Variation in epigenetic state correlates with gene expression across nine inbred strains of mice

# Abstract

It is well established that epigenetic features, such as histone modifications and DNA methylation, are associated gene expression across cell types. However, it is not well known how variation in genotype affects epigenetic state, or to what extent such variation contributes to variation in gene expression across genetically distinct individuals. Here we investigated the relationship between heritable epigenetic variation and gene expression in hepatocytes across nine inbred mouse strains. Eight of the inbred strains were founders of the diversity outbred (DO) mice, and the ninth was DBA/2J, which, along with C57Bl6/J, is one of the founders of the BxD recombinant inbred panel of mice. We surveyed four histone modifications, H3K4me1, H3K4me3, H3K27me3 and H3K27ac, as well as DNA methylation. We used ChromHMM to identify 14 chromatin states representing distinct combinations of the four measured histone modifications. We found that variation in chromatin state mirrored genetic variation across the inbred strains. Furthermore, epigenetic variation was correlated with gene expression across strains. The correspondence between epigenetic state and gene expression was replicated in an independent population of DO mice in which we imputed local epigenetic state. In contrast, we found that DNA methylation did not vary across inbred strains and was not correlated with variation in expression in DO mice. This work suggests that chromatin state is highly influenced by local genotype and may be a primary mode through which expression quantitative trait loci (eQTLs) are mediated. We further demonstrate that the mid-range resolution of chromatin states, between that of SNPs and haplotypes paired with gene expression, is useful for annotation of functional regions of the mouse genome. Finally, we provide, to our knowledge, the first data resource to document variation in chromatin state across genetically distinct individuals.

# Introduction

Epigenetic modifications to DNA and its associated histone proteins influence the accessibility of DNA to transcription machinery, and are associated with up- and down-regulation of gene expression [26704082, 22641018, 22781841]. Across cell types, unique combinatorial patterns of histone modifications mark chromatin states that establish cell type-specific patterns of gene expression [20657582, 21441907]. Similarly, the methylation of CpG sites around gene promoters and enhancers influences transcription in a cell type-specific manner [21701563, 20720541].

These patterns of histone modifications and DNA methylation are established during development. The result is a canonical epigenetic landscape for coordination of major patterns of gene expression for each cell type [sources about development]. As an organism ages and responds to its environment, patterns of both histone modifications [citation] and of DNA methylation change [citation]. Such changes have been linked to scenescence [Horvath clock] and cancer [citations].

Epigenetic modifications coordinate the usage of a single genome to be used for many different types of cells with diverse morphology and physiology. This amazing feature of epigenetic modifications has been intensely studied, and the variation in epigenetic landscapes across cell types has been extensively documented [citations]. Less well understood, however, is the role that genetic variation plays in determining epigenetic landscapes.

Across genetically diverse populations of humans or mice, individual cell types, such as hepatocytes, or cardiomyocytes, have globally similar gene expression profiles that define their role within the greater organism. However, it is also true that across individuals, gene expression varies widely within the global constraints of cell type. This variation can increase or decrease an organism’s risk of developing disease. Variation in gene expression has been extensively mapped to variation in genetic loci, or expression quantitative trait loci (eQTL). Large, coordinated efforts, such as the Genotype-Tissue Expression (GTEx) Project [32913073, 32913075] have identified and catalogued many such loci in humans, and countless independent studies have identified eQTL in mice and other model organisms.

Although the link between genetic variation and gene expression has been well studied, there is relatively little known about inter-individual variation in epigenetic modifications, and how these variations are related to variations in genotype and gene expression. The generation of a more complete picture of inter-individual variation in epigenetic modifications has the potential to increase our understanding of the mechanisms of gene regulation, provide insights into the mechanisms establishing cell type-specific epigenetic landscapes, and to improve the functional annotation of the genome as it relates to the regulation of gene expression. The vast majority of SNPs associated with human disease traits are located in non-coding regions, suggesting that they influence gene regulation, rather than protein function [citation]. However, annotation of these regions is difficult without additional genomic features, such as histone modifictions and DNA methylation. Overlaying a map of variation in epigenetic features has the potential to provide a picture of how genetic variation changes functional elements, like enhancers and insulators, in the genome [citation].

Advances in chromatin immunoprecipitation (ChIP) and sequencing technologies now enable genome-wide surveys of histone modifications with relatively few cells [20077036], thus opening the door to the possibility of cataloging epigenetic variation across cell types and individuals. Here, we performed a survey of epigenetic variation in hepatocytes across nine inbred mouse strains. We included the eight founders of the Diversity Outbred/Collaborative Cross (DO/CC) [citation] mice, as well as DBA/2J, which, along with C57Bl/6J, is one of the founders of the widely used BxD recombinant inbred panel of mice [citation]. We assayed four histone modifications (H3K4me1, H3K4me3, H3K27me3, and H3K27ac), as well as DNA methylation. We used ChromHMM [citation] to identify 14 chromatin states, classified by unique combinations of the four histone marks, and investigated the association between variation in these states and variation in gene expression across the nine strains. We separately investigated the relationship between DNA methylation and gene expression across strains.

We further investigated the relationship between epigenetic state and gene expression by imputing the 14 chromatin states and DNA methylation into a population of DO mice. We then mapped gene expression to the imputed epigenetic states to assess the extent to which eQTLs are driven by variation in epigenetic modification. We thus linked genetically controlled variation in epigentic modifications to variation in gene expression in mice, and we provide the first resource documenting epigenetic variation across a wide panel of genetically diverse mice.

# Materials and Methods

## Inbred Mice

information about housing, animal use, etc.

### Hepatocyte acquisition

Samples were taken from 12-week female mice of nine inbred mouse strains: 129S1/SvImJ, A/J, C57BL/6J, CAST/EiJ, DBA/2J NOD/ShiLtJ, NZO/HlLtJ, PWK/PhJ, and WSB/EiJ. Eight of these strains are the eight strains that served as founders of the Collaborative Cross/Diversity Outbred mice [REF]. The ninth strain, DBA/2J, will facilitate the interpretation of existing and forthcoming genetic mapping data obtained from the BxD recombinant inbred strain panel [REF]. Mice were aged and processed in groups to maintain a steady sample preparation workflow. Mice were housed, born, and aged in the same mouse room, with uniformity in timing, diet, and all other possible conditions. Female mice were used for all experiments due to potentially confounding effects from variation in testosterone among males that can affect liver gene expression, as well general experience that female expression is less variable than male in multiple tissues. This will also facilitate the analysis of maternal effects on offspring in later studies. Three mice were used from each strain.

### Liver perfusion

To purify hepatocytes from the liver cell population, the mouse livers were perfused with collagenase to digest the liver into a single-cell suspension, and then isolated using centrifugation. Mice were harvested at 9:00 AM and sacrificed by cervical dislocation. Mice were placed over a stack of paper towels in preparation to catch excess liquid, and the appendages were pinned out to hold the body in place. to keep the fur from contaminating the liver sample later, the fur was wiped down with 70% ethanol. The mouse skin was then cut open and peeled back to the appendages to allow clear access to the abdominal cavity. The fascia was cut open and back to the ribs, being careful to not nick the liver. Moving the intestines and stomach to the right side, the vena cava and hepatic portal vein should be clearly visible below the liver.

For the perfusion, a 23G x ’’ BD Vacutainer Safety-Lok needle (REF 367297) was attached to 1.6mm ID BioRad Tygon tubing (R-3603) connected to a Pharmacia peristaltic pump that allows a flow of up to 8 ml/min. The liver will be processed with three solutions: 5mM EGTA in Leffert’s buffer, Leffert’s buffer wash, and 87 CDU/mL Liberase collagenase with 0.02% CaCl2 in Leffert’s buffer. The three solutions were at 37C before perfusion.

The needle was placed into the vena cava for the perfusion superior to the kidneys and inferior to the liver. With the peristaltic pump running slowly, the vena cava was pierced at shallow 15 angle and the needle was inserted to a shallow depth (around 2-3mm of the needle tip in the vena cava). Once the needle is inserted into the vena cava, the volume on the peristaltic pump is increased to 5-7mL/min. The liver will immediately blanch, and the hepatic portal vein is immediately severed to allow flushing of the liver.

The 1x EGTA buffer was used to flush the blood out of the liver and start the digestion of the desmosomes connecting the liver cells. To help with the perfusion, pressure was applied to the hepatic portal vein for 5 second intervals causing more solution to be forced through the liver, which can be seen visually by the liver swelling. After 35ml of the 1x EGTA solution is passed through the liver, the solution was switched to the 1x Leffert’s buffer. The pump was turned off during the switch to prevent air from being sucked into the tubing while the tubing is transferred to the new solution. To wash, 7-10ml of the Leffert’s buffer was passed through the liver to flush out the EGTA, which otherwise chelates the calcium ions necessary for collagenase activity in the next buffer. The pump was turned off again to switch to the Liberase solution. To digest the liver, 25-50mL of Liberase solution ( wunsch units) was passed through the liver. Throughout the perfusion process, periodic pressure was applied to the hepatic portal vein to help pump the buffers more completely through the liver. As the liver was digested with the Liberase, it will swell and look soggy and limp. Over-digestion leads to increased contamination with non-hepatocyte cell types, and further reduces cell viability.

After perfusion, which takes around 15-20min to complete, the liver was carefully cut out of the abdominal cavity and placed in a petri dish with 35 mL ice-cold Leffert’s buffer with 0.02% CaCl. The digested liver was passed through Nitex 80 m nylon mesh (cat #03-80/37) into a 50mL conical, using additional ice-cold Leffert’s buffer with 0.02% CaCl2 if necessary, and a rubber policeman. After the liver cells from both animals were collected, they were put through two wash and spin cycles to purify the hepatocytes and remove other types of cells. To isolate the hepatocytes, the much larger size of the hepatocyte cells was exploited in very slow 4 min, 50 x g spins that leave smaller other cell types in suspension. After each spin, the solution was decanted as waste, and the enriched cell pellet of hepatocytes was resuspended in 30ml ice-cold Leffert’s buffer with 0.02% CaCl. After the second spin, the solution should be almost clear, indicating that other cell types have been removed. The hepatocytes are resuspended in room temperature PBS, counted, and volume adjusted to cells/mL.

We aliquoted cells for each RNA-Seq and bisulfite sequencing, and the rest were cross-linked for ChIP assays. Two aliquots (5mLs) of liver cells were removed into two 15mL conicals. These were spun down at 200 rpm for 5 min, and resuspended in RTL+BME (for RNA-Seq) or frozen as a cell pellet in liquid nitrogen (for bisulfite sequencing). Meanwhile, 37% formaldehyde in methanol (VENDOR) were added to the remaining cells to a final concentration of 1%. The cells were rotated at room temperature for 5 min to cross-link protein complexes to the DNA bound to them. After cross-linking, 10x glycine (VENDOR) is added to a final concentration of 125 mM and rotated for 5 min to quench the formaldehyde and stop cross-linking. The cells were spun down at 2000 rpm for 5 min, decanted, and resuspended in PBS to cells/mL. The cells were divided into aliquots in 2mL tubes. The tubes were spun down again at 5000 x g for 5 min, decanted, and the cell pellets frozen in liquid nitrogen. All cell samples were stored at -80°C until used.

### Hepatocyte histone binding and gene expression assays

Hepatocyte samples from 30 treatment and control mice were used in the following assays:

1. RNA-seq to quantify mRNA and long non-coding RNA expression, with approximately 30 million reads per sample.
2. Reduced-representation bisulfate sequencing to identify methylation states of approximately two million CpG sites in the genome. The average read depth is 20-30x.
3. Chromatin immunoprecipitation and sequencing to assess binding of the following histone marks:
4. H3K4me3 to map active promoters
5. H3K4me1 to identify active and poised enhancers
6. H3K27me3 to identify closed chromatin
7. H3K27ac, to identify actively used enhancers
8. A negative control (input chromatin) Samples are sequenced with million reads per sample.

The samples for RNA-Seq in RTL+BME buffer were sent to The Jackson Lab Gene Expression Service for RNA extraction and library synthesis.

### Histone chromatin immunoprecipitation assays

The H3K4me1 and H3K4me3 histone chromatin immunoprecipitation assays were performed on cross-linked hepatocytes using similar protocols. For all histone ChIP assays, the crosslinked chromatin was prepared the same way. First, the aliquot of hepatocyte cells was lysed to release the nuclei by rotating the sample in hypotonic buffer for 20 min at C. The cells were pelleted by spinning for 10min, 10K x G, at C. The cells were resuspended in 130ul MNase buffer with 1mM PMSF (VENDOR) and 1x protease inhibitor cocktail (Roche VENDOR) to prevent histone protein degradation, then digested with 15U of MNase. The micrococcal nuclease digests the exposed DNA, but leaves the nucleosome-bound DNA intact. After 10min of incubation at C, the chromatin was digested into primarily mononucleosomes. This was confirmed by DNA-purification of the MNase-digested chromatin run out on an agarose gel, which yielded mostly 150bp fragments, and few 300bp fragments. The MNase digestion was stopped by adding EDTA to 10mM, and incubating on ice for 5 min. The digested chromatin was purified by spinning out insoluble parts at top speed for 10 min at C. The chromatin was transferred to a new tube and spun again to further remove impurities and reduce background in the ChIP assays. The final chromatin was transferred to a fresh tube, and used immediately in the ChIP.

To prepare for the ChIP, cells Dynabead Protein G beads were aliquoted into an Eppendorf tube. A magnetic tube holder was used to attract the beads to the wall of the tube, and then the solution was carefully pipetted off, leaving only the beads behind. The beads were washed twice with buffer to prepare them for binding to the antibody. For this binding step and the chromatin binding step, the buffer used was either RIPA buffer for the H3K4me3 and K3K27me3 ChIPs, or ChIP buffer (VENDOR) for the H3K4me1 ChIP. The ChIP buffer was gentler and less stringent than RIPA buffer, which was better for the weaker binding of the H3K4me1 antibody that was used. The buffers were supplemented with 50 mg/mL BSA (VENDOR) and 0.5 mg/mL Herring Sperm DNA, both of which are blocking agents that reduce background and non-specific binding. The ChIP assays also varied in the amount of input chromatin and corresponding size of the reaction that was necessary to yield sufficient DNA for sequencing. H3K4me3 ChIP needed only cells, and H3K4me1 and K3K27me3 ChIP used cells. To perform the ChIP, L of Dynabeads per cells is incubated with of histone antibody for min in cells RIPA (or ChIP) buffer supplemented with 50 mg/mL BSA, 0.5 mg/mL Herring Sperm DNA, 1xPIC, and 1mM PMSF. The antibodies used were (XXX). Once the antibody was bound to the Dynabeads, the beads were washed twice with cells RIPA buffer with BSA and Herring Sperm DNA.

Next, the MNase-digested chromatin were added, which was at a concentration of cells/. The ChIP reaction was incubated overnight with rotation at C, to allow the histone protein to bind to the antibody, which was bound to the magnetic beads. In order to calculate enrichment for each ChIP sample, a known amount ( or ) of MNase-digested input chromatin was saved.  
The next morning, the ChIPs underwent a series of washes to remove unbound chromatin. The H3K4me3 and H3K27me3 ChIPs were washed 3x with cells RIPA buffer, and the H3K4me1 ChIP was washed with a low salt wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8, 150 mM NaCl), a high salt wash (0.1% SDS, 2% Triton X-100, 2mM EDTA, 20mM, Tris-HCl, pH 8, 500mM NaCl), and a LiCl wash (0.25 MLiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl pH 8). After three washes, the ChIPs were washed twice with TE buffer and transferred to a new tube during the last TE wash to reduce background. At this point, the histone of interest and the histone-bound DNA fragment had been purified from the MNase-digested, cross-linked chromatin, and was bound by histone-specific antibody to the magnetic Dynabeads. In the next step, a high-salt elution buffer is used to degrade the antibody binding interactions to the beads and the histone, and concurrently, proteinase K is added to digest the protein away from the DNA-protein complexes. The ChIP was incubated with the elution buffer and proteinase K at C for hours to liberate the DNA. At the same time, the saved input chromatin was also digested in the same buffer. Afterwards, the beads were removed using the magnet, and the DNA was purified using the Qiagen PCR purification kit. Quantification was performed using the Qubit quantification system, which is accurate to and only requires a small amount of sample to measure concentration. The ChIP sample was enriched for only DNA that was bound to the histone of interest. The goal for each ChIP was to yield 10 ng of ChIP DNA for sequencing. Not all samples met this criterion, and the H3K4me1 ChIPs often had a total yield of of DNA.

To test the efficiency of the ChIPs, quantitative PCR using QuantiFAST was performed. Two sets of primers were used, one set in a known region of histone binding (positive control), and one set in a region without histone binding (negative control). The qPCR was performed both on the ChIP DNA and the input DNA. Then the relative enrichment of positive vs negative assays was compared between the ChIP and input DNA.

The ChIP DNA was submitted to The Jackson Lab GES service for library preparation and sequencing. Libraries were made using the Kapa Hyper Prep kit with adapters at . The libraries were amplified by 10 cycles of PCR. These libraries were not size selected, although most fragments were bp due to MNase-digestion. The samples were sequenced with 40 or more million reads per sample, which is almost 2x more reads than the ENCODE project, which sequenced using 20 million reads.

## Diversity Outbred mice

We used previously published data from a population of diversity outbred (DO) mice [Svenson et al. 2012] to compare to the data collected from the inbred mice. The DO population included males and females from DO generations four through 11. Mice were randomly assigned to either a chow diet (6% fat by weight, LabDiet 5K52, LabDiet, Scott Distributing, Hudson, NH), or a high-fat, high-sucrose (HF/HS) diet (45% fat, 40% carbohydrates, and 15% protein) (Envigo Teklad TD.08811, Envigo, Madison, WI). Mice were maintained on this diet for 26 weeks.

### Genotyping

All DO mice were genotyped as described in Svenson et al. (2012) using the Mouse Universal Genotyping Array (MUGA) (7854 markers), and the MegaMUGA (77,642 markers) (GeneSeek, Lincoln, NE). All animal procedures were approved by the Animal Care and Use Committee at The Jackson Laboratory (Animal Use Summary # 06006).

Founder haplotypes were inferred from SNPs using a Hidden Markov Model as described in Gatti2014. The MUGA and MegaMUGA arrays were merged to create a final set of evenly spaced 64,000 interpolated markers.

### Tissue collection and gene expression

At sacrifice, whole livers were collected and gene expression was measured using RNA-Seq as described in (Chick, Munger et al.~2016, and Tyler et al.~2017). Transcript sequences were aligned to strain-specific genomes, and we used an expectation maximization algorithm (EMASE) to estimate read counts ().

## Data Processing

### Sequencing

The raw sequencing data from both RNA-Seq and ChIP-Seq was put through the quality control program FastQC. FastQC identifies problems or biases in either the sequencer run or the starting library material. The FastQC readout includes total number of reads, sequence quality, duplication level, and overrepresented sequences. All of our samples had comparable quality levels and no outstanding flags. However, the ChIP-Seq data was flagged for having a high level of duplicate reads. This can be explained by the use of MNase to shear the DNA into 150 bp fragments. If the binding positions of nucleosomes are fixed, then the MNase enzyme will cleave the DNA in the same place in multiple cells, resulting in duplicate pieces of DNA. Despite evidence that the duplication rate has a biological explanation, duplicates were removed before downstream analysis, as is typical in sequencing workflows, to avoid potential biases caused by starting libraries that have less diversity.

For the sequence analysis, reads from each sample were mapped to strain-specific pseudogenomes that integrate known SNPs from each strain. While the B6 samples were aligned directly to the reference mouse genome, the other samples were from genetically different strains. Strain-specific sequence variation in transcripts can affect alignment quality and result in biased estimates of abundance. To counteract potential strain biases, sequencing data from each strain were aligned to a custom strain pseudogenome, allowing a more precise characterization of gene expression and histone binding. The pseudogenomes were created using the EMASE computational program [REF] designed to construct customized genomes based on known SNP and indel attributes. The resulting custom genomes are called pseudogenomes, because they are based on inserting small known variations into the reference genome, but do not attempt whole genome sequencing for each strain and complete rebuild the entire genomic sequence from the scaffold up. The strain-specific pseudogenomes were then used in the Bowtie mapping algorithm to align and map reads from the RNA-Seq and ChIP-Seq experiments.

## Quantifying gene expression

Once the sequencing data was mapped to the custom genomes, edgeR is used to quantify transcripts. The edgeR program uses a Trimmed Mean of M-values (TMM), which adjusts each sample for library size and RNA composition using the assumption that most genes are not differentially expressed. The output is sample read count for each of the ENSMUSG transcript ID’s. Next, transcripts with less than 1 CPM in two or more replicates were filtered to remove lowly expressed genes. Also, the data were trimmed to include only protein-coding transcripts.

### ChIP-Seq quantification:

After the ChIP-Seq sequencing data were mapped to the custom pseudogenomes, peaks were called in each sample using MACS 1.4.2 [18798982], with a significance threshold of . In order to compare peaks across strains, the MACS output peak coordinates were converted to common B6 coordinates using g2g tools (<https://churchill-lab.github.io/g2gtools/>).

## Quantifying DNA methylation

RRBS data were processed using a bismark-based pipeline modified from Thompson et al. 2018 [30348905]. The pipeline uses Trim Galore! 0.6.3 (<https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/>) for QC, followed by the trimRRBSdiversityAdaptCustomers.py script from NuGen for trimming the diversity adapters. This script is available at: <https://github.com/nugentechnologies/NuMetRRBS>

All of our samples had comparable quality levels and no outstanding flags. Total number of reads was 45-90 million, with an average read length of about 50 bp. Quality scores were mostly above 30 (including error bars), with the average above 38. Duplication level was reduced to for about 95% of the sequences.

High quality reads were aligned to a custom strain pseudogenome, using bowtie2 as implemented in Bismark 0.22 [21493656]. The pseudogenomes were created by incorporating strain-specific SNPs and indels into the reference genome using g2gtools (<https://github.com/churchill-lab/g2gtools>), allowing a more precise characterization of methylation patterns. Bismark methylation extractor tool was then used for creating a bed file of estimated methylation proportions for each animal, which was then translated to the reference mouse genome (GRCm38) coordinates using g2gtools. Unlike other liftover tools, g2gtools does not throw away alignments that land on indel regions. B6 samples were aligned directly to the reference mouse genome.

## Analysis

## Filtering transcripts

For all gene expression data, we remove transcripts with extremely low read counts, by filtering out those whose mean read count across all individuals was less than five.

We used the R package sva [REF] to perform a variance stabilizing transformation (vst) on the RNA-Seq read counts from both inbred and outbred mice. In the inbred mice we used a blind transformation, while in the outbred mice, we included DO wave and sex in the model. For eQTL mapping, we performed rank Z normalization on the RNA-Seq read counts across transcripts from the outbred mice.

## Analysis of histone modifications

### Identification of chromatin states

We used ChromHMM [29120462] to identify chromatin states, which are unique combinations of the four chromatin modifications, for example, high levels of both H3K4me3 and H3K4me1, and low levels of the other two modifications. We conducted all subsequent analyses at the level of the chromatin state.

To ensure we were analyzing the most biologically meaningful chromatin states, we calculated chromatin states for all numbers of states between four and 16, which is the maximum number of states possible with four binary chromatin modifications (). We aligned states across the models by assigning each to one of the sixteen possible binary states using an emissions probability of 0.3 as the threshold for presence/absence of the histone mark. We then investigated the stability of three features across all states: the emissions probabilities (Supp Fig1), the abundance of each state across transcribed genes (Supp Fig2), and the effect of each state on transcription (Supp Fig3). Methods for each of these analyses are described separately below. All measures were remarkably consistent across all models, but the 14-state model was characterized by a wide range of relatively abundant states with relatively strong effects on expression. We used this model for all subsequent analyses.

### Genome distribution of chromatin states

We investigated genomic distributions of chromatin states in two ways. First, we used the ChromHMM function OverlapEnrichment to calculate enrichment of each state around known functional elements in the mouse genome. We analyzed the following features:

* **Transcription start sites (TSS)** - Annotations of TSS in the mouse genome were provided by RefSeq [26553804] and included with the release of ChromHMM, which we downloaded on December 9, 2019 [29120462].
* **Transcription end sites (TES)** - Annotations of TES in the mouse genome were provided by RefSeq and included with the release of ChromHMM.
* **Transcription factor binding sites (TFBS)** - We downloaded TFBS coordinates from OregAnno [26578589] using the UCSC genome browser [12045153] on May 4, 2021.
* **Promoters** - We downloaded promoter coordinates provided by the eukaryotic promoter database [27899657,25378343], through the UCSC genome browser on April 26, 2021.
* **Enhancers** - We downloaded annotated enhancers provided by ChromHMM through the UCSC genome browser on April 26, 2021.
* **Candidates of cis regulatory elements in the mouse genome (cCREs)** - We downloaded cCRE annotations provided by ENCODE [22955616] through the UCSC genome browser on April 26, 2021.
* **CpG Islands** - Annotations of CpG islands in the mouse genome were included with the release of ChromHMM.

In addition to these enrichments around individual elements, we also calculated chromatin state abundance relative to the main anatomical features of a gene. For each transcribed gene, we normalized the base pair positions to the length of the gene such that the transcription start site (TSS) was fixed at 0, and the transcription end site (TES) was fixed at 1. We also included 1000 bp upstream of the TSS and 1000 bp downstream of the TES, which were converted to values below 0 and above 1 respectively.

To map chromatin states to the normalized positions, we binned the normalized positions into 41 bins defined by the sequence from -2 to 2 incremented by 0.1. If a bin encompassed multiple positions in the gene, we assigned the mean value of the feature of interest to the bin. To avoid potential contamination from regulatory regions of nearby genes, we only included genes that were at least 2kb from their nearest neighbor, for a final set of 14048 genes.

### Chromatin state and gene expression

We calculated the effect of each chromatin state on gene expression. We did this both across genes and across strains. The across-gene analysis identified states that are associated with high expression and low expression within the hepatocytes independent of strain. The across-strain analysis investigated whether variation in chromatin state across strains contributed to variation in gene expression across strains.

For each transcribed gene, we calculated the proportion of the gene body that was assigned to each chromatin state. We then fit a linear model separately for each state to calculate the effect of state proportion with gene expression:

where is the rank Z normalized gene expression of the full transcriptome in a single inbred strain, and is the rank Z normalized proportion of each gene that was assigned to state . We fit this model for each strain and each state to yield one coefficient with 95% confidence interval. The effects were not different across strains, so we averaged the effects and confidence intervals across strains to yield one summary effect for each state.

To calculate the effect of each chromatin state across strains, we first standardized transcript abundance across strains for each transcript. We also standardized the proportion of each chromatin state for each gene across strains. We then fit the same linear model, where was a rank Z normalized vector concatenating all standardized expression levels across all strains, and was a rank Z normalized vector concatenating all standardized state proportions across all strains. We fit the model for each state independently yielding a coefficient and 95% confidence interval for each state.

In addition to calculating the effect of state proportion across the full gene body, we also performed the same calculations in a position-based manner. This second analysis yielded an effect of each state at multiple points along the gene body and a more nuanced view of the effect of each state.

### Selecting the most biologically meaningful model

We performed the above analyses on all states from the four-state model to the 16-state model to find the most meaningful clustering of histone modifications. Across all models, the states were remarkably stable. As we increased the number of states detected by the model, new states appeared, but previously detected states were not disrupted. This stability was apparent in all state measures: emissions probability (Supp Fig 1) patterns, overall abundance (Supp. Fig. 2), and effect on expresssion (Supp Fig 3). This analysis revealed interesting patterns in the detected states. For example, one highly abundant state (present in 65% of transcribed genes) detected first in the four-state model was split into two distinct states in the 10-state model. These resulting states were also highly abundant (appearing in 40% and 41% of transcribed genes), and had distinct emissions probabilities (Supp. Fig. 1). These two states remained stable with increasing numbers of clusters through to the 16-state model. States arising after the 10-state model were of lower abundance, appearing in 2% or less of transcribed genes.

All of the higher abundance states were established in the 10-state model. However, as we moved toward higher numbers of clusters, the resolution on the lower-abundance states improved in terms of the emissions probabilities profiles, and strength of the correlation with gene expression. For example, the 14-state model better resolved a state that had appeared in the 10-state model but was not strongly correlated with gene expression. In the 14-state model, the emission patterns were closer to binary, and the strength of the correlation with expression was increased. Beyond 14 clusters, the new states identified were extremely rare (1% of transcripts or less), and were not strongly correlated with gene expression. We thus selected the 14-state model and the model with the most biologically meaningful clusters.

## Analysis of DNA methylation

### Creation of DNA methylome

We combined the DNA methylation data into a single methylome cataloging the methylated sites across all strains. For each site, we averaged the percent methylation across the three replicates in each strain. The final methylome contained 5,311,670 unique sites across the genome. Because methylated CpG sites can be fully methylated, unmethylated, or hemi-methylated, we rounded the average percent methylation at each site to the nearest 0, 50, or 100.

### Distribution of CpG sites

We used the enrichment function in ChromHMM described above to identify enrichment of CpG sites around functional elements in the mouse genome. We further performed a gene-based analysis of abundance similar to that in the chromatin states. As a function of relative position on the gene body, we calculated the density of CpG sites as the average distance to the next downstream CpG site, as well as the percent methylation at each site.

### Effects of DNA methylation on gene expression

As with chromatin state, we assessed the effect of DNA methylation on gene expression both across genes and across strains. We used the same linear model described above, except that became the rank Z normalized percent methylation either across genes or across strains. Because the effect of DNA methylation on gene expression is well-known to be dependent on position, we only calculated a position-dependent effect on expression.

## Imputation of genomic features in Diversity Outbred mice

To assess the extent to which chromatin state and DNA methylation are responsible for local expression QTLs, we imputed local chromatin state and DNA methylation into a population of diversity outbred (DO) mice described above and in Svenson et al. 2012. We compared the effect of the imputed epigenetic features to imputed SNPs.

All imputations followed the same basic procedure: For each transcript, we identified the haplotype probabilities in the DO mice at the genetic marker nearest the gene transcription start site. This matrix held DO individuals in rows and DO founder haplotypes in columns (Supp. Fig. ).

For each transcript, we also generated a three-dimensional array representing the genomic features derived from the DO founders. This array held DO founders in rows, feature state in columns, and genomic position in the third dimension. The feature state for chromatin consisted of states one through 14, for SNPs feature state consisted of the genotypes A,C,G, and T.

We then multiplied the haplotype probabilities by each genomic feature array to obtain the imputed genomic feature for each DO mouse. This final array held DO individuals in rows, the genomic feature in the second dimension, and genomic position in the third dimension. This array is analagous to the genoprobs object in R/qtl2 [30591514]. The genomic position dimension included all positions from 1 kb upstream of the TSS to 1 kb downstream of the TES. SNP data for the DO founders in mm10 coordinates were downloaded from the Sanger SNP database [1921910, 21921916], on July 6, 2021.

To calculate the effect of each imputed genomic feature on gene expression in the DO population, we fit a linear model. From this linear model, we calculated the variance explained () by each genomic feature, thereby relating gene expression in the DO to each position of the imputed feature in and around the gene body.

# Results

Gene expression varies widely and reproducibly across inbred strains of mice. This is seen as a clustering of individuals from the same strain in a principal component plot of the hepatocyte transcriptome across strains (Figure A). Patterns of DNA methylation (Figure B) and individual histone modifications (Figure C-F) cluster in a similar pattern. This suggests that these epigenetic features may relate to gene expression in a manner that is consistent with genetic background.

## Chromatin state overview

To investigate this association, we used ChromHMM to identify 14 chromatin states composed of unique combinations of four histone modifications in the hepatocytes of nine inbred strains of mice. Panel A in Figure shows the representation of each histone modification across the states.

The states were distributed non-randomly around known functional elements in the mouse genome (Figure B). The majority of the states were enriched around the TSS, and other TSS-related functional elements, such as promoters and CpG islands. Two states (states 2 and 1) were primarily found in intergenic regions. Three states (states 9, 13, and 11) were enriched around known enhancers, and one (state 6) was enriched predominantly near the TES. The majority of these states were also associated with variation in gene expression. The colored bars in Figure C) show the effect of each state on gene expression across the inbred strains. For reference, the paired tan bars show the effect of each chromatin state on gene expression in hepatocytes. These effects tend to be of the same sign and greater magnitude than the across-strain effects.

The states in Figure are shown in order of their effect on expression, which helps illustrate several patterns in the data. The state with the largest negative effect on gene expression, state 1, is the absence of all measured modifications. The next few states all contain the repressive mark H3K27me3, and are all associated with reduced gene expression. The states with the largest positive effects on expression all have some combination of the activating marks, H3K4me3, H3K4me1, and H3K27ac. The repressive mark is less commonly seen in these activating states.

By merging the information from Figure A-C), we were able to suggest annotations for many of the 14 chromatin states (Figure D). States with the strongest effects on expression had the clearest annotations, while states with weaker effects remained unannotated.

## Spatial distribution of epigenetic modifications around gene bodies

In addition to looking for enrichment of chromatin states near annotated functional elements, we characterized the fine-grained spatial distribution of each state around gene bodies (Figure A-B). We similarly characterized the distribution of CpG sites and their percent methylation at this gene-level scale (Figure C-D).

The spatial patterns of the individual chromatin states are shown in (Figure A), and an overlay of all states together (Figure B) emphasizes the difference in abundance between the most abundant states (states 14, 12, and 1), and the remaining states, which were relatively rare.

Each chromatin state had a characteristic distribution pattern relative to gene bodies. For example, state 1, which was characterized by the absence of all measured histone modifications, was strongly depleted near the TSS, indicating that this region is commonly subject to histone modification. However, its abundance increased steadily through the gene to a peak at the TES. In contrast, states 12 and 14 were both concentrated at the TSS. State 12 was very narrowly concentrated right at the TSS, whereas state 14 was more broadly abundant both upstream and downstream of the TSS. Both were associated overall with increased expression in the inbred mice (indicated by red shading), suggesting promoter or enhancer functions. The third state in this group of high-expressing states, state 13, was depleted nere the TSS, but enriched within the gene body, suggesting that this state may mark active intragenic enhancers.

States with weaker effects on expression (indicated by grayer shades) were of lower abundance. However, they still had distinct distribution patterns around the gene body suggesting the possibility of distinct functional roles in the regulation of gene expression.

There were similarly dramatic spatial patterns in DNA methylation (Figure C-D). Across all genes, the TSS had densely packed CpG sites relative to the gene body (Figure C). As expected, the median CpG site near the TSS was consistently hypomethylated relative to the median CpG site in intergenic regions (Figure D). CpG sites within the gene body were slightly hypermethylated compared to intergenic CpGs.

## Spatially resolved effects on gene expression

The distinct spatial distributions of the chromatin states and methylated CpG sites around the gene body raised the question as to whether the effects of these states on gene expression could also be spatially resolved. To investigate this possibility we tested the association between both chromatin state and DNA methylation and gene expression with spatially resolved models (Methods). We tested the effect of each chromatin state on expression across genes within hepatocytes (Figure A) and the effect of each chromatin state on the variation in gene expression across strains (Figure B).

All chromatin states demonstrated spatially dependent effects on gene expression within hepatocytes. For many of the states, the effects on expression were concentrated at or near the TSS, while in the other states effects were seen across the whole gene. The direction of the effects matched the overall effects of each state seen previously (Figure ). Remarkably, the spatial effects were recapitulated for almost every state when we measured across strains. That is, variation in chromatin state across strains contributed to variation in gene expression in the same manner that cell-type expression was being established. One notable exception was state 9, whose presence upregulated genes within hepatocytes, but did not contribute to expression variation across strains.

We also examined the effect of percent DNA methylation across genes within hepatocytes, and across strains (Figure ). As expected, hypomethylation at the TSS was associated with lower expression in hepatocytes. However, percent DNA methylation did not contribute at all to expression variation across strains, implying that although percent DNA methylation is used in gene regulation within a cell type, it is not heritable and does not contribute to variation in gene expression across genetically diverse individuals.

## Imputed chromatin state explained expression variation in diversity outbred mice

Thus far, we have used inbred strains of mice to identify correlations between local chromatin state and gene expression. However, we cannot establish causality in this population. For that we need a mapping population in which we can associate genetic or epigenetic variation at a single locus with changes in gene expression. A mapping population also allows us to establish the extent to which variation in epigenetic factors contributes to observed expression quantitative trait loci (eQTL).

To compare the contribution of genetic and epigenetic features to eQTLs in a gentically diverse population, we imputed chromatin state, DNA methylation, and SNPs into a population of DO mice described previously [Svenson, Tyler] (Methods). Chromatin state is largely determined by local genotype, especially early in life [REF], and can thus be reliably imputed from local genotype. Further, we have shown here that local chromatin state correlates with variation in gene expression across inbred strains. DNA methylation, on the other hand, is known not to be highly heritable [REF], and thus cannot be reliably imputed from local genotype. We have also shown here that DNA methylation is not correlated with variation in gene expression across inbred strains. The imputation of DNA methylation thus serves as an estimate of a lower bound the ability of a feature imputed from local haplotype to explain gene expression in a new population.

For each transcript in the DO population, we imputed the local chromatin state across the gene body based on the gene’s local founder haplotype and the chromatin state at the corresponding position in the inbred mice. We did the same for DNA methylation and SNPs.

After imputing each genomic feature into the DO population, we mapped gene expression to the imputed features and calculated the variance explained. Examples of each genomic feature and the mapping results for the gene are shown in Figure . There are two particularly interesting regions in this gene. One is at the TSS and the immediately surrounding area, and the other is just downstream of the TSS.

These two regions are colored red, indicating that they are marked by chromatin states with a positive effect on gene expression. The order of the rows in this panel helps illustrate that the strains with the most red in chromatin state space contributed the highest-expressing alleles to the DO (Figure E). The two haplotypes with the strongest negative effect on gene expression in the DO have mostly blue chromatin states in these two regions. These two strains also had the lowest expression among the inbred mice (Figure F). The concordance between chromatin state and gene expression in the DO is seen as the blue pluses in Figure A that are aligned with the two red regions, which we suggest are putative enhancer regions.

The spatial patterns in the SNPs only partially mirror those in chromatin state (Figure Figure C). SNPs underlying the putative enhancer regions could potentially influence gene expression by altering chromatin state. But SNPs downstream of this region underly invariant chromatin.

Percent DNA methylation does not vary across the strains in either of these putative enhancer regions, and does not contribute to variation in expression across genetically distinct individuals (Figure D).

The overall distributions of variance explained by each feature across all transcripts is shown in Figure . These distributions show the haplotype effect for the marker nearest each transcript compared with the maximum effect across the gene body for each of the other imputed features. Overall, local haplotype explained the largest amount of variance of gene expression in the DO (). The variance explained by local chromatin state was very highly correlated with that of haplotype (Pearson ) and explained almost as much variance in gene expression in the DO as local haplotype ().

The mean variance explained by SNPs was lower () than that explained by haplotype and was not as highly correlated with local haplotype as chromatin state was (Pearson ). DNA methylation, the lower bound for variance explained by a feature imputed from local haplotype, explained the lowest amount of expression variance in the DO population (), and had a much lower correlation to haplotype than either chromatin state or SNPs (Pearson ).

# Discussion

In this sudy we showed that variation in histone modifications in inbred mice mirrors genetic variation, and we further showed that this variation was highly related to variation in gene expression across strains. These observations suggest that cell type-specific patterns of histone modifications are determined by local genotype, and may be a major mechanism through which expression QTL (eQTL) are generated. This hypothesis was supported by the high concordance between chromatin state, which was imputed from local genotype, and gene expression in an independent outbred population of mice.

The high resolution of the chromatin states combined with spatial patterns of abundance and effect on gene expression offers opportunities for the annotation of functional elements in and around genes. For example, the chromatin state patterns in the gene , suggest two enhancers – one at the TSS, and the other just downstream of the TSS inside the gene body. The positive effects of these putative enhancer regions in the inbred mice were replicated in outbred mice suggesting that these effects are robust and contribute to variation in gene expression seen in diverse populations.

The putative enhancers are not apparent in the SNP patterns or in the patterns or DNA methylation, which suggests that chromatin modification is the primary mechanism through which gene expression is regulated by these regions. Further, the richness of the information in this chromatin state layer provides data with which to further annotate the effects of SNPs underlying these regions. There are SNPs throughout the gene, as seen in Figure , and many of them are associated with variation in gene expression. However, while the SNPs within the putative enhancer regions may change expression by altering histone modifications placed in those regions, SNPs futher downstream may work through another mechanism, such as through directly dirsupting transcription, or by altering the transcript such that it is processed differently post transcriptionally. The intermediate resolution of the chromatin state between that of SNPs and haplotype thus provides a highly informative layer of information between genotype and gene expression.

In contrast to chromatin state, percent DNA methylation was not associated with variation in gene expression across inbred strains or in the outbred population. This was largely due to a lack of variation in methylation across strains. An example of this observation is shown in panel D of Figure . Despite strain variation in both genotype and chromatin state at the TSS of , DNA methylation is invariant – the CpG island at the TSS is unmethylated in all strains. Thus, although chromatin state appears to be highly influenced by local genotype, percent DNA methylation is not.

Similar observations have been made in human studies [33931130]. Multiple twin studies have estimated the average heritability of individual CpG sites to be roughly 0.19 [27051996, 24183450, 22532803], with only about 10% of CpG sites having a heritability greater than 0.5 [24183450, 22532803, 24887635]. Trimodal CpG sites, i.e. those with methylation percent varying among 0, 50, and 100%, have been shown in human brain tissue to be more heritable than unimodal, or bimodal sites (), and roughly half were associated with local eQTL [20485568]. Here, we did not see an association between trimodal CpG sites and gene expression across strains (Supplemental Figure XXX).

The diversity in the effects observed in the 14 chromatin states highlights the importance of analyzing combinatorial states as opposed to individual histone modifications. To illustrate this point, consider the three states with the largest positive effects on transcription. Each of these three states had a distinct combination of the three histone marks associated with transcriptional activation: H3K4me1, H3K4me3, and H3K27ac. State 12 was characterized by high levels of H3K4me3 and H3K27ac, and low levels of H3K4me1. State 13 was characterized by high levels of H3K4me1 and H3K27ac, and low levels of H3K4me3. And state 14 was characterized by high levels of all three activating marks (Figure XXX). Although all three states were associated with increased gene expression, each had a completely distinct spatial distribution. State 12 was distributed in a very narrow band centered on the TSS, while state 14 was distributed across a much broader region centered upstream of the TSS. State 13 had a completely different distribution – it was depleted at the TSS, and most abundant within the gene body and near the TES. This variation in spatial distribution was mirrored in the spatial effects on transcription. State 12, which we annotated as an active promoter, was positively associated with transcription when it was present at the TSS. In contrast, states 13 and 14, which we annotated as enhancers, were associated with increased transcription when present anywhere in the gene body (Figure XXX). We would not be able to detect such patterns if analyzing the histone modifications in isolation. These results highlight the complexity of the histone code and the importance at analyzing combinatorial states.

While we were able to annotate several states, particularly those with the strongest effects on gene expression, other states were more difficult to annotate. This raises the intruiguing possibility of identifying new modes of expression regulation through histone modification. One of these unannotated states, state 6, had a weak, but consistent negative effect on gene transcription centered within the gene body, downstream of the TSS. This state was characterized by high levels of H3K4me3 and low levels of the other three modifications.

The modification H3K4me3 is most frequently associated with increased transcriptional activity [citation], so the association with state 6 with reduced transcription is a deviation from the dominant paradigm. The physical distribution of this state is also interesting. It was depleted at the TSS, and enriched just upstream and just downstream of the TSS (Figuree XXX). It was also enriched just downstream of the TES, although it did not appear to influence transcription at this location (Figure XXX). The group of genes marked by state 6 were enriched for functions such as stress response, DNA damage repair, and ncRNA processing suggesting that this state may be used to regulate subsets of genes involved in responses to environmental stimuli.

There were other states that we were able to annotate, but were not necessarily expecting to see in this study. We detected two bivalent states, which are states that combine an activating histone modification and a repressing histone modificaction and are usually associated with undifferentiated cells [citation]. Here we identified two bivalent states in adult mouse hepatocytes, and annotated them as a poised enhancer (state 3) and a bivalent promoter (state 4). Both states were associated with downregulation across inbred strains when present near the TSS; however this effect was not replicated in the outbred mice. The lack of replication was perhaps because the effect was too weak to detect given the number of animals in the population.

Both bivalent promoters and poised enhancers are dynamic states that change over the course of differentiation and in response to external stimuli [citation]. Bivalent promoters have been studied primarily in the context of development. They are abundant in undifferentiated cells, and are typically resolved either to active promoters or to silenced promoters as the cells differentiate into their final state [23788621, 22513113]. These promoters have also been shown to be important in the response to changes in the environment. Their abundance increases in breast cancer cells in response to hypoxia [27800026]. Poised enhancers are also observed during differentiation and in differentiated cells [32432110]. In concordance with these previous observations, the genes marked by states 3 and 4 were enriched for vascular development and morphogenesis. That we identified these states in differentiated hepatocytes may indicate that a subset of developmental genes retain the ability to be activated under certain circumstances, such as during liver regeneration in response to damage. It is also possible these states were induced in the inbred strains in respose to stress, rather than genetically coded. This could explain why the negative effect on gene expression was not replicated in the outbred mice. However, given that we detected this state in all nine inbred strains in relatively equal proportions, this latter hypothesis seems less likely.

Broadly, local variation in chromatin state was highly correlated with variation in gene expression across individuals, an observation that was replicated in an independent population of genetically diverse, outbred mice. The percent variance explained by chromatin state closely matched that of haplotype, and exceeded that of individual SNPs. These results suggest two things. First, a large portion of the effect of local haplotype on gene expression in mice is likely mediated through variation in chromatin state. Second, the intermediate resolution of chromatin state between that of individual SNPs and broad haplotypes carries important imfornation that cannot be resolved at the other levels. Individual SNPs, although, sometimes causally linked to trait variation, are highly redundant and cannot be readily used to annotate functional elements in the genome. Haplotypes aggregate genomic information over broad regions and are a powerful tool to link genomic variation to trait variation. However, they are usually too broad to be used to annotate regions less than a few megabases in length. By combining the mapping power of haplotypes, the high resolution of SNPs, and the intermediate resolution of chromatin states, we can begin to build mechanistic hypotheses that link genetic variation to variation in physiology. Understanding the role that genetic variation plays in modifying the chromatin state landscape will be critical in making these links. Through this survey we are providing one of the first rigorous resources that explores the connection between genetic variation and epigenetic variation.

work this paragraph in… That states 1 and 2 were associated with reduced gene expression both within hepatocytes and across strains suggests that there may be differential epigenetic silencing of genes in hepatocytes across strains. Further, the majority of chromatin states were associated with variation in expression across strains, suggesting that epigenetic regulation of gene expression through histone modification may contribute substantially to variation in gene expression across genetically distinct individuals. That most states have the same effects across genes within a cell type and across strains suggests that the mechanisms that are used to regulate cell type specificity also contribure to variation in genetically distinct individuals.

# Acknowledgements

This work was funded by XXX.

# Data and Software Availability

All data used in this study and the code used to analyze it are avalable as part of a reproducible workflow located at… (Figshare?, Synapse?).

# Figure Legends

# Supplemental Figure Legends

# References