Transposon-mediated, cell type-specific transcription factor recording in the mouse brain

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Abstract

Transcription factors (TFs) play a central role in the regulation of gene expression, controlling fundamental cellular processes such as fate decisions and activity-dependent transcription. Recent efforts to profile TF genome occupancy in a variety of cell lines and tissues have illuminated the diversity of TF binding amongst cell types, however there remains a gap in our understanding of cell type-specific TF regulation in complex, heterogenous tissues, such as the mammalian brain. Widely-used, bulk level TF profiling methods (e.g. ChIP-seq) yield only an averaged picture of TF binding across all cell types present within a harvested tissue, so it is challenging or impossible to determine how a TF might bind different regions of the genome in different cell types, or even to identify its binding events at all in less abundant cell types. Thus, there remains a need for a method to selectively profile TF occupancy in specific cell types in *in vivo* model systems. Here we present a versatile methodology, FLEX calling cards, for *in vivo*, cell type-specific TF occupancy recording. In this method, the TF of interest is fused to a hyperactive *piggyBac* transposase (hypPB), and this bipartite gene is delivered, along with donor transposons, to mouse tissue via a Cre-dependent adeno-associated virus (AAV). The fusion protein is conditionally expressed in Cre expressing cells where it inserts transposon “calling cards” near to TF binding sites. These transposons permanently mark TF binding events and can be mapped to the genome using high-throughput sequencing. Alternatively, unfused hypPB interacts with and records the occupancy of the bromodomain and extra-terminal (BET) domain protein, Brd4, allowing for cell type-specific super enhancer profiling, which we validate by identifying several new astrocyte-specific small enhancers. Finally, by continually inserting transposons while expressed and active, FLEX calling cards integrates TF binding events across time, allowing for the detection of transient or infrequent TF binding events. To demonstrate the FLEX calling card method, we first show that calling card reagents can be efficiently delivered to the mouse brain with AAV and that transposon insertion profiles report TF occupancy. Then, using a Cre-dependent hypPB virus, we show utility of this tool in defining cell type-specific super enhancer profiles in multiple cell types of the brain. Finally, we demonstrate utility of FLEX calling cards in longitudinal, integrative recording of the promoter-associated TF, SP1, providing a historical record of SP1 occupancy across time. This approach will enable important cell type-specific studies of TF-mediated gene regulation in the brain and may yield valuable insights into brain development, homeostasis, and disease.

Introduction

Proper cellular development and function is a complex process established by elaborate gene expression networks. These networks are fundamentally regulated by transcription factors (TF), which bind to regulatory elements (RE) across the genome and facilitate gene expression through a variety of mechanisms, including recruitment of transcriptional co-factors and modulation of chromatin state[1]. Extensive efforts to profile TF genome occupancy and identify active REs across the genome have highlighted the profound diversity of TF binding amongst cell types, providing important insights into cell type-specific gene regulation[2–5]. Indeed, it is of no surprise that a large portion of genetic variation associated with improper cellular function or disease is found in REs that are active only in disease-relevant cell or tissue types (e.g. Alzheimer's disease-associated variants occurring preferentially in brain-specific REs)[3,6–10].

Several methods exist for profiling TF occupancy across the genome in whole tissues. Chromatin immunoprecipitation followed by sequencing (ChIP-seq), which works by first crosslinking TFs to DNA and then isolating, sequencing, and mapping TF-bound DNA, is widely used and has provided numerous insights into the cellular functions of TFs[3,4,7]. Notably however, this method requires the availability and individual optimization of TF-specific antibodies, limiting the throughput and breadth of genome-wide TF profiling. Further, while robust for non-cell type-selective, tissue-level analyses, it is often challenging to interpret ChIP-seq data obtained from complex tissues such as the brain, which is comprised of many different interconnected cell types. Because of this limitation, efforts have recently been made to develop methods for profiling cell type-specific TF occupancy. One such approach is to modify ChIP-seq for cell type-specific use, either through physical nuclear sorting[10,11], or conditional expression and subsequent isolation of tagged nuclei[5,12] or chromatin-associated enzymes[13] prior to ChIP. However, these methods thus far have been limited to highly abundant targets, such as histone modifications[5,10] and transcriptional coactivators[13], and may be complicated by potential disassociation-related artifacts[14]. Therefore, it is unclear if ChIP-seq is feasible from sorted or isolated nuclei for less abundant TFs. Finally, ChIP-seq provides only a snapshot of TF activity at the moment of cell lysis and thus may be inefficient at detecting transient or infrequent TF binding events.

An alternative approach is to record TF binding events by fusing the TF of interest to DNA tagging enzymes[15,16]. Prominent among these are two techniques: DNA adenine methylation identification (DamID)[15], which records TF binding through local adenine methylation by an *E. coli* Dam methylase fused to a TF of interest, and calling cards[16,17], in which a TF is fused to a transposase enzyme and binding events are recorded through transposon insertion proximal to the TF binding site. These approaches offer the potential for cell type-specificity through conditional expression of the TF-enzyme fusion protein, as well as the ability to record and integrate occupancy information across time through longitudinal DNA methylation or transposon insertion[18], while requiring very little starting material[19]. In this way, DamID has been successfully implemented for cell type-specific profiling[20], primarily in *Drosophila*[19] but also with some studies in cultured mammalian cells[21–23] and embryos[22]. Meanwhile, calling cards has also been successfully applied to yeast[24] and

mammalian cell[16] model systems. However, neither of these methodologies has to date been implemented for TF recording in postnatal mammalian model systems, such as mice.

Here we present FLEX calling cards, a virally-delivered system for recording longitudinal TF occupancy in specific cell types from heterogenous living tissues, *in vivo*. FLEX calling cards, in the mold of traditional calling card technologies[16], works by first expressing the *hyperPiggyBac* (hypPB) transposase within a cell and providing donor transposons. HypPB inserts donor transposons at TTAA sites throughout the genome, leaving permanent marks, or calling cards, at these loci. These transposons can later be sequenced and mapped to the genome to record the history of hypPB localization across the genome. HypPB-mediated insertions can be used to asses TF binding in two ways: 1) hypPB may be fused to a TF of interest, so that the TF directs the insertion of transposons near its genomic binding sites[16], or 2) unfused hypPB directly interacts with the bromodomain and extra-terminal domain (BET) protein, Brd4, and directs transposon DNA into Brd4-associated genomic regions[25,26], most prominently active super enhancers[7]. Importantly, FLEX calling cards is a genetically-encoded system, allowing for cell type-specificity through conditional Cre recombinase-dependent expression and does not require TF-specific antibodies. The delivery of FLEX calling cards via adeno-associated virus (AAV) allows for simple and fast adaptation to a variety of mammalian model organisms, and the multitude of readily available Cre-driver mouse lines allows for TF profiling in a variety of cell types. Furthermore, through continued transposon insertion, FLEX calling cards can record and integrate TF binding events over extended time periods following viral delivery, potentially providing insights into transient TF activity that would be otherwise missed with endpoint measures such as ChIP-seq. Finally, FLEX calling cards implements the newly-developed “self-reporting transposon”, or SRT[27], through which transposon insertion events are reported via RNA transcription; thus, this system offers the added advantage of multimodal TF-recording and RNA expression analyses, such as translating ribosome affinity purification (TRAP)[28], in the same cellular populations.

We demonstrate that FLEX calling card systems can be delivered in an efficient and widespread manner to the mouse brain via AAV and that these components successfully record TF occupancy. We then use this system to generate cell type-specific Brd4 occupancy profiles and showed that these cell type-specific REs preferentially regulate cell type-enriched genes for each respective cell type. Finally, by fusing hypPB to the promoter-binding TF, SP1, we demonstrate integrated SP1 occupancy recording across time in the brain, allowing for detection and retrospective analysis of transient binding events.

Results

Intracranial delivery of calling cards via AAV invokes widespread transposon insertion in the mouse cortex

In order to perform transposon calling cards in mammalian cells, two basic components are required: the hypPB transposase (or a TF-hypPB fusion) and donor transposons[16]. We sought to develop an *in vivo* method to efficiently deliver calling card components throughout the mouse brain to identify TF-associated sites. We first tested AAV as a means for calling card reagent delivery, as viral *piggyBac* delivery methods have been successful in other organ systems previously[29,30]. We packaged hypPB and donor transposons carrying TdTomato reporter genes into separate adeno-associated viruses with serotype 9 (AAV9), which efficiently transduce neuron and astrocyte populations[31], and intracranially injected these vectors into the cortices of postnatal day 1 (P1) mice. Animals were sacrificed at P21 for analysis (**Fig 1A**). We analyzed hypPB expression with *in situ* hybridization (**Fig 1SA**) and transposon-derived TdTomato immunofluorescence (**Fig 1B**) across the brain and observed widespread viral transduction in the neocortex, hippocampus, and inner brain structures. As expected with the AAV9 serotype[31], the vast majority of transduced cell types were neurons and astrocytes (**Fig 1C-D**). Finally, by co-delivering hypPB with an AAV version of the BrokenHeart transposon[32], which expresses TdTomato only when transposons are successfully inserted into the genome, we demonstrated functionality of the calling card system *in vivo* (**Fig 1SB**). These results demonstrate that calling card reagents can be efficiently delivered to the mouse brain by AAV, and establish that AAV episomes can provide donor transposons for insertion into the genome.

Earlier implementations of the calling cards method (e.g. BrokenHeart[32]) mapped transposon insertions by directly sequencing genomically-inserted transposon DNA[16,17] (**Fig 2SA**). However, our group recently developed a specialized calling cards donor transposon, termed a “self-reporting transposon” (SRT), which allows for amplification of each insertion via RNA transcription and highly efficient mapping of transposition events through deep sequencing of transposon-derived RNA[27] (**Fig 2SB**). We first sought to directly compare traditional DNA calling cards to SRT calling cards in AAV systems. To do this, we intracranially injected P1 mice with AAV::hypPB and either AAV::BrokenHeart FLEX or AAV::SRT. At P21, we isolated DNA or RNA from cortex samples and generated calling cards sequencing libraries (**Fig 2SA-B**). After sequencing, we mapped the reads and found that the majority (80.7%) of SRT reads mapped to the mouse genome (mm10) and had the sequence hallmarks of true insertion events. In contrast, reads from BrokenHeart were much less efficiently recovered from the genome (12.3%; **Fig 2SC**), with the majority aligning to the original AAV episomal sequences. Next, we mapped transposon insertions from these reads. While insertions from these two methods reliably mapped to similar genomic locations (**Fig. 2SE**), we were able recover an order of magnitude greater total number of insertions in SRT compared to BrokenHeart. From two mice receiving AAV::SRT, we recovered 3,732,694 insertions at mean read coverage of 3.84 reads/insertion, while only 198,981 insertions were recovered at mean read coverage of 8.98 reads/insertion from three mice receiving AAV::BrokenHeart FLEX (**Fig 2SD**). In summary, we found that SRTs provide a much greater sensitivity for

recovering insertion events from AAV than traditional DNA methods. Importantly, relative to control (RFP only) viruses, AAV SRT calling card reagents did not result in excess degeneration, weight loss, or behavioral/developmental defects, suggesting the calling cards system does not introduce significant toxicity or deleterious effects to the animal (**Fig 1SC-N**). Finally, we tested whether SRT calling cards could also be delivered efficiently via adult intraparenchymal cortical injection. Indeed, after delivery of AAV::hypPB and AAV::SRT to three P137 adult mice, we observed a high mapping rate of reads to mm10 (76.5%; **Fig. 2SC**), from which we recovered 4,114,106 insertions at mean read coverage of 5.92 reads/insertion (**Fig. 2SD**) which mapped to similar genomic locations as in P1 delivery (**Fig. 2SE**). Thus, AAV calling cards systems are functional, efficient, and safe *in vivo* and can be delivered to the mouse brain at various timepoints.

Unfused hypPB delivered with AAV profiles active super enhancers in the brain

Brd4 acts as a "chromatin reader" by binding to acetylated lysine residues on histones[33–35] and regulating gene transcription[36,37]. Accordingly, Brd4 is strongly associated with active, TF-bound REs, most prominently super enhancers[7,38]. Importantly, Brd4 has a known affinity for the unfused hypPB protein[25], and consequently unfused hypPB insertions are greatly enriched at known Brd4 binding sites[25] and super enhancers[26]. Thus, we aimed to test the hypothesis that unfused hypPB transposon insertion can be used to identify active super enhancers in the brain.

We first analyzed the sensitivity and specificity of unfused hypPB insertions for identification of active super enhancers in a pure, *in vitro* cellular population of neuroblastoma (N2a) cells. To do this, we transfected these cells with the AAV::hypPB and AAV::SRT plasmids, from which we collected a total of 806,653 insertions. We then downsampled this library into randomly selected pools of various insertion totals and used peak calling to identify regions of significantly enriched insertion density in each pool, at a range of significance thresholds. Using a previously published N2a H3K27ac ChIP-seq dataset[39] to independently define active super enhancers in this population, we assayed sensitivity and specificity of calling card insertion peaks for identifying these regions. From this, we observed that calling card peaks are highly specific for active super enhancers across a range of sensitivities, with a high area under the receiver-operator characteristic curve (0.82; **Fig. 3SA**). Further, we observed a high sensitivity for super enhancer identification, even at low insertion totals (e.g. sensitivity of up to 0.8 from only 10,000 insertions), that increases steadily with increasing number of insertions (**Fig. 3SB**). Thus, unfused hypPB calling card profiles can be used to identify active super enhancers *in vitro*.

We next asked whether AAV::hypPB could identify active REs, including super enhancers, in the brain. To do this, we combined all 3,732,694 insertions collected from two mice injected with AAV::hypPB and AAV::SRT at P1 and defined significantly enriched insertion peaks (7,031 peaks; $p<10^{-30}$). We first observed that insertion density at these peaks was highly correlated (R = 0.994) between the two animal replicates, indicating a high reproducibility of this method (**Fig 2A**). To validate that insertion peaks represented active REs, we compared our calling card data to ENCODE ChIP-seq datasets[31] of enhancer-associated histone modifications[4] in the developing mouse cortex. At the 7,031 significantly enriched insertion peaks, we observed

a strong enrichment of active enhancer-associated histone modifications H3K27ac and H3K4me1 and a de-enrichment of the repressive mark H3K27me3 (**Fig 2B-E**). We then used a previously published[40] P14 H3K27ac ChIP-seq data from mouse cortex to independently define active enhancers and super enhancers and asked whether calling card peaks significantly overlapped these regions. We observed that the majority (69.2%; [4,867/7,031]) of the 7,031 insertion peaks intersected with H3K27ac-defined enhancers, significantly higher than when insertion peak coordinates are randomized (9.2% [647/7,031]); χ^2 test with Yates correction: $p<0.0001$) (**Fig 2F**). Similarly, calling card peak intersection with H3K27ac-defined super enhancers is also significantly more likely than by chance (10.0% [702/7,031] v. 1.4% [97/7,031]; χ^2 test with Yates correction: $p<0.0001$) (**Fig 2G**). As expected for a Brd4-mediated mechanism, unfused hypPB calling card profiles identify only a subset of all H3K27ac-defined enhancers (22.2% [4,774/21,509] v. 3.2% [679/21,509]; χ^2 test with Yates correction: $p<0.0001$), but do intersect the majority of H3K27ac-defined super enhancers (71.7% [530/739] v. 12.7% [94/739]; χ^2 test with Yates correction: $p<0.0001$) (**Fig 2H-I**). Of note, this overlap analysis was performed using our standard, highly rigorous significance threshold for peak calling ($p=10^{-30}$), however we have also performed these analyses at a range of p-value thresholds to confirm the finding is robust to this parameter (**Fig 3SC-D**). Together, these data support that AAV-mediated calling card insertion profiles of unfused hypPB can be used to identify putative REs, particularly super enhancers, in the brain.

FLEX calling cards system allows for Cre-dependent, cell type-specific profiling of REs in the brain.

We next generated a Cre-dependent calling cards system, termed FLEX calling cards, and tested the ability of this system to record cell type-specific RE activity or TF binding in complex tissues without isolation of the cell population of interest. In the FLEX system, the reverse complement of the hypPB or TF-hypPB gene is positioned downstream of a strong, ubiquitous promoter and is flanked by two sets of loxP sites. In the presence of Cre, the transposase gene is flipped to the correct orientation and is expressed. To confirm Cre-dependence of the FLEX system, we co-transfected the Cre-dependent hypPB virus, AAV::hypPB FLEX, into HEK293T cells along with the BrokenHeart reporter plasmid. We observed BrokenHeart-derived TdTomato fluorescence only in cells that received both the FLEX calling card constructs and a Cre expression plasmid, demonstrating that this system is Cre-dependent (**Fig 4SA**).

As a proof of principle, we focused on two prominent and well-studied Cre-driver mouse lines, Syn1::Cre and GFAP::Cre, which direct expression to neurons and astrocytes, respectively. We packaged the AAV::hypPB FLEX plasmid into the AAV9 vector and intracranially co-injected it along with AAV::SRT into P1 mouse pups of either the Syn1::Cre or GFAP::Cre genotype, along with Cre(-) littermates as controls. We sacrificed the animals at P28, isolated cortical RNA, and sequenced and mapped insertions across the genome (**Fig 3A**)**.** Immediately apparent upon sacrifice was that brains of Syn1::Cre positive animals were noticeably more red than their negative littermates, even to the naked eye (**Fig 4SB**), a result of the transposition-enhanced TdTomato reporter expression derived from the AAV::SRT construct. This change in color was striking for Syn1::Cre brains, but not as apparent in GFAP::Cre animals, an observation that is consistent with the relative abundances of transduced neurons and astrocytes (**Fig 1C-D**). In Syn1::Cre brains,

we analyzed TdTomato expression with immunofluorescence and noted a marked increase in neurons of Cre(+) animals but not Cre(-) littermates (**Fig 4SC**). We then sequenced insertions in Cre(+) and Cre(-) littermates from each line and observed a significant increase in insertion events in positive animals as compared to their negative littermates (**Fig 4SD**).

We next sought to test whether FLEX calling cards could identify cell type-specific REs. To do this, we identified insertion peaks that were differentially enriched in either Syn1::Cre over GFAP::Cre or GFAP::Cre over Syn1::Cre by a count-based statistical comparison and asked whether genes near these differentially enriched peaks are more likely to be expressed in neurons or astrocytes, using a previously published and widely used cell type-specific RNA-seq dataset[41]. As predicted, we found that as our significance threshold for defining differentially enriched insertion peaks became more stringent, the RNA expression of nearest gene sets became more cell type-specific (**Fig 4SE-F**). At a stringent significance threshold of $p=10^{-7}$, we compared all nearest genes to Syn1::Cre or GFAP::Cre enriched insertion peaks, and found significant differences in astrocyte versus neuron expression in the expected directionalities (**Fig 3C-D and Fig 4SG**). We then inputted these gene sets into an unbiased cell type-identification tool (CSEA[42]) and successfully identified cortical astrocyte and neuron populations for genes near GFAP::Cre and Syn1::Cre enriched insertion peaks, respectively (**Fig 4SH-I**). Together, these data indicate that peaks derived from FLEX calling cards insertion profiles recorded by unfused hypPB represent cell type-specific REs responsible for driving expression of cell type-enriched genes.

Lastly, we sought to functionally validate the enhancer activity of a subset of the novel astrocyte-enriched REs by testing whether these regions could enhance the expression of a dsRed reporter gene in astrocytes *in vivo*. We chose 4 candidate astrocyte-enriched REs, based on their size, cell type-specific activity, and astrocyte/neuron RNA expression of their nearest genes (**Fig 5SA-D and Fig 5SG**). We then cloned these candidate REs upstream of the hsp68 minimal promoter driving a dsRed reporter gene. As a positive control, we also cloned the canonical GFAP promoter (pGFAP)[43] into the same location upstream of hsp68::dsRed. To test the functional enhancer activity of these REs *in vivo*, we delivered these plasmids, along with a separate plasmid carrying a CFP reporter under the GFAP promoter for astrocyte identification, via postnatal astrocyte labeling electroporation (PALE)[44]. At P7, mice were euthanized and brains were collected for immunohistochemistry. This method successfully targeted radial glial populations, including astrocytes in the cerebral cortex. As expected, the positive control (pGFAP hsp68::dsRed) plasmid exhibited enhanced dsRed fluorescence in astrocytes, relative to a negative control plasmid carrying only hsp68::dsRed, that approached statistical significance ($p=0.055$; **Fig 3E-F and Fig 5SE-F**). We then quantified enhancer activity of our candidate REs and observed a significant enhancement of dsRed fluorescence for three of the four candidates (**Fig 3E-F and Fig 5SE-F**). Thus, these astrocyte-enriched REs display active enhancer activity in astrocytes in the mouse brain at P7. However, as there were many radial glia still present at this age, we repeated this experiment allowing mice to age to P21 to allow further astrocyte maturation prior to euthanasia. To our surprise, at this timepoint we observed a change in localization of dsRed expression in brains receiving the minimal promoter construct, with fewer GFAP:CFP(+) astrocytes and a new population of NeuN(+) neurons

labeled with dsRed in the cortex (**Fig 6SA**). This suggests that the PALE method delivers some plasmids to neurons or neural progenitors in addition to astrocyte progenitors, but also that expression via the hsp68 promoter in neurons does not arise until later in postnatal development. Regardless, in contrast to this neuronal activity, in animals that received pGFAP hsp68::dsRed or any of the RE candidate plasmids, dsRed expression was largely contained to GFAP:CFP(+) astrocytes. Indeed, even *eMms22l*, which was not yet active at P7, becomes active at P21. Together this further confirms these are astrocyte-specific REs and indicates that in addition to enhancing expression in astrocytes, these sequences may also repress activity of hsp68 in neurons (**Fig 6SA**). Overall, these data indicate that cell type-specific REs derived from FLEX calling cards are functional in defining cell type-specific gene expression in the brain.

Coupling FLEX calling cards to translating ribosome affinity purification (TRAP) enables parallel cell type-specific profiling of REs and actively translated RNA in less abundant cell types.

A remaining question with FLEX calling cards is the ability to profile RE activity and TF binding in less abundant cell types, where insertion number is likely to be diminished and signal-to-background ratios are lower. Indeed, during our analysis of FLEX calling cards in neurons and astrocytes we observed the presence of some background insertions present in Cre(-) animals, particularly in the GFAP::Cre line (**Fig 4SD**), which may impinge on our ability to detect active REs in more rare cell populations. While the cause of these Cre-independent insertions is unclear, we reasoned that we could substantially mitigate non-cell type-specific background by selecting for the RNA that comes from these populations prior to SRT library preparation, thus enhancing sensitivity when profiling less abundant cell types. To do this, we employed translating ribosome affinity purification (TRAP)[45,46], a method that enriches for cell type-specific transcripts via pull down of a GFP-tagged large ribosomal subunit (Ribosomal Protein L10a; Rpl10a), in conjunction with FLEX calling cards. Further, because both TRAP and SRT-based FLEX calling cards library preparations originate from RNA, this allows for simultaneous analysis of TF binding and actively translated RNA in the same targeted cell populations.

As a proof-of-principle for combinatorial TRAP/FLEX calling cards, we chose to analyze a relatively rare cortical cell population, layer V pyramidal neurons, labeled with Rbp4::Cre. These experiments were carried out in litters from a Rbp4::Cre line that was crossed with a Cre-dependent TRAP reporter mouse[47], which expresses the GFP-tagged Rpl10a subunit in the presence of Cre. As before, we intracranially injected P1 Rbp4::Cre positive and negative littermates with AAV::hypPB FLEX and AAV::SRT, sacrificed animals at P21, and collected brain tissue. As expected, we observed a strong enrichment of SRT-derived TdTomato signal in Rbp4(+) neurons, but some background in Rbp4(-) cell types (**Fig 7SA**). We then performed TRAP immunoprecipitation with anti-GFP coated beads to isolate ribosome-bound mRNA, including SRT transcription products, specifically from Rbp4::Cre(+) cells (**Fig 4A**). As expected, we were able to successfully isolate ribosome-bound RNA from Cre(+) animals but not from Cre(-) littermates, indicating an enrichment of cell type-specific RNAs (**Fig 7SB**). From the Cre(+) TRAP samples, we sequenced insertions with our standard SRT

protocol and observed a total of 171,833 insertions across 4 animals, noting that these were collected from Cre(+) RNA only and represent Rbp4(+) layer V pyramidal neuron-specific insertions.

As super enhancer regulation is critical to cell identify and function[7,38], we next aimed to identify Rbp4-enriched super enhancers. To do this, we first defined significantly enriched insertion peaks with our background-free peak calling method from the 171,833 Rbp4(+) insertions and observed 223 significant peaks ($p<10^{-30}$; **Fig 4B**). As expected, the majority of Brd4 signal was observed at peaks displaying typical super enhancer features[7,38], with the top 25% most enriched peaks harboring >50% of total insertions (top 25%: 9200 insertions; bottom 75%: 7472 insertions) and having a median length more than one order of magnitude larger than the bottom 75% (top 25%: 9.1×10^4 basepairs; bottom 75%: 6.3×10^3 basepairs) (**Fig 4C**). To define super enhancers specifically enriched in Rbp4(+) cells, we next identified insertion peaks differentially enriched in Rbp4::Cre animals as compared to C57BL/6J wild-type mice profiled with the Cre-independent AAV::hypPB (detailed in **Fig 2**) and identified 65 differentially enriched peaks ($p<10^{-7}$). Of these peaks, 43.1% (28/65) intersected with a known super enhancer defined via whole cortex H3K27ac ChIP-seq data[40] and thus represent Rbp4(+) enriched super enhancers (**Fig 4D**). Similarly to our previous Syn1::Cre and GFAP::Cre analyses, we then asked whether genes near Rbp4-enriched REs were more likely to be expressed in Rbp4(+) neurons than other cell types. To do this, we utilized a recently published Rbp4 TRAP dataset generated by our group[48], in which ribosome-bound RNA isolated from Rbp4::Cre(+) cells was profiled and compared to pre-TRAP RNA input from the same mice. As expected, we observed that the majority of peak-proximal genes (29/47; 62%) exhibit increased expression in TRAP RNA over pre-TRAP input (**Fig 7SC**), with a median logFC of 0.287. This mirrors our results in Syn1:Cre and GFAP::Cre (**Fig 4SG**), and suggests that these peaks represent cell type-specific REs in the Rbp4(+) layer V pyramidal neuron population.

Fusion of hypPB to the promoter-binding transcription factor SP1 records SP1 occupancy

A key feature of calling cards is the ability to record binding of a TF of interest using TF-hypPB fusions. To demonstrate calling card TF recording *in vivo*, we fused hypPB to a sequence-specific DNA binding general TF, SP1, which binds to gene promoters and is involved in transcription[49,50], and cloned this fusion gene into the FLEX calling cards system for cell type-specific use (**Fig 5A**). As full length SP1 is too large to be efficiently packaged into AAV, we instead used a truncated version of SP1 containing the C-terminal 621 amino acids, which includes the DNA-binding domain and has been shown to be sufficient to replicate sequence-specific binding of full length SP1[51]. To test this system, we intracranially co-injected AAV::SP1(621C)-hypPB FLEX along with AAV::SRT into P1 mice of the Syn1::Cre line, sacrificed animals at P28, generated and sequenced SRT libraries from cortical RNA samples, and compared insertion profiles to that of unfused hypPB. Consistent with the affinity of SP1 for proximal promoters, we found that insertions were significantly enriched upstream of TSS, as compared to unfused hypPB insertion profiles (**Fig 5C**). Next, we defined differentially enriched insertion peaks in SP1(621C)-hypPB profiles over unfused hypPB ($p<10^{-15}$) and found that the majority of significant enrichments occur in gene promoters (**Fig 5D-E**). Finally, at these SP1 peaks, we performed motif

discovery and were able to identify enrichment of the canonical SP1 binding motif, GGGCGGGG[16] (**Fig 6E**). Thus, fusion of SP1 to hypPB and delivery via AAV identifies SP1 binding sites in the mouse brain.

FLEX calling cards provides historical TF binding information through longitudinal TF recording

An intriguing potential use of calling card technologies is in the recording of TF binding over an integrated period of time. Such a method, which is not possible with endpoint TF profiling methods such as ChIP-seq, could empower novel studies in which historical TF binding information would be useful, such as during cellular development or differentiation. Further, by integrating signal over time, longitudinal calling cards may report transient binding events which would be otherwise missed with endpoint-only measures.

To test whether FLEX calling cards could report integrated, historical TF occupancy, we asked whether we could recover transient SP1 promoter binding events and successfully read them out at a later date. Importantly, consistent with the known role of SP1 in regulating gene expression[16,49,50], we observed that expression of genes genome-wide was on average correlated with the number of SP1-directed promoter insertions (**Fig 6A-B**). Thus, we predicted that should a gene be expressed early, but not late, in the lifetime of the animal, this transient event could be marked by SP1 binding and be recoverable via SP1 calling cards at a later timepoint.

To test this hypothesis, we intracranially co-injected AAV::SP1(621C)-hypPB and AAV::SRT into two separate cohorts of P1 mice. The first cohort was sacrificed at P10, while the second cohort was allowed to continue to record SP1 occupancy until P28 (**Fig 6C**). This time period of postnatal brain development involves several key neurodevelopmental processes[52], including substantial hippocampal neurogenesis[53] as well as glial and synaptic maturation[52], development of the extracellular matrix[52], and closing of critical periods[52], which are accompanied by numerous changes in gene and protein expression[54]. For these analyses, we utilized a previously-published cortical RNA-seq dataset[55] with postnatal timepoints of 1 week (Wk1; ~P7) and 4 weeks (Wk4; ~P28). From these expression data, we then derived and tested three separate predictions (**Fig 6C**). First, for genes expressing at Wk1 but not Wk4, (i.e. “early genes”) we would observe equivalent SP1 binding at promoters in both cohorts. Second, for constitutive genes that express equally at Wk1 and Wk4, we would observe continued integration of SP1 binding in the P28 cohort, resulting in increased SP1 insertion density at promoters. And third, for genes expressing only at Wk4 (i.e. “late genes”), we would observe SP1 promoter binding only in the P28 cohort. We defined early genes as having a log(Wk4/Wk1 FPKM) of less than -0.5 (n = 292), late genes as greater than 0.5 (n = 285), and all genes in the middle as constitutive (n = 4413; **Fig 6D**). Indeed, at the promoters of these gene sets, we observed SP1 promoter occupancy to be consistent with our three predictions (**Fig 6E-G**). Perhaps most importantly, at the promoters of early genes, we observed no loss of SP1 calling card signal in the P28 cohort, despite these genes only being transiently expressed; thus, this system is capable of permanently recording transient TF binding events for retrospective read out at a later date. Together, these data support that TF-hypPB fusions integrate signal over time and provide a historical, integrated picture of TF occupancy.

Discussion

This work describes the development of FLEX calling cards, a versatile method for TF and RE mapping in the brain in a cell type-specific manner. This technology builds on previously developed *in vitro* calling card methodologies and represents a step forward in our ability to investigate epigenetic regulation in the brain. Here we implemented FLEX calling cards in the mouse brain and further, as a proof-of-principle, demonstrated the effectiveness of this protocol for cell type-specific RE profiling in multiple cell types. Our use of SRTs now also enable calling cards for single cell analyses[27], which expands and highlights the versatility of this toolkit in future studies.

Calling cards technologies, as genetically-encoded systems, have several unique features that provide advantages over biochemical TF profiling methods such as ChIP-seq for certain applications[16,17,32]. In FLEX calling cards, we have retained these important advantages and translated them to an *in vivo*, viral system which will be applicable to a range of animal models from brain development to disease. One key feature of our methodology is that there is no requirement for physical isolation of cell types for cell type-specific analyses. This allows for the same protocol to be used for any cell type of interest, the identify of which is determined by the Cre-driver mouse line used, and avoids potential disassociation-related artifacts[14]. Secondly, while not explored here, one could envision simple manipulations of the FLEX calling cards system to allow for temporal control of the system[41]. Such adaptations could allow for innovative studies in which TF binding is recorded only during defined windows of time[18]. In a similar vein, we have demonstrated here the ability of FLEX calling cards to integrate TF binding information over time, which will allow for retrospective analysis of historical TF activity in cells. By applying this unique utility to SP1, we identified promoter regions of genes with accumulating SP1 binding across postnatal development and captured transient SP1 binding events at early-expressed genes.

FLEX calling cards does not require a TF-specific antibody, allowing for TF profiling for, in theory, any packagable TF, simply by fusing it to hypPB. Also, this being a virally-mediated system allows for simple and rapid application to animal models without the need for expensive and time-consuming breeding. Intracranial injection for a standard size litter of mice can be completed in under an hour. Further, simply changing the viral serotype[31] or promoter[56,57] could allow for similar analyses in cell types not explored here. Finally, the non-Cre dependent versions of the system should be equally applicable in other species of interest such as rats and primates. Reagents, cloning strategies, and user-friendly analysis pipelines are available upon request, making FLEX calling cards readily available for neuroscience research.

Of course, there are caveats to be considered as well. Most notably, there is potential for induced mutation, given the tendency for transposons to insert into or near critical gene regulatory regions. Indeed, transposon technologies are often used in mutagenesis screens in which transposon-mediated gene disruption can be deleterious[58]; however in such studies, the transposons are specifically engineered with splice-site gene or enhancer traps, while the SRT used in FLEX calling cards only drives expression of a reporter gene and the genomic sequence immediately downstream of its insertion site. Consistent with this, we observed no

excess degeneration or behavioral/developmental deficits in AAV calling card-injected animals, beyond that induced by needle stick. Further, the transposition rate of the *piggyBac* transposase is inherently low (<20 per cell[59]), suggesting that it is highly unlikely for insertions to disrupt regulatory regions on both alleles in the same cell, and in general, calling card technologies have not exhibited marked deleterious effects in previous reports[16,17]. Nevertheless, it remains possible that a subset of calling cards transposition events could perturb nearby gene expression. Finally, it is important to recognize that while we do see a clear Cre-induction of this system, we also observed some background insertion events in the absence of Cre, particularly in the GFAP::Cre line, which could be limiting for profiling of rare cell types in which signal is likely to be reduced. One possible solution that we explored in this study is combining the FLEX calling cards system with TRAP[46] to isolate RNAs from specific cell types, adding an extra layer of specificity. Here, we employed TRAP and FLEX calling cards in parallel and demonstrated RE profiling in a less abundant cell type, Rbp4(+) layer V pyramidal neurons. Perhaps more interestingly, this bimodal approach allows for profiling of TF binding and RNA expression in tandem from the same cellular populations. However, this method does require an additional breeding to a transgenic TRAP reporter mouse line, and thus future adaptations of the viral FLEX calling card method could be improved through the advent of conditional AAV expression systems with tighter control of gene expression.

In summary, we have introduced FLEX calling cards as a viable method for recording TF binding and active REs *in vivo* in a cell type-specific manner and demonstrated its effectiveness in profiling cell type-specific and historical TF and RE activity in the brain. Future applications of this technology to animal models of development and disease could unlock important insights into epigenetic gene regulation in a variety of neuroscience disciplines.

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Author Contributions

Study designed by AJC, AM, TL, JC, MJV, RDM, and JDD. AJC, AM, JC, JH, XC, MNW, and MH designed and generated DNA constructs. AM developed SRT analysis pipelines. AJC, TL, JC, MJV, MS, KM, XC, and RDM generated and analyzed data. Manuscript written by AJC and edited by AM, TL, JC, MJV, TMM, RDM, and JDD.

Declaration of Interests

JDD has received royalties related to TRAP in the past. RDM, AM, and MNW have filed a patent application on SRT technology. No other authors have disclosures to report.

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Methods

Animals

All animal practices and procedures were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee (IACUC) in accordance with National Institutes of Health (NIH) guidelines. Transgenic mouse strains used in this study include β-actin(ActB)::Cre (RRID:IMSR_JAX:019199), Synapsin 1 (Syn1)::Cre (RRID:IMSR_JAX:003966), glial fibrillary acidic protein (GFAP)::Cre (RRID:IMSR_JAX:024098), retinol binding protein 4 (Rbp4)::Cre (RRID:MMRRC_037128-UCD)[60], and TRAP reporter mice CAG::EGFP/Rpl10a (RRID:IMSR_JAX:022367). All mice were bred to the C57BL/6J background, with the exception of animals used for adult stereotactic injections, which were wild-type animals of the FVB/N6 background (RRID:IMSR_JAX:001800). At indicated endpoints, mice were anesthetized with isoflurane and perfused with 15ml of cold saline (PBS) prior to tissue collection. Animals in the Brd4 Syn1::Cre, Brd4 GFAP::Cre, and P28 SP1 Syn1::Cre cohorts received pentylenetrazole-induced seizures immediately prior to sacrifice. Unless otherwise noted, brains were either dissected and flash frozen in liquid nitrogen (for molecular analyses) or fixed in 4% paraformaldehyde 24-48 hours, exchanged into 30% sucrose, and either directly frozen at -80°C or cryoprotected in O.C.T. (for immunofluorescence).

Cell culture and transfections

HEK293T and N2a cells used in this study were cultured in 1X DMEM with 10% fetal bovine serum (FBS) and grown under standard conditions (37°C; 5% CO_2). Plasmid transfections in HEK293T cells were carried out with Fugene® 6 Transfection Reagent (Promega, Madison, WI, USA) with the manufacturer's protocol. Calling cards constructs were delivered to N2a cells via either Fugene 6 or Neon Electroporation (ThermoFisher #MPK10025) with the following settings: 1050V, 30ms, 2 pulses.

Immunofluorescence and imaging

10μm-thick (for co-localization studies), 40μm-thick (for imaging of AAV::BrokenHeart) fixed-frozen sagittal or coronal brain sections were washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Non-specific binding was blocked with 5% normal donkey (Jackson ImmunoResearch, West Grove, PA, USA) or goat (Vector Laboratories, Burlingame, CA, USA) serum for 30-60 minutes at room temperature. After blocking, slides were exposed to primary antibody overnight at 4°C, washed three times with PBS, and then incubated with secondary antibodies for 1 hour at room temperature. Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) and coverslips were applied with ProLong Gold Antifade (ThermoFisher, Waltham, MA, USA) or Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) mounting media. Immunofluorescent images of brain sections were acquired with a Nikon A1Rsi confocal microscope and imported into ImageJ (v. 1.51s) for manual cell counts and quantification. For analyses of hypPB expression in various cell types, 5 mice were used, and co-localization was quantified in 2 cortical images from a single section per animal. Antibodies used for immunostaining included chicken anti-

GFP (Aves Labs GFP-1020) at 1:1000 dilution, mouse rabbit anti-RFP at 1:400 or 1:500 dilution (Rockland 600-401-379), anti-NeuN at 1:100 dilution (Millipore-Sigma MAB377), rabbit anti-cMyc at 1:250 dilution (Sigma C3956), and goat anti-GFAP at 1:500 dilution (Abcam ab53554).

Cells transfected with AAV::hypPB FLEX for testing Cre-dependence were live imaged for TdTomato on a Leica DMI 3000B tissue culture microscope. All images were acquired with equal conditions and exposure times for direct comparison.

In situ hybridization and imaging

10μM-thick, 4% paraformaldehyde fixed-frozen sections were cut and slide-mounted. mRNA encoding for *hyperPiggyBac* (VF1-20268-01) was detected using a custom probe-set designed by Affymetrix (now ThermoFisher) using the Affymetrix ViewRNA ISH Tissue 1-Plex kit (ThermoFisher, QVT0050) and chromogenic signal amplification kit (ThermoFisher VT0200) with the following modifications: Slides were immersed in 4% paraformaldehyde overnight at 4°C prior to *in situ* hybridization, then the baking, deparaffinization, and heat pretreatment steps were omitted (steps 1-3, 5) because sections were not embedded in paraffin. Slides were hybridized either with anti-hypPB or no-probe controls. Following the *in situ* labeling protocol, sections were labeled for 5 minutes with DAPI (1:20,000, Sigma D9542), washed with PBS, and a drop of prolong gold (ThermoFisher P36934) was added while applying the coverslip. Slides were then imaged at 20x magnification on a Zeiss LSM 700 confocal microscope using a Cy3 filterset to detect FastRed fluorescence.

Analysis of brain degeneration following viral injection

Mice were exposed to either the calling cards viruses (AAV::hypPB + AAV::SRT) or RFP-only virus (as a control) via P1 injection and sacrificed 28 days later for silver staining (a marker for cells irreversibly committed to cell death). Briefly, animals were heavily sedated and perfused with TRIS fixative in 4% paraformaldehyde followed by vibratome sectioning of brains at 75μM in the coronal plane. Every eighth section across the rostrocaudal extent of the brain was then silver stained as described previously[61]. The number of degenerating neurons was quantified for each animal by a rater blind to treatment who counted the total number of silver positive neurons in the dorsal cortex of each section.

Mouse behavior, developmental milestones, and sensorimotor battery

Mouse behavior and development was monitored and compared between animals injected calling cards AAV reagents (AAV::hypPB and AAV::SRT) or AAV::RFP only. In addition to weight, which was measured at P8, P14, P25, tests were administered to assess attainment of developmental milestones (P14 righting from back), anxiety related behavior (P25 1 hour behavior, recording time spent in the edge or center of cage), and balance/strength/coordination (P25/26 sensorimotor battery). Procedures were done as previously described[62,63], with two trials per animal. A break was allowed after the completion of the first set of test trials to avoid exhaustion effects, and the test order was reversed for the second trial for all animals.

Walking initiation, ledge performance, platform performance, and pole performance were all administered at P25, while 60° inclined screen test, 90° inclined screen test, and inverted screen test were administered on P26. Prior to testing, mice were given a routine health check, and from this, two animals were excluded; one for runtiness and one for severe hydrocephaly likely derived from needle stick. Further, one litter of RFP-only animals was not able to complete all timepoints (cage flooding) and was thus also excluded. In total, 21 animals were included in the calling card group (11M/10F) and 24 in the RFP-only group (10M/14F), all of which were used for downstream analyses. Test administrators were blinded from treatment group identity during all testing.

Virus generation and injections

Transposase and donor transposon constructs were cloned into Cre-dependent (FLEX) or Cre-independent AAV transfer vectors and used for *in vitro* transfection or viral packaging. Plasmids were packaged into AAV by the Hope Center Viral Vectors Core at Washington University School of Medicine. For *in vivo* experiments involving P1 delivery, transposase and donor transposon viruses (mixed equally by volume) or undiluted RFP-only virus were intracranially injected into the cortex of postnatal day 1 (P1; 3 sites per hemisphere, 1μl viral mix per site). For adult injections, viruses were delivered to 3.5 month-old animals intraparenchymally with stereotactic surgery, as previously described[39]. Two sites were chosen for direct, unilateral cortical injection with coordinates relative to bregma of 1.25mm rostral; 1.5mm lateral; 0.55mm depth; and 1.06mm caudal; 1.5mm lateral; 0.55mm depth. 2μl of viral mix was delivered at a rate of 0.2μl/minute.

Viral titers (viral genomes per milliliter) were as follows:

AAV::hypPB	$1.0x10^{13}$ - $1.1x10^{13}$ vg/ml
AAV::hypPB FLEX	$8.0x10^{12}$ - $1.0x10^{13}$ vg/ml
AAV::SP1(621C)-hypPB FLEX	$1.0x10^{13}$ vg/ml
AAV::SRT	$1.6x10^{13}$ - $2.2x10^{13}$ vg/ml
AAV::BrokenHeart FLEX	1.4×10^{13} vg/ml
AAV::RFP only	$1.6x10^{13}$ vg/ml

SRT and BrokenHeart library preparation, sequencing, and mapping

SRT libraries were prepared from cortex RNA samples. Prior to library preparation, cortex samples were dissected into 10 separate pieces, from which RNA was independently isolated with the manufacturer's protocol (Qiagen RNeasy kit, Germantown, MD, USA). This allows for identification of up to 10 independent insertion events into any TTAA site, given that these insertions occur in spatially separate samples. From these RNA samples, transposon sequencing libraries were generated with our bulk SRT protocol[27]. In brief, RNA samples were first reverse transcribed, from which self-reporting transcripts, including flanking genomic sequences, were amplified via PCR. These amplicons were then tagged with universal Illumina sequencing

overhangs, with separate indexes for libraries from each dissected piece, allowing for 10 'barcodes' per sample and sequenced on Illumina HiSeq 2500, NextSeq 500, or MiniSeq platforms (Illumina, San Diego, CA, USA).

BrokenHeart libraries were prepared from cortical DNA samples, as previously described[16]. A set of 20 individually barcoded BrokenHeart transposons were pooled and packaged into AAV. Thus, independent insertions into the same TTAA can be uniquely identified via barcode, removing the need to dissect and process tissue samples in separate pools as with SRT. Extracted DNA was self-ligated, amplified with inverse PCR, and sequenced with the Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA).

Sequencing reads obtained from SRT and BrokenHeart libraries were stringently filtered for features of true insertion events (presence of *piggyBac* terminal repeat sequence; intact sequencing adapters, barcodes, and indexes; and a TTAA site) and mapped to build version mm10 of the mouse genome with Novoalign 3 (Novocraft Technologies) (for SRT) or Bowtie2[64] (for BrokenHeart). Reads aligning to the same TTAA with separate barcodes were considered unique insertions, and all analyses in this report considered all unique insertions equally, independent of read depth. We used BEDtools intersect to count the number of insertions directed to introns, exons, 3'- and 5'-UTRs, and intergenic regions, using annotations from the HOMER package[65] (**Fig 2SE**).

Significant insertion peak calling and motif discovery

Significantly enriched insertion peaks were identified via a count-based statistical comparison as previously described[27]. In brief, this pipeline first segments the genome into blocks of constant insertion density. For each block, it calculates the p-value of insertion enrichment relative to a background model assuming uniformly distributed insertions. A user-defined significance threshold is defined, and all blocks surpassing this threshold are considered "significantly enriched insertion peaks". This "background-free" method for unbiased identification of all significantly enriched genomic regions in a single experimental sample, used here in **Fig 2** and **Fig 4C**, is expected to identify all Brd4-bound regions within the parameters of the calling cards system.

Alternatively, we can define differentially-bound regions between two experimental samples, as was done in **Fig 3, Fig4S, and Fig 5S** for astrocyte- or neuron-specific Brd4 peaks, **Fig 4** for Brd4 peaks enriched in Rbp4(+) layer V projection neurons, and **Fig 5** for SP1 peaks over unfused hypPB. In this analysis, the pipeline again segments the genome into blocks, but then assigns a p-value to each block based on the differential enrichment between the two samples. As with the background-free pipeline, a user defined p-value threshold is chosen, below which all blocks are considered significantly enriched.

TF motifs were identified with MEME-ChIP motif discovery software[66,67] with -zoops -meme-minw 6 -ccut 250.

Defining enhancers and super enhancers

Since H3K27ac is a known marker of active enhancers[4,68] and super enhancers[7,38], we utilized published P14 mouse cortex[40] and N2a[39] H3K27ac ChIP-Seq datasets to define cortical and N2a enhancers

super enhancers, respectively. As previously described[7,38], we used the rank ordering of super enhancers (ROSE) pipeline and the model-based analysis for ChIP-Seq (MACS) version 1.4.1 peak finding algorithm[69] with a p-value enrichment threshold of 10^{-9} to define enhancers and super enhancers. We then used the BEDtools suite[70] to compare the coincidence of enhancers and super enhancers with our unfused hypPB calling cards insertion peaks (**Fig 2, Fig 4, Fig 3SC-D**).

Additionally, for qualitative measures of histone modification enrichment at calling cards Brd4 insertion peaks (**Fig 2**), we used publicly available P0 mouse forebrain ChIP-Seq datasets from ENCODE[70]; specifically, H3K27ac (ENCSR094TTT), H3K4me1 (ENCSR465PLB), and H3K27me3 (ENCSR070MOK).

Super enhancer in vitro sensitivity and specificity

Sensitivity and specificity of calling cards peaks were assessed for super enhancer identification in N2a cells that were either transfected or electroporated with AAV::hypPB and AAV::SRT plasmids (**Fig 3S**). RNA was separately isolated from a total of 33 wells (i.e. barcodes) from 6-well plates, and SRTs were sequenced, generating 806,653 unique insertions, though of note, the majority (651,631) were derived from 12 barcodes that received plasmids via electroporation. 800,000 unique insertions were randomly selected from the total pool of 806,653 insertions, from which significantly enriched peaks were defined using our background-free peak calling method at a range of significance thresholds. These peaks were intersected (with BEDtools intersect) with known N2a super enhancers defined via a previously published N2a H3K27ac ChIP-seq dataset[39], and sensitivity was defined as the percentage of peaks intersecting super enhancers for each peak calling significance threshold. To then define specificity, we identified the “true negative” space of the genome, and assessed the percentage of true negative peaks intersected by calling cards peaks. To do this, we first identified any possible active enhancer region of the genome with MACS peak finding using a low-stringency significance threshold of 10^{-1} and subtracted these peaks from the mouse genome, creating a “true negative” genome. We then sampled peaks (with BEDtools shuffle) within this true negative genome of the same size distributions as the list of active super enhancers until we collected an average of 1X coverage across the genome. With a true negative space of 2,616,503,093 basepairs and a total super enhancer size of 23,143,876 basepairs, this required 114 random samplings, resulting in 85,158 true negative peaks. Finally, we intersected our calling card peaks with these true negative peaks, and specificity was defined as the percentage of true negative peaks not intersected by a calling card peak. Of note, we expect that unfused hypPB is driven to super enhancers via interaction with Brd4; thus, sensitivity and specificity measurements may be higher if compared to Brd4 occupancy rather than H3K27ac.

Analysis of enhancer- and promoter-associated gene expression

Gene expression has been shown to be preferentially regulated by proximal enhancer elements[4,68,71]. Thus, since a cell type-specific mapping of enhancers to the genes they regulate is not available, we used proximity as an imperfect[6] albeit widely used[3] proxy. In our analyses of cell type-specific expression of genes near cell type-enriched Brd4 calling cards peaks (**Fig 3, Fig4S** and **Fig 4**), we first defined the nearest gene (or

genes, if multiple intersected a calling cards peak) to each significant calling cards peak. These gene sets were then filtered and the remaining genes were used for subsequent analyses. Gene sets were filtered as follows: 1) genes greater than 10,000 bases away from a differential insertion peak were removed, 2) genes near or overlapping multiple insertion peaks counted once, and 3) genes for which cell type-specific RNA expression data were unavailable in our comparison dataset were removed.

Unbiased cell type identification was completed with the Cell-type Specific Expression Analysis (CSEA) tool[42] (http://genetics.wustl.edu/jdlab/csea-tool-2/) using candidate gene sets near either GFAP::Cre enriched or Syn1::Cre enriched insertion peaks. For each set, we analyzed genes near the most enriched peaks for each cell type. For GFAP::Cre, this included 131 genes ($p<10^{-21}$ for associated insertion peaks), of which 114 were present in CSEA reference sets and used for analysis. For Syn1:Cre, this included 123 genes ($p<10^{-11}$), with 110 present in reference sets.

For comparison of SP1 binding and gene expression in **Fig 6**, we utilized the mm10_knownCanonical gene set and mm10_TSS coordinates from the UCSC genes table. We defined promoter-proximal regions as +/-1000 bases from the TSS. We first filtered mm10_knownCanonical gene set to remove duplicates (<3% of total genes) and then intersected gene coordinates with promoter proximal regions. After manually filtering to assign true promoters to each transcript (i.e. immediately upstream from TSS), we generated a list of unique promoter/gene combinations (24,528 unique genes) and compared insertion density and gene expression at these coordinates.

For comparison of P28 and P10 SP1 promoter insertions to RNA expression in **Fig 6C-G**, we utilized a previously published RNA-seq dataset[55] with RNA expression data available for week 1 (Wk1) and week 4 (Wk4), which correspond to ~P7 and ~P28, respectively. Before assessing P28 or P10 insertion density at promoters, insertion profiles were downsampled such that each cohort had exactly 240,000 insertions per library (80,000 per mouse for P10, 3 mice; 60,000 per mouse for P28, 4 mice); thus, insertion totals could be directly compared without any normalization to library size. Further, this downsampling procedure eliminates the possibility that any given observed increase in insertion density at P28 was due to an overall increase in insertion total over time. We then calculated number of insertions at each unique promoter (using the list of unique promoter/gene combinations generated above) and removed any gene with no insertions at either timepoint (19,046 / 24,528 unique genes remaining). A pseudocount of 1 was added to promoter insertion totals for each gene at each timepoint prior to analysis. To eliminate noise due to low RNA expression and/or random low-frequency insertion events, we next removed any gene with <6 insertions combined between the P28 and P10 datasets (including the 2 pseudocounts) or <1 FPKM combined between Wk1 and Wk4 RNA-seq expression, leaving a final total of 4991 unique gene/promoter combinations which were used in subsequent analyses. This list of 4991 genes were divided into three categories, based on their RNA expression at Wk1 and Wk4: (1) early genes; log(Wk4/Wk1 FPKM) < -0.5, (2) constitutive genes; -0.5 < log(Wk4/Wk1 FPKM) < 0.5, and (3) late genes; log(Wk4/Wk1 FPKM > 0.5. Within these categories, SP1 occupancy was compared between the P28 and P10 cohorts.

Validation of astrocyte enhancer candidates with PALE[44]

Candidate astrocyte-enriched enhancers were selected from the list of GFAP::Cre-enriched insertion peaks in **Fig 3** and **Fig 5S** based on size, enrichment over Syn1::Cre, and astrocyte specific expression of their nearest genes. These candidates were PCR amplified with primers listed in the table below and with an MluI overhang adapter (TGTAGGACGCGT) on either end, and cloned into the miniP-dsRed plasmid with MluI, upstream of the hsp68 minimal promoter. As a positive control, the canonical GFAP promoter[43] was also cloned into this plasmid, in the same location.

To test efficacy of the candidates for enhancing dsRed expression, each plasmid was electroporated into lateral ventricle-proximal cells, along with a separate plasmid containing CFP driven by the canonical GFAP promoter. P0-1 pups were placed on a wet towel on wet ice for 10 minutes to anesthetize. Then plasmids were delivered to the lateral ventricle via intraventricular injection along with Fast Green FCF dye (Sigma, 2353-45-9), with coordinates approximately equidistant from the lambdoid suture and the eye, 2mm lateral to the sagittal suture, and 2mm depth to ensure lateral ventricle penetration. 1μl of DNA was delivered into one hemisphere for each mouse, at a concentration of 1μg/μl for GFAP::CFP and 0.5μg/μl for dsRed plasmids. Electroporation was induced for 5 square pulses, 50ms per pulse at 100V and 950ms inter-pulse intervals, sweeping the electrodes from the dorsal to lateral using ~25° angle intervals.

Pups were sacrificed at P7 or P21 and brains were collected and analyzed with immunohistochemistry for CFP and dsRed (CFP and dsRed stained with GFP and RFP antibodies, respectively, as indicated in methods). Individual astrocytes from 3 brains (34-42 cells) per condition were imaged for analysis using equivalent exposure settings. A region of interest (ROI) was defined around the astrocyte based on CFP only, and dsRed and CFP fluorescence was quantified within the ROI using ImageJ (v. 1.51s). The ratio of CFP:dsRed was calculated for each cell and averaged and compared across conditions for final assessment of dsRed enhancement.

eCandidate	Forward primer	Reverse primer
eRasa2	TTCATGAACTCTGTTACTAGTTTGT	TTTTAAACAGATGAGCTGGAGCC
eTaf4b	ATATTGGATCTCACTGGAGTTGC	TCAAAGTCTAGATTTAGGCATGAT
ePla2g7	ACAGAACAGACTCACATAAACTTGT	CCATTGTCACATCTAGTCATCAGT
eMms22l	GCTTATTTAAAATGAAAAGA	AAATTCCCTTAAACCCCCCTG

Translating Ribosome Affinity Purification (TRAP)

TRAP was used to isolate cell type-specific, ribosome-bound RNAs from Rbp4(+) layer V pyramidal neurons in **Fig 4** as previously described[43,46]. In brief, Cre positive and negative animals were processed by harvesting brains at P21 and splitting down the midline, creating two “barcodes” per animal. Tissue samples were homogenized according to modified protocols previously described[46]. Immunoprecipitation (IP) of transcripts was achieved via biotinylated-protein L (ThermoFisher Scientific 29997)-coated streptavidin

magnetic beads (ThermoFisher Scientific 65602) that were coupled with two custom monoclonal anti-EGFP antibodies (Htz-19f7, Htz-19c8)[46]. Input samples were collected prior to IP and remaining homogenate was incubated on an inverted mixer for 4 hours at 4°C. Beads were then washed several times with buffers and RNA purification was performed on eluted beads (Zymo RNA Clean & Concentrator -5, R1013, Zymo Research, Irvine, CA, USA). RNA quality and concentration were determined via Agilent BioAnalyzer prior to sequencing.

Statistical analyses

Statistical tests were done with GraphPad Prism v8.0 and are detailed in figure legends.

Figure legends

Figure 1. Co-AAV9 intracranial injection efficiently delivers calling cards to the cortex.
(A) Experimental paradigm and AAV constructs. Arrows represent approximate AAV injection sites. (B) Coronal section of brain injected unilaterally at P1 with AAV::hypPB and AAV::SRT, displaying widespread expression of SRT-derived TdTomato fluorescence throughout the brain (C,D) Co-immunofluorescence showing hypPB expression in neurons and astrocytes. (C) Representative images display co-localization of hypPB with neuronal (NeuN) and astrocyte (GFAP) markers. Insets show examples of hypPB-positive astrocytes. (D) Majority of hypPB-positive cells transduced with AAV9 are NeuN-positive neurons and GFAP-positive astrocytes (n=1005 myc(+) cells, counted across cortical image fields from 5 independent mice).

Figure 2. Unfused hypPB-directed calling cards insertions identify active enhancers and super enhancers in the brain.
(A) Normalized insertion depth in two littermate C57BL/6J mice (Rep1 and Rep2) at 7031 significantly-enriched insertion peaks ($p<10^{-30}$) displaying high correlation between replicates (R=0.994). (B-E) Unfused hypPB-directed insertions are highly enriched for the active enhancer marks H3K27ac and H3K4me1 and depleted for suppressive mark H3K27me3. Representative image (B), heatmaps, and enrichment plots (C-E) of H3K27ac, H3K4me1, and H3K27me3 density at 7031 significantly-enriched insertion peaks in two littermate mice. In (B), top track of each insertion replicate displays unique insertions, where each circle = 1 unique insertion and y-axis represents number of reads supporting each insertion on a log10 scale, and bottom track displays normalized local insertion density across the genome (insertions per million per kB). Y-axis of ChIP-seq data represents read depth with smoothing filter applied. Heatmaps and enrichment plots are centered on insertion peaks and extend 10kB in either direction. Relative enrichment quantifications displayed in log2(fold-change over ChIP-seq input). (F,G) Percentage of 7031 significantly-enriched insertion peaks with at least 1 basepair (bp) intersection with a H3K27ac-marked enhancer or super enhancer. Gray bar represents intersections after randomizing genomic coordinates of insertion peaks. Chi^2 test with Yates correction: $p<0.0001$. (H,I) Percentage of H3K27ac-marked enhancers and super enhancers with at least 1 bp intersection with a significantly-enriched insertion peak. Chi^2 test with Yates correction: $p<0.0001$.

Figure 3. FLEX calling cards system generates cell type-specific RE profiles.
(A) AAV constructs and experimental design in Syn1::Cre and GFAP::Cre animals. (B) Examples of differentially enriched insertion peaks near genes preferentially expressed in neurons (right) or astrocytes (left). (C,D) Quantifications of neuron and astrocyte specific expression of genes near GFAP::Cre (C) or Syn1::Cre (D) enriched insertion peaks at a stringent peak-calling significance threshold ($p=10^{-7}$) showing significant preferential expression in the expected cell type (Mann-Whitney U test: $p<0.0001$). (E) A GFAP::Cre enriched insertion peak nearby the *Pla2g7* gene (ePla2g7; see Fig 5S for peak coordinates) was cloned into a plasmid upstream of the hsp68 minimal promoter and a dsRed reporter gene and co-delivered along with a GFAP::CFP

plasmid to ventricle-proximal radial glia, including astrocytes, postnatal astrocyte labeling electroporation (PALE)[44]. Expression of dsRed is enhanced by both the canonical GFAP promoter (pGFAP; positive control) and ePla2g7. (F) Quantification of dsRed expression enhancement in CFP(+) astrocytes by pGFAP and ePla2g7. n=34-42 CFP(+) cells from 3 brains per condition. Comparisons made with unpaired Student's t-test, (****p<0.0001 corrected with Dunnett's multiple comparisons test). Error bars represent SEM.

Figure 4. Combinatorial FLEX calling cards/TRAP allows for RE profiling in Rbp4::Cre(+) layer V pyramidal neurons.

(A) Experimental design of combinatorial FLEX calling cards and TRAP for cell type-specific TF profiling in Rbp4(+) neurons. (B) Representative differentially enriched peak in Rbp4::Cre mice over wild-type. (C) Characteristic RE "hockey stick" graph of all Rbp4(+) background-free insertion peaks sorted by insertion density. Top 25% most insertion-enriched peaks (highlighted in dark purple) display increased median peak length relative to bottom 75% (highlighted in light purple) and harbor >50% of total insertions. (D) Out of 65 differential insertion peaks in Rbp4::Cre over wild-type, 28 intersect a known H3K27ac super enhancer (SE).

Figure 5. Fusion of SP1 to hypPB in FLEX calling cards system records SP1 occupancy.

(A) Schematic of AAV::SP1(621C)-hypPB FLEX construct. (B) Fusion of the promoter-binding TF SP1 to hypPB directs insertions to promoter-proximal TTAA sites. (C) Percentage of total insertions within 1000 basepairs (bp) of a transcription start site (TSS), displaying increased promoter-directed insertions upon SP1 fusion as compared to unfused hypPB (n = 3-4 mice per group; unpaired Student's t-test: p<0.001). Total SP1(621C)-hypPB insertions: 1,083,099. Total unfused hypPB insertions: 2,484,133. (D) Percentage of significant SP1 insertion peaks differentially enriched over unfused hypPB ($p<10^{-15}$; 1596 intersecting out of 2316 total peaks) intersecting promoter-proximal regions (1000bp on either side of TSS) compared to randomized peak coordinates (78/2316). Chi^2 test with Yates correction: p<0.0001. (E) Representative insertion peak displaying significantly increased insertion density near the TSS of the *Plrg1* gene. (F) Highest information content motif in the sequences flanking the center of significantly enriched SP1(621C)-hypPB insertion peaks ($p<10^{-15}$) displays the canonical SP1 binding motif (GGGCGGGG; $p<10^{-42}$).

Figure 6. Longitudinal SP1 profiling reports integrated, historical record of SP1 binding.

(A-B) Normalized number of SP1(621C)-hypPB directed insertions at promoter proximal regions after subtraction of unfused hypPB insertions, versus neuron-specific gene expression for all genes, binned and averaged into 100-gene bins. In (A), left y-axis represents number of promoter insertions normalized to 10^6 total insertions in the sample and right y-axis displays neuron-specific RNA expression from Zhang et al., *Journal of Neuroscience*, 2014. (B) displays strong correlation of SP1(621C)-hypPB promoter insertions with gene expression after subtraction of unfused hypPB insertions (R=0.96, p<0.0001). (C) Experimental paradigm and predicted temporal SP1 occupancy for early, constitutive, and late expressing genes. (D) (top) Distribution of Wk4/Wk1 expression ratios for all expressed and SP1-bound genes and (bottom) categorization into "early"

(log(Wk4/Wk1 FPKM) < -0.5), “constitutive” (-0.5 < log(Wk4/Wk1 FPKM) < 0.5), and “late” (log(Wk4/Wk1 FPKM) > 0.5) gene sets. RNA-seq from Lister et al., *Science*, 2013. (E) SP1-derived promoter insertions for early, constitutive, and late gene sets, demonstrating efficient capture of transient SP1 binding events at early gene promoters and continued integration of constitutive and late gene promoters in the P28 cohort relative to the P10 cohort. Error bars represent SEM. ****p<0.0001, unpaired Student’s t-test (F) Example of early expressed gene (*Idh1*) displaying equivalent SP1 binding in both cohorts. (G) Example of late expressed gene (*Gjb6*) displaying SP1 binding only in the P28 cohort.

Supplemental figure legends

Figure 1S. Co-AAV9 intracranial injection efficiently delivers calling cards to the cortex and does not result in excess degeneration or behavioral/developmental deficits.

(A) *In situ* hybridization of hypPB RNA and no probe control displaying widespread cortical expression of hypPB after delivery of AAV. (B) Immunofluorescence showing TdTomato signal after co-delivery of hypPB and BrokenHeart FLEX via AAV to ActB::Cre positive and negative littermates. Presence of TdTomato in Cre(+) animals indicates functional transposition of BrokenHeart transposons into the genome. (C) Mouse pups were intracranially injected at P1 with the (top) red fluorescent protein (RFP) or (bottom) calling cards viruses (AAV::hypPB and AAV::SRT) and labeled with silver staining to screen for degeneration. (D) Quantification of silver-positive cells in dorsal cortex revealed injection with either virus produces limited neurotoxicity that did not significantly differ between groups (unpaired Student's t-test, $p > 0.05$). (E-N) Behavioral and developmental assessments of mice injected at P1 with SRT calling cards or control (RFP only) viruses revealed few or no developmental, sensorimotor, or anxiety-related deficits in calling card animals relative to control. All group comparisons were done with unpaired Student's t-test, with Bonferroni corrected $\alpha=0.05$ as a significance threshold. **p<0.01 (Bonferroni corrected).

Figure 2S. Comparison of neonatal and adult AAV delivery of traditional DNA calling card and SRT calling card systems.

(A) DNA calling cards library preparations were carried out as previously described in Wang et al., *Genetics*, 2012. TF-hypPB fusions insert transposon DNA (e.g. BrokenHeart) near TF binding sites. Genomic DNA is then harvested and digested with restriction enzymes that cut near the end of the transposon and in downstream genomic sequence. These fragments are subsequently self-ligated and circularized. From these, transposons and their flanking genomic sequences are amplified with inverse PCR, using primers that contain Illumina sequencing primers and adapters. Final products are sequenced and aligned to the mouse genome to map transposons to genomic locations. (B) Schematic of Self-Reporting Transposon (SRT) library preparation protocol. HypPB inserts SRTs near TF binding sites, which then transcribe and report their genomic locations via RNA. RNA is reverse transcribed and PCR amplified, and Illumina adapters are added for sequencing. (C) Read mapping rates for BrokenHeart DNA calling cards and SRT RNA calling cards libraries after P1 or adult cortical delivery, showing increased efficiency of recovery for genome-mapping reads in SRT relative to BrokenHeart. (D) Number of unique insertions observed from BrokenHeart DNA calling cards and SRT RNA calling cards libraries (n=3 mice for P1 BrokenHeart; n=2 mice for P1 SRT; n=3 mice for adult SRT), displaying greatly increased recovery with SRT. (E) Mapping locations of transposon insertions in various genomic regions from BrokenHeart and SRT calling card libraries.

Figure 3S. In vitro and in vivo sensitivity of unfused hypPB calling card libraries for active REs.
(A-B) Sensitivity and specificity of super enhancer (SE) identification for unfused hypPB calling cards libraries in N2a cells. (A) Receiver-operator characteristic (ROC) curve for identification of active super enhancers using unfused hypPB peaks called from 800,000 unique insertions. Area under ROC curve: 0.82. (B) Super enhancer sensitivity at various significance thresholds and insertion totals, demonstrating high super enhancer sensitivity at even very low (10^4) insertion totals. (C-D) Intersections between significantly-enriched insertion peaks derived from cortical unfused hypPB calling cards libraries and H3K27ac-marked (C) enhancers or (D) super enhancers at a range of insertion peak significance thresholds. Red line represents total number of significant peaks at each p-value threshold. Quantifications in Fig. 2F-I represent intersections at $p=10^{-30}$ significance threshold.

Figure 4S. Supplemental to: FLEX calling cards system generates cell type-specific RE profiles.
(A) Transfection of BrokenHeart transposons (Cre-independent version), hypPB FLEX plasmid, and Cre recombinase into HEK293T cells. n=3 wells per condition, 5 image fields per well, representative images shown. No TdTomato reporter from reconstituted BrokenHeart transposons observed in the absence of Cre or hypPB FLEX. (B) Representative images of Syn1::Cre and GFAP::Cre positive and negative littermate brains, displaying increased SRT-derived TdTomato reporter signal in Syn1::Cre positive animals. (C) Representative images displaying preferential expression in Neu(+) neurons in CA3 hippocampal regions of Syn1:Cre(+) mice, but not negative littermates. TdTomato images taken at constant exposure times for direct comparison. (D) Quantifications of unique insertions in Syn1::Cre or GFAP::Cre positive and negative littermate mice at sequencing depth downsampled to equal read depth and normalized to AAV::SRT viral titer (3X coverage per insertion; unpaired Student's t-test: * $p<0.05$, **** $p<0.0001$). (E) Number of genes near differentially enriched insertion peaks in Syn1::Cre and GFAP::Cre animals at a range of significance thresholds. (F) Normalized neuron-to-astrocyte expression ratio [Neuron FKPM/(Neuron FPKM + Astrocyte FKPM] for genes near Syn1::Cre or GFAP::Cre enriched peaks at a range of significance thresholds for defining differentially enriched insertion peaks. As significance threshold becomes more stringent, expression of nearby genes becomes more cell type-specific. RNA-seq expression from Zhang et al., *Journal of Neuroscience*, 2014. (G) Astrocyte versus neuron expression of all genes near Syn1::Cre or GFAP::Cre enriched insertion peaks at a stringent peak-calling significance threshold ($p=10^{-7}$). Percent of genes on either side of the y=x midline shown. (H-I) Graphical representation of cortical cell type enrichment based on gene sets near either (H) Syn1::Cre ($p<10^{-11}$; top 123 genes) or (I) GFAP::Cre ($p<10^{-21}$; top 131 genes) enriched insertion peaks. Legend displays Benjamini Hochberg corrected Fisher's Exact Test p-value for overlap of reference cell type-specific gene sets and Syn1::Cre or GFAP::Cre candidate gene sets. Stringencies for enrichment for each pre-defined reference set are represented by size of each hexagon, with the outer ring being the least stringent set and inner ring being the most stringent set.

Figure 5S. Functional validation of enhancer activity for GFAP::Cre enriched unfused hypPB peaks.
(A-D) Candidate GFAP::Cre enriched insertion peaks that were chosen for functional enhancer validation. Each candidate RE (highlighted in blue) was separately cloned into a plasmid upstream of the hsp68 minimal promoter and a dsRed reporter gene for *in vivo* testing. (B) Candidate RE reporter constructs were co-delivered along with a GFAP::CFP plasmid to ventricle-proximal radial glia, including astrocytes, via PALE[44]. Expression of dsRed was enhanced by both the canonical GFAP promoter (pGFAP; positive control) and three of the four candidate REs (all but eMms22l). (F) Quantification of dsRed expression enhancement in GFAP::CFP(+) astrocytes by pGFAP and candidate RE constructs. n=34-42 GFAP::CFP(+) cells from 3 brains per condition. Comparisons made with unpaired Student's t-test, (*p<0.05; **p<0.01; ****p<0.0001; p-values corrected with Dunnett's multiple comparisons test). Error bars represent SEM. (G) Chromosomal coordinates and lengths of candidate REs.

Figure 6S. Candidate astrocyte enhancers identified with FLEX calling cards direct cell type-specific expression in astrocytes by P21.
(A) Candidate astrocyte enhancers derived from FLEX calling cards enrichment (see Fig 5S) were electroporated into P1 mouse pups via PALE[44] and animals were sacrificed at P21 for IF analysis. In animals receiving a plasmid containing dsRed driven by a minimal promoter only (hsp68::dsRed), dsRed expression in the cortex was evident in a population of NeuN(+) neurons (white arrows) that was not observed at P7 (see Fig 5S). In contrast, in animals receiving either the pGFAP-driven positive control plasmid or plasmids containing the candidate enhancers, dsRed expression was largely limited to GFAP::CFP(+) cells, indicating that the enhancer candidates facilitated cell type-specificity of gene expression.

Figure 7S. Supplemental to: Combinatorial FLEX calling cards/TRAP allows for RE profiling in Rbp4::Cre(+) layer V pyramidal neurons.
(A) Immunofluorescence of SRT-derived TdTomato signal reveals a strong enrichment in Rbp4(+) neurons (labeled with GFP), but some background in Rbp4(-) cell types (white arrow heads). TdTomato and GFP images taken with equal exposures for direct comparison. (B) Post-immunoprecipitation (IP) RNA yields are observed in Rbp4::Cre(+) but not Rbp4::Cre(-) samples. Each point represents post-TRAP IP yield from one barcode. (C) Proximal genes to differential insertion peaks are preferentially expressed in Rbp4 TRAP RNA samples compared to preIP RNA input in similar ratios observed in neurons v astrocytes (Fig 4SG).

Figure 1

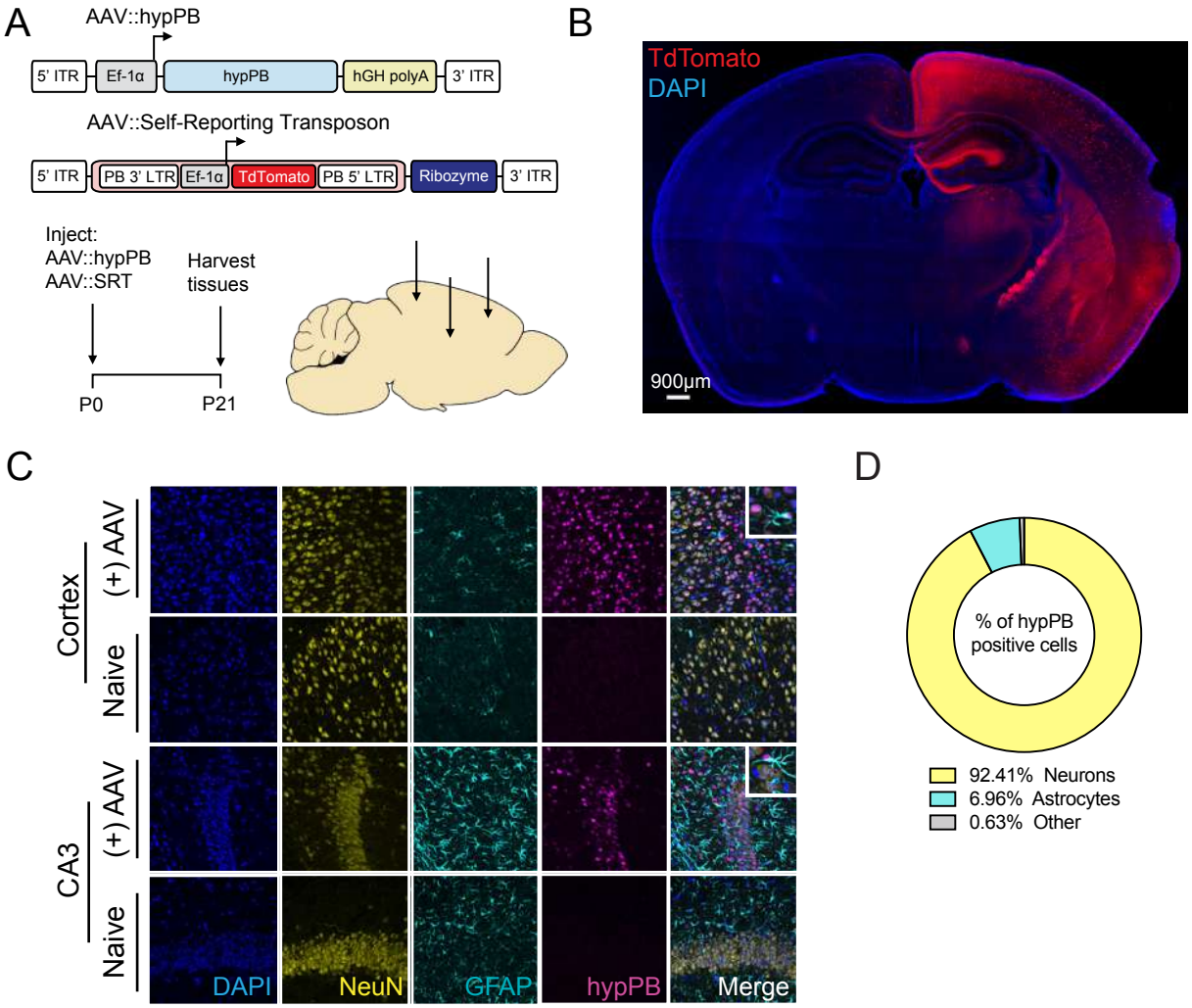

Figure 2

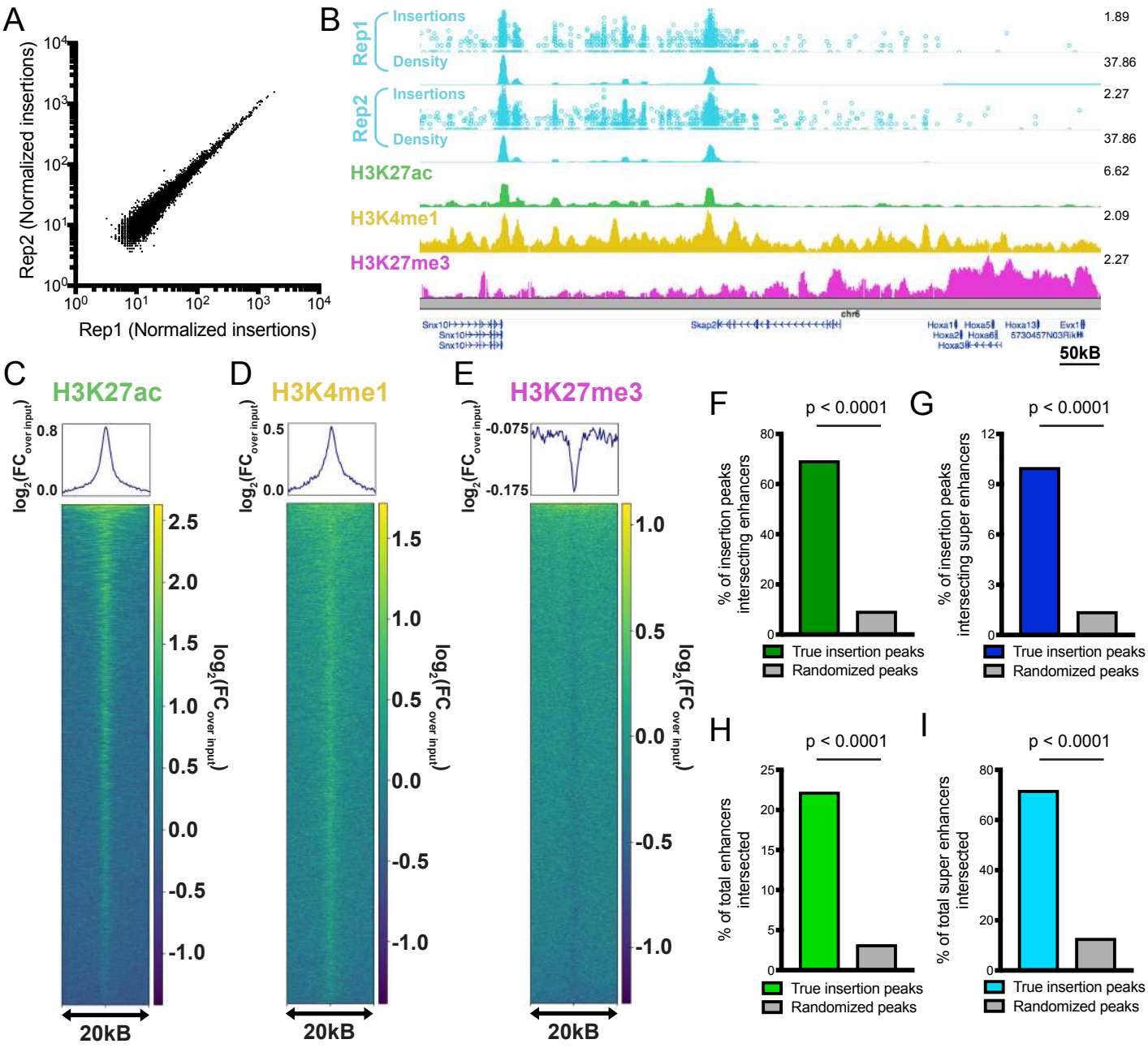

Figure 3

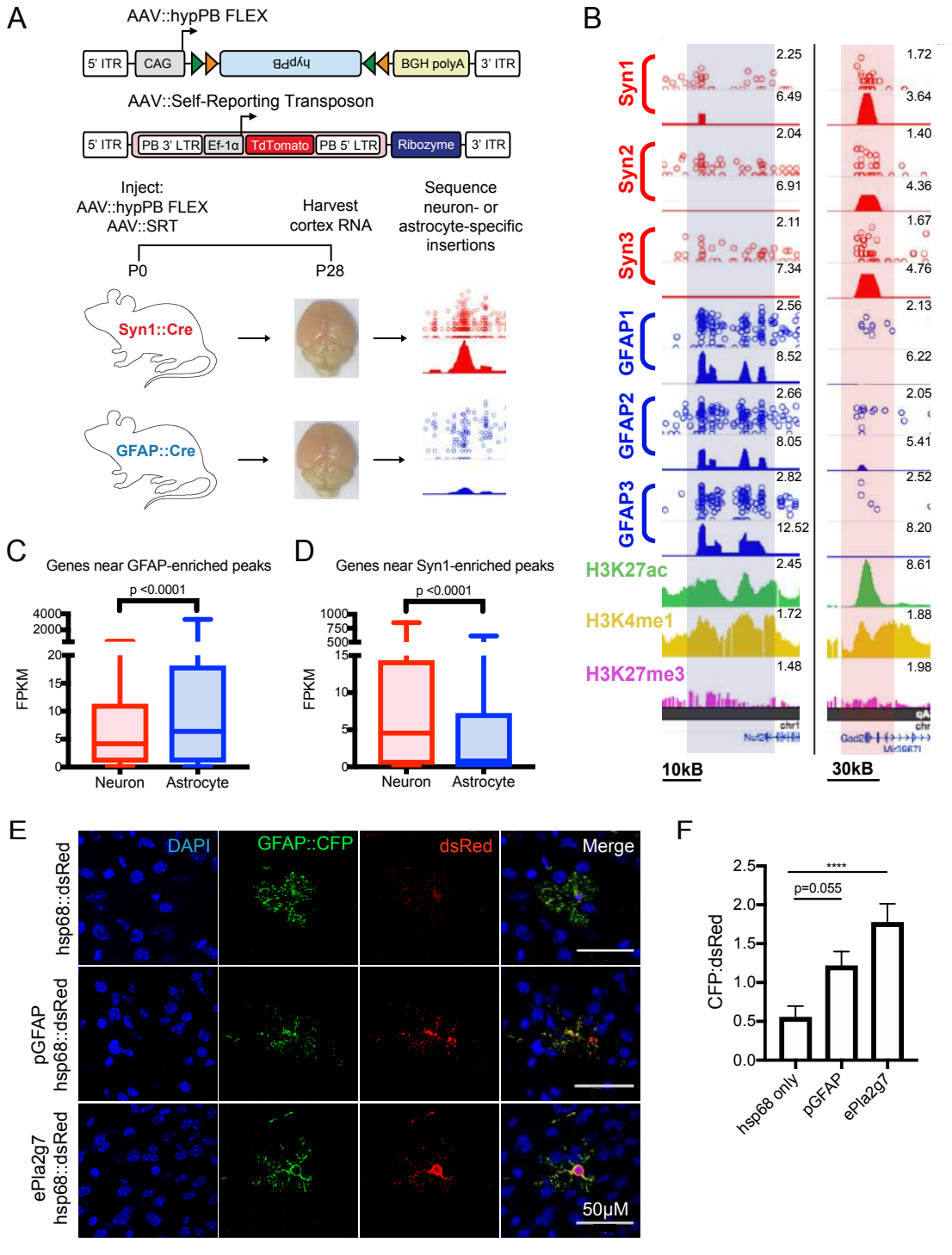

Figure 4

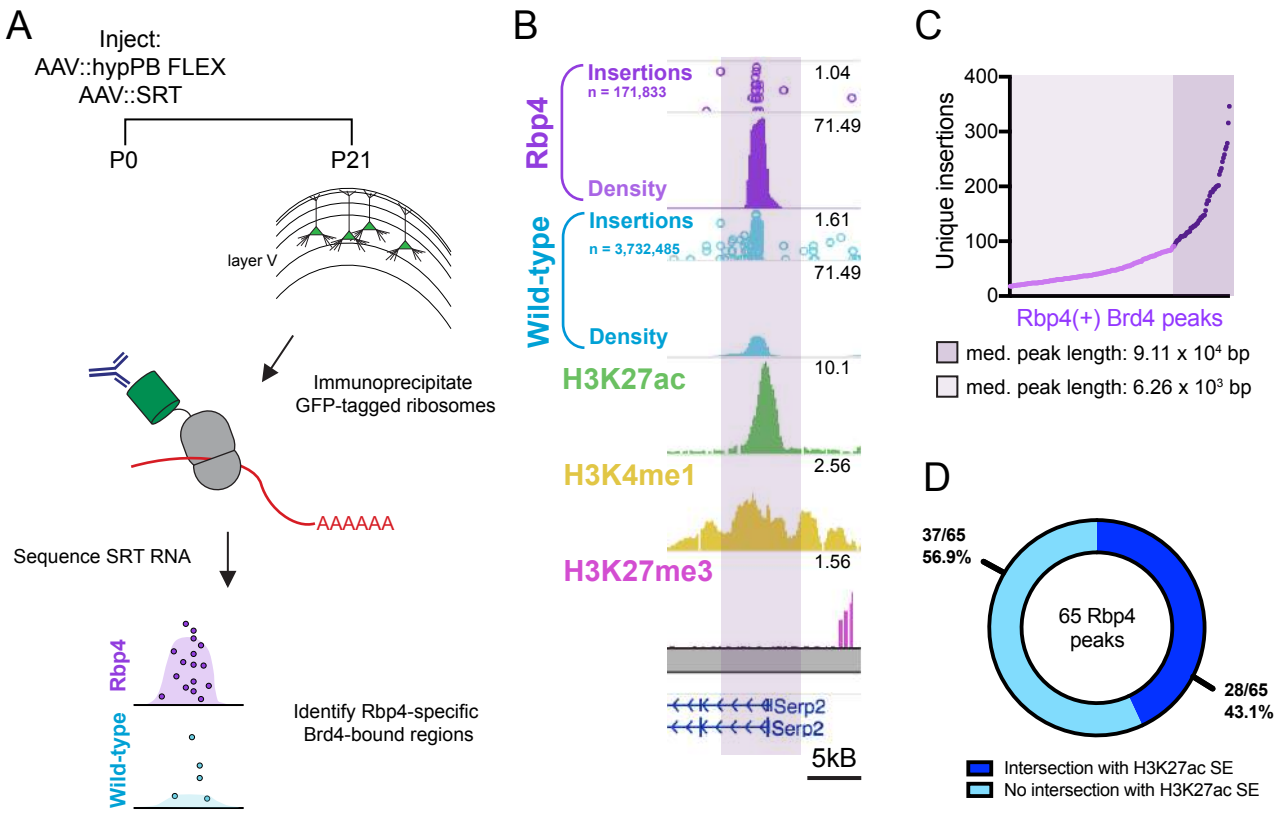

Figure 5

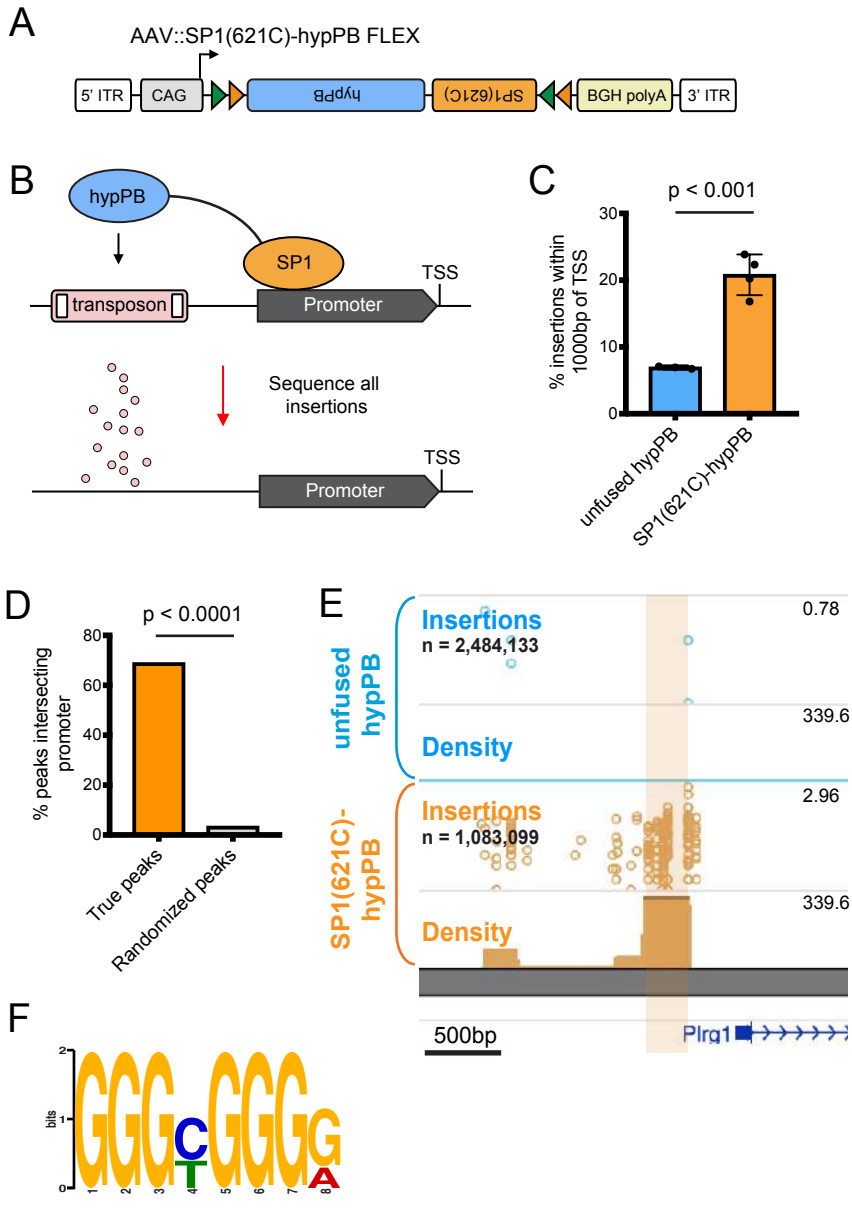

Figure 6