# Measuring Transcription Factor Binding and Gene Expression using Barcoded Self-Reporting Transposon Calling Cards and Transcriptomes

Matthew Lalli[1,2,3], Fengping Dong[1,2], Xuhua Chen[1,2], Jeffrey Milbrandt[1], Joseph Dougherty[1,4], Robi Mitra[1,2,*]

1. Department of Genetics, School of Medicine, Washington University in St. Louis School of Medicine, Saint Louis, MO 63110.

2. Edison Family Center for Genome Sciences and Systems Biology Washington University in St. Louis School of Medicine, Saint Louis, MO 63110.

3. Present address: Seaver Autism Center for Research and Treatment, Icahn School of Medicine at Mount Sinai, New York, NY 10027.

4. Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110.

* Corresponding author: rmitra@wustl.edu

## Abstract

Calling cards technology using self-reporting transposons enables the identification of DNA-protein interactions through RNA sequencing. By introducing a DNA barcode into the calling card itself, we have drastically reduced the cost and labor requirements of calling card experiments in bulk populations of cells. An additional barcode incorporation during reverse transcription enables simultaneous transcriptome measurement in a facile and affordable protocol. We demonstrate that barcoded self-reporting transposons recover in vitro binding sites for four basic helix-loop-helix transcription factors with important roles in cell fate specification: ASCL1, MYOD1, NEUROD2, and NGN1. Further, simultaneous calling cards and transcriptional profiling during transcription factor overexpression identified both binding sites and gene expression changes for two of these factors. RNA-based identification of transcription factor binding sites and gene expression through barcoded self-reporting transposon calling cards and transcriptomes is a novel and powerful method to infer gene regulatory networks in a population of cells.

## Keywords

Calling cards, transcription factors, transposons, barcoding, transcriptomics, pioneer factors

## Introduction

Calling cards is a uniquely powerful method to genetically record interactions between a protein of interest and the genome[1,2]. Briefly, a protein of interest is fused to a transposase which can insert a transposon 'calling card' into the genome at sites of DNA-protein interaction such as transcription factor binding sites (TFBS). A recent technical innovation termed the 'self-reporting transposon' (SRT) allows for the facile recovery of calling cards through RNA sequencing (RNA-seq)[3]. RNA-mediated mapping of transposon insertions is more efficient than previous DNA-based protocols[1], and this protocol enables the simultaneous identification of TFBS and changes in gene expression in single cells[4]. However, in bulk experiments on populations of cells, the RNA-mediated protocol is technically cumbersome, requiring a large number of replicates[4]. Here, we present two crucial modifications of the SRT technology and protocol to facilitate its use and to enable joint recording of TFBS and gene expression in populations of cells: barcoded SRTs and barcoded transcriptomes.

Current implementations of the mammalian calling card protocol employ a hyper-active *piggyBac* transposase[5]. An inherent constraint of this transposase is its requirement for a 'TTAA' tetranucleotide sequence for transposon insertion. As a result, multiple independent calling card insertions often occur at the same genomic location in different cells, particularly when there is a strong TFBS nearby. This can affect the dynamic range of bulk calling card experiments, because, at most, only two independent insertions into the same genomic location can be distinguished (i.e. if the two transposons are inserted in different orientations). To improve the quantitative readout, insertion counts are summed across all 'TTAA' sites within a given genomic window and then again across multiple replicate experiments (typically 8-12). Multiple replicates are especially needed to assess TF binding at genomic regions with few 'TTAA' sequences, but experimental cost and labor scale linearly with the number of replicate experiments.

As an alternative approach, we sought to embed a unique barcode within the self-reporting calling card which would enable multiple insertions at the same site across a population of cells to be counted independently within a single experimental replicate. Devising a barcoding strategy for the SRT is challenging in several technical and biological ways. The single-cell calling card method relies on cell barcodes introduced during droplet-based reverse transcription[3], but this strategy is incompatible with bulk experiments. The SRT consists of a promoter driving a selectable reporter flanked by the transposon terminal repeat sequences (TR). Introducing a barcode between the reporter gene and the TR, an approach used in our previous inverse PCR based DNA protocols[1], would no longer be compatible with our SRT recovery protocol because the barcode would be ~300 bases away from the transposon-genome junction in the final PCR and having a long stretch of shared sequence present in all amplicons would lead to extensive barcode swapping[6,7]. Therefore, we sought to introduce a barcode directly into the TR itself, as close to the transposon-genome junction as possible to minimize the risks of barcode swapping. A potential problem with this strategy is that all published sequences of the most terminal region of the *piggyBac* TR are completely invariant. This suggests strong sequence constraints on this region for proper transposition[8–12] which might preclude barcode insertion.

Here, we performed targeted mutagenesis of the *piggyBac* terminal repeat sequence to identify sites that could accommodate or serve directly as a barcode in calling card experiments. We discovered at least four consecutive nucleotides within the TR that were tolerant of a range of mutations without majorly reducing transposition efficiency. As a resource to the scientific community, we have developed a set of barcoded *piggyBac* SRT plasmids and we have modified the calling card analysis software to utilize these barcodes. We demonstrate that barcoded SRT calling cards can map the genomic binding sites of transcription factors (TFs) involved in cell fate specification and transdifferentiation in vitro. Lastly, we combined barcoded SRT calling cards with bulk RNA barcoding and sequencing (BRB-seq) to simultaneously identify TFBS and accompanying transcriptional changes from multiple TFs in an easy and affordable protocol[13].

These innovations simplify bulk SRT calling card experiments, enable barcoding of experimental conditions, and allow for pooled library preparations that substantially reduce cost and labor. This simple protocol for simultaneously measuring transcription factor binding and gene expression changes will facilitate the inference of gene regulatory networks for TFs involved in development, cellular reprogramming, and disease.

## Results

### *Identifying Candidate Regions for Barcode Insertion in piggyBac Terminal Repeat*

*piggyBac* and other transposases recognize and bind their cognate terminal repeat sequences that flank the transposon on both sides, and this interaction is necessary for transposition[8]. The SRT calling card captures the junction of the *piggyBac* TR and the adjacent genome to map insertion sites (Figure 1A)[3]. To maximize compatibility with calling cards library preparation and short-read next generation sequencing, an ideal barcode would be incorporated as close to the genomic insertion site as possible. A barcode introduced outside of the TRs will not be inserted into the genome (Fig. 1A, site 1). A barcode inserted between the reporter gene and the TR would be ~300 bp away from the transposon-genome junction, complicating readout by short-read next generation sequencing (Fig. 1A, site 2). This strategy would also include a long stretch of shared sequence present in all amplicons that would lead to extensive barcode swapping during the SRT amplification PCR step in library preparation[6,7]. Therefore, we sought to introduce a barcode directly into the TR itself (Fig. 1B, site 3), directly adjacent to the TR-genome junction. Such a strategy would eliminate almost all intervening sequence between the barcode and the genome junction which has two major advantages compared to other approaches. First, a barcode in this position could be captured in the same sequencing read as the transposon-genome junction which simplifies sequencing. Second, without a long constant intervening sequence, there is little risk of introducing aberrant chimeric PCR products during sequencing library preparation[6,7]. Whereas modifications to TRs from other transposases such as *SleepingBeauty* have been successfully engineered[9], similar efforts have revealed extensive sequence constraints on *piggyBac* TRs for efficient transposition[10,11]. Nevertheless, we sought to identify candidate regions within the TR that might accommodate a DNA barcode.

The minimal *piggyBac* TR consists of a 19-bp internal repeat (IR), a 3-bp spacer, and a 13-bp terminal invert repeat[12] (Fig. 1B). These sequences are critical for *piggyBac* recognition, cleavage, and transposition. Notably, all published sequences of the 13-bp terminal invert repeat

in the *piggyBac* TR are completely invariant. DNase I footprinting of *piggyBac* binding to its TRs revealed strong binding across much of this region[8], yet a few bases were less protected and therefore might be a candidate region for inserting a barcode (Fig. 1B, underlined nucleotides, gold).

*Targeted Mutagenesis Generates Mutant SRTs with High Transposition Efficiency*

We developed a simple and rapid screening protocol to generate and identify mutant *piggyBac* TR sequences capable of successful transposition (Fig. 1C). We designed primer sequences to introduce single point mutations into our candidate region using PCR. Purified PCR products encoding puromycin-resistance SRTs flanked by mutated TRs were directly transfected into HEK293T cells along with unfused hyper-active *piggyBac*. If mutated amplicons are compatible with transposition, they will be inserted into the genome and confer puromycin resistance. We selected for transposition events after 4 days by adding puromycin. RNA was extracted 3 days after this, and bulk SRT libraries were prepared according to established protocols with modifications described[4] (Methods).

We sequenced calling card libraries using RNA-seq and mapped genomic transposition events from at least two independently generated mutant SRT pools for each position. Each library yielded 75,000-150,000 unique insertion sites providing a representative view of genomic insertion efficiency for mutant SRTs. Analysis of transposition events revealed that all 3 candidate positions within the *piggyBac* TR accommodated mutations without greatly diminishing transposition ability (Fig. 1D-F). Each of the three mutagenized positions tolerated all 4 nucleotides at similar frequencies, hence generating at least 12 unique transposon barcodes.

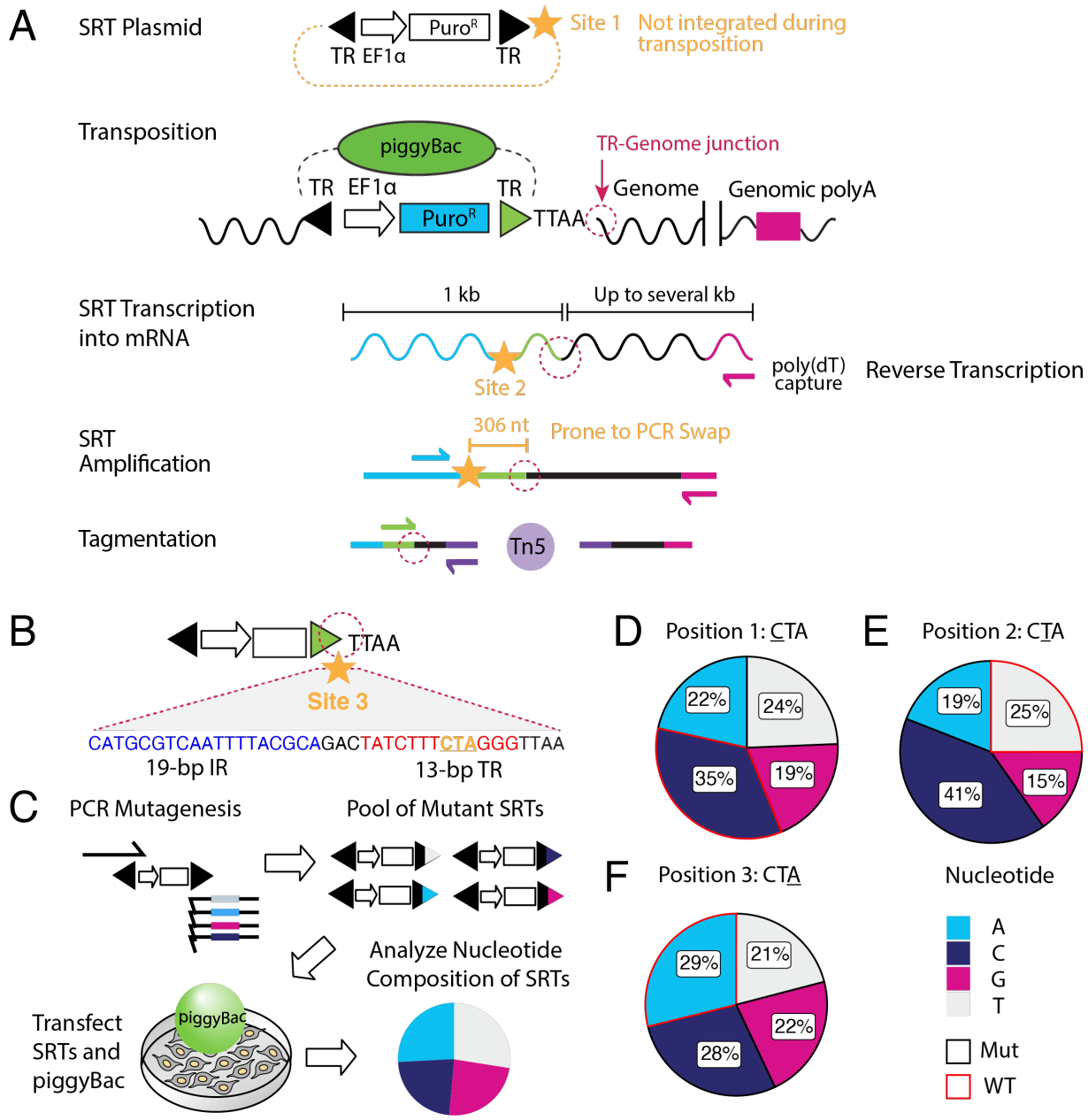


Figure 1: Barcoding the self-reporting transposon. A) Schematic overview of the SRT construct, Calling Card method, and sequencing library preparation. Candidate sites for barcode insertions are indicated with gold stars. The TR-Genome junction, used to map transposon insertions, is circled in dotted magenta line. B) Barcode site 3 is within the *piggyBac* TR sequence, immediately adjacent to the TR-Genome junction. Underlined nucleotides in the 13-bp terminal inverted repeat region ('CTA', gold) were targeted for mutagenesis by mutagenic PCR. C) Overview of calling card rapid mutagenesis scheme. Mutant amplicons were transfected into cells with *piggyBac* transposase and integrated calling cards were collected. Nucleotide frequency for each mutagenized position of integrated SRTs were calculated. Nucleotide frequency at D) position 1, E) position 2 and F) position 3 of integrated mutated SRTs. Wild-type sequences are outlined in red. All four possible nucleotides were well-represented at all three mutated positions. IR: inverted repeat. TR: terminal repeat. EF1a: eukaryotic translation elongation factor 1 α promoter. SRT: self-reporting transposon. nt: nucleotide. kb: kilobase. Puro$^R$: puromycin resistance cassette. WT: wild-type. Mut: mutant.

Having obtained successful transposition of SRTs with single mutations, we next tested whether multiple mutations within this region could be tolerated. Using PCR, we introduced 3 consecutive mixed bases (Ns, where N can be A,C,G, or T) into this region to generate a total of 64 barcoded SRTs. We transfected pools of these mutant SRT PCR amplicons into cells and again prepared calling card libraries after puromycin selection. Analysis of hundreds of thousands of transposition events showed that all 64 mutant transposons could be integrated into the genome, albeit at varying degrees of efficiency (Figure 2A). To better understand sequence preferences governing transposition efficiency, we generated a sequence motif from the top 30 most abundantly inserted transposons. Cytosine was slightly favored in the first two positions, and thymine was strongly disfavored from the third position (Supplementary Figure 1). Among mapped transposition events, we also observed the presence of mutations at a fourth nucleotide position immediately adjacent to our targeted bases, leading us to test whether this position could also be modified. Following the same approach, we generated SRTs with mutations in this position and prepared calling card libraries from two independently transfected sets of cells. As with the other single nucleotide SRT mutants, we found that this position could also tolerate all 4 nucleotides (Fig. 2B).

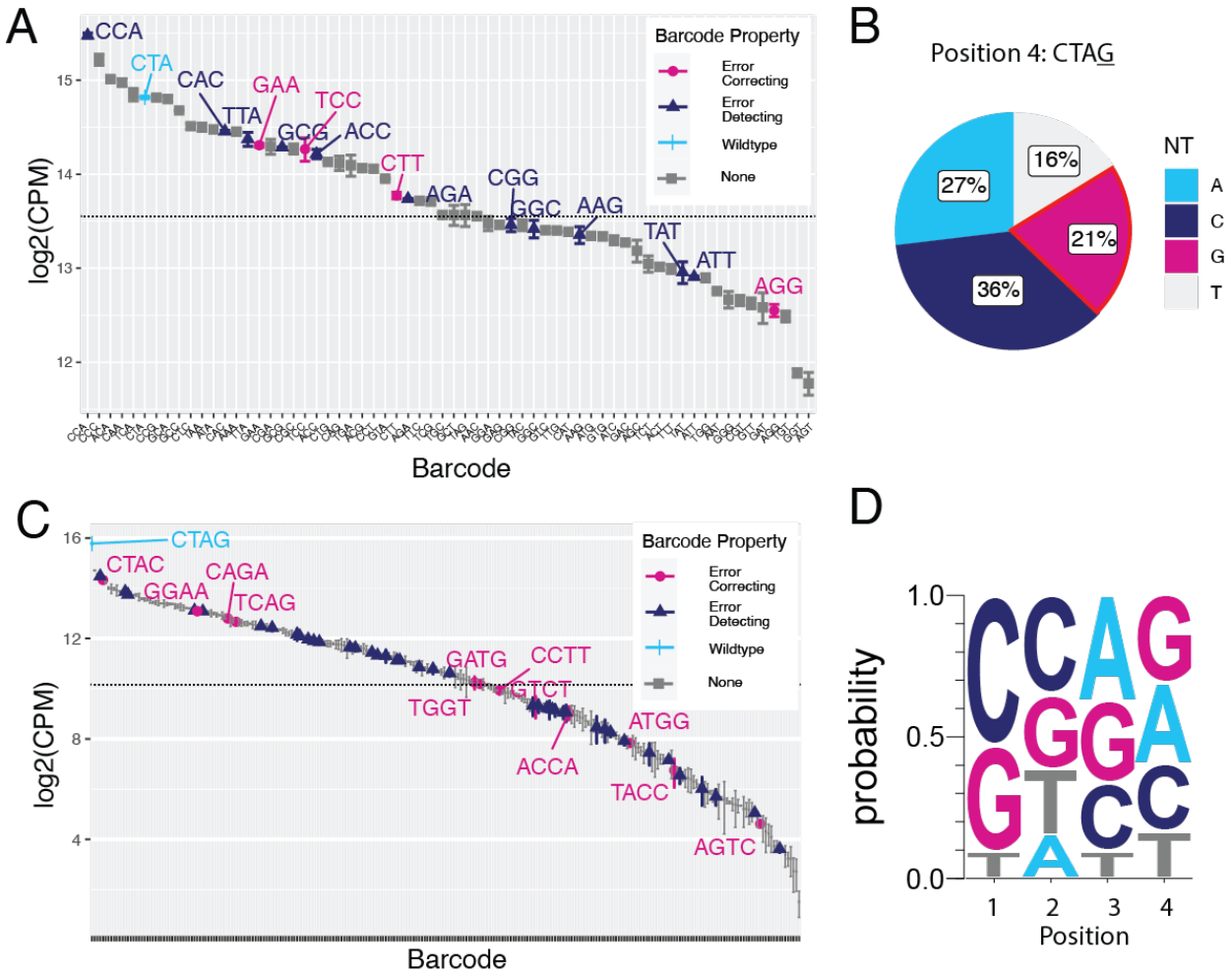


Figure 2: Multi-nucleotide mutagenesis in *piggyBac* terminal repeat yields integration-competent SRTs. A) Normalized counts of integration of events for 64 possible combinations of 3 nucleotide barcodes at the targeted

region are shown (log2 counts per million (CPM)). All 64 barcoded SRTs could integrate into the genome. Black dotted lines indicate 50$^{th}$ percentile of read counts. Data are plotted as mean and SEM from two independent replicates. B) Targeted mutagenesis at a fourth position in the terminal repeat identified another site that could tolerate all 4 nucleotide substitutions while retaining integration-competence. Wild-type sequence ('G') is outlined in red. C) Normalized counts (log2 CPM) of insertions for 256 combinations of 4-nt barcodes. All 256 barcodes were present at varying degrees of insertional efficiency. Wild-type sequence is colored cerulean. Error-detecting and error-correcting barcodes are colored respectively in magenta and midnight blue. D) Sequence logo of the top 100 most abundantly inserted 4-nt barcoded SRTs reveals modest sequence preference for integration efficiency. CPM: counts per million sequencing reads.

Longer barcodes are preferable in sequencing applications as they not only increase the number of unique sequences available but can also have advantageous properties including error detection and error correction[14]. A four-nucleotide barcode yields 256 unique sequences including 40 error-correcting and 12 error-detecting barcodes. To generate a pool of 256 mutant transposons, we introduced 4 consecutive mixed bases (Ns) into the TR using a degenerate primer. We collected and analyzed over 160,000 unique transposition sites in the genome and found all 256 possible mutated transposons were inserted into the genome (Fig. 2C). We analyzed the nucleotide composition of the top 100 most abundantly inserted transposons to reveal sequences mediating transposition efficiency (Fig. 2D). Overall preferences were modest except for a strong favoring of C/G in the first position and a disfavoring of thymine in the third position. These results suggest that the exact sequence of these 4 nucleotides is not recognized by *piggyBac* for binding and transposition.

Given the compatibility of mutations in this region of the TR with transposition, we tested whether we could insert a single nucleotide in this region to further increase barcode length. We generated mutant SRTs with a single nucleotide insertion and prepared calling card libraries after transfection. We observed that very few cells survived selection and consequently few transposition events were recovered from this experiment. Among the recovered transposition events, the most prevalent sequence matched the wild-type SRT with no insertion. Of the recovered SRTs that did contain an inserted nucleotide, many of the sequences also contained a nearby 1-nt deletion which may imply a strict TR length constraint for successful transposition (Supplementary Table 1). The inserted nucleotide may have disrupted any step of *piggyBac* recognition, cleavage, and transposition by changing the sequence, shape, or flexibility of the transposon[8,15]. Experiments that can discern among these possibilities might enable the accommodation of longer barcodes at this site. As a resource to the community, we individually cloned the top 20 integration-competent error-detecting barcodes into two self-reporting vectors. These SRT vectors include an adeno-associated viral (AAV) vector carrying a tdTomato reporter SRT compatible with in vivo calling card experiments[16] and a non-AAV SRT vector encoding the puromycin resistance gene.

*Using barcoded SRTs to map binding sites of transcription factors involved in cell fate specification*

To demonstrate that barcoded SRTs facilitate TFBS recording in cellular populations, we performed calling card experiments for four TFs using this method. We chose to record the binding of four members of the basic helix-loop-helix (bHLH) family: Achaete-scute homolog 1 (*ASCL1*), Myogenic Differentiation 1 (*MYOD1*), Neuronal Differentiation 2 (*NEUROD2*), and Neurogenin 1 (here referred to as *NGN1*). These TFs are implicated in cell fate specification and

cellular reprogramming[17–22]. Interestingly, all four TFs recognize the same canonical E-box motif *in vivo*, bind some overlapping and unique sites in the genome, and regulate distinct gene expression programs[23]. To perform calling card experiments, we first created mammalian expression vectors containing fusion proteins of each of the four TFs to the N-terminus of hyperactive *piggyBac* separated by an L3 linker[24]. We transfected HEK293T cells expressing fused or unfused *piggyBac* with wild-type or barcoded versions of SRTs encoding either tdTomato or puromycin-resistance reporters and harvested RNA after ~1 week. We prepared and sequenced SRT calling card RNA-seq libraries and analyzed the data to identify transposon insertions in the genome. Calling card peaks were called as described[1,25] and analyzed for enriched motifs and neighboring genes using HOMER[26]. We then performed Gene Ontology enrichment analysis on sets of genes located near TFBS[27].

For each of the four bHLH factors, we recovered hundreds of thousands of genomic insertion events and called thousands of calling card peaks (Supplementary Table 2). Motif enrichment analysis for each factor recovered several enriched bHLH E-box motifs, including the known motifs for Ascl1, MyoD, and NeuroD1 (Figure 3A). This motif recovery suggests barcoded calling cards identified bona fide TFBS for these factors. For NEUROD2, the top 3 enriched motifs belonged to specific neuronal bHLHs including NeuroD itself (Fig. 3A). Likewise for MYOD1, the top 3 enriched motifs belonged to myogenic bHLHs of the MyoD family (Fig. 3A), indicating specificity of the calling card peaks for the TFs of interest. This result supports that while the core E-box motif is common to all factors, nucleotides flanking this motif may confer binding specificity[28]. For ASCL1, in addition to recovering bHLH motifs, we also observed an enrichment of Jun/Fos and other basic zipper (bZIP) motifs which might indicate the binding of additional TFs at these sites.

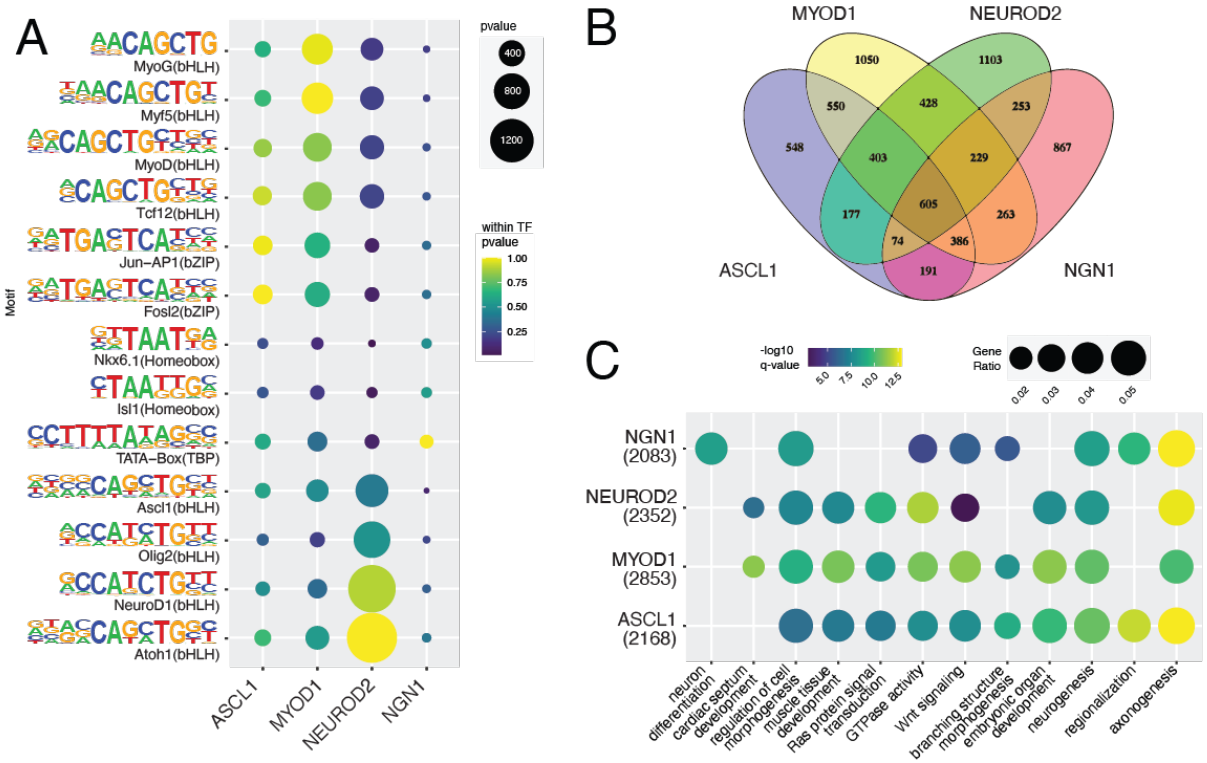


Figure 3: Calling cards experiments using barcoded SRTs recover known binding motifs for bHLH factors near genes related to known TF functions. A) Top binding motifs for each motif were retrieved from DNA sequences in calling card peaks. These sites are enriched for the canonical E-box motif as well as bHLH TFs including or related to each TF. B) Venn diagram of genes proximal to called peaks for each TF indicates both shared and distinct binding of these TFs. C) Gene Ontology enrichment analysis reveals terms related to neurogenesis and myogenesis.

Next, we identified genes located near TFBS, and found shared and differential binding of bHLH TFs (Fig. 3B)[29]. To gain insight into the potential functions of these TFs, we performed Gene Ontology enrichment analysis on sets of genes located near TFBS identified by barcoded SRTs[27]. Gene Ontology terms identified for genes proximal to the neurogenic TFs ASCL1, NEUROD2, and NGN1 were enriched for neuronal pathways including axonogenesis and neuron projection development, consistent with their roles in neuronal reprogramming (Fig. 3C)[20,30,31]. MYOD1 binding sites were located near genes strongly enriched for roles in cardiogenesis and muscle development (Fig. 3C, Supplementary Fig. 2). Consistent with prior findings of MYOD1 binding some neuronal targets[18], we found some enrichment for binding at genes enriched for neurogenic pathways. The observed enrichment of neuronal and muscle genes is particularly notable given the calling card assay was performed in human embryonic kidney cells which do not natively express any of the assayed TFs. That all factors are able to recognize and bind specific genes enriched for their known functions implies either a permissive binding environment in HEK293T cells or cell-type independent target access by these TFs. This also highlights that subtle differences in nucleotide sequences flanking the common core E-box motif can confer binding specificity at functionally enriched genes[28].

*Barcoded SRTs and Transcriptomes Enable Simultaneous Mapping of TFBS and Gene Expression*

SRTs were specifically invented to enable simultaneous readout of gene expression and transcription factor binding in single cells[3] but can also be used to map TFBS in populations of cells as demonstrated here and previously[3,16]. Because SRTs are amplified from polyadenylated (poly(A)) RNA, preparing standard poly(A) mRNA sequencing libraries in parallel from the same sample is trivial. To reduce cost and labor of library preparation, multiple poly(A) mRNA-seq libraries can be barcoded during reverse-transcription then pooled for library preparation and sequencing[13]. We have previously modified this barcoding protocol to employ the 10x Genomics single cell 3' chemistry which enables turnkey analysis of RNA-seq data using CellRanger[32,33].

To facilitate simultaneous preparation of SRT calling card and poly(A) 3' RNA-seq libraries, we introduced a sample barcode and unique molecular identifier (UMIs) into the poly(dT) capture oligonucleotide (Figure 4A). Each experimental replicate is reverse transcribed with a uniquely barcoded capture oligo, then multiple samples can be pooled for ultra-affordable transcriptomic analysis[13]. SRT experiments can be designed such that experimental replicates use distinctly barcoded SRTs (individual barcodes or sets of barcodes) so that the same pool of cDNA can then be used to amplify SRTs and total RNA in parallel reactions. Otherwise, SRT libraries can be amplified individually. Sequencing libraries of amplified products are then prepared by tagmentation[34].

*Simultaneous Mapping of TFBS and Gene Expression of Pioneer TFs*

ASCL1 and MYOD1 belong to a special class of transcription factors called pioneer factors that can access both open and closed chromatin and reprogram cell fate from pluripotent stem cells or fibroblasts to neurons and muscle cells respectively[19,35–37]. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) after overexpression of these factors in mouse embryonic fibroblasts revealed a surprising degree of overlapping binding sites between these factors[18]. Gene expression profiling of TF overexpression, however, revealed differing transcriptional outcomes for these two factors[18]. As many TF binding events have no or small effects on gene regulation[38–41], integrating ChIP-seq with mRNA-seq is a powerful method to decipher cis-regulatory modules and identify functional TFBS[40,42–44]. Typically, multi-omic measurements are collected from different populations of cells using separate protocols. In contrast, barcoded SRT calling cards and transcriptomes can be collected simultaneously from the same cells which may improve the ability to link TF binding to changes in gene expression.

As a proof-of-principle of this method, we transiently overexpressed unfused hyperactive *piggyBac* or fusions with ASCL1 or MYOD1 in HEK293T cells, then collected SRT calling cards and transcriptomes after one week. Cells transfected with ASCL1 and MYOD1 were co-transfected with non-overlapping pools of 12 barcoded SRTs to enable pooled SRT and transcriptome library preparation. Such experimental design therefore enables multiplexed TF profiling in a single pooled experiment. We performed 4 independent transfections for each factor. Compared to the recommended protocol for the original bulk RNA calling cards method for the same experiment[4], barcoded SRT calling cards and transcriptomes reduces material cost and labor of experiments by over 10-fold (Table 1).

| | Replicates (n) | | Cost ($USD) | |
|---|---|---|---|---|
| | **Original** | **Barcoded** | **Original** | **Barcoded** |
| Transfections | 36 | 12 | 720 | 240 |
| RNA isolation and reverse transcription | 36 | 12 | 180 | 60 |
| Amplification | 72 | 2 | 216 | 6 |
| Bead Cleanup, Tapestation | 72 | 2 | 360 | 10 |
| Tagmentation | 72 | 2 | 2160 | 60 |
| Bead Cleanup, Tapestation | 72 | 2 | 360 | 10 |
| Sequencing | *Same* | | | |
| | | | 3996 | 386 |

Table 1: Drastic cost and labor reduction of barcoded SRT and transcriptomes compared to original protocol. 'Original' calculations use the recommended 12 replicates per TF[4]. This experiment assayed 3 TFs (unfused hyper *piggyBac*, ASCL1, and MYOD1). Transfection costs are based on NEON or nucleofector transfection device reactions. Tagmentation costs assume a library is prepared for each of the 12 replicates for both calling cards and transcriptomes.

Using the pooled barcoded SRT approach, we recovered hundreds of thousands of genomic insertion sites for each factor (Supplementary Table 3). Compared to unfused *piggyBac* binding sites, barcoded calling card peaks for ASCL1 and MYOD1 were again enriched for bHLH motifs including Ascl1 and MyoD (Fig. 4B). Comparing genes near identified TFBS, we again observed ASCL1 and MYOD1 had shared and distinct binding profiles (Fig. 4C) consistent with previous studies[17,18]. The genomic insertion sites recovered strongly overlapped those from our earlier experiments with unpooled sequencing library preparations, but the total number of sites was lower. This could reflect reduced library complexity after pooling and future experiments to understand the decreased peak recovery would further improve this methodology.

Next, to identify transcriptional consequences of TF overexpression, we analyzed 3' gene expression profiles that were simultaneously captured with SRTs. Supporting the approach of pooling of barcoded first strands for 3' library preparation, all 12 samples were well-represented in the sequencing data. Neither the average number of genes detected, nor the total RNA counts differed across factors and samples clustered by experimental condition (Supplementary Figure 3). We performed differential gene expression analysis on transcriptomes of cells transfected with ASCL1 or MYOD1 fusions compared against unfused *piggyBac* and identified 182 and 666 genes differentially expressed respectively. Of the differentially expressed genes, 170 and 480 were upregulated in ASCL1 and MYOD1 cells respectively (Fig. 4D), consistent with known roles of these transcription factors as activators of gene expression. Gene Ontology analysis of upregulated genes recapitulated some relevant pathways in ASCL1 transfected cells, but many pathways were not related to neurogenic or myogenic pathways (Fig. 4E). Further, while some differentially expressed genes overlapped with genes near TFBS identified by barcoded SRTs, they were not enriched for such overlap. This is consistent with previous studies showing poor correlation between TF binding and gene expression[38–41]. Because this experiment used transient transfection, the low number of differentially expressed genes we observed might stem from the

loss of TF overexpression by the time of RNA collection. Future experiments shortening the collection period or prolonging the transgene overexpression may increase the number of differentially expressed genes and improve the concordance of these with TFBS. Nevertheless, these results demonstrate a novel method to simultaneously collect TFBS and gene expression changes from the same SRT calling card experiment which may facilitate the inference of functional TFBS.

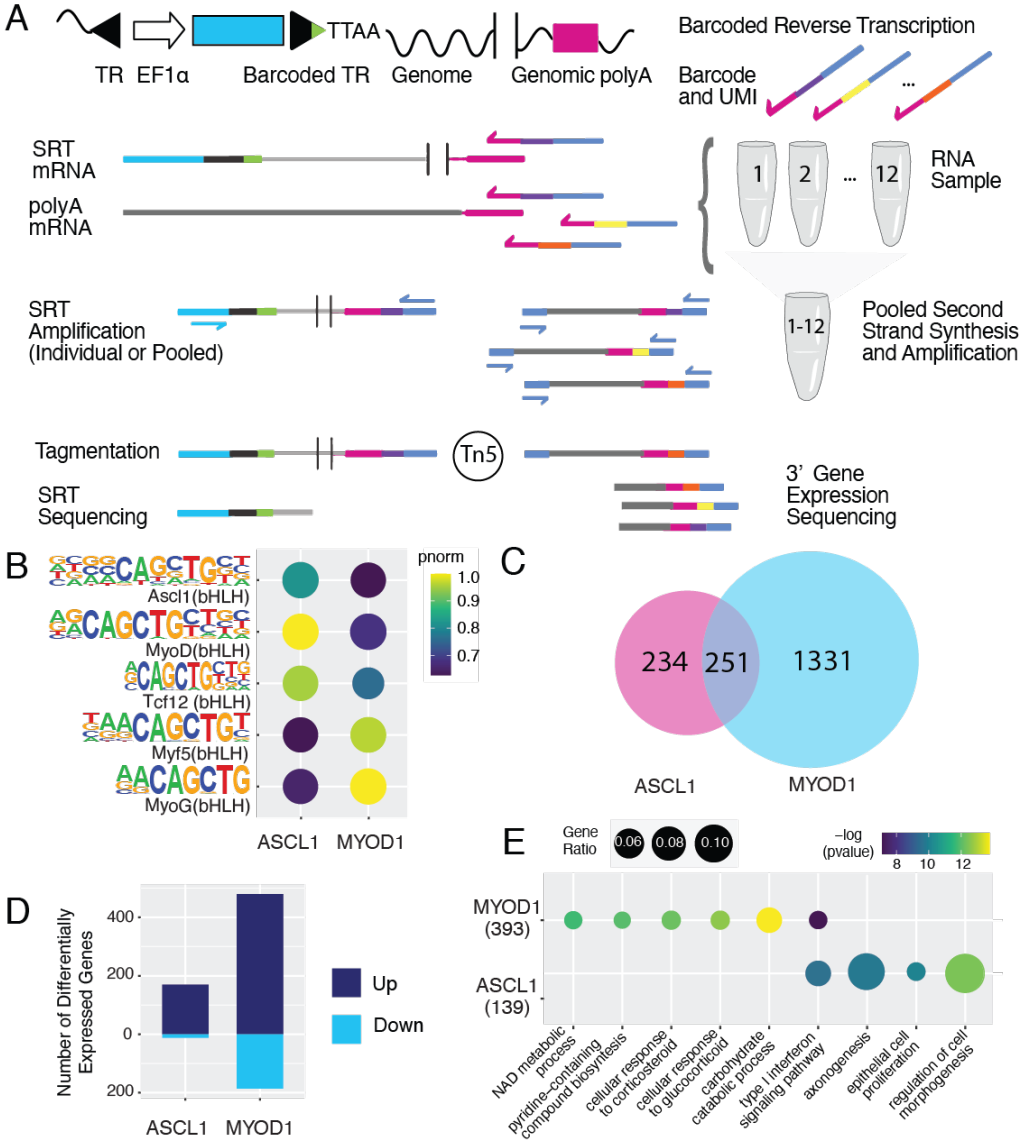


Figure 4: Barcoded SRT calling cards and transcriptomes enables joint measurement of TFBS and gene expression. A) Schematic overview of barcoded sequencing library preparation. Sample-specific barcode with unique molecular identifiers (UMI) is introduced during reverse-transcription of poly(A) RNA including SRTs and mRNA. Reverse transcription products (cDNA) can then be pooled for second strand synthesis and amplification. Libraries are prepared for SRTs and transcriptomes in parallel. B) Barcoded SRT experiments recover binding motifs for ASCL1 and MYOD1. C) Venn Diagram showing genes near ASCL1 and MYOD1 binding sites. D) Transcriptomes profiled by bulk RNA-seq with barcodes revealed differential gene expression for ASCL1 and MYOD1, compared to cells transfected with unfused *piggyBac*. E) Gene Ontology of differentially expressed genes in ASCL1 and MYOD1 cells.

## Discussion

Understanding where and how TFs bind in the genome to orchestrate gene expression is a central goal in genomics[40,42–44]. Calling cards is a powerful functional genomics method to identify the binding sites of TFs and other chromatin-associated factors in mammalian cells both in vitro and in vivo[1–3,16,25]. The recently invented 'self-reporting transposon' converts the calling card recordings of TF binding to an RNA readout, enabling simultaneous profiling of gene expression and binding in single cells[3]. Here, we present two crucial modifications of the SRT technology and protocol to enable parallel recording of TFBS and gene expression in populations of cells: barcoded SRTs and barcoded transcriptomes. Besides enabling transcriptomic measurement, these improvements also drastically reduce the experimental cost and labor of calling card experiments.

First, we performed targeted mutagenesis of the *piggyBac* transposon TR region. We coupled a simple PCR mutagenesis method with SRT calling cards to rapidly screen for positions in the *piggyBac* TR that could be mutated while retaining compatibility with transposition. Through this, we discovered four consecutive nucleotides within the TR that were tolerant of a range of mutations, both singly and in combination, without markedly reducing transposition efficiency. To our knowledge, these are the first reported mutations within the *piggyBac* terminal invert repeat that are compatible with transposition. We note the wild-type TR sequences are inserted at the highest frequency compared to any 4-nt barcoded SRT so our targeted mutagenesis approach did not improve overall transposition efficiency. Nevertheless, for applications where the number of integrations is not paramount such as mutagenesis screens[45], cellular lineage tracing[46] and human gene therapy[47] we anticipate that barcoded *piggyBac* transposons will have broad utility beyond calling cards.

As a resource to the community, we have individually cloned the top 20 integration-competent error-detecting barcodes into two versatile self-reporting transposon vectors compatible with in vitro and in vivo experiments. Barcoded error-detecting SRT vectors will allow experimental conditions (*e.g.* timepoint, drug treatment) to be uniquely barcoded, pooled, and accurately demultiplexed without sample mis-assignment due to sequencing errors. Of note, multiple transcription factors can be assayed simultaneously in a pooled experiment by using non-overlapping sets of barcoded SRT for each TF.

We demonstrated that barcoded SRT calling card experiments could identify TFBS for four bHLH transcription factors involved in cell fate specification and transdifferentiation: ASCL1, MYOD1, NEUROD2, and NGN1. We identified shared and unique binding sites for each factor and recovered binding motifs that matched known motifs for these factors. Supporting the identification of bona fide TFBS by barcoded SRT calling cards, we found that genes near TFBS were enriched for functions related to known functions of the assayed TFs. Barcoded SRT vectors reduced the experimental cost and labor of the calling card protocol which allowed us to easily measure TFBS for these four transcription factors in 293T cells. Remarkably, although HEK293T cells do not normally express any of the assayed TFs, all 4 TFs were able to recognize their cognate consensus motifs, and these motifs were located near genes with functions associated with each TF.

Identifying TFBS is a first step toward understanding gene regulatory networks but many TF binding events have no or small effects on gene regulation[38–41]. Integrating multi-omics datasets, such as TFBS and mRNA-seq, is therefore necessary to identify functional TFBS governing gene expression[40,42–44]. Often, these multi-omics datasets are generated from different populations of cells and vastly different protocols that can introduce biases and batch effects. Since SRTs are expressed and collected as RNA, TFBS and gene expression data can be simultaneously generated from the same RNA sample in a calling cards experiment. While this method has been demonstrated in single-cell experiments[3], bulk calling card experiments required modification to allow such joint measurement in bulk experiments. Specifically, we barcoded the SRT through a mutagenesis screen and introduced an additional barcode during reverse-transcription for barcoding mRNA. Combining barcoded SRT calling cards with bulk RNA barcoding and sequencing (BRB-seq) therefore enabled simultaneous identification of TFBS and gene expression in a protocol with drastically reduced cost and labor[13].

We demonstrate that the combined protocol can recover TFBS and gene expression during TF overexpression of the pioneer factors ASCL1 and MYOD1. Calling cards with barcoded SRTs and transcriptomes is therefore a novel and powerful method to infer functional TFBS in populations of cells. Technical and experimental optimizations of this method may improve its utility in future experiments. This versatile method is also compatible with cell-type specific recording of TFBS in a mixed populations of cells in vitro or in vivo[16]. Using an inducible *piggyBac* system[24,48] will enable temporal measurement of binding and expression changes especially during the time course of cellular reprogramming experiments.

Finally, our simple mutagenesis method will be useful for introducing barcodes to other DNA transposons such as Sleeping Beauty and Tol2. Transposons are widely used for transgenics, mutagenesis, and functional genomics experiments[49]. As the SRT protocol can easily scale to recover millions of genomic integration sites, insertion preferences for other transposons can be readily ascertained using this method. Each transposon has its own preferences for genomic integration which can have complementary uses. Further, insertion profiles can depend on chromatin state[50] so SRTs can potentially be used to read out chromatin status and histone modifications. For example, unfused *piggyBac* has an insertion preference at super-enhancers which are a class of enhancers regulating genes linked to cell identity[50,51], and it has been used to read out these important regulatory elements[3,16,52]. Joint measurements of *piggyBac* insertions and gene expression with this method may help link super-enhancers to gene regulatory networks.

In conclusion, barcoded SRTs simplify bulk calling cards experiments, enable barcoding of experimental conditions, and allow for pooled library preparations that drastically reduce cost and labor. Incorporating barcoded transcriptomes into the library preparation enables joint measurement of transcription factor binding and gene expression from the same biological sample. This method will facilitate the inference of gene regulatory networks for TFs involved in development, cellular reprogramming, and disease.

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## Methods

*Transposon Mutagenesis*

PCR mutagenesis was performed in a 50 µL reaction containing: 25 µl 2X Kapa HiFi HotStart ReadyMix, 1 µl of 10 µM SRT Mutagenesis Forward Primer (either puro or tdTomato version), 1 µl of 10 µM SRT Mutagenesis Reverse Primer, 100 ng of SRT DNA (either PB-SRT-puro or PB-SRT-tdTomato), and 22 µl of $ddH_2O$. PCR reactions were performed following thermocycling parameters: 95ºC for 3 minutes, 10 cycles of: 98ºC for 20 seconds, 60ºC for 30 seconds, 72ºC for 2 minutes, then 72ºC for 10 minutes, and 4ºC forever.

Importantly, we used low (10-12) cycles during PCR mutagenesis of SRTs to minimize the occurrence of any PCR duplications or 'jack-potting' events. PCR reactions were performed in duplicate. Each pool of mutant amplicons was purified with NucleoSpin Gel and PCR Clean-up (Macherey and Nagel). Products were transfected into separate wells of HEK293T cells to minimize any artifacts.

>SRT Mutagenesis Reverse Primer
tgcatctcaggagctcttaacc**NNNN**aaagatagtctgcgtaaaattgac

> SRT Mutagenesis Forward ($puro^R$)
GCGGAAGGCCGTCAAGGCC

> SRT Mutagenesis Forward (tdTomato)
CACGAGACTAGCCTCGAtcaaggcgcatttaaccctagaaagataa

*Cell Culture*

HEK293T cells were maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were passaged every 3–4 d by enzymatic dissociation using trypsin.

*Cloning*

ASCL1, MYOD1, NEUROD2, and NGN1 were amplified from lentiviral cDNA expression vectors using 2X Kapa HiFi HotStart ReadyMix. A nuclear localization sequence was added to the 5' end of each gene, and an L3 linker (amino acid sequence KLGGGAPAVGGGPKAADK) [24] was inserted between the TF and hyper-active *piggyBac.*

EF1a_ASCL1, MYOD1, and NEUROG1_P2A_Hygro_Barcode were gifts from Prashant Mali (Addgene plasmid #120427, #120464, and #120467). phND2-N174 was a gift from Jerry Crabtree (Addgene plasmid #31822).

*Calling Card Experiments*

Calling card experiments are performed as described with minor modifications[3,16,53]. Twenty-four hours before transfection, 250,000 HEK293T cells are plated per well in a 12 well plate. The next day, cells are transfected using PEI (Polysciences) with 1 ug of total DNA comprising 500 ng of *piggyBac* (fused or unfused) and 500 ng of donor SRT (purified PCR product or miniprepped DNA). Medium is changed 24 hours after transfection. Three days after transfection, each well is trypsinized and replated into a T25 flask. For puromycin-resistance SRTs, puromycin is added 24 hours later (2 ug / mL). Three days after puromycin selection, total RNA is harvested using Direct-zol RNA MiniPrep kit (Zymo Research).

*Calling Card Library Preparation*

Calling card libraries were prepared as described with minor modifications[3,4,16]. We performed first-strand reverse transcription reactions in 20 µL total volume using 2 µg of RNA from each sample. RNA mixed with water and dNTPs was hybridized to oligo-dT primers (1 µL of 50 µM SMART_dTVN) by incubation at 65 ˚C for 5 minutes and immediately transferred onto ice. 0.5 µL of Maxima H Minus Reverse Transcriptase, RNasin RNAse inhibitor, and 5X RT buffer were added and samples are incubated at 50 °C for 60 min for reverse transcription.

*Barcoded Calling Card and Transcriptome Library Preparation*

Bulk RNA Barcoding and sequencing (BRB-seq) was performed with minor modifications[13,32]. We performed first-strand reverse transcription reactions in 20 µL total volume using 2 µg of RNA from each sample. Barcoded BRB-seq_dT30VN primers modified to mimic the 10x Genomics v2 chemistry. RNA mixed with water and dNTPs was hybridized to barcoded oligo-dT primers (2 µL of 25 µM stock) by incubation at 65 ˚C for 5 minutes and immediately transferred onto ice. 1 µL of template switch oligo (TSO_SMART), 0.5 µL of Maxima H Minus Reverse Transcriptase, RNasin RNAse inhibitor, and 5X RT buffer were added and samples were incubated at 50 °C for 60 min for reverse transcription.

For barcoded SRT and transcriptome experiments studying ASCL1, MYOD1, and unfused *piggyBac*, 3 µL of reverse-transcription product from 4 replicates for each factor were pooled together for transcriptome analysis. 4 replicates of each factor (5 µL per replicate) were pooled and purified in parallel for calling card library preparation. Pooled samples were purified using NucleoSpin Gel and PCR Clean-up (Macherey and Nagel) and eluted with 30 µL of elution buffer. We designed barcoded oligoDT-VN oligos to mimic 10x Genomics v2 chemistry (partial seq1, 16 bp cell barcode extracted randomly from the 10x Genomics safelist, and a 10 bp UMI (5N + 5V). Barcoded primer sequences are provided in Supplemental Table S1.

Barcoded, pooled first-strand reactions (23 µL) were mixed with 1 µL partial seq1 primer (10 µM), 1 µL SMART primer (10 µM), and 25 µL 2X KAPA HiFi HotStart ReadyMix (Roche). 10 cycles of PCR with a long extension time (98˚ 20 seconds, 60˚ 30 seconds, 72˚ 6 minutes) were performed.

cDNA was purified with 0.6X AMPure XP (Beckman Coulter) magnetic beads. DNA was eluted with 20 µL water and concentration was measured using the Tapestation D5000 ScreenTape (Agilent). 600 pg of product were tagmented with barcoded N7 primers and P5-index-seq1 primers using the Nextera XT kit (Illumina). BRB-seq libraries were sequenced on a Novaseq 6000 paired-end with 28 x 91 reads.

*Primers*

>SMART_dT18VN
AAGCAGTGGTATCAACGCAGAGTACGTTTTTTTTTTTTTTTTTTTTTTTTTTVN

>BRB-seq_dT30VN
CTACACGACGCTCTTCCGATCTCTGATAGCATGGTCATNNNNNVVVVVTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTVN

>SRT_PAC_F1
CAACCTCCCCTTCTACGAGC

>SRT_tdTomato_F1
TCCTGTACGGCATGGACGAG

> SMART_TSO
AAGCAGTGGTATCAACGCAGAGTACrGrGrG

>SMART
AAGCAGTGGTATCAACGCAGAGT

>Partial Seq1
CTACACGACGCTCTTCCGATCT

Barcoded *piggyBac* primers, for example:

>OM-PB-ACG (barcode sequence is underlined)
AATGATACGGCGACCACCGAGATCT[optional_index]ACACTCTTTCCCTACACGACGC
TCTTCCGATCTACGCGTCAATTTTACGCAGACTATCTTT

>P5-index1-Seq1 (index sequence is underlined)
AATGATACGGCGACCACCGAGATCTACACAGGACAACACTCTTTCCCTACACGACG
CTCTTCCGATCT

>Nextera_N701 (index sequence is underlined)
CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG

*Library Prep and Sequencing*

Purified PCR product was measured on Tapestation D5000. cDNA samples were diluted to 600 pg / µL and 2 µL of this was used for tagmentation with Nextera XT kit.

Agencourt Ampure XP (Beckman Coulter)
Nextera XT DNA Library Preparation Kit (Illumina, Inc)
High Sensitivity D1000 ScreenTape (Agilent Technologies)

*RNA-seq Analysis*

Sequencing data corresponding to barcoded bulk RNA transcriptomes were processed using the 10x Genomics software package Cell Ranger (v 2.1.0). The output filtered gene expression matrices were imported into R (v 3.5.1) for further analysis[54]. Gene counts were used directly in edgeR for standard bulk RNA-seq analysis[55].

*Calling Card Analysis*

Sequencing and analysis:

Bulk barcoded RNA calling card libraries were sequenced and analyzed as described with modifications to utilize the SRT barcode[3]. Calling card reads begin with a 3-nucleotide library barcode, the barcoded transposon TR, the insertion motif TTAA, then the genome at the site of insertion. Reads are checked for the library barcode, TR sequence, and TTAA and these sequences are trimmed. SRT barcodes are extracted by UMI-tools[56], and appended as a sequence tag to the read. Any remaining Nextera adaptors are trimmed before mapping the reads to the human genome (hg38) using NovoAlign. Aligned reads are validated as insertions if adjacent to a TTAA site in the genome. Bona fide insertions are then converted to qBED format (née .ccf)[57]. SRT barcodes were incorporated into the barcode column of the qBED. If non-overlapping barcode sets were used to define experiments, qBED files can be demultiplexed by this barcode field.

Peak calling: Calling card peaks were called as described [1,25] using in-house peak calling software (CCF tools, https://gitlab.com/rob.mitra/mammalian_cc_tools). Specifically, peaks were called using the call_peaks_macs python script, which follows the algorithm used by

MACS to call ChIP-Seq peaks[58] modified for the analysis of calling card data. The main peak calling function is passed an experiment frame, a background frame, and an TTAA_frame, all in qBED/ccf format[57]. It then builds interval trees containing all of the background and experiment hops (insertion events) and all of the TTAAs. Next, it scans the genome with a window of window_size and step size of step_size and looks for regions that have significantly more experimental hops than background hops (poisson w/ pvalue_cutoff). It merges consecutively enriched windows and computes the center of the peak. Next it computes lambda, the number of insertions per TTAA expected from the background distribution by taking the max of lambda_bg, lamda_1, lamda_5, lambda_10. It then computes a *p*-value based on the expected number of hops = lamda * number of TTAAs in peak * number of hops in peak. Finally, it returns a frame that has Chr, Start, End, Center, Experiment Hops, Fraction Experiment, Background Hops, Fraction Background, Poisson *p*-value as columns. We used parameters: -pc 0.001 --peak_finder_pvalue 0.01 --window 1000 --step 500 --pseudocounts 0.2 for peak calling.

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