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Genomic comparison of DBA/2J and C57Bl/6J strains of *Mus musculus* and best practice of genome alignment for bioinformatics analyses

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioinformatics at Virginia Commonwealth University

by

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> Virginia Commonwealth University Richmond, Virginia July 21, 2023

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# Contributions

I am honored to have received aid from several colleagues during the course of my thesis. Any contributions not listed in the text are found below.

### Data Preparation

Dr. Mikhail Dozmorov assisted in the generation of count data from the D2 mouse annotation file provided by Dr. Keane. This proved to be a very tricky task, but we managed to accomplish it together.

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# **Tools and Programs**

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STAR Aligner	https://github.com/alexdobin/STAR
MultiQC	https://multiqc.info/
Deseq2	https://bioconductor.org/packages/release/bioc/html/Deseq2.html
ToppFun	https://toppgene.cchmc.org/enrichment.jsp
Revigo	http://revigo.irb.hr/
DEXSeq	https://bioconductor.org/packages/release/bioc/html/DEXSeq.html
RegTools	https://regtools.readthedocs.io/en/latest/
rMats	https://RNA-Seq-mats.sourceforge.net/
List Comparison	https://rnact.crg.eu/compare
List Comparison	https://comparetwolists.com/
BioVenn	https://www.biovenn.nl/
	https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12/topi
Pheatmap	<u>cs/pheatmap</u>
Ggplot2	https://ggplot2.tidyverse.org/

### Abstract

Alcohol use disorder is known to have significant genetic components that contribute to an individual's susceptibility to the disease. Mouse models are commonly used to study the mechanisms underlying alcohol use disorder, with C57BL/6J (B6) and DBA/2J (D2) being two of the more prominently used inbred strains. Research in the Miles Laboratory has used these two strains, and genetic panels of mice derived from them, to identify potential genes associated with variance in ethanol-related behaviors using quantitative trait loci (QTL) analysis. For example, Ninein (Nin) was identified as a potential candidate gene for the anxiolytic effects of ethanol, discovered because it resides in the confidence interval for a QTL and shows mRNA expression differences between B6 and D2 mice. This differential expression was identified using counts of RNA-Seq reads that have been aligned to a reference genome, specifically the B6 reference genome. Due to the known genetic differences between the two strains, it is possible that the D2 samples could benefit from being aligned to a D2 genome instead of the B6. This would lead to better results overall due to improved read alignment and identification of novel splicing events that might be seen in D2 mice. To test this hypothesis, a dataset consisting of deep (150 million reads) sequencing of RNA from nucleus accumbens of both B6 and D2 mice was used for multiple bioinformatics analyses (differential expression, gene ontology, semantic similarity, differential exon utilization, splice site location, and alternative splicing) with both B6 aligned D2 counts and D2 aligned D2 counts. End results of each analysis were then compared for significant differences in outcomes. The results of this analysis show that when aligning D2 samples to the D2 genome a majority of differentially expressed genes and differentially utilized exons are retained from the B6 aligned analysis while many new genes and exons are identified that are unique to the D2 aligned analysis.

## **Chapter 1: Introduction and Background**

## Introduction

#### Alcohol Use Disorder

Alcohol Use Disorder (AUD) describes the spectrum of problematic alcohol consumption that affects over 29 million people in the United States (SAMHSA, 2021). AUD includes increased alcohol consumption over time and binge alcohol consumption, though it encompasses any kind of problematic alcohol use. All forms of AUD relate to the inability to regulate or stop alcohol use despite external pressures such as negative social, health, or occupational consequences. AUD also leads to multiple alcohol-related end-organ diseases, affecting virtually every organ system, including such prevalent problems as fetal alcohol syndrome and alcoholic liver disease. It is estimated that 140,000 people die from AUD every year (SAMHSA, 2021), and alcohol use costs the United States \$249 billion annually, with \$28 billion of that coming only from healthcare costs (Sacks et al., 2015). Furthermore, less than 10% of people suffering from AUD in the past year received any form of treatment for it (Han et al., 2021), thus highlighting the need for improved understanding of the disorder so as to develop new therapeutic agents. The study of AUD has revealed it to have a genetic component, with twin studies being used to estimate that 50% of the risk of developing AUD is due to genetic factors (Kranzler et al., 2019). Single nucleotide polymorphism (SNP) based estimates are closer to 12%, and it is believed that AUD's genetic heritability is a result of many genes having small effects (Kranzler et al., 2019). Very few variants that cause changes to protein structure and

function have been identified, and variants that regulate gene expression have been put forward as a potential mechanism that affects these complex traits. Alcohol produces long lasting cellular changes in the brain, and it is these changes that can eventually lead to AUD (Egervari et al., 2019) However, studying gene expression in the human brain is difficult due to the complexity of the human brain, and the scarcity of human brain tissue. Because of these limitations, model organisms are used instead.

#### Mus musculus as a model organism and its use in AUD research

Model organisms have been extensively studied and have well-characterized genetic, physiological, and behavioral traits. One of the more commonly used model organisms for AUD research is the house mouse, *Mus musculus*. Mice make appealing model organisms due to their genetic, physiological, anatomical, and reproductive similarities to humans, as well as more practical reasons such as the relative ease of caring for them in a laboratory environment and the vast wealth of tools and resources available for working with mice (García-García, 2020).

Inbred mice are defined as being the product of at least 20 generations of brother X sister mating, with all individuals being derived from a single breeding pair. Inbred mice have several traits that make them ideal for research purposes. They are isogenic, and homozygous at each genetic locus. They have very unified phenotypes due to this stability. Due to this, inbred strains have very well documented traits, allowing for specific strains of mice to be selected for specific types of research (Blake et al., 2021). In AUD research, the C57BL/6J and DBA/2J inbred strains of mice are commonly used. This is due to several of the known traits that differ between the two strains being ideal for alcohol research, including their high variance in baseline ethanol consumption, with C57 consuming much more alcohol voluntarily than D2, and .

C57BL/6J, more commonly referred to as C57 or B6, are the most widely used inbred strain. They are often used as a background strain for behavioral genetic studies in alcohol research due to their facile self-administration of high amounts of alcohol. B6 mice were the DNA source for the first high quality draft sequence of the mouse genome and thus were the first strain to have their genome sequenced (Waterston et al., 2002). Due to this, their genome is one of the most well studied, and is widely used as the standard alignment sequence for genomic analyses. As Figure 1.1 shows, they are particularly useful for alcohol research as they voluntarily consume large quantities of alcohol (Lê et al., 1994).

DBA/2J, or D2, are the oldest of all inbred strains. They are used as a contrast to B6 mice in alcohol research, as they do not voluntarily consume large amounts of alcohol (Lê et al., 1994). Because they are so often used, the behavioral and genetic differences between the two strains are well documented, especially when it comes to alcohol research. He et al. (1997) performed an examination of these differing traits and their genetic components.



**Figure 1.1.** Alcohol intake (g/kg) by C57BL/6, BALB/c (another inbred strain of mice), and DBA/2 mice during the l-h daily access to alcohol solution. The concentration of alcohol solution was 3% w/v for the first 8 days, 607o for the next 12 days, and 12070 for the remaining 16 days. N = 17-18 mice per strain. Vertical lines indicate positive or negative halves of the SEs. Figure and description from Lê et al. (1994).

#### RNA-Sequencing

RNA-Sequencing is a technique used to measure gene expression in cells or tissues. The output of RNA-Sequences is a series of reads that represent the expression levels of individual genes. These reads are often short and fragmented, which makes it difficult to know where they

came from in the genome. In order to utilize these reads, they must be aligned to a reference genome. A reference genome must be a high quality, well-annotated representation of the genome. Through the alignment process, the locations that the reads originated in the genome can be identified. Once the reads are aligned, the total reads that overlap between the sample and the reference genome are counted, which quantifies the expression level of each gene in the sample (Martin & Wang, 2011).

#### Previous research and inspiration for this study

Miles laboratory studies have included extensive genome-level expression studies (Kerns et al., 2005a) (Agarwalla et al., 2020) and behavioral genetic analyses across the B6, D2 and recombinant (BXD) mice. Behavioral genetic analysis across the BXD recombinant inbred panel was used to identify genetic quantitative trait loci (QTL) modulating the anxiety-reducing actions of ethanol (Putman et al., 2016). Microarray gene expression across the BXD mice was further used to identify possible candidate genes for the QTL (Wolen & Miles, 2012). This analysis has recently shown that the gene Ninein (Nin), located within a highly significant behavioral quantitative trait locus (QTL) contributing to the anxiolytic-like properties of ethanol, was differentially expressed between B6 and D2 mice and that there was possible differential exon utilization for Nin expression between the two strains (Putman et al., 2016). Ninein is a gene that codes for a microtubule binding protein that is important in axonal development and is known to interact with Gsk3β. (Srivatsa et al., 2015). It was suggested to be a possible candidate gene for alcohol's anxiolytic effects (Putman et al., 2016).

#### Statement of significance

The B6 genome has been used as the reference genome for the majority of mouse studies, and virtually all RNA-Seq analysis, as it is the highest quality and best annotated genome available. However, there are known genetic differences between the B6 and D2 genomes. In addition to the research done in the Miles laboratory, initial sequencing efforts of the D2 genome have identified over five million single-nucleotide polymorphism and insertion/deletion differences between B6 and D2 mice (Doran et al., 2016). These genetic differences may lead to lower quality alignment when sequencing data from D2 are aligned to the B6 reference genome, compared to when data from B6 are aligned to the same reference. Which in turn may lead to biased results for downstream analyses. In particular, this difference may complicate studies on differential exon utilization.

#### Roadmap and Hypothesis for this study

The genetic variation and differential expression shown between the two strains provides a basis for the hypothesis that aligning D2 mice to their own genome will show a significant difference in outcomes when compared with aligning D2 mice to the B6 genome.

Using a recent deep-sequencing RNA-Seq dataset obtained in the Miles laboratory for B6 and D2 mice, I analyzedseveral kinds of bioinformatics studies between B6 and D2 reads aligned to the B6 reference genome versus results using D2 reads aligned to a recently derived D2 reference genome. The analyses to be performed are differential expression, gene ontology, differential exon utilization, and differences in splicing. If there are significant differences in results using D2 aligned D2 samples compared to the results using the B6 aligned D2 samples, then this will allow for better analyses by aligning to the D2 genome instead of the B6. If there are significant effects caused by aligning to the D2 genome, that also opens more avenues of research into other strains of mice.

#### Specific Aims

This project has two specific aims, both furthering the overall goal of comparing analyses run with D2 aligned D2 samples to those run with B6 aligned D2 samples. First, there will be a comparison of differential gene expression and gene ontology between the two strains of mice and an analysis of how aligning the D2 mice to their own genome changes those results. This will further the understanding of the effect alignment has on the results of gene expression analyses. The gene ontology will be used to compare the results of the two differential expression analyses at a functional level, and a semantic similarity analysis will continue that goal to further compare the semantic groupings of gene ontology categories.

Second, I will be comparing differential exon utilization and alternative splicing between the two strains, analyzing how aligning the D2 mice to their own genome changes those results. The comparison will be using DexSeq (Differential EXon and Transcript analysis for RNA-Seq) to compare exon utilization and alternative splicing, respectively. DexSeq is a computational method for detecting differential exon usage in RNA-Seq data, and is an extension of differential expression analysis, instead identifying differences in the usage of individual exons or groups of exons between samples.

This will show the abundance of alternative splicing events, and will focus on specific genes to showcase the differences in alternative splicing on a gene level caused by aligning D2 mice to the D2 genome. A gene ontology of the genes with differentially expressed exons will also be performed. This ontology will determine the most specific functions of each gene with differentially utilized exons, to further understand the differences caused by the change in alignment.

This research can lead to future studies to determine the impact of differential exon utilization on the proteome. Analyses can be conducted to determine how many different proteincoding sequence elements are derived when aligning to the D2 genome vs the B6 genome. Other future goals include a deeper look into the alternative splicing and changes in splicing events between the B6 and D2 aligned analyses, and a breakdown of the differentially utilized exons by size and other factors to determine if there is a pattern in the exons missed or picked up by the two analyses.

# **Chapter 2: Sample and Data Preparation**

# Introduction

#### Sample preparation

The samples for this study were prepared before this study began, following the procedures outlined in the methods section. The RNA-Seq data that came out of that work was the inspiration for this project, as the deep sequencing allowed for a robust analysis of the differences between the B6 and D2 strains.

#### Alignment and Count Generation using D2 genome

D2 alignments have been attempted before in the Miles Laboratory, but the relatively low quality of the prior existing D2 sequence data and genome annotations has made them less efficient than the consensus B6 annotations for RNA-Seq alignments. In some cases, the lower quality of the D2 annotations made certain analyses impossible to perform with samples and counts aligned to them. In this study a new high quality D2 genome sequence and annotation was provided by Dr. Thomas Keane from the Sanger Center. This sequence and annotation are of a high enough quality to allow for alignments at a similar level to those using the B6 genome. The annotation initially had Ensembl IDs corresponding to the D2 genome, whereas the B6 aligned counts had IDs corresponding the B6 genome. This issue was rectified using the annotation file, which contained gene names mapped to the D2 IDs. These gene names were mined from the file using a series of python scripts (Appendix 2 – ID Conversion Scripts), then converted to B6 Ensembl IDs, and mapped to the D2 IDs. The D2 IDs in the newly generated count files were

then replaced with their corresponding B6 Ensembl IDs, in order to perform Deseq2 and DexSeq analyses.

### **Methods and Materials**

#### Sample Preparation

In initial studies conducted on ethanol regulation of Ninein gene expression by Jessica Jurmain during the course of her M.S. thesis work in the Miles Laboratory (2020), eight-week old male C57Bl/6J and DBA/2J mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed in cages on ventilated racks with Teklad Sani-Chip bedding (currently Envigo, Cumberland, VA) and cotton nesting material. Four mice were housed in each cage. A 12-hour light dark cycle was maintained at all times and the mice were fed ad-libitum with Teklad LM-485 7012 standard rodent chow and tap water. Two weeks after the mice had arrived, they were given 0.9% saline, 1.8 g/kg or 4 g/kg ethanol via intraperitoneal injection and then euthanized 4 hours later by cervical dislocation and decapitation. This was done to obtain brain tissue from the nucleus accumbens for dissection and subsequent molecular studies. This tissue was the source of RNA used for the RNA-Seq studies that form the basis of this work. All procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee in accordance with National Institute of Health guidelines.

Immediately following decapitation, the entire brain was removed and microdissected as described by Kerns et al. (2005). Briefly, the whole brain tissue was chilled on ice for 1 minute in 1x phosphate buffer then dissected by sectioning and micropunch to isolate tissue from 7 regions of the brain, including the nucleus accumbens. The tissue samples were then placed in

individual tubes, flash frozen using liquid nitrogen, and stored at -80 degrees Celsius until RNA extraction.

RNA was extracted from the nucleus accumbens tissue using homogenization in STAT-60 (Tel-test, Inc., Friendswood, TX, USA) and purified with a Qiagen RNeasy Mini Kit (Qiagen, Redwood City, CA, USA). A ThermoFisher Nanodrop 2000 Spectrometer was used to assess RNA concentration by measuring the UV-Vi's absorbance at 260 nm. The sample quality was assessed using Agilent Technologies Agilent RNA 6000 Nano Kit. Samples with RNA quality indicator (RQI) values less than 7.0 were not used. The control samples (saline-treated) from B6 and D2 mice (n=5/strain) were then prepared for RNA-Sequencing at the VCU genomics core facility by Emma Gnatowski in in the Miles Laboratory and provide the resource for the analysis performed in this study.

#### D2 Annotation File Preparation

The annotation file provided by Dr. Keane was initially in GFF3 format. While this format will work with STAR aligner (Dobin et al., 2013) for the generation of counts, the SAMSORT (Danecek et al., 2021) and DexSeq (Anders et al., 2012) applications both require GTF files. In order to convert the GFF3 file to a GTF file, a docker environment was created and AGAT (Another Gtf/Gff Analysis Toolkit) (Dainat et al., 2020) was used to convert the GFF3 file to a GTF file. This worked for SAMSORT, but for DexSeq an additional step was required. GTF files do not usually contain parent relationships for the genes and transcripts contained within, but GFF3 files do. This causes the "Parent" attribute to conflict with the "gene\_ID" and "transcript\_ID" attributes. Removing the "Parent" attributes leaves the file with the same attributes as a normal GTF file, which was needed to prepare the DexSeq counts.

#### DESeq2 Count Generation

The following steps were performed using the VCU Group high performance computing cluster. The FASTA file for the D2 genome taken from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/view/GCA\_921998315.2) was modified so that the headers matched the chromosome names of the GFF3 file. Then STAR aligner was used to generate an index file for the count generation process using the FASTA file and GFF3 file (Appendix 2: submit02a\_STAR\_index.sh). Next, the samples were aligned to the D2 genome using STAR aligner, generating BAM files (Appendix 2: submit02b\_STAR.sh). The indexed BAM files were then sorted with SAMSORT (Appendix 2: SortScript.sh). These sorted BAM files would be used directly in the DexSeq count preparation, explained further in chapter 4. Feature counts would then be generated for the Deseq2 analysis using the converted GTF file and the sorted, indexed BAM files, explained further in chapter 3.

### Results

#### D2 Annotation File Preparation

The initial GFF3 (Appendix 1: DBA\_2J\_v3.2.gff3) file was successfully converted to a GTF file using AGAT. The resulting file is DBA\_2J\_v3.2\_3\_14\_23.gtf (Appendix 1). It was then successfully prepared for DexSeq analysis, with the resulting file being DBA\_2J\_v3.2\_3\_14\_23\_filtered.gtf (Appendix 1).

#### Deseq2 Count Generation

The headers of the FASTA file were successfully changed to match the GFF3 and GTF file chromosome names (Appendix 1:

GCA\_9219983152\_FASTA\_Converted\_DZ\_3\_23\_23.fasta). STAR aligner successfully generated the index files (Appendix 1 – Index Files) followed by the BAM files (Appendix 1 – BAM Files). Finally, SAMSORT successfully sorted the BAM files (Appendix 1 – Sorted Files). MultiQC was run on the indexed BAM files to determine the percentage and number of uniquely mapped reads, the STAR alignment scores, and gene counts of each sample (Figures 2.1, 2.2, 2.3). At this stage MulitQC was also performed on the generated feature counts used in the DESeq2 analysis. This was then compared to the MultiQC results of the B6 aligned B6 and B6 aligned D2 samples (Table 2.2) using a T-test.

These same steps were performed on the B6 mouse samples that were aligned to the B6 genome, successfully generating BAM files (Appendix 1 – BAM Files). RNA-Seq samples were aligned to release 108 of the B6 reference genome using STAR aligner (Dobin et al., 2013) on the VCU group server. The B6 reference genome (https://ftp.ensembl.org/pub/release-110/fasta/mus\_musculus/dna/Mus\_musculus.GRCm39.dna.primary\_assembly.fa.gz) and annotation (https://ftp.ensembl.org/pub/release-

110/gtf/mus\_musculus/Mus\_musculus.GRCm39.110.gtf.gz) were taken from Ensembl (European Microbiology Laboratory - European Bioinformatics Institute, Cambrige, UK). The D2 samples were then aligned to a D2 reference genome (Assembly GCA\_921998315.2) taken from the European Nucleotide Archive

(https://www.ebi.ac.uk/ena/browser/view/GCA\_921998315.2) and annotation (DBA\_2J\_v3.2.gff3) provided by Dr. Thomas Keane. The resulting BAM files were checked for quality using MultiQC, and compared to the MultiQC results of the B6 alignments (Tables 1 & 2.)

**Table 2.1:** D2 aligned D2 samples MultiQC results showing uniquely mapped reads, both

 alignment percentage and millions of reads (M) and the assignments of feature counts in

Sample Name	% Assigned	M Assigned	% Aligned	M Aligned
D11N_S1_001	72.80%	111	93.60%	139.1
D13N_S8_001	71.60%	103	92.80%	127.1
D22N_S7_001	72.40%	108.4	94.10%	139.4
D32N_S10_001	72.10%	107.6	93.90%	133.7
D34N_S6_001	72.60%	99.2	94.70%	141.8
Average	72.3%	105.84	93.82%	136.22

percentage assigned and millions of reads assigned.



Figure 2.1: STAR alignment scores of D2 aligned D2 samples, in millions of reads.



Figure 2.2: STAR gene counts of D2 aligned D2 samples, in millions of reads.



**Figure 2.3:** MultiQC of feature counts of D2 aligned D2 samples, showing the number of assigned features and the number of unassigned features with the reason they were not assigned.

**Table 2.2:** B6 aligned B6 and D2 samples showing uniquely mapped reads, both alignment

 percentage and millions of reads (M) and the assignments of feature counts in percentage

Sample	%	Μ	%	Μ
Name	Assigned	Assigned	Aligned	Aligned
B14N_S9	74.40%	111.5	92.90%	133.6
B21N_S5	74.90%	104	91.60%	124.1
B24N_S3	75.40%	97	90.20%	114.4
B31N_S4	74.60%	101.4	90.90%	120.9
B32N_S2	75.90%	108.2	92.00%	127.5
D11N_S1	75.00%	116.8	92.50%	138.7
D13N_S8	73.60%	108.7	92.40%	131.6
D22N_S7	74.40%	114.3	92.40%	136.9
D32N_S10	74.20%	113.2	91.60%	136.2
D34N_S6	74.60%	104.4	91.20%	124.9
B6 Average	75.04%	104.42	91.52%	124.1
D2 Average	74.36%	111.48	92.02%	133.66

assigned and millions of reads assigned.



Figure 2.4. STAR alignment scores of B6 aligned B6 and D2 samples, in millions of reads.



Figure 2.5. STAR gene counts of B6 aligned B6 and D2 samples, in millions of reads.



**Figure 2.6.** MultiQC of feature counts of B6 aligned B6 and D2 samples, showing the number of assigned features and the number of unassigned features with the reason they were not assigned.

## Discussion

The D2 aligned D2 samples showed significantly less percentage and total number of uniquely mapped reads assigned with 72.3% compared to 74.36% in the B6 aligned D2 (p < p(0.0001) and (105.84) million compared to (111.48) million total reads in the B6 aligned D2 (p < 0.0001). However, the D2 aligned D2 showed significantly more percentage of reads aligned with 93.82% compared to 92.02% in the B6 aligned D2 (p = 0.0272). The difference between the total reads aligned between D2 aligned D2 and B6 aligned D2 was not significant, with 136.22 million compared to 133.66 million total reads aligned (p = 0.5354). STAR aligner suggests that 80-90% alignment is acceptable, and their benchmark for experimental data is 94% aligned (Dobin et al., 2013). These results fall inside that window, and therefore the alignment percentage is acceptable. The alignment results are also higher than those used in previous differential gene expression studies that were aligning to the B6 genome (Bottomly et al., 2011), (Mortazavi et al., 2008), and with a significantly higher alignment percentage this should improve results of analyses done using these counts. The D2 alignment did produce a lower percentage of assigned reads than the B6 aligned. This could be due to several reasons, such as the complexity of the genomic regions or genetic variation between the samples and the D2 reference genome, though the most likely reason is that the D2 reference genome is less complete than the B6 reference genome. Regions that aren't well represented in the D2 reference genome would cause their associated reads to be assigned at a lower rate or not at all. However, the % assignment is still high. There is no guideline for what an acceptable assignment percentage is, however, being within 2% of the B6 aligned results is good enough to proceed. The benefits of aligning to the D2 genome, such as increasing the future analyses' ability to
detect SNPs and small indels, and potential allele specific expression differences outweigh the slight decrease in assignment percentage moving forward.

# **Chapter 3: Differential Expression Analysis and Gene Ontology**

# Introduction

The process of information taken from a gene being used to create a functional product is called gene expression. This leads to the related phenotypes being shown in the resulting organism, and therefore in any kind of genetic research understanding gene expression is extremely important. Gene expression is tightly regulated (Ptashne & Gann, 1997) as any dysregulation can quickly lead to disease (Esteller, 2007). Differential gene expression is when a gene in two or more samples has a statistically significant difference in expression levels, or read counts (Anjum et al., 2016). RNA-seq data is commonly used to identify differentially expressed genes (Li & Xie, 2013) by their read counts.

Deseq2 is an R package developed by Love et al. (2014) that performs differential expression analysis on RNA-seq feature count data using a negative-binomial (Gamma-Poisson) distribution. The input data required are some form of gene identifier, Ensembl IDs were used in this study, and read counts for each sample. It goes through three steps to perform the analysis, first normalizing the data by estimating size factors, then estimating the dispersion, then running the negative binomial test. The relevant output of the analysis are p-values indicating whether a gene is significantly (p < 0.05) differentially expressed between the sample groups, and a log2fold change, indicating the magnitude of the differential expression (Love et al., 2014). Deseq2 is used in this analysis to compare 5 B6 aligned B6 samples and 5 B6 aligned D2 samples, then again to compare those same 5 B6 aligned B6 samples with 5 D2 aligned D2 samples, resulting in a list of significantly differentially expressed genes between the two strains.

B6 and D2 mice are known to have differential expression of genes between them and previous analyses have been run aligning to the B6 genome as it was the only available mouse genome (Bottomly et al., 2011). Being able to align the D2 mice to their own genome allows for a differential expression analysis to be performed with more accurate results, as aligning to the D2 genome will account for genetic variation (SNPs, indels) specific to that strain. In addition, reliance on a single reference genome can cause bias in downstream analyses. It can also result in the analysis missing important genetic variants if they occur in regions not present in the reference genome (Kim et al., 2019).

Gene ontology (GO) categorizes genes based on the function of their products. There are three main categories, biological processes, molecular function, and cellular components. Each category contains a hierarchy of terms, with the most specific terms at the bottom and broader terms at the top. Genes are associated with the most specific term that accurately describes their products. GO is particularly useful when comparing genes across species or, in this case, strains within a species, as it allows for a comparison of function in a set of genes (Ashburner et al., 2000). In this study, gene ontology is used to compare the functions of the significantly differentially expressed genes between B6 and D2 samples, and between the B6 and D2 aligned analyses.

Revigo is a tool that was developed by Supek et al. (2011) that is designed to take an input of gene ontology terms and their significance levels in the form of p-values and return a reduced, clustered visualization of those terms based on their semantic content. This quantifies how much the terms share a common meaning, and uses SimRel to assign a score to each based on their semantic similarity, with scores of .9 or higher indicating high similarity. This reduces the number of gene ontology terms into larger categories, making for easier visualization and

comparison. SimRel is a functional similarity measure used to compare two GO terms with each other (Shlicker et al., 2006) (Figure 3.1). It is based on SimRes, Resnik's semantic similarity algorithm (Resnik, 2011) and SimLin, Lin's semantic similarity (Lin, 1998). Resnik's method focuses on the most informative common ancestor of the GO terms, and Lin's approach adds a focus on the shared information between the two terms. SimRel combines these approaches to incorporate relevance similarity (Schlicker et al., 2006).

$$sim(t_1, t_2) = \frac{2 * \log p(MIA)}{\log p(t_1) + \log p(t_2)} * (1 - p(MIA))$$

**Figure 3.1.** SimRel algorithm.  $t_1$ ,  $t_2$  refer to the gene ontology terms being compared, which are the most specific terms possible for each gene.  $p(t_1)$  and  $p(t_2)$  refer2 to the probability of those terms being found in the GO dataset, and p(MIA) refers to the probability of finding the common ancestors of terms  $t_1$  and  $t_2$  in the GO dataset. This is then weighted with 1-p(MIA) because the relevance of a term decreases with increasing probability. Equation taken from Schlicker et al.,

### 2006

This section of the study focuses on comparing the differential gene expression between the two strains using Deseq2, then generating GO terms using ToppFun and reducing them for visualization with Revigo. This analysis will be run twice, once using B6 aligned D2 samples and once using D2 aligned D2 samples. The results of both analyses will then be compared using 2 tailed t-test to determine if there is a significant difference in the magnitude of the LFCs of filtered significantly differentially expressed genes (p < 0.05, FDR 0.1) and list comparison to determine the changes in differentially expressed genes identified when aligning D2 samples to the D2 genome. GO terms will then be compared using list comparison and Revigo clustering to determine if aligning the D2 samples to the D2 genome causes a significant difference in the functions of those genes' products.

### Methods

### Differential Gene Expression

The paired end counts generated in the previous step were run through a differential expression analysis using Deseq2 (Bioconductor) as described by Love et al. (2014). First the B6 counts that were aligned to the B6 genome were compared to D2 counts that were aligned to the B6 genome in terms of log2fold change (LFC) using Deseq2. This showed to what degree each gene was differentially expressed between the two genomes. Then, B6 counts that were aligned to the B6 genome were compared to D2 counts that were aligned to the B6 genome were compared to D2 counts that were aligned to the B6 genome were compared to D2 counts that were aligned to the B6 genome were compared to D2 counts that were aligned to the D2 genome in terms of log2fold change. Finally, the significantly differentially expressed genes from each comparison were compared to each other using a two tailed t-test to determine if there was a significant difference in LFC between the two sets of differentially expressed genes, then using rnact.crg.eu's list comparison feature to determine the differences in which genes were differentially expressed.

Genes with median counts of less than 1 across all 10 samples were filtered out of the data. The counts were normalized using Deseq2's median of ratios method (Love et al., 2014), and pairwise correlation values were calculated for these samples. These were visualized using a hierarchal heatmap of correlation data created using pHeatmap. The pairwise correlation values for all samples were visualized using multiple scatterplots. A principal component analysis of the

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variance was run on the top 500 and top 10,000 genes by counts. Then the differential expression between the strains was calculated using Deseq2. The data was filtered again, taking only genes that were significant at p = 0.05 and filtered using an FDR of 0.05, again using Deseq2. These were visualized using both a volcano plot made with GGplot2 and a heatmap made with pHeatmap.

### Gene Ontology and Semantic Similarity Analysis

The filtered, significantly differentially expressed genes from the previous step were used to run a gene ontology analysis using ToppFun (ToppGene, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA). The results of this gene ontology in the biological process, cellular component, and molecular function categories were then put through Revigo's semantic similarity analysis as described in Supek et al. (2011) to better visualize the groupings of genes inside those categories. Scatterplots were created using the GGPlot2 R package and treemaps were created using the treemap R package.

This was repeated for the comparison of B6 counts aligned to the B6 genome to D2 counts that were aligned to the D2 genome.

### Comparison of Results

The resulting differentially expressed genes from both the analysis using B6 aligned D2 and the analysis using D2 aligned D2 were compared using simple list comparison metrics. The differentially expressed genes were compared in both number and name, with similarity being measured by how many genes were differentially expressed in both comparisons and by which genes were differentially expressed. The gene ontology results were compared to each other directly, with the total number in each category being compared as well as how similar the individual genes' functions were. This was accomplished by comparing the names directly and seeing what percentage of overlap there was between the two studies. The positive and negative sets of LFC values was determined to have significantly unequal variance (p < 0.05) and as such the t-tests used were Welch's t-tests, assuming unequal variance. Running a 2 tailed t-test on the significantly expressed genes from each analysis with positive LFC values, and a 2 tailed t-test on the significantly expressed genes from each analysis with negative LFC values. These were separated as the overall average of LFCs from both analyses was nearly zero, and as such would not be a good comparison. Finally, the Revigo results were compared, to see if the gene ontology results fell into similar or different broad categories.

**Results** 

### Aim 1a – Differential Gene Expression

### Differential Gene Expression Between B6 Aligned B6 and B6 Aligned D2

The initial correlation data of the counts showed that the two strains were closely related, with a minimum correlation value of .992. There was also clear delineation between B6 and D2, with each sample having significantly higher correlation with samples of the same strain than samples of the other strain (Figure 3.2). The principal component analysis of the top 10,000 most abundant genes by counts showed that strains were clustered together by 80% variance (Figure 3.3). However, both Figure 3.2 and Figure 3.3 indicate that 2 B6 samples showed slight variance compared to the other B6 samples in terms of correlation and PC2 grouping. We elected to not exclude these from further analysis since their overall correlation was more similar to B6 than D2 samples, and they clustered tightly with B6 samples on hierarchical clustering and principal

component analysis (PC2). A MA plot of genes with differential expression (FDR  $\leq$  0.05) was used to visualize results, showing the log10 fold-change (LFC) of all genes plotted versus the mean of normalized counts (Figure 3.4). 6,210 genes were differentially expressed (D2 vs. B6), with 3,257 having a positive log2fold change and 2,953 having a negative LFC. A positive LFC indicates that the gene showed higher expression in the D2 strain than the B6 strain. A heatmap of LFCs by genotype was generated to show the differences in LFC for each gene and each individual (Figure 3.5), with positive LFC values indicating higher expression in D2 mice. The top 20 differentially expressed genes exhibited no bias towards either positive or negative LFC (Figure 3.6), suggesting adequate normalization of the data and no systematic errors biasing the analysis.



### **B6** Aligned Heatmap of Correlation Data

**Figure 3.2.** Heatmap of count correlation data of the B6 aligned analysis. B6 and D2 correlate more with themselves than with each other. There are no major outliers. The overall high levels of correlation between B6 and D2 (.992 to 1) shows clear separation between two closely related strains. Two samples, B24N and B32N, showed slightly lower correlations with the other B6 samples but still clustered tightly with the remaining B6 samples.



# Principal Component Analysis of the Top 10,000 Most Abundant Genes, B6 Aligned

Analysis

**Figure 3.3.** Principal component analysis of the top 10,000 most abundant genes. Substrains are clustered together along the X axis (PC1) while some variation between samples within a strain are differentiated on the Y axis (PC2).



**B6** Aligned Log2Fold Change by Mean of Normalized Counts

**Figure 3.4.** MA plot of the LFC against the mean of normalized counts for all genes. Blue indicates genes that were significantly differentially expressed between the two strains (FDR <0.05) and grey indicates genes that were not significantly differentially expressed.



Figure 3.5. Heatmap of hierarchical cluster analysis of differentially expressed genes between
B6 aligned B6 and B6 aligned D2 and the log2fold changes (LFCs) of each gene. A positive LFC (Red) indicates higher expression in D2. The 2-dimensional cluster analysis reveals robust consistency across the samples for differential expression analysis.



**Figure 3.6.** Top 20 significantly differentially expressed genes and their normalized counts in each strain. 12 genes have positive LFC values, and 8 have negative LFC values for D2 expression versus B6 expression.



### Principal Component Analysis of the Top 10,000 Most Abundant Genes

**Figure 3.7.** Principal component analysis of the top 10,000 most abundant genes when using the D2 aligned D2 counts. Substrains are clustered together along the X axis.



**Figure 3.8.** MA plot of the LFC against the mean of normalized counts for all genes. Blue indicates genes that were significantly differentially expressed between the two strains (FDR <0.05) and grey indicates genes that were not significantly differentially expressed.

Heatmap of LFC of Results



Figure 3.9. Heatmap of hierarchical cluster analysis of differentially expressed genes between
B6 aligned B6 and D2 aligned D2 and the log2fold changes (LFCs) of each gene. A positive
LFC (Blue) indicates higher expression in D2. The 2-dimensional cluster analysis reveals robust
consistency across the samples for differential expression analysis.



Figure 3.10. Top 20 significantly differentially expressed genes and their normalized counts in each from the D2 aligned analysis.

### Comparison of Results

The comparison of results from the B6 aligned D2 analysis and the D2 aligned D2 analysis has been sorted into groups containing only significantly differentially expressed genes. These genes were further sorted by LFC, with positive and negative Log2Fold being compared separately. This is because while the overall LFC of the D2 aligned analysis was significantly higher (B6 aligned D2 = 0.0274, D2 aligned D2 = 0.3270, p < 0.0001), the QC performed showed that the distribution was still even (Figure 3.8). With an even distribution of positive and negative LFCs, the two sets of positive and two sets of negatives were compared to each other to better illustrate the differences between the two comparisons. Positive LFCs indicate increased expression in D2 mice. These analyses have two parts, the comparison of which genes are differentially expressed in each analysis focusing on unique differentially expressed genes, and the comparison of overall LFCs.

The comparison of the negative LFCs showed that 85.05% (2770/3257) of the genes that were significantly differentially expressed in the analysis using B6 aligned D2 samples were also differentially expressed in the analysis using D2 aligned D2 samples (Figure 3.11). The analysis using D2 aligned D2 samples showed significantly more unique genes being differentially expressed than in the B6 aligned analysis (2544/487). The significantly differentially expressed genes with negative LFCs were significantly different in their average LFC (p <0.0001) with the D2 aligned results have a greater magnitude than the B6 aligned results (D2 aligned average negative LFC = -2.5930, B6 aligned average negative LFC = -0.9810).

The comparison of positive LFCs showed that 89.84% (2316/2934) of the genes that were significantly differentially expressed in the analysis using B2 aligned D2 samples were also differentially expressed in the analysis using D2 aligned D2 samples (Figure 3.12). The analysis

using D2 aligned D2 samples showed significantly more unique genes being differentially expressed than in the B6 aligned analysis (2544/487). The significantly differentially expressed genes with positive LFCs were significantly different in their average LFC (p < 0.0001) with the D2 aligned results have a greater magnitude than the B6 aligned results (D2 aligned average positive LFC = 3.1672, B6 aligned average positive LFC = 1.1465).



**Figure 3.11.** Comparison of significantly differentially expressed genes with negative LFCs resulting from differential expression analyses using B6 aligned D2 samples (Red) and D2 aligned D2 samples (Blue). 487 genes were found to be differentially expressed only in the analysis using B6 aligned D2, and 2,544 genes were found to be differentially expressed only in the analysis using D2 aligned D2.



**Figure 3.12.** Comparison of significantly differentially expressed with positive LFCs genes resulting from differential expression analyses using B6 aligned D2 samples (Red) and D2 aligned D2 samples (Blue). 618 genes were found to be differentially expressed only in the analysis using B6 aligned D2, and 3147 genes were found to be differentially expressed only in the analysis using D2 aligned D2.

# Gene Ontology and Semantic Similarity

### B6 Aligned D2 Analysis

The gene ontologies of the significantly differentially expressed genes with negative LFCs are shown below, followed by those with positive LFCs. The analysis was run using ToppFun using probability density function to calculate the p value. An FDR correction of 0.05 was used and a gene limit of 3 was set to filter the data. Revigo's semantic similarity analysis was used to cluster and visualize the gene ontology results in the biological processes, molecular function and cellular component categories as scatterplots (Figures 3.13, 3.15, 3.17) and as tree maps (Figures 3.14, 3.16, 3.18). It is important to keep in mind when reading these scatterplots that the axes have no intrinsic meaning. Revigo uses Multidimensional Scaling (MDS) to reduce the dimensionality of a matrix of the GO terms pairwise semantic similarities. This may lead to the result being non-linear, though semantically similar groups will be clustered together. When repeating this analysis, keep in mind that the clusters may appear in different sections of the plot, but the same terms will be clustered together.



### **B6** Aligned Semantic Similarity of Biological Processes

**Figure 3.13.** Scatterplot of the semantic similarity analysis performed on the biological processes results from the significantly differentially expressed genes from the analysis using B6 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

			[	36 Aligned	Significant LFC Se	emantic S	Similari	ty of E	Biological	Processes					
regulation of organelle organization	positive regulation of receptor-mediated endocytosis	positive regulation of stress fiber assembly	regulation of mitochondrial fission	regulation of transferase activity	glial cell differentiation	circulatory system development developmental growth involved in morphogenesis		item nt	tube development	glycosamino metabolic p	oglycan a rocess me	aminoglycan tabolic process	cellular catabolic proces	carbohydrate derivative catabolic process	
	regulation of cellular	regulation of	regulation of	negative regulation				tal red esis		ncRNA metabolic	protein	protein modification by small	oxoacid metabolic process	organic acid metabolic process	
regulation of actin filament–based	regulation of org biogenesis	anelle organiza metabolic process	tionetabolic process	of cell projection organization	anatomical structure glia formation involved	l cell differ system	entiation syste	nji e em	endothelial cell	glycosamine	oglycan meta	bolic process conjugation	cellular ca sulfur	bolic process cyclic nucleotide	
process	regulation of anatomical structure size	regulation of membra	on re ane ac	gulation of tin filament	in morphogenesis de	development dev		opment developn		DNA metabo process	lic	phospholipid biosynthetic	catabolic process	catabolic process	
regulation of protein localization	regulation of cellular	regulation of p	protein positiv	ve regulation of	development	dentinogenesis		of a p	ogenesis iolarized helium	protein	autouxiquitina	process	heparan sulfate	ganglioside catabolic process	
	component size	external	iocess prote	in sunioyiauon	peripheral nervous system axon ensheathment	microglial cell activation		leukocyt invo nflammat	e activation olved in ory response	monoubiquitinat	ion cyclic metab	nucleotide olic process	catabolic process	process	
organelle assembly	supramolecular fiber organization	encapsulatir structure organizatio	ng orga	nelle fission	establishment of	inorganic ion transmembrane transport inorganic cration transport transport transport transport		vesicle-mediated transport vacuolar transport alization		response to wounding	o response	e to oxidative	<sup>D</sup> monoatomic cation	c actin filament-based s process	
	ovtra collular	cell projectio assembly	on sti a	ress fiber ssembly	protein localization					res	ponse to wo	unding	homeostasis		
actin cytoskeleton organization	maorganelle organization	assembly		coll	monoa <b>establishm</b> transport					response to axon	double-stra break repa	nd ir respons to	e carbohydrate derivative metabolic	estabilehment or establishment or	
	extracellular	organization	phagoson maturatio	n junction assembly				pr locali cell p	otein zation to eriphery	injury	cellular respo to extracellu stimulus	lar hormon	process	of cell cell polarity	
cytoskeleton organization	structure organization	vacuole organization	mitocho	ndrial fission	monoatomic ion transmembrane transport			lysosomal transport		mitotic cemitotic ce		chromosome /Clezation involved n meiotic cell cycle	oxygen species metabolic process	cell-substrate adhesion	

Figure 3.14. Treemap of the semantic similarity analysis performed on the biological processes results from the significantly differentially expressed genes from the analysis using B6 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.

### **B6** Aligned Semantic Similarity of Molecular Functions



**Figure 3.15.** Scatterplot of the semantic similarity analysis performed on the molecular function results from the significantly differentially expressed genes from the analysis using B6 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected

species in the EBI GOA database.



Figure 3.16. Treemap of the semantic similarity analysis performed on the molecular function results from the significantly differentially expressed genes from the analysis using B6 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.



**B6** Aligned Semantic Similarity of Cellular Components

**Figure 3.17.** Scatterplot of the semantic similarity analysis performed on the cellular component processes results from the significantly differentially expressed genes from the analysis using B6 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

					B6 A	igned Sign	ficant l	LFC S	Semantic Si	milarity of Cellular Comp	onents					
	Golgi apparatus	us perinuclear r of cytoplas		perinuclear region of cytoplasm		mitochondrial envelope microtu		microtubule cytoskeleton		collagen-containing extracellular matrix extrace		extracellular matrix		nal ulating	neuron projection	
										plasma	a membra	ane region	structure		neuror neuron spin	projection main axon
	lysosome	vacuole	vacuole secretory		/ granule	granule striated mus thin filame		uscle cortical nent cytoskeleton		plasma membrane region	cell cortex		leading edge membrane		node	of Ranvier
			Go		tus	s organelle		endoplasmic								
		cytoplasmic vesicle membrane		olasmic vacuolar l membrane		subcompartmen		ent reticulum lumen		synapse	memb		ane main	mye	lin sheath	cell leading edge
	microbody							exon-exo junction com	exon-exon action complex		vesicle ethering complex	synapse				
	peroxisome	catalytic com	Ilytic complex contracti		ie tiber					neuromuscular junction				supra co	molecular mplex	somatodendritic compartment
		centrosom	ne	Golgi mer	mbrane	collagen trimer	HAU	JS blex	ficolin-1-rich granule	membrane raft		envelope		Schmidt	-Lanterman cisure	stereocilia coupling link

Figure 3.18. Treemap of the semantic similarity analysis performed on the cellular component results from the significantly differentially expressed genes from the analysis using B6 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.

### D2 Aligned D2 Analysis

The gene ontologies of the significantly differentially expressed genes with negative LFCs are shown below, followed by those with positive LFCs. The analysis was run using ToppFun using probability density function to calculate the p value. An FDR correction of 0.05 was used and a gene limit of 3 was set to filter the data. Revigo's semantic similarity analysis

was used to cluster and visualize the gene ontology results in the biological processes, molecular function, and cellular component categories as scatterplots (Figures 3.19, 3.21, 3.23) and as tree maps (Figures 3.20, 3.22. 3.24).



**D2** Aligned LFC Semantic Similarity of Biological Processes

**Figure 3.19.** Scatterplot of the semantic similarity analysis performed on the biological processes results from the significantly differentially expressed genes from the analysis using D2 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

					D2 /	Aligned Significa	nt LFC Semant	tic Similarity of	Biological Pr	ocesses					
translation at	at translation at synapse		nstation at synapse cytoptasmic		negative regulation of RNA metabolic process	regulation of protein depolymerization	positive regulation of type I interferon production	regulation of protein stability	regulation of Wnt signaling pathway	regulation of calcium ion-dependent exocytosis	intracellular transport		vesicle docking	establishment of protein localization to organelle	
presynapse				translation		negative regulation	tumor necrosis factor-mediated signaling	positive regulation of I-kappaB kinase/NF-kappaE signaling	regulation of cilium assembly	tachykinin receptor signaling pathway	positive regulation of cilium movement	intracellula exocytic process		protein localization to chromosome,	organelle
						organization	pathway negative re	positive gulation of RNA	regulation of metabolic proce	ss regulation	Wet cignaling			telomeric region	
protein modifie by small pro	cation itein	tion protein in deubiquitination noval		sial	ylation	regulation	response to	DNA-templated transcription	transcription factor activity	of catabolic process	pathway	suppression of	DNA		
conjugation or r	emoval					of I-kappaB kinase/NF-kappaE signaling	retinoic acid	positive regulation of DNA-binding transcription facto	regulation of viral process	positive regulation of protein	activation of NF-kappaB-inducing kinase activity	viral release by host suppressio release b	damag respon n of viral by host	ie cilium se cilium	movement movement
ubiquitin-dependent	translation at macromolecule catabolic process		presynapse peptidyl-L-cys S-palmitoyla	rse protein cysteine toylation ubiquition		negative regulation of viral	I-kappaB kinase/NF-kappaB	regulation of	protein	localization positive regulation of microglial	negative regulation of transcription	innate immune response <sub>i</sub>	macropha activatio involved mmune resp	ige n ax in as ponse	oneme sembly
process				ubiquitination		transcription	aignaing	autopnagy	stabilization	cell mediated cytotoxicity	initiation by RNA polymerase II			proc	ess
protein	pro polyubiq	protein RNA splicing polyubiquitination		ng pepti ma	dyl-cysteine odification	vesicle organization	membrane fusion	fertilization	membrane organization	actin filame depolymeriza	nt system organization	autophagy		utilizing autophagic mechanism	
palmitoylation	prote	olveis	DNA	tRNA methylation				vesicle organiz	synaptonema	ıl		intracellu monoatomic	lar anion		
	prote					homologous			organization	ribosom	al synaptic	homeostasis		reproductio	on membrane docking
ganglioside biosynthetic process	ncRNA metabolic process		DNA metabolic process	type I interferor productio	camera-type n eye n development	chromosome pairing at meiosis	DNA topological change	organelle assembly	organelle fusio	small subuni biogenes	vesicle membrane sis organization	cilium or flagellum-dependent cell motility		microtubu proc	ile-based ess

Figure 3.20. Treemap of the semantic similarity analysis performed on the biological processes results from the significantly differentially expressed genes from the analysis using D2 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.



**D2** Aligned Semantic Similarity of Molecular Functions

**Figure 3.21.** Scatterplot of the semantic similarity analysis performed on the molecular function results from the significantly differentially expressed genes from the analysis using D2 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected

species in the EBI GOA database.

					D2 Alig	gned Si	gnificar	nt LFC Sema	ntic Similarity of M	lolec	ular Functior	าร														
	palmitoyltransferase activity	sialyltransferase activity		sialyltransferase activity		sialyltransferase activity		sialyltransferase activity		sialyltransferase activity		sialyltransferase activity		ub protei alyitransferase activity		ıg	ubiquiti enzy	n conjugating me activity	sequence-specific DNA binding sequence		transcription regulatory region nucleic acid binding cific DNA bind	double-stranded DNA binding	anded ding		ing transit	lion metal
			palmitoyltran		-glutamine ctivitysferase acyltr		isferase	alpha-N-acetylneuramina alpha-2,8-sialytransfara activity	transcription cis-regulatory region binding		rRNA b	indina				binding										
	protein-cysteine S-palmitoyltransferase activity		acyltransferase activity	sferase <sup>ty</sup> aminoacy ac		poly(A) RNA polymerase activity		beta-galactoside (CMP alpha-2,3-sistaytransfera activity	cysteine-type deubiquitinase activity cysteine-type deub	ubiquitin-like protein peptidas activity ubiquitinase activit		DNA topoison activity DNA topoison DNA topoison type I (single cut, ATP-indep activity	enerase pomerase activiti nerase activi strand endent)	farase y	structura constituer of riboson	i structural constituent of cytoskeleton										
	SNARE binding tumor necrosis factor receptor binding		syntaxin bindin SNARE b		axin binding ubiquit protein SNARE binding		e ubiquitin protein Ig ligase binding		cysteine-type pepti		se activity	mechanosensitivePera monoatomic ionalium channel activity_ivity		ed 1-phosphalidylinositol-3-kina: regulator activity 1-phosphalidylinositol-3-kina: regulator activity metalloendopeptidas		AP receptor activity										
			ubiquitin binc	ling	frizzled bin	nding n	polyubiquitin nodification-depe protein bindin	actin <sup>Indent</sup> binding	DNA-bi transcri factor a	nding iptior ctivit	transcription coactivator activity	calcium-d phospholip	ependent id binding	inhibitor activ acid-amino a ligase activi	cid act ty grou	doreductase tivity, acting on a sulfur up of donors										

Figure 3.22. Treemap of the semantic similarity analysis performed on the molecular function results from the significantly differentially expressed genes from the analysis using D2 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.



D2 Aligned Semantic Similarity of Cellular Components



species in the EBI GOA database.

	D2 Aligned Sign	ificant LFC Semantic Sim	ilarity of Cellular Com	ponents			
cytosolic ribosome	ribosomal subunit	SNARE complex	nuclear telomere cap complex	ribosome	male germ cell nucleus	phosphatidylinositol 3-kinase complex	
		cytoplasmic side of endoplasmic reticulum membrane	meiotic nuclear membrane microtubule tethering complex	synaptonemal complex	spectrin	endoplasmic reticulum-Golgi intermediate compartment membrane	plasma membrane urothelial plaque
cylosolic large ribosomal subunit		cytosolic ribosome rough endoplasmic reticulum membrane	microtubule organizing center attachment site	germ cell nucleus	9+2 motile cilit	IM PML body	
	myofilament	exocytic vesicle	nuclear membrane protein complex	vesicle tethering complex	tubular endosome	nuclear body	stereocilia coupling
cytoplasmic vesicle membrane	autophagosome	polysome	inner acrosomal membrane	early phagosome	SAGA-type complex	small-subunit processome	link

Figure 3.24. Treemap of the semantic similarity analysis performed on the cellular component results from the significantly differentially expressed genes from the analysis using D2 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.

# Discussion

### Alignment

Aligning D2 reads to the D2 genome produced a higher alignment percentage than aligning them to the B6 genome (Table 2). This will lead to more accurate gene quantification, increased sensitivity of the analysis with respect to genes and transcripts with low expression, and a reduction in background noise from unaligned reads (Oshlack et al., 2010). This again is promising, as it serves to show that there is a benefit to aligning to the D2 reference genome, though determining the scope and scale of that benefit is still in progress.

### Differential expression and comparison of results

Results of the differential expression analyses have shown that the differential expression between B6 aligned B6 and B6 aligned D2 is significant (Figure 3.5.) This is in keeping with the results found by Putman et al. (2016) and Kearns et al. (2005), and show that the basis for this study is well founded. By analyzing the resulting LFCs, there was a lack of bias in expression direction, with similar numbers of genes having positive and negative LFCs (Figures 3.11 and 3.12). The top 20 most significantly differentially expressed genes showed this same trend, indicating that the analysis was run correctly. If there was a significant bias towards positive or negative LFC values, then that would indicate a problem either in sample preparation, leading to one set of mice to have consistently higher or lower gene expression, or a problem in the analysis itself such as during normalization.

The analysis run using D2 aligned D2 samples showed similar quality control metrics (Figures 3.7-3.8) as the B6 aligned (Figures 3.2-3.4). There was a lack of bias in expression
direction observed in the resulting LFCs, and the top 20 most significantly differentially expressed genes again showing no bias towards positive or negative LFC (Figure 3.10). This indicates that the D2 aligned D2 samples are meeting the same quality control metrics as the B6 aligned D2 samples, and the comparison between the two sets of results can be done with confidence in the preparation and setup of the analyses.

The analysis run using D2 aligned D2 samples found significantly more significantly differentially expressed genes (p < 0.05) than the analysis run using the B6 aligned D2 samples, with 10,778 differentially expressed genes found in the D2 aligned D2 sample compared to 6,191 in the B6 aligned D2 analysis. In both the positive and negative LFCs a large amount of overlap was seen between the results of the two analyses (Figures 3.11 and 3.12). However, the D2 aligned results showed significantly more uniquely differentially expressed genes in both positive and negative directions (3,147 and 2,544 respectively). The D2 aligned average negative LFC was -2.5930 and the B6 aligned average negative LFC was -0.9810. The D2 aligned average positive LFC was 3.1672, and B6 aligned average positive LFC was 1.1465 This, along with the very high overlap of the B6 aligned results, where 85.05% (Negative LFC) and 89.84% (Positive LFC) were seen to be differentially expressed in the same direction as the D2 aligned results, shows that when aligning to the D2 genome there is a significant increase in the total number of differentially expressed genes while retaining a majority of the differentially expressed genes seen in the B6 aligned analysis.

This indicates that aligning to the D2 genome provides a 74.26% increase in significantly differentially expressed genes with negative LFCs and a 109.20% increase in significantly differentially expressed genes with positive LFCs. The total increase in significantly differentially expressed genes identified is 90.17%.

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# Gene ontology and semantic similarity

The gene ontology categories across both analyses showed similar levels of significance, with most sitting between -2.5 and -5 on a log10 scale of the p values. There were a few outliers, but the outliers were more significant than the average, with no low significance outliers. This indicates that the gene ontology categories can be considered reliable indications of the functions of the genes involved.

When comparing the categories between the B6 aligned analysis and the D2 aligned analysis, there is a low amount of overlap in the semantic clusters. There were overlaps, for example in the biological processes, gliogenesis showed the same amount of differentially expressed genes in both analyses. However, none of the other categories overlapped, which is likely at least partially caused by the large increase in differentially expressed genes in the D2 aligned analysis. This theme continues throughout the analyses, with there being some overlap but not overlap completely. One important category seen in the D2 aligned results that was not seen in the B6 aligned data was ribosomal protein gene expression. Categories such as "translation at synapse", "translation at postynapse", etc. This suggests that the D2 RNA-seq data shows a decrease in expression of the expression of ribosomal mRNAs when analyzed with the D2 alignment. Decreased ribosomal protein expression reflects a decreased capacity for protein translation, so this could be a biologically relevant difference discovered by aligning to the D2 genome that would not have been identified while aligning to the B6 genome.

# Chapter 4: Differential Exon Utilization and Alternative Splicing Introduction

Differential exon utilization occurs when exons within a gene are included in or excluded from the final RNA transcripts produced by a cell when compared between two experimental groups. DEXSeq (Anders et al., 2012) is an R/Bioconductor package that uses a statistical method to test for differential exon usage in RNA-seq data. It uses generalized linear models to do this, and takes biological variation into account to control false discoveries. It also identifies differences in splicing and translation.

DEXSeq requires exon count data which is prepared using scripts provided in the package. To generate this count data, RNA-Seq data, a genome fasta file, and an annotation GTF file are required. Indexed BAM files are generated first, using the fasta file and the annotation, then the annotation needs to be flattened for use with DEXSeq. These exon counts are then used in DEXSeq's analysis of differential exon utilization.

Gene ontology can be run on the genes with differentially utilized exons, providing an understanding of their functions. These GO categories reflect the functions of the genes with differentially utilized exons, and when comparing results of multiple analyses Revigo can be used to reduce the data and make visualization easier.

This study is running two different DEXSeq analyses, one using B6 aligned D2 samples and one using D2 aligned D2 samples. This will allow for a comparison using Welch's t-tests and direct list comparison of the genes with differentially utilized exons, as well as a comparison of the exons. The LFCs of both analyses will be used to compare the magnitude of the differentially expressed exons, broken in to positive and negative LFC groups.

# Methods

# Count Data Preparation

The RNA-Seq data was prepared for DexSeq analysis using the VCU Group Server on the VIPBG Cluster System First the GTF file converted in the previous chapter (See chapter 2) needed to be flattened for use with DexSeq (Bioconductor) (Anders et al., 2012). The script dexseq\_prepare\_annotation.py (Appendix 2) requires a GTF file as input. However, the process of converting the GFF3 file to a GTF file left certain attributes that were not compatible with the conversion script. With the aid of Dr. Mikhail Dozmorov, the specific attribute (parent) causing errors was identified and removed. The parent attribute is part of GFF3 files, indicating the parent transcript for each entry. It is not present in GTF files, and was not removed by AGAT in the conversion to GTF. Removing it does not change the function of the annotation file, but allows it to function in the dexseq\_prepare\_annotation.py. The resulting GTF file (DBA\_2J\_v3.2\_3\_14\_23\_filtered.gtf) was then run through the aforementioned script, resulting in a flattened GFF file (DBA\_2K\_v3.2\_flattened.gff). This flattened file was then used with the script dexseq\_count.py, to generate the dexseq counts. The BASH script used to run this on the VCU group server was DexSeqCounts.sh. The B6 aligned counts (both B6 and D2) were previously prepared using the same sample data by Emma Gnatowski.

## DexSeq Analysis

DexSeq was run using R version 4.3.1 (DEXSeq\_Analysis\_script.R) on the B6 aligned B6 and the B6 aligned D2 counts, and the significant results (FDR of 0.1) analyzed for expression patterns and gene ontology. This was repeated using B6 aligned B6 counts and D2 aligned D2 counts.

# Gene Ontology Analysis

The gene ontology of the genes showing differential exon utilization was run using ToppFun using a probability density function to calculate p values, then filtering for a false discovery rate of 0.05 and gene limits of 3 or more.

## Comparison of Results

The results of both DexSeq analyses were then compared to each other using direct comparisons to determine the total number of differentially utilized exons and differentially expressed genes, and t-tests to determine differences in the magnitude of those changes, measured by LFC. The LFC comparison was broken down into positive and negative LFC groups, as the overall average for both analyses was nearly zero. The gene ontology categories were compared using Revigo to reduce the number of categories and cluster by semantic terms. Lastly, specific genes of import were taken to use as examples of how aligning to the D2 genome can improve differential exon utilization results. The genes chosen for this were Ninein, Gabra2, and Gsk3b. These genes were chosen both for their import in ongoing AUD research and for how the D2 alignment affected them.

# Results

# Count Data Preparation

The GTF and count data files were successfully prepared, leading to the generation of count data (Appendix 1, B6 Aligned DexSeq Counts, D2 Aligned DexSeq Counts).

# B6 Aligned DexSeq Analysis

21,223 significantly (p < 0.05, FDR 0.1) differentially utilized exons were identified in the B6 aligned DexSeq analysis. There were 6,650 genes with differentially utilized exons. There were 10,566 exons with positive LFC indicating higher expression in D2, and 10,652 with negative LFC. These exons had an average positive LFC of 0.8323 and an average negative LFC of -1.2138.



**Figure 4.1.** Heatmap of B6 aligned D2 correlation data. There are 10 samples used as input, but 20 columns used in this analysis. The first 10 correspond to the number of reads mapping to out exonic regions, and the last 10 correspond to the sum of the counts mapping the rest of the exons

from the same gene on each sample. Samples 1-5 are B6 counts, specifically B14, B21, B24, B31, and B32, and samples 6-10 are D2 counts, specifically D11, D13, D22, D32, and D34. 11-

15 are B6, the same samples, and 16-20 are D2 aligned, the same samples.

**B6** Aligned Analysis LFC vs Mean Expression



**Figure 4.2.** LFC of differentially expressed exons compared to mean expression for the analysis using B6 aligned D2 samples. Red indicates significantly differentially expressed genes.

# D2 Aligned DexSeq Analysis

81,206 significantly (p < 0.05, FDR 0.1) differentially utilized exons were identified in the D2 aligned DexSeq analysis. There were 13,521 genes with differentially utilized exons. 43,775 of the exons had a positive LFC and 37,089 had a negative LFC. The average positive LFC was 1.5800 and the average negative LFC was -1.3011.



D2 Aligned Analysis Hierarchical Heatmap of Correlation Data

**Figure 4.3.** Heatmap of D2 aligned D2 correlation data. There are 10 samples used as input, but 20 columns used in this analysis. The first 10 correspond to the number of reads mapping to out exonic regions, and the last 10 correspond to the sum of the counts mapping the rest of the exons

from the same gene on each sample. Samples 1-5 are B6 counts, specifically B14, B21, B24, B31, and B32, and samples 6-10 are D2 counts, specifically D11, D13, D22, D32, and D34. 11-

15 are B6, the same samples, and 16-20 are D2 aligned, the same samples.

# D2 Aligned Analysis LFC vs Mean Expression



**Figure 4.4.** LFC of differentially expressed exons compared to mean expression for the analysis using D2 aligned D2 samples. Red indicates significantly differentially expressed genes.



# **B6** Aligned Biological Processes

**Figure 4.5.** Scatterplot of the semantic similarity analysis performed on the biological processes results from the genes with significantly differentially utilized exons from the analysis using B6 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

					B6 Ali	gned	Signific	ant D	exSeq	Sema	ntic Simila	arity of E	Biologica	al Pro	cesses	3						
regulation of organelle	regulation of cell	regulation of neurotransmitter levels	regulation of GTPase activity	e postsynap membrar potentia	of regul tic of syr e ves recy	ation reg aptic of cle ca cling pr	tabolic nocess	positive egulation of amide netabolic process	positive regulation of cell morphogenesis involved in differentiation	regulation of cell morphogenesi involved in differentiation	mRNA	RNA splicing	peptidyl- ac modific	-amino id f sation	ncRNA netabolio process	C small p conjug	tein ation by protein gation	protein atabolic process	cytoskele	eton	actin ytoskeleto	n protein-containing complex assembly
organization	inorphogenesis	regulation	regulation of postsynaptic membrane neurotransmitter	regulation of actomyosin structure organization	positiv regulatio translati	e regu n of m on pro	lation pr RNA regu abolio sod pess tra	lation of lum ion	nderallide – exclusion regiseir	protein stabilizatior	macromolecule	peptide metaboli	C polyadenylation	DNA biosyntheti process	C TNN aplicing, via tamender/fication reactions	profinin phosphorylation	poglidyd-thoson yfragharylada	And Come-contents and roading matcher process	Ulganiza	c	rganizatio	n
chemical synaptic	of cellular	process	negulation of microtubulo-based	glutamate receptor signaling	reputation of 0 protein-couple receptor signaling	regulatio of protei	n positive regulation of dendrite	regulatio of synapt vesicle	in regulation of neuro	n response to endoplasmi reticulum	catabolic process	amide	histone	histone mRNA catabolic	internal peptidyt-tysine acctylation	protein dephere hersteller	RNA 3'-end	amino acio metabolio	synapse	e m	embrane	cell junction
transmission	regulation	of actin filament-based process	double-strand break	regulation of DNA metabolic	cell cycle checkpoir	positive regulation dendritic sp	extension regulation of neurotransmitte ineurotransmitte	Rac prote	in regulatio	regulation of actin h cytoskalation	DNA	process	RNA-template	octuur ntropen A proce	septityl-series	peptidyl-serine modification	proteolysi	is DNA-template	organizati cyt	on org	anization on <mark>organiz</mark>	organization ation
regulation of trans-synaptic	of cellular component biogenesis	reregulation	n of organ	elle orga	signaling of RNA	developme guiation of glial cell differentiation	nt AF	Signal orbit	in secondice solved in the response spannys	reorganization position regulation of triglyceride biosynthesis	process	autophag	DNA replication	process transcription elongation by ICNA	DNA recombination put	an resolution concerning activ	nino idid vation	ntente passaia tele tele	supramolecular fiber organization	telomero organizati	endomembrane on ayalaam organization	organelle organelle disasently fusion
signaling	regulation of small GTPase	organization regulation of	recombinational repair	regulation of mRNA	splicing regulation of anatomical structure	regulation of translational initiation	rej national parts of national parts of national parts	gulation intro system cro rocess n	epair aloga	process or of please tion tion tion tion tion tion tion tion	mRNA metabolic	RNA polyadenylatic	translational initiation	DNA-templater transcription alongation	small molecule catabolic process	histone ubiquitination	suffer compound catabolic process	icicar DNA lication	telomere	protein polymerizat	ion Golgi organization	organelle fission
modulation of chemical synaptic	mediated signal transduction	double-strand break repair	regulation of dendritic spine	processing regulation of cell-substrate	size TORC1	regulation of receptor clustering	regulation of protein binding	TOR analing	iet regulation policity on est in process alle process 10.7500	Regulation of Rec protein signal transduction	process protein	peptidyl-lysin modification	HNA phosphodiester bond hydrolysis. exosualization	purine-combining compound methodic process	RNA	organic acid metabolic	process	pyruvate nofiNA melaholio cataboli process process	maintenance	cellular compone disassem	nt milochondrial Dly fission	clathrin membrane coat assembly
transmission	DNA damage response	of synapse structure or	regulation of cell	adhesion regulation of nervous	ngelation of magnetic magnetic set	of neuron apoptatic process regulation	codative stress	patien of Toy nearlytion of t ngation by ubliqs DNA presnase 1 post	Notoria Alternation Des mysternet Mon mysternet	ng cylopiaeric ay translation	small protein conjugation or removal	peptidy(-threonin modification	mitochondria translation	histone mRNA metabolic process	Issuectption by RNA polymorase III	process mitochondrial gene expression	hexose catabolic process		mitochondrion organization	vesicle organizati	protein ON tatumerization	basement membrane rpanization pretein bescurencessitation
small GTPase mediated signal transduction	Ras protein signal	regulation of cellular component	cycle regulation of membrane	regulation of amide	regulation of cell-substrate	of phosphate metabolic process regulation of phosphorus	regulation of mail of mail of mail of the	patrice of cylopic acide by the second secon	apol	Wat		c	head levelopme	ne pro	euron jection	response i organonitro compoun	<sub>to</sub> resp <sub>gen</sub> a nd sti	bonse to Ibiotic imulus	to to to to	cellular cellula	repairent protynogie optimie optimie film	actin
	transduction	size	potential	process	organization Olgi m	process onoatomic	monoatomic	station to schendrion	incryants in	inorganio calica	morphog	enesis	chordate	heart	circulatory	respons nitroge	respo organo	onse to nitroge	to metal neu ion ho	iomeost iron cellular imeostasis	anito atid tomeoslasia	process
intracellular	localization	cytoskalaton –dapa intracellular trans	ndent neurotran sport transp	sont tran	sicie isport otein	cation transport Sodium	transport	calcium	n protein	tansmombrer Inwnsport		cell mor	sensory	tevelopment SIS	development	compou respons	se	pound	to to response mice	otubule-bas	neuron deneuro	neuron cell-substrate poptotic adhesion
transport	protein localization	microtubule-ba transport	synaj vesio	ptic loca cle jur	ization cell iction	ion transport protein	L-amino	ion transpo receptor to	rt to cilium	n maintenance of location fic to the second	neuron pro develop	ojection f	system development <sup>fo</sup> ilopodium assembly	system and colopment motion melipoctum genization and	Op Hanthan Strong Drong Hanthan Drong Hantha	to salt	t respo	ntensily sus	radiation	process	deat intrinsio apoptotic signaling	hashester nothed in Nocesse
vesicle-mediated	to membrane establishmen	int	tracellular ated transp	r transpo loca port to or	rttein ization ganelle	ocalization o synapse alcium ion	transport t	o synapse import	region trans linitecellular lipid trans	id rosona port rosona celuter authors			muscle structure development	sube svelcoment system	rankal situlatar am development	cell	cycle	entre	estab	dishment or Itenance	tablishment of cell	carbohydrate dertvative cell metabolic adhesion
transport	of organelle localization	transport is synapse	n alon microtu	ng ubule <sup>transn</sup> tra	embrane Isport r	nto cytosol naintenance of protein	venuer L-glutarrato	protein localization to axon	exty endoceme to be endoceme barrigent	nenentare sterio sterio	learning or memor	y cogr		sport ross vascu -brain transp rier	llar response port to pain	cell cell cell	cycle	bios	ynthetic po	f cell blarity	growth	methylation rhythmic process
localization within membrane	protein localization to cell periphery	protein-contain complex localization	<sup>ning</sup> expo from	ort sec cell by	retion cell	location vesicle fusion	import i nilictionanal transmentrane transport	plasma membrane amide ransport	lysosomal transport		behavior	Del managements provide terminal terminal		anismal disul ponso nyni nimes pros uscle po	uury muscle system cos process xdocyte cell	proce	SS mainterer fert mänisterer strategi		polyamine process 	ell ision	process utilizing autophagic	circadian rhythm membrane docking

**Figure 4.6.** Treemap of the semantic similarity analysis performed on the biological processes results from the genes with significantly differentially utilized exons from the analysis using B6 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.



# **B6** Aligned Molecular Functions

**Figure 4.7.** Scatterplot of the semantic similarity analysis performed on the molecular function results from the genes with significantly differentially utilized exons from the analysis using B6 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in

selected species in the EBI GOA database.

		B6 /	Aligned Sig	nificant D	exSeq S	Semantic S	Similarity of N	lolecular Fu	nctions					
adaptivitial protein hindling	protein domair specific bindin	cell adhe moleci g bindir	esion SH3 d ule bino 1g	SH3 domain calm binding bir		cadherin binding	transferase activity, transferring phosphorus-containing groups		protein serine kinase activity		calcium ion transmembrane transporter activity monoatomic	L-glutama transmembr transporte activity low-density lipoprotein	ite ane transr er trar a ipoprotein	salt membrane nsporter nctivity
	transmembrane transporter binding	SNARE binding	scaffold protein binding	RN polym II con	IA erase ub nplex pr	piquitin-specific rotease binding	phosphotransfera		se activity, acceptor ligase activity	acyltransferase activity	cation calcium ion tran transmembrane transporter activity inorganic	particle smembrane activity voltage-gated cal channel activity inv in cardiac muscle	transporte activity cium voltaç olved ch	ealcium er activity activity ge-gated hannel
	cytoskeletal microtubule plus-end	protein bindi phosphoprotein binding	ng actinin binding	modification-de	ling <sub>pendent</sub> ATF <sup>ling</sup> pro	P-dependent otein binding	alcohol group	as acceptor	protein-disulfide reductase activity	myristoyltransferase activity	molecular entity transmembrane transporter activity	action potentia phospholip transporte activity	ni ao Nid high voi er calciu a	ctivity Itage-gated Im channel Ictivity
actin binding	binding p53 binding	glutamate receptor	dynein light intermediate chain binding	ankyrin repeat binding	neuroligi family protein binding	n ubiquitin binding	transcription coregulator activity	RNA exonuclease activity, producing 5'-phosphomonoesters	phosphoric ester hydrolase activity	hydrolase on acid ribonucl	activity, acting anhydrides <sub>hy</sub> eoside triphospi	drolase	dependent	t activity
GTPase binding	transcription	binding ATPase	clathrin binding protein	basal transc machinery b	ription inding fac	NA-binding anscription ctor binding	transcription coregula catalytic activity, Cataly acting on RNA activity		activity transcription coactivator	phos ribon triph phospha	sphatase activity ucleoside osphate itase activity	n ester bonds pho:	spholipid b	binding
	factor binding	binding	homodimerization activity	imerization alpha-au ctivity bindir		polyubiquitin fication-dependent protein binding	exonuclease activity	a tRNA phospho hydrola	activity pric diester se activity	cytoske motor ac	letal activit	alytic y, acting	ligase activity, forming rbon-oxygen	transporter activity
purine ribonucleoside triphosphate binding	single-stran	ded DNA bind	single-s DNA b	tranded inding	DNA	RNA binding			ATPase	oxidored	ictase micro	filament ca	irboxy-lya:	se
			flavin a dinucleotio	denine le binding	telomeri DNA binding	FAD binding	GTPase	e regulator act	ivity activator activity	activity, a on the CH–C of donors, as acce	oxygen ptor ac	ar adaptor tivity	activity vase activit	lipid binding

**Figure 4.8.** Treemap of the semantic similarity analysis performed on the molecular function results from the genes with significantly differentially utilized exons from the analysis using B6 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.

# **B6** Aligned Cellular Components



**Figure 4.9.** Scatterplot of the semantic similarity analysis performed on the cellular component results from the genes with significantly differentially utilized exons from the analysis using B6 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

				B	6 Aligne	d Signif	ficant De	exSeq S	Semant	ic Sim	ilarity c	of Cellular	Componen	ts						
	microtubule cytoskeleton	nuclear protein-containir complex	ng endo	endosome		ome	e nuclear body		perinuclear body region of cytoplasm		plasmic egion	synapse				Schaffer collateral – CA1 synapse	I neuron projection		jection	dundrific stadi
		intracellular protein-containing complex	transferase complex	mitochor matri	idrial chron x re	nosomal gion	vesicle tethering complex	clathrin-co: pit	<sup>sarcol</sup>	lemma י	vacuole		syr	napse	e	excitatory synapse		neuron projection		
	cell cortex	cortical	actin filament	spindle p	ole ATPa com	ase plex c	HOPS complex	mitochondri protein-contai complex	nucl	lear sp orane	liceosomal complex	GABA-orgi	presynaptic active zone	postsynaptic	postsynapt	tic paranodal	main axon	dendrit branct	c ciliary rootlet	ciliary transition fiber
		cytoskeleton	nuclear	insulin-responsiv compartment	° actomyos	in cleava	uge conder W chromos	nsed nuc some peri	clear ohery	ribosome	fibrillar center	synapse	membrane	cytoskeleton	cytoskeleto	on junction	glial cell	non-motile	filopodium	central region of growth cone
	plasma membrane region	organelle subcompartment	micro	microtubule cyt actin mbrane		SW/SNF superfamily-	type microtub end	oule small-s	ubunit perio	centriolar aterial	cytosolic region	neuromuscula junction	synapse presynaptic	disc	contact zone photorecept	t presynaptic cytosol tor postsynaptic	apical	node of	dendritic growth cone	9+0 non-motile cilium
			coat	bundle nuclear	lumen	endoplasmic reticulum	cytoplasmic ubiquitin	basal plasma	ER ubiquitin	extrinsic component	microtubule		zone	synapse	ribbon synapse	endocytic zone	dendrite	Ranvier	dendrite	ciliary tip
	Golgi apparatus	nuclear speck	sarcomere	matrix	membrane	network	complex	membrane	complex	of plasma membrane	fcolin-1-rich			si	te of		oll body	supra	molecula	r myelin
		ribonucleoprotein granule	site of DNA	ligase complex	protein complex	vesicle	podosome	endoplasmic reticulum extrinsic	chromosome	membrane	e granule lumen	somat com	odendritic partment	polarize	site of polarized grow		Jen bouy	C	omplex	sheath
	catalytic complex	cutoplacmic	damage	chromosome centromeric region	sarcoplasm	microtubu	Ile <sup>autolysoson</sup> DNA	ne component o postsynapti membrane	complex	Iranaporter complex	complex							ell	outer	basal
	ri organelle envelope	ribonucleoprotein granule	site of double-strand break	organelle membrane	stress	microboo	dy repair complex	comple	taminin x complex	complex	granule lumen					mid	body divi	ision <sup>m</sup>	embrane	cell
		Golgi	contractile	contact site	sodium	peraxison extrinsic	ne nuclear chromosom	endonucleas complex	complex	r myosin K filamen	t cylenkeleten	cell lea	ading edge	env	elope	coa	ated comp	rinsic ponent	rodomain vi	ibentic In at Ibrane
l		membrane	fiber	vesicle	channel complex	component of microtu synaptic bund membrane		<sup>le</sup> ribosom	e deacetylas complex	giutamata Inanapori vesiti membrane	RSC-type complex					mem	brane men	of me	raft my	elin sheath xonal region

**Figure 4.10.** Treemap of the semantic similarity analysis performed on the cellular component results from the genes with significantly differentially utilized exons from the analysis using B6 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.



#### **D2** Aligned Biological Processes

**Figure 4.11.** Scatterplot of the semantic similarity analysis performed on the biological processes results from the genes with significantly differentially utilized exons from the analysis using D2 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

					D2	Aligne	d Sigr	nificant	DexS	eq Semantic S	imilarity	of Biolog	ical Proc	esses						
neuron projection development	organelle assembly	supramolecular fiber organization	regulation of neurotransmit levels regulation of anatomical	f vacuo organiza	e circul syst develo	em neuron neuron neuron recept organelle	tion of yneptic brane ansmitter or levels telomere	heart relopment a	complex susseembly regulation of synapse	regulation of organelle organization	regulation of protein catabolic process	n modulat of chem synapt transmis	ion positive regulation of cellula component sion biogeness outive regulation	regulation of small GTPas mediated nt signal transduction	regulation of cellular response to stress	regulation of actin filament-based process	DNA damag response	e GTPa: mediat signa transduc	ie <sub>Ras protein</sub> ed signal I <sup>traneductio</sup> tion	n nesponse to organonitrogen n compound
	cell junction	vesicle organization	structure size	behavior	developmen	fission	maintenance	organization	activity		regulation of cell morphogenesis	of growth	of prote modificat process	in of mitotic == cell cycle	poses activi	ase by process	response to resp nitrogen sti	biotic to to to to the second	pose topologically incorrect protein	response to radiation
cytoskeleton	organization	developmental growth involved in	protein polymerization	structure development	rganization mer pol organization mer	of proxis statilizatio ential spot muscle	organization r	potential muscle	e cytoskolato organizato	regulation of cellular	regulation	regulation of cell sorphogenesis involved in	ion of glutamate strate ion glutamate signaling ani pathway	positive plateton of se metabolic process development	regulation of segulation of segulation of segulation (second particular) (second parti	an of splated statuture is stat provinces, provinces from	response to DNA endoplasmic	A damage	response	ec pathén signal veducion
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**Figure 4.12.** Treemap of the semantic similarity analysis performed on the biological processes results from the genes with significantly differentially utilized exons from the analysis using D2 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.





**Figure 4.13.** Scatterplot of the semantic similarity analysis performed on the molecular function results from the genes with significantly differentially utilized exons from the analysis using D2 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.



**Figure 4.14.** Treemap of the semantic similarity analysis performed on the molecular function results from the genes with significantly differentially utilized exons from the analysis using D2 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.





**Figure 4.15.** Scatterplot of the semantic similarity analysis performed on the cellular component results from the genes with significantly differentially utilized exons from the analysis using D2 aligned D2 samples. Color indicates the log base 10 of the p value output during the ToppFun analysis, with blue indicating the most significantly differentially expressed genes. The size of each point (log\_size) indicates the log base 10 of the number of annotations for GO Term ID in selected species in the EBI GOA database.

			D2 Aligne	ed Signi	ificant Dex	Seq Ser	nantic S	Similar	ity of Cel	Iular Component	s						
catalytic complex	centrosome	ne Golgi apparatu		apparatus vesic		mitoc m	mitochondrial matrix		nsferase omplex	synapse		GAB/ Syr Cell COI	A-ergic apse kinoton -Cell presynaptic ttact active zone active zone membrane	neuron proj		pjection	
	organelle subcompartment	actin filamen	mitochondrial t protein-containir complex	ubiqui Iigas compl	itin e cortic cytoskel	il nucl ston chromo	ear <sup>mito</sup> osome me	ochondrial outer embrane	condensed chromosome	Synapse		pa fib Punki sym	allel er to intercalated nje cell disc apse bDON	glial cell	main	ciliary c	ciliary base
microtubule cytoskeleton		ATPase complex	chromosome, centromeric region	SWI/SNF erfamily-type complex	endoplasmic reticulum pr lumen	eribosome pr	nuclear eriphery	membrane protein complex	e replication fork	Schaffer collateral – CA1 synapse	excitatory synapse	, syr inh syr	iapse ortegeneration ibitory presynaptic optoaction	projection apical dendrite	axon ciliary transition zone	tip as calyx of no Held	Itrocyte ojection n-motile olium
	nuclear body	nuclear membrane	actomyosin dea c	nistone acetylase omplex	ytopiaamic muchargestain genesia	tile U2-type spliceoson complex	al mitochondr ribosome	ial organel riboson	lar spindle <sup>ne</sup> pole	plasma membrane region	sarcolemma	extrinsic component of plasma membrane	envelope		cell leading edge		ge
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organelle envelope	vacuolar membrane	melanosome	mothyltransferation complex	fiber docytic	soctoonada SWI/SNF complex	exosome (RNase complex) cleavage	ayacasome microt plus- protein complex	end sememican azurophi p	on reconne stasse precatalytic apliceosome primary tunacription etongation	perinuclear region	cytoplasn	nic		growth		Comp	
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**Figure 4.16.** Treemap of the semantic similarity analysis performed on the cellular component results from the genes with significantly differentially utilized exons from the analysis using D2 aligned D2 samples. Gene ontology categories are grouped by semantic similarity with closely related categories being clustered together.

## Comparison of Results

The first comparison is the number of exons found to have differential utilization, and the number of genes connected to those exons. In the B6 aligned analysis, there were 21,223 differentially utilized exons. Of those, 14,245 (67.12%) were also differentially utilized in the D2 analysis, with 6,978 (32.88%) being unique to the B6 aligned DexSeq analysis. Of the 81,206 differentially utilized exons identified in the D2 aligned DexSeq, 66,961 (82.46%) were unique to the D2 aligned analysis.

The B6 aligned analysis showed 6,650 genes with differentially utilized exons. 5,828 (87.64%) of those were also identified in the D2 aligned analysis, with 822 (12.36%) being unique to the B6 aligned analysis. The D2 aligned analysis had 7,693 (56.90%) unique genes identified only in the D2 aligned analysis.

The magnitudes of the LFCs were broken into positive and negative groups in order to compare them using a t-test. The average positive LFC was had a significantly larger magnitude in the D2 aligned analysis (p < 0.0001), and the average negative LFC also had a significantly larger magnitude in the D2 aligned analysis (p = 0.0021).



**Figure 4.17.** Comparison of significantly differentially utilized exons between the B6 aligned D2

analysis (red) and the D2 aligned D2 analysis (green).



**Figure 4.18.** Differentially expressed genes identified during the DexSeq analysis. This represents genes only, not exons. Green represents the D2 aligned D2 analysis, and red the B6 aligned D2.

# Comparison of Gene Ontology

The semantic similarity analysis run using Revigo shows that there is a large amount overlap in the clustered categories between the B6 aligned and D2 aligned analyses. An analysis

of the individual gene ontology terms was used to quantify this, with 49% or more terms overlapping in each category (Table 4.1).

**Table 4.1.** Number of gene ontology terms in each category for each analysis (B6 aligned andD2 aligned). Overlap is the number of terms found in both sets of results, with percentages for

each.

	DexSe	q Gene Ontolog	y Terms and	Overlap	
	B6 Aligned	D2 Aligned	Overlap	% Overlap (B6)	% Overlap (D2)
<b>Biological Processes</b>	973	1665	829	85.20	49.79
Molecular Function	134	140	82	61.19	58.57
Cellular Component	285	380	243	85.26	63.95

# Comparison of Specific Genes

The three specific genes chosen as examples show three different effects from the D2 aligned analysis. Ninein is a gene that has been shown to have differential exon utilization by other studies done in the Miles laboratory. The D2 aligned analysis (Figure 4.20) identified two of the differentially utilized exons (34, 41) that were identified in the B6 aligned analysis (Figure 4.19) with one exon being unique to the B6 aligned analysis (16). However, exon 41, while remaining significant, went from showing higher utilization in B6 in the B6 aligned analysis to showing higher utilization D2 in the D2 aligned analysis. The D2 aligned analysis also identified several unique exons (10, 30, 33, 37, 43, 53) that were not identified in the B6 aligned analysis.

Gabra2 is a gene that has a known differential splicing event between the B6 and D2 strains (Cite, Add specific exon). The D2 aligned analysis (Figure 4.22.) showed the same differentially utilized exons (4, 11, 12, 13, 14, 16, 17, 20, 21) as the B6 aligned analysis (Figure 4.21.) with one exception that was found only in the B6 aligned analysis (10). Two unique exons found only in the D2 aligned analysis (7, 8).

Gsk3b was not found to have differential exon utilization in the B6 aligned analysis, but was found to have 5 differentially utilized exons (3, 9, 10, 11, 14) in the D2 aligned analysis (Figure 4.23).



**Figure 4.19.** B6 aligned DEXSeq splicing event analysis for Ninein. Exons 16 and 41 showed low utilization in both strains, and were determined to be retained introns using a BLAST search.

Exon 33 was an alternative splicing even, showing higher utilization in both strains.



**Figure 4.20.** D2 aligned DEXSeq splicing event analysis for Ninein. Exon 16 no longer shows differential utilization, and there are multiple new significant events not shown in the B6 aligned analysis. Exons 41 and 34 both show differential utilization, however exon 41 shows lower utilization in B6 in the D2 aligned analysis compared to showing lower utilization in D2 in the B6 aligned analysis.



**Figure 4.21.** B6 aligned DEXSeq splicing event analysis for Gabra2. Gabra2 is has a well know differential splicing event between B6 and D2, a single deleted base pair in an intron, located

between exon 3 and 4.



**Figure 4.22.** D2 aligned DEXSeq splicing event analysis for Gabra2. Gabra2 is has a well know differential splicing event between B6 and D2, located between exons 3 and 4. It can be seen that the B6 and D2 aligned DexSeq analyses identified the same differentially utilized exons, with the

D2 aligned analysis identifying slightly more events.



**Figure 4.23.** D2 aligned DexSeq analysis of Gsk3b. Gsk3b was not shown to have differential exon utilization in the B6 aligned analysis, but the D2 aligned analysis identified several exons that were differentially utilized.

# Discussion

#### Count Data Preparation

With the aid of Dr. Dozmorov, the DexSeq counts were generated using the D2 aligned annotation provided by Dr. Keane. However, that annotation would not work for the DexSeq analysis, so the B6 annotation was used. Because the count data was generated using D2 aligned D2, and the resulting D2 Ensembl IDs were converted to their B6 counterparts, using the B6 annotation for the analysis is acceptable. In order to verify the validity of this step, a future analysis using the D2 annotation with the B6 ensemble IDs converted to their D2 counterparts should be run. The results of that should be very similar, though some variance is to be expected when using a different annotation. For now, this is an acceptable method of using the D2 aligned counts in DexSeq.

#### DexSeq Results – B6 Aligned and D2 Aligned

In both the B6 and D2 aligned analyses, the LFCs were evenly distributed between positive and negative, with the D2 aligned analysis skewing slightly towards the positive. This indicates that the analysis did not have significant bias towards either positive or negative LFC that would affect the results or indicate an error in the analysis.

In both the B6 (Figure 4.1) and the D2 (Figure 4.3) aligned analysis, there is high correlation between out exonic regions, with B6 and D2 correlating more to themselves than to each other, though the correlation between the two is still quite high. There is low correlation between the out exonic regions and the rest of the exons, though the "rest of the exons" correlate strongly to each other. Interestingly one of the B6 samples correlates more strongly to the D2 than to the B6, though not enough to be an outlier. This is to be expected, as the samples are taken from the same species, and this indicates that it is possible to differentiate between closely related substrains. The out exonic regions having low correlation with the rest of the exons also indicates that the analysis was done correctly.

## Comparison of Results

Similarly to the differential expression analysis, the D2 aligned analysis showed significantly larger numbers of differentially utilized exons, and a correspondingly larger number of genes with significantly differentially utilized exons. The retention among genes was as good as the differential expression analysis, with 87.64% being identified in both the B6 and D2 aligned analyses. The exon overlap was much lower, at 67.12%, but with a much larger number of unique differentially utilized exons. This indicates that the D2 aligned analysis does provide an improvement in the identification of differentially utilized exons, though the lack of retention from the B6 aligned analysis warrants further investigation. It may be related to the usage of the B6 annotation with the D2 aligned counts, and this will be tested in the future.

The magnitude of the LFCs was higher in the D2 aligned analysis for both positive and negative LFCs. This indicates that not only were more differentially utilized exons identified in the D2 aligned analysis, those exons were also significantly more expressed or less expressed in the D2 aligned analysis. This indicates potential improvement, as the analysis run using D2 aligned counts showed a significant difference from the B6 aligned counts. It is important to quantify which analysis is "better", however. Future analysis will look more into differential exons and gene expression, specifically focusing on splicing events to determine this. Emma Gnatowski has begun this analysis already. Another goal is to look more deeply into the exons

identified in both analyses, with a focus on the unique exons. If they follow a similar pattern to the overlapping exons in size and LFC, then that removes a potential factor causing the D2 aligned analysis to identify them. Eventually all factors other than the D2 alignment will be accounted for and a definitive answer will be found.

# Comparison of Gene Ontology

The high amount of overlap is what was expected and is encouraging to see. Because the D2 aligned analysis had much larger numbers of significant GO terms (Table 4.1.) while still having high overlap with the B6 aligned analysis, it can be inferred that aligning to the D2 provides a noticeable change in the results while not losing results found in the B6 aligned analysis.

# Comparison of Specific Genes

The specific genes compared are either currently being studied in the Miles laboratory or, in the case of Gabra2, have a known alternative splicing event.

With Ninein, the most interesting result is the flip of exon 41. In the B6 aligned analysis, it showed higher utilization in the B6 samples, whereas in the D2 aligned analysis it showed higher utilization in the D2 samples. This warrants further investigation, as this indicates not only a significant change in the magnitude of the event, but a complete reversal in the direction. The D2 aligned analysis also identified unique differentially utilized exons, and these should be tested as described above to determine if there are any other factors causing these to be missed in the B6 aligned analysis.

Gabra2 showed the same directionality of each change, and the D2 aligned analysis identified several new differentially utilized exons. This is in keeping with the results seen

before, and it is good to see that the differentially utilized exons from the B6 aligned analysis, including a known deletion that occurred and became fixed in the B6 line, located in the intro between exons 3 and 4 (Mulligan et al., 2019). This shows that the D2 aligned analysis is successfully identifying differentially utilized exons, and not simply giving false positives.

With Gsk3b, it was not found to have differential exon utilization in the B6 aligned analysis. The D2 aligned analysis did however find several exons with differential utilization. This shows why aligning D2 mice to the D2 genome for these analyses is important, as it can identify differentially expressed exons and genes that would have otherwise been missed.
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# **Appendix 1: Files and Data**

These files are presented as either dropbox links or pathways to the file on the VIPBG group server. The Dropbox links will take you to the folder where all of the listed files can be found.

### **Dropbox Links**

Data Preparation

Count Data Preparation

**B6** Aligned Counts

**D2** Aligned Counts

Differential Expression Analysis

Working Directory

<u>Results</u>

<u>B6 Aligned</u>

D2 Aligned

**Comparisons** 

Differential Exon Usage Analysis

<u>B6 Aligned DexSeq</u>

D2 Aligned DexSeq

Comparison of Results

### Paths to Group Server Files

/home/projects/MilesLab/teamshare/DZ\_B6\_Alignment/

/home/projects/MilesLab/teamshare/DZ\_D2\_Alignment/

/home/projects/MilesLab/teamshare/DZ\_D2\_Alignment\_DexSeq/

/home/projects/MilesLab/teamshare/B6D2\_DeepSeq/

## **Appendix 2: Code**

This appendix follows the same format as the previous. Dropbox links to folders that contain the scripts used, and paths to the scripts on the group server.

### **Dropbox Links**

#### Count Data Preparation

"C:\Users\zelif\Dropbox (MilesLab)\Miles and Dustin Z\Aim 1 - Differential Exon, Differential Expression, Gene Ontology, and Transcript Level\Data Preparation\Count Data Preparation\commonkeys\_first\_step\_of\_gene\_ID\_conversion.py"

"C:\Users\zelif\Dropbox (MilesLab)\Miles and Dustin Z\Aim 1 - Differential Exon, Differential Expression, Gene Ontology, and Transcript Level\Data Preparation\Count Data Preparation\Gene ID and Name extraction from gff3 script.py"

### Differential Expression Analysis

"C:\Users\zelif\Dropbox (MilesLab)\Miles and Dustin Z\Aim 1 - Differential Exon, Differential Expression, Gene Ontology, and Transcript Level\Differential Expression Analysis\Code\DZ\_DESeq2\_B6\_aligned\_script\_3\_19\_23.R"

"C:\Users\zelif\Dropbox (MilesLab)\Miles and Dustin Z\Aim 1 - Differential Exon, Differential Expression, Gene Ontology, and Transcript Level\Differential Expression Analysis\Code\GTF Conversion for DEX seq.py"

## Paths to Group Server Scripts

/home/projects/MilesLab/teamshare/DZ\_D2\_Alignment/scripts/

/home/projects/MilesLab/teamshare/DZ\_D2\_Alignment\_DexSeq/