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Regulation of SERPINA1 mRNA Expression by Environmental Conditions in Hepatocyte Cells

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REGULATION OF SERPINA1 MRNA EXPRESSION BY ENVIRONMENTAL CONDITIONS IN HEPATOCYTE CELLS

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Abstract

The *SERPINA1* gene encodes the critical protease inhibitor α-1-antitrypsin (A1AT). A1AT represses neutrophil elastase activity to protect lung tissue from inflammatory damage. A deficiency in α-1-antitrypsin can lead to chronic obstructive pulmonary disease (COPD). Pathogenic genetic variants in *SERPINA1* are also associated with A1AT protein misfolding and liver cirrhosis. The regulatory mechanisms of *SERPINA1* expression are not well understood, but previous studies suggest that alternative polyadenylation in the 3' untranslated region (3'UTR) affects A1AT protein expression. In this study, we used the liver cancer cell line HepG2 to determine how environmental conditions influence *SERPINA1* mRNA expression and post-transcriptional regulation. We stimulated HepG2 cells with interleukin-6 (IL-6), ethanol, and peroxide and performed 3' end RNA sequencing. Our findings reveal that IL-6 upregulates distal polyadenylation of *SERPINA1* mRNA in HepG2 cells. Additionally, we observed that changes in environmental conditions can lead to alternative polyadenylation in other genes. This study enhances our understanding of how environmental factors, such as IL-6, regulate *SERPINA1* expression, particularly through alternative polyadenylation. By clarifying these regulatory mechanisms, this research helps us better understand the development of A1AT related disorders and may guide the creation of new treatments.

Acknowledgment

Special thanks to every member of the Lackey lab in the Center for Human Genetics. I want to personally thank the faculty on my committee for providing me with mentoring and feedback during my studies at Clemson. Personal thanks to Heather Flanagan-Steet for all the advice on my research, Alex Feltus for mentoring my medical bioinformatics course, and Zhana Duren for all the statistical advice. A special thanks to Lela Lackey for supporting me so well as my advisor. Thank you to Vijay Shankar and the entire bioinformatics core for your support. Lastly, I want to thank my parents, Shuluun and Badma, for their financial support and love, and my wife Naqing, for all the support, encouragement, and understanding throughout this journey.

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Introduction and Background

The protein Alpha-1-AntiTrypsin (A1AT) is a protease inhibitor. The A1AT protein is encoded by the serine protease inhibitor family A1 (*SERPINA1*) gene. A1AT is mainly produced in the liver and reaches the lungs by diffusion from the circulation (Morrison, Afford, and Stockley 1984). Inherited variants in *SERPINA1* are associated with A1AT deficiency (A1ATD), characterized by reduced A1AT levels. The most common disease-causing variant involves a change of *SERPINA1* mRNA from guanine to adenine, resulting in a glutamic acid-to-lysine substitution at position 342 (Glu342Lys)(Mahadeva and Lomas 1998). This mutation leads to the production of a mutant protein, Z-A1AT, which has a tendency to misfold and aggregate (Werder et al. 2021). In an individual with A1ATD, Z-A1AT can form abnormal aggregates, which can trigger responses including autophagy, endoplasmic reticulum stress and apoptosis and contribute to the development of liver cirrhosis (Teckman et al. 2004). Alcohol exposure and viral infections are thought to contribute to development of liver related A1ATD symptoms (Bowlus et al. 2005). If insufficient levels of A1AT make it to the lung, neutrophil elastase becomes overly active. This imbalance between A1AT and neutrophil proteins can lead to lung disease, such as chronic obstructive pulmonary disease (COPD). Smoking strongly increases the chance that a person with A1ATD will develop COPD (Yang et al. 2005). Currently there is no effective cure for either liver cirrhosis or COPD other than organ transplant (Verleden and Gottlieb 2023).

The *SERPINA1* gene produces 11 different mRNA variants, all which code for the same protein but have different 5' UnTranslated Regions (UTRs) (NCBI RefSeq annotation). This makes it one of the top 5% most complex genes in the human

genome (Corley et al. 2017). Genome-wide association studies found COPD-related variants in the *SERPINA1* gene's UTRs, introns, and promoter region (Morgan, Scobie, and Kalsheker 1993; Sandford et al. 1997). Additionally, genetic variants that affect *SERPINA1* splicing were found in the introns of COPD patients (E.Laubach, Ryan, and Brantly 1993; Seixas et al. 2002). Alternation in the 5'UTR may impact the translation of the A1AT, possibly by affecting the structure of *SERPINA1* mRNA (Ortega et al. 2020). *SERPINA1* mRNA can undergo alternative splicing to generate three distinct 5'UTR isoforms, which exhibit tissue-specific expression (Matamala et al. 2015). The structure of *SERPINA1* transcript isoform NM_000295's 5'UTR promotes accessibility at the translation initiation site (Grayeski et al. 2022). Our previous study revealed alternative polyadenylation (APA) as a regulatory mechanism that can influence A1AT protein expression (Lackey et al. 2021). However, there remain gaps in our understanding of how different polyadenylation (polyA) sites are selected and the impact of different 3'UTR isoforms.

For mRNA, the maturation of precursor messenger RNA (pre-mRNAs) involves processing of the 3' end, which is important for the function and stability of mRNA (Mandel, Bai, and Tong 2008). This process is mediated by a group of proteins called the cleavage and polyadenylation specificity factor (CPSF) complex that recognize the polyA sites in nascent mRNA. The poly(A) sites are defined by specific sequence motifs, such as the canonical AAUAAA hexamer or its close variants (Proudfoot and Brownlee 1976). The CPSF complex interacts with the cleavage stimulation factor (CSTF) and cleavage factors I and II (CFI and CFII) to cleave the pre-mRNA (Shi et al. 2009; Wilusz and Shenk 1990). Cleavage occurs at the poly(A) site, typically located 10-30 bases downstream of the AAUAAA polyadenylation signal (F. Chen, MacDonald, and Wilusf

1995). Poly(A) sites are flanked by U-rich (not GU-rich) elements, both upstream (USE) and downstream (DSE) of the cleavage site, located around positions 0 to -50 and +20 to +60 relative to the poly(A) signal, respectively. The presence of a USE and DSE distinguishes true poly(A) sites from randomly occurring AAUAAA hexamers(Legendre and Gautheret 2003). However, RNA secondary structure can alter polyadenylation site selection, the usage of a poly(A) site could be inhibited by an internal RNA stem-loop structure (Brown, Tiley, and Cullen 1991). After the cleavage of the pre-mRNA, an essential step in the 3' end processing is the addition of a poly(A) tail. This process is carried out by the polyA polymerase (PAP) enzyme (Edmonds 1990). Polyadenylation protects the pre-mRNA from enzymatic degradation and facilitates nuclear export and translation (Guhaniyogi and Brewer 2001).

APA is a widespread mechanism that generates transcript isoforms with varying 3' untranslated regions (3'UTRs) from the same gene, which occurs when there is more than on polyA site in same transcript (Ren et al. 2020). APA results in the same pre-mRNAs producing mature transcript isoforms that contain different lengths of 3’UTR. Different 3'UTR isoforms contain regulatory elements that modulate various aspects of gene expression (Batra, Manchanda, and Swanson 2015). For instance, microRNAs (miRNAs) can differentially bind to 3'UTR isoforms, leading to translational repression or mRNA destabilization (Huntzinger and Izaurralde 2011). In addition, RNA binding proteins (RBPs) also interact with specific sequences in the 3'UTR, and can affect mRNA stability, localization, and translation (Vallejos Baier, Picao-Osorio, and Alonso 2017).

SERPINA1 transcripts undergo APA, producing two distinct mRNA isoforms, with long and short 3’UTRs (Lackey et al. 2021). The longer 3'UTR isoform of *SERPINA1*

mRNA is associated with reduced A1AT protein levels, suggesting a repressive role of the extended 3'UTR in A1AT expression. This mechanism may be mediated by the presence of regulatory elements within the 3'UTR that interact with trans-acting factors, such as RNA-binding proteins or microRNAs, to modulate mRNA stability and translation (Guo and Lin 2023).

A1AT is an acute phase protein, upregulated during the inflammatory response, that acts to prevent cellular damage. Interleukin-6 (IL-6) is an important pro-inflammatory cytokine known to upregulate *SERPINA1* expression and influence the transcription of a broad set of target genes involved in immune response, cell proliferation, acute phase response and chronic inflammation (Seif et al. 2017)(Gabay 2006). IL-6 mainly activates the JAK/STAT, pathway, where Janus kinases (JAKs) phosphorylate and activate Signal Transducers and Activators of Transcription (STATs), which then translocate to the nucleus and regulate the expression of target genes (Naka et al. 1997). IL-6 also activates MAPK signaling. through the MAP kinase. MAP kinase, in turn, phosphorylates and activates various transcription factors (Kang et al. 2019).

In the context of lung diseases , the higher level of IL-6 expression is associated the worse lung symptoms in A1ATD (EPI-SCAN Steering Committee et al. 2010). Patients with COPD often have elevated IL-6 levels, which are linked to disease progression (Lilov, Marinova, and Slavova 2018). Additionally, a decreased A1AT : IL-6 ratio is associated with poor survival in COVID-19 in patients (Philippe et al. 2022).IL-6 also plays a role in liver cirrhosis by regulating acute-phase proteins and systemic inflammation (Heinrich, Castell, and Andus 1990). High IL-6 levels are associated with the severity of hepatic encephalopathy in liver cirrhosis patients (Wiest et al. 2011).

Alcohol is a major risk factor for liver disease in general, and for liver cirrhosis in particular, especially for individuals with disease-associated variants in *SERPINA1* (Tsochatzis, Bosch, and Burroughs 2014). Chronic ethanol exposure significantly affects liver function, possibly due to oxidative stress and inflammation response in the liver (Hakucho et al. 2014; Lieber 2004).

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the ability of cells to detoxify or repair the resulting damage. This imbalance can lead to detrimental effect on cellular components and processes (Pizzino et al. 2017). ROS are involved in cell signaling pathways that regulate diverse physiological processes like cell proliferation, apoptosis, and immune responses(Amman, Jordan et al. 2018). ROS are highly reactive molecules derived from oxygen, which include the superoxide anion ($O_2\bullet-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), and singlet oxygen ($\bullet O_2$).(Sies and Jones 2020). They are formed during normal cellular metabolism, particularly in the mitochondria, as well as in response to environmental stresses, such as UV radiation, chemical exposure, and inflammation (Ray, Huang, and Tsuji 2012).

Peroxide (H_2O_2) is of particular interest because it can be used to study ROS more directly than ethanol, which has a complex effect on the cell. Peroxide can be generated during ethanol metabolism. Peroxide can activate the mitogen-activated protein kinase (MAPK) signaling pathways, which regulate cellular processes like inflammation, proliferation, and apoptosis (Bhat and Zhang 1999). Peroxide can increase the levels of malondialdehyde (MDA) and decreased activity of superoxide dismutase (SOD), characteristic of apoptosis or cell death, in HepG2 cells (Jiang et al. 2014).

All three drugs in the above study have effects on the liver, which is the main organ for secreting A1AT. IL-6 and other cytokines are known to control the transcription of *SERPINA1* (Ercetin et al. 2019). However, there is no direct evidence provided that exposure to ethanol or peroxide is associated with altered *SERPINA1* transcription. Also, the relationship between these drugs and APA of *SERPINA1* in liver cells is not explicitly established.

This study aims to explore how environmental conditions such as IL-6, ethanol and peroxide influence the alternative polyadenylation and expression of *SERPINA1* mRNA in HepG2 cells. By understanding these mechanisms, we hope to uncover novel regulatory pathways that could be targeted to treat A1AT-related diseases.

Results

We hypothesize that environmental conditions regulate *SERPINA1* mRNA expression in hepatocyte cells. Our previous research showed that lung tissue from individuals with COPD have an increase in the long 3'UTR isoform of *SERPINA1* compared to healthy individuals, suggesting that inflammatory and immune responses may alter the post-transcriptional processing of *SERPINA1* (Lackey et al. 2021). To test our hypothesis that environmental conditions affect *SERPINA1* in hepatocytes, we exposed HepG2 liver carcinoma cells with three different treatments: IL-6, ethanol, and peroxide. We chose these treatments based on our knowledge that A1AT, the protein product of *SERPINA1,* is a leading cause of lung and liver disease, which are known to be influenced by inflammation and alcohol consumption (Grander et al. 2021). We expect to validate known changes in gene expression, as previously published in IL-6 and ethanol treatment (Lukowski et al. 2015)(Pochareddy and Edenberg 2012) and using 3' specific RNA sequencing, test whether *SERPINA1* undergoes post-transcriptional regulation of polyadenylation in response to IL-6, ethanol or peroxide.

HepG2 liver cell response to IL-6 treatment

To verify that IL-6 treatment was working as expected, we measured the mRNA expression of *IL1R1* and *FGB* in HepG2 cells using qRT-PCR (Lukowski et al. 2015). Following IL-6 treatment, HepG2 cells exhibited a significant increase in *IL1R1* mRNA expression compared to the untreated control group at both 4 hours and 24 hours

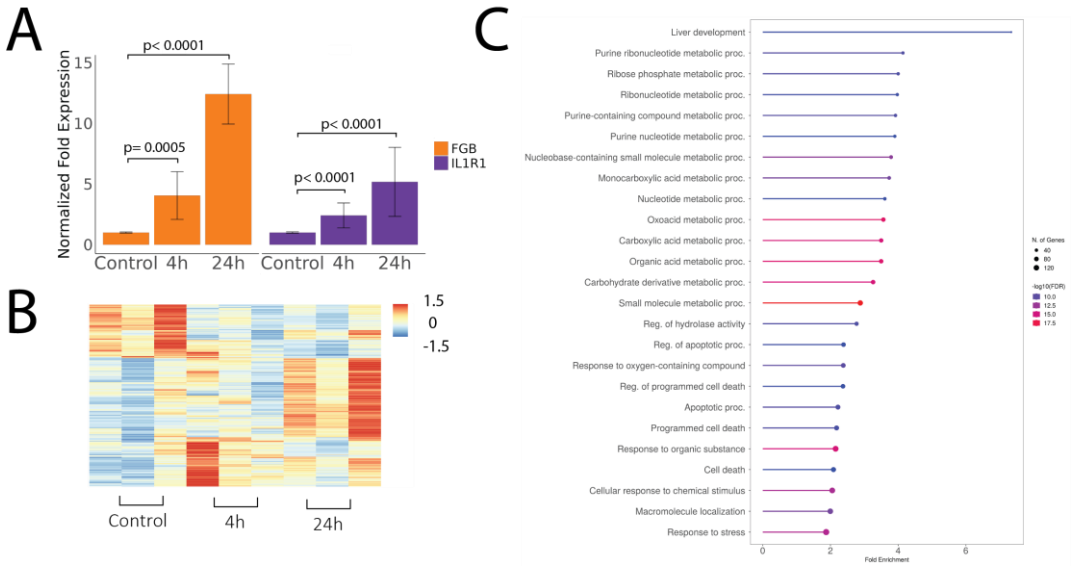


Figure 1. IL-6 exposure induces an inflammatory response in HepG2 liver cells.

HepG2 cells were exposed to IL-6 for 4 or 24 hours (A) qRT-PCR was used to measure the expression of the *FGB*, *IL1R1* and normalization control *GAPDH* (B) QuantSeq RNA sequencing libraries for differential gene expression analysis. (C) Genes identified as differentially expressed were analyzed for pathway enrichment.

(FDR < 0.05) (Figure 1A). This indicates that *IL1R1* is upregulated in response to IL-6 as expected. Similarly, the expression of *FGB* was significantly elevated in IL-6 treated HepG2 cells at both 4 hours and 24 hours (FDR < 0.05) (Figure 1A). This upregulation aligns with the expected response, as *FGB* is known to be involved in acute phase reactions (Róka et al. 2019). The increase in *IL1R1* and *FGB* expression indicates that IL-6 treatment induces the expected response in HepG2 cells.

To investigate gene expression changes we performed 3' end specific QuantSeq library preparation followed by RNA sequencing on HepG2 cells treated with IL-6 at

two time points (4 hours and 24 hours) compared to control conditions. We conducted Differential Gene Analysis (Robinson, McCarthy, and Smyth 2010) with these data. We found that after IL-6 treatment, 492 genes were identified as differentially expressed in response to IL-6 treatment at either 4 or 24 hours (Figure 1B). From Gene Ontology analysis performed by ShinyGo 0.80 (Ge et al., 2020), we found that these differentially expressed genes were significantly enriched in liver development and response to stress Signaling Pathways, indicating a broad impact of IL-6 on cellular function and stress responses. Our qRT-PCR and sequencing data confirm that IL-6 treatment alters RNA levels of genes associated with inflammation and cell growth.

IL-6 treatment changes alternative polyadenylation of *SERPINA1*

The QuantSeq library preparation protocol we used allows us to identify the specific 3'UTR end of transcripts, making it possible for us to analyze alternative polyadenylation of *SERPINA1* mRNA. We measured reads in the 3'UTR of *SERPINA1* in

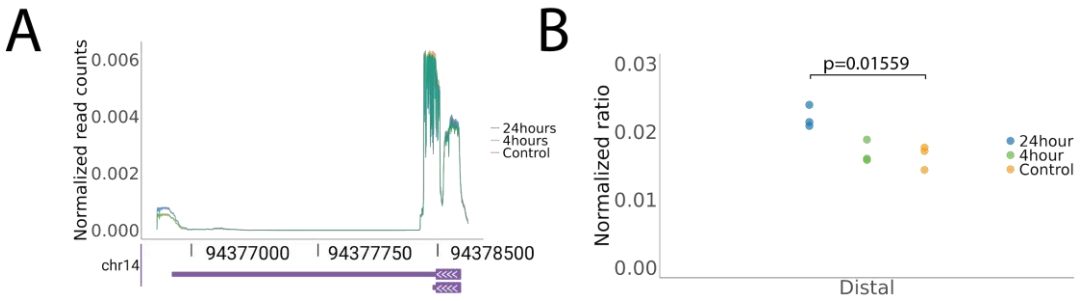


Figure 2. Increase in *SERPINA1* distal polyA site use after IL-6 treatment.

Using aligned sequencing readsfor quantifying the SERPINA1 3'UTR read coverage.
A) A) Prevalence of isoforms as measured by read density across the *SERPINA1* 3'UTR. A schematic from the UCSC Genome Browser for proximal and differentially expressed distal 3′ UTR is shown below the plots, thinner lines indicate 3'UTR and arrow indicates the direction of strand.
B) Quantification of reads in the proximal and distal *SERPINA1* 3'UTR, highlighting a significant increase in reads in the distal part after 24 hours of IL-6 treatment, normalized by region size.

HepG2 cells treated with IL-6 at 0, 4 and 24 hours (Figure 2A). Previous studies identified two polyA sites in the 3'UTR of *SERPINA1* (Lackey et al. 2021), which we validated (Figure 2A). In addition, we found evidence for an additional polyA site in the last exon of *SERPINA1*, before the coding sequence (Figure 2A). We calculated the length normalized number of reads in the proximal and distal 3'UTRs and found a significant increase (p value=0.01559) in read density in the distal part of the *SERPINA1* 3'UTR after 24 hours of IL-6 treatment (Figure 2B). This supports IL-6 mediated regulation of *SERPINA1* mRNA polyadenylation.

IL-6 treatment changes transcriptome-wide alternative polyadenylation

To reveal the impact of IL-6 treatment on transcriptome-wide alternative polyadenylation in HepG2 cells, we conducted analyses of alternative polyadenylation on our QuantSeq RNA sequencing data by using QAPA(Ha, Blencowe, and Morris 2018).

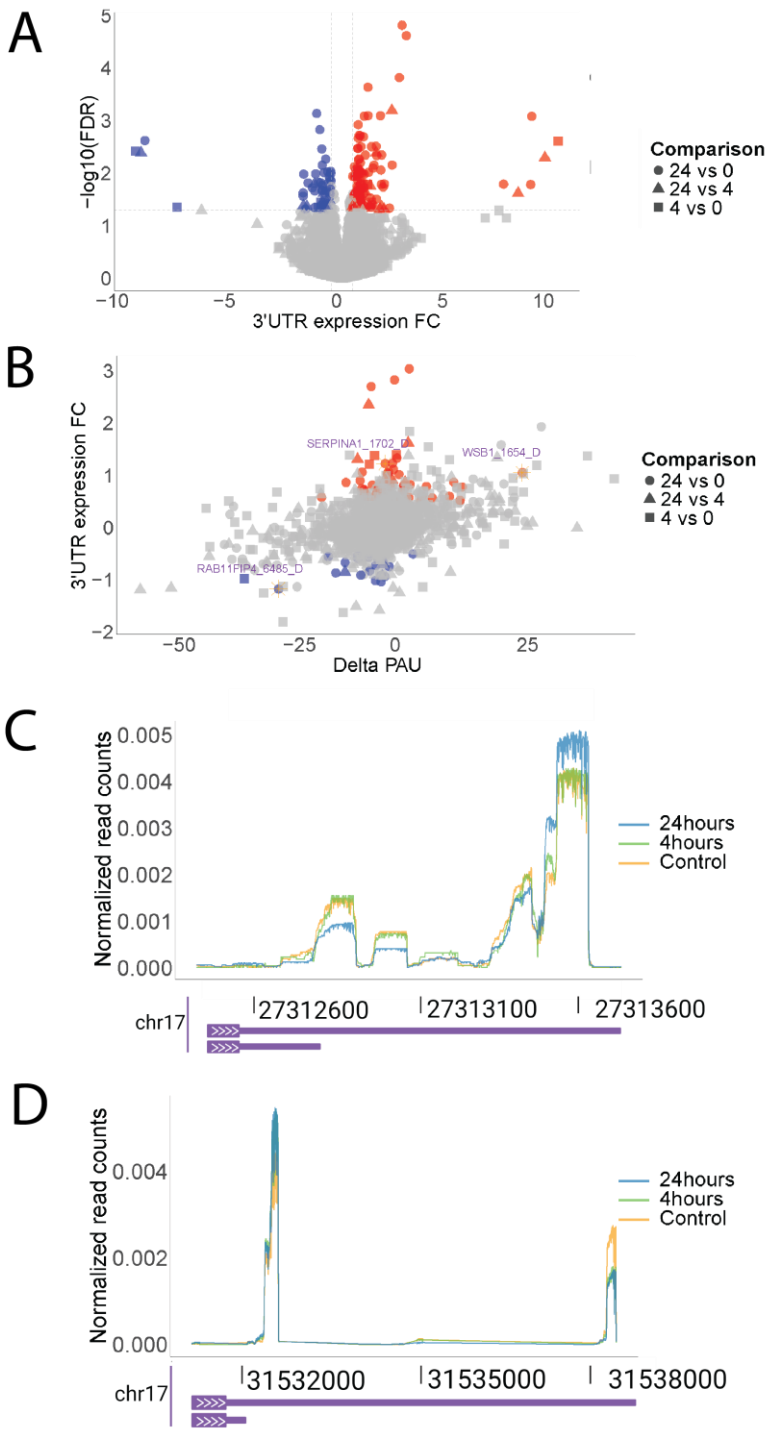


Figure 3. Impact of IL-6 treatment on alternative polyadenylation in HepG2 cells.

A) Volcano plot of differentially expressed genes, red dots indicate up regulated long 3'UTR isoforms, blue dots indicate down regulated long 3'UTR isoforms, dash lines indicate fold change and FDR cut off.
B) Correlation between distal polyadenylation usage (PAU) and isoform expression fold change. C) Coverage of mapped reads on gene *WSB1* 3'UTR. D) Coverage of mapped reads on gene *RAB11FIP4* 3'UTR. A schematic from the UCSC Genome Browser for proximal and differentially expressed distal 3′ UTR is shown below the plots, thinner lines indicate 3'UTR and arrow indicates the direction of strand.

QAPA calculates relative usage of alternative 3′ UTR isoforms based on transcript-level abundance. From the distribution of the percentage of long 3'UTR isoforms in the transcriptome, we did not find any significant transcriptome-wide changes between the control group and the treatment group. However, interestingly, 4 and 24 hours treatment with IL-6 resulted in a small increase in 60-70% of long isoforms (Supplementary

figure 1). We found 90 long 3'UTR isoforms that are differentially expressed in response to IL-6 treatment at either 4 or 24 hours. The correlation between significant value and 3'UTR isoform expression fold change is demonstrated in (Figure 3A). After 24 hours of IL-6 treatment the number of up-regulated and down-regulated long isoforms is basically the same. For ten of these differentially expressed isoforms, their proportion changed by more than 10%. Notably, the long isoform of gene *WSB1* increased by the highest percentage (28.95%) after 24 hours of IL-6 treatment and the long isoform of gene *RAB11FIP4* decreased by the lowest percentage (28.74%) after 4 hours of IL-6 treatment (Figure 3B). *WSB1* is implicated in tissue repair and fibrotic disease processes (Chong et al. 2024). Over-expression of *Rab11FIP4* is linked to worse outcomes and increased tumor growth in pancreatic cancer (He et al. 2017). We also performed coverage of mapped reads of these two genes (Figure 3C, Figure 3D) .This indicates that IL-6 not only alters the selection of *SERPINA1* poly(A) site selection, but IL-6 treatment can significantly influence the alternative polyadenylation of additional genes, potentially impacting their function and regulation.

HepG2 liver cell response to Ethanol

Next, we tested the impact of ethanol on RNA expression and polyadenylation. Ethanol is known to cause multiple, complex responses in cells (Wilke et al. 1994). To verify that ethanol treatment was working as expected, we measured the mRNA

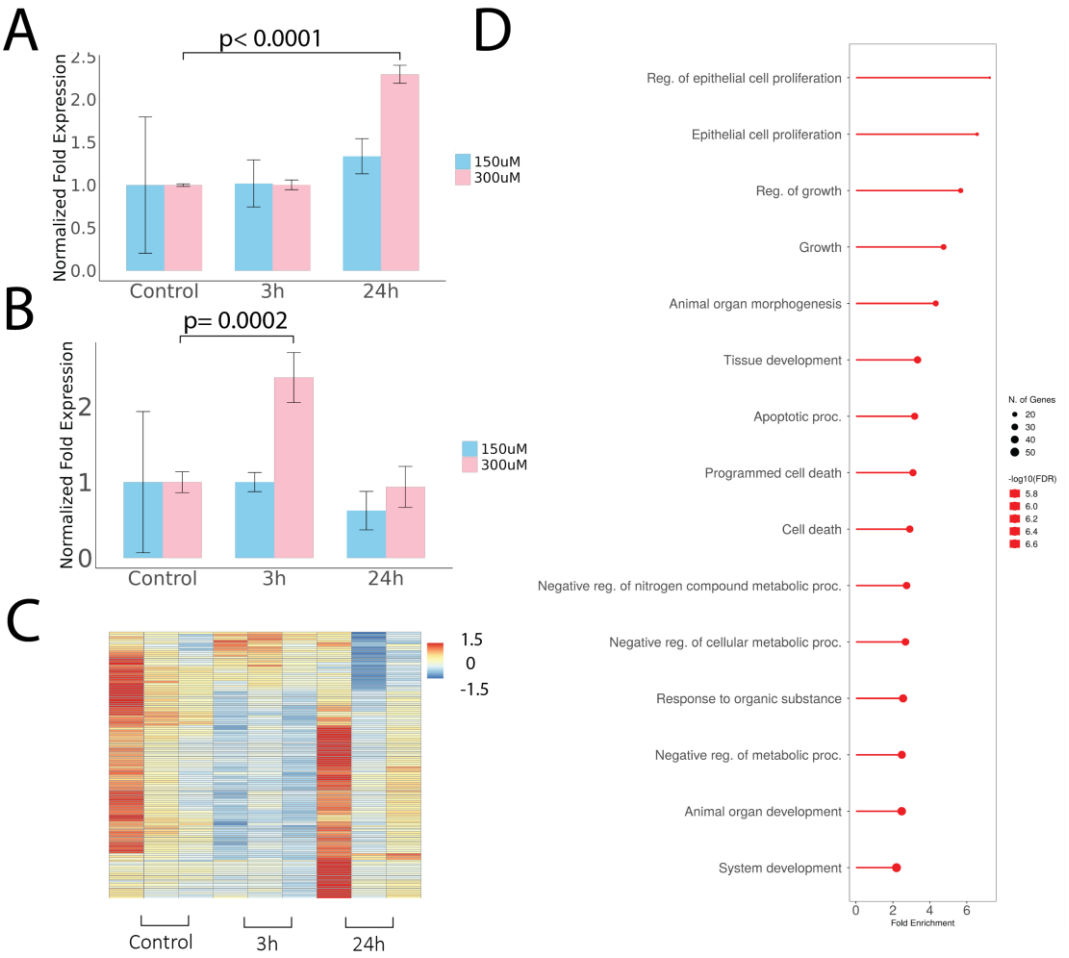


Figure 4. Ethanol induces differentially expressed genes in HepG2 liver cells.

HepG2 cells were exposed to ethanol for 3 or 24 hours. A) B) qRT-PCR was used to measure the expression of the *BCL2*, *GPX2* and normalization control *GAPDH* C) and used to make QuantSeq RNA sequencing libraries for differential gene expression analysis. D) Gene identified as differentially expressed were analyzed for gene ontology enrichment.

expression of several *BCL2* and *GPX2* in HepG2 cells using qRT-PCR (Casañas-Sánchez et al. 2016). Following ethanol treatment, HepG2 cells exhibited a significant increase in *BCL2* mRNA expression compared to the untreated control group at 3 hours with 300 mM ethanol (FDR < 0.05) (Figure 4A). This indicates that *BCL2* is upregulated in

response to ethanol as expected (C. Li et al. 2020).Similarly, the expression of *GPX2* was significantly elevated in ethanol treated HepG2 cells at 24 hours with 300mM ethanol (FDR < 0.05) (Figure 4B). This upregulation aligns with the expected response, as *GPX2* is known to be involved in antioxidant reaction reactions (Emmink et al. 2014). The increase in *BCL2* and *GPX2* expression indicates that ethanol treatment induces the expected response in HepG2 cells. Although we did not see a significant difference in RNA levels with 170 mM treatment, we found that high concentrations of alcohol caused cell death (unpublished data). Since the 170 and 300 mM treatments had similar trends, we decided to use the 170 mM treatment RNA sequencing and subsequent analysis.

To investigate ethanol induced gene expression changes we performed QuantSeq RNA sequencing on HepG2 cells treated with ethanol at two time points (3 hours and 24 hours) compared to control cells. We conducted Differential Gene Analysis (Robinson, McCarthy, and Smyth 2010) with these data. We found that after ethanol treatment, 136 genes were differentially expressed in response to ethanol treatment at either 3 or 24 hours (Figure 4C). From Gene Ontology analysis (Ashburner et al. 2000) we found that these differentially expressed genes were significantly enriched in the growth pathway , apoptotic process pathway and cell death pathway, suggesting that ethanol treatment impacts various growth pathways and regulation processes in HepG2 cells (Figure 4D).

Ethanol treatment on alternative polyadenylation of *SERPINA1*

To investigate alternative polyadenylation changes of *SERPINA1* in HepG2 cells treated with ethanol at 3 and 24 hours, we extracted read counts within the 3'UTR of *SERPINA1* (Figure 5A). We calculated the length normalized number of reads in the

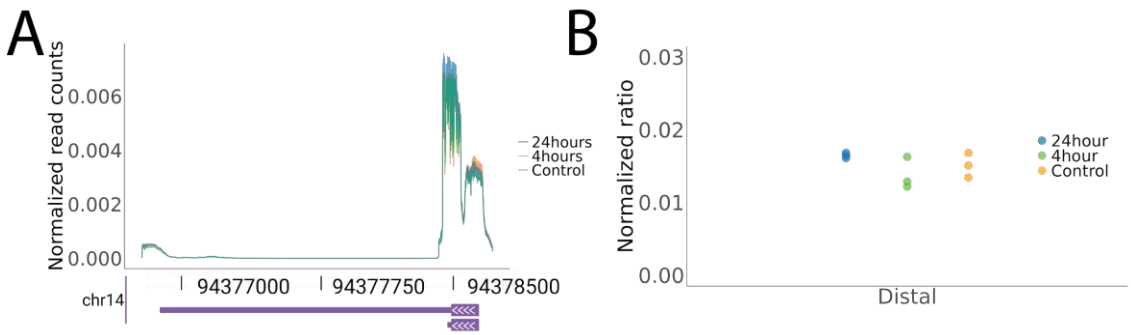


Figure 5. Coverage of mapped reads on *SERPINA1* and significance test on normalized ratio.

Using aligned sequencing reads for quantifying the SERPINA1 3'UTR read coverage.
A) Prevalence of isoforms as measured by read density across the *SERPINA1* 3'UTR. A schematic from the UCSC Genome Browser for proximal and differentially expressed distal 3′ UTR is shown below the plots, thinner lines indicate 3'UTR and arrow indicates the direction of strand.
B) Quantification of reads in the ethanol treatment and distal *SERPINA1* 3'UTR, normalized by region size.

proximal and distal 3'UTRs. We did not find any significant increase in read density in the distal part or proximal part of the *SERPINA1* 3'UTR after 3 hours or 24 hours of ethanol treatment (Figure 5B). This suggests that treatment with 170 mM of ethanol at either 3 or 24 hours does not influence *SERPINA1* mRNA polyadenylation.

Ethanol treatment has minimal impact on transcriptome-wide alternative polyadenylation

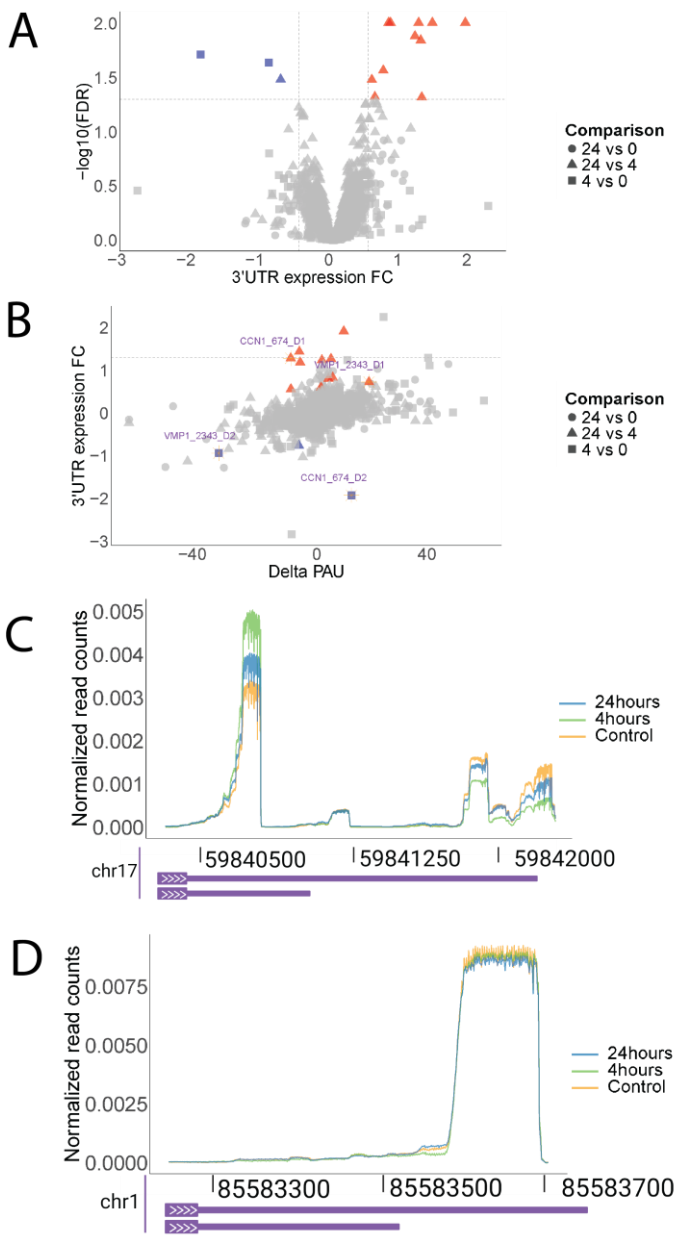


Figure 6. Impact of ethanol treatment on alternative polyadenylation in HepG2 cells.

A) Volcano plot of differentially expressed genes, red dots indicate up regulated long 3'UTR isoforms, blue dots indicate down regulated long 3'UTR isoforms, dash lines indicate fold change and FDR cut off. B) Correlation between distal polyadenylation usage (PAU) and isoform expression fold change C) Coverage of mapped reads on gene *VMP1* 3'UTR. D) Coverage of mapped reads on gene *CCN1* 3'UTR. A schematic from the UCSC Genome Browser for proximal and differentially expressed distal 3′ UTR is shown below the plots, thinner lines indicate 3'UTR and arrow indicates the direction of strand.

To reveal the impact of ethanol treatment on transcriptome-wide alternative polyadenylation in HepG2 cells, we conducted analyses of alternative polyadenylation on our QuantSeq RNA sequencing data by using QAPA(Ha, Blencowe, and Morris 2018). From the distribution of the percentage of long 3'UTR isoforms in the transcriptome, we did not find any significant transcriptome-wide changes between the control group and the treatment groups (Supplementary figure 2). There were 14 differentially expressed 3'UTR isoforms in response to ethanol treatment at 3hour ethanol

treatment (Figure 6A). The long isoform of gene *VMP1* decreased by the highest percentage (35.76%) after 3 hours of ethanol-6 treatment (Figure 6B), *VMP1* is involved in the formation of autophagosomes and interacts with various cellular components such as lipid droplets, Golgi, and endosomes (Wang, Kou, and Le 2020). The long isoform of gene *CCN1* was also flagged as decreasing over the same period by highest percentage (10.72%) (Figure 6B). *CCN1* promotes cell proliferation, chemotaxis, angiogenesis, and cell adhesion, and play a crucial role in wound healing (C.-C. Chen, Mo, and Lau 2001). We also performed coverage of mapped reads of these two genes (Figure 6C, Figure 6D), we observed after 24 hours treatment the percentage of long isoforms returned to number similar to control for both transcripts. Ethanol does not significantly influence transcriptome-wide alternative polyadenylation. but does affect specific genes, with the potential to impact their function.

HepG2 liver cell response to Peroxide

Ethanol is expected to induce reactive oxygen species (ROS) (Bailey, Pietsch, and Cunningham 1999). Another way to directly study ROS is to treat cells with

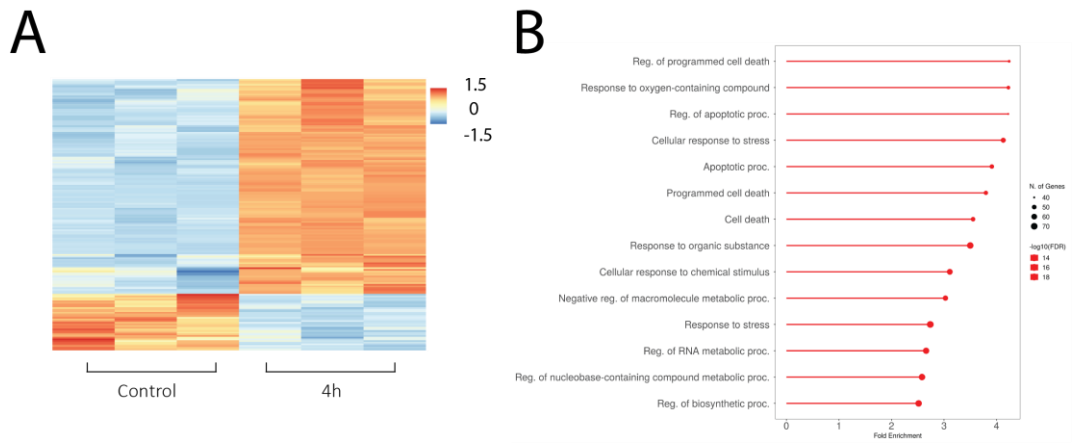


Figure 7. Peroxide induces differentially expressed genes in HepG2 liver cells.

HepG2 cells were exposed to peroxide for 4 hours and used to make QuantSeq RNA sequencing libraries for (A) differential gene expression analysis. (B) Gene identified as differentially expressed were analyzed for pathway enrichment.

peroxide (Xin, Gong, and Hong 2022). To investigate ROS-induced gene expression changes we treated HepG2 cells with peroxide and performed QuantSeq RNA sequencing after 4 hours. We conducted DEG (Robinson, McCarthy, and Smyth 2010) with control cells and cells treated for 4 hours with peroxide. We found that after peroxide treatment, 164 genes were identified as differentially expressed in response to peroxide treatment at 4 hours (Figure 7A). From Gene Ontology analysis (Ashburner et al. 2000) we found that these differentially expressed genes were significantly enriched in response to apoptotic pathway and response to stress and suggesting that peroxide both impact stress response pathways in HepG2 cells (Figure 7B).

Peroxide treatment on alternative polyadenylation of *SERPINA1*

To investigate alternative polyadenylation changes of *SERPINA1* in HepG2 cells treated with peroxide for 4 hours, we extracted read depths from our RNA-seq data (Figure 8A). We calculated the length normalized number of reads in the proximal and

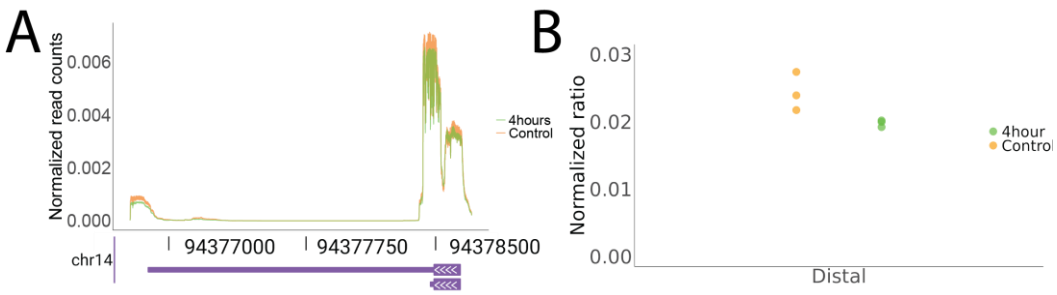


Figure 8. Coverage of mapped reads on *SERPINA1* and significance test on normalized ratio.

Using aligned sequencing reads for quantifying the SERPINA1 3'UTR read coverage.
A) Prevalence of isoforms as measured by read density across the *SERPINA1* 3'UTR. A schematic from the UCSC Genome Browser for proximal and differentially expressed distal 3′ UTR is shown below the plots, thinner lines indicate 3'UTR and arrow indicates the direction of strand.
B) Quantification of reads in the proximal and distal *SERPINA1* 3'UTR, normalized by region size.

distal 3'UTRs and did not find any significant increase in read density in the distal part or proximal part of the SERPINA1 3'UTR after 4 hours of peroxide treatment (Figure 8B). This suggests that peroxide and ROS may not influence regulation of *SERPINA1* mRNA polyadenylation during the timeframe studied.

Peroxide treatment changes transcriptome-wide alternative polyadenylation

To reveal the impact of peroxide treatment on transcriptome-wide alternative polyadenylation in HepG2 cells, we conducted analyses of alternative polyadenylation on our QuantSeq RNA sequencing data by using QAPA (Ha, Blencowe, and Morris 2018). From the distribution of the percentage of long 3'UTR isoforms in the transcriptome, we did not find any significant changes between the control group and the treatment group (Supplementary figure 3). We found 52 long 3'UTR isoforms were identified as differentially expressed in response to peroxide treatment at 4 hours (Figure 9A). For nine of

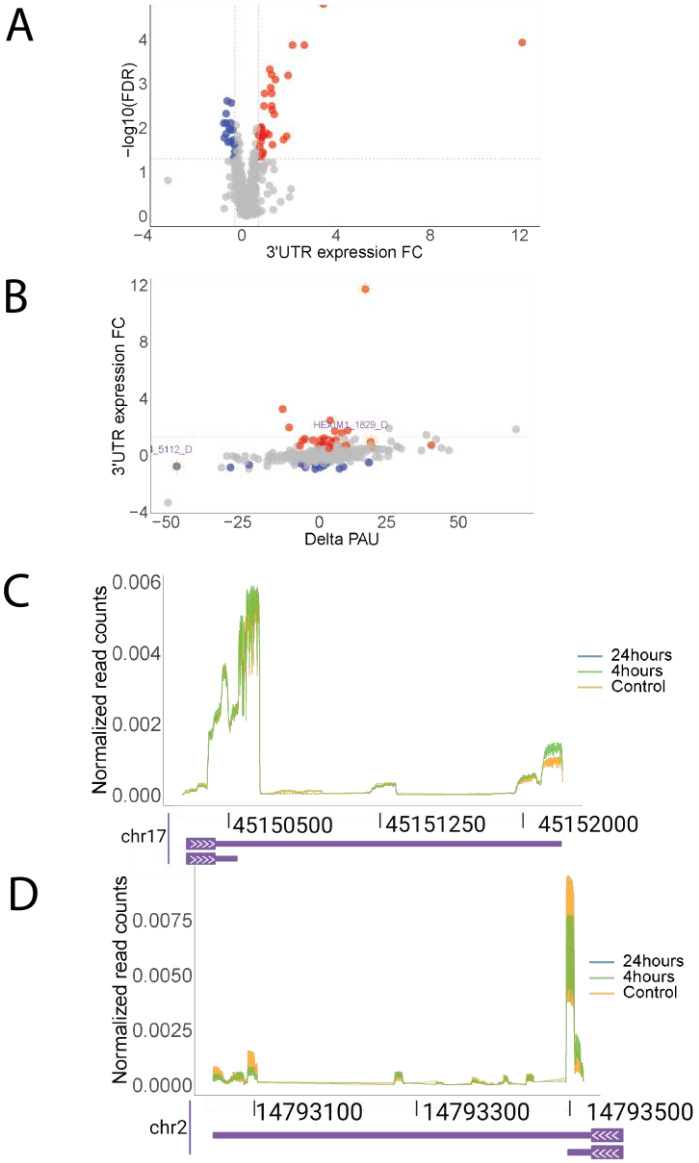


Figure 9. Impact of peroxide treatment on alternative polyadenylation in HepG2 cells.

A) Volcano plot of differentially expressed genes, red dots indicate up regulated long 3'UTR isoforms, blue dots indicate down regulated long 3'UTR isoforms, dash lines indicate fold change and FDR cut off. B) Correlation between distal polyadenylation usage (PAU) and isoform expression fold change C) Coverage of mapped reads on gene *HEXIM1* 3'UTR. D) Coverage of mapped reads on gene *ORC4* 3'UTR. A schematic from the UCSC Genome Browser for proximal and differentially expressed distal 3′ UTR is shown below the plots, thinner lines indicate 3'UTR and arrow indicates the direction of strand.

these isoforms, their proportion in the entire transcript changed by more than 10%. Notably, the long isoform of gene SNX6 increased by the highest percentage (37.36%) 4 hours of peroxide treatment, however, the differences between the three samples are large, so we chose to demonstrate the second highest increased percentage (16.27%) gene : *HEXIM1*(Fig 9B), *HEXIM1* is a protein-coding gene that functions as a general RNA polymerase II transcription inhibitor (Michels et al. 2004). The long isoform of gene *ORC4* decreased by the lowest percentage (51.19%) (Figure 9B), *ORC* complex acts as a nucleating center for the assembly of the pre-replication complex, which is essential for the initiation of DNA replication (Kara et al. 2015). We also performed coverage of mapped reads of these two genes (Figure 9C, Figure 9D). This result indicates that peroxide can significantly influence the alternative polyadenylation of specific genes.

Discussion

The cytokine IL-6 induces an inflammatory response in HepG2 cells and upregulates SERPINA1 mRNA expression (Piret et al. 2011). We found that IL-6 influences the usage of distal polyadenylation sites in *SERPINA1* transcripts, but also changes APA site usage in a transcriptome-wide manner. These findings indicate that IL-6 signaling can broadly impact gene expression programs through both transcriptional and post-transcriptional mechanisms. The modulation of APA patterns by IL-6 suggests a potential role for this post-transcriptional regulatory process in mediating cellular responses to inflammatory stimuli. The widespread changes in APA site usage induced by IL-6 may contribute to the regulation of various cellular pathways and processes, including those involved in the response to endoplasmic reticulum stress and response to Unfolded Protein Signaling Pathways. We observed that IL-6 could influence compared to other isoforms, the long isoform ENST00000262394 of gene *WSB1* expression is increased by the highest percentage, which is strongly associated with hypoxic conditions, and there is a research reveals its role in the cellular response to low oxygen levels(EPI-SCAN Steering Committee et al. 2010). In our GO analysis we also found pathway response to hypoxia is highly enriched, it suggests that IL-6 treatment could enhance the cellular adaptation to hypoxic stress by upregulating the expression of specific isoforms.

We will examine the identified differentially expressed genes from our experiment and narrow down the number of genes that may be involved in *SERPINA1* APA regulation by filtering regulatory factors from a list of genes related to APA site choice, using the TREND database(Ogorodnikov and Danckwardt 2021). We have found 8 TREND genes are overlapping with differentially expressed genes, here is the

gene list: HNRNPH1, HNRNPA2B1, SRSF1, ALYREF, PCNA, SRSF3, UPF1, SF1. Interestingly, from this overlapped list, only one gene is occurred in APA gene list, which is SRSF1. SRSF1 is important splicing factor, in our future research, we will take focus on this gene.

We are investigating the interaction between RBPs and *SERPINA1* 3'UTR. We are using biotinylated RNA to pull down proteins that interact with the 3'UTR and using Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to determine which RBPs specifically bind to the short or long 3'UTR of *SERPINA1* mRNA. In our previous study, we found that RBP Quaking (QKI) would suppresses A1AT protein expression by interacting with distal part of *SERPINA1* 3'UTRs (Lackey et al. 2021). From our investigation by mining eCLIP data (enhanced CrossLinking and ImmunoPrecipitation) from recent ENCODE (Encyclopedia of DNA Elements) experiments on RNA binding proteins carried out in HepG2 cell lines(Van Nostrand et al. 2016), we have found 9 RBP's binding motifs are highly enriched in distal 3'UTR of *SERPINA1* mRNA by using package HOMER (Heinz et al. 2010). In future, for those RBPs that found from bioinformatic study or UHPLC-MS/MS, using small interfering RNA or other RNA knockdown technology to identify which factors that impact the localization or translational efficiency of the long 3'UTR *SERPINA1* mRNA isoform.

Ethanol treatment increases ROS levels in HepG2 cells (Gutiérrez-Ruiz et al. 1999) altering the mRNA expression of genes involved in apoptosis and oxidative stress, such as *BCL2* and *GPX24*. Ethanol did not directly affect *SERPINA1* expression or APA in our data. However, the transcripts of *CCN1* and *VMP1* genes exhibited APA following ethanol treatment, favoring the usage of proximal polyadenylation sites.

Interestingly, we observed that the long isoform of the *VMP1* gene (ENST00000262291) is down-regulated after a 3-hour treatment with ethanol, indicating a potential influence of ethanol on alternative polyadenylation (APA) site choice in transcription. In contrast, treatment with interleukin-6 (IL-6) and hydrogen peroxide leads to upregulation of the long isoform of the *VMP1* gene, suggesting a distinct effect on APA site selection compared to ethanol. The differential regulation of the *VMP1* gene isoform by ethanol, IL-6, and hydrogen peroxide highlights the complex interplay between various signaling pathways and the polyadenylation machinery, resulting in distinct patterns of APA site choice and transcript isoform expression. Due to concerns about cell toxicity, we chose a lower concentration of ethanol for our cell treatment experiment. This could be the reason that we identified a relatively low number of differentially expressed genes (136) and fewer instances of transcriptome-wide APA. In future studies, we plan to use a higher concentration of ethanol (300mM) to treat the HepG2 cell line to further investigate the impact of ethanol on transcription of SERPINA1 and APA regulation.

Similar to ethanol, hydrogen peroxide (H_2O_2), is an exogenous ROS inducer and enhances ROS activity in HepG2 cells (Lister et al. 2020). Like ethanol, we did not see direct effects of H_2O_2 on *SERPINA1* expression or APA. The transcripts of *HEXIM1* and *ORC4* genes exhibited alternative polyadenylation in response to H_2O_2 treatment, with *HEXIM1* favoring the usage of distal PA sites and *ORC4* favoring proximal PA sites. In general, a larger set of transcripts were alternatively polyadenylated after peroxide treatment compared to ethanol treatment. This may be due to our decision to use lower levels of ethanol to avoid cell toxicity.

In summary, this study demonstrated that IL-6 treatment in HepG2 cells induces an inflammatory response, alters the expression of genes involved in various cellular pathways, and modulates alternative polyadenylation patterns of specific transcripts, including *SERPINA1*. These results provide insights into the broad impact of IL-6 signaling on gene expression regulation and suggest a potential role for APA in mediating cellular responses to inflammatory stimuli.

Methods

Cell culture and treatments

HepG2 cells were sourced from Synthego. Both cell lines were seeded in 10cm^2 plates and grown at 5% CO2 and 37 C. The grown media was Eagle's Minimum Essential Media (EMEM, ATCC #30-2003) supplemented with 5% FBS (Thermo Scientific #A3160602) and 0.5% Penicillin-Streptomycin (MilliporeSigma #P4333-100mL). For exposure treatments, both cell lines were seeded into at 3.0 x 10^5 cells in 6-well plates. Cells were incubated for 24 hours then serum-starved using 1 mL serum-free EMEM for another 24 hours. After media starvation, the cells were treated in 1 mL serum-free media with 170mM ethanol diluted in PBS (EtOH, 3, and 24 hours. Fisher Scientific, #BP2818-4), 150 uM hydrogen peroxide diluted in PBS (H_2O_2, 4 hours. Fisher Scientific, #H325-500), and 20ng/mL interleukin-6 diluted in 0.1% of BSA at (IL-6, 4, and 24 hours. Fisher Scientific, #200-06-20UG).

RNA extraction

Total RNA was extracted using Trizol (Thermo Fisher) according to manufacturer's recommendations for adherent cell lines. After RNA extraction via trizol reagent, RNA was purified using RNA clean up columns (NEB, #T2030L). Genomic DNA was removed using TURBO DNase (Fisher Scientific) according to the given protocol. RNA was quantified via NanoDrop (Thermo Scientific ND-8000).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on a QuantStudio 3 (Thermo Scientific) system using iTaq Universal SYBR Green One-Step Kit (Biorad). For EtOH and peroxide experiments, 150 ng of total RNA was loaded, in biological triplicate, into a total reaction of 20 uL. For IL-6 treatment experiments, 150 ng of total RNA was loaded, also in biological

triplicate, was used in a total reaction volume 20uL. qRT-PCR run parameters were as follow: 10 minutes reverse transcription at 50 C, 1 minute polymerase activation and DNA denaturation at 95 C, followed by 40 cycles involving denaturation at 95 C for 15 seconds, annealing/extension/plate read at 60 C for 1 minute, and a melt curve analysis (95 C, 15 sec; 60 C, 1 min; 95 C, 1 sec with 0.15C /s ramp rate). The sequences for the primers used for genes *BCL2*, *GPX2*, *GAPDH*, *ACTB*, *FGB*, *IL1R1* are shown in Table [SUPPLEMENTARY TABLE 1].

Quantification of qRT-PCR results

Cq values were analyzed for variability and outliers (+/-0.5 deviation from mean Cq per group) were removed. Relative quantity (RQ) was calculated as $2^{\Delta Cq}$ Normalized expression was calculated as RQ (test gene)/ RQ (geometric mean of the associated reference gene). Fold change was calculated as $\log_2{}^{(\text{Normalized expression})}$and significance was calculated by t-test and p value is adjusted by Benjamini-Hochberg. (Taylor et al. 2019).

RNA library preparation and sequencing

RNA samples were prepared using the QuantSeq 3' mRNA V2 Library Prep Kit V2 (Lexogen), which captures polyadenylated RNA. Quality assurance for loading samples for sequencing was performed using a Qubit dsDNA High Sensitivity Assay (Thermo Scientific) on a Qubit 4 fluorometer (Thermo Scientific), and a High Sensitivity D1000 Screentape (Agilent) on a Tapestation 4150 (Agilent). The Lexogen QuantSeq samples were sequenced on a NovaSeq 6000 (Illumina) using a 2x100 cycle cartridge. A custom sequence primer was used for Read 1 according to instructions from Lexogen and Illumina.

Quantification of *SERPINA1* 3'UTR reads

Coverage of mapped reads of certain regions are conducted by Samtools mpileup (H. Li et al. 2009), each position's normalized read counts is calculated by dividing total read counts of region from the read counts of each individual position. The read ratio for each genomic region was calculated by dividing the read counts by the length of the region, and the normalized read depth ratio was subsequently calculated by dividing the read ratio of each region by the sum of the read ratios of all regions.

Differential gene expression analysis

For differential gene expression analysis, transcripts were exported as .txt files into R. The edgeR pipeline used for TMM normalization and quantification was based on the pipeline made by Vijay Shankar and is available on his GitHub (https://github.com/vshanka23/snakemake_rnaseq). The modified script used, "RNA-seq with edgeR", is available on GitHub (https://github.com/bmunn99/Clemson-Grad-Rscripts). The edgeR package was used to normalize library sizes and quantify expression levels for individual gene (Robinson, McCarthy, and Smyth 2010). Genes considered differentially expressed were those that passed the following filters: FDR < 0.05 and $\log_2$CPM > 6 and $\log_2$FC > 0.5.

Gene ontology and pathway analysis

Differentially expressed genes (FDR < 0.05 and $\log_2$CPM > 6 and $\log_2$FC > 0.5.) were input into the ShinyGo 0.80 website (http://bioinformatics.sdstate.edu/go/) for biological pathway analysis (T. Wu et al., 2021), gene sets were filtered by FDR<0.05. Dot plots were made using ggplot2 (Wickham et al., 2019).

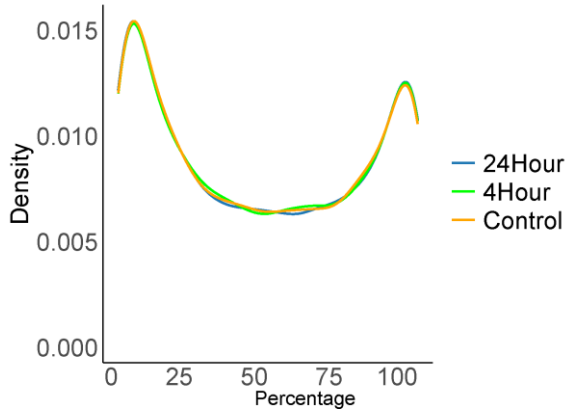
Transcriptome-wide alternative polyadenylation analysis

We prepared the necessary gene and poly(A) annotation files from Ensembl Biomart andused QAPA build to create the 3' UTR library. We used the quantification tool salmon to quantify 3’ UTR Isoform Usage. We used QAPA to create the raw count for isoform expression and PolyAdenylation Usage (PAU) and Transcript Pre Million (TPM) matrix. For differential gene expression analysis, transcripts were exported as .txt files into R. The edgeR pipeline used for TMM normalization and quantification was based on the pipeline made by Vijay Shankar and is available on his GitHub (https://github.com/vshanka23/snakemake_rnaseq). Isoforms considered differentially expressed were those that passed the following filters: FDR < 0.05 and $\log_2$CPM > 6 and $\log_2$FC > 0.5. The PAU matrix shows the TPM proportion of each isoform in its overall gene expression. The percentage change in the long isoform is calculated by subtracting the mean value of PAU in the control set from the mean value of PAU in the treatment set.

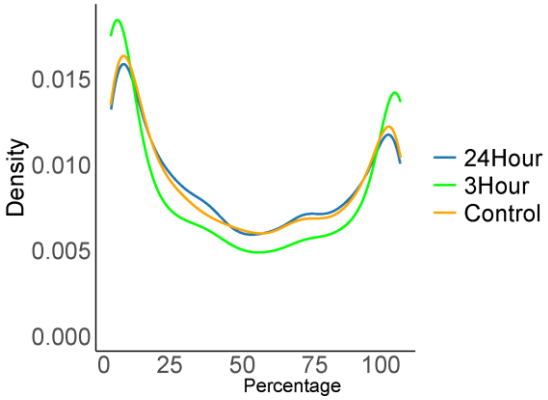
Appendix

Supplementary Table 1: Primers used for qRT-PCR. Primers were designed using Geneious software and made by Integrated DNA Technologies (IDT), F indicates forward primer, R indicates reverse primer. Melting temperatures were confirmed by IDT.

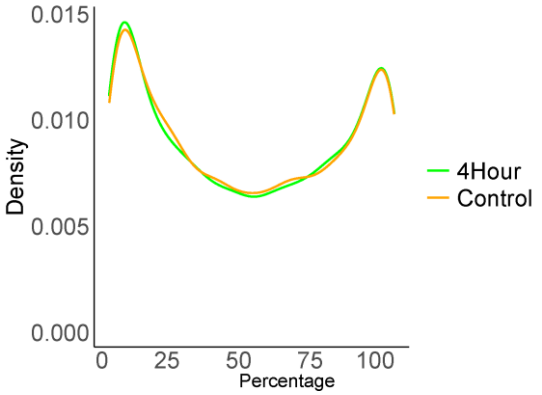
Primers	Sequence 5’ to 3’	Source
ACTB_F	CTGGAACGGTGAAGGTGACA	IDT
ACTB_R	AAGGGACTTCCTGTAACAATGCA	IDT
IL1R1_F	ATGAAATTGATGTTCGTCCCTGT	IDT
IL1R1_R	ACCACGCAATAGTAATGTCCTG	IDT
FGG_F	TTATTGTCCAACTACCTGTGGC	IDT
FGG_R	GACTTCAAAGTAGCAGCGTCTAT	IDT
GAPDH_F	GGTGGTCTCCTCTGACTTCAACA	IDT
GAPDH_R	GTTGCTGTAGCCAAATTCGTTGC	IDT
BCL2_F	AGGAAGTGAACATTTCGGTGAC	IDT
BCL2_R	GCTCAGTTCCAGGACCAGGC	IDT
GPX2_F	GGTAGATTTCAATACGTTCCGGG	IDT
GPX2_R	TGACAGTTCTCCTGATGTCCAAA	IDT



Supplementary figure 1. Distribution of the percentage of long 3'UTR isoforms in the transcriptome between the control group and IL-6 treatment



Supplementary figure 2. Distribution of the percentage of long 3'UTR isoforms in the transcriptome between the control group and the ethanol treatment



Supplementary figure 3. Distribution of the percentage of long 3'UTR isoforms in the transcriptome s between the control group and the peroxide treatment

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