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 everything from cell fate decisions to activity dependent gene expression. However, widely-used methods for TF profiling *in vivo* (e.g. ChIP-seq) yield only an aggregated picture of TF binding across all cell types present within the harvested tissue; thus, it is challenging or impossible to determine how the same TF might bind different portions of the genome in different cell types, or even to identify its binding events at all in rare cell types in a complex tissue such as the brain. Here we present a versatile methodology, FLEX Calling Cards, for the mapping of TF occupancy in specific cell types from heterogenous tissues. 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 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 using high-throughput sequencing. Alternatively, unfused hypPB interacts with and records the binding of the super enhancer (SE)-associated bromodomain protein, Brd4. To demonstrate the FLEX Calling Card method, we first show that donor transposon and transposase constructs can be efficiently delivered to the postnatal day 1 (P1) mouse brain with AAV and that insertion profiles report TF occupancy. Then, using a Cre-dependent hypPB virus, we show utility of this tool in defining cell type-specific TF profiles in multiple cell types of the brain. This approach will enable important cell type-specific studies of TF-mediated gene regulation in the brain and will provide 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 binding and identify active REs across the genome have highlighted the profound diversity of TF occupancy 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)[3,6–9].

Several methods exist for profiling TF occupancy across the genome in whole tissues. Chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq), which works by crosslinking, 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. While efforts have been made to manipulate ChIP-seq for cell type-specific use[5,10,11], these approaches are limited by the need for physical cell sorting[11] or breeding to a knock-in mouse line[5,10] overexpressing a potentially toxic nuclear envelope protein[12]. Further, these methods have only been successfully applied to the detection of highly abundant histone proteins, and it is unclear if ChIP-seq is feasible from sorted or purified nuclei for many less abundant TFs. Finally, ChIP-seq is inherently destructive, requiring the lysis and biochemical manipulation of the tissues or cells being analyzed and thus provides only a snapshot of TF activity at the moment of cell sacrifice. Thus, ChIP-seq cannot provide historical TF binding information and may be inefficient at detecting transient TF binding events.

Because of these limitations, efforts have recently been made to develop genetically-encoded profiling techniques which are able to record longitudinal, cell type-specific TF occupancy through use of conditional expression systems. Prominently among these is DNA adenine methylation identification (DamID), which records TF binding through targeted adenine methylation by an *E. coli* Dam methylase-TF fusion protein. DamID has been successfully implemented for cell type-specific profiling in Drosophila[13] and recently mammalian cell lines[13], however, overexpression of Dam methylase is highly cytotoxic and thus DamID has yet to be successfully implemented in *in vivo* mammalian model systems. Further, targeted adenine methylation is transient and cannot be recovered after cell division. Thus, there remains a need for cell type-specific TF recording in live mammalian tissues.

Here we present FLEX Calling Cards, a virally-mediated method for recording TF binding profiles in specific cell types from heterogenous living tissues, *in vivo*. FLEX Calling Cards, in the mold of traditional Calling Cards technologies[14], 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[14], 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[15,16]. Brd4 is a TF which binds to acetylated histones and is associated with REs[17], most prominently enhancers and super enhancer (SEs)[7]. Importantly, FLEX Calling Cards is a genetically-encoded system, allowing for several advantages, including cell type-specificity through conditional Cre recombinase-dependent expression, as well as the ability to record and integrate TF binding events over extended time periods between viral delivery and animal sacrifice, all without the requirement of TF-specific antibodies. Further, 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 means that FLEX Calling Cards can be immediately used to study TFs in a variety of different cell types. Finally, FLEX Calling Cards implements the newly-developed “self-reporting transposon”, or SRT, (Moudgil *et al.*, *in preparation*), 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)[18], in the same cellular populations.

Here we demonstrate that donor transposons and hypPB or hypPB-TF fusions can be delivered in an efficient and widespread manner to the mouse brain via AAV and that these components successfully record TF occupancy. With the FLEX Calling Cards system, we then generated multiple cell type-specific Brd4 occupancy profiles and showed that these cell type-specific Brd4-bound REs preferentially regulate known cell type-enriched genes for each respective cell type.

Results

Generation of the FLEX Calling Cards viral system

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[14]. We cloned both Cre-independent and dependent (i.e. "FLEX") versions of the transposase as well as donor transposon constructs into adeno-associated virus (AAV) transfer vectors (**Fig 1A**). 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 AAV::hypPB FLEX into HEK293T cells with a transposon donor plasmid called "BrokenHeart", which acts as a reporter of transposase activity[19]. BrokenHeart contains a piggyBac transposon flanked by a split TdTomato reporter gene; prior to its transposition into the genome, the transposon disrupts the reporter gene, rendering it non-functional. However, upon transposition, the TdTomato gene is reconstituted seamlessly, causing the cell to report functional transposition through red fluorescence. We observed BrokenHeart fluorescence only in cells that received both the FLEX Calling Card constructs and a Cre expression plasmid, demonstrating that this system is Cre-dependent *in vitro* (**Fig 1B**).

Earlier implementations of the Calling Cards method mapped transposon insertions by directly sequencing genomically-inserted transposon DNA[14,20]. 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 therefore more efficient mapping of transposition events (Moudgil *et al.*). Thus, we have adopted this technology for use with FLEX Calling Cards. Briefly, SRTs contain a ubiquitous promoter that drives the transcription of a TdTomato reporter gene with no 3' poly-A termination signal. When the TdTomato gene is transcribed episomally in the context of the AAV genome (i.e. prior to transposition), a downstream self-cleaving ribozyme is incorporated into the transcript, destabilizing it and ultimately causing its degradation (**Fig 1A**). However, once the transposon is inserted into the genome, the SRT is no longer affiliated with the ribozyme, and the TdTomato transcript becomes stabilized and extends to include flanking genomic sequence in its 3' UTR. In this fashion, the transposon "self-reports" its location via RNA. Through subsequent deep sequencing of the transposon-derived RNA, we are then able to record the locations of SRTs across the genome (**Fig 1C**). We found that SRTs provide a much greater sensitivity for recovering insertion events than traditional methods (Moudgil *et al.*), and thus we have employed this system for all subsequent *in vivo* work here.

Intracranial delivery of Calling Cards via AAV invokes widespread transposition in the mouse cortex and records genome-wide TF binding events

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. We individually packaged the AAV::hypPB and AAV::SRT constructs into adeno-associated virus serotype 9

(AAV9), which efficiently transduces neuron and astrocyte populations[21], and intracranially co-injected them directly into the cortices of postnatal day 1 (P1) mice. Animals were sacrificed at P21 for analysis (**Fig 2A**). We analyzed the expression pattern of hypPB across the brain with immunofluorescence, using a myc-tag incorporated in hypPB, and observed widespread transduction throughout the cortex and hippocampus. As expected with the AAV9 serotype[21], the vast majority of transduced cell types were neurons and astrocytes (**Fig 2B-C**). These results demonstrate that Calling Card reagents can be efficiently delivered to the mouse brain by AAV.

To demonstrate TF recording with AAV Calling Cards, we next measured the genome-wide occupancy of the chromatin-interacting TF Brd4. Brd4 acts as a "chromatin reader" by binding to acetylated lysine residues on histones[22,23] and regulating gene transcription[24,25]. Accordingly, Brd4 is strongly associated with REs such as enhancers and SEs[7,26]. Importantly, Brd4 has a known affinity for the unfused hypPB protein, and consequently hypPB insertions are greatly enriched at known Brd4 binding sites[15], such as SEs[16]. Thus, we aimed to test the hypothesis that unfused hypPB transposon insertion can be used to identify Brd4-bound REs *in vivo*. To do this, we isolated RNA from P21 cortex samples of two mice that were injected at P1 with AAV::hypPB and AAV::SRT and generated SRT sequencing libraries. After sequencing, we mapped the reads and found that the majority (80.7%) mapped to the mouse genome and had the sequence hallmarks of true insertion events. We combined all *piggyBac* insertions (3,732,694 total) collected from both mice and used peak calling to identify regions of significantly enriched insertion density. This resulted in 7,031 significantly ($p<10^{-30}$) enriched regions (or insertion peaks), which we predict to be biologically important, Brd4-bound REs. To test reproducibility of this method, we asked how many insertions were derived from each mouse at all 7,031 peaks and found that insertion density was highly correlated ($R = 0.994$) between the two replicates (**Fig 3A**). To validate that insertion peaks represented Brd4-bound enhancers and SEs, we compared our Calling Card data to ENCODE ChIP-seq datasets[21] 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 3B-E**). We next used P14 H3K27ac ChIP-seq data from a previously published dataset[27] to independently define enhancers and SEs and asked whether Calling Cards 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 3F**). Similarly, Calling Cards peak intersection with H3K27ac-defined SEs 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 3G**). As expected for a Brd4-mediated mechanism, Calling Cards 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 SEs (71.7% [530/739] v. 12.7% [94/739]; χ^2 test with Yates correction: $p < 0.0001$) (**Fig 3H-I**). It is important to note that these intersections were observed despite that the H3K27ac ChIP dataset was derived from a P14 sample, while Calling Cards insertions represent Brd4 occupancy integrated over the postnatal lifetime of the animal. Further, the ChIP dataset is representative of whole brain tissue, whereas

Calling Cards insertions account for only transduced cell types, due to the viral serotype. It is also noteworthy that 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 3SA-B**). Together, these data indicate that co-AAV delivery of Calling Cards mediates widespread neuronal and glial expression in the mouse brain and support that insertion profiles of unfused hypPB represent Brd4 occupancy and thus can be used to identify putative enhancers and SEs in the brain.

FLEX Calling Cards system allows for Cre-dependent, cell type-specific profiling of TFs 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 TF binding in complex tissues without cell population purification. 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 intracranially co-injected AAV::SRT with the Cre-dependent hypPB virus, AAV::hypPB FLEX, 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 4A**). 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 4SA**). This fluorescence is the result of transposition-dependent TdTomato reporter expression in 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 2B-C**). 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 S4B**).

We next sought to validate that Calling Card peaks represented cell type-specific Brd4-bound REs in these lines. We first identified significantly enriched insertion peaks across the genome with our background-free peak calling method from the combined profiles of Syn1::Cre and GFAP::Cre animals and asked whether the Brd4 Calling Cards enrichment at these peaks would cluster by genotype. Indeed, across 11,393 significantly enriched peaks, we found that Brd4 insertion density was more similar within genotype than across genotypes (**Fig 4SC**). Next, we identified insertion peaks that were differentially enriched in either Syn1::Cre over GFAP::Cre or GFAP::Cre over Syn1::Cre by count-based statistical comparison (see Methods) and asked whether genes near these differentially Brd4-bound REs are more likely to be expressed in neurons or astrocytes, using a previously published and widely used cell type-specific RNA-seq dataset[28] (**Fig 4B**). As expected, 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 4C and 4SD-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 4D-F**). Finally, we inputted nearest gene sets into an unbiased cell type-identification tool (CSEA; http://genetics.wustl.edu/jdlab/csea-tool-2/) and successfully identified cortical

astrocyte and neuron populations for genes near GFAP::Cre and Syn1::Cre enriched insertion peaks, respectively (**Fig 4G-H**). Together, these data indicate that FLEX Calling Cards profiles are Cre-dependent and that insertion profiles recorded by unfused hypPB represent cell type-specific Brd4-bound REs responsible for driving expression of cell type-enriched genes.

A key feature of Calling Cards is the ability to record binding of any TF of interest using TF-hypPB fusions. To demonstrate flexible TF recording, we fused hypPB to a sequence-specific DNA binding TF called SP1, which binds to many gene promoters and is involved in regulation of gene expression[29,30], 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[31]. To test this system, we intracranially co-injected AAV::SP1(621C)-hypPB FLEX (see **Fig 1A** for construct design) 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 promoter affinity of SP1, we found that insertions were significantly enriched upstream of TSS, as compared to unfused hypPB insertion profiles (**Fig 5B**). Next, we defined differentially enriched insertion peaks in SP1(621C)-hypPB profiles over unfused hypPB and found many significant enrichments in gene promoters (**Fig 5C-D**). At these SP1-enriched peaks, we performed motif discovery and were able to identify an enrichment of the canonical SP1 binding motif, GGGCGGGG[14] (**Fig 5E**). Finally, as SP1 regulates expression of its associated genes[14,29,30], we asked whether SP1 binding at gene promoters would correlate with resulting neuronal gene expression. As predicted, we found that expression of genes genome-wide was tightly correlated with the number of SP1-directed promoter insertions (**Fig 5F-G**). We also observed a significant correlation between gene expression and unfused hypPB (i.e. Brd4) promoter insertions, which is consistent with role of Brd4 in recruiting protein complexes such as positive transcription elongation factor b (P-TEFb) to gene promoters and promoting transcriptional elongation[32], particularly in the brain, where Brd4 has been shown to regulate transcription of genes critical to learning and memory[33]. However, the overall magnitude of promoter insertion density was substantially lower than in SP1 (**Fig 5F-G**). In summation, these data demonstrate that FLEX Calling Cards is capable of recording cell type-specific TF binding *in vivo* through TF-hypPB fusions.

Coupling FLEX Calling Cards to translating ribosome affinity purification (TRAP) enables parallel cell type-specific profiling of TF binding and actively translated RNA in rare cell types.

A remaining question with FLEX Calling Cards is the ability to profile TF binding in rare 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 a substantial number of background insertions present in Cre(-) animals, particularly in the GFAP::Cre line (**Fig S4B**), which may impinge on our ability to detect TF-bound REs in a more rare cellular population. While the cause of these low frequency, Cre-independent insertions is unclear, we reasoned that we could substantially mitigate non-cell type-specific background by simply selecting for the RNA that comes from these rare populations prior to SRT library

preparation, thus potentially allowing for profiling of rare cell types. To do this, we employed translating ribosome affinity purification (TRAP)[34,35], a method that enriches for cell type-specific transcripts via pull down of a GFP-tagged large ribosomal subunit (Ribosomal Protein L10a; Rpl10a) that is also specifically expressed in these cells, in parallel with FLEX Calling Cards. In addition to potentially reducing FLEX Calling Cards background, because both TRAP and FLEX Calling Cards library preparations originate from RNA, this allows for simultaneous analysis of TF binding and actively translated RNA in the same 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[36], which expresses the GFP-tagged Rpl10a subunit. 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. 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 6A**). 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 6B**). 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 SE regulation is critical to cell identify and function[7,26], we next aimed to identify Rbp4-enriched, Brd4-bound SEs. Importantly, because SEs are highly enriched for Brd4 binding, Calling Cards sensitivity for SE identification is quite high even with lower insertion totals (Moudgil *et al.*). To profile Brd4-bound SEs, we defined significantly enriched insertion peaks with our background-free peak calling method from the 171,833 Rbp4(+) insertions and observed 223 significant peaks at the $p<10^{-30}$ cutoff. As expected, the majority of Brd4 signal was observed at peaks displaying typical SE features[7,26], 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 6C**). As Rbp4(+) neurons are a subset of all neurons, and by extension brain cells overall, we expect that many of these REs would be shared amongst cell types, with a subset likely Rbp4-enriched. To define Brd4-bound regions specifically enriched in Rbp4(+) cells, we next identified insertion peaks differentially enriched in Rbp4::Cre animals as compared to C57wt mice profiled with the Cre-independent AAV::hypPB (detailed in **Fig 3**) and identified 65 differentially enriched peaks (**Fig 6D)**. Of these peaks, 43.1% (28/65) intersected with a known SE identified via whole cortex H3K27ac ChIP-Seq data[27] and thus likely represent Rbp4(+) enriched SEs (**Fig 6E**). Similarly to our previous Syn1::Cre and GFAP::Cre analyses, we then asked whether genes near differentially Brd4-bound peaks 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[37], 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 6F**), with an

overall median logFC of 0.287. This mirrors our results in Syn1:Cre and GFAP::Cre (**Fig 4D-F**), and suggests that these peaks represent cell type-specific, Brd4-bound REs in the rare population of Rbp4(+) layer V pyramidal neurons.

Discussion

This work describes the development of FLEX Calling Cards, a versatile method for TF mapping in the brain in a cell type-specific manner. This technology builds on previously developed *in vitro* Calling Cards methodologies and represents a substantial step forward in our ability to investigate epigenetic regulation in the brain. We have successfully 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 TF profiling in multiple cell types. Also of note, a parallel report (Moudgil *et al.*) from our group details adaptation of Calling Cards for single cell analyses, 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[14,19,20]. 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. Secondly, while not explored here, one could envision simple manipulations of the FLEX Calling Cards system to allow for temporal control of the system[28]. Such adaptations could allow for innovative and powerful studies in which TF binding is recorded only during defined windows of time. Another advantage of FLEX Calling Cards over ChIP-seq is the lack of requirement of a TF-specific antibody, allowing for TF profiling for, in theory, any TF, simply by fusing it to hypPB. Finally, 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 once proficient. Further, simply changing the viral serotype 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 important caveats to be considered as well. Most notably, there is potential for induced toxicity, 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[38]; however in such studies, the transposons are specifically engineered with splice-site gene or enhancer traps, while the transposons used in FLEX SRT Calling Cards only drive expression of a reporter gene and the genomic sequence immediately downstream of its insertion site. Nevertheless, it remains possible that a subset of Calling Card transposition events could perturb nearby gene expression. That said, in general, Calling Cards technologies have not exhibited marked deleterious effects in previous reports[14,20]. Further, the transposition rate of the *piggyBAC* transposase is inherently low, suggesting that it is highly unlikely for insertions to disrupt regulatory regions on both alleles in the same cell[39]. Finally, it is important to recognize that while we do see a clear Cre-induction of this system, we also observed a

substantial number of 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[35] 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 Brd4 profiling in a rare cell type, Rbp4(+) layer V pyramidal neurons. Perhaps more interestingly, because FLEX Calling Cards is mediated via RNA, this allows for profiling of TF binding and RNA expression in tandem from the same cellular populations, which could yield a variety of future studies moving forward.

In summary, we have introduced FLEX Calling Cards as a viable method for profiling TF binding events *in vivo* in a cell type-specific manner and demonstrated its effectiveness in profiling cell type-specific TF-bound regions 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, MJV, RDM, and JDD. AJC, AM, JH, XC, and MNW designed and generated DNA constructs. AM developed SRT analysis pipelines. AJC, TL, MJV, and MS generated and analyzed data. Manuscript written by AJC and edited by AM, TL, 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 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)[40], and TRAP reporter mice CAG::EGFP/Rpl10a (RRID:IMSR_JAX:022367). All mice were bred to the C57BL/6J background. At indicated endpoints, mice were anesthetized with isoflurane and perfused with 15ml of cold saline (PBS) prior to tissue collection. Brains were either dissected and flash frozen in liquid nitrogen (for molecular analyses) or fixed in 4% paraformaldehyde overnight, followed by 2 daily exchanges of 30% sucrose, and cryoprotected in O.C.T. (for immunofluorescence analyses).

As part of a separate study, all animals in the Syn1::Cre and GFAP::Cre experiments were given chemically-induced seizures immediately prior to sacrifice. In brief, mice were injected intraperitoneally (i.p.) with 65mg/kg of the GABA-antagonist pentylenetetrazole (PTZ) and seizures were recorded with a video camera for 15 minutes, after which animals were immediately anesthetized and sacrificed. All analyses described in this report were strictly cell type-to-cell type comparisons and were not related to seizure administration, and thus we do not expect these results to be affected by this extraneous procedure.

Cell culture and transfections

HEK293T 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 were carried out with Fugene® 6 Transfection Reagent (Promega, Madison, WI, USA) with the manufacturer's protocol.

Immunofluorescence and imaging

10µm-thick fixed-frozen sagittal 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 serum (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour 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 mounting media (ThermoFisher, Waltham, MA, USA). Immunofluorescent images of brain sections were acquired with a Nikon A1Rsi confocal microscope and imported into ImageJ (ver 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 mouse 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.

Virus generation and injections

Calling Cards hypPB, SP1(621C)-hypPB, and SRT 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 all *in vivo* experiments, transposase and donor transposon viruses were mixed equally by volume and then intracranially co-injected into the cortex of postnatal day 1 (P1) mouse pups, 3 sites per hemisphere. Viral titers were as follows:

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

SRT library preparation, sequencing, and mapping

Prior to library preparation, cortex samples were divided into 10 separate pieces, or “barcodes”, 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 separate, barcoded samples. From these RNA samples, transposon sequencing libraries were generated with our bulk SRT protocol, which is described in detail in a parallel report from our group (Moudgil *et al.*, in preparation). 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 (Illumina, San Diego, CA, USA) and sequenced on Illumina HiSeq 2500 or NextSeq 500 platforms.

Sequencing reads obtained from SRT libraries were stringently filtered for features of true insertion events (presence of *piggyBac* terminal repeat sequence, intact sequencing adapters and indexes, and a TTAA site) and mapped to build version mm10 of the mouse genome with Novoalign 3 (Novocraft Technologies). Reads aligning to the same TTAA in separate barcodes were considered unique insertions, and all analyses in this report considered all unique insertions equally, independent of read depth. We refer readers to a parallel report from our group (Moudgil *et al.*, in preparation) for a detailed description of the bulk SRT sequence analysis pipeline.

Significant insertion peak calling and motif discovery

Significantly enriched insertion peaks were identified via a count-based statistical comparison, as described in Moudgil *et al.* 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 3, Fig 4S,** and **Fig 6**, 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 4** for astrocyte- or neuron-specific Brd4 peaks, **Fig 5** for SP1 peaks, and **Fig 6** for Brd4 peaks specific to Rbp4(+) layer V projection neurons. 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 in significantly enriched SP1 insertion peaks (**Fig 4**) were identified with MEME-ChIP motif discovery software[41,42].

Defining enhancers and SEs

Since H3K27ac is a known marker of active enhancers[4,43] and SEs[7,26], we utilized a published P14 mouse cortex H3K27ac ChIP-Seq dataset[27] to define cortical SEs. As previously described[7,26], 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[44] with a p-value enrichment threshold of 10^{-9} to define enhancers and SEs. We then used the BEDtools suite[45] to compare the coincidence of enhancers and SEs with our Brd4-directed Calling Cards insertion peaks (**Fig 3** and **Fig 6**).

Additionally, for qualitative measures of histone modification enrichment at Calling Cards Brd4 insertion peaks (**Fig 3**), we used publicly available P0 mouse forebrain ChIP-Seq datasets from ENCODE[45]; specifically, H3K27ac (ENCSR094TTT), H3K4me1 (ENCSR465PLB), and H3K27me3 (ENCSR070MOK).

Analysis of enhancer- and promoter-associated gene expression

Gene expression has been shown to be preferentially regulated by proximal enhancer elements[4,43,46]. 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 4** and **Fig 6**), 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 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 (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 5**, 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.

Translating Ribosome Affinity Purification (TRAP)

TRAP was used to isolate cell type-specific, ribosome-bound RNAs from Rbp4(+) layer V pyramidal neurons in **Fig 6** as previously described[35,47]. 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[35]. Immunoprecipitation (IP) of transcripts was achieved via biotinylated-protein L (ThermoFisher Scientific; cat# 29997)-coated streptavidin magnetic beads (ThermoFisher Scientific; cat# 65602) that were coupled with two custom monoclonal anti-EGFP antibodies (Htz-19f7, Htz-19c8)[35]. 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 was determined via Agilent BioAnalyzer prior to sequencing.

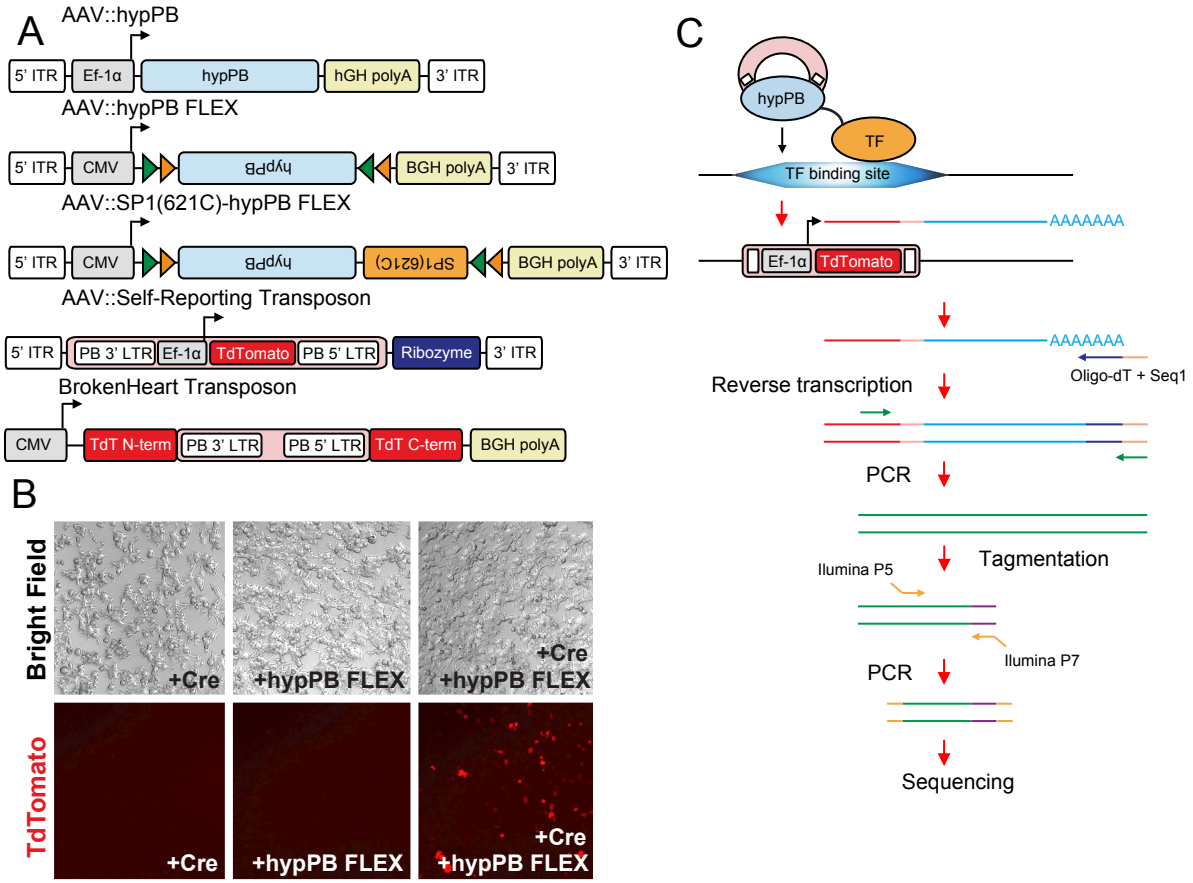

Figure 1. Constructs and library preparation. (A) Constructs used in AAV Calling Cards system, including Cre-dependent ("FLEX") and independent hypPB and TF-hypPB as well as donor transposon expression plasmids. (B) Transfection of BrokenHeart transposons, hypPB FLEX plasmid, and Cre recombinase into 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. (C) Schematic of library preparation protocol. HypPB inserts Self-Reporting Transposons (SRTs) near TF binding sites, which then transcribe and report their genomic locations via RNA. RNA is reverse transcribed, PCR amplified, and Illumina adapters are added for sequencing.

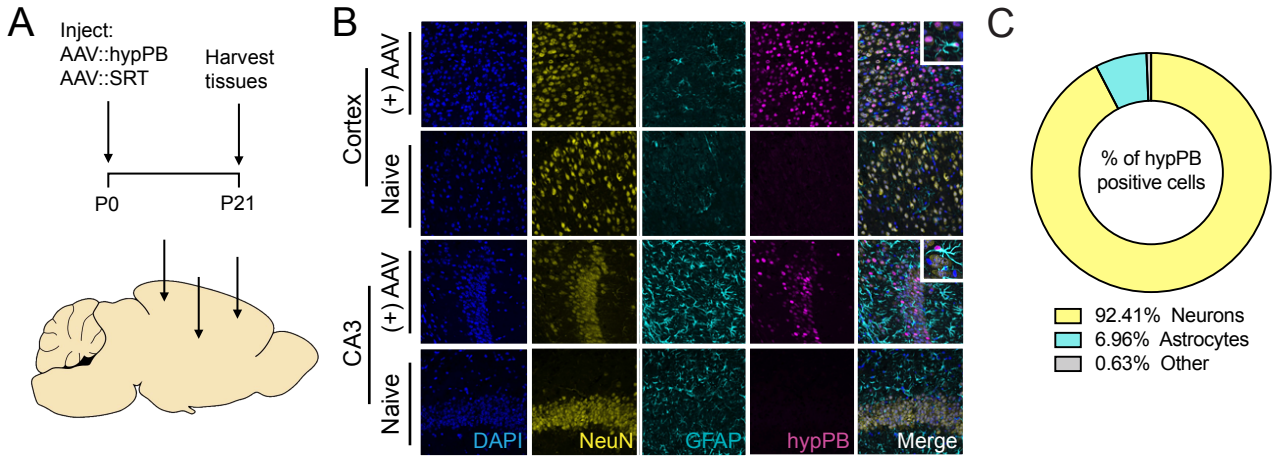

Figure 2. Co-AAV9 intracranial injection efficiently delivers Calling Cards to the cortex. (A) Experimental paradigm. (B,C) Co-immunofluorescence showing hypPB expression in neurons and astrocytes. (B) Representative images display co-localization of hypPB with neuronal (NeuN) and astrocyte (GFAP) markers. Insets show examples of hypPB-positive astrocytes. (C) 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 independent 5 mice).

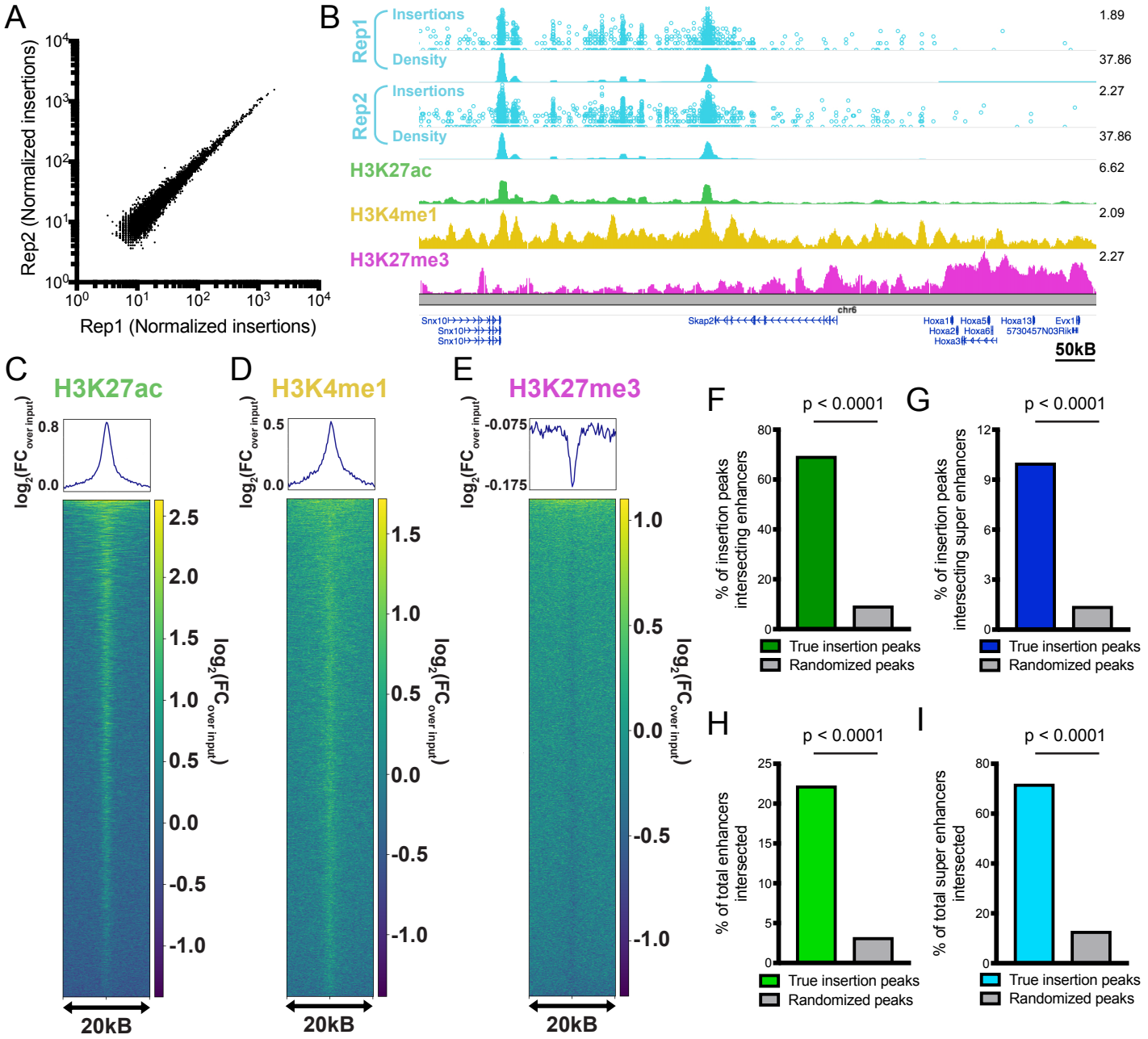

Figure 3. Unfused hypPB-directed Calling Cards insertions identify Brd4-bound active enhancers and super enhancers in the brain. (A) Normalized insertion depth in two littermate C57BL/6 mice at 7031 significantly-enriched insertion peaks displaying high correlation between replicates (R = 0.994). (B-E) Unfused hypPB-directed insertions are highly enriched for the active enhancer marks H3K27ac and H3K4me1 and depleted for suppresive 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 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 $\log_2$(fold-change over ChIP-seq input). (F,G) Percentage of 7031 significantly-enriched insertion peaks with at least 1 base-pair 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 base-pair intersection with a significantly-enriched insertion peak. Chi^2 test with Yates correction: $p < 0.0001$.

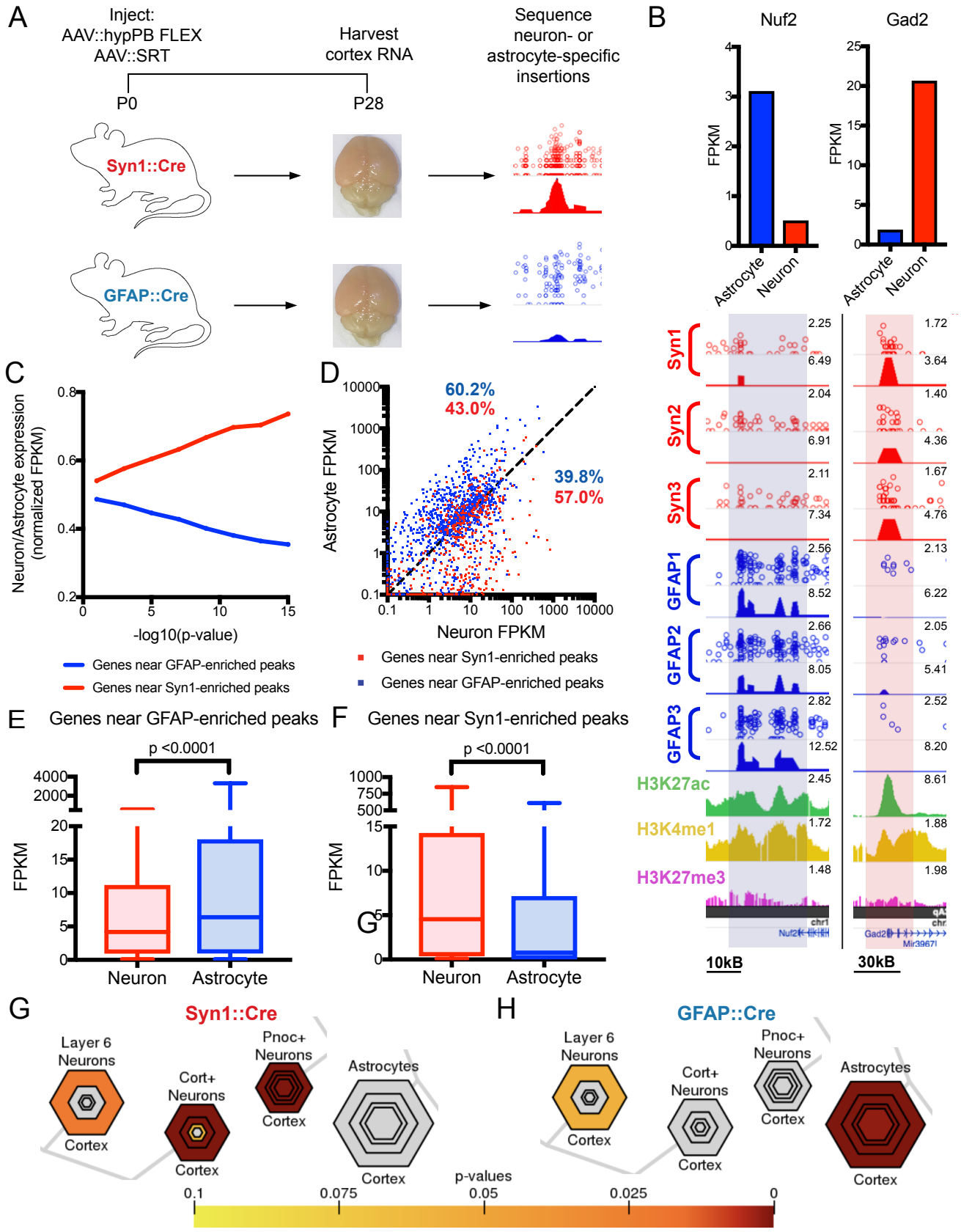

Figure 4. FLEX Calling Cards system generates cell type-specific Brd4 profiles. (A) Experimental design in Syn1::Cre and GFAP::Cre animals. (B) Examples of differentially enriched insertion peaks near genes preferentially expressed in neurons (right) or astrocytes (left). RNA-seq expression from Zhang et al., Journal of Neuroscience, 2014. (C) 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 signficance thresholds for defining differentially enriched insertion peaks. As significance threshold becomes more stringent, expression of nearby genes becomes more cell type-specific. (D) Astrocyte vs 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. (E,F) Quantifications of neuron and astrocyte specific expression of genes near GFAP::Cre (E) or Syn1::Cre (F) enriched insertion peaks displayed in (D), showing significant preferential expression in the expected cell type (Mann-Whitney U test: $p<0.0001$). (G,H) Graphical representation of cortical cell type enrichment from CSEA, based on gene sets near either (G) Syn1::Cre ($p<10^{-11}$; top 123 genes) or (H) 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.

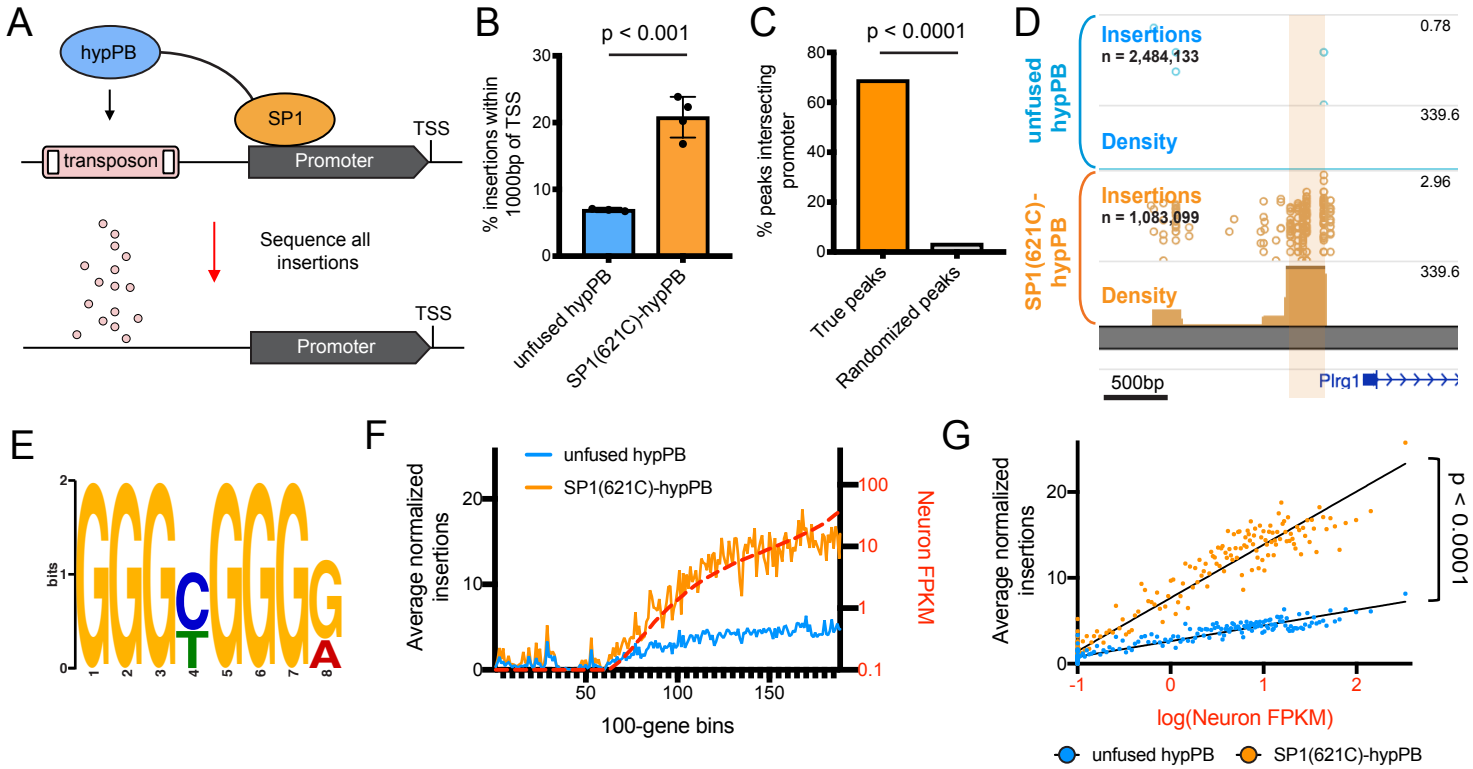

Figure 5. Fusion of SP1 to hypPB in FLEX Calling Cards system records neuron-specific SP1 occupancy profile. (A) Fusion of the promoter-binding TF SP1 to hypPB directs insertions to promoter-proximal TTAA sites. (B) Percentage of total insertions within 1000 basepairs of a transcription start site (TSS), displaying increased promoter-directed insertions upon SP1 fusion as compared to unfused hypPB (n = 3-4 mice per group; unpaired t-test: p<0.001). Total SP1(621C)-hypPB insertions: 1,083,099. Total unfused hypPB insertions: 2,484,133. (C) 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. (D) Representative insertion peak displaying significantly increased insertion density near the TSS of the *Plrg1* gene. (E) 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 ($p<10^{-42}$). (F-G) Normalized number of SP1(621C)-hypPB and unfused hypPB directed insertions at promoter proximal regions and neuron-specific gene expression for all genes, binned and averaged into 100-gene bins. In (F), 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 obtained from Zhang et al., Journal of Neuroscience, 2014. (G) displays strong correlation of both normalized SP1(621C)-hypPB and unfused hypPB directed promoter insertions with gene expression (SP1(621C)-hypPB: R = 0.96, p <0.0001; unfused hypPB: R = 0.94, p <0.0001), but SP1(621C)-hypPB insertions have a significantly higher slope (analysis of covariance (ANCOVA): p < 0.0001).

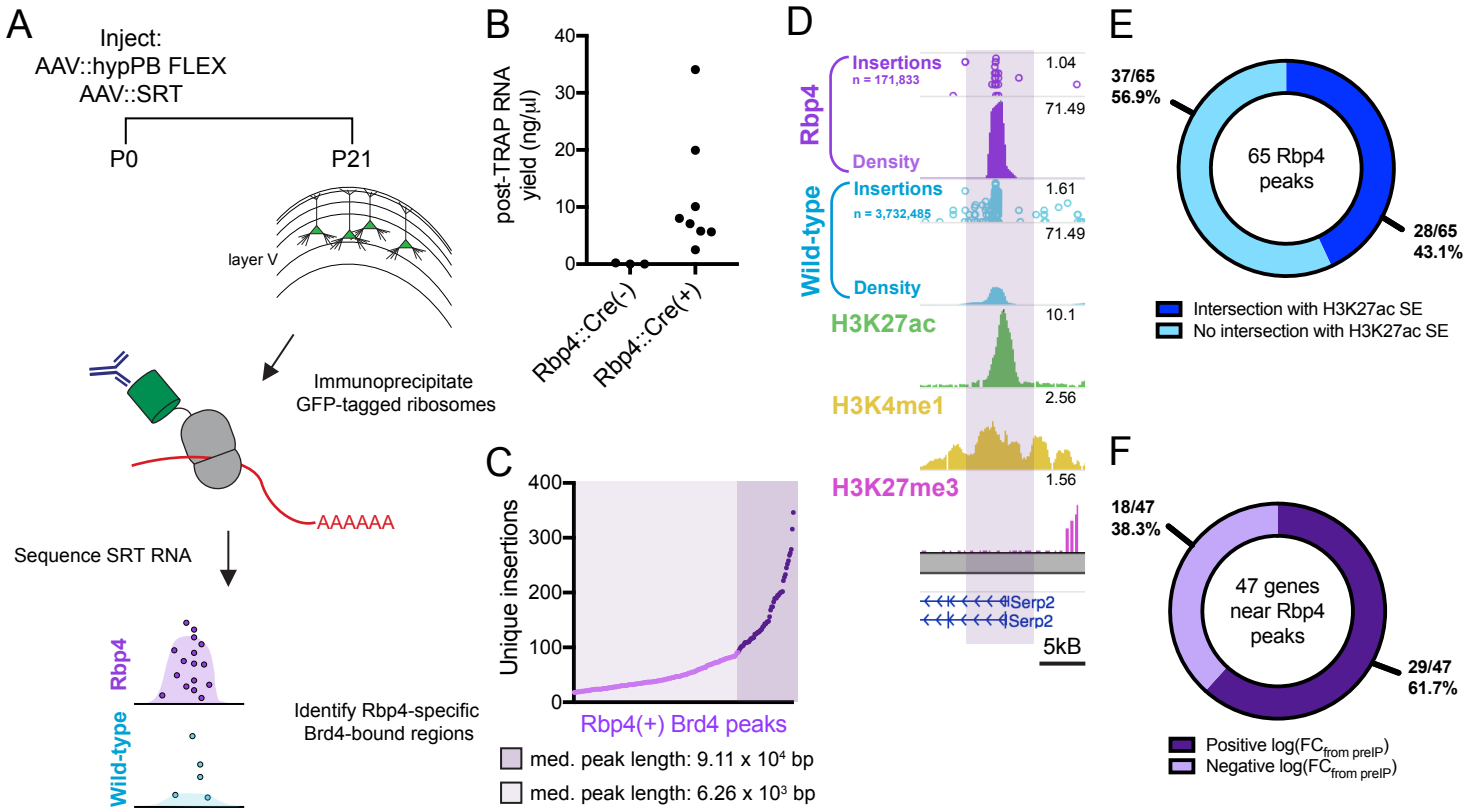

Figure 6. Combinatorial FLEX Calling Cards/TRAP allows for TF profiling in Rbp4::Cre(+) layer V projection neurons. (A) Experimental design of combinatorial FLEX Calling Cards and TRAP for cell type-specific TF profiling in Rbp4(+) neurons. (B) Post-immunoprecipitation (IP) RNA yields are observed in Rbp4::Cre(+) but not Rbp4::Cre(-) samples. Each point represents post-TRAP IP yield from one barcode. (C) Characteristic "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) Representative differentially enriched Brd4 peak in Rbp4::Cre mice over wild-type. (E) Out of 65 differential insertion peaks in Rbp4::Cre over wild-type, 28 intersect a known H3K27ac super enhancer (SE). (F) Proximal genes to differential insertion peaks are preferentially expressed in Rbp4 TRAP RNA samples than preIP RNA input in similar ratios observed in neurons v astrocytes (Fig. 4).