, some of them are completely methylated while others could be completely unmethylated. With this approach, we observed the number of bipolar methylated loci increased dramatically during early stages of brain development and brain bipolar methylated loci were enriched for GWAS variants associated with neurological disorder-related diseases/trait. Interestingly, genes associated with brain bipolar methylated loci are involved in neuronal differentiation, cell migration and cell morphogenesis. In this study, we explored the epigenetic regulatory mechanism underlying the birth of bipolar methylated loci and identified EGR1 as a key mediator involved in brain epigenome programming during postnatal development. Our study provides the first compelling data demonstrating EGR1 recruits TET1 to demethylate EGR1 binding sites. Our results implicate the interaction between transcription factors (TFs) and epigenetic machinery as a general mechanism to achieve locus-specific epigenetic regulation upon neuronal activation.

**Results**

**EGR1 peaks lose methylation during brain development.** To explore epigenetic regulatory mechanisms during brain development, we followed our previous approach (see “Methods” for details) to re-analyze methylomes for frontal cortices at different developmental stages and identified a total of 11,178 (human) and 4692 (mouse) bipolar methylated loci within 10 kb upstream and downstream from transcription start sites (TSSs). For these bipolar methylated loci, we determined the methylation correlations between all possible pairs (Supplementary Fig. 1a and 1d) and identified five major co-methylated modules showing distinct methylation profiles during brain development and neural cell specification (Supplementary Fig. 1b and 1e). For instance, in mouse frontal cortices, the bipolar methylated loci in module I and II were hypomethylated in neurons. In contrast, the bipolar methylated loci in module III and IV were found to be hyper-methylated in non-neuronal cells, while the bipolar methylated loci in module V tended to show age-related methylation. Using HOMER, we determined the motifs for TFs enriched in each co-methylated module (Supplementary Fig. 1c, 1f and Supplementary Data 1) and identified Egr1 is associated with module I, the largest module for both human and mouse. More interestingly, the CpG dinucleotides within the EGR1 binding motifs are gradually demethylated during postnatal brain development and the methylation losses are limited in neurons (Supplementary Fig. 2).

To validate such a computational prediction, we performed ChIP-seq for EGR1 in duplicate with mouse frontal cortices at 6 weeks and identified 12,014 high-confidence peaks (Supplementary Fig. 3). Independent ChIP-qPCR assays were performed for six loci to confirm the significant enrichment of EGR1 binding in peaks identified (Supplementary Fig. 4a). From the sequences of EGR1 peaks, we determined the most significantly enriched motif as “GGCGGGGGGCGG” (Fig. 1a, E-value = 1.1e−522), which is similar to the canonical EGR1 response element reported previously. A total of 81.8% of EGR1 peaks localize in gene promoters (from TSSs to 2 kb upstream of TSSs) or within genomic regions (Fig. 1b), and the frequency of EGR1 binding sites increases when approaching transcription start sites (Supplementary Fig. 4b). We further integrated EGR1 peaks with ChIP-seq datasets for histone modifications and observed that active enhancer mark H3K27ac and active promoter mark H3K4me3 are strongly enriched in the vicinity of EGR1 peaks (Fig. 1c). More specifically, 46.9% and 44.4% of EGR1 binding sites overlap with H3K27ac peaks and H3K4me3 peaks, respectively. Additional ChIP-qPCR assays validated that the H3K27ac mark is enriched at the six genomic loci with EGR1 binding (Supplementary Fig. 4c). These results suggest that EGR1 mainly binds active promoters or enhancers to activate the expression of downstream genes in the mouse frontal cortex.

To examine methylation dynamics of EGR1 peaks, we made use of embryonic forebrain methylomes recently released by ENCODE to obtain the methylation profiles for 12,014 EGR1 peaks during brain development. 51.0% of EGR1 binding sites show constant hypomethylation (methylation level <= 0.2) throughout all developmental stages with methylomes available from E11.5 to 22 m (Supplementary Fig. 5). On the other hand, 34.3% of EGR1 binding sites (n = 4,125) exhibit methylation dynamics during development with the maximum methylation difference between stages greater than or equal to 0.2 (The range of methylation was defined from 0 to 1 in this study). The
majority of these EGR1 binding sites show decreased methylation changes during development and become hypomethylated in neurons (Fig. 1d). We next focused on the comparisons of DNA methylation between d0 and 6-week-old frontal cortices, and between NeuN+ and NeuN− cells at 7 week. Out of the 4,125 EGR1 binding sites with methylation variation, 2,106 (51.1%) showed methylation decrease by at least 20% during postnatal development (d0 to 6 week) and 3,451 (83.7%) showed methylation decrease by at least 20% in neurons compared with those in glial cells. In contrast, only 111 (2.7%) and 37 (0.8%) of EGR1 binding sites showed methylation increase by at least 20% during development or in neurons, respectively. We next asked if the methylation changes on EGR1 binding sites during development and between the two cell types were correlated. We found that 1,925 out of 2,106 (91.4%) EGR1 binding sites hypomethylated in adult frontal cortex were also hypomethylated in neurons. In addition, the methylation changes in d0 vs. 6 week, and glia vs. neuron were significantly positively correlated (Fig. 1e, Pearson’s R = 0.35, p-value = 1.31e−122). To determine if the 1,925 EGR1 peaks are linked to specific functions, we performed Gene Ontology (GO) enrichment analysis and found that genes with TSS flanking 10 kb of these EGR1 peaks are significantly enriched in the regulation of ion membrane transport, which is important for membrane potential formation and action potential propagation in neurons (Supplementary Fig. 6 and Supplementary Data 2).

**EGR1 interacts and recruits TET1 to its target binding sites.** A recent report indicated that a member of EGR family, WT1 (Wilms tumor suppressor gene 1) may recruit TET2 to demethylate its binding sites in leukemia cells. More specifically, the zinc-finger domain (residues 323–449) of WT1 binds directly to the CD domain (C-terminal region) of TET2 enzyme. WT1 and EGR1 share a similar structure and bind to a same consensus DNA sequence. Interestingly, the three TET family members
also share significant homology\textsuperscript{36}. These findings raise the possibility that EGR1 may interact with TET enzymes to program the brain methylome. To test this hypothesis, we reanalyzed RNA-seq data\textsuperscript{17} for mouse frontal cortices to examine the expression profiles of \textit{Egr1} and \textit{Tet} gene family (\textit{Tet1}-3) during mouse brain development. \textit{Egr1} transcript in mouse frontal cortices rapidly increased during fetal to 2-weeks, maintaining at a higher level throughout later developmental stages (Supplementary Fig. 7a). The levels of \textit{Tet2} and \textit{Tet3} expression gradually decrease during development while \textit{Tet1} shows an increased expression level during the second postnatal week (Supplementary Fig. 7b). We further examined the methylation profiles of EGR1 binding sites in \textit{Tet2} knockout mice\textsuperscript{17}. It has been reported that 19.7% of regions hypo-methylated in adult frontal cortex vs. fetal are with increased methylation in adult \textit{Tet2} knockout mice\textsuperscript{17}. Interestingly, we found that EGR1 binding sites show no significant methylation difference (Wilcoxon rank-sum test) between \textit{Tet2}\textsuperscript{−/−} and wild-type mice (Supplementary Fig. 8). This indicates that \textit{Tet2} is not required for the demethylation of EGR1 binding sites.

The expression of mouse full-length TET1 protein (2,039 aa, \~220 KDa) is restricted to early embryonic and primordial germ cells, and a short isoform TET1s (residues 654–2,039, \~170 KDa) is expressed in somatic tissues including brain\textsuperscript{37}. To further determine whether TET1 may participate in the demethylation of EGR1 binding sites, we performed co-immunoprecipitation assays (Co-IP) using mouse frontal cortices. The short isoform TET1s was found in the EGR1 immunoprecipitated complex (Fig. 2a) and EGR1 could be precipitated together with TET1s (Fig. 2b). To narrow down the binding regions responsible for the EGR1-TET1s interaction, we first conducted Co-IP in HEK293T cells with Flag-tagged EGR1 and HA-tagged TET1 deletion mutants as shown in the schematic illustration were co-expressed in HEK293T cells. Protein-protein interactions were examined by IP-western blot using the antibodies indicated. Source data are provided as a Source Data file.

\textbf{Fig. 2} Identification of the protein-protein interaction between EGR1 and TET enzymes by co-immunoprecipitation. \textit{a, b} Endogenous association of EGR1 and TET proteins. EGR1 was immuno-precipitated from mouse frontal cortex, followed by western blot to detect TET1 (\textit{a}). TET1 was immuno-precipitated from mouse frontal cortex, followed by western blot to detect EGR1 (\textit{b}). Normal rabbit IgG served as a negative control for immunoprecipitation. IP, immunoprecipitation. \textit{c} Interactions between full-length EGR1 (EGR1-FL) and TET1 deletion mutants. Flag-tagged EGR1-FL and HA-tagged TET1 deletion mutants as shown in the schematic illustration were co-expressed in HEK293T cells. \textit{d} Interactions between TET1s-CD and EGR1 deletion mutants. HA-tagged TET1-CD and Flag-tagged Egr1 deletion mutants as shown in the schematic illustration were co-expressed in HEK293T cells. Protein-protein interactions were examined by IP-western blot using the antibodies indicated. Source data are provided as a Source Data file.
involved in neuron differentiation

significantly decreased in the frontal cortices derived from Egr1 knockout mice (Egr1KO) compared with those from wild-type mice. Only eight EGR1 peaks were found to overlap with these 751 TET1 peaks and no EGR1 peak overlaps with the rest 104 peaks identified in Egr1KO only. However, 61 EGR1 peaks overlap with the 699 TET1 peaks identified in WT but not in Egr1KO. GO terms including “axon guidance” and “cell morphogenesis involved in neuron differentiation” were enriched for genes associated with TET1 peaks identified in WT mice but not in Egr1KO mice (Supplementary Fig. 11). We further examined the influence of Egr1 loss on the distribution of TET1 peaks. Compared to those in WT control, TET1 peaks in Egr1KO mice shift away from their adjacent EGR1 peaks (Fig. 3e). Altogether, these results indicate that EGR1 may attract TET1 to genomic regions flanking EGR1 binding sites.

EGR1 coordinates with TET1 in gene expression regulation. We examined whether the interaction between EGR1 and TET1 would have epigenetic regulatory effects. We started with two loci within Galnt9 and Npas4 genes, which were identified to be EGR1 binding sites by our ChiP-seq and validated by ChiP-qPCR to be enriched for H3K27ac enhancer mark (Supplementary Fig. 4c). To test their enhancer activities in primary culture of cortical neurons (Supplementary Fig. 12), the genomic fragments were cloned to the upstream of EF1 promoter in the pCpG-free vector, respectively. Compared with the control vector, Npas4 and Galnt9 loci significantly promoted the gene expression from the basal EF1 promoter in the enhancer luciferase assays (Fig. 4a). To further examine whether their enhancer activities are under epigenetic control, prior to transfection, the constructs containing two loci were methylated in vitro with CpG methyltransferase, M.SssI. The methylation of these loci greatly reduced their enhancer activities (Fig. 4b). We utilized the unmethylated and methylated reporter constructs to examine their enhancer activities with or without EGR1 or/and TET1 overexpression. For unmethylated reporter constructs, EGR1 overexpression alone could significantly increase luciferase signals of reporters,
consistent with the fact that EGR1 as a transcriptional factor can induce the enhancer activities of its binding sites. By contrast, TET1 overexpression alone displayed no increase in luciferase signal of these reporter vectors (Fig. 4b–c). For methylated reporter constructs, we observed increases in the enhancer activities when EGR1 was co-overexpressed with TET1 ($p = 0.01$ for *Galnt9* and $p = 0.08$ for *Galnt9*). This result suggests that EGR1 and TET1 may cooperate to activate the expression of EGR1 downstream gene by DNA demethylation.

To further investigate the epigenetic effect of EGR1 and TET1 on their binding sites, we examined the methylation statuses of *Galnt9* and *Npas4* loci in primary neurons with Egr1 or/and Tet1 knockdown. Primary mouse cortical neurons isolated at E16.5 were knocked down with their corresponding shRNA constructs (Supplementary Fig. 13b). To explore whether Egr1 or Tet1 alone displayed no increase in luciferase activity when EGR1 was co-overexpressed with TET1 ($p = 0.01$ for *Galnt9* and $p = 0.08$ for *Galnt9*). This result suggests that EGR1 and TET1 may cooperate to activate the expression of EGR1 downstream gene by DNA demethylation.

To further investigate the epigenetic effect of EGR1 and TET1 on their binding sites, we examined the methylation statuses of *Galnt9* and *Npas4* loci in primary neurons with Egr1 or/and Tet1 knockdown. Primary mouse cortical neurons isolated at E16.5 was adopted as in vitro neuronal activity model for KCl knockdown. Western blot showed that EGR1 increased was adopted as in vitro neuronal activity model for KCl knockdown. Western blot showed that EGR1 increased dramatically upon neuronal activation, while TET1 protein level remains relatively constant (Supplementary Fig. 13a). Importantly, methylation level of *Galnt9* and *Npas4* loci significantly reduced in E16.5 cortical neurons upon KCl stimulation (Supplementary Fig. 13b). To explore whether Egr1 and Tet1 mediate methylation changes at these two loci, Egr1 and Tet1 were knocked down with their corresponding shRNA constructs (Supplementary Fig. 13c). Pyrosequencing results for bisulfite converted DNA indicated that Egr1/Tet1 shRNA knockdown or the double knockdown significantly increased their methylation levels (Supplementary Fig. 13d). The RT-qPCR results showed that the mRNA level of *Galnt9* was reduced with Egr1 knockdown, and significantly lower with Egr1 and Tet1 double knockdown. However, no significant change in *Npas4* mRNA level was observed (Supplementary Fig. 13e). This may be due to the incomplete knockdown of *Egr1/Tet1* and the subtle methylation changes (~3%) achieved on *Npas4* locus over a short period of stimulation. Collectively, these data suggest that EGR1 is able to coordinate with TET1 to epigenetically regulate its target loci but the significant alteration in expression of some downstream genes may require repeated stimulations over a long period of time.

**EGR1KO and TET1KO mice share aberrant methylation profiles.** It has been documented that Egr1KO mice show impaired long-term memory. Recent studies show Tet1 knockout mice (Tet1KO) exhibited significant deficiency in memory retention, abnormal long-term depression and impaired memory extinction. To examine the epigenetic effects of *Egr1* or *Tet1* loss, we performed methylation and transcriptome analyses for the frontal cortices derived from Egr1KO and Tet1KO mice. The genotypes of Egr1KO and Tet1KO mice were confirmed by the read coverage among *Egr1* and *Tet1* loci (Supplementary Fig. 14). Since EGR1 binding sites are enriched in promoters and CG rich regions, we performed reduced representation bisulfite sequencing using restriction enzymes Msel and MluCI to remove AT-rich regions. For four methylation, we generated 211 to 287 million read pairs with an average of 140 million read pairs uniquely mapped to mouse reference genome (Supplementary Data 4). On average, we obtained methylation information for 48.9% of all CpG dinucleotides in the mouse genome and 18.1% of all CpG sites covered by at least 10 reads. Based on spiked-in unmethylated λ DNA control, the bisulfite conversion rates for four libraries were determined as 99.0% on average.

We observed strong correlations between biological replicates for two Egr1KO mice and two Tet1KO mice, respectively (Supplementary Fig. 15). For the corresponding four transcriptomes, we generated 39 million read pairs on average, 86.0% of which were unambiguously mapped (Supplementary Data 4). We determined pairwise Pearson’s correlation at gene expression level and validated the consistency of RNA-seq results for biological replicates (Supplementary Fig. 16). Compared with the methylation of the frontal cortex from wild-type mice, we identified 49,991 differentially methylated sites (DMRs) in Egr1KO with 34,747 (69.5%) hypermethylated and 15,244 (30.5%) hypomethylated, and 113,488 DMSs in Tet1KO with 94,862 (83.6%) hypermethylated and 18,626 (16.4%) hypomethylated. To examine the association between EGR1 binding and methylation changes in KO mice, we determined the distribution of DMSs at the flanking of EGR1 binding sites. The density of hypermethylated DMSs in Egr1KO increases when approaching to the centers of EGR1 peaks, while hypomethylated DMSs in both Egr1KO and Tet1KO are depleted from EGR1 peaks (Supplementary Fig. 17a). When DMSs were clustered into differentially methylated regions (DMRs), the increased enrichment in EGR1 peaks was observed for hypermethylated DMRs from both Egr1KO and Tet1KO mice (Supplementary Fig. 17b). We next focused on the aforementioned 1,925 EGR1 binding sites, which display methylation loss from d0 to 6 weeks. Approximately 83.0% and 84.5% of these loci show increased methylation in KO mice; particularly, 19.4% and 24.7% loci are with hypermethylated DMRs in Egr1KO and Tet1KO mice respectively. These results indicate that EGR1 and TET1 are indispensable for the demethylation of some EGR1 binding sites during brain development.

Compared to wild-type mice, 322 and 2,373 DMRs were identified in the frontal cortices of Egr1KO and Tet1KO mice...
respectively and these DMRs are significantly overlapped (Hypergeometric test, \( p \)-value = 8.36e-14). In addition, the methylation correlation of the overlapping 184 DMRs between Egr1KO and Tet1KO is 0.88 (Pearson’s \( r \)). The knockout of Tet1 has a broader and more severe impact on the methylomes compared to the loss of Egr1. Intriguingly, for DMRs identified in Tet1KO mice only, moderate changes in methylation were often observed in Egr1KO mice as well, and vice versa (Fig. 5a). We further divided DMRs into hypermethylated or hypomethylated in either Egr1KO or Tet1KO and obtained their methylation profiles across developmental stages and in neuronal cell types (Supplementary Fig. 18). Methylation loss during development and in neurons was observed for around 78.0% and 56.2% of DMRs identified in Egr1KO and Tet1KO mice, respectively. Interestingly, 9.1% Egr1KO DMRs and 13.9% Tet1KO DMRs were found to be constantly hypomethylated across different stages.

Fig. 5 Correlations of DNA methylation and gene expression profiles between Egr1KO and Tet1KO frontal cortices. Methylation correlations (a) and gene expression correlations (b) between Egr1KO and Tet1KO mice. c Aberrant DNA methylation on Galnt9 and Npas4 loci. Each CpG is represented by a circle; yellow in circles indicates the percentage of methylation in each CpG site. The statistical significance of methylation differences between Egr1/Tet1KO and WT mice was evaluated with the Wilcoxon rank-sum test. d The correlations between DNA methylation levels of Galnt9, Npas4 loci and corresponding gene expression during brain development from embryonic day 11.5 (E11.5) (denoted in blue color) to 22 months (22 mo) (denoted in red color).
developmental stages but with increased methylation in KO mice. This result suggests EGR1 and TET1 are required for the maintenance of demethylation statuses for some genomic loci, which are not or lowly methylated since early brain development (at E11.5 or earlier).

Compared to wild-type controls, 896 and 1,359 differentially expressed genes (DEGs) were determined in Egr1KO and Tet1KO mice respectively with 529 of them shared (Fig. 5b). Similar to what observed for methylomes, for these 529 genes, the correlation in the expression changes vs wild type is 0.94 (p-value = 1.14e-256) between Tet1KO mice and Egr1KO mice. For all DEGs identified either in Egr1KO or Tet1KO mice, strong correlations were observed between the two kinds of KO mice. GO annotation analyses showed that both Egr1KO and Tet1KO DEGs are involved in several biological processes (BPs) related to central nervous system development or neural tube development, including potassium ion transport and Notch signaling pathway which plays critical roles in brain development39 (Supplementary Fig. 19 and Supplementary Data 5). To explore the relationship between DMR methylation and gene expression, we calculated the Spearman’s correlation coefficients of hypomethylated DMRs identified in Egr1KO or Tet1KO mice (Supplementary Fig. 20). Negative correlations between methylation level and gene expression were observed for DMRs in 5’-UTR, Promoter and Distal Promoters. In addition, significant increases in methylation were observed in KO mice for the three EGR1 binding sites of these loci, Galnt9 and Npas4 genes (Fig. 5c). The methylation levels of these loci, Galnt9 in particular, are negatively correlated with gene expression (Fig. 5d).

Lastly, hypermethylated DMRs identified in either Egr1KO or Tet1KO show low methylation in excitatory neurons compared with PV and VIP neurons (Supplementary Fig. 18). This prompts us to make use of single-cell brain methylome data40 for additional bioinformatics analyses on the cell-subtype-specific function of EGR1 bindings. We confirmed that the hypermethylated DMSs identified in Egr1KO mice are significantly enriched on excitatory-neuron-specific hypomethylated regions determined in single cell analyses (Supplementary Fig. 21), especially for excitatory-neuron-subtype mL5-1 (odds ratio = 1.4, Binomial test, p-value = 4.8e-42). EGR1 binding sites are significantly enriched on excitatory-neuron-specific hypomethylated regions but excluded from inhibitory-neuron-specific ones (Supplementary Fig. 22). For instance, the enrichment of EGR1 binding sites on hypomethylated regions in excitatory-neuronal subtype mL4 is highly significant (odds ratio: 1.7, Binomial test, p-value = 6.7e-83). In addition, the enrichment of EGR1 binding is correlated with the bindings of other early response genes including TFs induced by neuronal activity, such as JUNB, FOSB, CFOS, and Npas4 (Supplementary Fig. 23).

**Discussion**

The link between epigenetic changes and neuronal activity has been well established, together with the gradual recognition of critical roles of TET DNA demethylases in learning and memory6–9. Apparently, epigenetic changes upon neuronal activation are not random but TET enzymes do not display DNA binding specificity. Our study shows how TET1 gains its specificity via the interaction with EGR1, a sequence specific DNA binding protein. On the other hand, as a key member of immediate early genes, Egr1 has been known for decades to play an essential role in transcriptional response to environmental stimuli. Egr1 is an important mediator of the effects of early-life experience and directly regulate genes controlling synaptic plasticity in both physiological and pathological conditions1,20,23,38. Egr1 expression has been widely used as a marker for neuronal activation but how it leaves memory trace remains elusive. In this study, we provided a key piece of evidence that may help in solving this puzzle at the epigenetic level. Although neurogenesis and neuronal migration are largely completed at birth in mouse, postnatal brains continue forming synapses and neural circuits and undergo activity-dependent refinements. Egr1 has been shown to control newborn neuron selection and maturation during the critical period of a few weeks after birth21. The decoding of epigenetic machineries during this developmental period is critical for a complete understanding of the mechanisms that underlie late-stage refinement of maturing neuronal circuits. Of note, Egr1 gene continues to have functions in the adult brain and may have pathological significance in Alzheimer’s disease41.

Our study provides several key evidences for the interaction between EGR1 and TET1. First, our results reveal that extensive DNA demethylation occurs in thousands of EGR1 binding sites during the postnatal frontal cortex development. Second, the C-terminals of EGR1 and TET1 are required for their interaction. The co-occupancy of EGR1 and TET1 at target loci were confirmed with sequential ChIP analyses. In the presence of EGR1, TET1 is capable to achieve locus-specific demethylation and activate the expression of EGR1 downstream genes. Third, both EGR1 and TET1 are indispensable for the demethylation of a common set of EGR1 binding sites that show aberrant DNA methylation in Egr1KO and Tet1KO mice. Altogether, our data support a model that links environmental stimuli to brain methylome programming (Fig. 6). At birth, a subset of Egr1-mediated and neuronal activity-induced genes are silenced with methylated EGR1 binding sites. During early postnatal development, the overexpression of Tet1 and Egr1 upon neuronal activation demethylate EGR1 binding sites and shift the genes to either “Poised” or “ON” states. DNA methylation cannot block EGR1 binding but may prevent the bindings of other transcription factors, which bind to the regions adjacent to EGR1 binding sites. Thus, the demethylation of EGR1 binding sites may facilitate the formation of stronger transcription enhancesomes.

![A simplified model for EGR1 and TET1 interaction linking environmental stimuli to brain methylome programming. At birth, Egr1-mediated and neuronal activity-induced genes are silenced with methylated EGR1 binding sites. During postnatal development and upon neuronal activity, the increase in expression of Tet1 and Egr1 leads to the demethylation of EGR1 binding sites to facilitate the binding of co-factors and shifts the genes to either “Poised” or “ON” states. DNA methylation cannot block EGR1 binding but may prevent the bindings of other transcription factors, which bind to the regions adjacent to EGR1 binding sites. Thus, the demethylation of EGR1 binding sites may facilitate the formation of stronger transcription enhancesomes.](image-url)
demethylation of EGR1 binding sites may facilitate the formation of stronger transcription enhanceosomes.

Our study raises a few interesting questions. Could EGR and TET interaction become a general mechanism for various kinds of cells to keep epigenetic memory in response to stimuli? EGR family members are involved in a variety of BPs and the epigenetic memory may not be limited to the nervous system. For instance, Egr1, Egr2 and Egr3 have been shown to be critical for the response to external signals and to direct lineage differentiation in immune system22,23. More dedicated study is needed to fully address how TET and EGR family members may interact with each other and to understand their combinatorial interactions in various BPs. In our study, we found Egr1KO and Tet1KO have significant effects but limited to less than 30% of the 1,925 EGR1 binding sites which show demethylation during postnatal brain development. Compared with the loss of Egr1, the Tet1 knockout has a much more severe outcome and broader impact in epigenome. These results suggest a compensatory mechanism to rescue the loss of Egr1 or Tet1 in the frontal cortex, and TET1 may have other partners to recognize genomic loci where EGR1 can’t bind. Since EGR1 is a CREB activity induced TF, EGR1 protein level is very low in neurons at rest. TET1 protein levels remains relatively constant before and after KCl stimulation. This suggests the majority of TET1 proteins are not necessarily bound by EGR1 at all times. Even with the massive EGR1 expression pattern in activated neurons, the interaction between TET1 and EGR1 could be transient. As reflected in our TET1 ChiP-seq result, 51.8% of TET1 peaks identified in WT mice were present in Egr1KO mice. In this study, we found that the genome distribution of TET1s binding in brain is biased toward intergenic regions, compared to those full length TET1 binding sites reported in mouse embryonic stem cells.27,44,45. The short isofrom TET1s expressed in mouse brain lacks CXXC and BC domain (“before CXXC”). The CXXC domain helps the binding of full length TET1 to CpG rich regions while the BC domain assists in its global chromatin affinity. Thus, TET1s shows low chromatin affinity and increased presence in soluble fraction instead of chromatin57. Our finding is also consistent with a recent study with cancer cells that the full length TET1 protects CpG islands from methylation while TET1s mediates demethylation outside CpG islands and the recruitment of TET1s to chromatin likely needs specific factors46. Additional effort is required to identify other proteins associated with TET1s to gain a better understanding on how it functions in brain. Lastly, the integrated bioinformatics analyses with single neuron methylomes suggest the methylation changes of EGR1 binding sites are largely restricted to a subset of excitatory neurons. It would be interesting to explore whether different kinds of neurons would adopt distinct epigenetic programming mechanisms in future.

Methods

Animals. All animal experiments were performed according to guidelines of the Institutional Animal Care and Use Committee at Virginia Tech (Blacksburg, VA, USA). The Egr1 heterogeneous mouse strain (B6; 129-Egr1tm11ml/l), the Tet1 heterogeneous mouse strain (B6; 129S4-Tet1tm1111/l) were purchased from The Jackson Laboratory. Genomic DNA was isolated from tail biopsies and genotyped by PCR according to The Jackson Laboratory’s instructions. Media was changed at 24 h after transfection. Infectious plasmids encoding Flag-tagged EGR1 N-terminal (amino acids 1–318), EGR1 C-terminal (amino acids 318–533), EGR1A2 (amino acids 1–335, 417–533) were generated by subcloning of the DNA fragments into E16.5 mouse cortical neurons and HEK293T culture. Brieﬂy, E16.5 mouse cortical neurons were seeded on 8-well chamber and cultured in vitro for 7 days (DIV7). The neurons were rinsed once with PBS and ﬁxed with 4% paraformaldehyde in PBS for 15 min at room temperature. After washing three times with PBS, cells were permeabilized with 0.2% TritonX-100 in PBS for 10 min. Cells were then washed three times with PBS, blocked with 5% Normal Goat Serum (ThermoFisher) in 1×PBS for 1 h, followed by incubation with primary antibodies at 4 °C overnight. After washing 3 times with 1×PBS, cells were incubated with corresponding secondary antibody for 1 h at RT. After another wash, cells were mounted with DAPI-Fluoromount-G®. Primary mouse cortical neurons and HEK293T (ATCC, CRL-11268™) cells were maintained in Dulbecco’s Modiﬁed Eagle’s Medium (DMEM) (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Corning) and 1% penicillin/streptomycin (Gibco). All cells were cultured at 37 °C in a humidiﬁed atmosphere of 5% CO2 incubator.

Antibodies. Rabbit anti-TET1 (Millipore, 09–872), mouse anti-TET1 (Active Motif, 91171), rat anti-TET1 (Active Motif, 61741, refer to as S6), mouse anti-HA (Invitrogen, 26183), mouse anti-Flag (sigma, F1804 and F7425), mouse anti-Tuj1 (Biologend, 801201), rabbit anti-GFAP (Sigma, HPA05630) antibodies were purchased commercially. Rabbit anti-EGR1 antibody (sc-189), mouse anti-EGR1 antibody (sc-101033), rabbit normal IgG (sc-2027) and mouse normal IgG (sc-2025) were purchased from Santa Cruz Biotechnology. For western blot analysis, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Invitrogen, 65–6120) was used at a 1:5000 dilution, goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma, A8924) was used at a 1:10000 dilution. The antibodies used for the IP and western blot experiments were summarized in Supplementary Data 6.

Plasmid construction. Flag-tagged mouse EGR1 expression vector was obtained from Addgene (plasmid #11729). Plasmids encoding Flag-tagged EGR1 N-terminal (amino acids 1–318), EGR1 C-terminal (amino acids 318–533), EGR1A2 (amino acids 1–335, 417–533) were generated by subcloning of the DNA fragments into E16.5 mouse cortical neurons and HEK293T culture. In order to clone truncated versions of EGR1 plasmid DNA was synthesized from mRNA of C57BL/6 mice. Plasmids encoding HA-tagged Tet1s (amino acids 654–2039), Tet1s-N (amino acids 654–1366), Tet1s-CD domain (amino acids 1367–2039), Tet1s-CDΔc (amino acids 1358–2039) were generated by subcloning of the DNA fragments into BamHI and XhoI of pcDNA3 vector. For shRNA cloning, pLKO.1 puro, a gift from Bob Weinberg (MIT) (Addgene plasmid # 4545) was digested with AgeI and EcoRI for 4 h at 37 °C. Digested plasmid was excised from the gel and purified with GeneJET gel extraction kit (ThermoFisher). Oligos were designed for the following target sequences and annealed (Tet1 shRNA GCTCATGGAGCATGTTTGG, Egr1 shRNA AGGCGTAGACCATAGGT) as described previously28,48. Annealed oligos were ligated into the digested vector with T4 ligase and filled with NcoI and EcoRI restriction enzymes to generate scrambled shRNA. Scramble shRNA was a gift from David Sabatini (MIT) (Addgene plasmid # 1864).

For luciferase constructs, two selected EGR1 binding loci: Galnt9 and Nps4 loci were amplified from genomic DNA of C57BL/6 mice using the primers listed in Supplementary Data 7. After enzymatic digestion for at least 4 h, PCR-amplified products were cloned into pcPlFire-promoter-Lucia Vector (Invivogen). All inserts were verified by Sanger sequencing.

Lentivirus generation and transfection. Dishes were plated with 50 μg/mL poly-D-lysine (Sigma) and HEK293T cells (ATCC® CRL-3216™) were plated at ~70% confluence before transfection. Helper and shRNA constructs were transfected at a 1:3 ratio using X-tremeGENE® G (Roche) according to the manufacturer’s instructions. Media was changed at 24 h after transfection. Infectious media containing virus was collected 40 h later and filtered with a 0.45μM PES membrane filter (Millipore). Viral media was concentrated at 100,000 g for 90 min at 4 °C (Beckman Optima Max tabletop ultracentrifuge). Viral pellets were resuspended in sterile PBS overnight at 4 °C. Lentiviral particles (IU/mL) were determined by real-time qPCR (BioRad CFX96) using the qPCR Lentivirus kit (Roche).
Titration(Titer) Kit (ABM) according to the manufacturer’s instructions using iTaq™ Universal SYBR® Green Supermix (BioRad). Lentivirus particles were added to cortical neuron cultures at DIV5 with final concentration of 1.0 × 10⁶ IU/mL. After incubation for 24 h, the medium was replaced with fresh neuronal culture medium and continue culture for another 24 h prior to KCl stimulation.

Luciferase reporter assays. Luciferase reporter constructs were either mock-treated or methylated in vitro with M.SssI methylase (NEB) for at least 4 h at 37 °C and purified with PureLink PCR Purification Kit (Qiagen). E16.5 mouse cortical neurons were seeded at 3 × 10⁵ cells/well in 24-well plates overnight, then transected with FuGENE HD (Promega) or Lipofectamine 2000 (Invitrogen). For each sample, triplicate transfections were carried out. 48 h after transfection, cell lyses and the medium were assayed for luciferase activity by Dual Luciferase Reporter Assay (Promega). Lucifer luciferase activity of individual transfections was normalized to firefly luciferase activity and analyzed relatively to empty pCPG-free promoter vector58.

qRT-PCR analysis. Total RNA was extracted using the RNaseasy kit (Qiagen) and cDNA was generated using a high-capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR experiments were performed using GoTaq® qPCR Master Mix (Promega) on StepOnePlus™ Real-Time PCR Systems. Relative expression levels were determined by comparative ΔΔCt method with beta-actin as an internal reference control.

Co-immunoprecipitation and western blotting. HEK293T cells were transfected with plasmids by Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. Cell lysis, immunoprecipitation, and western blot analysis was performed as previously described59. Briefly, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) supplemented with protease inhibitor cocktail (Thermo scientific). Immunoprecipitation was carried out by incubating specific antibody coupled Dynabeads Protein A or Protein G (Life Technologies) at 4 °C overnight. The samples were washed four times with ice-cold lysis buffer, and then suspended in 30 μl loading buffer (Life Technologies). After boiling at 95 °C for 5 min, the samples were analyzed by western blot with specific antibodies.

ChIP-seq data analysis. All reads for ChiPseq libraries were first trimmed according to their sequencing qualities, then the trimmed reads were mapped to the mouse reference (mm10) following the procedure described previously50. Second, at each cluster which include at least 5 CpG sites, at least 80% of significant peaks were considered as DMR candidates. Second, after bisulfite conversion, the single-stranded uracil-containing DNA was subjected to 150-bp paired-end read sequencing on the Illumina HiSeq 4000 platform with 75 bp paired end mode (Illumina). Methylation analysis was performed using the script findMotifs.pl in HOMER with parameter “–mset vertebrates”. TET1 peaks were determined with MACS2 using broad parameters including the cutoff for fold change as 2 and the cutoff for q value as 1E-5.

Methylome analysis to identify bipolar methylated loci. The bipolar DNA methylation inference was performed by pooling all human and mouse brain methyolome datasets together, and then bipolar DNA methylation loci with at least 100Xs read coverage were identified following the procedure described previously50. After merging of the overlapped loci, a total of 39,114 and 21,946 bipolar DNA methylation loci were identified for human and mouse brain methylomes, respectively.

RRBS library construction and data analysis. Genomic DNA from mouse frontal cortex was extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). Five microgram mouse genomic DNA was spiked with 0.02% unmethylated chlorpyrifos (ChP) and Lambda DNA (Promega) and sonicated into 200 bp fragments with Covaris M2 (Covaris). After purification (PureLink PCR Purification Kit, Invitrogen), DNA fragments were then subjected to end repair with the end repair enzyme mix (NEN), 4A tailing using Klenow 3 ’-5 ’-exo- (NEB) with NEB. Ligation with cytosine-methylylated Illumina TruSeq DNA adapters were performed at 16 °C using T4 DNA ligase (NEB) overnight. The adapter-ligated DNA was then digested with MseI and MluCI (NEB) at 37 °C for 1 h. After purification, DNA fragments were subject to bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen). After bisulfite conversion, the single-stranded DNA was subjected to 12 cycles of PCR reaction with Illumina TruSeq PCR primers and 2.5 U Pf TurboCox Hotstart DNA polymerase (Agilent) to recover enough DNA for sequencing on the HiSeq 4000 platform with 75 bp paired end mode (Illumina). A total of 1,000 permutations were performed for each CpG site. The number of true null hypotheses (m0) was estimated by a histogram method37. Based on the estimated m0, the adjusted p-value for each CpG site was calculated. DMSs were identified with adjusted p-value lower than or equal to 0.05. To determine DMRs, we developed a two-step method. First, any two adjacent DMSs with at most 50 bp length were merged into a cluster. In each of clusters which include at least 5 CpG sites, at least 80% of DMSs are prone to be methylated or unmethylated in one of the conditions. All clusters filled requirements above will be considered as DMR candidates. Second, at least 80% of CpG sites in a candidate DMR are prone to be methylated or unmethylated in one of conditions and each CpG site was required to have at least 0.1 methylation differences.

RNA-seq library construction and data analysis. Total RNA from mouse frontal cortex was extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). RNA-seq libraries were constructed using the TruSeq Stranded mRNA Library Preparation Kit (Illumina) following the manufacturer’s instructions. Briefly, the poly-A-containing mRNA molecules were enriched from 500 ng total RNA via two rolls of oligo-dT magnetic beads purification. The resultant mRNA was fragmented and primed into first strand CDNA using reverse transcriptase and random primers, followed by the removal of the RNA template and synthesis of the second strand to generate blunt-ended ds CDNA. Then a single ‘A’ nucleotide was added to the 3’ ends of the blunt fragments and indexing adapter was ligated to the ends of the ds CDNA. Those DNA fragments with adapter molecules on both ends were then enriched by PCR amplification for 12 cycles. After Amplification, the PCR product was size-selected with the range from 220 to 500 bp on 2% dye-free agarose gel using pippin recovery system (Page Scientific). The recovered libraries were sequenced on HiSeq 4000 platform with 75 bp paired end mode (Illumina). After trimming bases of low quality and removing adapters, reads were mapped to mm10 and TE326 with Bowtie2. The raw counts were employed to identify differentially expression genes by DESeq279. The definition of differentially expression genes includes two requirements: (1) the p-value adjusted is less than
0.05, and (2) there are at least 1.5 fold changes. The visualized data normalized to 1 million was generated with Bedtools19.

GO analysis. GO analysis was performed via the Gene Functional Annotation Tool at the DAVID119 website (https://david.ncifcrf.gov/, version 6.8). Default parameters were used for the enrichment analysis for BP, cellular component (CC), and molecular function (MF). The resulting GO terms and the corresponding p-values were then processed using REVIGO11 to remove redundancy. The ten most significant BP categories were shown.

Reporting summary. Further information on research design is available in the Nature Reporting Summary linked to this article.

Data availability

The datasets generated during and/or analysed during the current study are available in the NCBI Gene Expression Omnibus (GEO), GSE108768 (including GSE108750, GSE108762, and GSE124671). Publicly available brain eQTL file.

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Author contributions

H.X. conceived and designed the study; Z.S. and A. Mu conducted ChIP-seq, Co-IP experiments and luciferase assays; X.X. constructed the RRBS, RNA-seq libraries; M.S., E.X., X. We and J. H. analyzed ChIP-seq, RNA-seq, RRBS data; A.M.P. generated shRNA lentivirus constructs/particles; X. Wa, E.M., X.I., L.L., J.Z., J.C., A. Mo and M.H.T. collected and processed neural stem cells and tissue samples; Z.S., X.X., J.H., M.S. and H. X. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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