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Identification of Alternatively Spliced Genes in Metabolic Disease Pathways

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A capstone project submitted for Graduation with University Honors

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Abstract

As of 2022, one-third of US adults experience metabolic diseases. Current therapies treat symptoms but do not address disruptions in signaling pathways of the liver that lead to the development of metabolic diseases. It is now recognized that many genes involved in metabolic disease pathways are alternatively spliced. This research aims to identify real alternative splicing events at genes that can serve as therapeutic targets. Alternative splicing is a critical process by which exons within pre-mRNA are either included or removed to generate diverse mRNAs and proteins. Transcriptomic data from the livers of both male and female mice under several different conditions–fed versus fasted, wildtype, and α7HMZ mice were analyzed for splicing events using RNA-seq program, DEXSeq. α7HMZ mice express an alternative form of the transcription factor HNF4a, a critical liver and metabolism regulator. Current RNA-seq programs cannot distinguish alternative splicing from other activity occurring at the gene locus, so manual curation is necessary. Using a curation criterion, I manually analyzed 177 genes identified by the program for alternative splicing events. My analysis identified splicing events at mitochondrial genes usually expressed during fasting conditions and genes whose loss-of-function is implicated in obesity, hyperglycemia, and hypertension. Future research will analyze the mechanistic roles of these mitochondrial genes in various metabolic disease models.

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Introduction

Millions of people across the world today suffer from debilitating metabolic diseases. According to the NIH, as of 2022, 1 in 3 US adults meet the criteria for metabolic syndrome (Metabolic Syndrome. NHLBI, NIH.), which includes a cluster of biological factors characterized by abdominal obesity, dyslipidemia, hypertension, and type 2 diabetes (Moore, 2017). With a predicted further increase in the incidence of metabolic syndrome, more people are at risk for developing more serious chronic conditions and comorbidities, making it urgent to understand metabolic disease pathways and identify improvements for current interventions. While current treatments and medications for these diseases have saved lives by treating symptoms, future therapies must address the metabolic dysregulation that occurs when tightly controlled signaling pathways in the liver go awry and lead to disease.

Recent studies have now recognized that much of the dysregulation involved in metabolic disease pathways result from alternatively spliced (AS) genes; thus, identifying these is critical to elucidating disease mechanisms (Baralle et al., 2017). We utilize the bioinformatics programs, DEXSeq and DESeq2, to analyze RNA-seq data from the livers of male and female mice under different conditions: fed versus fasted states; wildtype (WT) versus HNF4a7 HMZ. The α7HMZ mice express an isoform of the transcription factor HNF4a, a critical liver and metabolism regulator, that has been found to be upregulated in liver cancer, in the livers of mice fed high-fat diets, and alcoholic livers (Fekry et al. 2018; Argemi et al, 2019). RNA-seq programs called 177 genes as loci of alternative splicing events. My research aims to manually verify the programs' identification of 177 alternative splicing events and identify those involved in metabolic disease pathways to screen for potential therapeutic targets for metabolic diseases.

Signaling Pathways and the Liver

Studying alternative splicing in the liver is important for understanding metabolic disease because the liver plays a critical role in regulating metabolism. The liver maintains metabolic homeostasis during fed and fasted states by employing tightly controlled signaling pathways (Kalra et al., 2017). During a prolonged fast, a drop in blood sugar decreases the need for insulin. A decrease in insulin increases glucagon and catecholamines, which activate AMP-activated protein kinase (AMPK), and in turn activates the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) (Pawlak et al., 2013). As shown in Figure 1, PPARα subsequently increases the expression of genes involved in gluconeogenesis and glycogenolysis, processes to maintain glucose levels in the blood for vital organs such as the brain. As fasting continues, the liver also increases fatty acid oxidation, the process to produce ketone bodies for use as an alternative fuel source for the brain and other tissues (Cahill et al., 2006). If gluconeogenesis or ketogenesis is disrupted, it can lead to the onset of metabolic conditions such as hypoglycemia or ketoacidosis (Shimano, 2001).

In contrast, during the fed state, the liver shifts its focus to storing excess nutrients. Insulin levels increase, which promotes glucose uptake and glycogen synthesis in the liver. In individuals with insulin resistance, excess glucose and fatty acids accumulate in the liver, leading to the development of Non-Alcoholic Fatty Liver Disease (NAFLD), a condition characterized by fat accumulation in the liver. The liver also increases de novo lipogenesis to convert excess glucose into triglycerides, which are stored in adipose tissue or exported as very low-density lipoprotein (VLDL) particles (Cahill et al., 2006). This process is regulated by several transcription factors, including sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) (Shimano, 2001). Dysregulation of

these transcription factors (SREBP-1c and ChREBP) can contribute to the development of metabolic diseases (Shimano, 2001).
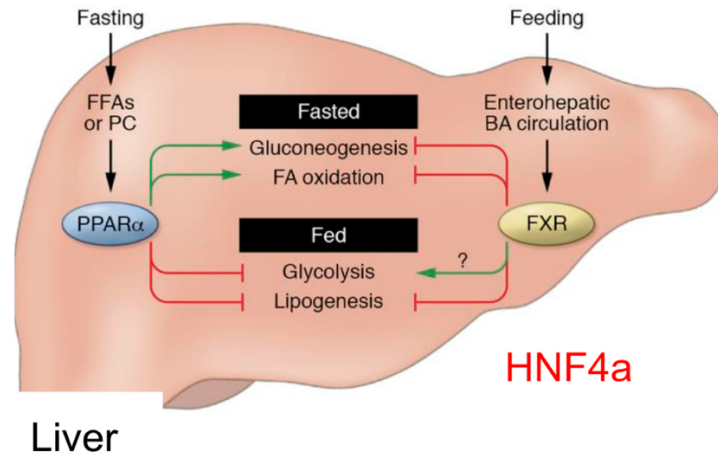

Figure 1. Liver's Pathway Mechanism of Action in Fed and Fasted states
(modified from Preidis et al., 2017)

HNF4a and the Liver

Hepatocyte Nuclear Factor-α (HNF4α) is a highly conserved transcription factor. It is expressed in the liver and recognized as the master regulator of liver-specific genes as it is essential for the proper functioning of the adult and fetal liver (Battle et al., 2006.; Torres-Padilla et al., 2001). It is also expressed in other tissues including the kidney, pancreas, stomach, and intestine. The *HNF4A* gene consists of two promoters, P1 and P2, and along with alternative splicing processes, produces multiple variants or isoforms of the HNF4α protein (Ko et al., 2019). The two promoters are expressed under different conditions and play distinct roles in different tissues.

When the P1 promoter is expressed, the HNF4α1/α2 isoform of the protein is produced. When the P2 promoter is expressed, it gives rise to the HNF4α7/α8 (Chellappa et al., 2016) protein isoform. Under normal conditions, mice engineered to produce only P1-driven HNF4a proteins and mice producing only P2-driven HNF4a proteins appear healthy. However, when subjected to treatments that cause colitis or colon cancer, mice that produce only P2- HNF4a

proteins experienced more colitis and developed more tumors than untreated mice due to increased expression of pro-inflammatory factors (Chellappa et al., 2016). This finding explains how the dysregulation of HNF4α in the liver can cause defective glucose homeostasis, dyslipidemia, and hepatic defects, many of which are observed in metabolic diseases (Hayhurst et al., 2001).

Alternative Splicing

Alternative splicing is a very common occurrence in the human genome. In fact, nearly 95% of genes with multiple exons undergo alternative splicing (Bhadra et al., 2020). This is the process by which 25,000 genes can produce 90,000+ proteins (E et al., 2013). As shown in Figure 2, introns in pre-mRNA are spliced out and the remaining exons in mRNA can be spliced together in different orders to produce different proteins. Since alternative splicing is critical to generating protein diversity, changes in splicing patterns have led to abnormal gene expression. This abnormal gene expression can later contribute to the pathogenesis of metabolic diseases such as diabetes, obesity, and non-alcoholic fatty liver disease (NAFLD) (Bhadra et al., 2020).
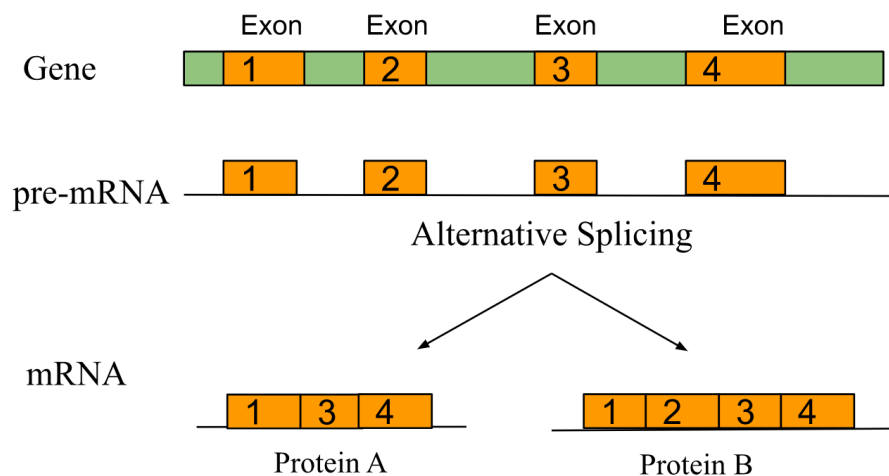

Figure 2. Alternative Splicing Mechanism. Alternative Splicing is a process by which exons within pre-mRNA are either included or removed to generate differentially spliced mRNAs. Splicing events generate diversity in protein products and can result in pathogenic isoforms that contribute to metabolic disease.

In the liver, alternative splicing is key to regulating the expression of *HNF4a* and other glucose and lipid metabolism genes. Specifically, studies have shown that changes in the splicing pattern of HNF4a can lead to dysregulation in the expression of genes involved in lipid metabolism, contributing to the development of NAFLD (Battle et al., 2006). Splicing of *HNF4a* can result in isoforms with different transactivation activities, suggesting a role in the regulation of liver-specific gene expression in metabolic disease pathways (Briançon et al., 2006).

Methods

Young adult (16 to 20 weeks) male WT and HNF4a exon swap mice (α7HMZ) (Briançon and Weiss, 2006) (see Figure 3) were fed a standard lab chow (LabDiet, #5001, St. Louis, MO) and maintained in an SPF vivarium. Mice were fed or fasted for 12 hours from 10:30 PM to 10:30 AM (lights off at 7:30 PM, lights on at 7:30 AM) and euthanized by CO_2 asphyxiation at 10:30 AM followed by tissue harvest. Their livers were examined for changes in gene expression by RNAseq using DESeq2 software and Illumina sequencing (Deans et al., 2021). The RNAseq results were aligned to the mouse genome (mm39) and analyzed for alternative splicing using DexSeq software (Martinez-Lomelli, J., 2022). RNA-Seq #1 is from WT and a7HMZ exon swap mice in a mixed 129/Sv plus C57BL/6 background. RNA-Seq #2 is from WT and a7HMZ exon swap mice backcrossed into C57BL6/N. Liver samples from three mice per condition (triplicates) were prepared in an identical fashion. Both sets of data were analyzed in an identical fashion and compared for commonly splicing transcripts. Care and treatment of the animals were in strict accordance with guidelines from the Institutional Animal Care and Use Committee at the University of California, Riverside.

Statistical Modeling Software

DESeq2 uses a statistical model to accurately detect differences in gene expression by considering the relationship between the amount of gene expression and the variability in the data. The differential expression analysis involves comparing gene expression levels between two or more samples and identifying genes that are significantly upregulated or downregulated. DexSeq detects splicing events that are differentially used between two or more sample groups by modeling differences in read counts across exons of a gene and identifying exons that have significantly different usage between the samples being compared. This software was used to identify isoform-level changes in gene expression and understand how alternative splicing events may be associated with disease or other biological processes.

Exon-swap mice

HNF4 exon swap mice (α7HMZ) express an alternative form of *HNF4A* that is not expressed in normal adult liver (Braincon and Weiss, 2006). This isoform has been identified as a major isoform upregulated in liver cancer, in the livers of mice fed high-fat diets, and alcoholic livers making it a relevant model to study metabolic diseases (Fekry et al. 2018; Argemi et al, 2019). As shown in Figure 3, α7HMZ mice have the exon 1A coding sequence deleted at the P1 promoter and replaced by that of exon 1D using a plasmid construct so that they are homozygous for exon 1D and referred to as 'α7HMZ-only' mice (Chellappa at al., 2016). This minimal intervention makes it so the mice do not have obvious phenotypic defects or lethal defects as HNF4a was not completely knocked out.
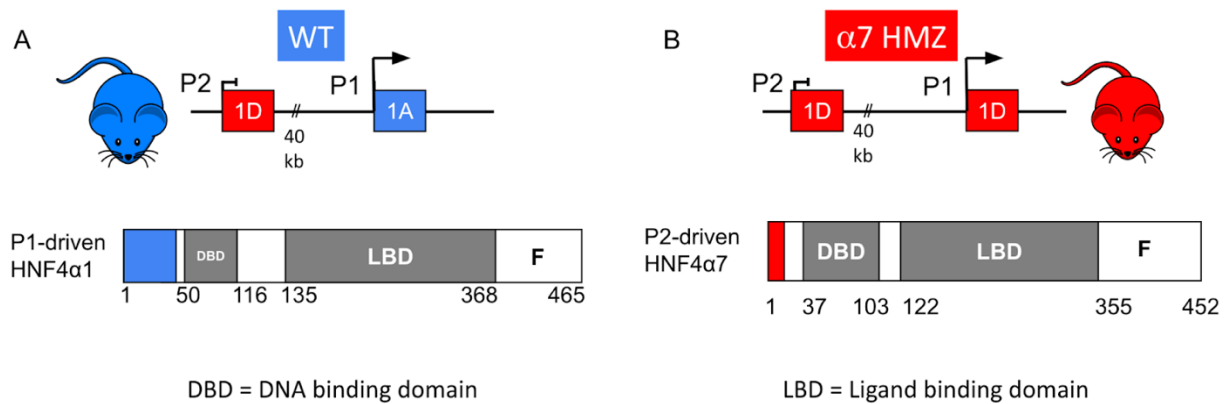
Figure 3. HNF4a exon swap mice.
Schematic of mouse *HNF4a* gene in (A) WT and (B) a7HMZ mice showing the two promoters, P1 and P2, and the P1- and P2-driven *HNF4a* isoforms they express (bottom). DBD, DNA binding domain; LBD, ligand binding domain. Exon 1D and Exon 1A encode Activation Function (AF-1) and distinguish the two isoforms.

Fasting HNF4a7 HMZ mice and comparing them to fasted WT HNF4a mice is important for understanding alternative splicing in metabolic disease because fasting is a metabolic stressor that induces changes in gene expression and splicing events in the liver. By comparing the alternative splicing events in the livers of these two mouse models under fasting conditions, we can identify splicing events that are specifically involved in metabolic regulation and may be dysregulated in metabolic diseases.

Following the generation of transcriptomic (RNAseq) data from the livers of male mice under several different conditions – fed versus fasted; wildtype (WT) versus α7HMZ – the RNAseq data was analyzed for differential expression (DESeq2) and splicing events (DEXSeq) (Figure 4). It was observed in RNA-Seq dataset #1 that the greatest number of potential splice variants was between the WT and a7HMZ mice that had been fasted for 12 hours (Martinez-Lomelli, J., 2022). Therefore, in the two RNA-Seq datasets, we compared the differentially expressed genes (DEGs) to the alternatively spliced genes (DASGs) in 12h-fasted mice as well as WT vs a7HMZ mice. RNA-Seq #1 identified 5,877 genes (3782 DASG + 2095 DEG) with an overlap of 1,238 genes classified as a DEG and DASG. RNA-Seq #2 identified 3,787 genes

(2019 DASG + 1768 DEG) with an overlap of 586 genes classified as a DEG and DASG. We then looked at the overlap between the two triplicate RNA-seq datasets (1238 genes from RNA-seq #1 and 586 genes in RNA-seq #2, n=6) to identify 177 DASGs found in both datasets to increase the probability of identifying real splicing events.
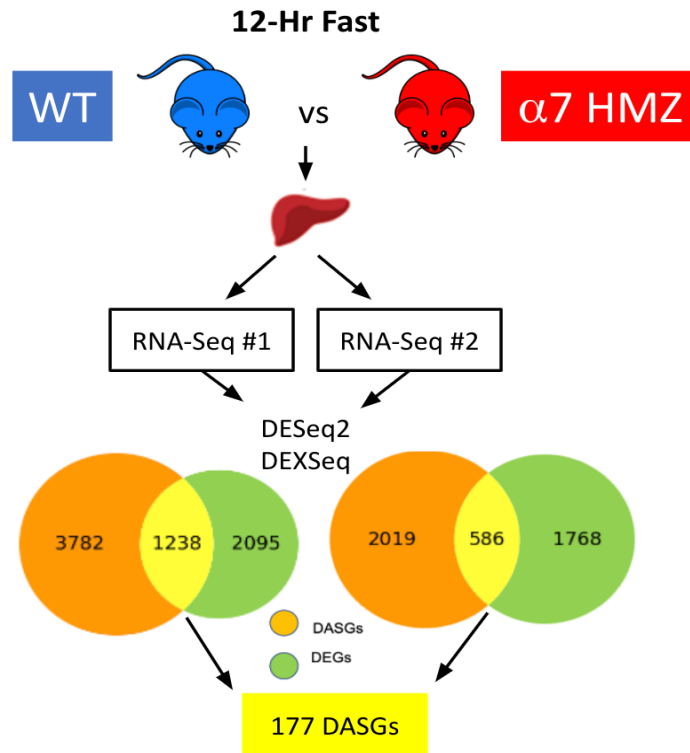

Figure 4. Experimental Design.
Gene expression in livers of male mice on 12-hr fasts was analyzed by RNA-seq using DESeq2 software and alternative splicing was analyzed using DexSeq. Alternative splicing 177 genes that were both alternatively spliced (DASGs) and alternatively expressed (DEGs) in both data sets from male mice were manually curated.

Results

Improvements to Statistical Modeling Software

DESeq2 and DexSeq are powerful tools for analyzing gene expression and identifying differential usage of exons in alternative splicing events. However, like any computational programs, they have certain limitations that can impact their ability to detect alternative splicing

accurately. Through the course of these project, I was able to develop suggestions for improvement on their end:

1. Read mapping biases: Both DESeq2 and DexSeq heavily rely on the accuracy of read mapping to reference genomes. However, mapping biases can occur due to the presence of repetitive regions, genomic structural variations, or errors in the reference genome itself. These biases can lead to misalignment of reads, potentially affecting the detection of alternative splicing events. To overcome this limitation, incorporating alternative aligners or optimizing existing aligners to handle challenging regions would enhance the accuracy of read mapping.

2. Transcript annotation quality: Accurate annotation of transcripts is crucial for correctly identifying alternative splicing events. However, existing transcript databases may be incomplete or outdated, lacking information on tissue-specific or condition-specific isoforms. Improving transcriptome annotation by integrating multiple sources of evidence, such as RNA-seq data, proteomics data, and long-read sequencing technologies, would enhance the sensitivity and specificity of alternative splicing detection.

3. Lowly expressed isoforms: DESeq2 and DexSeq perform better when analyzing highly expressed genes. Lowly expressed isoforms often have lower read coverage, making it challenging to detect significant differences in their expression levels accurately. Applying specialized algorithms or statistical methods specifically designed for low-count data, such as single-cell RNA-seq analysis tools, may improve the detection sensitivity for lowly expressed isoforms.

4. Complexity of alternative splicing: Alternative splicing is a complex and diverse process, involving multiple splicing events, including exon skipping, alternative 5' or 3' splice site usage, intron retention, and more. DESeq2 and DexSeq primarily focus on exon-level differential expression and usage. To comprehensively capture alternative splicing events, incorporating additional algorithms or tools that specifically target different splicing events would provide a more comprehensive analysis.

5. Experimental design considerations: DESeq2 and DexSeq rely on the experimental design and sample size to detect significant differences. Insufficient sample size or unbalanced groupings can limit the statistical power of these tools, potentially leading to false negatives or limited sensitivity. Careful consideration of the experimental design, including sample size determination, appropriate control groups, and biological replicates, is crucial to ensure accurate and reliable detection of alternative splicing events.

6. Validation of detected events: Computational tools like DESeq2 and DexSeq provide valuable insights, but their predictions should be experimentally validated. Incorporating experimental techniques, such as RT-PCR, Iso-Seq, or nanopore sequencing, to confirm the alternative splicing events identified by these programs would strengthen the reliability of the results.

While DESeq2 and DexSeq are valuable tools for analyzing gene expression and alternative splicing, they have limitations that can affect their accuracy. Addressing these limitations through improvements in read mapping, transcript annotation, specialized algorithms, experimental design considerations, and validation techniques would enhance their ability to detect alternative splicing events accurately and comprehensively.

Manual Curation Criteria

Between the two RNA-seq datasets we generated, 177 genes were called alternatively spliced (DASGs). These were then manually curated using the following inclusion and exclusion criteria to identify genes with real alternative splicing events. The genes were visualized for consistency by comparing the gene body to normalized exon counts from DEXSeq using UCSC Genome Browser (provides a visual representation of the gene body, including genes of interest, regulatory regions, and other important features to explore gene expression and modifications). Genes that were marked by the program as loci where differential expression occurred were included if they fell under the following inclusion criteria and disqualified if they fell under the following exclusion criteria:

Inclusion Criteria:

1. Differential number of reads mapped to alternatively spliced exons in α7HMZ and WT
2. Alternatively spliced exons were called consistently across both datasets
3. Alternatively spliced exon has >100 normalized reads

Exclusion Criteria:

1. One genotype being consistently higher than the other across all exons
2. Values of biological triplicates vary widely
3. Small difference in normalized counts between genotypes
4. Expression levels <100 normalized reads
5. Putative alternatively spliced exon overlaps with another gene
6. Reads not mapping to known exon
7. Reads into introns

One example of a gene analyzed using these criteria is shown below (Figure 5). DNA-directed

RNA polymerase II subunit, Rbp4 is a gene that met the negative criteria of one genotype being

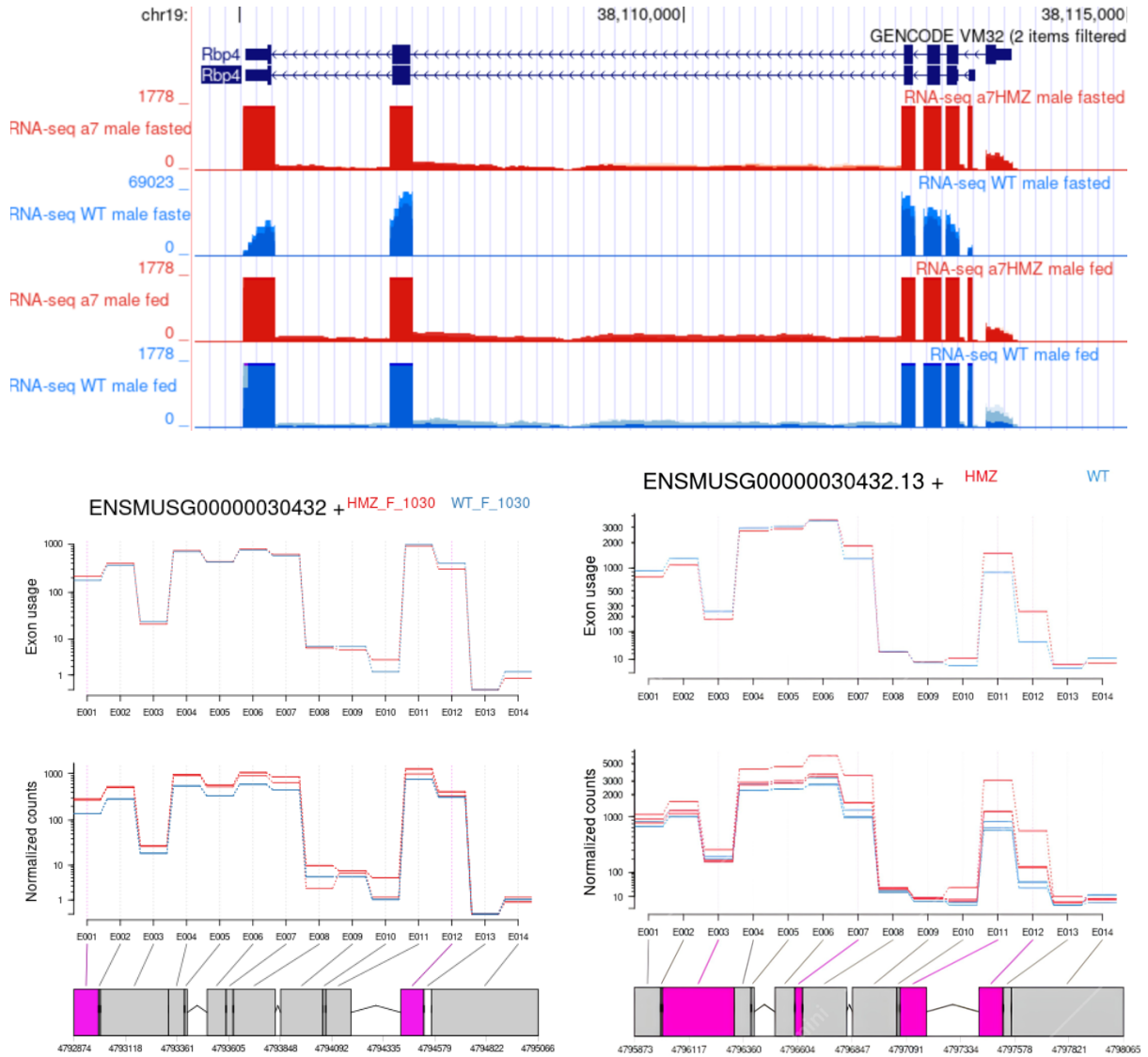consistently higher than the other, suggesting differential expression but not alternative splicing.

Figure 5. Example of a gene that falls within the exclusion criteria.
Expression levels are displayed in UCSC Genome Browser(top) by red and blue bars of male α7 and WT mice in fasted conditions (RNA-Seq #1, 2). Gene body(top) has noticeably fewer exons than shown by Dexseq maps(bottom), making mapping challenging; Red circled exons: called alternatively spliced across both datasets by DexSeq. Expression levels are <100 normalized counts, falling under the significance cutoff.

The results of my study concluded that all 177 genes analyzed fell into the following disqualifying

categories: small differences in splice levels (<100); putative alternatively spliced exon overlaps

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with another gene; RNA-seq reads that did not map to known exons; and RNA-Seq reads went through into introns of highly expressed genes. Out of 177 DASGs, there were only 16 genes that were not disqualified by the negative criteria but did not meet all the inclusion criteria to label it a splicing target (Pafah2, Ywhae, Rpl28, Itih3, Fau, Bbox1, Hpd, Gstk1, Acaa1b, mtarc2, Ndufb10, Qprt, C8g, Ugt2b36, Cyp3a11, Serpina1e). Although the results of my research demonstrated no gene fully satisfied the positive criteria to count as a significant alternative splicing event, these genes may still play a role in liver dysregulation and metabolic disease pathways that the criteria do not account for. In the fasted mice, my results also identify numerous genes related to mitochondrial function as potential splice sites. These mitochondrial genes are known to be expressed during fasting conditions and when these genes undergo loss-of-function, people can develop obesity, hyperglycemia, and hypertension.

Discussion

Genes Identified as Potentially Alternatively Spliced

1. Pafah2 (Platelet-activating factor acetylhydrolase 2): Pafah2 is involved in the degradation of platelet-activating factor (PAF), a phospholipid mediator that has been implicated in inflammation, lipid metabolism, and fibrosis in the liver (Karidis et al., 2006). Dysregulation of Pafah2 activity may contribute to liver dysfunctions associated with metabolic diseases.

2. Ywhae (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon): Ywhae is a member a highly conserved protein family involved in numerous cellular processes, including metabolism, signaling, and cell cycle control. In the liver, it has been linked to insulin signaling and glucose metabolism (Martens et al., 2010). Altered

Ywhae expression or function may disrupt these processes, potentially leading to metabolic disorders such as insulin resistance.

3. Rpl28 (Ribosomal protein L28): Rpl28 is a component of the ribosome, the cellular machinery responsible for protein synthesis. While Rpl28's specific role in liver dysregulation and metabolic disease pathways is not well-characterized, dysregulation of ribosomal proteins can impact protein synthesis, potentially affecting hepatic metabolism and leading to metabolic diseases (Genecards).

4. Itih3 (Inter-alpha-trypsin inhibitor heavy chain 3): Itih3 is a member of the inter-alpha-trypsin inhibitor (ITI) family, involved in extracellular matrix stabilization and modulation of inflammation. In the liver, Itih3 has been associated with fibrosis and inflammation (Nakamura et al., 2019). Dysregulated Itih3 expression may contribute to hepatic fibrosis and metabolic dysfunction.

5. Fau (FAU ubiquitin like and ribosomal protein S30 fusion): Fau is a protein involved in multiple cellular processes, including cell growth regulation and apoptosis. While the precise role of Fau in liver dysregulation and metabolic diseases is not well-defined, dysregulated Fau expression has been linked to cancer and cellular stress responses, suggesting a potential involvement in liver pathologies (Pickard et al., 2009).

6. Bbox1 (Bifunctional apoptosis regulator): Bbox1 plays a role in apoptosis regulation and redox homeostasis. Dysregulation of Bbox1 has been associated with oxidative stress and cellular dysfunction in various tissues (Yang et al., 2020). In the liver, Bbox1 may contribute to metabolic disorders through its impact on redox balance and apoptosis regulation.

7. Hpd (4-hydroxyphenylpyruvate dioxygenase): Hpd is involved in the tyrosine catabolic pathway and is essential for the breakdown of tyrosine to fumarate. Mutations in Hpd can lead to hereditary tyrosinemia type III, a metabolic disorder characterized by elevated tyrosine levels and liver dysfunction (Rüetschi et al., 2000). Dysregulated Hpd activity may disrupt hepatic tyrosine metabolism, contributing to metabolic diseases.

8. Gstk1 (Glutathione S-transferase kappa 1): Gstk1 belongs to the glutathione S-transferase (GST) family, which in the liver, are involved in the metabolism of various substances, including drugs, toxins, and reactive oxygen species (Prysyazhnyuk et al., 2021). Dysregulation of Gstk1 expression or activity may compromise hepatic detoxification processes, potentially leading to metabolic diseases and liver damage.

9. Acaa1b (Acetyl-CoA acyltransferase 1B): Acaa1b is an enzyme involved in the degradation of fatty acids through the beta-oxidation pathway. In the liver, beta-oxidation is a key process for energy production and lipid metabolism. Dysregulated Acaa1b expression or activity may disrupt fatty acid metabolism and contribute to the development of metabolic disorders, such as non-alcoholic fatty liver disease (NAFLD) (Kim et al., 2022).

10. Mtarc2 (Mitochondrial aminoacyl-tRNA synthetase complex-interacting multifunctional protein 2): Mtarc2 interacts with the mitochondrial aminoacyl-tRNA synthetase complex and is involved in mitochondrial translation and aminoacylation processes. Although the specific role of Mtarc2 in liver dysregulation and metabolic diseases is not well-established, mitochondrial dysfunction has been implicated in the pathogenesis of metabolic disorders (Genecards). Altered Mtarc2 function may impact mitochondrial processes and contribute to hepatic metabolic dysfunction.

11. Ndufb10 (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10): Ndufb10 is a subunit of mitochondrial complex I, a key component of the electron transport chain involved in oxidative phosphorylation. Impaired mitochondrial electron transport and ATP production have been associated with metabolic disorders and liver dysfunction (Genecards). Dysregulation of Ndufb10 expression or function may disrupt mitochondrial energy metabolism, contributing to metabolic diseases.

12. Qprt (Quinolinate phosphoribosyltransferase): Qprt is an enzyme involved in the kynurenine pathway of tryptophan metabolism, regulating the production of quinolinic acid, a neuroactive metabolite. Dysregulated tryptophan metabolism and increased quinolinic acid levels have been implicated in liver inflammation and metabolic disorders (Hestad et al., 2022). Altered Qprt activity may contribute to dysregulated tryptophan metabolism and associated liver dysfunctions.

13. C8g (Complement component 8 gamma chain): C8g is a component of the complement system, a crucial part of the immune response. Dysregulation of the complement system has been implicated in liver diseases, including non-alcoholic steatohepatitis (NASH) and liver fibrosis (Genecards). Altered C8g expression or activity may disrupt immune responses in the liver, contributing to inflammatory and metabolic liver diseases.

14. Ugt2b36 (UDP glucuronosyltransferase 2 family, member B36): Ugt2b36 is an enzyme responsible for glucuronidation reactions, a key step in the metabolism and elimination of xenobiotics and endogenous compounds. In the liver, Ugt2b36 and other UGT enzymes play a crucial role in drug metabolism and detoxification processes (Kurita et al., 2017). Dysregulation of Ugt2b36 expression or activity may compromise hepatic detoxification capacity and contribute to metabolic disorders, such as drug-induced liver injury.

15. Cyp3a11 (Cytochrome P450 family 3 subfamily A member 11): Cyp3a11 belongs to the cytochrome P450 enzyme family, which plays a critical role in the metabolism of endogenous and exogenous compounds, including drugs, toxins, and steroids. In the liver, Cyp3a11 is involved in the metabolism of numerous drugs and environmental chemicals (Li et al, 2009). Dysregulation of Cyp3a11 expression or activity can lead to altered drug metabolism, potentially contributing to liver dysregulation and metabolic diseases.

16. Serpina1e (Serpin family A member 1E): Serpina1e is a member of the serine protease inhibitor family, which regulates protease activity. While the specific role of Serpina1e in liver dysregulation and metabolic diseases is not extensively studied, other members of the serpin family have been implicated in liver inflammation, fibrosis, and cancer (Genecards). Dysregulation of Serpina1e expression or function may contribute to hepatic protease imbalance and associated pathologies.

Limitations and Future Directions

DexSeq called over 3000 differentially spliced and expressed genes. Having two comparative data sets was helpful as it allowed for narrowing down to 177 DASGs by finding common genes in the two datasets. However, manual curation of these DASGs showed it may not be worth following up with wet bench testing. Of these, 16 genes did not fall into the negative criteria and could be followed up with alternate RNAseq programs to analyze the gene locus for any other forms of differential usage. Livers were harvested at the same time of day following the same fasting regime, so differences in the two DexSeq-generated maps for the two RNAseq datasets may be a result of the background or strains of the mice in the two datasets as well as any RNAseq noise. Different sequencing protocols were used in the two RNAseq datasets: 75-bp single-end versus 150-bp paired-end which could account for some of that difference. Current

RNAseq programs do not account for all the different variables that could go into making an accurate alternative splicing call. DexSeq was selected for this purpose because it gave the greatest number of calls, but future research could use alternate programs to analyze gene targets more accurately.

While aligning the DexSeq-labeled exons to exons on the gene body in UCSC Genome Browser, there were differences in the number of exons identified and the orientation of the gene. It made aligning these exons difficult. This was exacerbated by exon maps in DexSeq not coinciding with the orientation of the gene in UCSC genome browser. The program called many mitochondrial genes usually expressed during fasting conditions and implicated in obesity, hyperglycemia, and hypertension. With this research, we were able to support a role for alternative splicing in metabolic disease that should be followed up with wet-lab testing. Further research could be conducted to analyze the roles of those mitochondrial genes in similar metabolic disease models.

Additionally, with intermittent fasting being a recommended intervention to improve liver health in patients with metabolic disease, studying the role of the liver master regulator, *HNF4A* and its isoforms with transcriptomics data from mice as done in our study suggests can clarify essential molecular and cellular mechanisms that regulate liver metabolism during fasting and feeding (Bolotin et al., 2019; Lu et al.,2016). Previous studies have shown intermittent fasting alters HNF4a activity (Hatchwell et al., 2020). Thus, studying subsequent gene expression changes using exon-swap mice and RNAseq data could clarify the gene expression pathways by which new diet interventions address metabolic disease and liver health to inform preventative nutrition changes or drug therapies.

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