Region specific knock-out reveals distinct roles of chromatin modifiers in adult neurogenic niches

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ABSTRACT

Histone methyltransferases (HMTs) are present in heterogeneous cell populations within the adult brain including neurogenic niches. Yet the question remains whether loss of HMTs and the resulting changes in histone methylation after cell fate in a region-specific manner. We utilized stereotaxic injection of Cre recombinant protein into the adult neurogenic niches, the subventricular zone (SVZ) adjacent to the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus. We confirmed that Cre protein was enzymatically active in vivo and recombination events were restricted to the vicinity of injection areas. In this study, we focus on using Cre mediated recombination in mice harboring floxed HMT: enhancer of zeste homolog 2 (EZH2) or suppressor of variegation homolog (Suv4-20h). Injectable Cre protein successfully knocked out either EZH2 or Suv4-20h, allowing assessment of long-term effects in a region-specific fashion. We performed meso-scale imaging and flow cytometry for phenotype analysis and unbiased quantification. We demonstrated that regional loss of EZH2 affects the differentiation paradigm of neural stem progenitor cells as well as the maintenance of stem cell population. We further demonstrated that regional loss of Suv4-20h influences the cell cycle but does not affect stem cell differentiation patterns. Therefore, Cre protein mediated knock-out a given HMT unravel their distinguishable and important roles in adult neurogenic niches. This Cre protein-based approach offers tightly-controlled knockouts in multiple cell types simultaneously for studying diverse regulatory mechanisms and is optimal for region-specific manipulation within complex, heterogeneous brain architectures.

Introduction

Methylation of lysines in histone tails is a critical post-translational modification that regulates diverse biological processes including cell cycle, development, differentiation, metabolism, and multipotency of stem cells. Conventional promoter-driven knock-out mouse models have demonstrated the importance of histone modifications in specific cell types in the adult brain. However, histone methylation occurs in all cells types and is vulnerable to regional changes in metabolism-associated methyl influx due to environmental effects or pharmaceutical inhibitors of methylation. Since metabolic effects or drugs generally do not function in a cell type-specific manner, we developed a method allowing knockout of genes of interest in multiple cell types using a penetrating peptide tagged Cre protein. We used this approach to examine the functional consequences of regional loss of histone methylation by performing regional knock-out of two independent methyltransferases in the adult neurogenic niches.

Multipotent neural stem progenitor cells (NSPCs) exist in two adult neurogenic niches, the subventricular zone (SVZ) adjacent to the lateral ventricle and the subgranular zone of the dentate gyrus (SGZ/DG). Neural stem cells (NSCs) give rise to progeny including neuroblasts, which undergo cell fate transition toward neuronal lineages [1–3]. The balance between NSC self-renewal and differentiation is maintained, in part, by epigenetic repression to prevent transcriptional noise of lineage-specific genes [4–9]. One such mechanism to inhibit transcription is post-translational modifications on histone tails, including trimethylation at histone 3 lysine 27 (H3K27me3) and trimethylation at histone 4 lysine 20 (H4K20me3). H4K20me3 is catalyzed by suppressor of variegation 4–20 homologs (Suv4-20h1/Suv4-20h2; human homologs KMT5B/KMT5C), collectively described as Suv4-20h. Our previous genomic study using endogenous NSPCs isolated from baboon brain identified putative targets of H4K20me3 that function in cell cycle, metabolism, and immune response [10]. Germline deletion of Suv4-20h1/Suv4-20h2 by Mox2-Cre showed that Suv4-20h impaired the maintenance of lymphoid progenitors [11]. To date, there is no report assessing the long-term effect of loss of Suv4-20h in adult neurogenic niches. H3K27me3 is catalyzed by enhancer of zeste homolog 2 (EZH2) [12], which is ubiquitously expressed in a wide range of cell types including NSPCs.
residing in the SVZ and SGZ/DG of murine and non-human primate baboon brains [10]. Our previous study using a non-human primate baboon model suggests that H3K27me3 represses developmentally critical regulators involved in differentiation [10]. A study using a GFAP-Cre mediated conditional Ezh2 knockout found that EZH2/H3K27me3 is critical for postnatal neurogenesis in the mouse SVZ [13]. Using Nestin-CreERT2 and tamoxifen inducible system to deplete Ezh2 in the SGZ/DG showed that Ezh2 is important for learning and memory capabilities in 6-week old mice [14]. However, considerable evidence has shown that tamoxifen administration is neurotoxic, resulting in impaired learning in rodents and impaired decision making in humans, even at dosages far below those used for recombination studies [15–18]. Such toxicity can affect long-term phenotypic analysis, requiring an alternative approach to overcome these confounding factors.

As EZH2/H3K27me3 and Suv4-20h/H4K20me3 are present in heterogeneous populations within adult neurogenic niches [10], we developed an alternative method to gain insight into the roles of EZH2/H3K27me3 or Suv4-20h/H4K20me3 in a wide range of adult NSPCs. Using conditional Ezh2 and Suv4-20h mouse models, we delivered penetrating peptide tagged Cre protein into the adult SVZ and SGZ/DG via stereotaxic injection to assess the long-term in vivo effect of region-specific loss of EZH2/H3K27me3 or Suv4-20h/H4K20me3. Following injection, Cre protein crosses the plasma and nuclear membranes via an engineered transduction peptide (CTP) and a nuclear localization sequence (NLS), respectively. The Cre protein consists of bacteriophage P1 Cre recombinase, which specifically recognizes 34 base-pair loxP sequences flanking a DNA sequence corresponding to the enzymatic domain of Ezh2 or Suv4-20h, resulting in their deletion. We demonstrated that Cre protein is readily and locally taken up by cells following stereotaxic injection into targeted brain regions to decrease EZH2 or Suv4-20h abundance. After region-specific reduction of EZH2 in the adult SVZ, we utilized the dDISCO protocol [19] for meso-scale imaging of mouse brains by light sheet fluorescent microscopy (LSFM) and found a drastic effect on doublecortin-positive (DCX⁺) neuroblasts. After Cre protein locally reduced EZH2 in the SGZ/DG, both immunohistochemistry (IHC) and flow cytometry demonstrated that loss of EZH2 affects the maintenance of NSPCs. To determine if this phenotype is specific in response to the abundance of Ezh2, we stereotaxically injected Cre protein in the SGZ/DG of conditional Suv4-20h mice and utilized imaging flow cytometry to provide comprehensive quantification of recombination efficiency along with phenotype analysis. Imaging flow cytometry also allowed co-localization studies of cell markers while providing a means for unbiased cell counting that has not been reported in any study relevant to adult hippocampal neurogenesis. We found regional loss of Suv4-20h influences the cell cycle. Hence, Cre protein mediated knock-out of either Ezh2 or Suv4-20h underscores the importance of distinguishing roles of EZH2/H3K27me3 from Suv4-20h/H4K20me3 in a region-specific manner. Taken together, we demonstrated a useful method for localized injection of cell-permeable Cre recombinase into mouse brain for the study of conditional knockouts in mosaic contexts. Importantly, this region-specific protein-based approach for genetic manipulation enables future in vivo studies to understand the role of diverse epigenetic regulators distributed in a range of cell types within complex tissue architectures.

Results

Recombinant Cre protein induces homologous recombination in vitro and in vivo

The development of cell-penetrating peptide (CPP) tagging-Cre fusion proteins has provided an alternative approach to introducing Cre into a cell, in addition to transgenic or viral transduction methods [20,21]. As a proof of principle, this Cre fusion protein containing a cell transduction peptide (CTP: APWHLSSQYSRT) and a nuclear localization sequence (NLS) has been successfully delivered to cardiac muscle via ultrasound guided injection [22]. To simplify the description of this NLS-Cre-CTP fusion protein, Cre protein is the denotation used throughout the text.

To evaluate the long-term utility of Cre protein induced recombination in vivo, we delivered Cre protein via stereotaxic injections into the brains of adult R26R-lacZ or R26R-EYFP (denoted by ROSA26) (Supplementary Fig. 1A-1E). Injection coordinates were empirically derived using fluorescent red retrobeads (Supplementary Fig. 1F-1H). Within the SVZ of R26R-lacZ mice injected with Cre protein, constitutive β-galactosidase (β-gal) expression was restricted to injection sites indicating that Cre protein injection induces recombination within targeted sites in a highly localized manner (Supplementary Fig. 2). Injection sites were largely demarcated from surrounding areas, as only minor β-gal expression was observed in cells outside the injection site. Using image flow cytometry analysis, we confirmed the high efficiency of Cre-mediated recombination within the injected areas of R26R-EYFP mice (Supplementary Fig. 3). Immediately following dissection of hippocampal tissue for flow cytometry, cells from Cre protein injected hemispheres displayed YFP expression in regions corresponding to injection sites (Supplementary Fig. 3B, 3C). Cells from Cre injected hemispheres increased 519% in YFP⁺ populations compared to hemispheres injected with vehicle solution (20% glycerol in PBS) (Supplementary Fig. 3D-3G). The ability of injected Cre protein to induce recombination localized to injection sites presents a novel approach in which genes can be genetically manipulated in vivo in a spatially-restricted manner. Importantly, our method leads to applications involving conditional knockout of small populations of cells and also genes expressed across multiple cell types simultaneously, such as examining the long-term regional effect of chromatin modifiers within neurogenic niches.

Conditional loss of EZH2 or Suv4-20h function in adult SVZ and SGZ/DG by recombinant Cre protein

The chromatin modification enzymes EZH2 and Suv4-20h, as well as resulting histone modifications, H3K27me3 and H4K20me3, respectively, are ubiquitously present in a wide range of cell types in the adult SVZ and SGZ/DG. Within these neurogenic niches, such cell types include NSCs expressing GFAP and/or Nestin, and DCX⁺ neuroblasts (NBs)
(Supplementary Fig. 4–6). The SVZ/DG also contains differentiated cell types including βIII-Tubulin+ immature neurons and NeuN+ mature neurons (Supplementary Fig. 5–6). To delineate which subpopulations are most impacted by the loss of Ezh2 or Suv4-20h, we utilized stereotaxic injection of Cre protein into targeted sites within the adult SVZ and SGZ/DG of conditional Ezh2 or Suv4-20h1 mouse models. These mouse models harbor floxed alleles of genomic sequence encoding the enzymatic SET domain of Ezh2 (Ezh2<sup>fl/fl</sup>)<sup>12</sup> or Suv4-20h1 (Suv4-20h1<sup>fl/fl</sup>)<sup>13</sup> mice (Supplementary Fig. 4–6). To monitor knock-out efficiency, we bred Ezh2<sup>fl/fl</sup>;ROSA26<sup>lox/lox</sup> and Suv4-20h1<sup>fl/fl</sup>;ROSA26<sup>lox/lox</sup> mice for experiments. Following stereotaxic injection, the Cre protein was capable of crossing the plasma membrane of adjacent cells, importing into the nucleus, and initiating recombination at loxP flanked target sites. Such Cre mediated recombination resulted in excision of the SET domain of Ezh2 (or Suv4-20h) as well as the stop cassette upstream of β-galactosidase (β-gal) or yellow fluorescent protein (YFP). The crosses resulted in homozygous alleles denoted by Ezh2<sup>fl/fl</sup>;ROSA26 and Suv4-20h1<sup>fl/fl</sup>;ROSA26<sup>lox/lox</sup> mice compared to controls (p-value = 0.002) following knockdown of Ezh2<sup>fl/fl</sup>;ROSA26<sup>lox/lox</sup> mice compared to controls (Figure 2(B–J), Supplementary Fig. 8). However, 32 days post Cre injection into Ezh2<sup>fl/fl</sup>;ROSA26<sup>lox/lox</sup> mice did not show a significant change in either GFAP+ or Nestin+ populations compared to controls (Cre injected into ROSA26 mice). Immunohistochemistry (IHC) results from 10 day post-Cre protein injected SVZ showed a decrease in DCX+ NBs along the RMS, suggesting NBs migrate away from the SVZ earlier than 10 days. In this regard, a shorter time point of 5 days post-Cre protein injection was chosen to detect early NBs migration, using sagittal sections (Supplementary Fig. 9). Compared to vehicle injected controls, Cre injected hemispheres showed a moderate decrease in DCX+ NBs at the dorsal SVZ and substantial increases in DCX+ NBs along the RMS and migrating tangentially into the olfactory bulb (OB) (n = 4). To further assess the migratory capacity of NBs along the RMS to the OB by meso-scale imaging, we utilized a modified iDISCO tissue clearing protocol and LSF to examine DCX+ populations originating from the SVZ. Consistent with our short- and long-term IHC data, iDISCO showed DCX+ populations substantially increased in the dorsal SVZ and along the RMS upon Cre-mediated loss of Ezh2/ H3K27me3 after 5 days (Figure 3(B–D), Supplementary Fig. 10A-10E) while a moderate increase in DCX+ cells was still detected in animals injected with Cre compared to vehicle (Figure 3(E–G), Supplementary Fig. 10F–10G) after 32 days post-injection. In addition, βIII-Tubulin+ and NeuN+ populations increased in the OB 10 days after loss of Ezh2/H3K27me3 (Supplementary Fig. 11), as did the interneuron marker Calbindin (Supplementary Fig. 12).

The ubiquitous abundance and distribution of H3K27me3 among NSPCs within the SVZ/DG (Supplementary Fig. 5) suggested distinct regulation by H3K27me3 in this adult neurogenic niche and compelled further investigation. We assessed the effect of loss of Ezh2/H3K27me3 at 19, 32, or 46 days after stereotaxic injection of Cre into the SVZ/DG, which corresponded to the times required for NSPC proliferation, differentiation/migration, and integration into neuronal circuit, respectively [2,3]. In the SVZ/DG, we observed a notable increase in NeuN+ (Figure 4(A,B)) and a moderate increase in Nestin+ cells but no change in GFAP+ cells at 19 days post-Cre injection compared to vehicle injected controls (Supplementary Fig. 13). Although the populations of DCX+ or βIII-Tubulin+ populations were not substantially changed 19 days after Cre injection (Supplementary Fig. 14), unusual colocalization of DCX+/NeuN+ cells was more abundant in the Cre injected SVZ/DG compared to vehicle injected SVZ/DG 19 days after injections (Figure 4(C–G)). Consistent with the 19 days immunohistochemistry data, flow cytometry analysis of SGZ/DG 32 days after Cre injection demonstrated an increase in NeuN+ neurons (Figure 5). Intriguingly, 46 days after Cre injection...
Figure 1. Loss of EZH2 affects neuroblasts within the subventricular zone. (A) Schema of coronal sectioned mouse brain containing subventricular zone (SVZ). Black boxes indicate regions imaged. Horizontal bars depict stepwise cell fate transition of NSPC. (B) Bar graph of the percentage of H3K27me3-positive cells present in the dorsal SVZ of ROSA26 or Ezh2fl/fl;ROSA26 mice, 10 days post-injection of Cre. n = 3 mice per experimental group is indicated at base of bar graph, p-value = 0.007, determined using a Welch two sample t-test. (C) Percentage of DCX+H3K27me3+ and DCX+H3K27me3− cells within the dorsal SVZ of ROSA26 or Ezh2fl/fl;ROSA26 mice, 10 days post-injection of Cre. n = 3 mice per experimental group, standard error of the mean displayed on bars. (D-K) Immunostaining for doublecortin (DCX) and H3K27me3 in coronal sections obtained from the SVZ of a ROSA26 mouse, 10 days post-injection of Cre, 40X. (L-S) Immunostaining for doublecortin (DCX) and H3K27me3 in the SVZ of an Ezh2fl/fl;ROSA26 mouse, 10 days post-injection of Cre, 40X. Scale bars = 20 μm.
into SGZ/DG, Nestin+ populations were substantially increased, while a moderate decrease in NeuN+ neurons was detected compared to vehicle injected SGZ/DG (Supplementary Fig. 15). Findings from three time-point analyses suggest that regional loss of EZH2/H3K27me3 in the adult SGZ/DG induced early differentiation at 19- and 32-day while Nestin+ cells replenished NSPCs in response to declined NeuN+ population at extended 46-day. Collectively, our results showed distinguishable cellular phenotypes, in which the DCX+ population in the SVZ and the NeuN+ population in the SGZ/DG were the most affected upon regional loss of EZH2/H3K27me3.

Figure 2. Sustained loss of EZH2 affects neuroblasts within the subventricular zone. (A) Bar graph of the percentage of H3K27me3-positive cells present in the dorsal SVZ of ROSA26 or Ezh2fl/fl;ROSA26 mice, 32 days post-injection of Cre. n = 5 mice per experimental group is indicated at base of bar graph, p-value = 0.002, determined using a Welch two sample t-test. (B) Percentage of DCX+H3K27me3+ and DCX+H3K27me3− cells within the dorsal SVZ and RMS of ROSA26 or Ezh2fl/fl;ROSA26 mice, 32 days post-injection of Cre. n = 5 mice per experimental group, standard error of the mean displayed on bars. (C-F) Immunostaining for doublecortin (DCX) and H3K27me3 in coronal sections obtained from the SVZ of a ROSA26 mouse, 32 days post-injection of Cre, 40X. (G-J) Immunostaining for doublecortin (DCX) and H3K27me3 in the SVZ of an Ezh2fl/fl;ROSA26 mouse, 32 days post-injection of Cre, 40X. Scale bars = 20 μm.
Suv4-20h/H4K20me3 affects cell cycle of the adult NSPCs

Our previous study implicated Suv4-20h/H4K20me3 in regulating the cell cycle of NSPCs in the SVZ and inspired assessment of potential regulatory roles in the SGZ/DG. Using the Cre protein approach, we assessed changes in NSPC populations and proliferation in Suv4-20h1flox/flox;ROSA26 mice following Cre protein (experimental) or vehicle (control) injection into the SGZ/DG at the time points of 19, 32, and 46 days post injection. Two hours EdU labeling is commonly utilized for embryonic and postnatal neurogenesis studies. However, 19, 32, and 46 days post-Cre injection resulted in no significant change in numbers of 5-ethynyl-2’-deoxyuridine (EdU) labeled cells for 2 hours post-EdU injections (Supplementary Fig. 16–18). As adult neurogenesis is a slow process, our validation of proliferation rate in the SGZ/DG of adult mice indicated that a 12-hour EdU labeling was deemed suitable for examining NSPC proliferation (Supplementary Fig. 19). Intriguingly, 46 days post-Cre injection resulted in marginally increased numbers of EdU labeled cells 12 hours after EdU injection (Figure 6). However, no significant change in mitosis markers (i.e. phospho-H3(S10) or phospho-S780-Rb) and cell type markers (i.e. GFAP+, DCX+, or NeuN+ cells) was observed in subpopulations of NSPCs within the SGZ/DG 19, 32, or 46 days after Cre or vehicle injection (Figure 6(H–K), Supplementary Fig. 16–18, 20). Taken together, proliferation was consistently affected upon loss of Suv4-20h. Intriguingly, long-term knockout of Suv4-20h/H4K20me3 in the adult neurogenic niche resulted in instances of seizures over the one year post-treatment period (Data not shown).
Discussion

A challenging and yet unresolved obstacle in genetic manipulations of mice is altering gene expression in tissue composed of heterogeneous cell types. Such heterogeneity is common in virtually all adult stem cell niches, such as bone marrow, germ cell niches, and neurogenic niches. In such environments, there are circumstances where it would be beneficial to globally knockout a gene of interest across all cell types and examine any resulting phenotypes. For cell-type specific studies, a well-defined promoter can be applied to target a cell type of interest. For region-specific studies, our method of stereotaxic delivery of recombinant Cre protein presents a novel approach for a temporally and spatially-restricted knockout of a gene of interest. Injectable Cre proteins will not supplant other methods of tissue specific gene manipulation, including transgenic promoter...
driven expression, viral expression systems, or tamoxifen/tetracycline inducible systems. However, our Cre protein approach is useful for gene manipulation within selected injection sites that results in Cre-induced recombination in targeted regions without exogenous Cre expression; the Cre gene was neither integrated into the host genome, nor maintained extra-chromosomally on a non-integrating vector. The high spatial precision of our technique for manipulating target cells offers

Figure 5. NSPC populations are altered following 32 day loss of EZH2 in the SGZ/DG. (A) Schematic of NSPC markers in the SGZ/DG. Horizontal bars depict markers expressed during stepwise cell fate transition of NSPCs in the SGZ/DG. (B-K) Representative flow cytometry analysis of isolated cells from SGZ/DG shows gating strategy based on SSC-A/FSC-A (B), single cells (C), and H3K27me3-negative population in Ezh2fllox/lox;ROSA26 mouse, 32 days post-injection of vehicle (control) (D-G) or Cre (Ezh2 KO) (h-k). (E-G) Vehicle injected hemisphere. Lineage specific H3K27me3 DCX+ (E), H3K27me3 NeuN+ (F), and H3K27me3 β-gal+ populations (G). (I-K) Cre injected hemisphere. Lineage specific H3K27me3 DCX+ (I), H3K27me3 NeuN+ (J), and H3K27me3 β-gal+ populations (K). (L) Bar graph presents abundance of lineage specific populations. n = 5 mice per experimental group, differences between conditions tested using a Welch two sample t-test.
Figure 6. Suv4-20h mediates S-phase progression of NSPCs within the adult hippocampus. (A) Table indicating gating scheme used for population analyses, including test populations, mean % of parent population, standard deviation of each population and p-value, if applicable, for control and Suv4-20h1 knockout experimental groups. (B) Histogram of YFP expression in SGZ/DG cells from vehicle or Cre injected hemispheres of Suv4-20h1fl/fl;ROSA26Y/ mouse, 46 days post-injection. (C-D) Histogram of EdU in SGZ/DG cells from unstained population (C), and vehicle or Cre (D) injected hemispheres of Suv4-20h1fl/fl;ROSA26Y/ mouse, 46 days post-injection. (E) Bar graph of the percentage of 12 hr. EdU-positive cells present in the DG of Suv4-20h1fl/fl;ROSA26Y/ mice, 46 days post-injection of vehicle or Cre. n = 4 mice per experimental group, standard error of the mean displayed on bars. (F-G) Representative images of 12 hr. EdU labeled cells from vehicle injected (F) or Cre injected (G) hemispheres of Suv4-20h1fl/fl;ROSA26Y/ mouse, displaying brightfield, YFP, EdU and merged channels. (H-J) NeuN and phospho-H3(S10) in SGZ/DG cells from unstained population (H), vehicle injected (I), and Cre injected (J) hemispheres of Suv4-20h1fl/fl;ROSA26Y/ mouse, 46 days post-injection. (K) Histogram of NeuN+ neurons from vehicle and Cre injected hemispheres of Suv4-20h1fl/fl;ROSA26Y/ mouse, 46 days post-injection.
enhanced modeling capabilities in murine models that would be useful for lineage tracing and cell fate studies at extended time-points. Future applications of such a system include region-specific gene knockout followed by single cell transcriptomic or microfluidic drug treatment to glean valuable insight into regulatory mechanisms at the molecular level within a heterogeneous population.

The combination of conditional HMT deletion and ROSA26 reporter expression co-localized to a given injection site has utility in dissecting the role of various HMTs in complex processes such as cell fate transition. Within injection sites of the SVZ or SGZ/DG, two distinct populations of cells were observed following Cre recombination: cells with elevated levels of β-galactosidase and decreased histone modifications (i.e. cells having undergone Cre recombination), and cells with endogenous β-galactosidase levels, but high enrichment of histone methylation (i.e. cells which have not undertaken Cre recombination). The co-occupancy of both populations within an injected region acts as an internal control, and becomes useful in probing the role of HMTs in cell autonomous or non-cell autonomous regulations within these neurogenic niches. Utilizing this novel region-specific gene manipulation in parallel with modified dISCO whole brain imaging and quantitative image flow cytometry, we have demonstrated that EZH2/H3K27me3 plays an important role in the maintenance of sub-populations within the SVZ and the SGZ/DG. Thus, our finding adds to a compendium of previous studies on the role of EZH2 in NSPCs using promoter-specific conditional knockout methods [5,6,13,14]. In addition, while cancer treatment using EZH2 inhibitors to target dysregulation of EZH2/H3K27me3 in glioblastoma subtypes is ongoing, our Cre protein-based manipulation uncovers long-term in vivo effects upon loss of EZH2. The concept of non-cell type specific manipulation described in this work can be applied to modeling long-term in vivo effect of other epigenetic modifiers prior to the development and implementation of drug inhibitors for therapeutics. Moreover, our study is the first demonstration that the cell cycle within adult neurogenic niches is regulated, at least in part, by Suv4-20h/H4K20me3.

Our approach is not limited to neurogenic niches, and can be successfully implemented in various models to address myriad tightly-controlled knockouts or knock-ons in many tissues or cells simultaneously. As a broader impact, our novel approach for gene manipulation is optimal for studying many other regulatory mechanisms in a site-specific fashion, and can be applied to additional brain regions and/or to targeted cell populations within complex, heterogeneous tissue architectures. In addition, this method would be particularly useful for investigating developmentally critical genes in localized regions of solid organs, where complete deletion in utero would result in embryonic or perinatal lethality. Furthermore, use of recombinant proteins for in vivo transduction of therapeutic protein has already been performed to protect against cerebral infarction, ischemic brain injury, and neural apoptosis, via intraperitoneal injection in mice [23,24]. Therefore, the cell permeable proteins like CTP-tagged protein used in this study could be potentially adapted for production of recombinant pharmaceutical proteins for therapeutic applications.

Methods

Animal models

Mice contained a floxed SET domain of EZH2 (Ezh2^{lox/lox}) or Suv4-20h (Suv4-20h^{lox/lox};Suv4-20h^{−/−}). Ezh2^{lox/lox} mice or Suv4-20h^{lox/lox};Suv4-20h^{−/−} mice were crossed to R26R-lacZ or R26R-EYFP reporter mice (Jackson laboratory) (denoted by ROSA26) with loxP sites flanking a stop cassette upstream of β-galactosidase (β-gal) or yellow fluorescent protein (YFP). The crosses resulted in Ezh2^{lox/lox};ROSA26 and Suv4-20h^{lox/lox};Suv4-20h^{−/−};ROSA26 mouse colonies for experiments. Genotyping utilized tissues from ear punch. Ear punch samples were lysed in 90 μL of fresh-made 50 mM NaOH shaking at 95.0°C for 10 min. After 10 min, samples were cooled on ice and NaOH was neutralized immediately with 10 μl 1M Tris HCl, briefly vortexing. Samples can be used immediately for genotyping or stored at −20°C. PCR cocktail includes: 25 μl Apex Taq RED Master Mix (Genesee Scientific, Cat #: 42–137), 1 μl per primer, 1 μl template DNA, bring to 50 μl with nuclease free water. Thermocycler program was as follows: 1 cycle: 95.0°C @ 3 min; 25–55 cycles: 95.0°C @ 20 sec, 58.0°C @ 40 sec, 72.0°C @ 1 min; 1 cycle: 72°C @ 7 min, hold at 4°C. Visualize amplicons using 1–1.5% agarose gel in 1X TAE with 0.5 μg/ml final concentration of EtBr in gel (100 V for 45–90 min for separation of bands). All mouse strains were congenic prior to experiments. Mice (males and females) between 12 and 24 weeks old were used. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas at San Antonio (UTSA).

Cre protein induction and purification

For Cre protein induction and purification, His-Cre-CTP was transformed into BL21(ED3)pLysS (Invitrogen) for protein expression. The overnight bacterial culture was inoculated at dilution of 1:80 in LB with 100 mg/mL ampicillin for 2 hours at 37°C and proteins were induced with 0.5 mM IPTG for 2 hr at room temperature. Total protein was extracted with lysis buffer (50 mM NaH2PO4, pH 8, 0.5 M NaCl, 10 mM imidazole, 0.2% Triton X-100, 6 mM 2-mercaptoethanol) and purified with Ni-NTA superflow cartridge (Qiagen). After wash with buffer containing 50 mM NaH2PO4, pH 8.0, 0.3 M NaCl, 20 mM imidazole, Cre protein was eluted with elution buffer (250 mM imidazole in PBS). The eluted protein fractions went through buffer exchange with PBS using 10 K Amicon centrifugal filter (Millipore) and then stored in 20% glycerol/PBS at −80°C. Protein concentration was determined by Bradford Assay (BioRad).

Stereotaxic injection of Cre-CTP protein and vehicle solutions

Experimental design: For external controls, ROSA26 "reporter" mice were utilized which only have the lacZ or YFP reporter (R26R-lacZ or R26R-EYFP), but do not have conditional alleles for Ezh2^{lox/lox} or Suv4-20h^{lox/lox};Suv4-20h^{−/−}. These mice were injected with Cre and the phenotypic effect were compared to animals harboring Ezh2^{lox/lox};ROSA26 or Suv4-
Gene expression analysis

For qRT-PCR validation, total RNA was extracted from the SGZ/DG using TRIzol (Invitrogen) reagent followed by aqueous/organic phase separation, isopropyl alcohol RNA precipitation, dissolving RNA with 25–50 uL nuclease free water and subsequently cDNA synthesis by iScript reverse transcriptase supermix (Bio-Rad, #170-8841) according to the manufacturer’s instructions. Briefly, 5 uL input RNA (1 pg – 1 ug) was combined with 4 uL iScript supermix and brought to 20 uL total volume with nuclease free water. RT reaction protocol is as follows: priming: 5 min @ 25°C; RT: 20 min @ 46°C; inactivation: 1 min @ 95°C. The transcript expression level was measured by Real-Time PCR with SYBR Select Master Mix (Life Technologies, #4472908) and detection on ABI7900HT system (Applied Biosystems). S16 RNA was used as internal control for normalization (∆Ct). The differential expression was measured by subtraction of ∆Ct between wildtype and Ezh2 knockout animals to obtain ∆∆Ct (log2). Primers were designed using ABI Primer Express Software version 3.0, PrimerBank (https://pga.mgh.harvard.edu/primerbank/), and Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). At least one primer pair per gene spanned 2 or more exons.

Immunohistochemistry

Mice were anesthetized with 450 mg/Kg Avertin, transcardially perfused with 1x PBS, and fixed with 4% paraformaldehyde. Following fixation, mouse brains were cryopreserved with 30% sucrose/PBS overnight and frozen in OCT compound. Frozen brains were sectioned at 12 µm, blocked with 10% goat serum for 30 minutes, and incubated 4°C overnight with combinations of antibodies specific for: H3K27me3 (Active Motif, Cat#39155;Lot#31814017, 1:1000), β-galactosidase (Promega, Cat#Z788;Lot#000040733, 1:750), GFP (Invitrogen, Cat#A11122, 1:500; Aves Labs, Cat#GFP-1020;Lot#1229FP08, 1:500), and cell type specific markers: GFAP (Millipore, Clone GA5, Cat#mab3402, Lot#2580632, 1:750), Nestin (AbCam, Cat#ab29752, 1:300; AbCam, Cat#ab134017, 1:250), DCX (Santa Cruz, Cat#sc-8066; Lot#10314, 1:200), βIII-Tubulin (Millipore, clone TU-20, Cat#MAB1637, Lot#JC1686015, 1:200), and NeuN (Millipore, clone A60, Cat#MAB377, Lot#NG1876252, 1:200). On the subsequent day, sections were washed with 0.5% TritonX-100/1X PBS. Following wash, antibody combinations needing only secondary antibodies (i.e. H3K27me3, GFAP, GFP, chicken anti-Nestin, and/or DCX) were incubated with AlexaFlour-488 or –594 conjugated secondary antibodies (anti-mouse: Life Technologies, Cat#A21200; Life Technologies, Cat#A21201; anti-rabbit: Invitrogen, Cat#A21441; Life Technologies, A21207, anti-chicken Life Technologies, Cat#A11039, anti-goat: Life Technologies Cat#A1055; Life Technologies, Cat#A11058). Sections probed with all other antibodies (rabbit anti-Nestin; mouse anti-βIII-Tubulin, NeuN, β-gal) were incubated with Biotinylated-rabbit or –mouse secondary antibody for 1 hour at room temperature (Vector Laboratories, Cat#BA-1000, 1:1000; Vector Laboratories, Cat#BA-2000, 1:1000), followed by Avidin-conjugated AlexaFlour-488 or Avidin-conjugated AlexaFlour-594 tertiary antibody for 1 hour (Life technology, Cat#A21370, 1:2000; Life technology, Cat#S32356, 1:2000). Analysis of thymidine analog incorporation involved administration of EdU (5-ethyl-2'-deoxyuridine) into mice by intraperitoneal (IP) injection 2 hours prior to perfusion. EdU was detected by Click-it EdU 488 or 647 imaging kits (Invitrogen, C10337; Invitrogen, C10640), following manufacturer’s instructions. All sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Cat# H-1200). Images were acquired on a either a Zeiss 510 or 710 confocal microscope with 20X, 40X, and 63X objectives and were processed using the associated analysis software, Zen. Scale bars were added by ImageJ (https://imagej.nih.gov/ij/).

Flow cytometry

Mice were anesthetized with 450 mg/Kg Avertin prior to decapitation. Freshly isolated SGZ/DG cells were fixed with 4%
parafomaldehyde, permeabilized, incubated 4°C overnight with primary antibodies (anti-GFP, Nestin, PSA-NCAM, Doublecortin, NeuN, H3K27me3, S100-β, and ALDH1L1), and subsequently labeled with BV421, PE, PE-Cy7 (BD Biosciences) and Alexa fluor 488, −594, −647 (Life Technologies) conjugated secondary antibodies. Primary antibody sources: Doublecortin (Cell Signaling #4604; Millipore clone2G5 MABN707, 1:500), Polysalic Acid Neural Cell Adhesion Molecule PSA-NCAM, clone 2-2b (Millipore #MAB5324, Lot# 1966892, 1:500), Giall Filibrillary Acidic Protein GFAP, clone GA5 (Millipore #MAB3402, Lot#1993774, 1:500; Abcam clone 2A5 #ab4648, 1:500), Nestin (Abcam #AB27952; 1:500), NeuN (Millipore MAB377, 1:500), H3K27me3 (Active Motif, 1:1000), S100-β (AbCam, Cat#ab41548 1:500), β-galactosidase (Promega, Cat#z378B, 1:750). Secondary antibody sources: BD Biosciences. Analysis of thymidine analog incorporation involved administration of BrdU (5-bromo-2′-deoxyuridine) and EdU (5-ethyl-2′-deoxyuridine) into mice by intraperitoneal (IP) injection 12 hours prior to dissection. EdU was detected by Click-IT Edu 488 or 647 imaging kits (Invitrogen, C10337; Invitrogen, C10340), following manufacturer’s instructions. Processed cells were imaged by Amnis ImageStreamX MK11 imaging flow cytometer and quantified with associated IDEAs software.

**Tissue clearing and light sheet microscopy**

The new iDISCO+ protocol (May 2016, https://idisco.info/idisco-protocol/update-history/) was followed for all steps and reagents used during tissue fixing, immunohistochemistry and clearing. Briefly, mice were perfusion fixed with PBS and 4% paraformaldehyde (PFA) followed by overnight incubation in 4% PFA in 4°C. Brains were cut into 2 mm sagittal blocks in a brain matrix (Braintree Scientific, BS-A 5000S) prior to clearing. Following overnight fixing with 4% PFA, brains were pretreated with methanol (Fisher A412-4) and bleached with 30% hydrogen peroxide (Sigma 216763-100ML) on an orbital rocker. For immunohistochemistry, doublecortin was detected using goat anti-doublecortin primary (Santa Cruz, sc-8066, 1:200) and Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, A11058, 1:200). NeuN detection utilized mouse anti-NeuN (Millipore, MAB377, 1:200), biotinylated secondary (Vector Laboratories, BA-2000, 1:200) and Alexa Fluor 488-conjugated avidin (Life Technologies, A21370, 1:200). For all steps prior to tissue clearing, incubation times (listed as “n” in iDISCO+ protocol) Solution volume followed table in iDISCO+ protocol and were as follows: 3 day/ 4 mL for 2 mm sagittal mouse sections. Tissue clearing involved methanol dehydration series and overnight 66%C:33% methanol: Dichloromethane (DCM) incubation, and clearing with 100% DCM (Sigma 270997-1L) and Dibenzyl Ether (DBE) (Sigma 108014-1KG). Cleared tissues were imaged on an Ultramicroscope, LaVision BioTec light sheet microscope (LaVision, Bielefeld, Germany) and were processed with Imaris software (Imaris x64 8.1.2).

**Image quantification**

All steps of light sheet imaging quantification were performed in Imagem following manufacturer’s example in “Area Measurements and Particle Counting” (https://imagej.nih.gov/ij/docs/pdfs/examples.pdf). Mean fluorescence intensity (MFI) was determined by splitting RGB Color images into 8-bit greyscale images using the Split Channels tool before auto-adjusting with the Threshold setting. After thresholds were set, the Analyze Particles tool detected the average pixel intensity per area within the threshold range. The sizes used for particle analysis were 100-Infinity.

**Statistics**

For flow cytometry, two-sample t-tests were performed in R (https://cran.r-project.org/). For differential expression, a moderated t-test was performed using empirical Bayes moderation of the standard errors using the ebays function of limma package in R/Bioconductor (https://bioconductor.org/packages/release/bioc/html/limma.html).
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Author contributions

CTR performed stereotaxic injection and preparation of figures and manuscript. GZ performed light sheet imaging and processing by Imaris. CTR, GZ, and SAH performed experiments including modified iDISCO, IHC, and confocal imaging. CTR, SMC, and AEC performed flow cytometry and analysis. CAL, CTR, and SAH performed Cre protein purification. MSB and VPL contributed to provision of materials, data interpretation, and manuscript preparation. CAL contributed to project management, provision of materials, conception and design of experiments. CAL wrote manuscript with comments from all authors.

Disclosure of potential conflict of interest

The authors declare no competing financial interests.

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