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Linking RNA Methyltransferases and Epitranscriptomic Marks to Diseases of the Brain and Pharmaceutical Responses

An honors thesis presented to the
Department of Biology,
University at Albany, State University of New York
in partial fulfillment of the requirements
for graduation with Honors in Biology
and
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Nicole Gaglia

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Research Mentor: Thomas Begley, Ph.D.

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Abstract

The Central Dogma of Molecular Biology states that DNA goes to RNA to proteins through the process of transcription and translation. tRNAs have numerous post transcriptional modifications that promote anticodon-codon interactionns and protein synthesis. The spectrum of RNA modifications is collectively known as the epitranscriptome, and modification defects can disrupt cytoplasmic and mitochondrial protein synthesis and are linked to human disease. RNA methyltransferases catalyzes the reaction between a methyl group to a nucleoside residue. Alkylation repair homolog 8 (ALKBH8) completes the formation of wobble uridine (U) modifications through the addition of a methyl group to promote the specialized translation of selenoproteins, which assist with the detoxification of reactive oxygen species (ROS). Deficiencies in Alkbh8 (*Alkbh8^{def}*) in C57BL/6J mice are known to decrease wobble U modifications, disrupt glutathione biology, promote sensitivity to ROS, disrupt stress responses, and are linked to cancer and intellectual disability. In my project, I used computational approaches and literature review to determine if Alkbh8 and other RNA modification enzymes can be linked to diseases of the brain and pharmaceutical responses. It has been reported that Alkbh8 defects are linked to intellectual disability. The pharmaceutical exposure that I specifically analyzed was acetaminophen (APAP), an over-the-counter medicine that decreases pain and fever in humans. The excess use of acetaminophen leads to death, emergency hospital visits, and even acute liver failure. After thorough analysis, p53 was up-regulated by a 5-fold change in WT livers treated with APAP vs. WT saline, consistent with stress induced gene regulation. Nrep was down-regulated by a 5-fold change in WT livers treated with APAP vs. WT saline. We also found that Sec24a was up-regulated in *Alkbh8^{def}* treated livers related to WT treated, which is linked to APAP hepatotoxicity. Also in *Alkbh8^{def}* treated livers related to WT treated, we saw an upregulation with antigen processing and immunodeficiences that suggests antibody production decreased when mice received high dosages of APAP. Transcriptional analysis of the APAP reponse has identified that APAP can induce stress and some transcripts have altered regulation in *Alkbh8^{def}* treated livers.

Keywords: *Alkbh8, ROS, APAP*

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Introduction

A. The Central Dogma of Molecular Biology

The central dogma of molecular biology describes the stream of genetic information as it starts as DNA, which is transcribed to RNA, which is then translated into proteins. DNA holds all of the information that the body needs to produce proteins. A sequence of DNA on the template strand is transcribed into messenger RNA through the enzyme RNA Polymerase, when bound to the promoter sequence. RNA polymerases use ribonucleoside 5'-triphosphate to begin the chain and these nucleotides are used throughout transcription (Britannica, 2019). Every ribonucleotide that is placed is based on complementary base pairing rules. For example, a guanine molecule in DNA will be transcribed into a cytosine molecule in RNA. This is because of their available hydrogen bond donors and acceptors. In hydrogen bonding, electrostatics arises due to forces that electrical charges exert on each other (Strasser, 2006). A hydrogen bond is a bond between a hydrogen atom (donor) and an electro-negative atom such as fluorine, nitrogen, and oxygen (accepter). Hydrogen bonding occurs between complementary bases to allow transcription to occur (Rashid et al., 2015). The sequence of these nucleotides with precede until a termination signal causes the RNA polymerase to stop transcribing and release the primary transcript.

The primary transcript must now undergo processing through the addition of the 5' cap and poly-A tail to protect the RNA from degradation when traveling to the ribosome for translation. Splicing in the nucleus will also have to be done to remove introns that have non-coding regions and join exons that have coding regions (van den Hoogenhof et al., 2016). When splicing is complete, the mature mRNA will travel to the ribosome for translation, with the ribosome the primary site for protein synthesis.

In the ribosome of eukaryotic cells, there are at least eleven initiation factors that are needed to properly complete translation. Translation is divided into three steps: initiation, elongation and termination. Initiation commences when the methionyl-initiator tRNA is brought to the P site of the ribosome to the AUG (methionine). Then there is a recruitment of the 43S complex at the 5' of the mRNA by eIF3 and eIF4. Once the scanning of the 5' untranslated region until it approaches the AUG codon is complete, the 80S ribosome can be assembled (Poulin et al., 1998).

In elongation and with the aid of initiation and elongation factors, tRNA and the anticodon are matched to the codon on the mRNA through base pairing. Once the next tRNA is in the A site, a peptide bond is formed to connect the second amino acid to the methionine from the first tRNA. This step subsequently causes the mRNA to move through the ribosome by exactly one codon to allow for the first tRNA to leave through the E site. This also pushes the second codon to go to the P site and a new codon in the A site to create a polypeptide chain so that the methionine forms the N-terminus side (Dever & Green, 2012). Every three nucleotides in mRNA (i.e., codon) bind to a matching tRNA anticodon while the other end of the RNA carries the amino acid (Berg et al., 2002). Each codon comprising the genetic code corresponds to one of the twenty amino acids and each amino acid is added to the growing polypeptide chain (Alberts et al., 2002). There are four significant components of the genetic code. The first is that the genetic code is degenerate, meaning that more than one triplet codon can specify for one amino acid. The next is that the genetic code is unambiguous, so each triplet (codon) is specific to only one amino acid. The genetic code is also comma-less, meaning that once translation begins, the codons are read sequentially without a break. Lastly, the code is non-overlapping, so any ribonucleotide within mRNA is part of only one triplet (Lodish et al., 2000). Note there are some exceptions to these rules in the translation of viruses and for stop codon recoding.

The last step of translation is called termination. Translation will stop once one of the three stop codons enters the A site (UAG, UAA, UGA). One of the three stop codons will be recognized by proteins known as release factors that fit into the P site to disrupt the enzyme that typically forms the peptide bonds to connect the polypeptide chain. Instead, they add a water molecule to hydrolyze the last amino acid of the chain to isolate it from the tRNA and allow the protein to leave the ribosome (Proudfoot, 2016). Since we now have a finished polypeptide chain, the polypeptides could undergo further modifications such as altering amino acids or phosphorylation, if necessary. Some proteins need help folding through chaperones and other processing adjustments (Darling & Uversky, 2018).

B. Epitranscriptome

In the Central Dogma, RNA is translated into proteins, but the process of translation is regulated at many levels. The epitranscriptome refers to all of the biological and chemical modifications of RNA in our cells. The study of this is properly known as epitranscriptomics and focuses on the broad spectrum of RNAs that include tRNAs, mRNAs, rRNAs, and snRNA. (Saletore et al., 2012). In the same way that proteins can have post-translational modifications such as methylation or phosphorylation, RNA can be modified post transcriptionally. Called RNA modifications these epitranscriptomic marks permit functional changes to the transcript without disrupting the ribonucleotide sequence. The epitranscriptome has the ability to regulate the quantity and timing of when translation occurs.

In more recent years, studying the epitranscriptome has become crucial because it allowed scientists to address and understand the effects it has in our lives. The rejuvenated interest in epitranscriptomics is due to two expansions in the field. First, new biochemical approaches and RNA sequencing allow for the observation of RNA modifications for many different types of

RNA. With this type of development, the dynamics of the modification, and the sequence context of specific methylated sites can be assessed and their effect on protein synthesis can be determined (Schwartz, 2016).

The second aspect that caused the revitalization of the field of RNA modifications is the understanding of enzymes involved in the epitranscriptome. The encoding and decoding are known for the “writing” and “erasing” enzymes, respectively, that alter the modification level and “readers” that recognize or bind the chemical marks made to methylated proteins (Meyer & Jaffrey, 2017). “Writers” function as methylases to add methyl groups to RNA, “erasers” demethylate RNAs while “readers” are proteins that identify and bind to the methylated RNAs (Cheung, 2021).

The benefit of studying these “readers”, “writers”, and “erasers” is because of their lasting effects that are linked to various diseases and syndromes (AAAS Custom Publishing Office, 2018). “Studies also support that mutations associated with more than half of the known RNA modifications and RNA modifying enzymes are involved in major human diseases like cancer, neurological disorders, cardiovascular diseases, metabolic diseases, genetic birth defects and mitochondrial-related defects” (Kadumuri & Janga, 2018). With the continued advancement of RNA modification enzymes, RNA modification target sites, and the discovery of new writers and erasers and how they function to regulate RNA and their outcomes, we will be able to further connect the epitranscriptome with its RNA modifications to disease (Baysal, 2017).

C. Stop Codon Recoding

During mRNA translation, stop codon recoding can occur when the stop codon UGA is reassigned to selenocysteine. The recoding requires the cis-acting Sec insertion sequence that’s encoded in mRNAs and a Sec-tRNA^{Sec}-specific elongation factor called SelB. To be encoded,

selenocysteine must be delivered to the ribosome by SelA, SelB, and Sel D (Figure 1). Once its aminoacyled by SerRS and converted to Sec-tRNA^{Sec} by SecA, selenophosphate is produced (Kotini et al., 2015). For a UGA stop codon, selenocysteine has the ability to be incorporated into proteins through recoding. A naturally- occurring process occurs in the cells to direct this recoding and includes the need for biosynthesis on tRNA. The products of this translation from recoding a UGA codon into a selenocysteine protein are a specialized elongation factor called SBP2 and a downstream mRNA hairpin, which is known as the ¬cis-acting Sec insertion sequence (SECIS) (Bröcker et al, 2014).

Selenoproteins are known for helping to detoxifying reactive oxygen species (ROS). ROS are made by the mitochondria through oxidative damage and influences retrograde signaling from the mitochondria to the cytosol and nucleus. The electron transport chain involves several redox reactions, in which electrons are passed from one complex to the next to eventually reduce oxygen to produce water (Murphy, 2009). This oxidoreductase function is critical in organisms where it is not abundant. ROS serves as a feedback regulator for cell concentrations since it has the ability to cause damage to cells, RNA, DNA, buildup of free radicals, and cause cell death (Ray et al., 2012).

In the brain, oxidative stress can lead to neurodegenerative diseases (Chen et al., 2012). An enzyme in the Krebs cycle called alpha-ketoglutarate dehydrogenase (alpha-KGDH), has the ability to generate ROS. The formation of reactive oxygen species through alpha-KGDH is controlled by NADH/NAD+ ratio through the inhibition of complex I. Since alpha-KGDH is both a generator and a target for ROS, researchers have found it to be a key component to how ROS can be induced in cells (Adam-Vizi, 2005). This corresponds directly to ALKBH8 because in organisms where they are *Alkbh8* deficient or expression is not full, there is a limit on selenoprotein synthesis, which thus intensifies ROS levels (Lee et al., 2020).

Modifications in the wobble position of tRNA enhances the efficiency of codon reading and prevents mistranslation. Eukaryotes have several different modifications including 5-methoxycarbonylmethyluridine (mcm^5U) that are found in 11 out of 13 wobble uridine-containing tRNAs. The mcm^5U in $tRNA^{Sec}$ regulates selenoprotein production such as glutathione peroxidase 1 (Gpx1) (Handy et al., 2006). Gpx1 as well as the rest of the glutathione peroxidase family contribute to reducing the amount of hydrogen peroxide and lipid peroxide to protect cells against oxidative damage (Songe-Møller et al., 2010). A Sec-containing protein called Trxr1 assists ribonucleotide reductase enzymes through the maintenance of the family of thioredoxins (Leonardi et al., 2019). These detoxifying proteins act in the cell to help reduce the amount of reactive oxygen species in the cell to limit their damaging effects. Because we are equipped with this machinery, our cells can respond oxidative stress conditions. When ROS detoxifying proteins are not working effectively or other factors are introduced, the cell is stressed. Alkbh8 catalyzes the reaction of a methyl group in 5-carboxymethyl uridine (cm^5U) to 5-methylcarboxymethyl uridine (mcm^5U). Alkbh8 belongs to a family called AlkB with nine other proteins. Alkbh1-8 and fat mass and obesity (FTO) all have a 2-oxoglutarate-Fe(II) oxygenase domain (Endres et al., 2015). These proteins are demethylases that rely on both iron and α-ketoglutarate to catalyze the demethylation on a substrate (Xu et al., 2020). The Alkb proteins function as dioxygenases to repair alkylation damage or methylation of either DNA or RNA. In *E. coli*, Alkb can protect against damage from a SN2-alkylating agent. This reaction needs an AlkB- bound non-heme Fe^{2+}, O_2, and an α-ketoglutarate for the oxidization of a methyl group. This results in the subsequent release of carbon dioxide, formaldehyde, succinate, and the fixed piece of DNA or RNA (Holland & Hollis, 2010). The specificity for this reaction by E. coli AlkbB is limited to the N1 position of purines and the

N3 position of pyrimidines (Begley & Samson, 2003). This differs from DNA glycosylases that remove the entire base via cleavage of the N-glycosidic bond (Sedgwick & Lindahl, 2002).

Mammalian Alkylation Repair Homolog 8 (Allkbh8) is a writer on cm^5U to make mcm^5U at the wobble position of the anti-codon loop on tRNA through its methyltransferase domain. This methyltransferase domain forms the wobble nucleoside 5-methylcarbonylmethyluridine (mcm^5U) from the former 5-carboxymethyluridine (cm^5U). It has been studied that the (R) and (S)-5-methoxycarbonylhydroxymethyluridine are found in mammalian tRNA arginine (UCG) and selenocysteine (UGA) and hydroxylated forms of mcm^5U are found in mammalian tRNA glycine (UCC) (van den Born et al., 2011). This shows that there are diastereomers of modified RNA nucleosides and that Alkbh8 oxygenases function beyond DNA and RNA repair and expand on the information of wobble uridine modifications.

Alkbh8 acts as an epitranscriptome writer, where it is needed to form the modified wobble uridine on $tRNA^{Sec}$. It was found that when human ALKBH8 interacts with mcm(5)U tRNAs, purified ALKBH8 complexes can methylate RNA. A decrease in ALKBH8 in human cells reduces the levels of mcm(5)U in RNA and increases the cell's sensitivity to reactive oxygen species (Fu, 2010). Modifications in the anti-codon loop allow tRNAs to expand the genetic code post-transcriptionally. The RNA modifications that are found with the anticodon and surrounding nucleotides in the anticodon stem-loop influence not only to the accuracy of translation, but the promptness of it through the stabilization of interactions between both the anticodon and codon (Nguyen et al., 2019).

ALKBH8 is linked to a variety of different cancers. For example, ALKBH8 is highly expressed in bladder cancer. In mouse models, ALKBH8 induced survivin, an antiapoptotic factor that regulates survival in bladder cancer cells. In mouse models, bladder cancer progresses at a

faster rate, in terms of tumor mass and invasiveness, when ALKBH8 expression is high (Ohshio, 2016). As of now, the mechanism of the antiapoptotic factor remains unknown, it still provides support that ALKBH8, specifically at high levels, is critical for growth and progression of bladder cancer (Doudican et al., 2005).

It has also been shown in vitro and in vivo that ALKBH8 promotes the development and continued existence of bladder cancer (Janin et al., 2020). The silencing of ALKBH8 decreased the amount of invasion and angiogenesis in vivo (Shimada et al., 2009). This also shows the correlation between bladder cancer and high ALKBH8 levels. $Alkbh8^{def}$ mice were made by using an insertional mutagenic method that instituted a "gene trap" into intron 7. This generated a fusion transcript with a premature stop codon of exons 8-11, which not only shortens the methyltransferase domain but also a part of 2OG- Fe(II) (Endres et al., 2015). Alkbh8 also has an effect on embryogenesis. Embryogenesis is what effects the transition from the embryo to the multicellular generation. It encompasses the synchronized advancement of organs and tissues facilitated by the changes of spatiotemporal expression of genes (Yi et al., 2010). In mice that were Alkbh8 deficient, they saw that their mouse embryonic fibroblast cells grew approximately two times slower compared to wildtype mice. This information suggests that the Alkbh8 deficient mice were working under stressful conditions. The study also shows increased apoptosis phenotypes compared to the wildtype mice in this study. Cells that have a greater apoptotic index are predicted to express cell death effectors. To continue with this reasoning, the study also provided results with a known p53 gene target and apoptotic effector called PERP. There were higher levels of PERP as well as p53 protein levels in the Alkbh8 deficient mice, drawing a correlation between the slow growth phenotype is probably due to the increased prevalence of apoptosis (Endres et al., 2015).

It has also been reported that when Alkbh8^{def} mice are exposed to the environmental pollutant naphthalene, there is increased ROS, DNA damage and disrupted glutathione levels *in vivo*. Naphthalene is commonly found in gasoline, mothballs and cigarette smoke and is classified as a possible carcinogen (Leonardi et al., 2020). Disrupted ALKBH8 epitranscriptomic system leads to dysfunctional translation of selenoprotein and sensitizes mice to naphthalene. In this study, the lungs from Alkbh8^{def} mice were more sensitive to the naphthalene than the wild-type, therefore demonstrating that ALKBH8 plays a protective role against naphthalene induced lung damage (Leonardi et al., 2020).

Senescence is the progressed deterioration of cells and is linked to many degenerative diseases. The role of selenium in senescence in vitro shows that it extends the life of cells. A deficiency in ALKBH8 in cells shows modified selenoprotein levels, senescence, and mitochondrial reprogramming. Alkbh8^{def} mouse embryonic fibroblasts (MEFs) demonstrate signs of senescence that includes senescence associated β-galactosidase, heterochromatic foci and the senescence associated secretory phenotype (SASP) (Lee et al., 2019).

A significant portion of the research done on Alkbh8 has to deals with intellectual disability. There are two homozygous truncating ALKBH8 mutations that cause intellectual disability (ID) in people. With the investigation of the analysis of the tRNA from the affected people that show a lack of these modifications, it was observed that there was brain sensitivity. In this study, an individual had a brain MRI, and it showed a completely normal brain except for lesions in the right transverse sinuses, suggesting an arachnoid granulation. Another individual who was tested at 12 years old with ID, epilepsy GDD (global developmental delay) with a thickened tri-leaflet aortic valve with mild regurgitation showed an age equivalency of 66 months and a severe linguistic impairment. After analyzing the modification status from the previously

described individuals compared to their families, the impaired individuals show that the wobble modifications mcm^5s^2U, (R)-$mchm^5U$ and (S)-$mchm^5U$, and mcm^5Um were found in the RNA of the unaffected family members while they were undetected from the affected individuals. These findings support that these loss-of-function ALKBH8 mutations in humans are correlated to ID and caused by defects in tRNA modification (Monies et al., 2019).

D. Pharmaceutical Response

Acetaminophen is a common OTC drug that humans take to decrease pain and fever and helps with headaches, arthritis, toothaches etc. since 1951. Researchers are still unsure how acetaminophen works in the body, but one of the leading explanations is that it acts through the cyclo-oxygenase (COX) pathway, where NSAIDs act. The NSAIDs inhibit production of prostaglandins and exert consequent effect and influence a vasoconstrictor such as TXA_2. The majority of the research has centered on its inhibition of the COX enzyme primarily because of its analgesic and antipyretic effects (Anderson, 2008).

Acetaminophen blocks COX enzymes that help produce prostaglandins, which signal pain and inflammation. In a study done by Clinical Pharmacology & Therapeutics, acetaminophen can only be an effective COX inhibitor if there are decreased levels of hydro-peroxide- containing compounds in cells. The levels of these compounds vary throughout the body, so it works better in some cells than others (Aranoff et al., 2006). The effects of the continued use of acetaminophen could potentially be harmful. While there are 500 deaths and 50,000 emergency department cases each year due to excess acetaminophen use, (Gerriets et al., 2020) it is also the most universal drug-related source of acute liver failure (Wang et al., 2017). Liver damage and/or failure shows symptoms similar to the flu but could really cause issues when combined with other antibiotics, high blood pressure medication, high cholesterol and more (American Addictions Centers, 2020).

With this, acetaminophen toxicity causes increased ROS. In a paper that studied oxidative stress in acetaminophen hepatoxicity, it was observed through fluorescence measurements that H_2O_2 was formed during the metabolism phase of acetaminophen *in vivo* (Wendel et al., 1976). Another paper demonstrated that acetaminophen hepatotoxicity is caused by lipid peroxidation. This is significant because the P450-mediated breakdown of xenobiotics promotes ROS and that the inducers and inhibitors of P450 enzymes are responsible for oxidative stress and cell death (Du et al., 2016).

It is important to recognize that the age of the mice can affect how severe they react to high dosages of acetaminophen. In a study where they wanted to distinguish the susceptibility of injuries between different aged mice when injected with APAP (300mg/kg), the younger mice were harmed more than the adult mice. They found that there were higher numbers of hepatic drug metabolic enzymes in immature mice, such as hepatic CYP2E1, which is the most important enzyme that breaks down acetaminophen into NAPQI than in adult mice. GSH (glutathione) is also digested with NAPQI and serves to prevent damage to cells including through free radicals, peroxides, and ROS (Lushchak, 2012). APAP toxicity occurs when GSH resources are consumed, which imposes the covalent binding of NAPQI to APAP-cysteine adducts which interrupts cellular responsibilities (Cohen et al., 1997). These observations help prove that younger mice are more affected by hepatotoxicity compared to their adult counterparts (Lu et al., 2017).

Alkbh8^{def} mice may express their genes differently in response to stressful conditions with acetaminophen. The results from a paper show how the livers of rats react to high dosages of APAP (Powell et al., 2006). “The sub-toxic dose of APAP (150 mg/kg) does induce oxidative stress as demonstrated by the significant accumulation of nitrotyrosine protein adducts, 8-OH-dG DNA lesions, and the reduction in GSH content 6 hrs after treatment” (Powell et al., 2006). In other

words, this verifies that changes in gene expression can function as an effective and sensitive marker of APAP toxicity.

The importance of the results from my resultant experiments and research will be to determine how acetaminophen affects not only the mice's biology, but also human biology. It is important to use mice as model organisms so that our results can subsequently help scientists learn more about Alkbh8 gene expression and the consequences of high dosages of acetaminophen.

E. Next-generation Sequencing

Next generation sequencing (NGS) is a technology that can take either a DNA or an RNA sequence and study its composition and components in its relation to diseases. Scientists can rapidly and thoroughly sequence genomes to uncover RNA splice variants, quantification, and expression. NGS works to find DNA/RNA variants and splice sites, epigenetic factors, and new pathways to study diseases or other phenomena (Qin, 2019). NGS technology allows researchers to produce a mass amount of data to closely look into the details of genomics and epitranscriptomics. Analyzed data can give you information about how an organism's genetics and molecular biology functions to survive (Kulski, 2016). It produces this data by removing the DNA/RNA from cells. The DNA/RNA transforms into cDNA through reverse transcription and the fragments become altered (Gupta & Verma, 2019). Ligated adapters are attached to the ends of the target fragments and quantification of the final product is available for sequencing (Figure 4).

To study the transcriptional response and APAP in wildtype and Alkbh8^{def} brains, we performed next-generation sequencing to study the biological and cellular associations to disease. We were also able to use our C57BL6/J mice for mRNA sequencing with three groups of mice: The first group of mice were wild-type and exposed to saline or acetaminophen (600 mg/kg, 6

hours). These mice had many 5-fold changes in gene expression, for upregulation and downregulation, and trends were identified in biological processes using the STRING database. The last two groups were comprised of mice (both WT and Alkbh8^{def}) exposed to an acute and a chronic dose of acetaminophen. My analysis identified links between RNA methyltransferases and epitranscriptomic marks to stress, which can inform on diseases of the brain and pharmaceutical responses.

Methods

A. Genotyping

In our mouse colony, we genotyped wild-type and $Alkbh8^{def}$ mice that were approximately 12 weeks old through using tail clippings. Genotyping was necessary to ensure we were using the correct models to accurately test our hypothesis of Alkbh8 having a protective role against acetaminophen toxicity.

B. RNA Isolation

Prior to the isolation of the mice RNA, we exposed C57/BL6J mice with acetaminophen. We performed RNA isolation to accurately measure the gene expression and analysis of RNA. We obtained the brain samples from dry ice and placed it in a weigh boat. We cut a piece of a brain that was approximately 50-100 mg. We placed the tissue in the 1 mL Trizol-filled Eppendorf tube and inserted the tissue homogenizer inside the Eppendorf tube and turned it on to allow the tissue to fully homogenize. After the tissues are fully integrated, we let the samples incubate for 5-10 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Then, we added 200 µL of chloroform to each tube and vortexed the samples for 15 seconds to allow full integration. We let the samples incubate for 2-3 minutes at room temperature and then centrifuged them for 15 minutes at 4°C 12,000 x g (rcf). After 15 minutes, phase separation was seen. We removed 500 µL of the clear, aqueous phase on top with a pipette without disturbing the lower phenol- chloroform layer. The aqueous phase has the RNA that we are trying to assess for their quantity and purity. We transferred 500 µL of isopropyl alcohol into our newly labeled Eppendorf tube and hand mixed. The tubes were incubated at room temperature for 10 minutes and then centrifuged again for 10 minutes at 4°C 12,000 x g (rcf). After the ten minutes, we observed an RNA precipitate in the form of a gel- like pellet. To obtain the pellet we removed the supernatant

and washed the pellet with 75 % ethyl alcohol followed by vortexing. We centrifuged one last time for 5 minutes at $4^{\circ C}$ 7,500 x g (rcf). Then, we removed all ethyl alcohol and dissolved the RNA in approximately 50 µL of nuclease- free water by passing the solution through the pipette until fully dissolved.   To assess RNA quality and quantity, we performed an analysis using nanodrop. We first wiped the nanodrop arm and stage with a kimwipe to prepare for the analysis of RNA and decontaminate. We took 1 µL of Thermofisher nuclease- free water and placed it on the stage opening. Then we performed a blank using another 1 µL of nuclease- free water so we can start at 0 absorbance. Then we pipetted 1 µL of our sample onto the stage opening to measure the concentration. We recorded the absorbance for $A_{260/280}$ and $A_{260/230}$ and the concentration is to determine the purity of our sample through Beer's Law. We then repeated this process for our other samples to measure their purity for analyzation.

C. qRT-PCR

To confirm the amount of gene expression our mice had, we used Taqman probe chemistry (Figure 2). Unlike regular PCR, qRT-PCR uses a probe that differ from primers since they cannot be extended by taq polymerase. There are two different Taqman probes: the reporter on the 5' end and a quencher on the 3' end. When Taq polymerase finds the primer and extends until it meets the probe, taq will cleave it and break it off into small pieces due to its nuclease activity. The goal of the chemistry is to split the reporter and the quencher probe, thereby activating a recordable signal, as concentration is proportional to fluorescence.

We first gathered our RNA from the Alkbh8 proficient and Alkbh8 deficient brains, along with reagents to test for the expression of our target genes and beta-actin, with the later stored at -80 °C. We then created a master mix for Alkbh8, gpx1, beta-actin and trixr to make 3 technical replicates by normalizing RNA samples to 100 ng using a formula. The reason that we use triple

replicates is so when we go to analyze the data, we will be able to have a more accurate measure of the mean Ct value since this experiment can be extremely sensitive. The first Master Mix included qPCR tough mix and Taqman Gene Expression Assay (20X). The calculations needed for Mastermix #1 and Mastermix #2 are found in Table 1. For example, we pipetted 0.55 (accounting for a 5% pipetting error from a 0.5 μL) and multiply 0.55 by three to get 1.65 to account for the three triple replicates. For the PCR toughmix, it's 5.5 μL (also accounting for pipette error) and multiple it by three for the triple replicate it to get a volume of 16.5 μL. To make Master Mix #2, we need the RNA template and Master Mix #1. To make this, we pipetted 100 ng of the RNA template and our first Master Mix for our 3 technical replicates into each individual gene to create a second Master Mix. This means, we took 15 μL of the Mastermix and pipetted it into a new tube for a specific gene while also adding 15 μL of the normalized RNA from the beginning to obtain a final volume of 30 μL. Before pipetting into the wells, we had eight tubes total: four for Alkbh8$^{+/+}$ and four for the Alkbh8 deficient mice.

We set up the qPCR plates by pipetting 10 μL of the second Master Mix into our wells in triplicates and sealed with an adhesive optical cover. We spun our samples at 2,000 rpm for 5 minutes and then placed them in the qPCR machine to allow the plate to run. Once the plate finished running, we inputted the data onto Microsoft Excel to observe and analyze the gene expression of ALKBH8 mice and ALKBH8$^{-/-}$ mice.

D. Acute and Chronic Exposure to Acetaminophen

Two different mRNA sequencing experiments were completed to distinguish the distinctions between acute exposure and chronic exposure to pharmaceutical response, in this case acetaminophen. These studies were performed by Sara Evke (5th year graduate student) and I have helped to analyze the gene expression data. The acute exposure was 600 mg/kg for 6 hours one

time, while chronic exposure was also 600mg/kg but for four consecutive days. Three mice were used in each respective experiment.

E. NGS/STRING Database

My computational work for this project was to take C57BL6/J mice that were exposed to APAP and saline (600 mg/kg, 6 hours) that the Begley lab generated and analyze the mRNA-seq data. mRNA-seq performed by Sara Evke generated a list of significantly regulated genes, with both groups of mice that were regulated in expression by 5-fold. Sequencing data was transferred to Microsoft Excel, where I sorted the data to identify these changes in fold expression. Data was categorized into two ways: through a fold expression either downregulated or upregulated, or through acetaminophen-treated samples (acutely or chronically) with wild-type and Alkbh8^{def} mice. The resultant data was entered into the STRING protein database to identify genes and biological trends specific to the response to acetaminophen.

Results and Analysis

Once the mice were genotyped (which were $Alkbh8^{+/+}$ and $Alkbh8^{Def}$), we were able to do our work on RNA isolation. The brains of our mice were all exposed to acetaminophen and saline chronically. We determined the absorbance for $A_{260/280}$, $A_{260/230}$, and the concentration in order to correctly assess the RNA quality and quantity from the total isolated RNA. The $A_{260/A280}$ ratio of absorbance indicates purity for DNA/RNA, as 260 is a marker for RNA absorbance. A number of approximately 2.0 is considered "pure" for RNA. If lower, it indicated contaminants that absorb strongly around the 280 nm mark. The other ratio needed for us to evaluate the RNA we isolated is $A_{260/A230}$. This ratio refers to another method of nucleic acid purity. A ratio from 1.98 to slightly above 2.0 is pure for RNA. The A_{230} absorption is used as second measure with the A_{260} to highlight the common impurities that are possibly present in the sample. Since these contaminants usually absorb at 230, if you have a ratio outside the approximate 2.0 bounds, it's easier to identify the impurity. The concentration of the RNA isolation is determined by the amount of constituent (in this case nanograms) divided by the total volume of our mixture measured in microliters.

The results from the isolated RNA with the experimental samples are shown in Table 3. Three of the mice were wild-type and three were Alkbh8 deficient mice. We were unable to complete the full round of experimental samples due to COVID-19 restrictions. The genes that we analyzed with the C57BL6/J mice were Alkbh8, Gpx1, and Trxr1 (Table 2). We ran a qRT- PCR plate with these six samples from the RNA that were isolated. After running the plate, we recorded our results in excel and calculated the fold expression by using the delta Ct and delta delta Ct. From there, we observed and analyzed differences in gene expression from the Alkbh8 wild type and Alkbh8 deficient mice. Table 3 displays the distinctions between the three genes. The wild-type mice that were used for this analysis were used and normalized as expressing 100% of their

respective gene for distinguishing purposes. Alkbh8 showed a significant decrease by only expressing 44% of the gene. Gpx1 was almost fully expressed at 93% and trxr1 expressed approximately 80% of its gene.

There was a lot of data produced from NGS. The analysis made from the STRING database for upregulation by 5-fold in WT mice exposed to APAP, relative to saline, showed that in both the gene ontology and in the KEGG Pathways there were connections to p53, a tumor suppressor protein (Table 4). P53 activation is induced when the cell is under stress, such as oxidative stress or DNA damage. In cells, p53 is manufactured as a transcriptional activator for p53- related genes, resulting in cell cycle arrest, senescence, and/or apoptosis. A paper written by AACR discusses the connection between p53 and ALKBH8. When exploring the role that ALKBH8 plays in bladder cancer development *in vitro*, they discovered that silencing ALKBH8 through siRNA transfection decreased ROS detoxification and induced apoptosis through the subsequent activation of p38 (Shimada et al., 2009). We know that p38 is connected to p53 because in an experiment done also done by AACR, when interfering with the production of p38, it prevented the stimulation of the transcriptional activity of p53 (Sanchez-Prieto et al., 2000). These findings that ALKBH8 could be linked to disease progression, including cancer.

The results from the STRING database for downregulation showed a network of proteins that were obtained from the 5-fold change for WT mice exposed to APAP relative to saline (Table 5). One of the proteins listed was Nrep (neuronal regeneration related protein), which is a protein that is highly expressed in the brain. In a research article, scientists conducted a meta-analyses of massive gene expression data to identify biomarkers indicative of pharmacotoxicity and found that Nrep has a possibility of being used as an efficient toxicity biomarker. Nrep had the largest fold-changes and showed significant amounts of depletion upon drug treatment when treated with toxic

concentrations of known toxic compounds (Kim et al., 2015). This realization shows that Nrep can be used as a biomarker for disease, infection, or pharmaceutical response.

The results from the acute exposure from acetaminophen with the wild-type and Alkbh8^{def} mice show a connection to Sec24a, a constituent of coat protein II (COPII)-coated vesicles that facilitate protein transport from the endoplasmic reticulum. In an experiment done in 2018, researchers evaluated extracellular vesicles following APAP overdose in hepatocytes in mice and explored the role of APAP-protein adducts. APAP-protein adducts are a crucial feature of APAP hepatoxicity and are thus used in bioassays for identifying overdoses in humans. The findings support that the number of APAP-protein adducts were much higher in the APAP overdosed mice than in the control (Duan et al., 2019). With this, APAP overdose enhances extracellular vesicle release in mice and humans.

The chronic exposure results with acetaminophen comprising the Alkbh8^{def} vs WT mice show that there is a connection to antigen processing and primary immunodeficiencies, which are disorders that disrupt the immune system. Antigen processing ensures that the body is able to take on the components of adaptive immunity. In a paper published in 2010, the authors tested common NSAID function, including Tylenol (APAP), through inhibition of cyclooxygenases 1 and 2 and examined the immune response. They found that Tylenol shows an aptitude to prevent antibody production when used at pharmacological doses (Bancos et al., 2009). Since the antibody processing of the mice in the 2010 study was disrupted, preventing antibody production in general could be one of the ways in which the body responds to high doses of acetaminophen.

Conclusions and Implications

The results from the isolated RNA demonstrate that we have relatively pure RNA in our samples, and we know this because of the absorbance numbers around 2.0. Since the absorbance for our samples is close to 2.0, our samples indicate very few contaminants. The majority of samples in general are acceptable above 1.8, but any lower could be a sign of contamination. It's important to recognize that nucleic acids have a peak absorbance of UV light at 260nm. Thus, the amount of light absorbed in this region can be used to determine the concentration of RNA in solution by its relation to the Beer-Lambert law. The Beer-Lambert Law correlates an important linear relationship with absorbance and concentration. It's qualitative and quantitative analysis of spectroscopic data allows a deeper understanding between light and matter. Deviations from a typical relationship (depending on the matter) indicates a change in standard structure which could be seen as a contaminant. The works of Pierre Bouguer, Johann Heinrich Lambert, and August Beer illustrate that the light intensity transmitted through a thin medium is proportional to the medium's thickness.

The results from the qRT-PCR plate for the six experimental samples were analyzed for gene expression differences between the Alkbh8^{def} mice and wild-type mice using the fold expression previously stated. Each of the three genes from the Alkbh8^{def} had decreased in expression when comparing the two groups. This confirms the decreased gene expression in Alkbh8^{def} mice.

The results from the saline vs treated 5-fold expression changes highlight the many roles that ALKBH8 could play in the cell. For upregulation, we observed a p38 and p53 connection, which links ALKBH8 to stress response and cancer prevention. For down regulation, the nrep protein may be an efficient biomarker for toxicity. This toxicity could be in terms of

acetaminophen, which is extremely relevant in today's world with so many people being harmed or possibly die from it.

The implications from the acute exposure from acetaminophen with the wild-type and Alkbh8^{def} mice show a link to Sec24a, which helps with extracellular vesicle transport. Now learning that high dosages of acetaminophen are linked to APAP adducts, a key feature of APAP hepatotoxicity, there is a possibility that scientists can use this to detect early signs of acetaminophen overdose. For the chronic exposure, the analysis led us to believe that this affects a significant amount of the immune system and antibody processing. When antibody synthesis is limited or ceased, NSAIDs can weaken the immune system, which poses a threat on too many people but especially the elderly, the immune-compromised, and children.

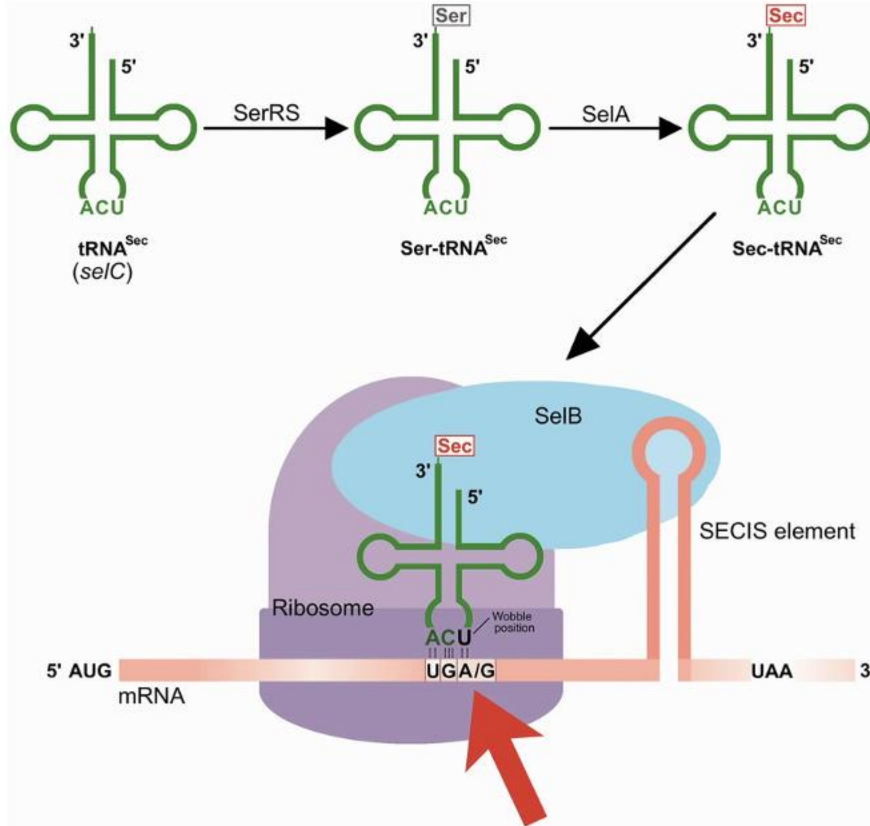

Figure 1 *Stop Codon Recoding*

Note. The above figure is from a study on the machinery in E. coli selenocysteine machinery (Xu et al., 2012). The mechanism the recodes UGA to selenocysteine incorporates SerRS, Sel A, Sel B, Sel D, and SECIS element in the wobble position.

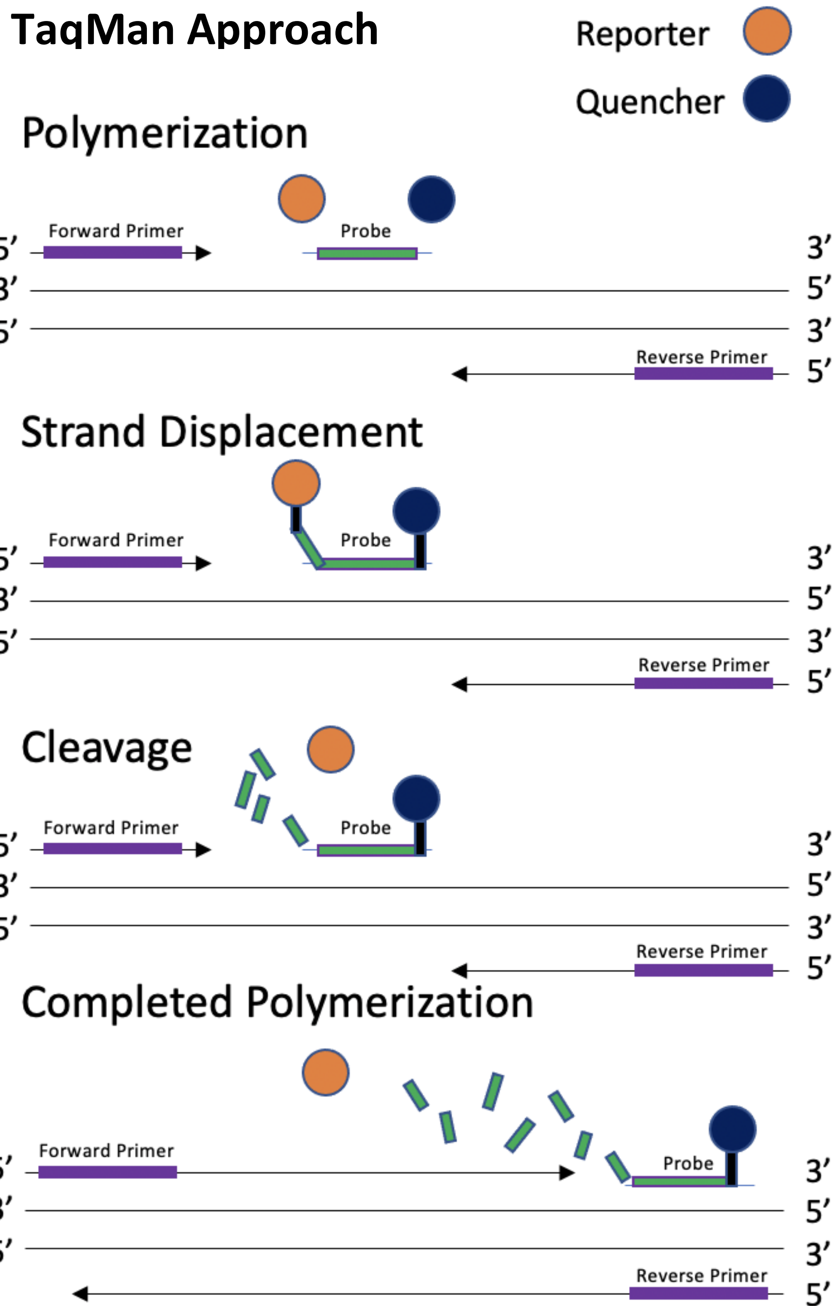

Figure 2 *TaqMan Approach*

Note. A visual representation of the chemistry used for qRT-PCR. Taqman chemistry consists of a reporter and a quencher that undergo four steps.

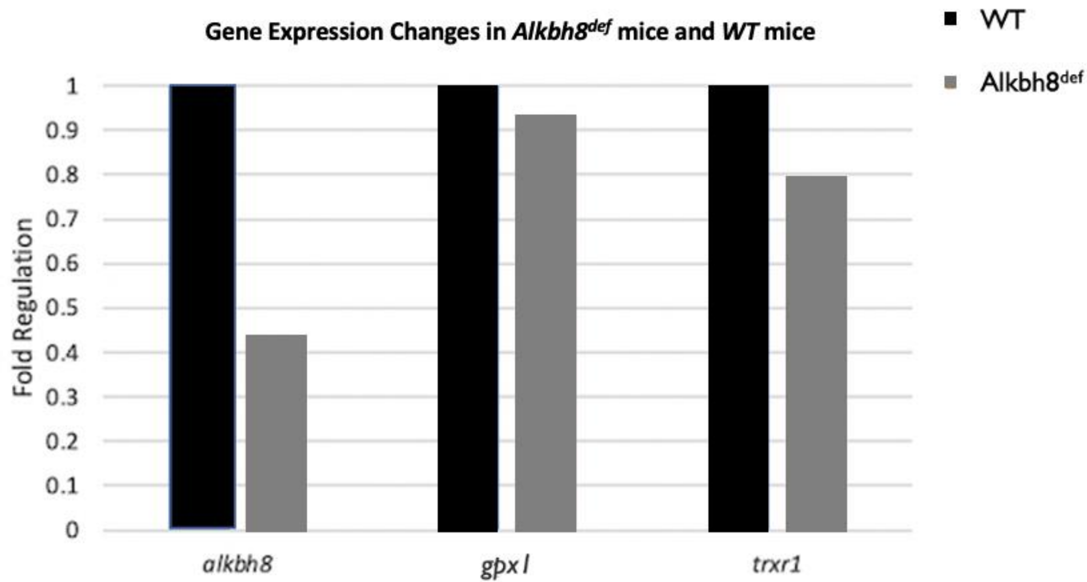

Figure 3 *Gene Expression Changes in Alkbh8^{def} mice and WT mice*

Note. The results from the q-PCR using the genes Alkbh8, Gpx1, and Trxr1. Blue refers to wild-type mice while orange refers to the Alkbh8^{def} mice.

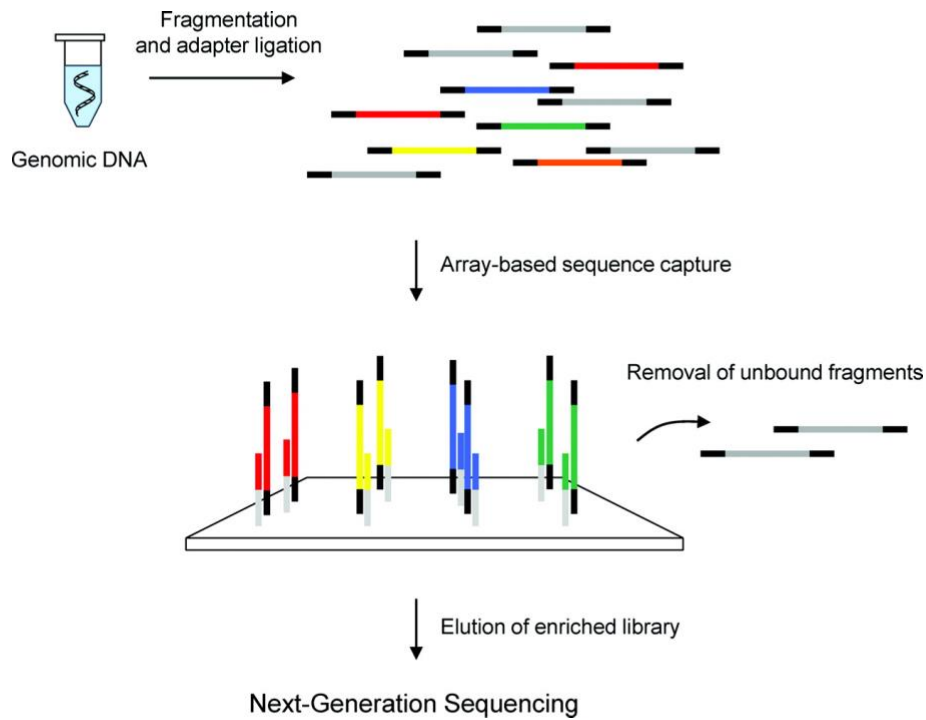

Figure 4 *Next-generation Sequencing*

Note. A brief overview of the next-generation sequencing process when using DNA (Meder et al., 2011).

Table 1 *Calculations for Mastermix #1 and Mastermix #2*

MasterMix #1

	1X
qPCR Tough Mix:	5 μL
Tagman Gene Expression Assay (20X)	0.5 μL
RNA Template (100ng)	4.5 μL

Mastermix #2

	3X
qPCR Tough Mix:	15 μL
Tagman Gene Expression Assay (20X)	1.5 μL

Note. These are the calculations performed during the experiment. These are the numbers right before implementing a 5% pipetting error.

Table 2 *Genes analyzed by qPCR*

Name	Protein Function
Alkbh8	Reduces the cell's sensitivity to reactive oxygen species.
Gpx1	Works to reduce the concentration of hydrogen peroxide and lipid peroxide to prevent oxidative damage to cells.
Trxr1	Reduces oxidative stress through the regulation of ribonucleotide reductase enzymes.

Note. These are the names and function of the genes examined during qRT-PCR.

Table 3 *RNA Isolation with Acetaminophen treated brain samples*

RNA Isolation with Acetaminophen treated brain samples

Sample	$A_{260/280}$	$A_{260/230}$	Concentration ($\frac{ng}{\mu L}$)
WT30A,C,BRNAi	2.08	2.19	1084.5
WT31A,C,BRNAi	2.06	2.04	1026.3
WT33A,C,BRNAi	2.09	2.22	1054.3
KO30A,C,BRNAi	2.06	1.9	1426.1
KO32A,C,BRNAi	2.08	1.83	733.8
KO33A,C,BRNAi	2.09	2.1	1024.5

Note. The six samples of our mice are displayed in this table. The results are thoroughly analyzed through absorbance and concentration.

Table 4 *Enriched functional categories for gene upregulation by 5-fold change in WT livers treated with acetaminophen relative to saline.*

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0006977	DNA damage response, signal transduction by p53 class me...	2 of 10	1.93	0.0035
GO:0060213	positive regulation of nuclear-transcribed mRNA poly(A) tail ...	2 of 12	1.85	0.0044
GO:0043619	regulation of transcription from RNA polymerase II promoter...	2 of 12	1.85	0.0044
GO:0071243	cellular response to arsenic-containing substance	2 of 14	1.78	0.0052
GO:0043555	regulation of translation in response to stress	2 of 14	1.78	0.0052

KEGG Pathways				
pathway	description	count in network	strength	false discovery rate
mmu04115	p53 signaling pathway	4 of 66	1.41	0.0013
mmu05216	Thyroid cancer	2 of 35	1.38	0.0217
mmu05219	Bladder cancer	2 of 40	1.33	0.0254
mmu04216	Ferroptosis	2 of 40	1.33	0.0254
mmu05214	Glioma	3 of 68	1.27	0.0078

Note. The tables are showing you the protein's function and what they are connected to. There were 53 items.

Table 5 *Downregulation by 5-fold change for WT livers treated with acetaminophen relative to saline*

Proteins
Asap3
Fam47e
Klhdc7a
Marveld1
Mid1ip1
Nrep
Nmrk1
Rorc
Tmem25
Wnt5b

Note. 13 genes were downregulated by a 5-fold change that amounted to 10 proteins.

Table 6 *A gene regulated in Alkbh8^{def} APAP- treated livers acutely, vs. WT treated*

Sequence	**Protein**	**Function**
ENSMUSG00000036391	Sec24a	Promotes production of vesicles from the endoplasmic reticulum.

Note. The table states the sequence, protein and function of Sec24a.

Table 7 *Enriched functional categories for gene upregulated by 5-fold for Alkbh8^{def} APAP- treated livers chronically, vs WT treated*

KEGG Pathways				
pathway	description	count in network	strength	false discovery rate
mmu05340	Primary immunodeficiency	2 of 34	2.07	0.00099
mmu04612	Antigen processing and presentation	2 of 78	1.71	0.0024
mmu04640	Hematopoietic cell lineage	2 of 90	1.65	0.0024
mmu04660	T cell receptor signaling pathway	2 of 100	1.6	0.0024
mmu04514	Cell adhesion molecules (CAMs)	2 of 158	1.4	0.0039

Note. The table shows the main KEGG Pathways for mice that were treated chronically with acetaminophen.

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