Developmental Regulation of Eed Complex Composition Governs a Switch in Global Histone Modification in Brain*

Se Young Kim1, Jonathan M. Levenson2, Stanley Korsmeyer1*, J. David Sweat3, and Armin Schumacher4

From the 1Department of Molecular and Human Genetics and 2Department of Neuroscience, Baylor College of Medicine, Houston, Texas 77030 and the 3Howard Hughes Medical Institute, Department of Pathology and Medicine, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Originally discovered as epigenetic regulators of developmental gene expression, the Polycomb (PcG) and trithorax (trxG) group of proteins form distinct nuclear complexes governing post-translational modification of histone tails. This study identified a novel, developmentally regulated interface between Eed and Mll, pivotal constituents of PcG and trxG pathways, respectively, in mouse brain. Although the PcG proteins Eed and EzH2 (Enhancer of Zeste protein-2) engaged in a common complex during neurodevelopment, Eed associated with the trxG protein Mll upon brain maturation. Comprehensive analysis of multiple histone modifications revealed differential substrate specificity of the novel Eed-Mll complex in adult brain compared with the developmental Eed-EzH2 complex. Newborn brain from eed heterozygotes and eed;Mll double heterozygotes exhibited decreased trimethylation at lysine 27 of histone H3, as well as hyperacetylation of histone H4. In contrast, adult hippocampus from Mll heterozygotes was remarkable for decreased acetylation of histone H4, which restored to wild-type levels in eed;Mll double heterozygotes. A physiological role for the Eed-Mll complex in adult brain was evident from complementatory defects in synaptic plasticity in eed and Mll mutant hippocampi. These results support the notion that developmental regulation of complex composition bestows the predominant Eed complex with the chromatin remodeling activity conducive for gene regulation during neurodevelopment and adult brain function. Thus, this study suggests dynamic regulation of chromatin complex composition as a molecular mechanism to co-opt constituents of developmental pathways into the regulation of neuronal memory formation in adult brain.

The evolutionarily conserved Polycomb group (PcG)5 and the trithorax group (trxG) pathways are required for mitotically stable inheritance of developmental gene expression programs (1). PcG and trxG proteins engage in nuclear complexes of varying composition and function as epigenetic repressors and activators of target gene transcription, respectively (2, 3). In support of their chromatin remodeling activity, PcG and trxG complexes interact with enzymes, such as histone methyltransferases or histone deacetylases (HDAC), which modify specific residues on histone tails (4–6). For example, the PcG protein EzH2 (Enhancer of Zeste protein-2) mediates gene silencing by methylation of histone 3 of lysine 27 (H3-K27) (7–10). In contrast, the trxG protein Mll (Mixed-lineage leukemia) methylates H3-K4, which delineates a transcriptionally active chromatin domain (11, 12).

In addition to the histone methyltransferase Ezh2, the Polycomb-repressive complex 2 (PRC2) encompasses the tryptophan-aspartic acid (WD) motif protein Eed, the zinc finger protein Suz12, as well as the histone-binding proteins RbAp46/RbAp48 (7–10, 13, 14). Biochemical analyses identified the 535-amino acid protein Eed (embryonic ectoderm development) as a critical component of PRC2 and related complexes, referred to as PRC2–4 (13, 14). Missense mutations in the Eed mRNA, direct the histone methyltransferase activity of Ezh2 to H3-K27 or H1-K26 in vitro (13). Missense mutations in the L7Rn51989SB null and L7Rn19989SB hypomorphic alleles map to the second WD motif (15) and disrupt the direct interaction of Eed with Ezh2, as well as HDAC1 and -2 (16–18). Indeed, global H3-K27 methylation defects in eed mutant embryonic and trophoblast stem cells provided testimony to the pivotal

* This work was supported in part by research grants from the National Institutes of Health (to D. S. and A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Deceased.
‡ Deemed.
§1 Supported in part by an NIGMS predoctoral training grant from the National Institutes of Health.
§2 Supported by a NINDS postdoctoral training grant from the National Institutes of Health. Present address: Dept. of Pharmacology, University of Alabama, Birmingham, AL 35294.
§3 Present address: Dept. of Neurobiology, University of Alabama, Birmingham, AL 35294.
§4 To whom correspondence should be addressed: Dept. of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-6865; Fax: 713-798-8985; E-mail: armins@bcm.tmc.edu.

5 The abbreviations used are: PcG, Polycomb group; trxG, trithorax group; HDAC, histone deacetylases; H3-K27, histone 3 of lysine 27; Mll, Mixed-lineage leukemia; H3-K4, histone 3 of lysine 4; Eed, embryonic ectoderm development; PRC, Polycomb repressive complex; H1-K26, histone 1 of lysine 26; CNS, central nervous system; P, postnatal day; α, antibody against; Mill-N, N-terminal region of Mill; Mill-C, C-terminal region of Mill; H4acK5,8,12,16, histone 4 acetylated at lysines 5, 8, 12, and 16; H3m3K27, histone 3 trimethylated at lysine 27; H3m2K27, histone 3 dimethylated at lysine 27; H3m1K27, histone 3 monomethylated at lysine 27; H3m2K4, histone 3 dimethylated at lysine 4; H3acK14, histone 3 acetylated at lysine 14; H3, histone 3; H4, histone 4; LTP, long term potentiation; I/O, input/output relationships; PPF, paired-pulse facilitation; IEPS, field excitatory postsynaptic potential; ACSF, artificial cerebral spinal fluid; NMDA-R, N-methyl-D-aspartic acid receptor; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor; CNQX, 6-cyano-7-nitroquinazoline-2,3-dione; HFS, high frequency stimulation; PTP, post-tetanic potentiation; ANOVA, analysis of variance; RT, reverse transcription; qRT, quantitative real time RT.
role of Eed in PRC2–4 function (19). Eed regulates multiple developmental processes, including anterior-posterior patterning of the primitive streak at gastrulation (20, 21), vertebral identity by means of Hox gene expression in somitic mesoderm (15, 22, 23), random and imprinted X chromosome inactivation (24–29), and genomic imprinting (30).

Aside from differentiating mouse embryonic stem cells (14), the developmental regulation of Eed complex formation and activity remains largely unknown. This study detected dynamic changes in Eed isoform expression, complex composition, and histone modifying activity in the developing central nervous system (CNS) in mice. Specifically, Eed co-immunoprecipitated with EzH2 during CNS development but formed a complex with the trxG protein Mll in adult brain. Mll represents a 3,868-amino acid protein with multiple domains, including AT-hook domains, PhD zinc fingers, and a bromodomain (31).

In addition, a C-terminal SET domain methylates H3-K4 in the regulatory regions of transcribed genes (12). Mll has been shown to interact directly with HDAC1 and -2 (32), providing an additional mechanism for Mll function in chromatin remodeling. In this study, the developmental switch from Eed–EzH2 to Eed–Mll complex formation resulted in stage-specific changes in global histone modification in eed and Mll mutant brains.

Furthermore, a distinct physiological role of the Eed–Mll complex in adult brain was evident from the interdependent function of Eed and Mll in the regulation of hippocampal synaptic plasticity. Thus, the combined biochemical, genetic, and physiological results demonstrate a novel molecular interface between Pcg and trxG function in mouse brain.

**EXPERIMENTAL PROCEDURES**

Mice—l7Rn533548R represents N-ethyl-N-nitrosourea-induced point mutation allele, which encodes an L290P substitu-tion in the second WD motif of Eed (15). Although homozygosity for this eed null allele causes lethality at gastrulation, heterozygous animals are viable (20, 21). The mutant Mll allele was generated by gene targeting and harbors a lacZ insertion in the third exon, resulting in a truncated protein (33). Similar to other Mll alleles (34, 35), homozygosity for the lacZ insertion causes embryonic lethality, whereas Mll heterozygotes are viable (33, 36). eed and Mll mutant mice were genotyped as described (15, 33). Single and double heterozygous brains were dissected at embryonic day 14.5 (E14.5) from timed mating, as described (15, 33). Single and double heterozygous brains were dissected from mice at 2–3 months of age.

**Immunoprecipitation and Western Blot Analysis**—Brain (E14.5, P0, P28, and adult) and spleen (adult) were sonicated in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 with Complete Mini Protease Inhibitor (Roche Applied Science)). One to two mg of protein lysate were incubated overnight with αEed antibody coupled to beads (ProFound mammalian co-immunoprecipitation kit (Pierce)). As a control, brain and spleen lysates were incubated with free beads. Elutions were loaded onto 8% SDS-polyacrylamide gels for Western blot analysis. Polyvinylidene difluoride membranes were blocked with 5% bovine serum albumin in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) or ReliAblot reagent (Bethyl Laboratories) for 2 h at room temperature and incubated overnight at 4 °C with αEed, αEzh2, αMll-N, or αMll-C antibody. Following incubation with horse-radish peroxidase-conjugated secondary antibodies (Vector Laboratories), (co-)immunoprecipitated proteins were detected by chemiluminescence (ECL reagent, Santa Cruz Biotechnology).

**Histone Preparation**—Bulk histone preparation from P0 brain and adult hippocampus was performed as described (37). Electrophoresis of histone preparations and transfer onto polyvinylidene difluoride membranes (Bio-Rad) was followed by blocking for 2 h at room temperature with 5% bovine serum albumin in TBST. Primary incubation with αH4acK5,8,12,16, αH3m3K27, αH3m2K27, αH3m1K27, αH3m2K4, αH3acK14, αH3, or αH4 antibody was completed overnight at 4 °C followed by anti-mouse horseradish peroxidase-conjugated secondary antibody incubation for 2 h at room temperature. Histone modifications were quantified as described previously (37). Levels of histone acetylation and methylation were normalized to the amount of total H3 or H4. Statistical analysis consisted of a one-sample t test with Bonferroni correction for multiple comparisons.

**Immunohistochemistry**—Dissected P0 and adult brain was fixed in Bouin’s fixative and embedded in paraffin for sectioning at 5–7 μm. Immunohistochemistry with αEed, αMll-N, or αMll-C antibodies was performed on mutant and wild-type sections mounted side by side on the same slide to allow for comparison of expression levels between genotypes. Standard streptavidin-biotin immunoperoxidase detection was performed as described (38).

**Antibodies**—The following antibodies were employed: αEed (rabbit polyclonal raised against residues 123–140, custom-generated by Bethyl Laboratories, and rabbit polyclonal from Upstate), αMll-N (mouse monoclonal, Upstate), αMll-C (mouse monoclonal, Upstate), αEzh2 (rabbit polyclonal, Upstate), αH4acK5,8,12,16 (rabbit polyclonal, Upstate), αH3m3K27 (rabbit polyclonal, Upstate), αH3m2K27 (rabbit polyclonal, Upstate), αH3m1K27 (rabbit polyclonal, Upstate), αH3m2K4 (rabbit polyclonal, Upstate), αH3acK14 (rabbit polyclonal, Upstate), αH3 (mouse monoclonal, Upstate), or αH4 (rabbit polyclonal, Upstate).

**Quantitative Real Time RT-PCR**—Total RNA was extracted from newborn brain or hippocampus from 3-month-old animals with the MELT total nucleic acid isolation system (Ambion). eed and Mll mRNA expression was evaluated by TaqMan One-step RT-PCR (Applied Biosystems) using the 7500 Fast System (Applied Biosystems). The following TaqMan MGB probes were employed: eed (exon junction 2–3, Mm469651_m1; Applied Biosystems), Mll (exon junction 28–29, Mm1179231_m1; Applied Biosystems) and 18 S riboso-mal RNA (4326313E; Applied Biosystems) as an endogenous control for each sample. The relative levels of eed and Mll mRNA expression were expressed as a ratio of the gene-specific probe to 18 S rRNA and normalized to a single wild-type ratio set to 1.

**Hippocampal Slice Electrophysiology**—Input/output (I/O) relationships, paired-pulse facilitation (PPF), and 100-Hz long
term potentiation (LTP) utilized 39 mice \( n = 10 \text{ eed}^{+/-}; \ Mll^{+/-}, 12 \text{ eed}^{+/-}; \ Mll^{+/-}, 8 \text{ eed}^{+/-}; \ Mll^{+/-}, \text{ and } 9 \text{ eed}^{+/-}; \ Mll^{+/-} \). For NMDA input/output and post-tetanic potentiation (PTP), three mice for each genotype were tested. Theta-burst LTP analysis employed \( n = 5 \text{ eed}^{+/-}; \ Mll^{+/-}, 6 \text{ eed}^{+/-}; \ Mll^{+/-}, 4 \text{ eed}^{+/-}; \ Mll^{+/-}, \text{ and } 5 \text{ eed}^{+/-}; \ Mll^{+/-} \) mice. Dissected brains were immersed in oxygenated (95:5% \( \text{O}_2/\text{CO}_2 \)) ice-cold cutting saline (CS: 110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 28 mM NaHCO3, 0.5 mM CaCl2, 7 mM MgCl2, 5 mM glucose, and 0.6 mM ascorbate) prior to isolation of the caudal portion containing the hippocampus and entorhinal cortex. Transverse slices (400 \( \mu \text{m} \)) were prepared with a Vibratome (The Vibratome Co., St. Louis, MO). During isolation, slices were stored in ice-cold CS. After isolation, cortical tissue was removed, and hippocampal slices were equilibrated in an oxygenated mixture of 50% CS and 50% artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, and 25 mM glucose) at room temperature for 30 min prior to transfer to the recording chamber.

Electrophysiology was performed in an interface chamber (Fine Science Tools, Foster City, CA). Oxygenated ACSF (30 °C) was perfused into the recording chamber at a rate of 1 ml/min. Electrophysiological traces were digitized and stored using a Digidata 1320A and Clampex software (Axon Instruments, Union City, CA). Extracellular stimuli were administered on the border of area CA3 and CA1 along the Schaffer-collaterals using Teflon-coated bipolar platinum electrodes. fEPSPs were recorded in stratum radiatum with an ACSF-filled glass recording electrode (1–3 megohms). The relationship between fiber volley and fEPSP slopes over various stimulus intensities was used to assess base-line synaptic transmission. All subsequent experimental stimuli were set to an intensity that evoked an fEPSP with a slope of 50% of the maximum fEPSP slope. To assess NMDA receptor (NMDA-R)-dependent synaptic transmission, the ratio of fEPSP slope versus fiber volley slope over a range of intensities was measured in a modified ACSF (same as above except 4 mM CaCl2 and no MgCl2) in the presence of the AMPA-R antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (20 \( \mu \text{M} \)). High frequency stimulation LTP was induced by administering two 100-Hz tetani (1 s), with an inter-tetanus interval of 20 s. Synaptic efficacy was monitored 20 min prior to and 180 min following induction by recording fEPSPs every 20 s (traces were averaged for every 2 min interval). Slices that did not exhibit stable fEPSP slopes during the first 20 min of recording were excluded from the study.

Input/output relationships were analyzed using a single exponential function \( Y = \text{slope}_{\text{max}} \times 1 - e^{-k \times X} \) or linear regression where appropriate. Parameters used to fit I/O relationships were compared across genotypes using an F test. PPF, LTP, and PTP data were analyzed via 2-way ANOVA with repeated measures. For analysis of LTP and PTP, data acquired before and after induction were analyzed separately. Post hoc comparisons after 2-way ANOVA were made using the method of Bonferroni. Total depolarization induced by a 100-Hz tetanus was analyzed by comparing the area under the curve with a Kruskal-Wallis ANOVA, followed by a post hoc Dunn’s multiple comparison test. Significance for all tests was set at \( p < 0.05 \).

**RESULTS**

**Developmental Regulation of Eed Isoform Expression in Brain**—Immunoprecipitation of Eed at various stages of brain development revealed four Eed isoforms of varying abundance in the 50–70-kDa molecular mass range, reminiscent of recent findings in 293 cells (13). As shown in Fig. 1, E14.5 brain and adult spleen demonstrated a similar pattern of Eed isoforms. At P0 and P28, the co-expression of developmental and adult brain isoforms at similar abundance suggested a gradual transition in Eed isoform expression. Interestingly, the isoforms identified by immunoprecipitation were undetectable in input lanes at both standard (Fig. 1) and maximum loading capacity (data not shown). However, it should be emphasized that two Eed antibodies with different epitopes detected the four Eed isoforms, confirming the specificity of the immunoprecipitation (data not shown). In addition, the Western blot results correlate with the low level of Eed expression demonstrated by immunohistochemistry (Fig. 3A).

**Developmental Transition from Eed-Ezh2 to Eed-Mll Complex Formation in Brain**—Differential Eed isoform expression correlated with developmental regulation of Eed complex composition. Ezh2 co-immunoprecipitated with Eed from E14.5

---

**FIGURE 1. Developmental transition from Eed-Ezh2 to Eed-Mll complex formation in brain.** Immunoprecipitation with an \( \alpha \text{Eed} \) antibody detected several Eed isoforms in the 50–70-kDa range at various stages of brain development, as well as in adult spleen. Asterisks denote isoforms of similar molecular weight in brain stages and spleen. Note the change in the relative abundance of Eed isoforms and the gradual transition from the Eed-Ezh2 complex to the Eed-Mll complex during brain development. Antibodies against N- or C-terminal Mll epitopes detected an ~180-kDa band. Input consisted of 10 \( \mu \text{g} \) of lysate. As a control for nonspecific binding, lysates were immunoprecipitated with free beads lacking the Eed antibody.
A Developmental Switch in Global Histone Modification in Brain—The transition from Eed-EzH2 to Eed-Mll complex formation suggested a developmental switch in histone modifying activity from newborn to adult brain. This would be evident from stage-specific aberrations in global histone modification in eed and/or Mll mutant brains. Based on previous studies in other cellular contexts, defects in Eed-Ezh2 interaction would decrease levels of H3-K27 methylation, whereas disruption of Mll SET domain activity would reduce H3-K4 methylation (12). Alternatively, given the known interaction of both Eed and Mll with HDAC1 and -2 (18, 32), mutant brains may exhibit histone acetylation defects. Global histone modification was analyzed from whole newborn brain. To circumvent the significant regional heterogeneity in adult brain as a confounding variable, histones were isolated from dissected hippocampus, which presents a defined cellular composition. In addition, electrophysiology was employed as a well-characterized assay for hippocampal function in wild-type and mutant brains.

At P0, eed heterozygotes exhibited hyperacetylation of histone H4 (p < 0.03) and decreased trimethylation of H3-K27 (p < 0.0001), consistent with decreased HDAC and Ezh2 histone methyltransferase activity, respectively, of the Eed complex (Fig. 2). No statistically significant changes in mono- or dimethylation of H3-K27, dimethylation of H3-K4, or acetylation of H3-K14 were detected (all comparisons, p > 0.05) (Fig. 2). Furthermore, eed;Mll double heterozygotes presented similar defects in global histone modification compared with eed heterozygotes (p < 0.0001) (Fig. 2). In contrast, P0 Mll heterozygotes did not manifest any defects in global histone modification, including H3-K4 methylation (all comparisons, p > 0.05). Thus, analysis of histone modifications in eed and Mll mutants supported the biochemical findings, wherein Eed and Mll did not engage in a common complex at P0.

Unlike P0 brain, adult hippocampus from eed heterozygotes did not exhibit significant changes in histone acetylation or methylation (all comparisons, p > 0.05) (Fig. 2). However, hippocampal histone preparations from Mll heterozygotes were remarkable for a significant decrease in H4 acetylation (p < 0.02). Defects in histone modification in adult Mll heterozy-

and P0 brain, as well as adult spleen (Fig. 1). In contrast, the two proteins did not co-immunoprecipitate from adult brain. Instead, Eed formed a complex with Mll, as evident from Eed immunoprecipitation and Western blot analysis using both N- and C-terminal Mll antibodies (Fig. 1). Similar to the Eed isoform pattern, the transition from the Eed-Ezh2 complex in the developing brain to the Eed-Mll complex in adult brain appeared gradual, and both complexes co-existed in P28 brain (Fig. 1). These results implicate Eed isoforms in stage- and tissue-specific recruitment of Eed complex constituents. Unlike in transiently transfected 293T cells (32), the Mll and Bmi1 did not co-immunoprecipitate from adult brain (data not shown). Furthermore, attempts to confirm a possible association of HDACs with Eed-Ezh2 and Eed-Mll complexes were inconclusive because of the high affinity of HDAC1 and -2 to agarose beads (data not shown).

A Developmental Switch in Global Histone Modification in Brain—The transition from Eed-Ezh2 to Eed-Mll complex formation suggested a developmental switch in histone modifying activity from newborn to adult brain. This would be evident from stage-specific aberrations in global histone modification in eed and/or Mll mutant brains. Based on previous studies in other cellular contexts, defects in Eed-Ezh2 interaction would decrease levels of H3-K27 methylation (7–10, 13), whereas disruption of Mll SET domain activity would reduce H3-K4 methylation (12). Alternatively, given the known interaction of both Eed and Mll with HDAC1 and -2 (18, 32), mutant brains may exhibit histone acetylation defects. Global histone modification was analyzed from whole newborn brain. To circumvent the significant regional heterogeneity in adult brain as a confounding variable, histones were isolated from dissected hippocampus, which presents a defined cellular composition. In addition, electrophysiology was employed as a well-characterized assay for hippocampal function in wild-type and mutant brains.

At P0, eed heterozygotes exhibited hyperacetylation of histone H4 (p < 0.03) and decreased trimethylation of H3-K27 (p < 0.0001), consistent with decreased HDAC and Ezh2 histone methyltransferase activity, respectively, of the Eed complex (Fig. 2). No statistically significant changes in mono- or dimethylation of H3-K27, dimethylation of H3-K4, or acetylation of H3-K14 were detected (all comparisons, p > 0.05) (Fig. 2). Furthermore, eed;Mll double heterozygotes presented similar defects in global histone modification compared with eed heterozygotes (p < 0.0001) (Fig. 2). In contrast, P0 Mll heterozygotes did not manifest any defects in global histone modification, including H3-K4 methylation (all comparisons, p > 0.05). Thus, analysis of histone modifications in eed and Mll mutants supported the biochemical findings, wherein Eed and Mll did not engage in a common complex at P0.

Unlike P0 brain, adult hippocampus from eed heterozygotes did not exhibit significant changes in histone acetylation or methylation (all comparisons, p > 0.05) (Fig. 2). However, hippocampal histone preparations from Mll heterozygotes were remarkable for a significant decrease in H4 acetylation (p < 0.02). Defects in histone modification in adult Mll heterozy-
Developmental Regulation of Chromatin Complexes in Brain

gotes were restricted to H4acK5,8,12,16 because all other sites exhibited normal levels of histone acetylation or methylation (all comparisons, \( p > 0.05 \)) (Fig. 2). Strikingly, H4 acetylation levels in eed;Mll double heterozygotes were indistinguishable from wild type (\( p > 0.05 \)). This suggests that Mll requires Eed for histone modification and supports the biochemical findings wherein Mll and Eed form a common complex in adult brain.

Increased Mll Protein Expression in Brain Is Consistent with a Gain-of-Function Allele—Newborn and adult brain from eed and Mll heterozygotes revealed normal gross morphology (data not shown). Immunohistochemistry detected widespread nuclear expression of Eed and Mll in wild-type P0 and throughout adult brain (data not shown), including hippocampus (Fig. 3). For comparison of Eed and Mll expression across genotypes, immunohistochemistry was performed on sections from wild-type, eed, and Mll mutant brains mounted side by side on the same slide. Eed protein levels in eed heterozygous and eed;Mll double heterozygous hippocampi were indistinguishable from wild type (Fig. 3A). Importantly, the Eed antibody

![Diagram of eed and Mll mutant mice](image)

**FIGURE 3.** Expression analysis in eed and Mll mutant mice. A, across all genotypes, immunohistochemistry detected similar levels of Eed expression in P0 brain and adult hippocampus. Nuclear expression of Eed in adult hippocampal pyramidal cells. B, although wild-type and eed \(^{-/-}\)Mll \(^{+/+}\) animals displayed low levels of Mll expression, both eed \(^{+/+}\)Mll \(^{+/+}\) and eed \(^{-/-}\)Mll \(^{+/+}\) animals exhibited significant Mll up-regulation throughout the P0 brain and adult hippocampus. The antibody employed in this reaction specifically recognized the Mll wild-type protein. Mll displayed nuclear expression in adult hippocampal pyramidal cells across all genotypes. A and B, eed \(^{-/-}\)Mll \(^{+/+}\) hippocampi showed normal hippocampal architecture. In contrast, eed \(^{+/+}\)Mll \(^{-/-}\) and eed \(^{-/-}\)Mll \(^{+/+}\) mice exhibited a subtle bilayer formation of pyramidal cells in area CA3 (arrowhead) in Mll heterozygotes as well as eed/Mll double heterozygotes. Genotypes are indicated above representative images. Stages and antibodies are denoted to the left and right of the panels, respectively. Scale bars: newborn cortex (0.1 mm), adult hippocampus low (0.2 mm), and high magnification (0.02 mm).

![Graph of mRNA levels](image)

**FIGURE 4.** Decreased Mll transcript levels in Mll mutants. eed and Mll mRNA expression levels in newborn brain and adult hippocampus were evaluated by quantitative real time RT-PCR using 18 S ribosomal RNA as an endogenous control. The relative amounts of eed or Mll mRNA levels were expressed as the ratio of the gene-specific probe to 18 S ribosomal RNA and normalized to a single wild-type ratio set to 1. Both in P0 brain and adult hippocampus, eed transcript levels were indistinguishable across genotypes, whereas Mll heterozygotes and eed/Mll double heterozygotes exhibited a significant reduction in Mll mRNA expression. Horizontal bars indicate the mean of eed or Mll mRNA levels.
employed in this study could not distinguish between wild-type Eed and the L290P mutant protein encoded by the eed null allele $\text{I}7\text{Rn}^5\text{EES}^5\text{G5B}$ (15). Nonetheless, because the L290P missense mutation renders the Eed protein nonfunctional (16, 18), wild-type Eed activity decreases to $\sim 50\%$ in eed heterozygotes and $\text{eed;Mll}$ double heterozygotes compared with wild type.

The mutant $\text{Mll}$ allele was created by in-frame insertion of a lacZ cassette into the third exon of $\text{Mll}$ (34). The N-terminal Mll/$\beta$-Gal fusion fragment truncates the Mll protein and removes the PhD zinc fingers, the bromodomain, as well as the SET domain at the C terminus. Therefore, expression analysis using an antibody against C-terminal epitopes detected activity of the wild-type Mll protein in Mll heterozygotes. Strikingly, immunohistochemistry on sections from Mll heterozygotes and $\text{eed;Mll}$ double heterozygotes revealed increased expression of the wild-type Mll protein in newborn and adult brain, including hippocampus (Fig. 3B). Similarly, an antibody against N-terminal epitopes of Mll manifested significantly higher Mll protein levels in Mll heterozygous and eed;Mll double heterozygous hippocampus compared with wild type (data not shown). This unexpected augmentation in wild-type Mll protein levels in Mll mutant brain should result in increased Mll activity, which is most consistent with a gain-of-function allele of Mll.

A Post-transcriptional Mechanism of Increased Mll Protein Expression—eed and Mll transcript levels were analyzed by quantitative real time RT-PCR (qRT-PCR) in newborn brain and adult hippocampus from mutant animals. At both stages, qRT-PCR did not detect significant changes in eed mRNA levels in mutant genotypes compared with wild-type littermates (Fig. 4), indicating stability of the $\text{I}7\text{Rn}^5\text{EES}^5\text{G5B}$ mRNA. In contrast, qRT-PCR with primers spanning an exon junction at the 3’ end of the coding region revealed an $\sim 50\%$ decrease in Mll mRNA levels in Mll heterozygous and eed;Mll double heterozygous animals in both P0 brain and adult hippocampus (Fig. 4). This statistically significant decrease in Mll mRNA expression is consistent with the presence of truncated mRNA caused by insertion of a lacZ cassette with SV40 transcription termination sequences into exon 3b of Mll (33). In addition, mRNA levels of the paralogous gene Mll2, whose encoded protein shares many domains with Mll (39), were indistinguishable between wild type and Mll mutants (data not shown). Taken together, decreased wild-type Mll mRNA levels suggested a distinctly post-transcriptional mechanism for augmentation of Mll protein expression in Mll mutant brain.

Interdependence of Eed and Mll Function in the Regulation of Hippocampal Synaptic Plasticity—Synaptic plasticity represents the leading cellular candidate mechanism for memory storage in the adult CNS (40). The distinct phases of synaptic plasticity involve different mechanisms, including gene transcription during the late phase. Given PeG and trxG function in transcriptional regulation via histone modification, analysis of LTP at Schaffer-collateral synapses in area CA1 in eed and Mll mutant hippocampi investigated a specific physiological role of the Eed-Mll complex in the induction of synaptic plasticity.

Prior to measuring synaptic plasticity, control experiments in mutant hippocampi determined basal synaptic transmission. For example, input/output (I/O) relationships measured postsynaptic depolarization (fEPSP slope) as a function of presynaptic depolarization (fiber volley slope). As indicated by I/O relationships (Fig. 5A), hippocampal slices from Mll heterozygous ($p < 0.2$) and eed;Mll double heterozygous animals ($p < 0.3$) displayed normal levels of synaptic transmission. Analysis of I/O in hippocampal slices from eed heterozygotes revealed a significant reduction in synaptic transmission (Fig. 5A; $p < 0.005$). To assess a potential mechanism for this defect, paired-pulse facilitation (PPF) was employed as an index of neurotransmitter release. Hippocampal slices from all genotypes exhibited normal PPF (Fig. 5B, eed, $p < 0.2$; Mll, $p < 1.0$; eed; Mll, $p < 1.0$). With regard to eed heterozygotes, normal PPF indicated that the defect in synaptic transmission was not because of a reduction in presynaptic neurotransmitter release. Mechanistically, heterozygosity for the eed null allele may reduce the expression and/or function of postsynaptic glutamate receptors.

Both I/O and PPF indicated that mutations in eed and Mll only minimally affected synaptic transmission. Subsequent experiments compensated for the reduced synaptic transmission in eed heterozygotes by stimulation at 50% maximal fEPSP for each particular animal. Therefore, every hippocampal slice presented the same potential for expression of synaptic plasticity, as assessed by long term potentiation with theta-burst stimulation and with high frequency tetanic stimulation. As shown in Fig. 5C, theta-burst LTP was normal in eed and Mll mutant hippocampus (all comparisons, $p < 0.1$), indicating that Eed and/or Mll do not affect the induction and/or expression of short term forms of synaptic plasticity.

Importantly, LTP induction with high frequency stimulation (2, 1-s, 100-Hz tetani) demonstrated a robust enhancement of early and late phase LTP in hippocampal slices from eed heterozygotes ($p < 0.0001$) (Fig. 6A). This LTP enhancement was apparent 40 min after induction and persisted for at least 180 min after induction. In contrast to eed, slices from Mll heterozygotes exhibited a significant deficit in LTP ($p < 0.0001$) wherein a gradual decrease in potentiation started at 20 min post-induction (Fig. 6B). Synaptic efficacy reached basal levels by 160 min after induction. Strikingly, LTP in hippocampal slices from eed;Mll double heterozygous animals was not significantly different from LTP in wild-type littermates ($p = 0.99$) (Fig. 6C). The lack of a significant difference between eed;Mll double heterozygous and wild-type slices could derive from complementary effects on induction and/or expression of late phase synaptic plasticity. However, the increase in variance in eed;Mll double mutants raises the possibility that other, as yet unidentified, mechanisms may contribute to the apparent complementation in LTP.

A series of control experiments determined whether heterozygosity for Eed or Mll affected induction of LTP. The first parameter entailed depolarization during a 100-Hz tetanus, which represents an integrated metric of all synaptic mechanisms involved in the induction of LTP. As shown in Fig. 6, A–C, insets, wild-type and mutant hippocampi revealed no differences in the magnitude of depolarization during a 100-Hz
FIGURE 5. Synaptic transmission in hippocampal neurons from *eed* and *Mll* mutant mice. A and B, I/O relationships and PPF were assayed at Schaffer-collateral synapses of hippocampal slices in vitro. A, I/O relationships measured basal synaptic transmission and were expressed as the relationship between fEPSP slope (post-synaptic depolarization) to fiber volley slope (pre-synaptic depolarization). Schaffer-collateral synaptic transmission was significantly reduced in *eed*/*Mll*/*H11001*/*H11002*; *Mll*/*H11001*//*H11001* animals relative to wild-type littermates. B, PPF, an index of presynaptic neurotransmitter release, was measured at Schaffer-collateral synapses of hippocampal slices in vitro. PPF was depicted as percentage of observed facilitation versus the interstimulus interval. PPF in *eed*/*Mll*/*H11001*/*H11002*; *Mll*/*H11001*//*H11001* animals was indistinguishable from wild type, suggesting that the reduction in synaptic connectivity in *eed*/*Mll*/*H11001*/*H11002* hippocampus (see Fig. 2A) was not because of a reduction in neurotransmitter release. Likewise, PPF was normal in *eed*/*H11001*//*H11002*; *Mll*/*H11001*//*H11002* and *eed*/*H11001*//*H11002*; *Mll*/*H11001*//*H11002* animals.

C, theta-burst LTP at Schaffer-collateral synapses of hippocampal slices in vitro. LTP was induced using a theta-burst stimulation paradigm (3 trains of 10 bursts, 4 stimuli/burst at 100 Hz, 200-ms interburst interval, 20-s intertrain interval). Synaptic efficacy was monitored 20 min before and 90 min after induction of LTP. Compared with wild type, theta-burst LTP appeared normal in *eed*/*Mll*/*H11001*/*H11002*, *eed*/*H11001*//*H11002*; *Mll*/*H11001*//*H11002*, and *eed*/*H11001*//*H11002*; *Mll*/*H11001*//*H11002* animals. Arrows indicate time of LTP induction. Representative fEPSPs are shown 2 min before (dashed) and 90 min after (solid) induction of LTP. Calibration bars indicate 2 mV and 5 ms. D, PTP at Schaffer-collateral synapses of hippocampal slices in vitro. Compared with wild type, there was no difference in PTP in any of the genotypes. Synaptic efficacy was monitored 2 min before and 3 min after administration of a 100-Hz tetanus (1 s) in the presence of DL-AP5 (50 mM). Arrows indicate time of tetanus. Representative traces are shown 3 s before (dashed) and 3 s after tetanus (solid). Calibration bars indicate 2 mV and 5 ms. A–D, error bars indicate S.E. I/O relationships were analyzed using an F test. Statistical analysis of PPF, LTP, and PTP experiments involved a two-way ANOVA with repeated measures followed by post hoc comparisons using the method of Bonferroni. Statistical significance for all tests was set at $p \leq 0.05$. 

*Developmental Regulation of Chromatin Complexes in Brain*

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 13 • MARCH 30, 2007

9968
Developmental Regulation of Chromatin Complexes in Brain

An intricate interplay between biochemically distinct PcG and trxG chromatin complexes specifies segmental identity in embryos by means of transcriptional repression and activation, respectively, of Hox gene transcription (2, 3, 41). Best characterized in Drosophila, PcG and trxG complexes frequently co-localize to adjacent but physically separable regions in the vicinity of transcription units (42–47). Although similar genomic elements await characterization in mammals, evolutionary conservation of antagonistic PcG and trxG function was evident from the rescue of reciprocal homeotic phenotypes in intercrosses between mutant alleles of Bmi1 and Mll (48) in mice.

This study identified a novel, developmentally regulated interface between PcG and trxG function in the CNS (see model in Fig. 7). In the developing brain, Eed formed a complex with the histone methyltransferase EzH2, reminiscent of PRC2–4 (13, 14), whereas Mll engaged in a physically separate complex of unknown function. In contrast, adult brain was remarkable for complex formation between Eed and Mll. The transition from the Eed-EzH2 complex to the Eed-Mll complex appeared gradual, as evident from their co-existence in P28 brain. Alternatively, the three proteins may be present in a single, transient complex at this stage. It should be emphasized that these findings do not exclude the existence of Eed-Mll and Eed-EzH2 complexes at lower abundance in the developing and adult CNS, respectively. However, the results underscore the importance of analyzing complex composition at various stages of pre- and postnatal development to detect dynamic regulation thereof.

Convergence from separate biochemical entities during CNS development to the presence of Eed and Mll in a common complex in adult brain correlates with a switch in Eed isoform expression. Previous studies implicated Eed isoforms in targeting EzH2 activity to H3-K27 or H1-K26 (13). Beyond regulation of histone methyltransferase activity,
the present findings suggested a broader role for Eed isoforms in directing the recruitment of Eed complex constituents, such as Mll. Although complex formation between Eed and Mll represents a novel molecular interface between these two proteins, there is precedence for physical association of other PcG and trxG constituents. Trxl (trithorax-like), the Drosophila homolog of the GAGA factor, was detected in a complex with multiple PcG proteins in pre-blastoderm embryos (49). In addition, studies in transiently transfected 293T cells identified direct interaction of Mll with a number of transcriptional regulators, including the PcG proteins Bmi1 and HPC2 (32). In support of context-specific PcG and trxG interaction, co-immunoprecipitation did not detect Bmi1 in the Eed-Mll complex in adult context-specific PcG and trxG interaction, co-immunoprecipi-
tation and, hence, elevated repressor activity of the Eed-Mll complex. Based on this notion, a 50% decrease in Eed wild-type dosage in adult hippocampus strongly suggests increased HDAC function and, hence, elevated repressor activity of the Eed-Mll complex.

Opposite allelic effects govern differential abundance of Eed-Mll complex constituents in adult brain. \textit{l7Rn53354SB}, a genetically and biochemically well characterized \textit{eed} null allele (15, 16, 18), reduces the Eed wild-type dosage in \textit{eed} heterozygotes by about 50%. In contrast, this study identified gain-of-function effects of the \textit{Mll} allele. This was evident from an increase in wild-type Mll protein expression, despite decreased \textit{Mll} mRNA levels in both newborn and adult brain from \textit{Mll} mutants, implicating a post-transcriptional mechanism in augmentation of Mll protein levels. Recent studies showed that Mll undergoes proteolytic cleavage, and heterodimerization of the resulting N- and C-terminal fragments confers increased fragment stability (50, 51). In addition, N-terminal fragments of human MLL exhibited significant stability following transfection into U937 cells (52). This suggests that the N-terminal Mll/\beta-galactosidase fusion fragment (derived from the mutant \textit{Mll} allele) may increase stability of wild-type C-terminal fragments (derived from the wild-type \textit{Mll} allele), augmenting Mll protein levels in \textit{Mll} heterozygous and \textit{eed;Mll} double heterozygous brain.

These findings form the basis for a model that reconciles the opposite effects of the \textit{eed} and \textit{Mll} loss- and gain-of-function allele, respectively, with the presence of Eed and Mll in a common complex and levels of histone H4 acetylation in adult brain. In this model, Mll represents the rate-limiting component of complex assembly and HDACs transiently or permanently associated with the Eed-Mll complex. Based on this notion, a 50% decrease in Eed wild-type dosage in \textit{eed} heterozy-
Developmental Regulation of Chromatin Complexes in Brain

gotes would be less likely to limit complex formation and, consequently, H4 acetylation remained at wild-type levels. Conversely, up-regulation of wild-type Mll protein levels should facilitate Eed-Mll complex formation. In strong support of this hypothesis, overexpression of EzH2 in 293 cells promoted formation of PRC4 (14), indicating a reservoir of Eed isoforms available for increased complex formation upon overexpression of a rate-limiting constituent. Concomitant with increased Eed-Mll complex formation, HDAC activity would increase, as reflected by a decrease in hippocampal H4 acetylation levels in Mll heterozygotes. A significant reduction in wild-type Eed dosage in eed;Mll double heterozygotes would curtail the increase in Eed-Mll complex formation because of Mll up-regulation. As a result, H4 acetylation is restored to approximately wild-type levels in eed;Mll double heterozygotes. Thus, genetic analysis suggests a tightly regulated dosage requirement for Eed-Mll complex formation. Whereas Mll constitutes the rate-limiting factor under wild-type conditions, in turn, a rather small reservoir of Eed isoforms limits complex formation upon overexpression of Mll.

Recent studies identified potential downstream targets of PcG complexes in mammalian embryonic stem cells and embryonic fibroblasts (53–55). Although specific target loci in brain remain unknown, it is intriguing to speculate that the Eed-EzH2 complex regulates genes concerned with neurodevelopment, whereas the Eed-Mll complex governs genes regulation in support of specific functions of the adult CNS. Electrophysiological studies provided a well-established experimental avenue toward elucidating the biological significance of Eed-Mll complex formation in mature brain. Indeed, interdependency of Eed and Mll function was evident from LTP induction at Schaffer-collateral synapses in hippocampal area CA1, a candidate mechanism for neuronal memory formation in vivo (40). Therein, eed and Mll heterozygotes exhibited opposite phenotypes in early and late phase LTP. Strikingly, eed;Mll double heterozygotes exhibited normal LTP, indicative of an intricate interplay between Eed and Mll in the regulation of synaptic plasticity.

Similar to other mutant strains, the question arises as to whether the aberrant synaptic plasticity in eed and Mll mutant mice derives from neurodevelopmental defects or reflects an acute requirement for Eed and Mll in neuronal function. In support of the latter, recent studies implicated changes in histone acetylation and phosphorylation in the induction of synaptic plasticity and long-term memory in brain (37, 56–59). Although not irreversible, methylation is considered a more permanent histone modification compared with acetylation (60). Hence, absence of the EzH2 histone methyltransferase from the predominant Eed complex could reflect a response to stage-specific functional requirements. Whereas the Eed-EzH2 complex would stably maintain gene repression during neurodevelopment, regulation of histone deacetylation by the Eed-Mll complex may provide the necessary molecular flexibility for the execution of higher order functions of the adult CNS, including synaptic plasticity. In conclusion, dynamic regulation of Eed complex composition may constitute a novel molecular mechanism to co-opt components of developmental chromatin-remodeling pathways into acute regulation of neuronal memory formation in the adult mammalian brain.

Acknowledgments—We are grateful to Arthur Beaudet, Juan Botas, and Huda Zoghbi for discussions and critical reading of the manuscript. Agnieszka Mlodnicka and Sadia Waheed provided excellent technical assistance. We acknowledge support by the Administrative, Mouse Neurobehavior and Mouse Physiology Cores of the Baylor Mental Retardation and Developmental Disabilities Research Center.

REFERENCES

1. Ringrose, L., and Paro, R. (2004) Annu. Rev. Genet. 38, 413–443
2. Otte, A. P., and Kwaks, T. H. J. (2003) Curr. Opin. Genet. Dev. 13, 448–454
3. Gramaud, C., Negre, N., and Cavalli, G. (2006) Chromosome Res. 14, 363–375
4. Simon, J. A., and Tamkun, J. W. (2002) Curr. Opin. Genet. Dev. 12, 210–218
5. Fischle, W., Wang, Y., and Allis, C. D. (2003) Curr. Opin. Cell Biol. 15, 172–183
6. Peterson, C. L., and Laniel, M. A. (2004) Curr. Biol. 14, R546–R551
7. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) Science 298, 1039–1043
8. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002) Genes Dev. 16, 2893–2905
9. Müller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O’Connor, M. B., Kingston, R. E., and Simon, J. A. (2002) Cell 111, 197–208
10. Czernin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Piriotta, V. (2002) Cell 111, 185–196
11. Kuo, M. H., Brownell, J. E., Sobel, R. E., Ranalli, T. A., Cook, R. G., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) Nature 383, 269–272
12. Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002) Mol. Cell 10, 1107–1117
13. Kuzmichev, A., Jenuwein, T., Tempst, P., and Reinberg, D. (2004) Mol. Cell 14, 183–193
14. Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T. S., Scher, M., Kirmizis, A., Ouyang, X., Brockdorff, N., Abate-Shen, C., Farnham, P., and Reinberg, D. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1859–1864
15. Schumacher, A., Faust, C., and Magnuson, T. (1996) Nature 383, 250–253
16. van Lohuizen, M., Tijms, M., Veenendaal, A. J., Cheung, P. H., Allis, C. D., and van Loo, P. (1994) EMBO J. 13, 3631–3637
17. Denisenko, O., Shnyreva, M., Suzuki, H., and Bomsztyk, K. (1998) Mol. Cell. Biol. 18, 5634–5642
18. van der Vlag, J., and Otte, A. P. (1999) Nat. Genet. 23, 474–478
19. Montgomery, N. D., Yee, D., Chen, A., Kalaria, S., Chamberlain, S. J., Otte, A. P., and Magnuson, T. (2005) Curr. Biol. 15, 942–947
20. Faust, C., Schumacher, A., and Magnuson, T. (1995) Development (Camb.) 121, 273–285
21. Faust, C., Lawson, R. A., Schork, N. J., Thiel, B., and Magnuson, T. (1998) Development (Camb.) 125, 4495–4506
22. Wang, J., Magee, J., Schneider, E., and Magnuson, T. (2002) Mammm Genome 13, 493–503
23. Kim, S. Y., Paylor, S. W., Magnuson, T., and Schumacher, A. (2006) Development (Camb.) 133, 4957–4968
24. Wang, J., Magee, J., Chen, Y., Schneider, E., Cross, J. C., Nagy, A., and Magnuson, T. (2001) Nat. Genet. 28, 371–375
25. Mak, W., Baxter, J., Silva, J., Newall, A. E., Otte, A. P., and Brockdorff, N. (2002) Curr. Biol. 12, 1016–1020
26. Plath, K., Fang, J., Mlyarczyk-Evans, S. K., Cao, R., Worringen, K. A., Wang, H., de la Cruz, C. C., Otte, A. P., Panning, B., and Zhang, Y. (2003) Science 300, 131–135
27. Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T. B., Webster, Z., Peters, A. H., Jenuwein, T., Otte, A. P., and Brockdorff, N. (2003) Dev. Cell 4, 481–495

MARCH 30, 2007 • VOLUME 282 • NUMBER 13
JOURNAL OF BIOLOGICAL CHEMISTRY 9971
28. Kalantry, S., and Magnuson, T. (2006) PLoS Genet. 2, 656–664
29. Kalantry, S., Mills, K. C., Yee, D., Otte, A. P., Panning, B., and Magnuson, T. (2006) Nat. Cell Biol. 8, 195–202
30. Mager, J., Montgomery, N. D., de Villena, F. P., and Magnuson, T. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8342–8347
31. Ma, Q., Alder, H., Nelson, K. K., Chatterjee, D., Gu, Y., Nakamura, T., and Korsmeyer, S. J. (1998) Blood 92, 108–117
32. Yu, B. D., Hess, J. L., Horning, S. E., Brown, G. A. J., and Korsmeyer, S. J. (1995) Nature 378, 505–508
33. Yagi, H., Deguchi, K., Aono, A., Tani, Y., Kishimoto, T., and Komori, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10632–10636
34. Yagi, H., Deguchi, K., Aono, A., Tani, Y., Kishimoto, T., and Komori, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10632–10636