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Ezh1 and Ezh2 differentially regulate PSD-95 gene transcription in developing hippocampal neurons

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ABSTRACT

Polycomb Repressive Complex 2 (PRC2) mediates transcriptional silencing by catalyzing histone H3 lysine 27 trimethylation (H3K27me3), but its role in the maturation of postmitotic mammalian neurons remains largely unknown. We report that the PRC2 paralogs Ezh1 and Ezh2 are differentially expressed during hippocampal development. We show that depletion of Ezh2 leads to increased expression of PSD-95, a critical plasticity gene, and that reduced PSD-95 gene transcription is correlated with enrichment of Ezh2 at the PSD-95 gene promoter; however, the H3K27me3 epigenetic mark is not present at the PSD-95 gene promoter, likely due to the antagonizing effects of the H3S28P and H3K27Ac marks and the activity of the H3K27 demethylases JMJD3 and UTX. In contrast, increased PSD-95 gene transcription is accompanied by the presence of Ezh1 and elongation-engaged RNA Polymerase II complexes at the PSD-95 gene promoter, while knock-down of Ezh1 reduces PSD-95 transcription. These results indicate that Ezh1 and Ezh2 have antagonistic roles in regulating PSD-95 transcription.

Introduction

Enzymes that alter the chromatin structure are central players in controlling the accessibility of transcription factors to target genes: they accomplish this by regulating DNA methylation and/or histone-tail modifications, including acetylation, methylation, sumoylation and phosphorylation, among others (Gardner et al., 2011; Kouzarides, 2007). These epigenetic modifications are known to have important roles in the fate regulation of neuronal stem cells; however, for postmitotic neurons, it is mostly DNA methylation and histone-tail acetylation, along with their relationship to synaptic function and animal behavior, that are studied extensively (Borrelli et al., 2008; Day and Sweatt, 2011; Graff et al., 2011). To date, an understanding of the role of histone tail methylation and phosphorylation in regulating gene expression in differentiated neurons remains elusive.

PcG group proteins mediate critical contributions to various biological processes, including maintenance and lineage specification of stem cells, from fruit flies to humans (Margueron and Reinberg, 2011; O'Meara and Simon, 2012; Sparmann and van Lohuizen, 2006). PcG group complexes comprise the two major Polycomb Repressive Complexes (PRCs) PRC1 and PRC2. The composition of PRC1 is more complex and variable compared to PRC2, and in mammals it can include RING1A/B, BMI1, MEL18, and NSPC1 (Gao et al., 2012; Lund and van Lohuizen, 2004; Margueron and Reinberg, 2011; O'Meara and Simon, 2012). Due to the heterogeneous nature of the PRC1 complexes, most studies have focused on the role of PRC2 (Margueron and Reinberg, 2011). The PRC2 complex contains several proteins, but its core is made up by enhancer of zeste homolog (Ezh), embryonic ectoderm development (Eed) and suppressor of zeste 12 (Suz12). *Drosophila* have only a single Ezh gene, while mammalian PRC2 complexes can contain either Ezh1 or Ezh2, the latter being the closest to its invertebrate homologs. Growing evidence indicates that PRC2 complexes are critical for the terminal differentiation of different cell types including skeletal muscle cells, intestinal cells, and heart cells (Benoit et al., 2012; He et al.,

Abbreviations: AraC, cytosine arabinoside; Aldh1L1, aldehyde dehydrogenase 1 family, member L1; ANOVA, analysis of variance; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; DIV, days in vitro; Eed, embryonic ectoderm development; EGS, ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester); Ezh1/2, enhancer of zeste homolog 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDAC2, histone deacetylase 2; JMJD3, jumonji domain-containing protein 3; PcG, polycomb group protein; PRC1/2, Polycomb Repressive Complex 1/2; PVDF, polyvinylidene difluoride; RNA Pol II, RNA polymerase II; S.E.M, standard error of the mean; Suz12, suppressor of zeste 12; UTX, ubiquitously transcribed tetratricopeptide repeat, X chromosome.

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2012; Margueron et al., 2008; Mousavi et al., 2012; Stojic et al., 2011). Once PRC2-Ezh2 is recruited to the chromatin, it catalyzes the trimethylation of histone-tail H3-lysine 27 residue (H3K27me3), which is associated with transcriptional repression via mechanisms that are not yet well defined (Margueron and Reinberg, 2011; Mikkelsen et al., 2007; O'Meara and Simon, 2012). Delineation of the function of PRC2-Ezh1 has been more challenging: some studies suggest that Ezh1 substitutes for the repressive function of Ezh2 (Ezhkova et al., 2009, 2011; Shen et al., 2008), and that this repressive function can be independent of its methyltransferase activity (Margueron et al., 2008). In contrast, recent studies in differentiating muscle cells conclude that binding of PRC2-Ezh1 complexes to target promoters is associated with the active H3K4me3 epigenetic mark, and that such binding promotes RNA polymerase (Pol) II elongation and mRNA transcription (Lau and Cheung, 2011; Mousavi et al., 2012; Stojic et al., 2011).

Little is known about the role of PcG proteins in the central nervous system. Components of PRC2 and PRC1 are expressed in the *Drosophila* brain, where they are required for dendritic targeting and arbor maintenance (Parrish et al., 2007; Tea and Luo, 2011). Here we analyze the expression pattern of the PRC2 proteins Ezh1 and Ezh2, and determine their contribution to the regulation of PSD-95 gene transcription in maturing mammalian hippocampal cells. PSD-95 has a fundamental role in regulating synaptic maturation and plasticity by scaffolding glutamate receptors with signaling proteins in the excitatory synapse; thus PSD-95 gene expression increases during development where it limits plasticity (Elias and Nicoll, 2007; Funke et al., 2005; van Zundert et al., 2004). Despite this important role of PSD-95, however, little is known about the molecular mechanisms that control its expression. PSD-95 gene transcription is regulated by the transcription factors Eos and NeuroD2 (Bao et al., 2004; Wilke et al., 2012), and recent analyses in HDAC2 knock-out mice suggest that histone acetylation epigenetically regulates the PSD-95 gene promoter (Guan et al., 2009). Here we show that Ezh1 and Ezh2 are differentially expressed in developing hippocampal cells, and have opposing influences on the control of PSD-95 gene transcription. We also document that during maturation, Ezh1 is recruited to the PSD-95 gene promoter to up-regulate gene transcription, while the presence of Ezh2 at the PSD-95 promoter is associated with silencing of the PSD-95 gene during early stages of development. Surprisingly, we find that Ezh2-mediated repression of the PSD-95 gene is independent of the H3K27 trimethylation at the proximal promoter region.

Results

Expression of PRC2 proteins during hippocampal development in vitro and in vivo

Previous studies show that PRC2 proteins Ezh1, Ezh2, and Suz12 are expressed in several tissues including skeletal muscles and heart, as well in primary cultures derived from these tissues. However the expression pattern of these proteins depends on the stage of tissue differentiation (He et al., 2012; Mousavi et al., 2012; Stojic et al., 2011). In mouse brain, levels of Ezh1 and Ezh2 mRNA are inversely correlated: Ezh2 mRNA is highly expressed during early development (embryonic days (E) 13 to E17) and then is significantly reduced with neuronal maturation, whereas Ezh1 mRNA expression is low during embryogenesis, and is significantly elevated in the adult brain (Laible et al., 1997). Recent studies that compare wild-type and Ezh2 knock-out mice also reveal that Ezh2 is expressed in neuronal progenitor cells (NPCs) and in neurons during embryonic stage. Ezh2 trimethylates H3K27 at target sequences, and is essential for controlling the balance between self-renewal and differentiation (Pereira et al., 2010; Sher et al., 2011). However, the expression pattern of the PRC2 proteins Ezh1 and Ezh2, and the role of these proteins in regulating transcription in the post-mitotic mammalian central nervous system, have not yet been established.

We used western blots of nuclear extracts and determined the expression pattern of these PcG proteins in neuronal tissue as well as in cultures derived from the hippocampus at several developmental stages. PRC2 proteins Ezh1, Ezh2 and Suz12 are detected both in vivo (Fig. 1a) and in vitro (Fig. 1b) in these brain cells, and their expression pattern depends on the stage of maturation of the cells. Ezh1 exhibits a strong signal at ~95 kDa in immature hippocampal tissue (E18) and cultures (2 days in vitro; DIV). While the intensity of this protein band gradually decreases with maturation, it nevertheless remains significantly high in mature cells (postnatal days (P) P30 and 20 DIV). Expression of Ezh2 is also high during early developmental stages, but declines with differentiation, and is no longer detectable in mature hippocampal cells (see also saturated band signals in an over-exposed western blot at the bottom of Fig. 1a and b). This decline in Ezh2 protein expression was confirmed by using two independent antibodies (see Supplementary Fig. S1). Expression of Suz12 also declines during development, but it remains detectable in mature hippocampal cells, a result that is in agreement with the role of Suz12 as the scaffolding component of PRC2 complexes, capable of interacting with both Ezh2 and Ezh1 proteins (Margueron et al., 2008; Stojic et al., 2011).

Using quantitative real-time reverse transcriptase PCR (qRT-PCR) analyses, we determined that reduction in mRNA levels for Ezh2 (Fig. 1c, d) parallels the decreased levels of this protein during development. In contrast, mRNA levels for Ezh1 increase slightly in hippocampal tissue (Fig. 1e) as well as in cultures (Fig. 1f) during maturation, even though levels of Ezh1 protein decrease. This suggests that post-transcriptional mechanisms may be preventing an efficient translation of this increased Ezh1 mRNA. The specificity in the recognition of the ~95 kDa Ezh1 protein band was confirmed by comparing its migration in gel electrophoresis and enrichment within neuronal nuclear extracts with that for rat-derived osteoblastic cells (ROS17/2.8), and for differentiated mouse skeletal muscle cells (C2C12) (Fig. 1g). We also knocked-down Ezh1 expression with use of high titer lentiviral particles carrying two specific short hairpin interference RNAs against Ezh1 (shRNA-Ezh1.1 and shRNA-Ezh1.2). Hippocampal neurons at 5 DIV were infected with the lentiviral particles, and nuclear extracts were prepared 48 and 96 h later to assess Ezh1 expression levels by western blot. We found that 96 h post infection, shRNA-Ezh1.1 (Fig. 1h, i) and shRNA-Ezh1.2 (Supplementary Fig. S2a) each produced a marked reduction in levels of Ezh1 protein (Fig. 1h) and mRNA (Fig. 1i). No change in the expression of RNA Pol II was observed with use of the shRNA-Ezh1 constructs (Fig. 1h), confirming specificity of the Ezh1 knock-down.

Collectively, our results indicate that two different PRC2 complexes are present in brain cells: PRC2-Ezh2 and PRC2-Ezh1 are both abundantly expressed in immature brain cells, whereas PRC2-Ezh1 is expressed predominantly in mature brain cells. Similar changes in protein and mRNA expression patterns for Ezh1, Ezh2 and Suz12 have been reported for differentiating muscle cells (Mousavi et al., 2012; Stojic et al., 2011).

Ezh1 and Ezh2 are expressed in hippocampal neurons and glial cells

Previous studies suggest that while Ezh2 is more abundantly expressed in proliferating cells, Ezh1 is preferentially present in post-mitotic cells (Margueron et al., 2008), raising the possibility that expression of the two proteins is associated with different cell types within our mixed hippocampal cell preparations. To test the possibility that Ezh1 is expressed in neurons (differentiated post-mitotic cells) and Ezh2 in astrocytes (proliferating cells), we established neuron-enriched and glia-enriched cell cultures (see Experimental methods). Whole cell extracts were prepared from hippocampal cultures at 5 or 12 DIV, and specific antibodies were used to analyze PcG