

Title:

**Inflammation Drives Alternative First Exon usage to Regulate Immune Genes including a
Novel Iron Regulated Isoform of Aim2**

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Abstract:

Determining the layers of gene regulation within the innate immune response is critical to our understanding of the cellular responses to infection and dysregulation in disease. We identified a conserved mechanism of gene regulation in human and mouse via changes in alternative first exon (AFE) usage following inflammation, resulting in changes to the isoforms produced. Of these AFE events, we identified 95 unannotated transcription start sites (TSS) in mice using a *de novo* transcriptome generated by long-read native RNA sequencing, one of which is in the cytosolic receptor for dsDNA and known inflammatory inducible gene, *Aim2*. We show that this unannotated AFE isoform of *Aim2* is the predominant isoform expressed during inflammation and contains an iron-responsive element in its 5'UTR enabling mRNA translation to be regulated by iron levels. This work highlights the importance of examining alternative isoform changes and translational regulation in the innate immune response and uncovers novel regulatory mechanisms of *Aim2*.

Summary Sentence:

Alternative first exon usage was the major splicing event observed in macrophages during inflammation, which resulted in the elucidation of a novel isoform and iron mediated regulatory mechanism of the protein coding gene, *Aim2*.

Keywords:

Innate Immunity, Macrophages, Alternative Splicing, *Aim2*, 5'UTR, Nanopore Sequencing

Introduction:

Macrophages are critical cells in the innate immune system that combat infection by initiating acute inflammatory responses. Acute inflammation is tightly coordinated and begins with the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which include Toll-Like Receptors (TLRs) (Medzhitov and Janeway, 1998; Pai et al., 2016a). These initial steps are followed by the activation of sequestered transcription factors, such as nuclear factor of kappa-B (NF- κ B) and interferon regulatory factors (IRFs), which orchestrate pro-inflammatory and antiviral response signals involved in pathogen clearance (Pai et al., 2016a). Once pathogens are cleared, macrophages express genes involved in the resolution of inflammation to return the host to homeostasis (Hamidzadeh et al., 2017). Dysregulation of these pro-inflammatory pathways can have devastating consequences, leading to unresolved inflammation and chronic inflammatory diseases (Zhou et al., 2016).

Recently the process of alternative splicing has emerged as another key mechanism by which the immune system is regulated. Alternative splicing is a regulated process enabling a single gene to produce many isoforms, thus increasing the complexity of gene function and the proteome (Boudreault et al., 2016; Ivanov and Anderson, 2013; Pai et al., 2016a; Wang et al., 2015). Much of this occurs in a cell-type-specific and signal-induced manner (Ergun et al., 2013; Wells et al., 2006). Previous studies have shown that mouse and human macrophages exposed to inflammatory stimuli undergo alternative splicing (Beyer et al., 2012; Bhatt et al., 2012; de Bruin et al., 2016; Haque et al., 2018; Janssen et al., 2020; Lin et al., 2016; Liu et al., 2018; O'Connor et al., 2015; Pai et al., 2016a; Pandya-Jones et al., 2013). Alternative splicing within the immune system can affect the type and magnitude of the inflammatory response, such as the production of a soluble form of TLR4 that is expressed upon LPS, which leads to inhibition of TNF α and

NFκB serving as a negative feedback mechanism (Lynch, 2004; Schaub and Glasmacher, 2017). Additionally, this mechanism has been characterized within signaling molecules (Blumhagen et al., 2017; Shakola et al., 2015), including TBK1 (Deng et al., 2008) and MyD88 (De Arras and Alper, 2013), that produce the alternative RNA splice forms, TBK1s and MyD88s respectively, which function to limit the extent of the pro-inflammatory response. Alternative splicing can also result in the production of inflammatory signaling molecules, such as TRIF (Han et al., 2010) and the proteins in the NFκB family (Wells et al., 2006) with altered activity or stability. Beyond changing the ORF of an mRNA molecule, elongating or shortening the first or last exon can impact post-transcriptional gene regulation and are important to consider when elucidating the regulatory mechanisms of immune genes (Carpenter et al., 2014; Ghiasvand et al., 2014; Leppek et al., 2018), specifically underlying motifs in 5'UTRs (Kramer et al., 2013; Resch et al., 2009) and 3'UTRs (Mariella et al., 2019; Mayr, 2016).

While inflammation-induced alternative splicing in both human and mouse macrophages has been investigated on a genome-wide scale (Beyer et al., 2012; Bhatt et al., 2012; de Bruin et al., 2016; Haque et al., 2018; Janssen et al., 2020; Lin et al., 2016; Liu et al., 2018; O'Connor et al., 2015; Pai et al., 2016a; Pandya-Jones et al., 2013), to our knowledge, long read RNA-sequencing has not been utilized to generate a *de novo* transcriptome for primary murine macrophages. Such an approach is necessary to fully appreciate the extent of alternative transcript isoform usage as we know most transcriptome annotations are incomplete (Workman et al., 2019), and isoforms generated are cell-type and treatment specific (Sapkota et al., 2019; Weirather et al., 2017; Workman et al., 2019; Wu et al., 2018).

Here we used both long and short-read RNA sequencing to uncover novel isoforms and classes of alternative splicing events following inflammation in human and murine macrophages.

Interestingly the dominant conserved class of alternative isoform usage observed following inflammation is alternative first exon usage (AFE), which involves alternative TSS usage coupled with alternative splicing. AFE events can have multi-level effects on protein diversity, regulating genes through alterations of the 5'UTR region, and directing the locality of proteins through alternative N-termini (Landry et al., 2003). We identified 95 unannotated AFE events in mice from native RNA sequencing, one of which is in the cytosolic receptor for dsDNA and known inflammatory inducible gene, *Aim2*. We show that this unannotated AFE isoform of *Aim2* is the predominant isoform produced during inflammation and contains an iron-responsive element in its 5'UTR, enabling mRNA translation to be controlled by iron levels. This work reveals that alternative transcript isoform usage plays a crucial role in shaping the transient nature of the inflammatory response. Isoform expression is an additional layer of regulation within the immune response and therefore a possible contributing factor to the development of auto-immune and inflammatory diseases. Understanding the exact isoforms of genes that are expressed during an inflammatory response will enable us to design better targets for therapeutic intervention of these diseases.

Results:

Global profiling of the cellular alternative splicing landscape in human and mouse macrophages post-inflammation.

To identify alternative splicing events following inflammation, we performed whole transcriptome analysis on human monocyte derived macrophages (MDMs) and murine bone marrow derived macrophages (BMDMs) with and without lipopolysaccharide (LPS) treatment (Fig.1A). We found that ~50% of splicing changes (corrected p-value ≤ 0.25 and $|\Delta\text{PSI}| \geq 10$) - were classified as alternative first exon (AFE) events following LPS activation in both human

and murine macrophages (Figure 1B, Figure1-figure supplement 1). A ranking analysis of the significant events, from both mouse and human datasets, revealed AFE events consistently comprised a large proportion of the top splicing changes (Figure 1-figure supplement 2-3). Additionally, analysis of previously published primary human macrophages stimulated with either *Listeria* or *Salmonella*, using our bioinformatic pipeline and with more stringent thresholds than the previous study, also revealed AFE events to be amongst the most significant prevalent alternative splicing events (corrected p-value ≤ 0.05 or 0.25 and $|\Delta\text{PSI}| \geq 10$) (Figure 1-figure supplement 4) (Table 4-5) (Pai et al., 2016b).

We next identified 11 conserved AFE splicing events between human and mouse (Figure 1-figure supplement 5A-B), the largest number of conserved event types amongst all alternative splicing event types (Figure 1-figure supplement 5). We validated the AFE changes upon stimulation on the already characterized *Ncoa7* (Figure 1-figure supplement 6A-C) (Singer et al., 2008; Yu et al., 2015) and *Rcan1* (Figure 1-figure supplement 6D-F) (Pang et al., 2018), as well as a previously uncharacterized inflammatory specific isoform of *Ampd3* (Figure 1-figure supplement 6G-I), in human and mouse primary macrophages using RT-PCR. Taken together, these results show the high prevalence and conservation of alternative first exon usage following inflammatory activation.

A caveat to our analysis thus far was the reliance on annotated transcriptome assemblies to identify first exons of genes (Brooks et al., 2015). In order to determine if there are additional splicing events that are not captured using the publicly available GENCODE M18 transcriptome annotation (Garalde et al., 2018; Hsieh et al., 2019; Pollard et al., 2018; Workman et al., 2019), we performed native RNA sequencing of murine macrophages with and without LPS treatment to build a *de novo* murine macrophage specific transcriptome with an average read-depth of 1

130 million. We identified isoforms using Full-Length Alternative Isoform analysis of RNA (FLAIR)
131 (Tang et al., 2020; Workman et al., 2019) that also had promoter support identified from
132 accessible chromatin (ATAC-seq) (Atianand et al., 2016; Tong et al., 2016). The FLAIR
133 isoforms were then merged with the GENCODE M18 assembly (mm10) (Frankish et al., 2019)
134 as a transcript reference to identify and quantify alternative splicing events using short-read
135 sequencing, which has increased read depth, denoting this transcriptome as ‘Gencode + *de novo*’
136 (Figure 1-figure supplement 7A). Overall, the incorporation of long-read sequencing to generate
137 a novel transcriptome, led to the identification of 95 novel and statistically significant AFE
138 events that occur following inflammation (Figure 1C-D). A comparison of significant AS events
139 between splicing events identified by using GENCODE M18 annotation and *de novo* FLAIR
140 transcriptome show an overall increase in the number of unique events (Figure 1-figure
141 supplement 7B-H). To gain confidence of the novel AFE events, we compared a comprehensive,
142 339 mouse sample, FANTOM CAGE-seq data (The FANTOM Consortium and the RIKEN PMI
143 and CLST (DGT), 2014) or annotated and unannotated TSSs as defined by the GENCODE M18
144 assembly. The caveat to this FANTOM dataset, is that there are no inflammatory stimulated
145 samples. Even with this caveat, the analysis revealed the similarity of the distributions between
146 CAGE scores associated with CAGE peaks overlapping known and those overlapping novel
147 TSSs of FLAIR isoforms further supporting the validity of the novel TSSs identified (Figure 1-
148 SFig8A-10B). Additionally, we utilized a dataset generated through application of machine
149 learning methods to classify CAGE-seq peaks as true or false, where true corresponds to CAGE-
150 seq peaks that overlap true TSSs (Kanamori-Katayama et al., 2011). Novel TSSs identified with
151 FLAIR showed a 45% overlap with CAGE-seq peaks classified as true, providing us with

confidence in the novel AFE events identified (Figure 1-figure supplement 8C). Together these approaches provide us with confidence in the novel AFE events identified by direct RNA-seq.

Interestingly, when identifying gene expression changes, we found that ~50% of genes with AFE usage were not differentially expressed following inflammation (Figure 1E-F) (Table 6-7), highlighting the importance of studying isoform usage for control of gene expression.

Among the most statistically significant novel AFE first exon events were *Denr*, *Arhgef7*, and *Aim2*, which we validated using RT-qPCR (Figure 1G-I, Figure 1-figure supplement 9).

Identification of an unannotated promoter for *Aim2*.

To better understand the potential functional consequence of AFE changes, we further examined the novel first exon event upregulated upon inflammatory activation in *Aim2*. *Aim2* is an interferon-stimulated gene (ISG), localized to the cytosol. *Aim2* is a dsDNA sensor, that upon recognition induces the formation of an inflammasome complex releasing IL1 β and IL18 from the cell as a defense mechanism to control infection (Wang and Yin, 2017). Chromatin Immunoprecipitation (ChIP)-seq for the myeloid pioneering transcription factor PU.1 in primary BMDMs (Figure 2A-B, top track, in black) (Lam et al., 2013) supported the presence of an additional promoter upstream of the canonical isoform for *Aim2* (NM_001013779.2).

Predominant isoforms ($\geq 10\%$ of total gene expression in a sample) assembled from native RNA sequencing with FLAIR identified the canonical isoform and five unannotated isoforms that use the inflammatory activated promoter, revealing a new longer 5'UTR (Figure 2C). Native RNA sequencing-based quantification provided additional support that the unannotated promoter usage is upregulated upon LPS stimulation. At steady-state, approximately 20% of reads map to *Aim2*'s transcript with the canonical promoter and 65% of reads map to transcripts with the upstream promoter, while following inflammatory activation, 14% of the reads map to transcripts with the

canonical promoter and 81% of reads map to transcripts with the upstream promoter (Figure 2D). To validate the change in *Aim2* AFE usage upon LPS stimulation, RT-qPCR was performed using exon spanning primers that were either specific to the annotated AFE or the unannotated AFE, in BMDMs (Figure 2E). The expression profile of the annotated first exon is not induced by LPS stimulation (Figure 2F), while the unannotated first exon and the CDS of *Aim2* are equally induced by LPS stimulation (Figure 2G-H). Therefore, these data show that it is the novel isoform of *Aim2* that is inflammatory-regulated and not the canonical isoform defined in GENCODE annotation, nor isoforms from the RefSeq annotation.

The novel inflammatory promoter of Aim2 is regulated by IRF3 and p65.

To gain insights into potential regulatory mechanisms controlling the expression of the 152 significant AFE events, we assessed changes in chromatin accessibility during inflammatory activation in BMDMs. Analysis of ATAC-Seq (Tong et al., 2016) revealed differential peaks at the promoter regions for 25% of genes with significant AFE events suggesting that chromatin remodeling is one mechanism driving the expression of the AFE events (Figure 3A). This regulation is not what controls isoform usage for *Aim2*. The annotated promoter is accessible in all cells while the novel *Aim2* promoter is specific to myeloid progenitors and monocytes (Lara-Astiaso et al., 2014; Tong et al., 2016) (Figure 3-figure supplement 1A). In addition, the accessibility of both the annotated and unannotated promoters remain open despite the cell's inflammatory status (Figure 3-figure supplement 1B). Therefore, the expression of the new isoform is not due to chromatin remodeling of either promoter region (Figure 3-figure supplement 1C).

Another potential mode of regulation that can drive AFE usage is transcription factor (TF) binding. We next analyzed ChIP-seq data of two major transcription factors that drive

inflammation downstream of LPS, nuclear factor of kappa-B (NFkB, p65) and interferon response factor 3 (IRF3) (Tong et al., 2016). We found that p65 and IRF3 specifically account for another 25% of the AFE events, including the novel *Aim2* isoform, which we confirmed using multiple ChIP-seq data sets (Figure 3A, Figure 3-figure supplement 2).

Further bioinformatic analysis of promoters associated with all AFEs, using HOMER TF motif enrichment, shows that there are 304 potential TFs that bind these promoters. By gene ontology analysis, we see that the majority of the TFs are associated with metabolism, as well as the immune system (Figure 3B) (Table 10). When specifically assessing the two promoter regions driving the annotated and unannotated isoforms of *Aim2*, we identified 106 individual transcription factor (TF) motifs within the annotated promoter and 121 motifs within the unannotated promoter (Table 11). Of these predicted motifs there were 44 motifs unique to the unannotated promoter (Figure 3C). By gene ontology (Huang et al., 2007) analysis of TFs specific to the annotated and unannotated promoter regions of *Aim2* confirms that the unannotated promoter is driven by inflammatory specific transcription factors including NF-κB and IRFs (Figure 3-figure supplement 2L). Use of ATAC-seq and ChIP-seq for specific TFs has enabled us to determine the regulatory pathways driving 50% of the AFEs in our data. For the remaining 50%, there could be additional TFs, RNA binding proteins (Lynch, 2004), or differential RNA stability driving their expression. Further work will be required to fully understand the complex regulation of all AFE events.

Unannotated 5'UTR of Aim2 negatively regulates translation through a single iron responsive element.

The novel inflammatory isoform identified here for *Aim2* acquired a longer 5' untranslated region (UTR) compared to the canonical isoform, however globally there are no

difference in AFE size between conditions (Figure 4A, Figure 4-figure supplement 1A-B). Previous studies have shown that longer 5'UTRs can affect the translation of a gene (Kramer et al., 2013; Senanayake and Brian, 1999). Using a GFP reporter system, the translational efficiency of the unannotated *Aim2* 5'UTR (767bp) was compared to the annotated 5'UTR (489bp) (Figure 4B). The unannotated 5'UTR showed significantly lower mean GFP fluorescence units, suggestive of lower translational efficiency, as assessed by flow cytometry 72 hr post transient transfection in 293T cells (Figure 4C-D), while equal mCherry fluorescence was observed for all co-transfected control constructs (Figure 4E). To explore the mechanism of how the unannotated 5'UTR results in decreased translational efficiency, we used RegRNA2.0 to predict RNA regulatory motifs in the 5'UTRs (Chang et al., 2013). We identified a single iron-responsive element (IRE) within the unannotated 5'UTR, while Musashi binding elements (MBE) motifs were identified in both 5'UTRs (Fig.4F) (Table 14). Globally, we find there are more predicted motifs in inflammatory-regulated first exons (inclusion exons) in comparison to basal exons (excluded exons), but the IRE motif is found only in *Aim2* (Figure 4-figure supplement 1C). The finding that there are structured motifs in the unannotated 5'UTR of *Aim2* is also supported by the RNAfold Vienna package (Gruber et al., 2008), which predicts the hairpin structure of the IRE element in the alternative 5'UTR (Figure 4-figure supplement 2). Since the IRE motif is unique to the unannotated 5'UTR of *Aim2*, we hypothesized that this motif is critical in regulating translational efficiency.

When cells are at homeostasis, Iron Binding Proteins (IRP1/2) bind to IRE elements located within the 5'UTR (e.g. ferritin) and can block translation, while IRE elements in the 3'UTR (e.g. transferrin receptor) can promote translation (Rouault, 2006; Wang et al., 2004). However, iron repletion results in the inactivation of IRP1/2 (Outten, 2017) (Figure 4G). To

experimentally test if the IRE motif within the unannotated 5'UTR acts as a translational repressor, we removed the element using site-directed mutagenesis which led to an increase in GFP expression by ~20% compared to the annotated 5'UTR (Figure 4H-I). Next, we exogenously added 100 uM ferric ammonium citrate (FAC) to overload the cells with iron and determined if this can rescue the observed decrease in translational efficiency from our unannotated 5'UTR. Upon FAC administration, the relative GFP expression of the unannotated 5'UTR plasmid increased by ~50%, while mCherry control was unchanged, suggesting that the translational efficiency of the unannotated 5'UTR can be rescued with iron supplementation (Figure 4J-K). From these results, we conclude that the predicted IRE motif within the unannotated 5'UTR of *Aim2* functions as an IRE to control translation.

To test if the IRE motif in the unannotated 5'UTR of *Aim2* acts as a translational regulator endogenously, we performed polysome profiling followed by RT-qPCR on primary BMDMs in the presence and absence of LPS for 18 hr to determine the translational competency of the isoforms of *Aim2* (Figure 4L-M). As a negative control, we examined *Neat1* (Nakagawa et al., 2014), a long non-coding RNA (lncRNA) that is not detected in polysomes, nor translated and this is not dependent on LPS (Figure 4N). The relative distribution of our positive control gene, *Gapdh*, which encodes a highly expressed housekeeping protein, is enriched in the high polysome fraction, with or without LPS, as expected (Figure 4O). Using isoform-specific primer sets (Figure 2E) for the annotated and unannotated *Aim2* isoforms, we find that the annotated isoform is enriched in the high polysome fraction with and without LPS treatment (Figure 4P), while the novel isoform is enriched in the low polysome fraction with and without inflammatory stimulation (Figure 4Q). Additionally, from our RNA-seq data we find that IRP1 and IRP2 are not transcriptionally regulated by inflammation, but based on Weiss *et al.* inflammation can

affect the binding affinity of IRPs in macrophages (Weiss et al., 1997) (Figure 4-figure supplement 3A-D). These data show that the unannotated *Aim2* isoform has a lower translational efficiency compared to the canonical form.

To further validate the effect the post-transcriptional mechanism of the unannotated *Aim2* isoform, we performed time-course LPS stimulations. In primary BMDMs a 72 hr, time-course stimulation with and without the treatment of iron (FAC) protein lysates were generated and *Aim2* expression was measured by western blot (Figure 4R-S). *Aim2* is expressed basally and significantly decreases upon LPS treatment at the 48 hr time point, most likely as a control mechanism to return the pathway to homeostasis and limit the inflammatory stimulation. When FAC is added to cells, *Aim2* expression does not decrease at the 48 hr time point suggesting that it is indeed the IRE element that is driving this decrease in *Aim2* observed in the wild type cells (Fig. 4R-S). Finally, we utilized the CRISPR/Cas9 technology to remove the *Aim2* IRE element endogenously (Figure 4-figure supplement 4A). In these immortalized BMDMs, RT-qPCR was performed with an LPS time-course. The annotated *Aim2* isoform shows no induction by LPS (Figure 4-figure supplement 4C), while the unannotated *Aim2* isoform and the CDS sequence are induced by 2-fold (Figure 4-figure supplement 4D-E). There is no significant difference in transcriptional expression at the *Aim2* locus between the WT and IRE KO cell lines (Figure 4-figure supplement 4D-E). Using these newly characterized WT and IRE KO clonal cell lines we performed a 72 hr time-course of LPS stimulation (Figure 4-figure supplement 4F-G). At the 48 hr timepoint there is a significant decrease in expression of *Aim2* protein in WT cells, but not in the IRE KO cells (Figure 4-figure supplement 4H), further indicating that endogenous *Aim2* is regulated through the IRE motif located in the AFE.

Discussion:

While we have come a long way in determining the transcriptomes of immune genes to better understand signaling pathways, very little work has focused on the role that mRNA isoforms play (Akira et al., 2006; Medzhitov and Janeway, 1998; Pai et al., 2016a). Over 95% of genes have more than one mRNA transcript due to alternative splicing but the regulatory importance of these splicing events are not fully understood (Carninci et al., 2006; Tian and Manley, 2017; Wang et al., 2008). On a gene-by-gene approach, alternative splicing has been shown to play a role in health and disease by shaping the proteome (Nishiyama et al., 2000; Yang et al., 2011; You et al., 2016). Globally, a number of labs have tackled the prevalence of alternative splicing *in vitro* and *in vivo* showing that alternative splicing can affect both the nature and duration of inflammation (Beyer et al., 2012; Bhatt et al., 2012; Janssen et al., 2020). To date no one has examined conservation of these mechanisms using primary cells or utilized long read sequencing to build the transcriptome *de novo* to obtain a complete understanding of the extent of alternatively expressed isoforms generated following an immune response.

In our study, we demonstrate a conservation of splicing, specifically alternative first exon (AFE) events, in both human and murine macrophages. We found that there are 11 genes that have AFE in both human and mouse macrophages (Figure 1-figure supplement 5A). Most studies to date have focused on isoform changes linked to genes that are differentially expressed following inflammation and interestingly these 11 genes would have been previously overlooked because many of them are not differentially expressed, emphasizing the importance in studying isoform expression in all conditions (Figure 1E-F). Of the 11 conserved genes, 7 AFE isoforms have been previously studied in some context including: *Rps6ka1* (Qin et al., 2018), *Ncoa7* (Singer et al., 2008; Yu et al., 2015), *Rcan1* (Pang et al., 2018), *Wars* (Liu et al., 2004; Miyanokoshi et al., 2013), *Arap1* (Kulzer et al., 2014), *Tsc22d1* (PMID: 21448135) and *Sgk1*

(Arteaga et al., 2007; Kobayashi et al., 1999; Lang et al., 2006). While this validates our technique, it is important to note that none of these genes had been connected to inflammation or formally shown to be conserved mechanisms of regulation, besides *Wars* (Liu et al., 2004; Miyanokoshi et al., 2013). This also highlights our method's ability to accurately identify inflammatory regulated RNA isoforms, in addition to the uncharacterized AFE events of *Ampd3*, *Snx10*, *Shisa5* and *Tspan4*. Furthermore, *Snx10* is studied outside the context of inflammation, has been implicated in chronic inflammatory disease and our study may suggest new insights into how alternative splicing could be regulating these genes (You et al., 2016). We further validated *Ncoa7*, *Rcan1* and *Ampd3* in human and murine macrophages using RT-PCR (Figure 1-figure supplement 6).

To overcome the current limitations of any transcriptome build, we used direct RNA nanopore technology on primary murine macrophages to build our own transcriptome *de novo* with the aim of identifying novel full length transcriptional isoforms that are expressed with and without inflammation (Tang et al., 2020; Workman et al., 2019a). The limitation of direct RNA sequencing is read depth and our average read depth was about 1 million reads per treatment. In a previous study of direct RNA sequencing, we found that increased sequencing depth will result in additional isoforms detected (Workman et al., 2019). Therefore, we expect to see additional unannotated AFE and other AS events with increased sequencing depth. However, even with 1 million reads, we were able to build a transcriptome that led to the identification of 154 novel AS events. Following this, we identified hundreds of novel isoforms resulting in 95 novel AFE events, supported by CAGE-seq (Figure 1, Figure 1-figure supplement 7-8), including an unannotated mRNA isoform of the well-studied gene protein absent in melanoma 2 (*Aim2*).

Aim2 is characterized as an interferon inducible gene (Johnstone and Trapani, 1999) (PMID: 10454530), functioning as a cytoplasmic dsDNA sensor leading to the formation of an inflammasome and eventual cleavage and release of pro-inflammatory cytokines of IL1 β and IL18 (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Our study highlights that it is an alternative mRNA isoform of *Aim2* that is inducible, and that this upregulated transcript is translated less efficiently compared to the canonical isoform. This novel finding goes against the existing assumption that induced gene expression results in induced protein expression (Figure 1I, Figure 2, Figure 4, Figure 4-figure supplement 4). FLAIR-identified transcripts (Workman et al., 2019) show three clear AFEs for *Aim2* and only one of those first exons is inflammatory inducible (Figure 2C-D). RT-qPCR further confirms that the annotated TSS of *Aim2* is not inducible, while the unannotated AFE of *Aim2* is LPS inducible (Figure 2E-H). This result highlights the need for cell-type and treatment specific transcriptome annotations if one is to have a complete understanding of the transcriptome and proteome of a given cell.

We further investigated what drove the expression of this novel *Aim2* isoform, as well as what drove the expression of all the AFE genes. Using ATAC-seq (Lara-Astiaso et al., 2014; Tong et al., 2016) and ChIP-seq (Tong et al., 2016) data sets, we were able to determine that 50% of the AFE events were driven partially by chromatin accessibility and inflammatory specific transcription factors (Figure 3A). Using HOMER, a TF motif enrichment tool, we identified that the majority of TFs that regulate the expression of the AFE genes are involved in metabolism pathways (Figure 3B). Additionally, another mechanism that can drive AFE usage is the splicing of internal exons that activate proximal upstream promoters (Fiszbein et al., 2019). Further analysis incorporating more transcription factors and coupling multiple splicing events

will be necessary to determine the definitive regulatory mechanism of all AFEs. Interestingly, the annotated *Aim2* promoter accessibility is constitutively open across all hematopoietic cells, while the unannotated *Aim2* promoter is only accessible in myeloid progenitors or terminally differentiated cells (Figure 3-figure supplement 1A), meaning that the novel *Aim2* isoform may be specific to myeloid cells. Furthermore, *Aim2* annotated and unannotated promoter usage is not driven by chromatin accessibility (Figure 3-figure supplement 1B-C) but is driven by the activation of inflammatory specific transcription factors (Figure 3C-E, Figure 3-figure supplement 2L).

There is no difference in the open reading frame of the novel isoform of *Aim2* when compared to the annotated transcript using NCBI ORFfinder, therefore we turned our attention to a possible regulatory mechanism within the 5'UTR (Mignone et al., 2002). Broadly, UTRs play crucial roles in the post-transcriptional regulation of gene expression, including alteration of the mRNA translational efficiency (van der Velden and Thomas, 1999), subcellular localization (Jansen, 2001) and stability (Bashirullah et al., 2001). Post-transcriptional regulatory mechanisms of *Aim2* have not been previously studied. Using RegRNA2.0 (Chang et al., 2013) globally we identify that LPS-activated AFEs contain more regulatory elements than basal first exons (Figure 4-figure supplement 1). More importantly, we identified an Iron Responsive Element (IRE), a regulatory motif unique to only the novel 5'UTR of *Aim2* (Figure 4F). Utilizing a GFP reporter plasmid, we were able to determine that the IRE motif was functional, by recapitulating the same experiments used on the protein Ferritin, the first functional IRE motif ever studied (Leedman et al., 1996). Then, we showed the inflammatory specific mechanism regulating *Aim2* protein expression by performing a western blot on primary BMDMs with and without Ferric Ammonium Citrate (FAC) during a 72 hr LPS time-course experiment. *Aim2*

protein is basally expressed and while the transcript is inducible, specifically the novel isoform, we do not observe an increase in expression of Aim2 protein by western blot. In fact, we find that Aim2 protein decreases following inflammation, and this can be reversed by iron supplementation. Finally, we confirmed the IRE mechanism by using CRISPR/Cas9 to generate an IRE KO clonal cell line. Once our cell line was characterized (Figure 4-figure supplement 5A-E), we performed an LPS time-course stimulation and measured Aim2 protein expression. At the 48 hr time-point we see a significant rescue of Aim2 protein expression in the IRE KO cell line (Figure 4-figure supplement 5F-H). This could be a critical regulatory step that has evolved to ensure the Aim2 pathway is switched off following its formation and activation of the inflammasome.

These results demonstrate that the inflammatory specific mRNA isoform of *Aim2* has lower translational efficiency than the canonical form and that protein translation can be increased by the addition of iron. Crane *et al.* (Crane et al., 2014) demonstrate that ROS can contribute to activation of Aim2 inflammasome in mouse macrophages. Our proposed mechanism of translational regulation of Aim2, is through an Iron Responsive Element (IRE), which is known to directly interact with IRP proteins (Outten, 2017; Rouault, 2006; Wang et al., 2004). Interestingly, IRP2 degradation can be driven not only through iron, but also through ROS and RNS (Hanson and Leibold, 1999), further supporting this novel IRE regulatory mechanism of Aim2 protein expression. Finally, Cheng *et al.* has shown that AIM2 is regulated by oxidative stress and show that over activation of AIM2 inflammasome can contribute to pancreatic tumorigenesis, all within the environment of mitochondrial iron overload (Li et al., 2018). This newly identified isoform, with an IRE specific translational mechanism provides mechanistic understanding to these recent studies of Aim2 (Crane et al., 2014; Li et al., 2018).

These findings could have significance for better understanding the mechanisms driving pathology in inflammatory disease such as systemic lupus erythematosus (SLE) (Corbett, 2018). AIM2 expression levels have been correlated with severity of inflammation in SLE patients (Zhang et al., 2013) and it is well known that iron is dysregulated in this disease (Vanarsa et al., 2012). It is possible that AIM2 levels remain high in SLE patients due to dysregulated iron; therefore, homeostasis in macrophages cannot be maintained.

In summary, signaling within macrophages enables us to fight infection but also can contribute to pathological inflammation associated with a wide variety of diseases. While there are multiple regulatory checkpoints in place to control inflammation, we propose that alternative splicing and translational regulation play critical roles in maintaining this type of control. A better understanding of the molecular mechanisms that control inflammatory-regulated genes, including *Aim2*, could provide new targets for therapeutic intervention of autoimmune and inflammatory diseases.

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Competing interests:

MA holds options in Oxford Nanopore Technologies (ONT). MA is a paid consultant to ONT. MA and ANB received reimbursement for travel, accommodation and conference fees to speak at events organized by ONT. MA is an inventor on 11 UC patents licensed to ONT (6,267,872, 6,465,193, 6,746,594, 6,936,433, 7,060,50, 8,500,982, 8,679,747, 9,481,908, 9,797,013, 10,059,988, and 10,081,835). MA received research funding from ONT.

Data and Materials Availability:

All data are available in the manuscript or the supplementary materials. RNA-seq raw data have been deposited in the NCBI Gene Expression Omnibus database under accession numbers GSE141754.

Figure Legends:

Figure 1: Global profiling of the cellular alternative splicing landscape in human and mouse macrophages post-inflammatory. (A) Diagram of RNA-seq library generation. **(B)** Categorization of significant splicing events in human and mouse macrophage. **(C)** Categorization of significant splicing events found in mouse BMDM +/- 6 hr LPS by using either the Gencode annotation or the Gencode + *de novo* annotation. **(D)** Venn diagram representing unique and common genes with AFE events found in RNA-seq of primary BMDMs post-inflammatory stimulation using the Gencode annotation or the Gencode + *de novo* annotation. **(E)** Volcano plots of all differentially expressed genes from RNA-seq of either human **(E)** or mouse

(F) macrophages. Genes highlighted in red undergo significant AFE changes following inflammation. Schematic of AFE inclusion and exclusion isoforms, followed by RT-PCR gel results and PSI calculation for *Argehf7* (G) *Denr* (H) and *Aim2* (I), were performed in biological triplicates, p-value assessed using student's t-test.

Figure 2: Identification of an unannotated promoter in *Aim2*. (A-B) The top track, in black, represents ChIP-seq data for a macrophage-specific transcription factor, PU.1. Peaks represent possible promoter regions; two distinct peaks of equal height are present at the annotated transcriptional start site for *Aim2* and about 1kb upstream of the transcriptional start site (TSS). The middle track, in orange, represents basal transcription in bone marrow-derived macrophages (BMDMs), while the bottom track, in purple, represents active transcription in BMDMs 6 hr LPS post treatment. (C) *Aim2* transcript isoforms identified in BMDMs by native RNA long-read sequencing through FLAIR analysis. Transcripts are categorized by promoter, denoted by grey, orange or purple. (D) The bar-chart represents data from long-read sequencing showing the abundance of each transcript isoform from BMDMs +/- 6 hr LPS. (E-H) qRT-PCR was performed in biological triplicate, on primary BMDM RNA extracts that had been stimulated with LPS for indicated time points.

Figure 3: Novel inflammatory promoter of *Aim2* is regulated by IRF3 and p65. (A) UpSet plot showing the number of AFE events out of 77 total that have differential transcription factor binding and differential chromatin accessibility and all combinations of these sets. (B) Pie chart representing the gene ontology of transcription factor (TF) motifs identified through analysis motif enrichment (AME) tool of all AFE promoter sequences. (C) Venn diagram of all motifs defined using HOMER analysis within the annotated and unannotated promoter regions. (D-E)

DAVID analysis examining the gene ontology of transcription factors at the annotated and unannotated promoters of *Aim2*.

Figure 4: Unannotated 5'UTR of *Aim2* negatively regulates translation through a single iron responsive element. (A) Schematic of annotated and unannotated 5'UTR of most prevalent *Aim2* isoforms in mouse macrophages. (B) Diagram of cloning strategy of *Aim2*'s 5'UTR in GFP plasmid. (C) Transfection strategy of 5'UTR-GFP plasmids co-transfected with an mCherry control plasmid at a 1 to 1 ratio in 293T cells. (D-E) Flow cytometry of 293T cells 72 hr post-transfection with control annotated and unannotated 5'UTR of *Aim2* to measure GFP and mCherry (Ctl) protein fluorescence. (F) Using RegRNA2.0 a single iron-responsive element (IRE) was found in the alternative 5'UTR, in addition to multiple musashi binding elements (MBE). (G) Diagram of how an IRE functions in the cytoplasm of a cell within a 5'UTR. With low or normal levels of iron, Iron Binding Proteins (IRP1 or IRP2) bind to IRE elements and block translation. During high levels of iron, within a cell, IRP1 is sequestered by iron-sulfur (Fe-S) clusters and IRP2 is degraded, therefore allowing translation of the protein. (H-I) Flow cytometry of 293T cells 72 hr post-transfection of mCherry (Ctl), along with an annotated 5'UTR-GFP plasmid, unannotated 5'UTR-GFP plasmid, or a GFP plasmid containing the unannotated 5'UTR without the defined iron-responsive element (IRE). (J-K) Flow cytometry of 293T cells +/- 100uM ferric ammonium citrate (FAC) 72 hr post-transfection of mCherry (Ctl), along with an annotated 5'UTR-GFP plasmid or unannotated 5'UTR-GFP plasmid. (L) Overview of the polysome profiling protocol to analyze translation activity. (M) Cytoplasmic lysates from +/- LPS treated cells were fractionated through sucrose gradients. Global RNA polysome profiles generated by the density gradient fractionation system are shown. A representative plot from stimulated primary BMDM fractionated samples is shown. The experiment was performed 3

times. **(N-Q)** The relative distribution of *Gapdh* mRNA, encoding a housekeeping protein, *Neat1* long non-coding RNA (lncRNA), annotated and unannotated *Aim2* mRNA were measured by RT-qPCR analysis of RNA. Each of the gradient fractions are calculated as relative enrichment when compared to unfractionated input mRNA, standard deviation represents technical triplicate.

(R) Protein lysates of time course LPS stimulation of 0 hr, 6 hr, 24 hr, 48 hr and 72 hr without and with 100uM of Ferric Ammonium Citrate (FAC, iron) added to immortalized WT BMDMs. Western blot performed on AIM2 and B-ACTIN. **(S)** Western blot quantification performed in FIJI, standard deviation represents biological triplicates, p-value assessed using student's t-test.

Supplemental File 1. Numerical source data from human macrophages +/- TLR4 RNAseq analyzed by JuncBASE using GENCODE transcriptome for Figure 1B.

Supplemental File 2. Numerical source data from mouse macrophages +/- TLR4 RNAseq analyzed by JuncBASE using GENCODE transcriptome for Figure 1B and Figure 1C.

Supplemental File 3. Numerical source data from mouse macrophages +/- TLR4 RNAseq analyzed by JuncBASE using GENCODE+denovo transcriptome for Figure 1C.

Supplemental File 4. Numerical source data from human macrophages +/- Listeria RNAseq analyzed by JuncBASE using GENCODE transcriptome for Supplemental Figure 4.

Supplemental File 5. Numerical source data from human macrophages +/- Salmonella RNAseq analyzed by JuncBASE using GENCODE transcriptome for Supplemental Figure 4.

Supplemental File 6. Numerical source data from human macrophages +/- TLR4 RNAseq analyzed by DESeq2 using GENCODE transcriptome for Figure 1E.

Supplemental File 7. Numerical source data from mouse macrophages +/- TLR4 RNAseq analyzed by DESeq2 using GENCODE transcriptome for Figure 1F.

513 **Supplemental File 8.** Numerical source data from mouse macrophages +/- TLR4 ATACseq
514 analyzed using a peak calling pipeline for Figure 3A.

515 **Supplemental File 9.** Numerical source data from mouse macrophages +/- TLR4 p65 and IRF3
516 ChIPseq analyzed using a peak calling pipeline for Figure 3A.

517 **Supplemental File 10.** Numerical source data from all AFE promoters analyzed by HOMER for
518 Figure 3B.

519 **Supplemental File 11.** Numerical source data from unannotated and annotated Aim2 promoter
520 analyzed by HOMER for Figure 3C.

521 **Supplemental File 12.** Numerical source data of inclusion AFE coordinates for Supplemental
522 Figure 12A.

523 **Supplemental File 13.** Numerical source data of exclusion AFE coordinates for Supplemental
524 Figure 12A.

525 **Supplemental File 14.** Numerical source data of unannoated and annotated Aim2 5'UTR
526 analyzed by RegRNA2.0 for Figure 4F.

527

528

529 ***Materials and Methods***

530 Maintenance of mice

531 UCSC and the Institutional Animal Care and Use Committee maintained mice under specific
532 pathogen-free conditions in the animal facilities of `University of California Santa Cruz (UCSC)
533 in accordance with the guidelines.

534 Human PBMC derived macrophage differentiation and in vitro stimulation

535 Human peripheral blood mononuclear cells (PBMC) were enriched by density gradient
536 centrifugation of peripheral blood from healthy human donors through a Ficoll-Paque PLUS (GE
537 Healthcare) gradient. Monocytes were isolated from PBMC by negative selection using the
538 EasySep™ Human Monocyte Isolation Kit (STEMCELL Technologies) according to the
539 manufacturer's instructions. To differentiate monocytes into macrophages, recombinant human
540 M-CSF (50ng/mL) was used in RPMI-1640 medium with 10% FBS, 2mM L-glutamine, 10mM
541 HEPES, 1mM sodium pyruvate, 100U/ml penicillin, and 100 µg/mL streptomycin. The culture
542 medium which contained fresh recombinant human M-CSF was replaced every 2 days.

543 Cell culture, Mouse Macrophage Differentiation and Stimulation

544 Cells were cultured in D-MEM with 10% fetal bovine serum (FCS) supplemented with
545 penicillin/streptomycin or ciprofloxacin. Primary BMDM were generated by cultivating
546 erythrocyte-depleted bone marrow cells in the presence of 30% L929 supernatant and the cells
547 were used for experiments 6-9 days after differentiation. J2Cre virus (Blasi et al., 1989) was used
548 on day 3/4 after isolation of bone marrow cells to establish transformed BMDM cell lines.
549 BMDMs were cultivated in the presence of J2Cre virus for 48 hr and L929 was then gradually
550 tapered off over 6-10 weeks depending on the growth pattern of transformed cells.

551 In vitro stimulation of macrophages

552 Bone derived macrophage cells were primed with 100uM of Ammonium Ferric Citrate (FAC)
553 for 24 hr prior to TLR stimulation. Bone marrow derived macrophage cells were stimulated with
554 Toll-like receptor (TLR) ligands for the indicated time points using the following concentrations:
555 Lipopolysaccharide (LPS) 200ng/ml (TLR4). For RNA and protein isolation, $1-2 \times 10^6$ cells were
556 seeded in 12-well format.

557 RT-PCR Validation

558 RT-PCR validation was completed using three biological replicates. KAPA HiFi HotStart
559 ReadyMix PCR Kit (Kapa Biosystems) and the manufacturer's suggested cycling protocol was
560 used to complete the PCR reaction with the following primers:

561 *Mse_Denr_F1*: ATCGCGATAAAGGCTCATTG

562 *Mse_Denr_F2*: GCTACCTGTCCTTTTCCCCA

563 *Mse_Denr_R*: AACTTGGCACTGTTCTTCGT

564 *Mse_Arhgef7_F1*: TGTTGTTCTGGGGTTTGTGA

565 *Mse_Arhgef7_F2*: CTGTGTGTTGCAGGTCTACC

566 *Mse_Arhgef7_R*: GTGTCACCAAGGAGCTGAGG

567 *Mse_Ncoa7_F1*: GTGGTGGAGAAGGAAGAGCT

568 *Mse_Ncoa7_F2*: TTCTATTGTGCCAGGCCTGA

569 *Mse_Ncoa7_R*: GCATGTTTTCCAGGAGTGCA

570 *Mse_Ampd3_F1*: CCCTACTGTAGATGAATCCCCTTA

571 *Mse_Ampd3_F2*: GCTGAGCTTTGTGTCTGTGT

572 *Mse_Ampd3_R*: GGGGACAGTAAACAGGGACA

573 *Mse_Rcan1_F1*: ACTGGAGCTTCATCGACTGC

574 *Mse_Rcan1_F2*: GACTGAGAGAGCGAGTCGTT

575 *Mse_Rcan1_R*: CATCGGCTGCAGATAAGGGG
576 *Hu_NCOA7_F1*: TGTTTCAGTGGTCTCCCGATGTCTATGG
577 *Hu_NCOA7_R*: GGGCCGTAGGACAGGCAGCA
578 *Hu_NCOA7_R2*: AGCGTGGCTACAAGTAACTGTGGTGT
579 *Hu_AMPD3_F1*: TATGCAAAACAGAGACCTCC
580 *Hu_AMPD3_R*: CACTTCAGAGATGTTCAGCT
581 *Hu_AMPD3_F2*: CCTGCTTGGTTTTAGAGGAT
582 *Hu_RCAN1_F1*: GACTGGAGCTTCATTGACTG
583 *Hu_RCAN1_R*: ATTCTGACTCGTTTGAAGCT
584 *Hu_RCAN1_F2*: TAGCGCTTTCACGTGTAAGAA

585 Band intensities were measured for each band in each condition and sample using FIJI
586 (Schindelin et al., 2012). The relative abundance of each isoform was calculated using the
587 equation to calculate PSI (PSI = inclusion/ (inclusion + exclusion)) in each condition and sample
588 to validate the computationally derived delta PSI values. A gel extraction was completed for
589 each band using the PCR clean-up Gel extraction kit (Machery-nagel). The PCR product was
590 confirmed using Sanger Sequencing.

591 RNA isolation, RT-qPCR

592 Total cellular RNA from BMDM cell lines or tissues was isolated using the Direct-zol™ RNA
593 MiniPrep Kit (Zymo Research) according to manufacturer's instructions. RNA was quantified
594 and controlled for purity with a nanodrop spectrometer. (Thermo Fisher). For RT-qPCR, 500-
595 1000 ng were reversely transcribed (iScript Reverse Transcription Supermix, Biorad) followed
596 by RT-PCR (iQ SYBRgreen Supermix, Biorad) using the cycling conditions as follows: 50°C for
597 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 45

598 sec. The melting curve was graphically analyzed to control for nonspecific amplification
599 reactions. Quantitative RT-PCR analysis was performed with the following primers listed below:
600 *Mse_Aim2_F_Annotated*: CCGCCATGCTTCCTTAACTA
601 *Mse_Aim2_F_Unannotated*: AGGCGGATGGTTTGAAGTCTCT
602 *Mse_Aim2_R_Exon2*: TTGAAGCAACTTCCATCTGC
603 *Mse_Aim2_CDS_F*: AGTACCGGGAAATGCTGTTG
604 *Mse_Aim2_CDS_R*: GAGTGTGCTCCTGGCAATCT
605 *Mse_Gapdh_F*: CCAATGTGTCCGTCGTGGATC
606 *Mse_Gapdh_R*: GTTGAAGTCGCAGGAGACAAC
607 *Mse_Neat1_F*: TTGGGACAGTGGACGTGTGG
608 *Mse_Neat1_R*: TCAAGTGCCAGCAGACAGCA
609 Cloning Strategy for 5'UTR GFP Plasmid
610 The GFP reporter plasmid was CMV-Zeo-t2A-GFP. Zeocin is flanked by NheI and AgeI. The
611 sequence of the annotated and unannotated 5'UTR were used as defined by the UCSC RefSeq
612 and our sequencing results to be. Using KAPA HiFi HotStart ReadyMix PCR Kit (Kapa
613 Biosystems) the two 5'UTRs were amplified from cDNA.

5'UTR	cDNA Sequence
Annotated	TTCCTGTCCTGTCTGCCGCCATGCTTCCTTAACTAGCTGCTAGGTTTT TTCCTTGTCGTGATGAAATCCACCCTCATGGACCTGTAAGTAAAATG TAGACTTGCATAGAGTGCTGTAATCTTACGGCCGAGGTTTCTTTTCA GGCTGATCCTGGGACTGTGAG

Unannotated	TATATCTAAAATACCTCTGGTTGAGACCTCACAGCTGGAGGAGAAAC TCTGCTGAGGCTTGTA AAAAGGAAACTGAAA ACTAGCATTTGCTTGG GCAGAGCCTTAATATATAATTATTTTGCCCCAGCATCAGGGTTTAGG ACTCAGCTATAGGGCCAGGACTAGCCAAGCTTCAAAGTGAAAGAAG ATAGTTGAGAGTACTTTCTGCTTTCTGTCTCCCAAGACCTGATTTTCA TGATTTTCATGTCCTACTACTCATAGTGAAAATCTTTGTGAGGCGGA TGGTTTGA ACTCTCAGGACATACACCAGTCCCTGAGTTGAGAACTAA GGCTGCTTTGGAGAGAAGAAAATCCCCTGAGGTAAGTAGACTTGCA TAGAGTGCTGTAATCTTACGGCCGAGGTTTCTTTTCAGGCTGATCCT GGGACTGTGAG
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614

615 Primers:

616 *cDNA_F_Annotated*: TTCCTGTCCTGTCTGCCG

617 *cDNA_F_Unannotated*: TATATCTAAAATACCTCTGGTTGAGACCTC

618 *cDNA_R_5'UTR*: CTCACAGTCCCAGGATCAGC

619 The 5'UTRs were then PCR amplified with primers containing restriction enzyme sites for AgeI
620 and NheI.

621 *NheI_F_Annotated*: GGT**GCTAGCT**TCCTGTCCTGTCTGCC

622 *NheI_F_Unannotated*: GGTGGT**GCTAGCT**TATATCTAAAATACCTCTGGTTGAGA

623 *AgeI_R_5'UTR*: GGT**ACCGGT**CTCACAGTCCCAGGATCAGC

624 PCR products, as well as the GFP plasmid, were then processed using AgeI and NheI restriction
625 enzymes overnight. These samples were run on a 1% agarose gel. A gel extraction was

completed for each band using the PCR clean-up Gel extraction kit (Machery-nagel). The PCR product was confirmed using Sanger Sequencing.

Site Directed Mutagenesis

Set up PCR reaction with 1ul 279 Plasmid, with unannotated Aim2 5'UTR, 5ul 10x Phu buffer, 1ul F primer (0.1ug/ul) [Remove_IRE_F - CCCTGATGCTGGGGCAAATAATTATAAATGCTAGTTTTTCAGTTTC], 1ul R primer (0.1ug/ul) [Remove_IRE_R - GAAACTGAAACTAGCATTTATAATTATTTTGCCCCAGCATCAGGG], 1ul dNTP (10nM), 1ul Phu polymerase and 40ul dH₂O. PCR program: 95°C 1min, 18 cycles of 95°C 30sec, 55°C 1min, 72°C 1min, then end PCR with 72°C 1min and 4°C hold. Add 0.5ul of Dpn1 (NEB) to 25ul PCR reaction. Incubate at 37°C for 1 hr to digest parental DNA. Transform digested and undigested plasmid into DH5 α competent cells. Pick ~10-15 colonies and start overnight cultures. Colony PCR plasmids using Dpn1_Colony_PCR_F - TTGGCTAGTCCTGGCCCTAT and Dpn1_Colony_PCR_R - GCTGGTTTAGTGAACCGTCAG to check for 20bp deletion on a 3% Agarose Gel. Grow up colonies that have deletion, miniprep plasmids and send to Sequetech for sequence verification.

Transfection of 5'UTR GFP and mCherry Plasmid

A 1:1 ratio of the GFP vector containing the mature sequence of Aim2 5'UTR (annotated or unannotated) or zeocin and a plasmid containing mCherry were transfected into 293T cells for 48-72 hr. A 6-well plate of HEK293Ts were plated the night before with a concentration of 2×10^5 . HEK293Ts cells were primed with 100uM of Ammonium Ferric Citrate (FAC) for 24 hr prior to transfection. Transfection was performed on HEK293Ts (+/- 100 uM FAC) using lipofectamine 2000, serum free OPTI-MEM media was used as a transfection reagent according

to manufacturer's instructions, and a (1:1) concentration of the 5'UTR GFP reporter plasmid and the mCherry control plasmid. HEK293Ts were visualized via flow cytometry 48-72 hr post transfection.

Polysome Profiling

Prior to lysis, cells were treated with cycloheximide (100 mg/mL), 10 min at 37°C, 5% CO₂. Cells were washed three times with ice cold PBS and lysed in ice cold buffer A (0.5% NP40, 20 mM Tris HCl pH 7.5, 100 mM KCl and 10 mM MgCl₂). Lysates were passed three times through a 23G needle and incubated on ice for 7 min. Extracts were then centrifuged at 10K rpm for 7 min at 4°C. The supernatant was collected as crude cytosolic extract. Cytosolic extracts were overlaid on 10%–50% sucrose gradients prepared in 20 mM Tris HCl pH 7.5, 100 mM KCl and 10 mM MgCl₂ buffer (prepared using the Gradient Station, Biocomp Instruments). Gradients were then ultracentrifuged at 40K rpm for 1 hr 20 min at 4°C using an SW41 in a Beckman ultracentrifuge. Individual polyribosome fractions were subsequently purified using a Gradient Station (Biocomp Instruments) and stored in (1:3) TRI Reagent.

IRE KO Cell Line Generation

The gRNA construct was constructed from a pSico lentiviral backbone driven by EF1a promoter expressing T2A flanked genes: puro and cherry. gRNAs were expressed from a mouse U6 promoter. Cloning of 20nt gRNA spacer forward/reverse oligos were annealed and cloned via the AarI site.

IRE_gRNA1_F: TTGGACTGAAAAGTAGCATTGCT

IRE_gRNA1_2: AAACAGCAAATGCTAGTTTTCAGT

IRE_gRNA2_F: TTGGCTGAAAAGTAGCATTGCTT

IRE_gRNA2_2: AAACAAGCAAATGCTAGTTTTCAG

672 *IRE_gRNA3_F*: TTGGGGCAAATAATTATATATTA

673 *IRE_gRNA3_2*: AAATAATATATAATTATTTTGCC

674 3µg of pooled gRNAs were electroporated using the Lonza Amaxa Mouse Macrophage

675 Nucleofector kit (VPA-1009). Electroporated cells were then selected using puromycin 5µg/ml

676 and grown to 80% confluency. Limited dilution series were seeded in 96-well plate, let to grow

677 for 3 weeks. Then the clonal cell lines were genotyped using:

678 E:GCAGGAAATAACTTTTGTGGAGT and R:TGGGAGACAGAAAGCAGAAAG. Then this

679 PCR product was sequenced, and KO efficiency was assessed using ICE Synthego

680 (<https://ice.synthego.com/#/>). Then the hairpin structure was assessed by RNAfold

681 (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>).

682 Protein Lysate and Western Blot

683 Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM

684 EDTA, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) containing protease

685 inhibitor cocktail (Roche) and quantified by the Bicinchoninic Acid Assay (BCA) assay (Thermo

686 Fisher). Equivalent masses (20µg) of each sample were resolved by SDS-PAGE and transferred

687 to a polyvinylidene difluoride (PVDF) membrane and Western blotted with either Aim2

688 (1:1,000; Cell Signaling #63660) and horseradish peroxidase-conjugated b-actin monoclonal

689 antibody (1:5,000, Santa Cruz Biotechnology) were used as a loading control. HRP-conjugated

690 goat anti-rabbit (1:1,500, Biorad) secondary antibodies were used. Image J (Schindelin et al.,

691 2012) was used for quantification of Western blots.

692 Alternative splicing quantification (percent spliced in, PSI)

693 After the identification of each alternative splicing event, JuncBASE counts reads supporting the

694 inclusion and exclusion isoform of each event. Isoform abundances are then calculated by

dividing the read counts for the isoform by the length of the isoform. Ψ -values for each splicing event are derived from the isoform abundances:

PSI Formula:

$$\text{PSI} = \frac{\text{Inclusion Isoform Abundance}}{(\text{Inclusion Isoform Abundance} + \text{Exclusion Isoform Abundance})}$$

Statistical Analysis

Error bars represent the standard deviation of biological triplicates. Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (* $p > 0.05$, ** $p > 0.01$, *** $p > 0.005$).

Illumina RNA Sequencing (Human)

RNA-seq libraries were prepared with the Illumina TruSeq RNA Sample Preparation kit (Illumina) according to the manufacturer's protocol. Libraries were validated on an Agilent Bioanalyzer 2100. Indexed libraries were equimolarly pooled and sequenced on a SE50 (single-end 50 base pair) Illumina HiSeq2500 lane, which yielded an average of about 30×10^6 reads/sample.

Illumina RNA Sequencing (Mouse)

For generation of RNASequencing libraries, RNA was isolated as described above and the RNA integrity was tested with a BioAnalyzer (Agilent Technologies) or FragmentAnalyzer (Advanced Analytical). For RNASequencing target RIN score of input RNA (500-1000ng) usually had a minimum RIN score of 8. RNASequencing libraries were prepared with TruSeq stranded RNA sample preparation kits (Illumina), depletion of ribosomal RNA was performed by positive selection of polyA⁺ RNA. Sequencing was performed on Illumina HighSeq or NextSeq machines. RNA-seq 50bp reads were aligned to the mouse genome (assembly GRCm38/mm10)

using TopHat (Trapnell et al., 2009). The Gencode M13 gtf was used as the input annotation. Differential gene expression specific analyses were conducted with the DESeq (Anders and Huber, 2010) R package. Specifically, DESeq was used to normalize gene counts, calculate fold change in gene expression, estimate p-values and adjusted p-values for change in gene expression values, and to perform a variance stabilizing transformation on read counts to make them amenable to plotting. Data was submitted to GEO GSE141754.

Gene Expression Analysis

DESeq2 v1.22.2 (Love et al., 2014) was used to create counts tables (Table 6-7) and complete differential gene expression analysis on RNA-seq data from Human monocyte-derived macrophage +/- 18 hr LPS and Mouse BMDM +/- 6 hr LPS experiments. The sample conditions used were “control” and “LPS.” Data was plotted using ggplot2 v3.1.1 (Wickham, 2009). Significance thresholds were set to $|\log_2FC| \geq 2$ and adjusted p-value ≤ 0.05 . The list of genes with significant AFE events were then highlighted on the appropriate graphs.

Nanopore Direct RNA Sequencing

Total RNA extraction: Total RNA was extracted according to Workman *et al.* (Workman et al., 2019). 5×10^7 frozen macrophages were resuspended in three ml of TRI-Reagent (Invitrogen AM9738) and vortexed for 5 min. The mixture was incubated at RT for 5 minutes, transferred to 1.5 mL tubes and spun down to remove debris. Supernatant was transferred to fresh tubes and chloroform extracted. The aqueous portion was mixed with an equal volume of isopropanol, incubated for 15 min at RT and centrifuged at 12,000 g at 4°C. Pellet was washed twice with 75% ethanol, air dried and resuspended in nuclease free water.

Poly(A) RNA isolation: One hundred μ g aliquots of total RNA preparation were brought to 100 μ l in nuclease free water and poly-A selected using NEXTflex Poly(A) Beads (BIOO Scientific

741 Cat#NOVA-512980) according to the manufacturer's instructions. The resulting poly-A RNA
742 solution was stored at -80°C.

743 *Library preparation and MinION run:* A native RNA sequencing library was
744 prepared following the ONT SQK-RNA001 using Superscript IV (Thermo Fisher) for the reverse
745 transcriptase step. Sequencing was performed using ONT R9.4 flow cells and the standard
746 MinKNOW protocol.

747 *Basecalling and sequence alignments:* ONT albacore version 2.1.0 was used to basecall
748 Nanopore direct RNA raw signal. We used minimap2 (Li, 2016) with default parameters to align
749 reads to the mm10 mouse genome reference. Following alignment, we used SAMtools (Li et al.,
750 2009) to filter-out reads with mapping quality (MAPQ) less than 30.

751 Alignment of Paired-end Mouse RNA-seq data

752 Bowtie2-build v2.3.1 (Langmead and Salzberg, 2012) was used to build the index files from
753 GRCm38.p6 mouse (mm10 assembly) genome sequence obtained from Gencode. The index files
754 were then used for completing paired-end alignment of each sample using TopHat2 v2.1.1 (Kim
755 et al., 2013) with parameters: *--segment-length 20, --library-type fr-firststrand, --no-coverage-*
756 *search.*

757 Identification of Splicing Events and Calculating PSI

758 Human monocyte-derived macrophage +/- LPS and Mouse bone marrow-derived macrophage
759 +/- LPS were each run through JuncBASE v1.2 (Brooks et al., 2011) to calculate percent spliced
760 in (PSI) values and identify splicing events. The JuncBASE parameters used for the
761 identification of splicing events and calculation of PSI in Human monocyte derived macrophage
762 +/- LPS are: *-c 1.0 -j [introns from Gencode v24 (hg19 assembly) (Frankish et al., 2019) --*
763 *jcn_seq_len 88.* The JuncBASE parameters used for the identification of splicing events and

764 calculation of PSI in Mouse bone marrow-derived macrophage +/- LPS are: *-c 1.0 -j [introns*
765 *from Gencode M18 (mm10 assembly)*(Frankish et al., 2019) *--jcn_seq_len 88*.

766 Differential Splicing Analysis

767 Differential splicing analysis was completed using DRIMSeq v1.10.1 (Nowicka and Robinson,
768 2016) and the compareSampleSets.py script within JuncBASE. CompareSampleSets.py applies
769 the statistical t-test and DRIMSeq applies the framework of the Dirichlet-multinomial
770 distribution for differential analysis. Each tool was used to apply the respective statistical method
771 in order to determine significant differentially spliced events between control (-LPS) and LPS
772 (+LPS) conditions. The AS_exclusion_inclusion_counts_lenNorm.txt JuncBASE output table
773 from the identification and quantification analysis of each experiment was used as the input table
774 for both compareSampleSets.py and DRIMSeq.

775 For all experiments, compareSampleSets.py was run using parameters: *--mt_correction BH --*
776 *which_test t-test --thresh 10 --delta_thresh 5.0*. The following parameters were used for the
777 differential splicing analysis of data from Human monocyte-derived macrophage +/- LPS with
778 DRIMSeq: *min_samps_gene_expr = 8, min_samps_feature_expr = 4, min_gene_expr = 10,*
779 *min_feature_expr = 0*. The following parameters were used for the differential splicing analysis
780 of data from Mouse bone marrow-derived macrophage +/- LPS with DRIMSeq:

781 *min_samps_gene_expr = 6, min_samps_feature_expr = 3, min_gene_expr = 10,*
782 *min_feature_expr = 0*. Following differential splicing analysis using each tool, genes with
783 significant differential splicing events were filtered for using thresholds of a corrected/adjusted
784 p-value ≤ 0.25 and a $|\Delta \text{PSI}| \geq 10$. Within each category of event type, the union of genes with
785 significant events identified using compareSampleSets.py and DRIMSeq within each experiment

was used for further comparison. Novel intron retention events, annotated with a “N,” were removed for further analyses.

Ranking Analysis

Following differential splicing analysis with JuncBASE, events were ordered by p-value. A subset of the top 5%, 10%, 15%, 20%, and 25% events were quantified by event type. Only events having a $|\Delta \text{PSI}| \geq 10$ were considered. The same analysis was completed following differential splicing analysis with DRIMSeq2.

Analysis of Listeria and Salmonella Datasets following 24 hr stimulation.

Data for the 24h time point was downloaded from GEO (GSE73502) for control and experimental conditions: Listeria and Salmonella (Pai et al., 2016b). JuncBASE and differential alternative splicing analysis was run using each pair of control and experimental samples and parameters as described above. Following differential alternative splicing analysis, alternative splicing events were categorized by event type using two significance thresholds: $|\Delta \text{PSI}| \geq 10$ and adjusted p-value ≤ 0.05 or $|\Delta \text{PSI}| \geq 10$ and adjusted p-value ≤ 0.25 . Only known (K) events were considered for intron retention events. Jcn_only_AD and jcn_only_AA events were not considered.

Identification of High-Confidence Isoforms from Nanopore Data

FLAIR (full length alternative isoform analysis of RNA) (Tang et al., 2020) was used to assemble the high-confidence isoforms from native RNA sequencing of Mouse BMDM +/- 6 hr LPS. FLAIR modules align, correct, and collapse were used for the assembly. Corresponding short read data was used when running the correct module in order to help increase splice-site accuracy. Putative promoter regions were obtained using ATAC-seq data from Smale *et al.* and

808 Atianand *et al.* (Atianand et al., 2016; Tong et al., 2016) converted to mm10 coordinates using
809 liftOver (Hinrichs et al., 2006), and used when running the collapse module.

810 Creating merged reference annotation files and incorporating nanopore

811 The *isoforms.gtf* output file from FLAIR collapse was combined with the Gencode M18 (mm10
812 assembly) basic annotation using cuffmerge from Cufflinks v2.2.1 (Trapnell et al., 2010) with
813 parameter: -s GRCm38.p6.genome.fa. Similarly, the *isoforms.gtf* output file was combined with
814 the Gencode M18 (mm10 assembly) comprehensive annotation with parameter: -s
815 GRCm38.p6.genome.fa. The resulting comprehensive annotation output file was used to
816 generate an intron coordinate file for the identification of splicing events and calculating PSI of
817 splicing events found in Mouse Bone-marrow derived macrophage +/- 6 hr LPS using
818 JuncBASE with parameters: -c 1.0, -j [intron coordinates from merged comprehensive
819 annotation], --jcn_seq_len 238. Parameters used for finding significantly differentially spliced
820 events using compareSampleSets.py from JuncBASE are: --mt_correction BH --which_test t-test
821 --thresh 10 --delta_thresh 5.0. Parameters used for finding significantly differentially spliced
822 events using DRIMSeq are: min_samps_gene_expr = 6, min_samps_feature_expr = 3,
823 min_gene_expr = 10, min_feature_expr = 0.

824 Creating and comparing gene lists

825 For each experiment, a table with the union of significant events found using DRIMSeq and
826 compareSampleSets.py was created. A list of genes with significant events was generated for
827 each experiment using this table. BioVenn (Hulsen et al., 2008) and DrawVenn (Li, 2016) were
828 then used to remove duplicate gene names and compare the lists of genes to find unique and
829 common genes between experiments.

830 Identification of Genes with Conserved Alternative Splicing Events

831 Following differential splicing analysis with JuncBASE and DRIMSeq2, gene names of
832 significant events ($|\Delta \text{PSI}| \geq 10$ and adjusted p-value ≤ 0.25) were curated by event type.
833 Overlaps were determined between Human and Mouse JuncBASE and DRIMSeq2 events
834 determined with and without the support of Nanopore-identified transcripts.

835 Comparison of all alternative splicing events Identified in Mouse using Gencode or *de novo* +
836 Gencode annotations

837 Following differential splicing analysis with JuncBASE and DRIMSeq2, gene names of
838 significant events ($|\Delta \text{PSI}| \geq 10$ and adjusted p-value ≤ 0.25) were curated by event type.
839 Overlaps were determined between Mouse JuncBASE and DRIMSeq2 events determined with
840 and without the support of Nanopore-identified transcripts.

841 Validation of FLAIR-identified TSS with CAGE data.

842 Coordinates corresponding to mouse CAGE peaks
843 (mm9.cage_peak_phase1and2combined_coord.bed) were downloaded from the FANTOM5
844 database (The FANTOM Consortium and the RIKEN PMI and CLST (DGT), 2014).
845 Coordinates were converted from mm9 to mm10 using liftOver. Transcription start sites (TSS)
846 of FLAIR-identified isoforms were extracted using the pull_starts.py script included in the
847 FLAIR software. TSS were extracted from the Gencode M18 annotation using a custom script
848 and used as the set of known TSS. TSS of FLAIR-identified isoforms were annotated as known
849 if they overlapped with known TSS using bedtools (v 2.25.0) tool intersectBed. TSS of FLAIR-
850 identified isoforms were annotated as being novel if they did not overlap with any known TSS.
851 These were also identified using bedtools tool intersectBed with parameter: -v. CAGE peaks that
852 overlapped with known and novel FLAIR-identified TSSs were identified using bedtools tool
853 intersectBed and the distribution of CAGE scores was plotted for each group.

Coordinates corresponding to CAGE peaks identified as true TSS by TSS classifier (TSS_mouse.bed) were downloaded from the FANTOM 5 database. Coordinates were converted from mm9 to mm10 using liftOver. The proportion of true known and novel FLAIR-identified TSS sites was identified by determining TSSs that overlapped with the CAGE peaks. Bedtools (v 2.25.0) tool intersect was used to complete this analysis.

Differential chromatin accessibility

Raw ATAC-seq fastq sequence files were published in Tong *et al.* (Tong et al., 2016) and pulled from the GEO accession number GSE67357. A bowtie2 (Langmead and Salzberg, 2012) index file was created from the GENCODE mm10 version M18 genome annotation file and the untreated and LPS treated ATAC-seq reads were aligned using the created index file with the default bowtie2 parameters. Peaks were then called separately by treatment type on untreated and treated samples using the ENCODE published ATAC-seq peak calling pipeline (<https://github.com/ENCODE-DCC/atac-seq-pipeline>) using the aligned reads as sequence input. Parameters that were used followed the basic JSON input file template, using an IDR threshold of 0.05. Peaks from both conditions were then merged using bedtools merge (Quinlan and Hall, 2010) if the tail ends were less than 10bp away from each other, in order to create a set of consensus peaks from both conditions. A GFF file was created from the merged peaks, assigning a unique ID to each peak. This GFF file was provided to HTSeq-count (Anders et al., 2015) along with the aligned reads for each condition in each replicate to count reads aligning to the unique peaks. The read count matrix was provided to DESeq2 (Love et al., 2014) to call differential peaks. All peaks were considered significant if $\log_2FC \geq 0.8$ and $p\text{-value} \leq 0.05$.

Differential transcription factor binding

ChIP-seq fastq sequencing files for the NF- κ B subunit p65 and interferon transcription factor Irf3 were downloaded from the GEO accession number GSE67357 published by Tong *et al.* (Tong et al., 2016). ChIP-seq samples for input control, untreated, and LPS treatment were aligned using bowtie2 (Langmead and Salzberg, 2012, p. 2) to the mm10 version M18 mouse genome annotation with default parameters. Peaks were separately called between untreated and treated conditions using the ENCODE published ChIP-seq peak calling pipeline (<https://github.com/ENCODE-DCC/chip-seq-pipeline2>) from the aligned reads. The aligned input control reads were input as genomic background to account for noise in ChIP-seq experiments. The basic JSON input template file was used, using an IDR threshold of 0.05. Differential peak analysis was done using the HOMER suite designed for ChIP-seq data (Heinz et al., 2010). Consensus peaks from both conditions were merged using mergePeaks within HOMER, reporting the direct overlap between peaks. Tag directories were created to count reads for each aligned sequence file with TagDirectory. The merged consensus peaks were then annotated for raw read counts using the tag directories for each replicate and condition with the annotatePeaks.pl tool. Annotated consensus peaks were provided to getDiffExpression.pl, normalizing to total read counts. Peaks were considered significant if they had a corrected p-value ≤ 0.05 and $\log_2FC \geq 1$.

Alternative splicing event overlap

To identify differential transcription factor binding and chromatin remodeling at the promoters of the observed alternative first exon events, the coordinates of the alternate first exon were determined from the statistical testing results. Significant (p-value ≤ 0.05) alternative first exon events were first filtered out from all results. For all significant results, if the inclusion exon had a $\Delta PSI \geq 10$, the inclusion exon coordinates from the JuncBASE table (Tables 1-3) were used as

the coordinates for that splicing event. If the inclusion exon had a $\Delta \text{PSI} \leq -10$, all other inclusion exons for that splicing event from the statistical testing (DRIMSeq or t-test) were considered, and any inclusion exon with $\Delta \text{PSI} > 10$ was used. Redundant events with the same exon coordinates were then filtered out, leading to a final set of exon coordinates. The coordinates were then extended to include 10kbp upstream of the exon. Overlap of differential chromatin accessibility and different transcription factor binding was done using bedtools intersect (Quinlan and Hall, 2010) with the significant differential peak coordinates and the alternative first exon 10kbp upstream region, returning the coordinates of the exon that show differential chromatin accessibility or transcription factor binding.

HOMER Promoter Analysis

HOMER (Boeva, 2016) was used to search for sequence motifs in promoter regions of LPS-specific AFEs. The start coordinates from the BED file were extended to include the 3kb upstream of the exon. These extended regions were used as input for HOMER's findMotifsGenome.pl script, along with the mm10 reference genome from within HOMER and the size parameter set to 'given'. findMotifsGenome.pl was run initially with the -prepare flag to parse the reference genome based on the size of the input sequences and then was ran after without the prepare flag in order to generate the motif output file (Table 10-11).

BedFile generation of Inclusion and Exclusion AFE

Significant (corrected p-value ≤ 0.25) alternative first exon events were first filtered out from all results. For all significant results, if the inclusion exon had a $\Delta \text{PSI} \geq 10$, the inclusion exon coordinates from the JuncBASE table were used as the coordinates for that splicing event. If the inclusion exon had a $\Delta \text{PSI} \leq -10$, all other exons for that splicing event from the statistical

921 testing (DRIMSeq or t-test) were considered, and any other exon with $\Delta \text{PSI} \geq 10$ was used

922 (Table 12-13).

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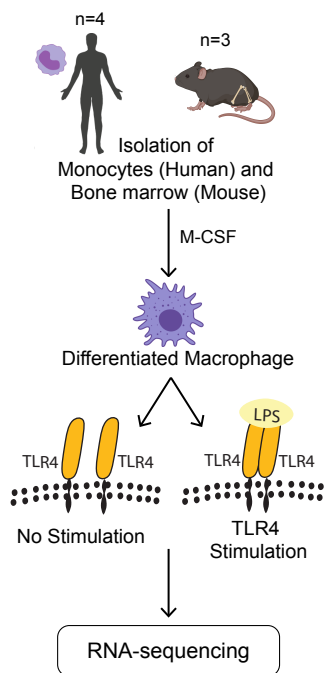
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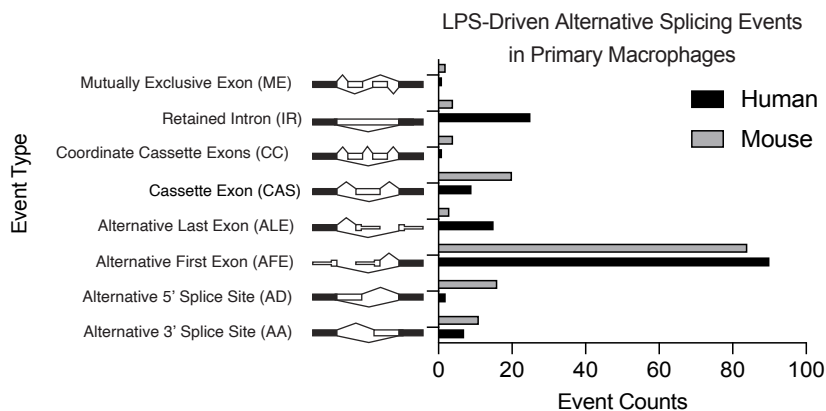
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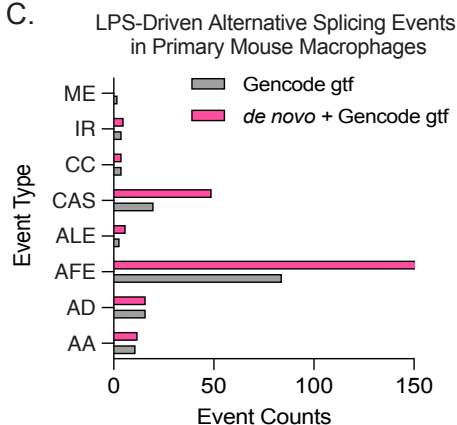
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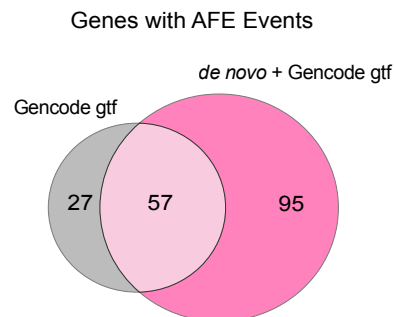
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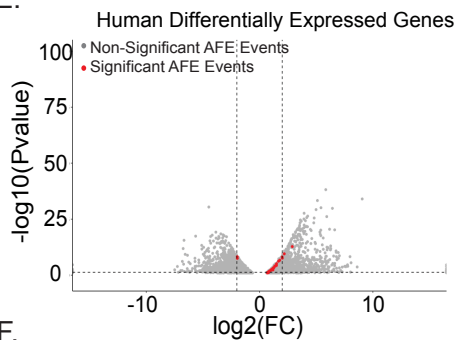
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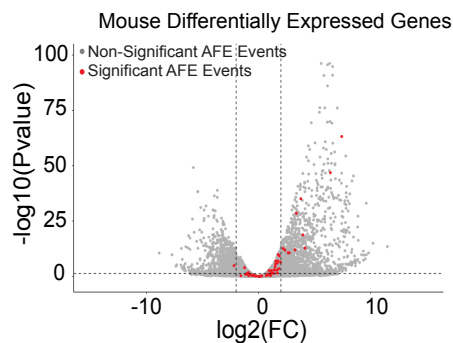
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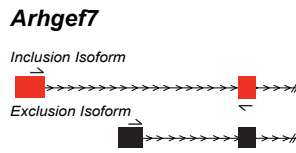
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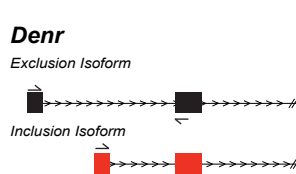
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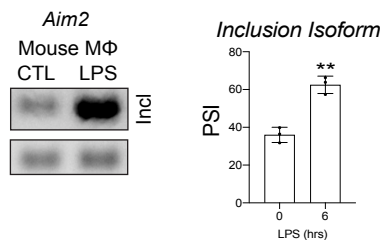
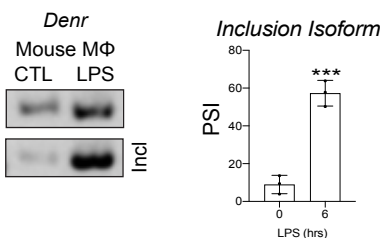
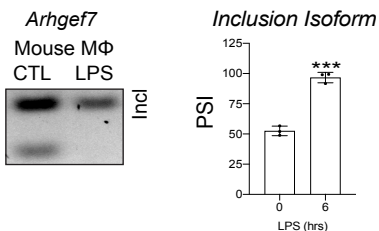
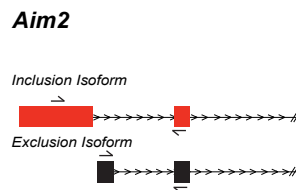
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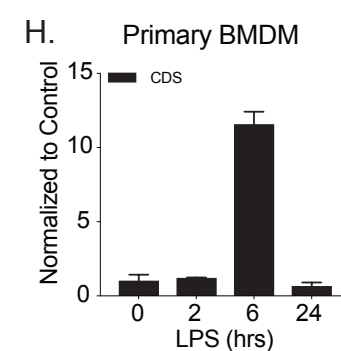
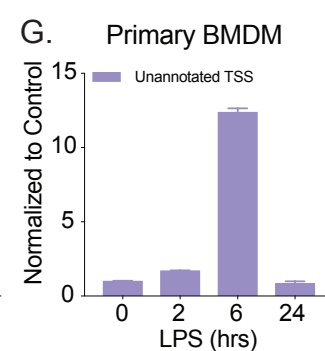
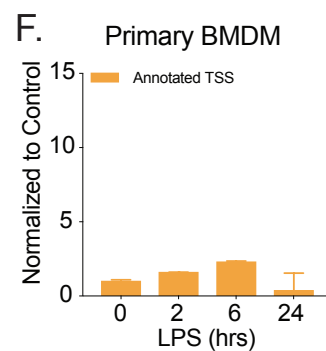
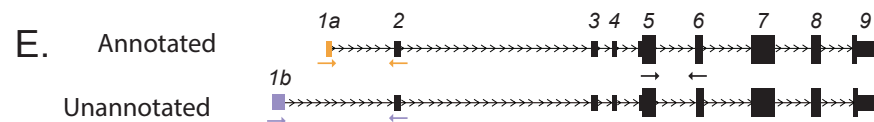
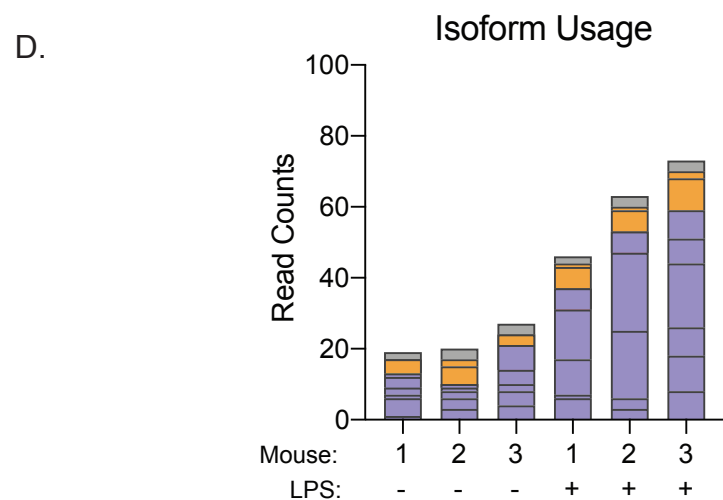
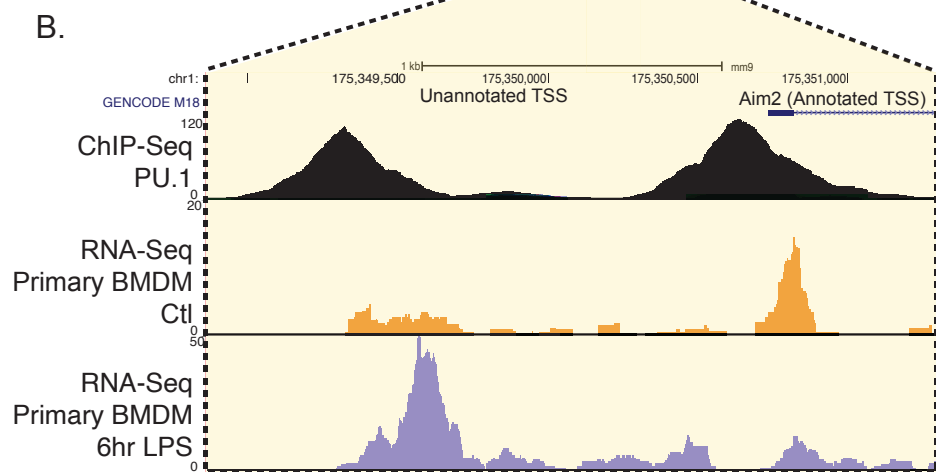
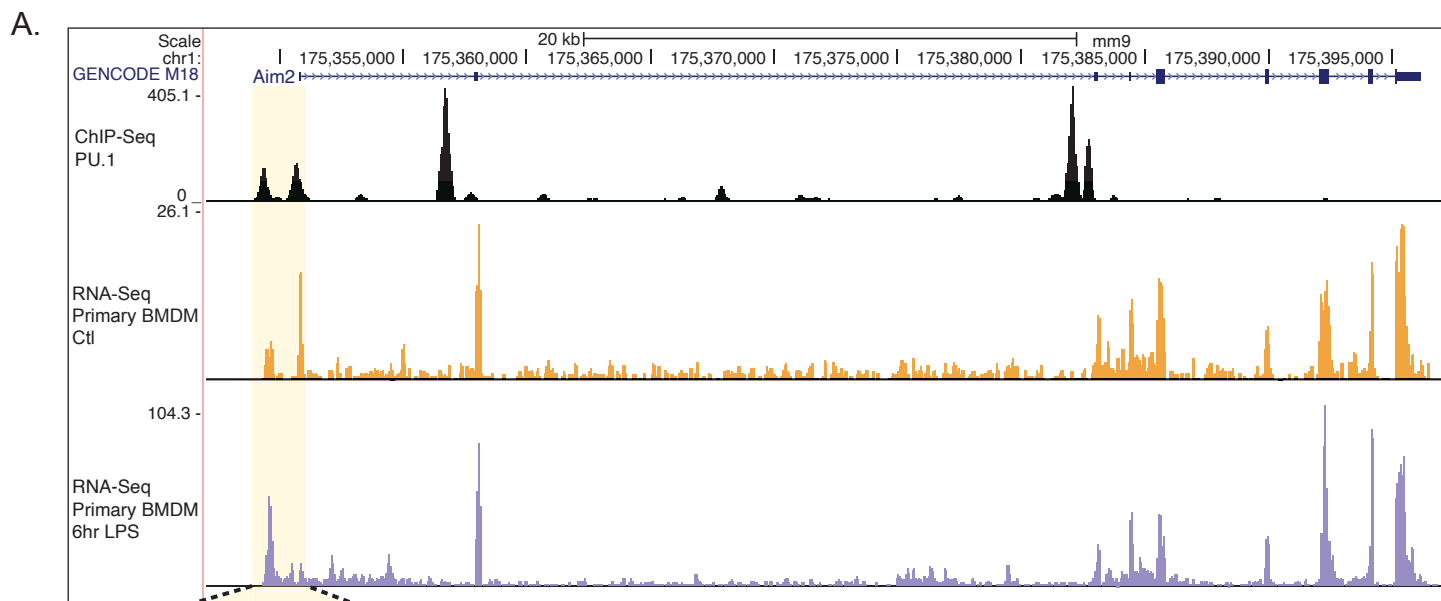


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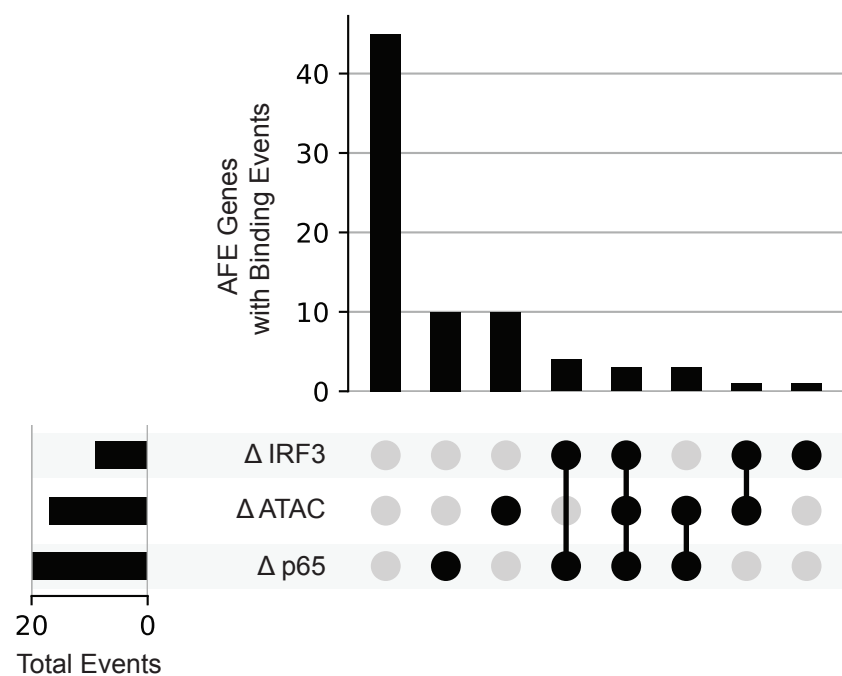


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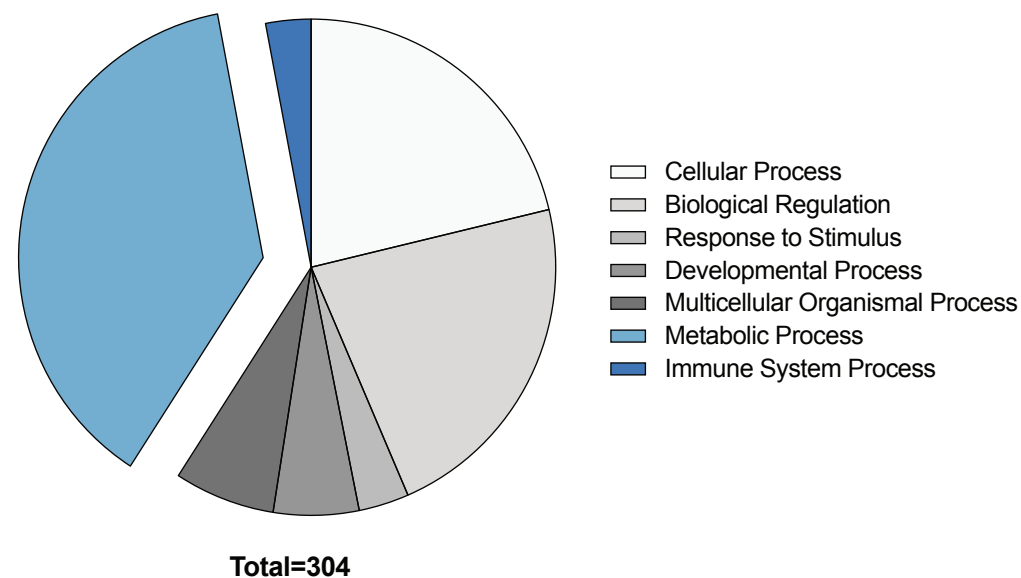


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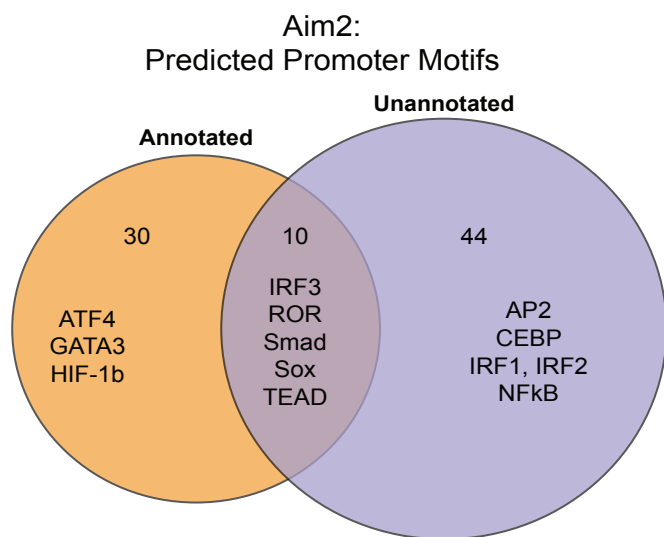


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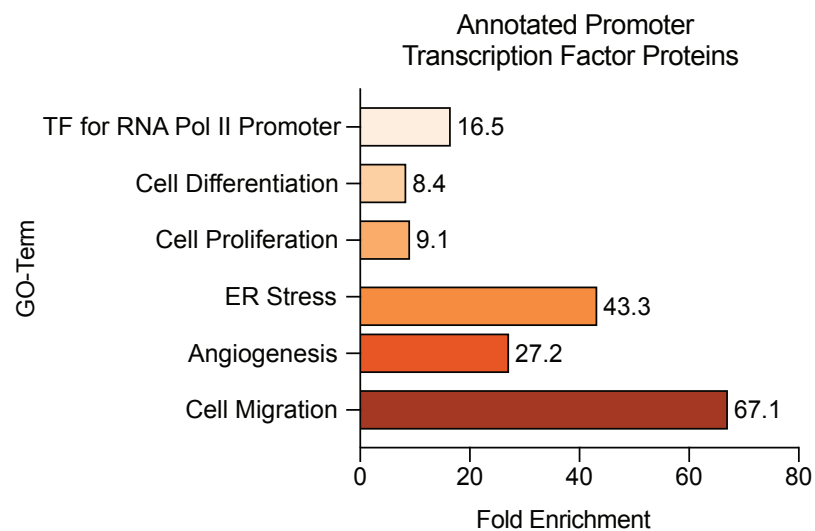
Gene Ontology Analysis of Predicted Promoter Motifs for all AFE genes



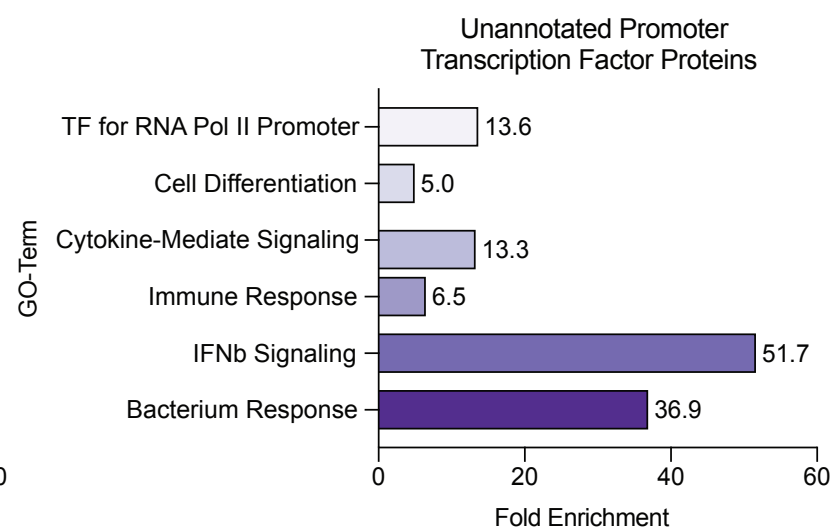
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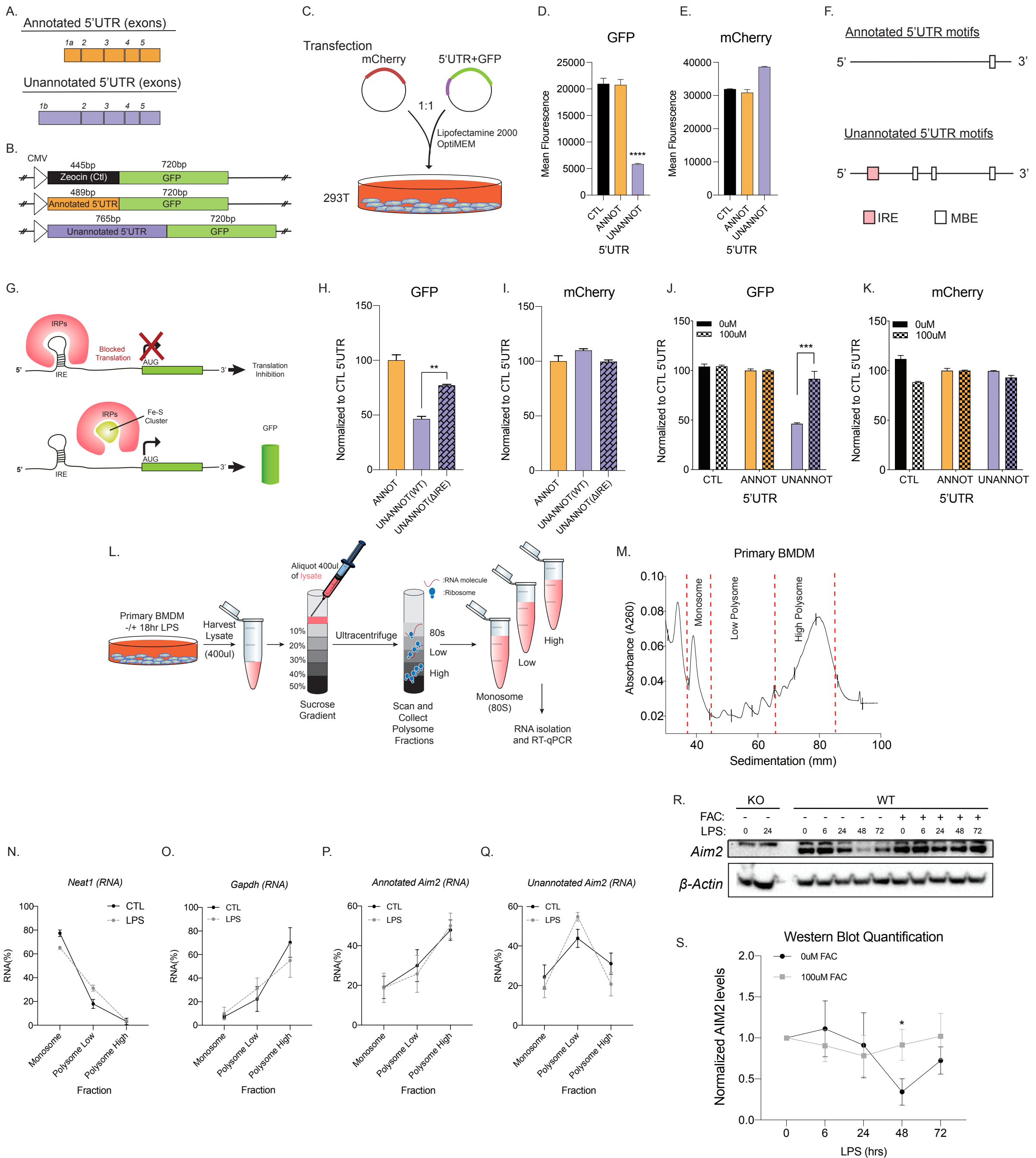


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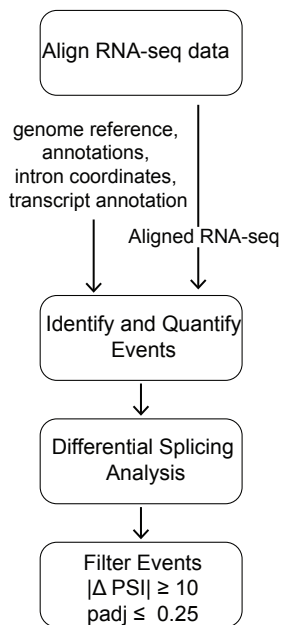


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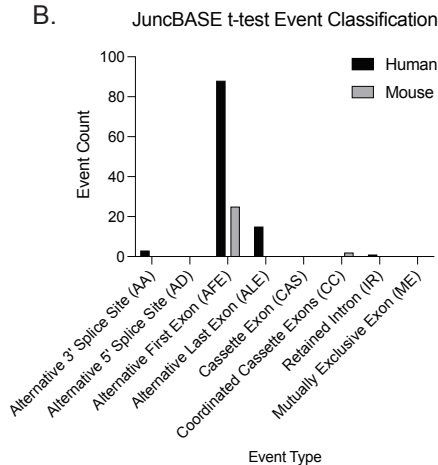




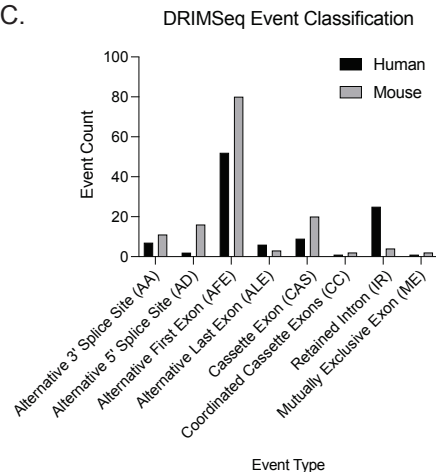
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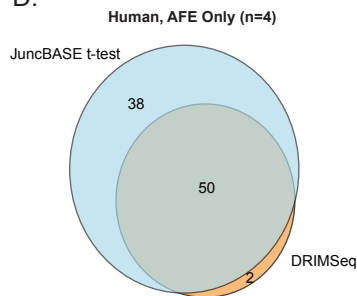
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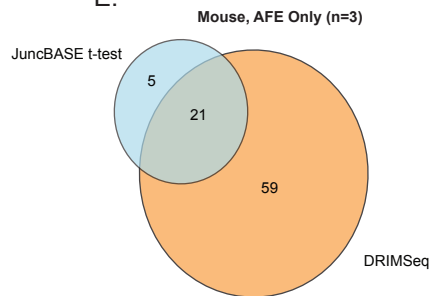
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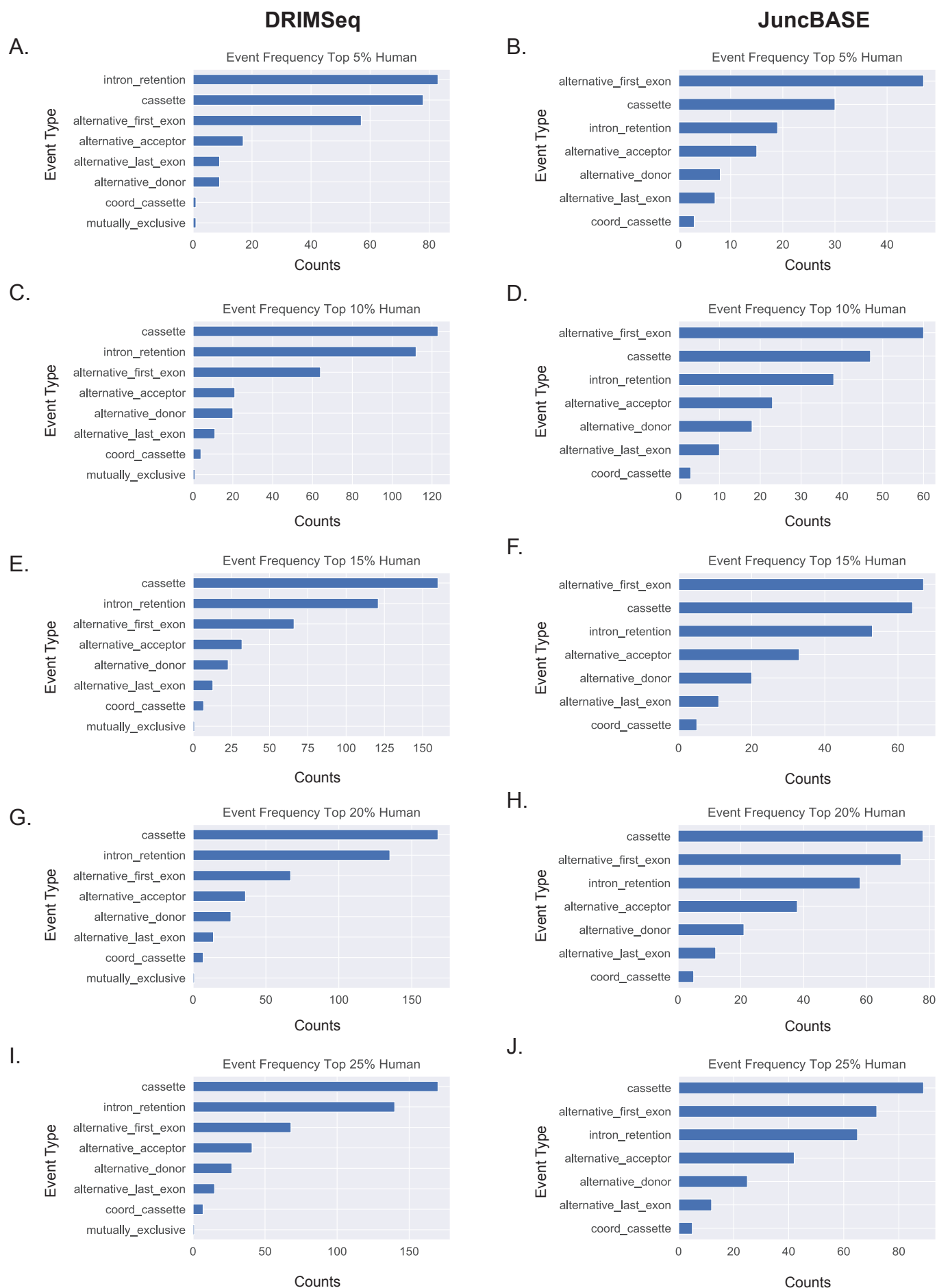
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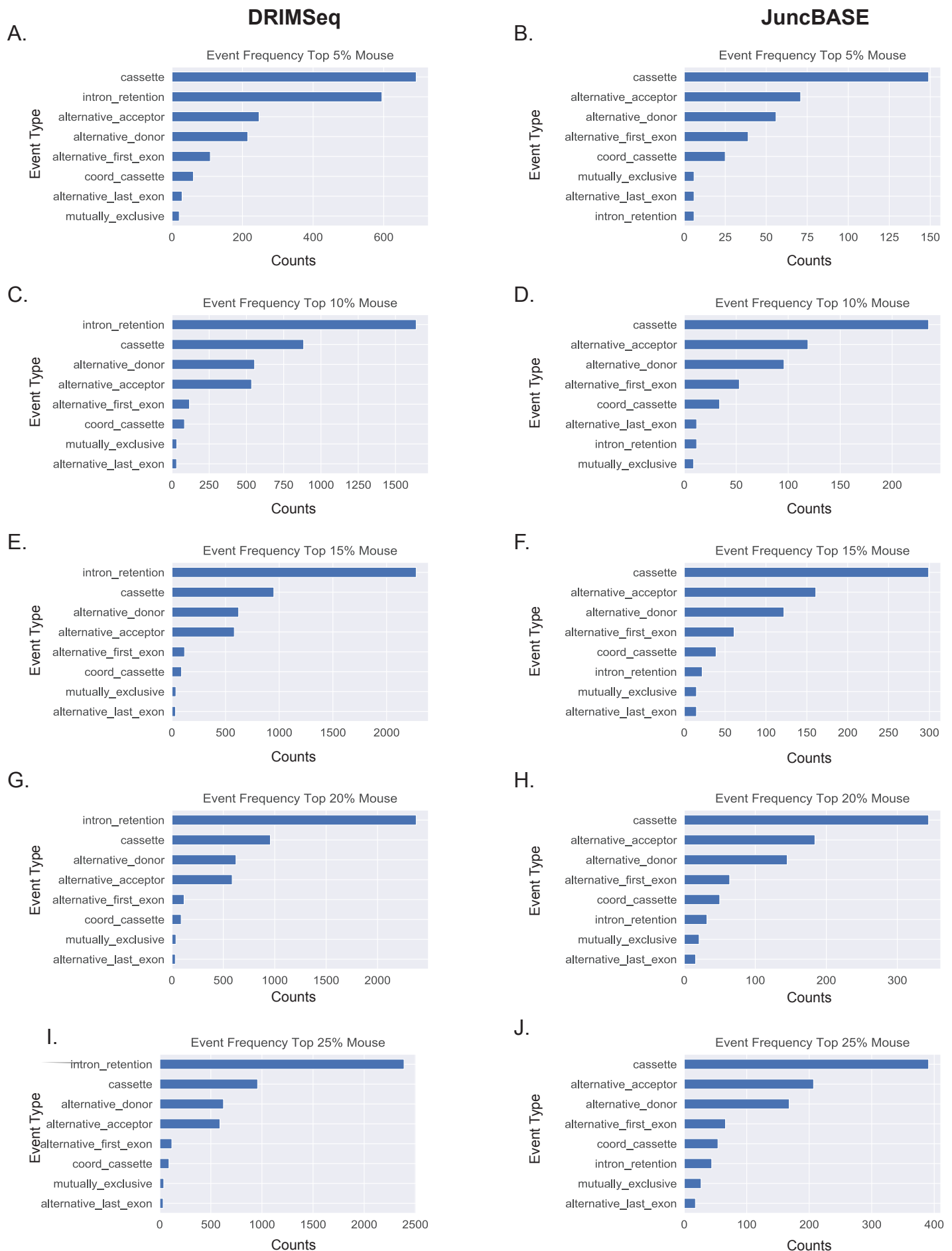
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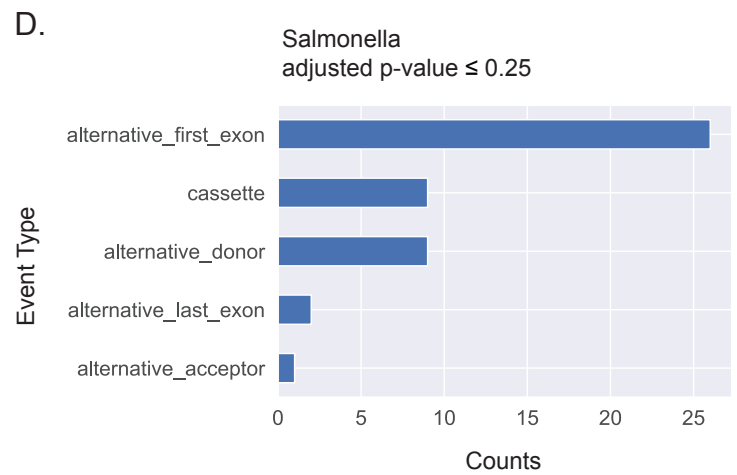
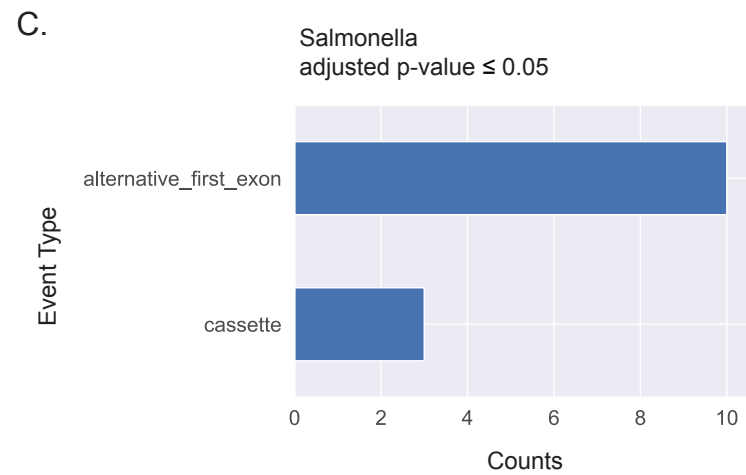
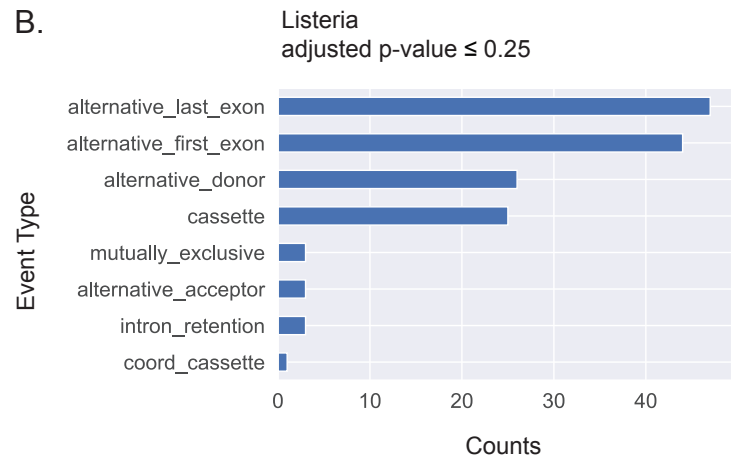
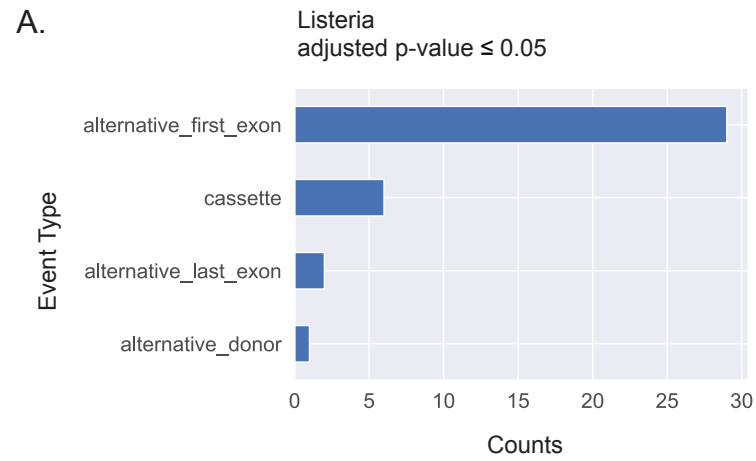
Supplemental Fig 1: Computational Pipeline and comparison of t-test and DRIMSeq alternative splicing events. (A) Bioinformatic pipeline for Human and Mouse RNA-seq data. Alternative splicing event type classification of significant differential splicing events ($|\Delta$ PSI| $\geq$ 10 and corrected p-value $\leq$ 0.25) in Human and Mouse macrophages +/- LPS as identified and quantified using the (B) t-test with JuncBASE or (C) the Dirichlet-multinomial framework applied by DRIMSeq. Venn Diagrams representing the unique and overlapping AFE events from (D) Human or (E) Mouse macrophage alternative splicing analysis.



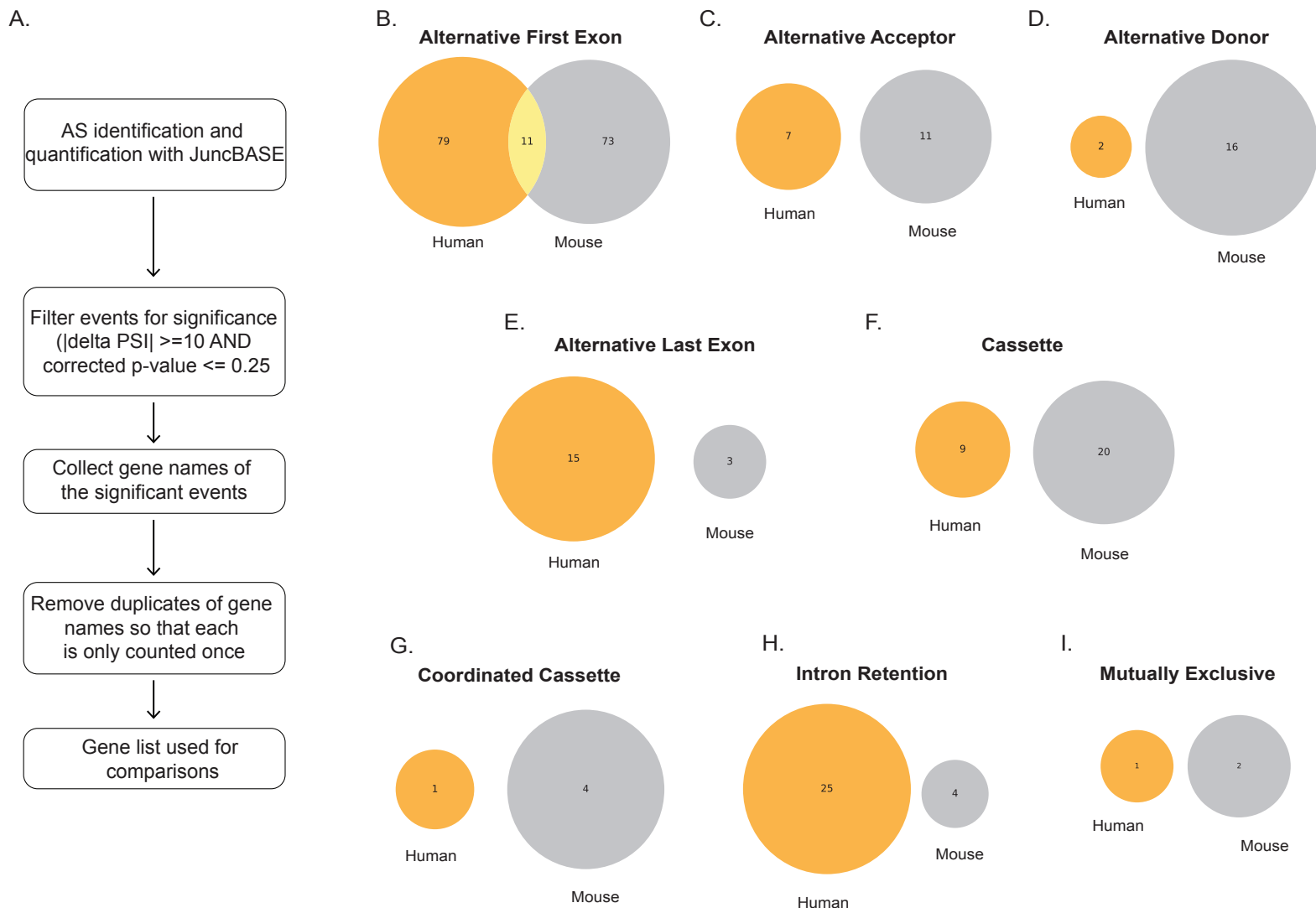
Supplemental Fig 2: AFE events remain prevalent amongst ranked alternative splicing events in human macrophages. (A-B) Ranking of the top 5%, (C-D) 10%, (E-F) 15%, (G-H) 20%, (I-J) 25% of alternative splicing events based on p-value of events as classified by JuncBASE and DRIMSeq in human macrophage cells following LPS stimulation.



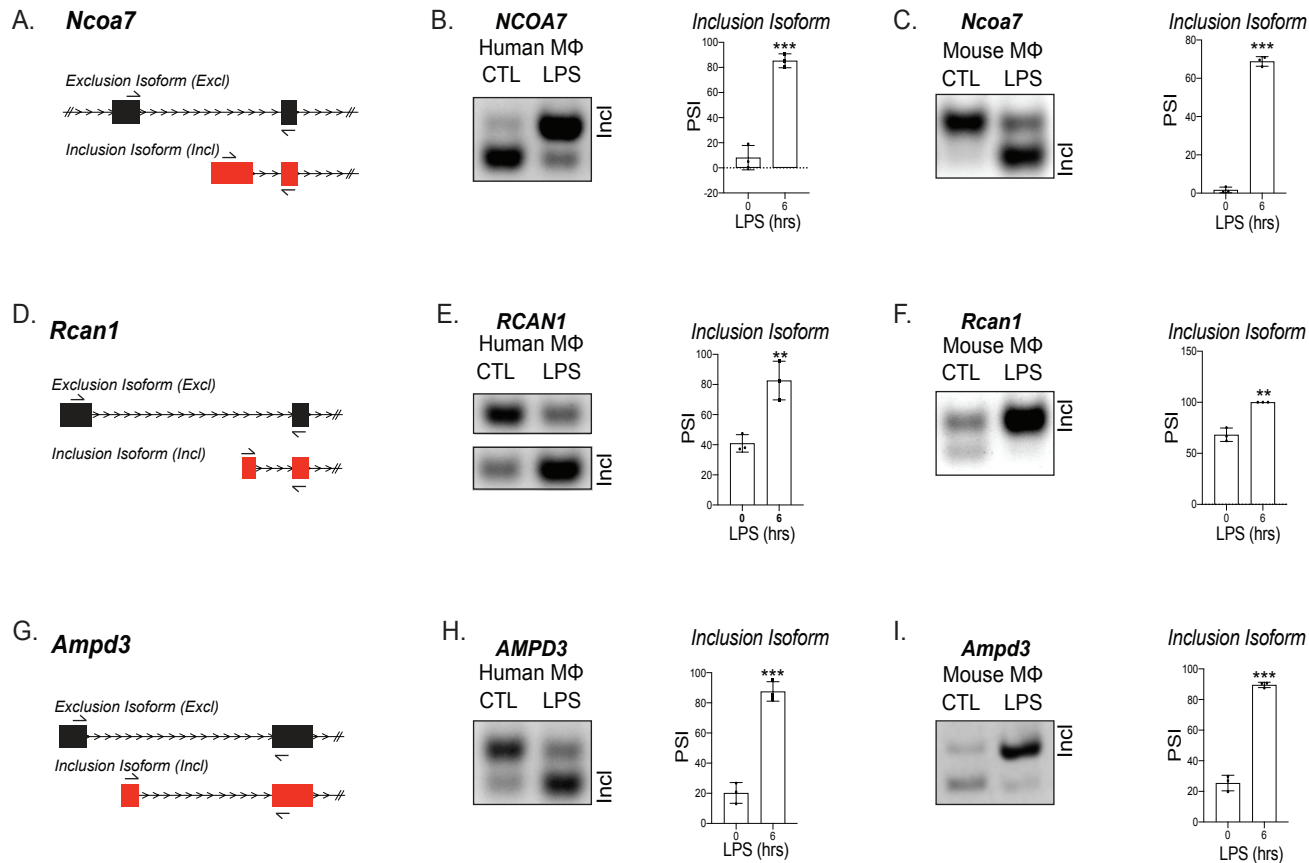
Supplemental Fig 3: AFE events remain prevalent amongst ranked alternativet splicing events in mouse macrophages. (A-B) Ranking of the top 5%, (C-D) 10%, (E-F) 15%, (G-H) 20%, (I-J) 25% of alternative splicing events based on p-value of events as classified by JuncBASE and DRIMSeq in human macrophage cells following LPS stimulation.



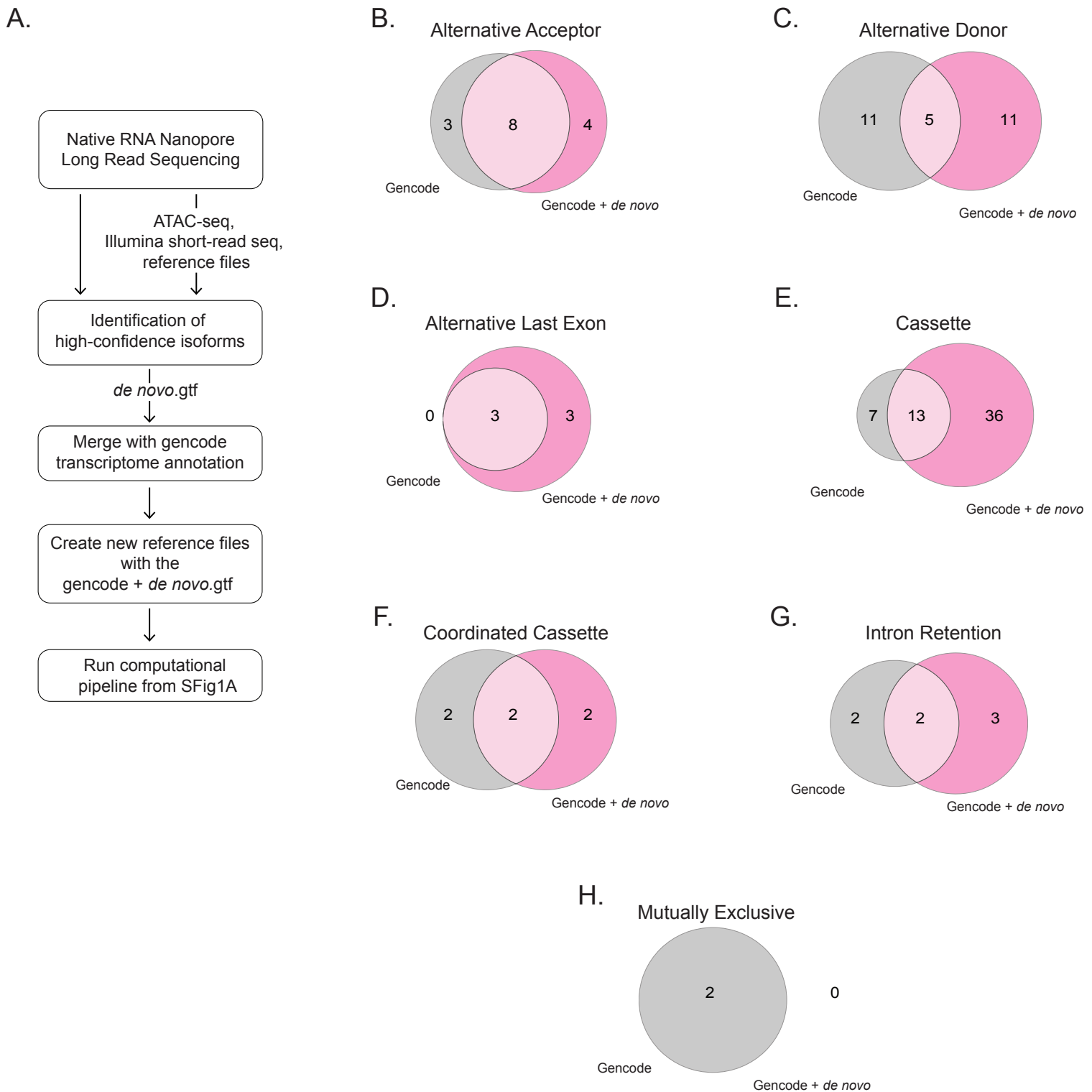
Supplemental Fig 4: Alternative First Exon usage is the top splicing event when using JuncBASE to identify and quantify alternative splicing events from primary human macrophages stimulated with Listeria and Salmonella for 24hrs. Significant alternative splicing events identified and categorized by event type using an adjusted p-value cutoff ≤ 0.05 (A) and a cutoff of ≤ 0.25 (B) following Listeria infection. Significant alternative splicing events identified and categorized by event type using an adjusted p-value cutoff of ≤ 0.05 (C) and a cutoff of ≤ 0.25 (D) following Salmonella infection. Data acquired from GSE73502.



Supplemental Fig 5: Conserved Genes identified through alternative splicing events between human and mouse using gencode transcriptome. (A) Schematic depicting how gene lists were generated from exon events. Venn digrams denoting unique and overlapping genes with significant splicing events in human (orange) and mouse (grey) for (B) Alternative First Exon, (C) Alternative Acceptor, (D) Alternative Donor, (E) Alternative Last Exon, (F) Cassette, (G) Coordinated Cassette, (H) Intron Retention and (I) Mutually Exclusive all have conserved splicing events when analyzing LPS stimulated primary macrophage.

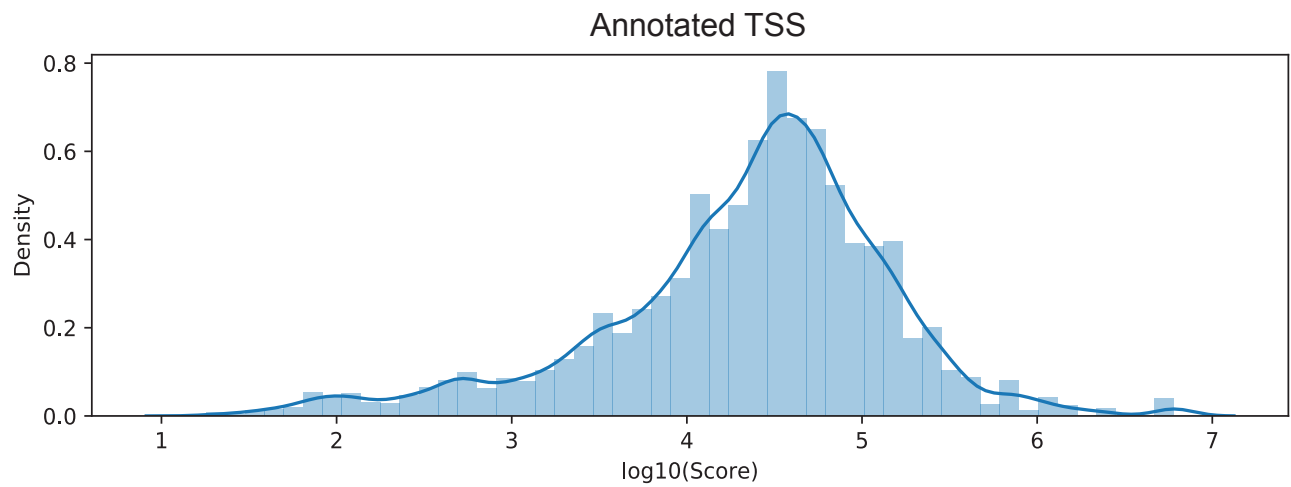


Supplemental Fig. 6: Validation of mouse and human alternative first exon events. (A) mRNA transcript diagram of the exclusion and inclusion isoform of *Ncoa7* for mouse and human. RT-PCR of Human (B) and mouse (C), *Rcan1* (D-E) and *Ampd3* (G-I) with PSI calculation.

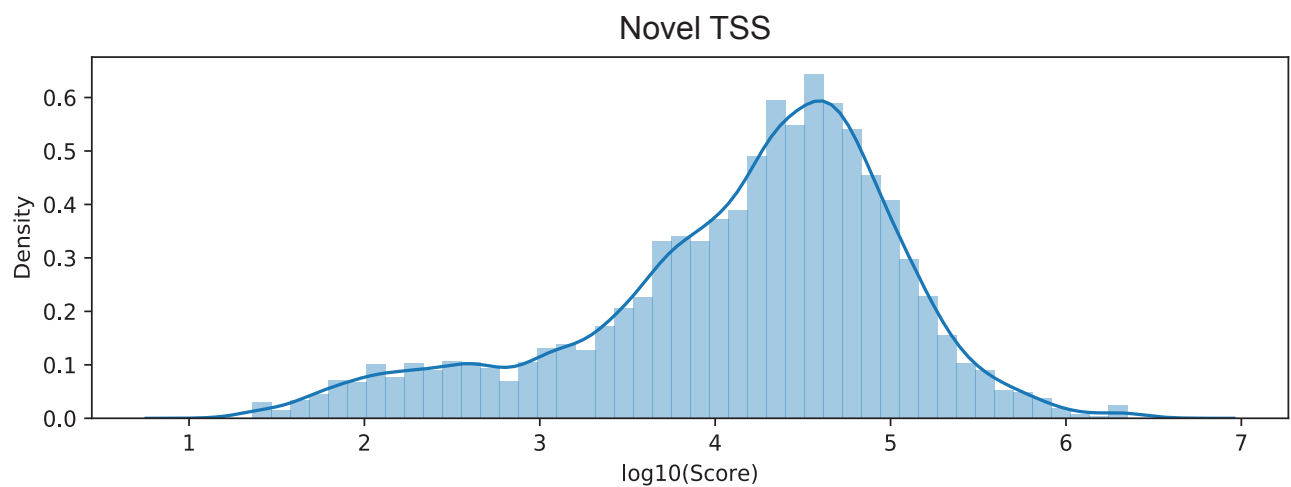


Supplemental Fig 7: Comparison of alternative splicing events identified using the gencode transcriptome or the gencode + *de novo* transcriptome. (A) Schematic describing the steps taken to analyze alternative splicing using either the publicly available gencode.gtf or our gencode + *de novo*.gtf. Venn diagrams show unique and common genes when mapping RNA-sequencing libraries using the publicly available gencode gtf file and the *de novo* transcriptome file using nanopore sequencing for alternative splicing events including: (B) alternative acceptor, (C) alternative donor, (D) alternative last exon, (E) cassette, (F) coordinated cassette, (G) intron retention and (H) mutually exclusive.

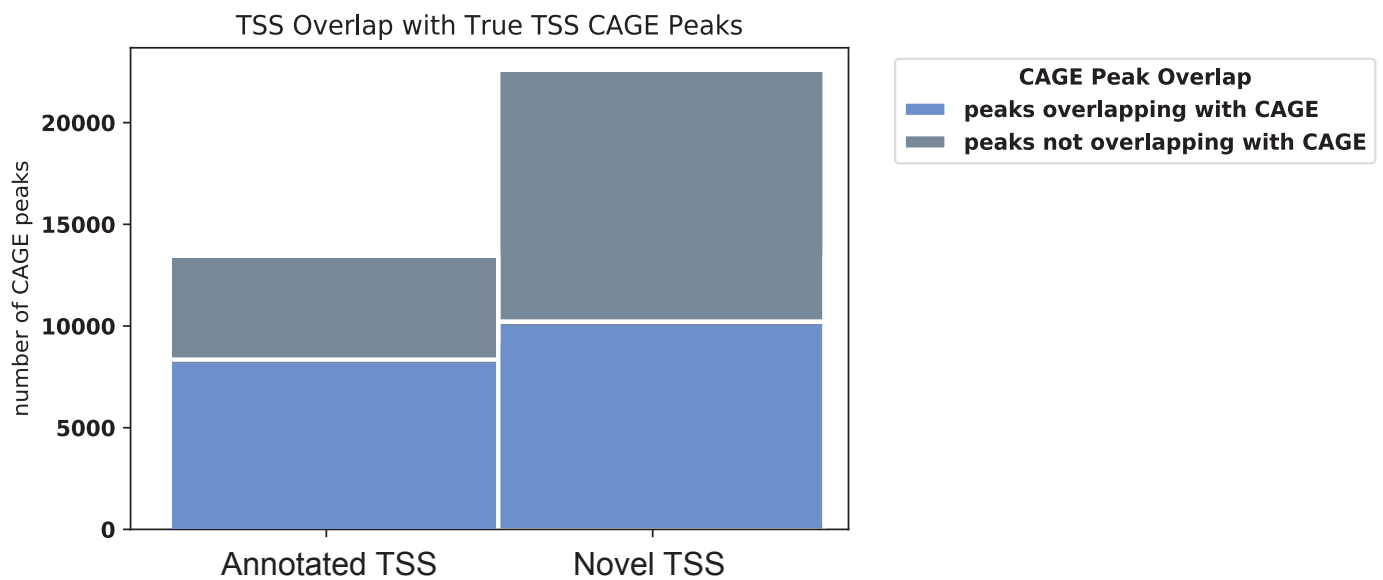
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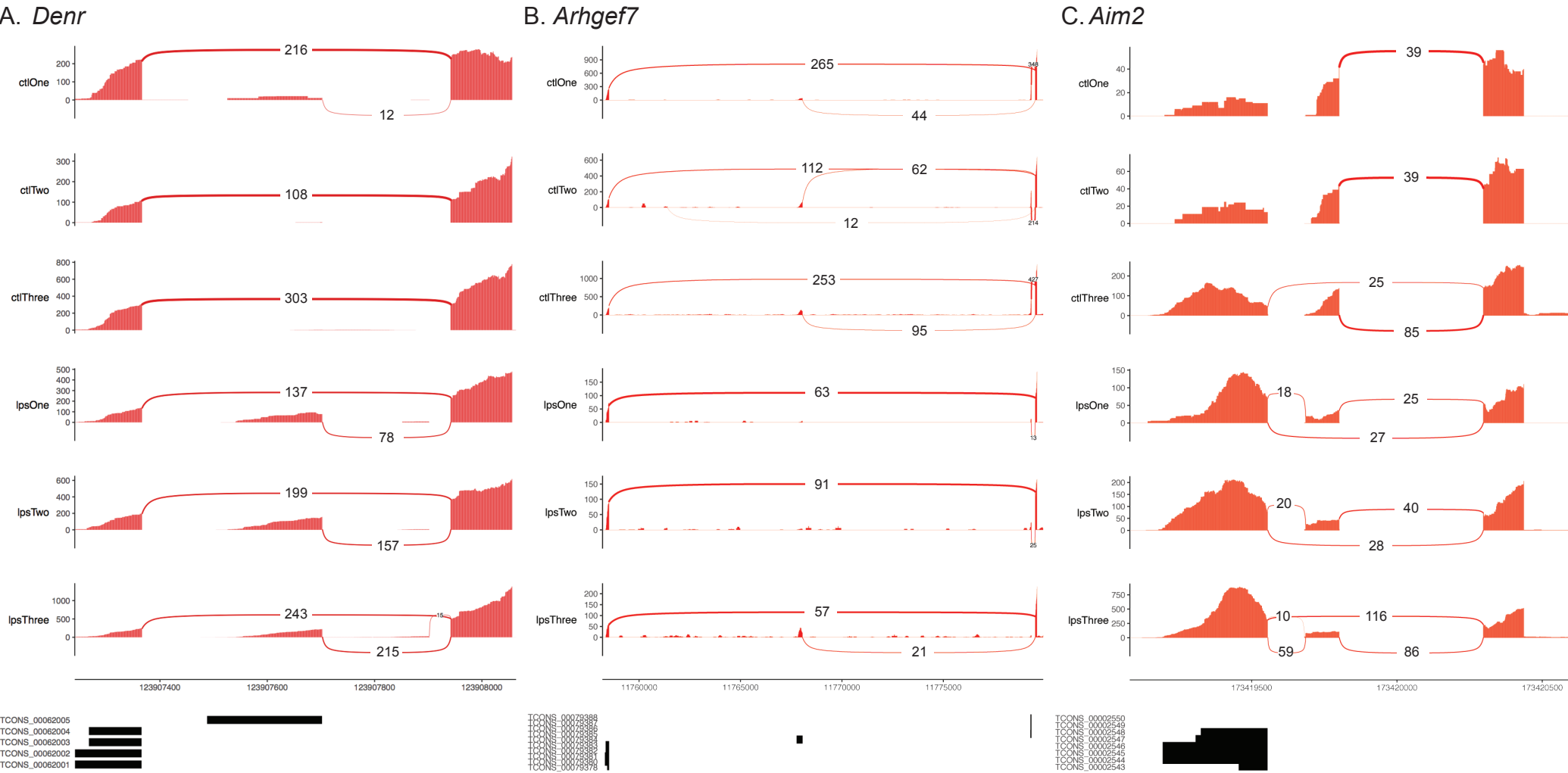
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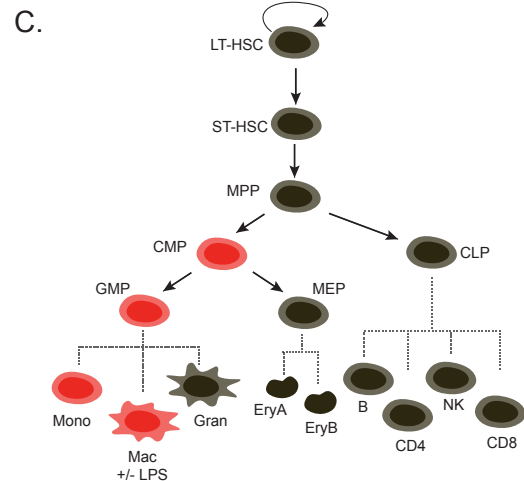
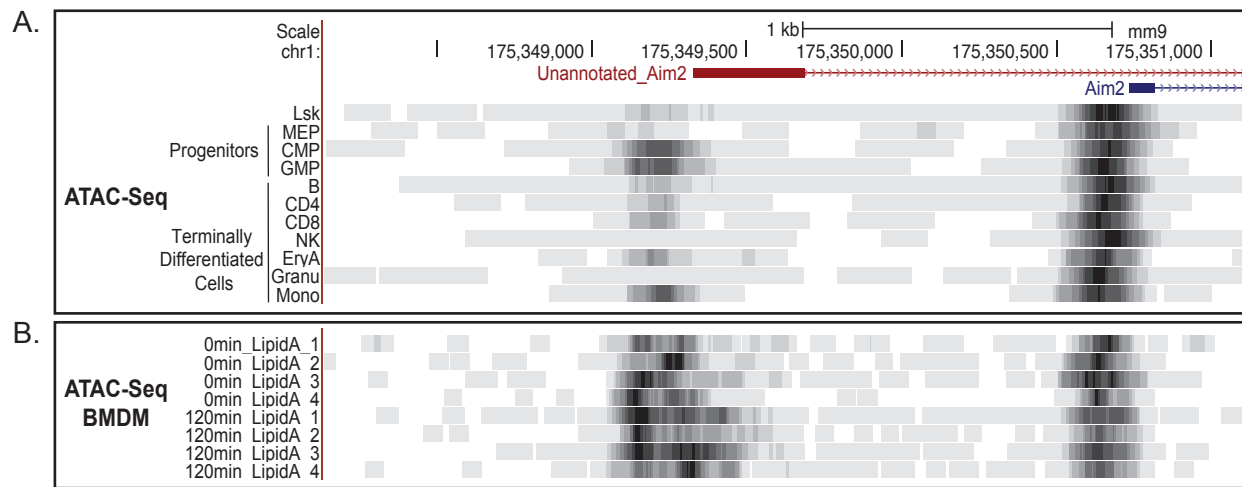


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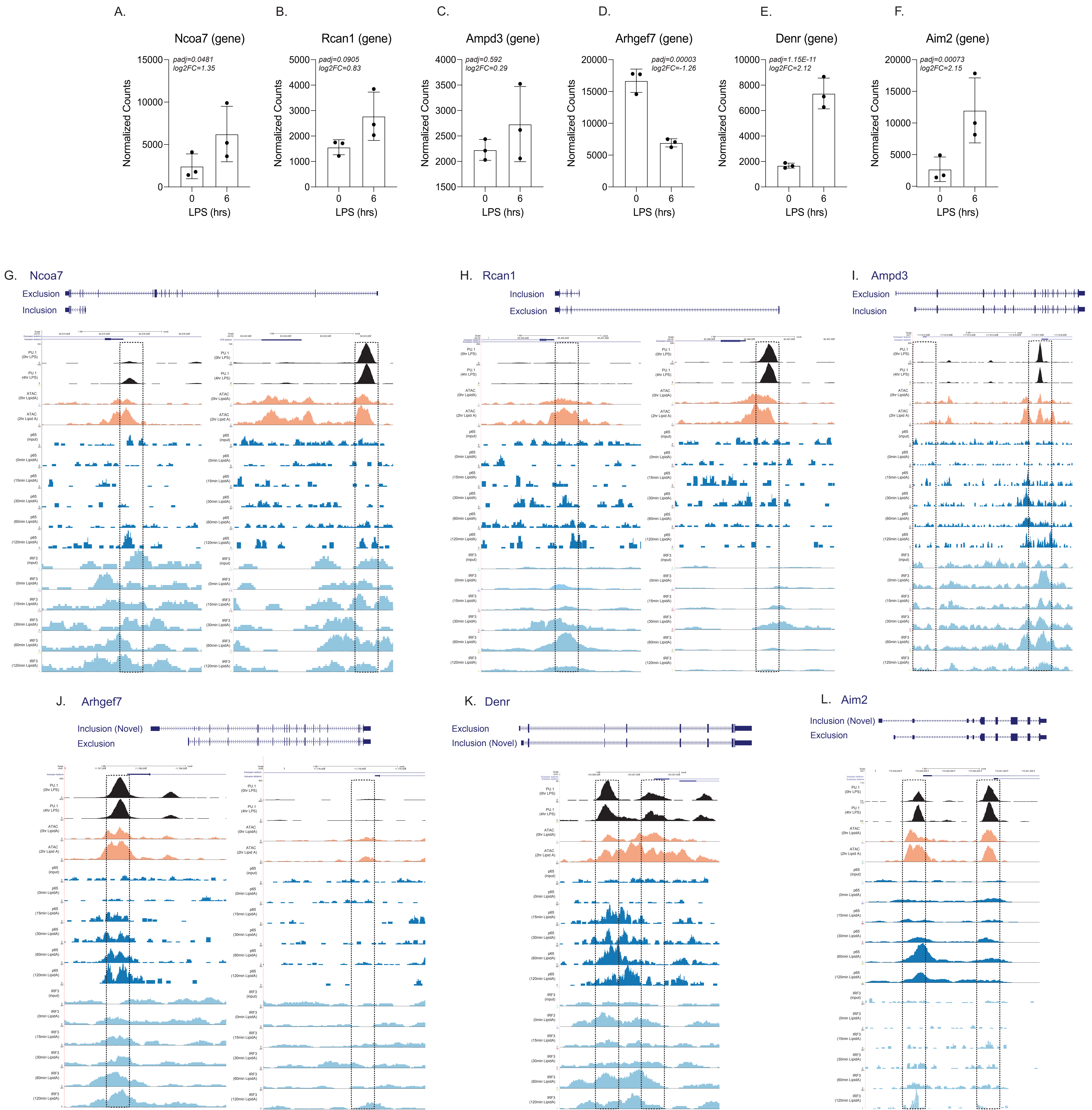


Supplemental Fig 8: CAGE Scores Support Validity of Novel TSS identified by Nanopore Sequencing. CAGE scores of CAGE peaks overlapping the 5'UTR region of (A) annotated and (B) novel transcript isoforms. (C) Bar graph of TSS peaks overlapping and not overlapping with CAGE data.

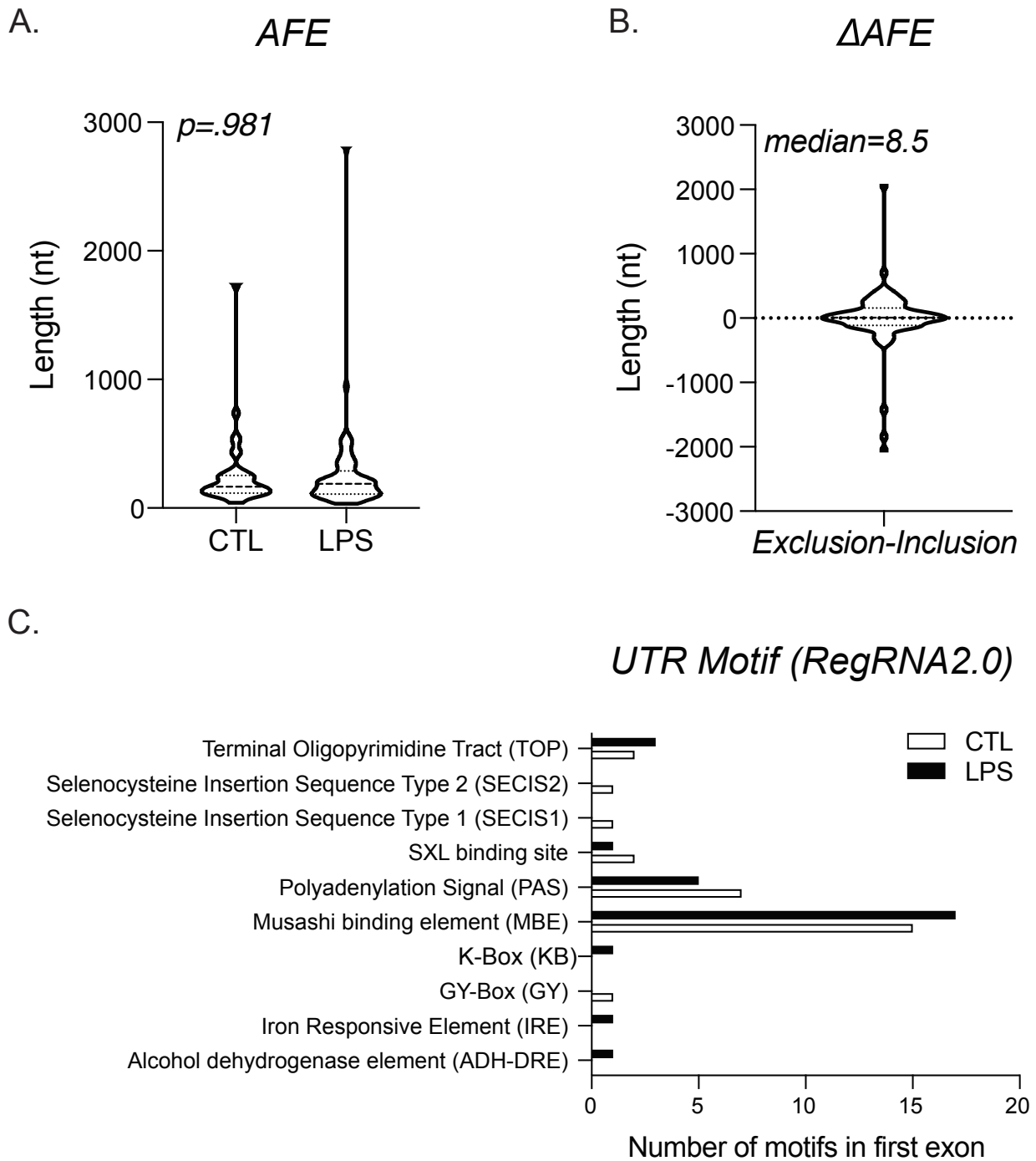




Supplemental Fig 10: Chromatin remodeling not a mechanism driving novel Aim2 isoform. (A) mm9 genome browser shot between chr1:175,348,283-175,351,422 indicating ATAC-seq of cells from hematopoietic tree. (B) mm9 genome browser shot between chr1:175,348,283-175,351,422 indicating ATAC-seq of BMDMs +/- Lipid A for 2 hrs. (C) Schematic summarizing promoter accessibility of novel Aim2 isoform, red=accessible and black=not accessible.

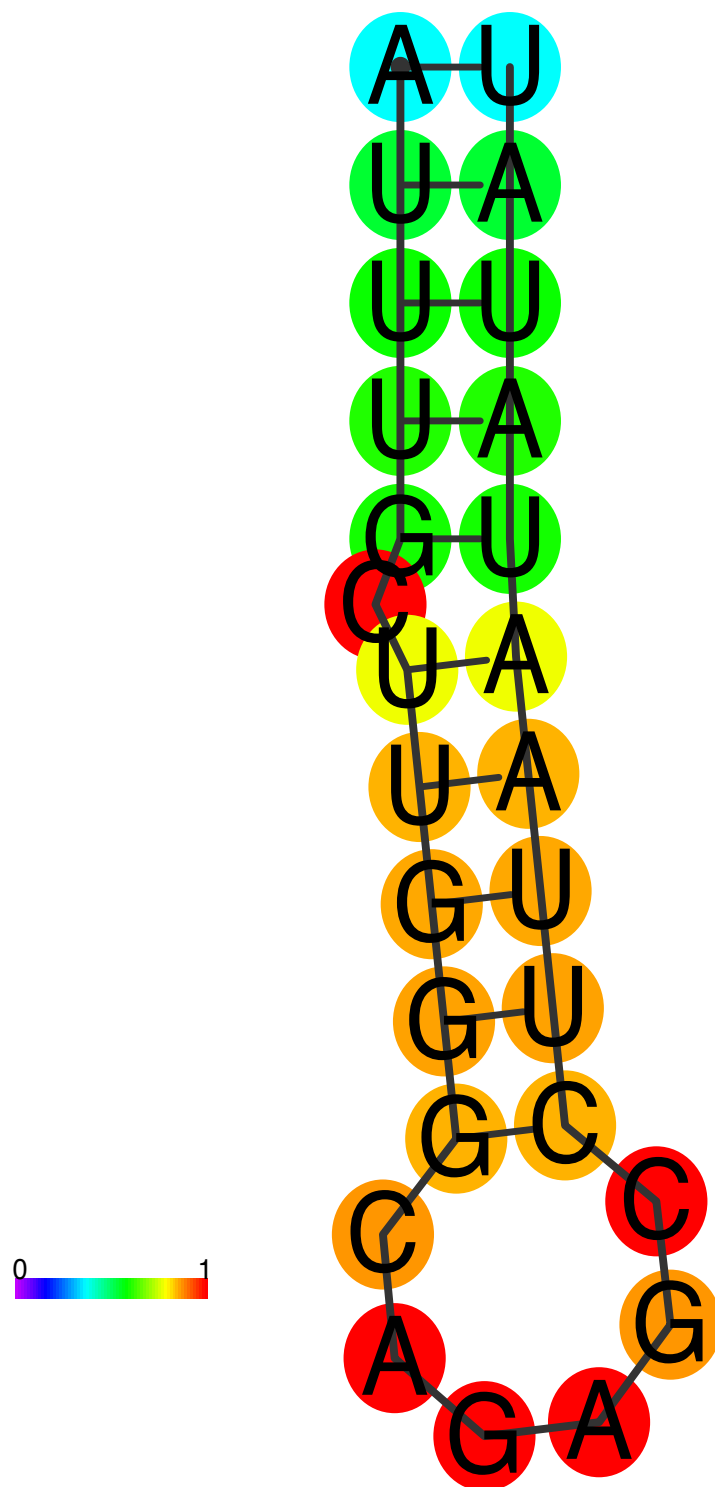


Supplemental Fig 11: Genome Browser images of ChIP-seq and ATAC-seq for both the promoters of CTL and LPS specific AFE isoforms. The normalized counts and DESeq2 analysis of of Ncoa7 (A), Rcan1 (B), Ampd3 (C), Arhgef7 (D), Denr (E) and Aim2 (F) are represented in a bar graph in triplicate. Genome browser images are taken for the CTL and LPS promoters highlighted in yellow of Ncoa7 (G), Rcan1 (H), Ampd3 (I), Arhgef7 (J), Denr (K), and Aim2 (L). All tracks are next-generation sequencing data from BMDMs. The top track (in black) is ChIP-sequencing data of PU.1 from unstimulated cells, while the second track is PU.1 ChIP-seq data from LPS stimulated cells (GSE109965). The next two tracks (in orange) are of ATAC sequencing data from either unstimulated or LPS stimulated cells (GSE74191). The next five tracks (dark blue) are p65 ChIP-sequencing data from the input, 0min KLA, 15min KLA, 30min KLA, 60min KLA or 120min KLA stimulation (GSE67343). Finally the last five tracks (in light blue) are IRF3 ChIP-sequencing data from the input, 0min KLA, 15min KLA, 30min KLA, 60min KLA or 120min KLA stimulation (GSE67343).

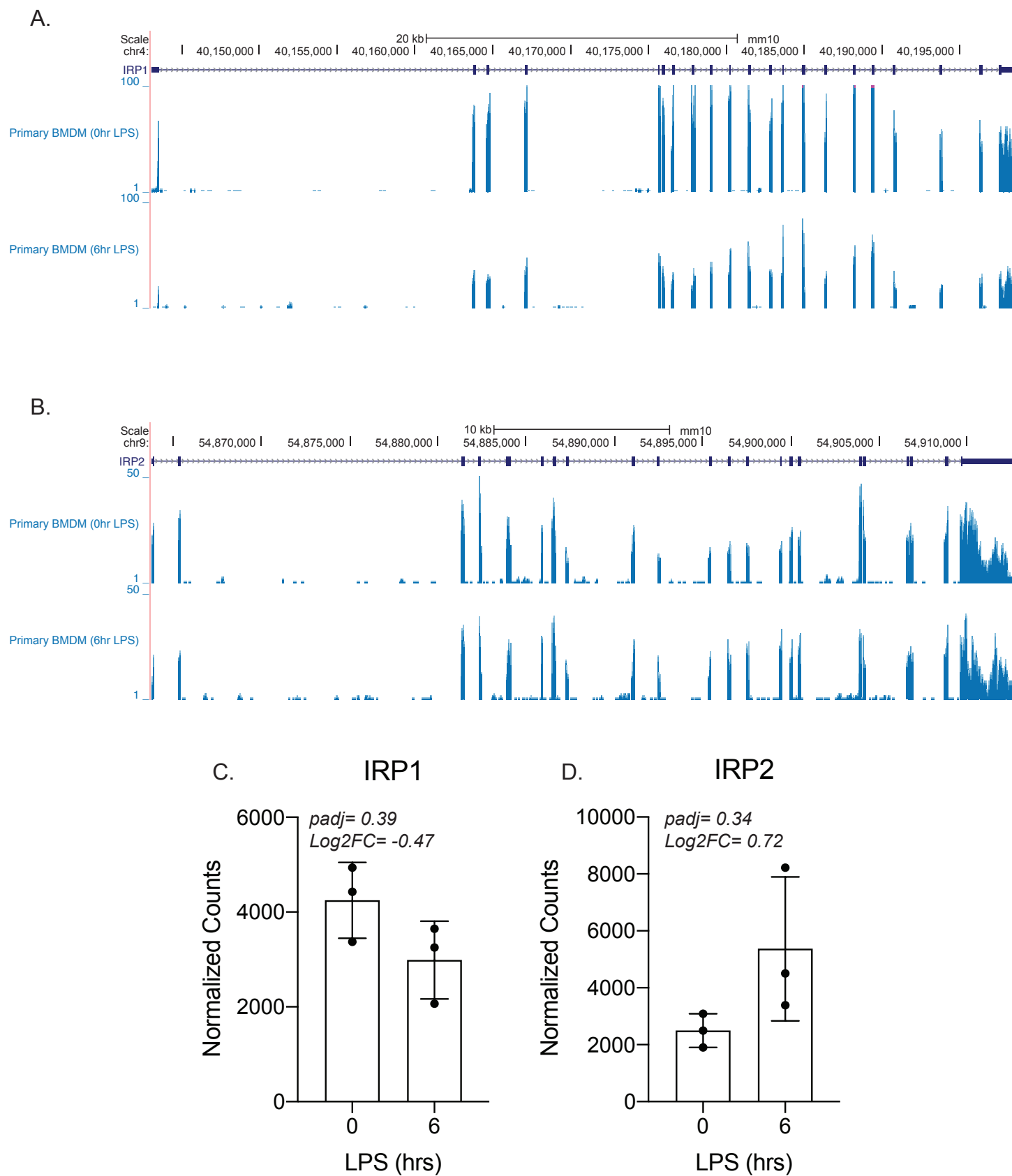


Supplemental Fig 12: Global characterization of AFE. (A) Length distribution of first exon from exclusion (CTL) exon and inclusion (LPS) exon. (B) Graph of the length difference between the exclusion and inclusion exons, also known as ΔAFE . (C) All potential UTR motifs were identified using RegRNA2.0 for either the exclusion (CTL) or inclusion (LPS) specific first exons.

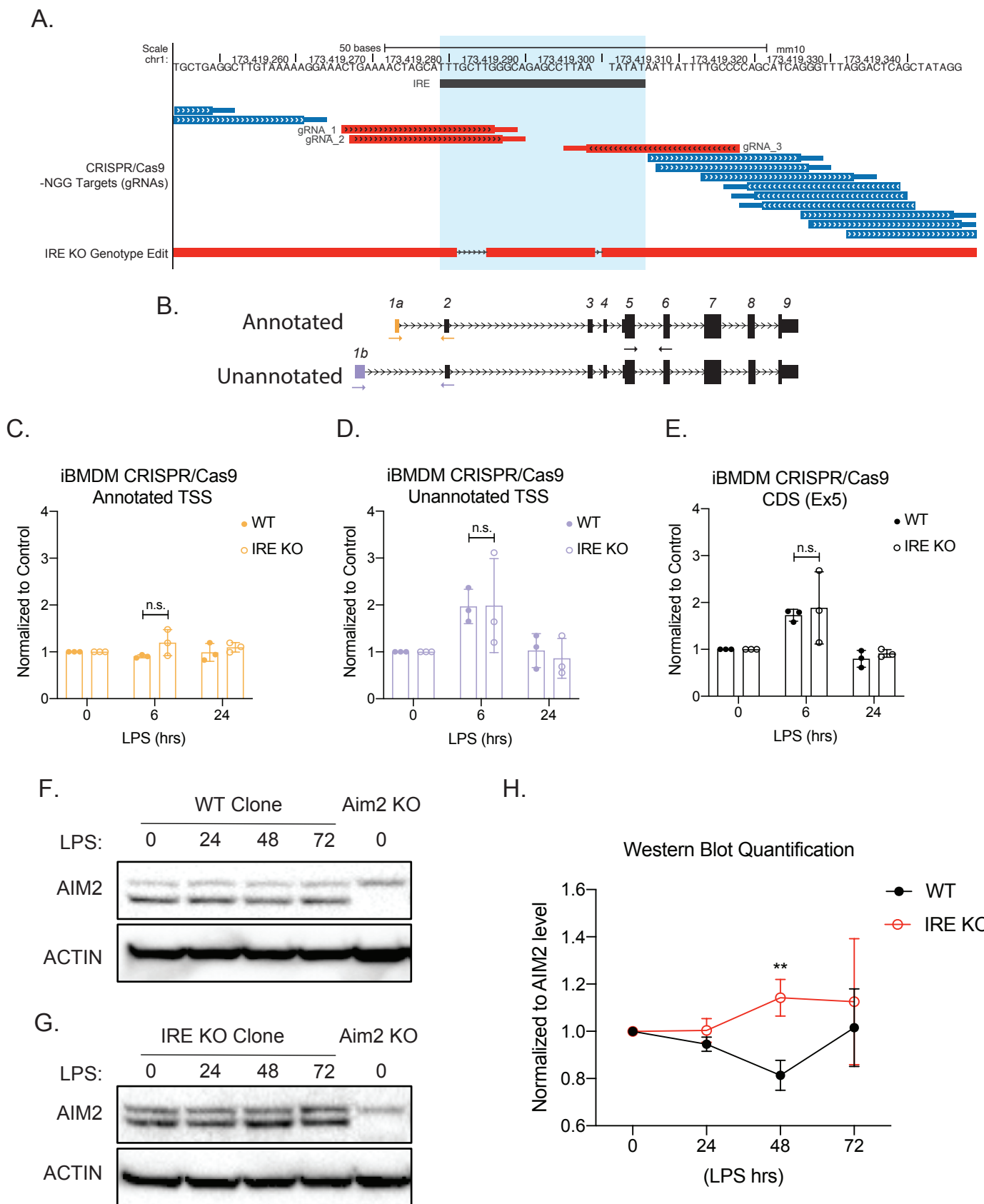
Mouse IRE sequence
Vienna RNAFold



Supplemental Fig 13: Aim2 IRE hairpin structure predicted. Using the Vienna RNAFold package, the IRE motif is folded into a hairpin, with strong base-pairing.



Supplemental Fig 14: IRP1 and IRP2 are not differentially expressed by LPS in bone marrow derived macrophages. Genome browser tracks showing the transcript of either (A) IRP1 or (B) IRP2, followed by two RNA-sequencing tracks from primary BMDMs +/- LPS for 6hrs. The normalized counts and DESeq2 analysis of IRP1 (C) and IRP2 (D) are represented in a bar graph in triplicate.



Supplemental Fig 15: CRISPR/Cas9 knock-out of IRE in Aim2 5'UTR leads to an increase of protein expression in mouse macrophages during inflammation. (A) Genome browser shot. Top track indicates chromosome position. Second track shows the location of the Aim2 IRE, highlighted in light blue. The third track shows all the possible gRNAs found using the CRISPR/Cas9 -NGG Target track and the chosen gRNAs indicated in red. The last track shows the specific edits in the IRE KO BMDM clonal cell line using the BLAT tool. (C-E) qRT-PCR was performed in biological triplicate, on WT or IRE KO immortalized CRISPR/Cas9 BMDM RNA extracts that had been stimulated with LPS for indicated time points. (F) WT or (G) IRE KO Protein lysates of time course LPS stimulation of 0hr, 24hr, 48hr and 72hr. Western blot performed on AIM2 and B-ACTIN. (H) Western blot quantification performed in FIJI, standard deviation represents biological triplicates, p-value assessed using student's t-test.

Aim2 Ab

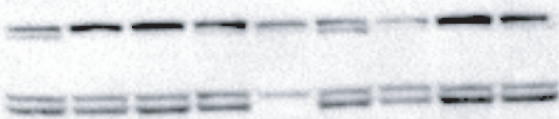
Aim2

IRE KO Clone

KO

0 24 48 72 0

Data not for paper



Actin Ab

IRE KO Clone

Aim2
KO

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Data not for paper



Aim2 Ab

Aim2

IRE KO Clone

KO

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Actin Ab

IRE KO Clone

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Aim2 Ab

Aim2

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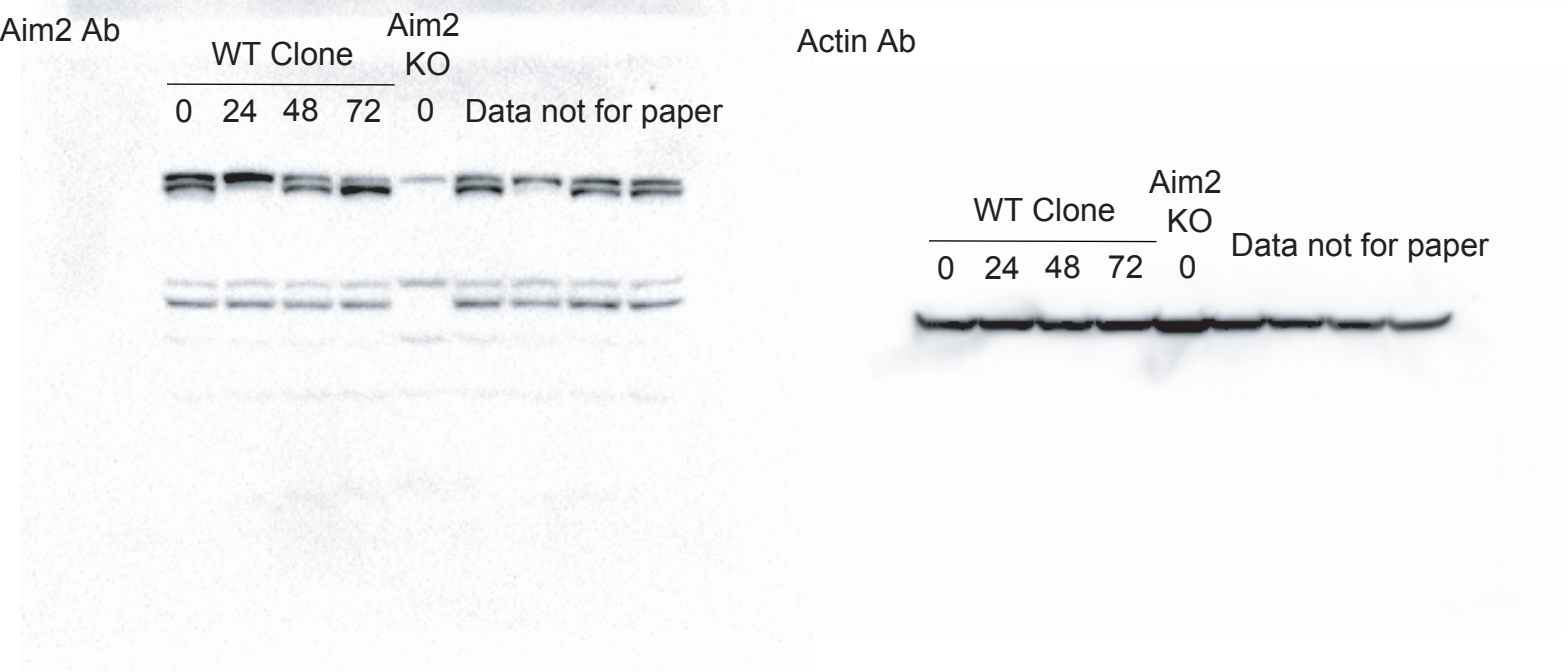
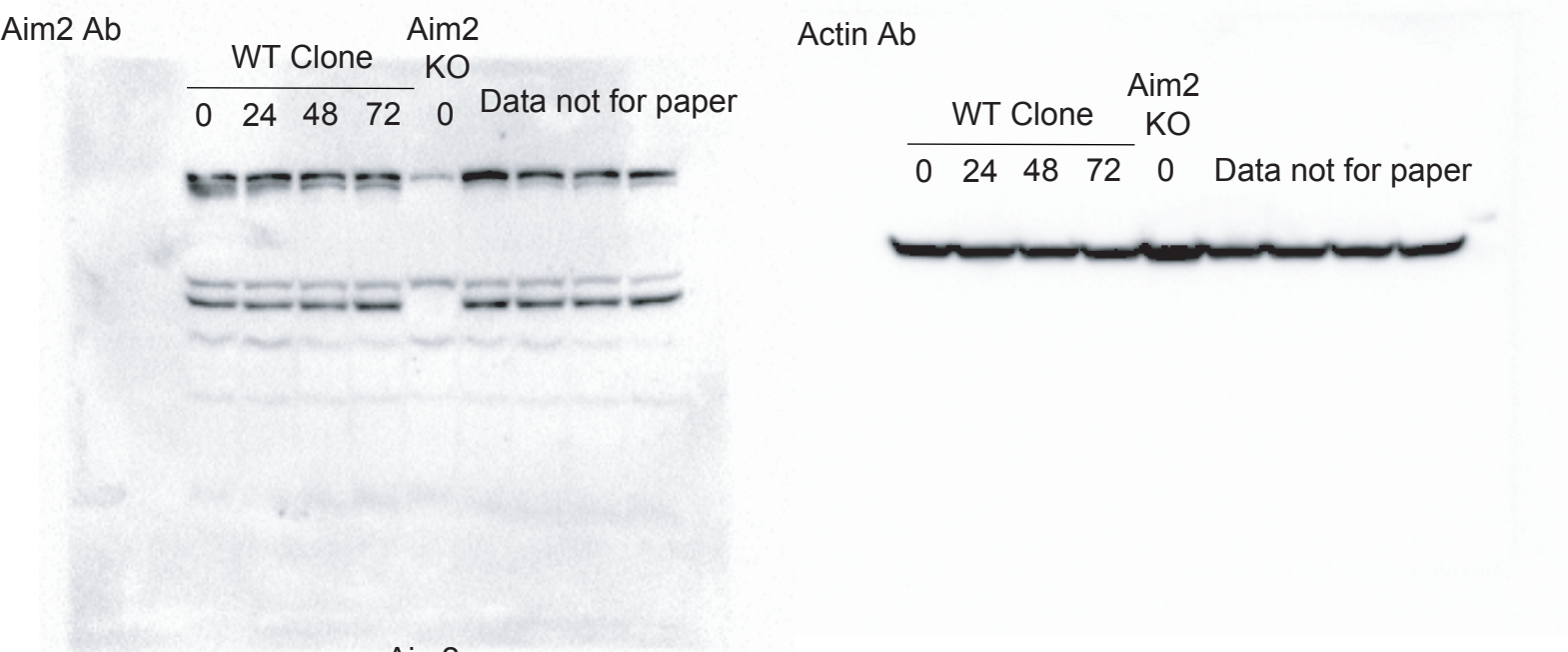
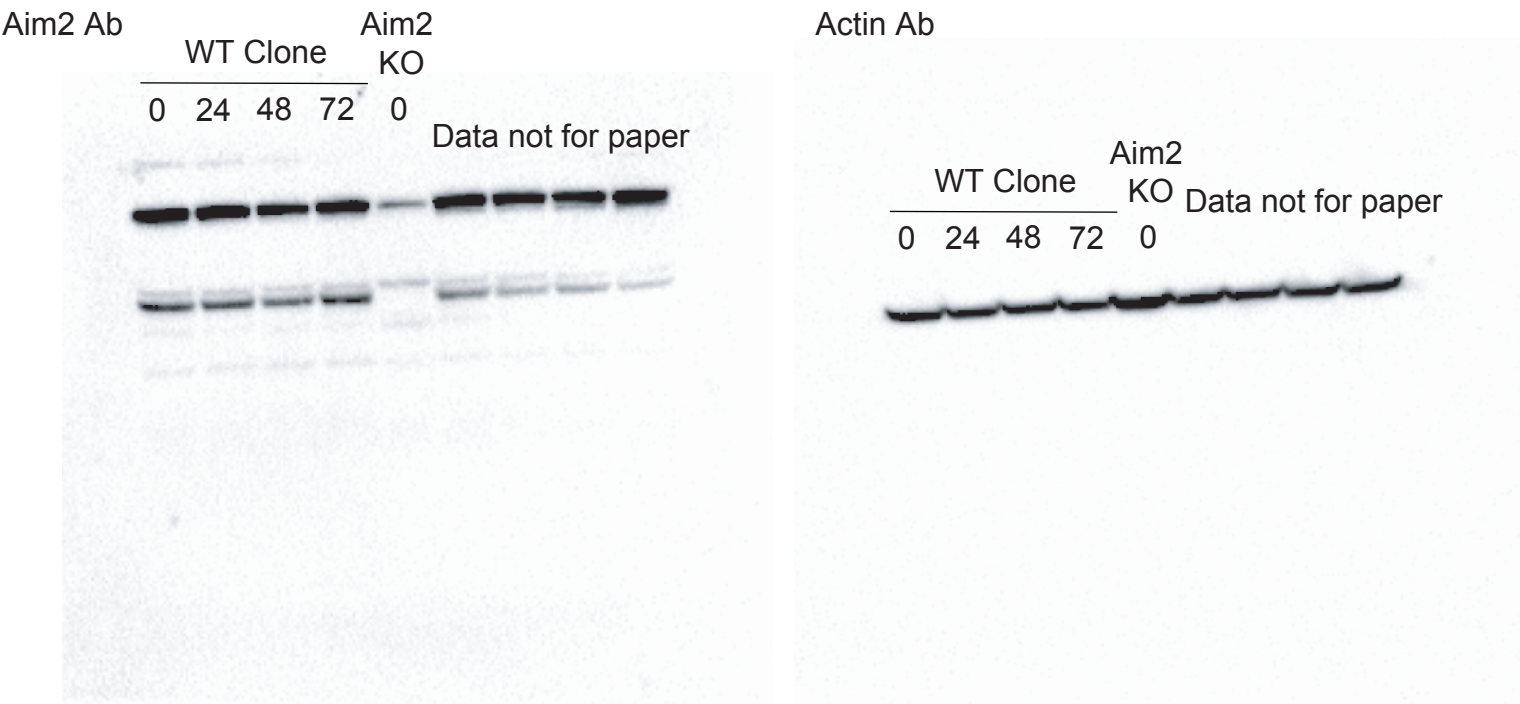
IRE KO Clone

Aim2
KO

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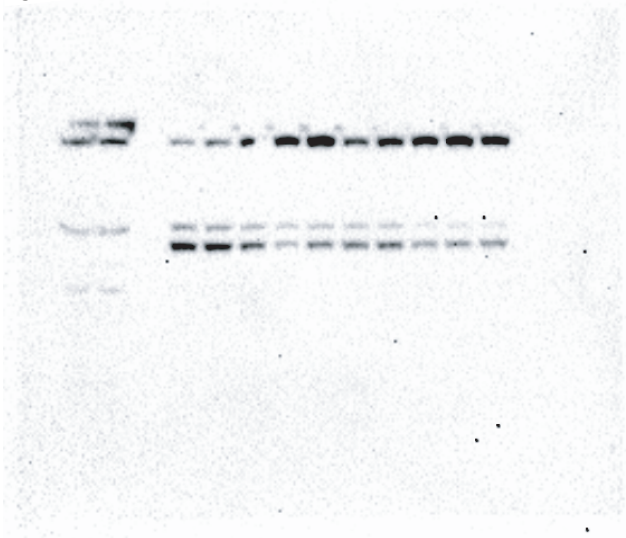
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Aim2 Ab

	KO		WT									
FAC:	-	-	-	-	-	-	-	+	+	+	+	+
LPS:	0	24	0	6	24	48	72	0	6	24	48	72



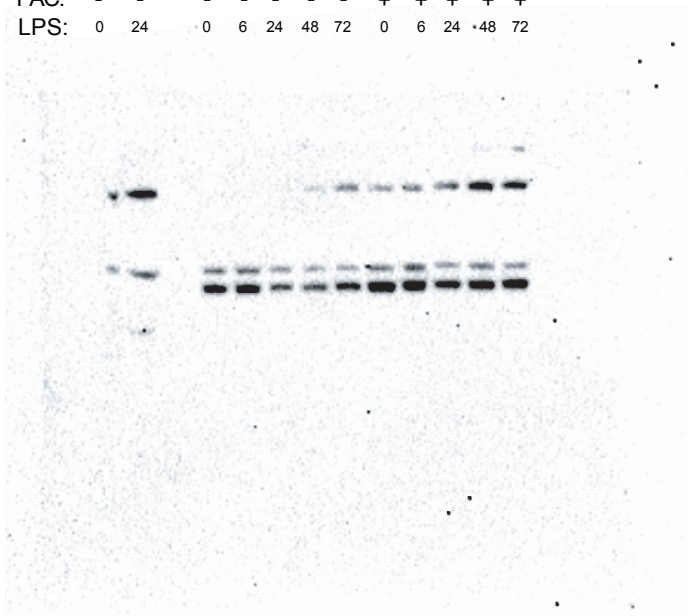
Actin Ab

	KO		WT									
FAC:	-	-	-	-	-	-	-	+	+	+	+	+
LPS:	0	24	0	6	24	48	72	0	6	24	48	72



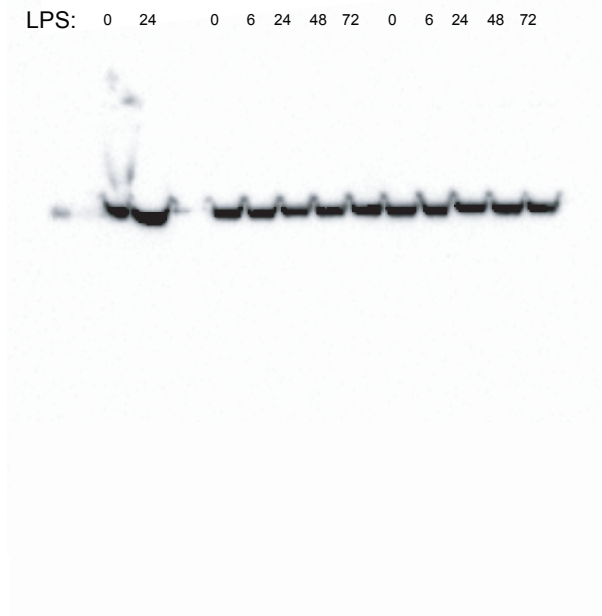
Aim2 Ab

	KO		WT									
FAC:	-	-	-	-	-	-	-	+	+	+	+	+
LPS:	0	24	0	6	24	48	72	0	6	24	48	72



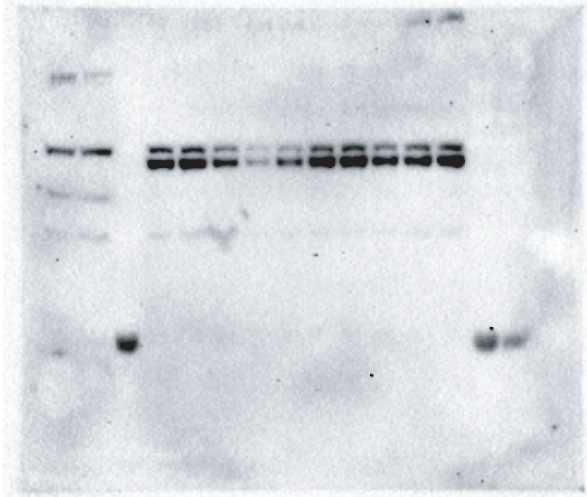
Actin Ab

	KO		WT									
FAC:	-	-	-	-	-	-	-	+	+	+	+	+
LPS:	0	24	0	6	24	48	72	0	6	24	48	72



Aim2 Ab

	KO		WT									
FAC:	-	-	-	-	-	-	-	+	+	+	+	+
LPS:	0	24	0	6	24	48	72	0	6	24	48	72



Actin Ab

	KO		WT									
FAC:	-	-	-	-	-	-	-	+	+	+	+	+
LPS:	0	24	0	6	24	48	72	0	6	24	48	72



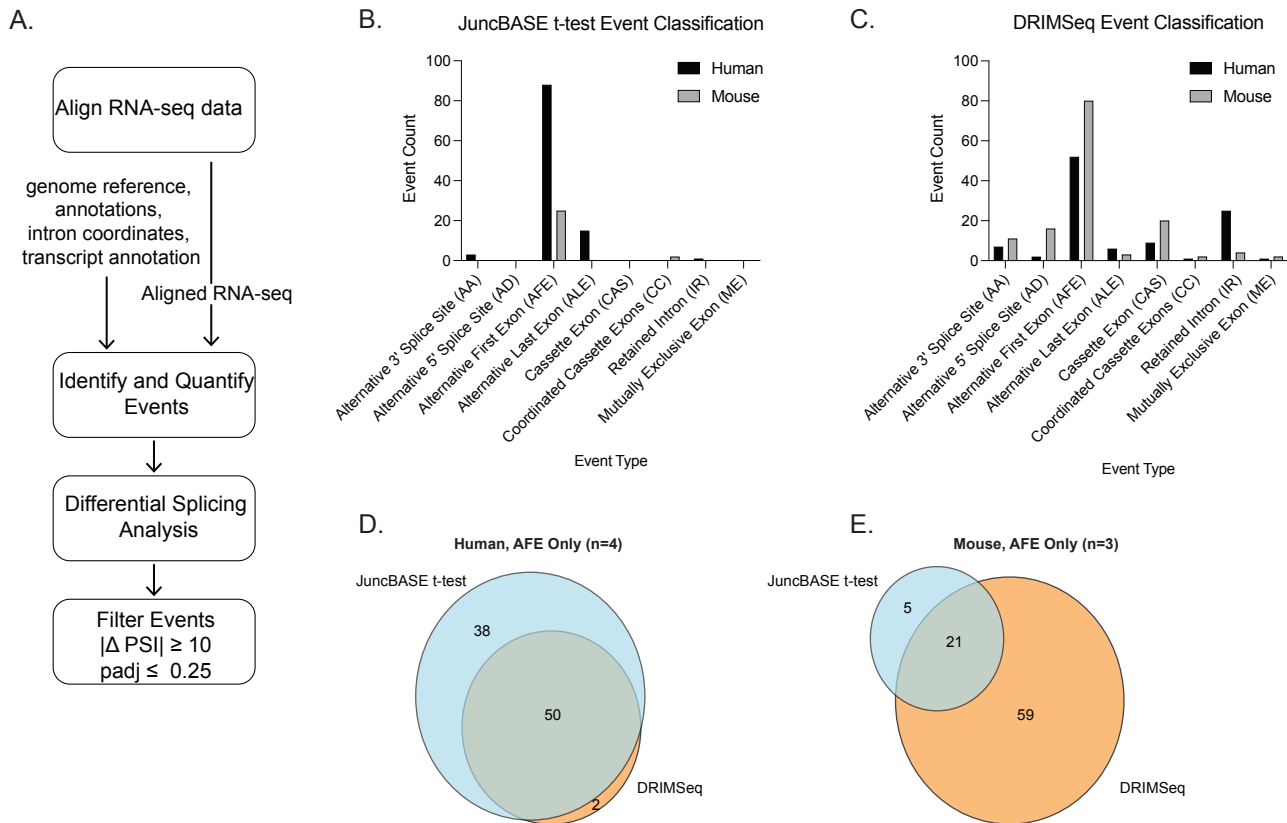


Figure 1 - Supplemental Figure 1: Computational Pipeline and comparison of t-test and DRIMSeq alternative splicing events. (A) Bioinformatic pipeline for Human and Mouse RNA-seq data. Alternative splicing event type classification of significant differential splicing events ($|\Delta\text{PSI}| \geq 10$ and corrected $p\text{-value} \leq 0.25$) in Human and Mouse macrophages +/- LPS as identified and quantified using the (B) t-test with JuncBASE or (C) the Dirichlet-multinomial framework applied by DRIMSeq. Venn Diagrams representing the unique and overlapping AFE events from (D) Human or (E) Mouse macrophage alternative splicing analysis.

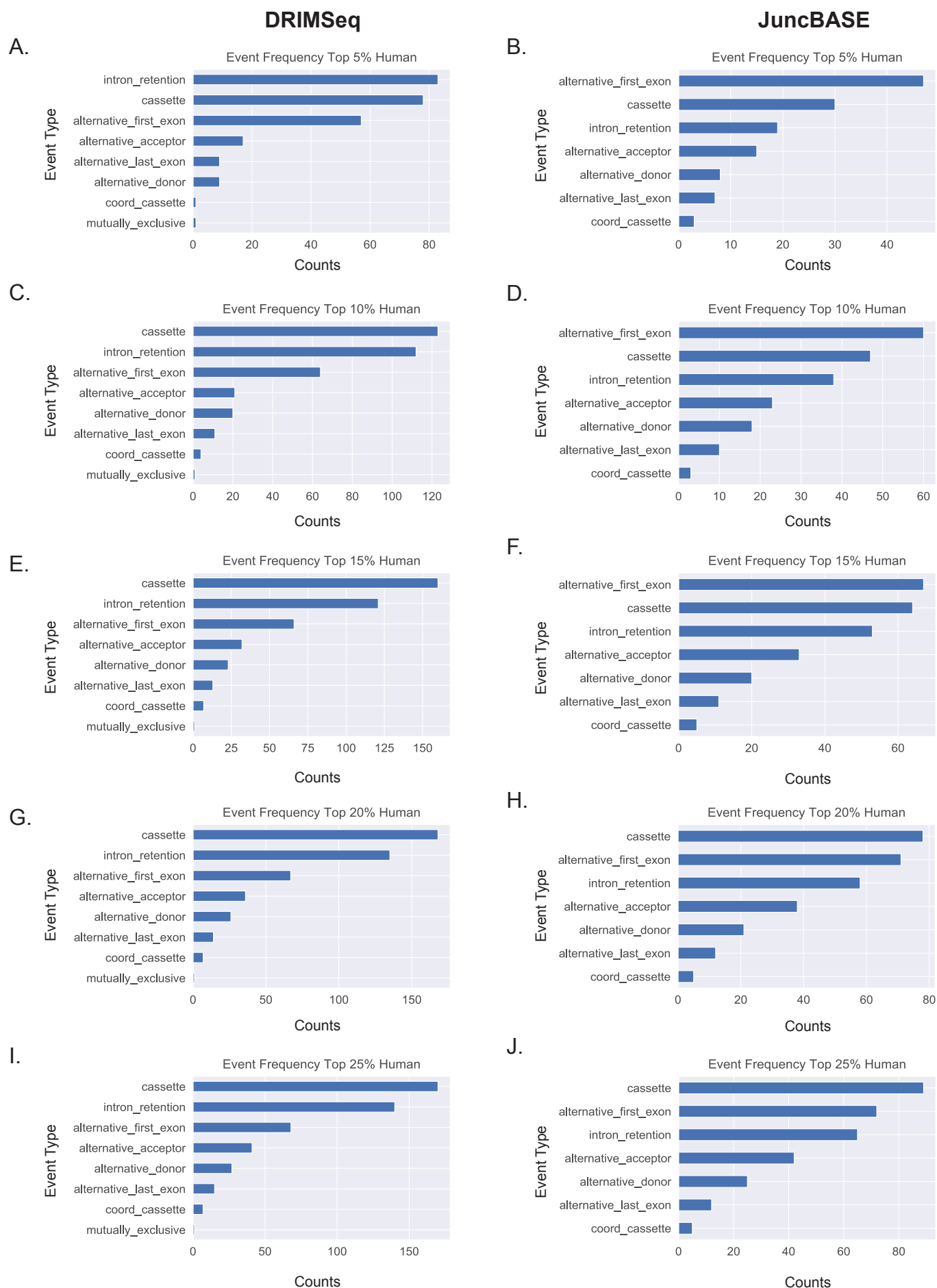


Figure 1 - Supplemental Figure 2: AFE events remain prevalent amongst ranked alternative splicing events in human macrophages. (A-B) Ranking of the top 5%, (C-D) 10%, (E-F) 15%, (G-H) 20%, (I-J) 25% of alternative splicing events based on p-value of events as classified by JuncBASE and DRIMSeq in human macrophage cells following LPS stimulation.

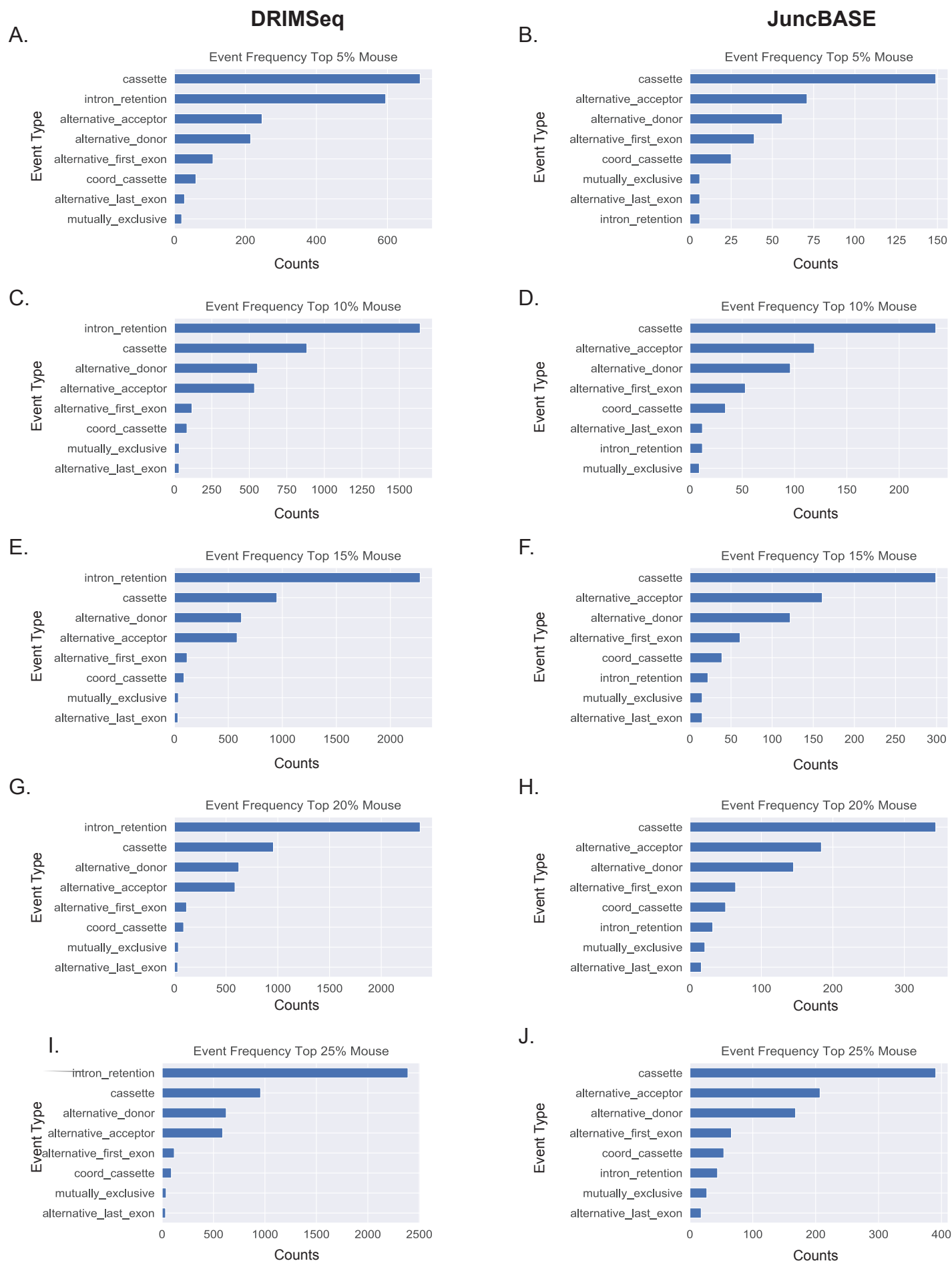


Figure 1 - Supplemental Figure 3: AFE events remain prevalent amongst ranked alternative splicing events in mouse macrophages. (A-B) Ranking of the top 5%, (C-D) 10%, (E-F) 15%, (G-H) 20%, (I-J) 25% of alternative splicing events based on p-value of events as classified by JuncBASE and DRIMSeq in human macrophage cells following LPS stimulation.

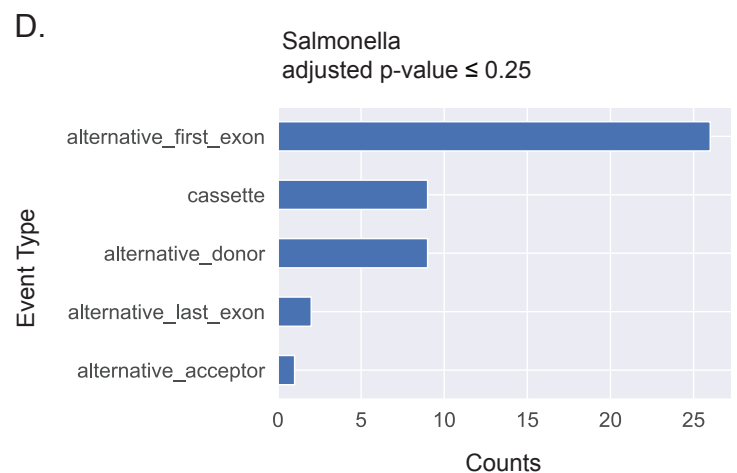
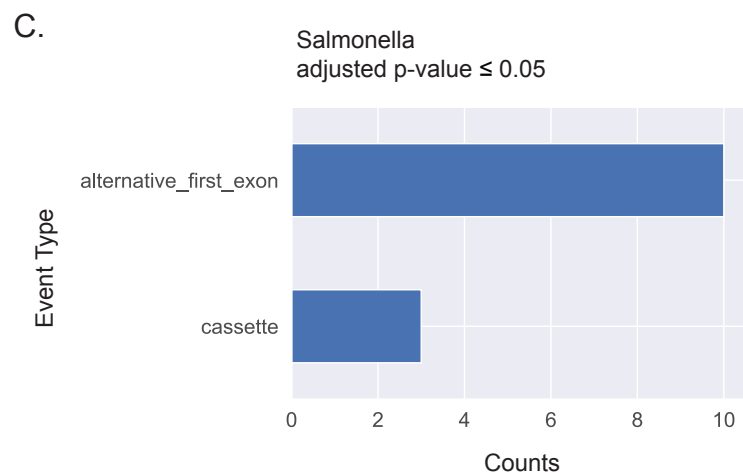
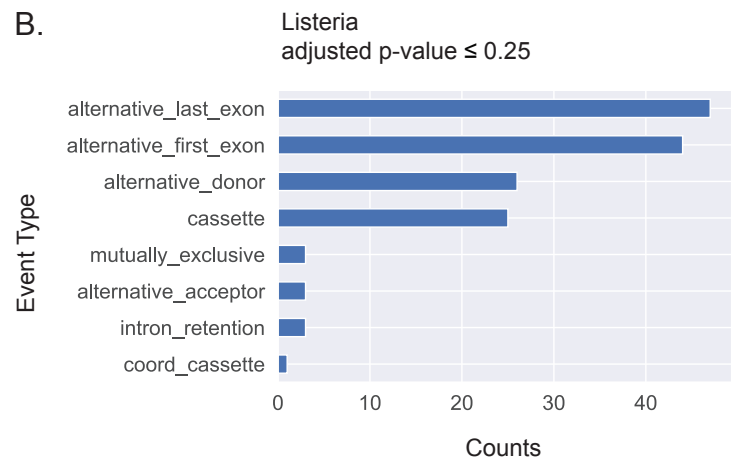
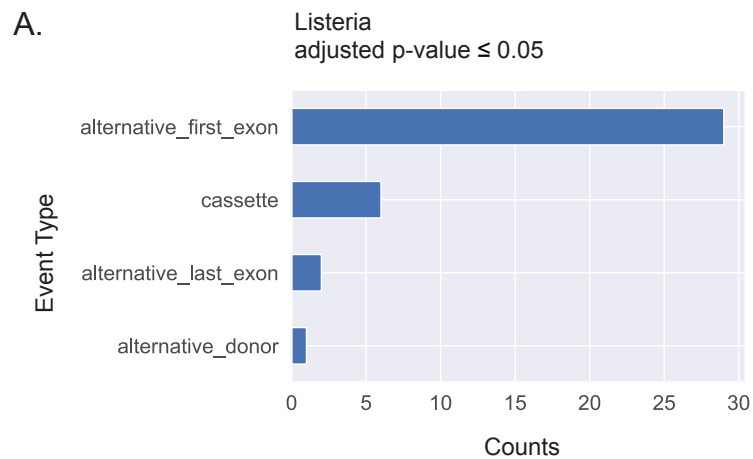


Figure 1 - Supplemental Figure 4: Alternative First Exon usage is the top splicing event when using JuncBASE to identify and quantify alternative splicing events from primary human macrophages stimulated with *Listeria* and *Salmonella* for 24hrs. Significant alternative splicing events identified and categorized by event type using an adjusted p-value cutoff ≤ 0.05 (A) and a cutoff of ≤ 0.25 (B) following *Listeria* infection. Significant alternative splicing events identified and categorized by event type using an adjusted p-value cutoff of ≤ 0.05 (C) and a cutoff of ≤ 0.25 (D) following *Salmonella* infection. Data acquired from GSE73502.

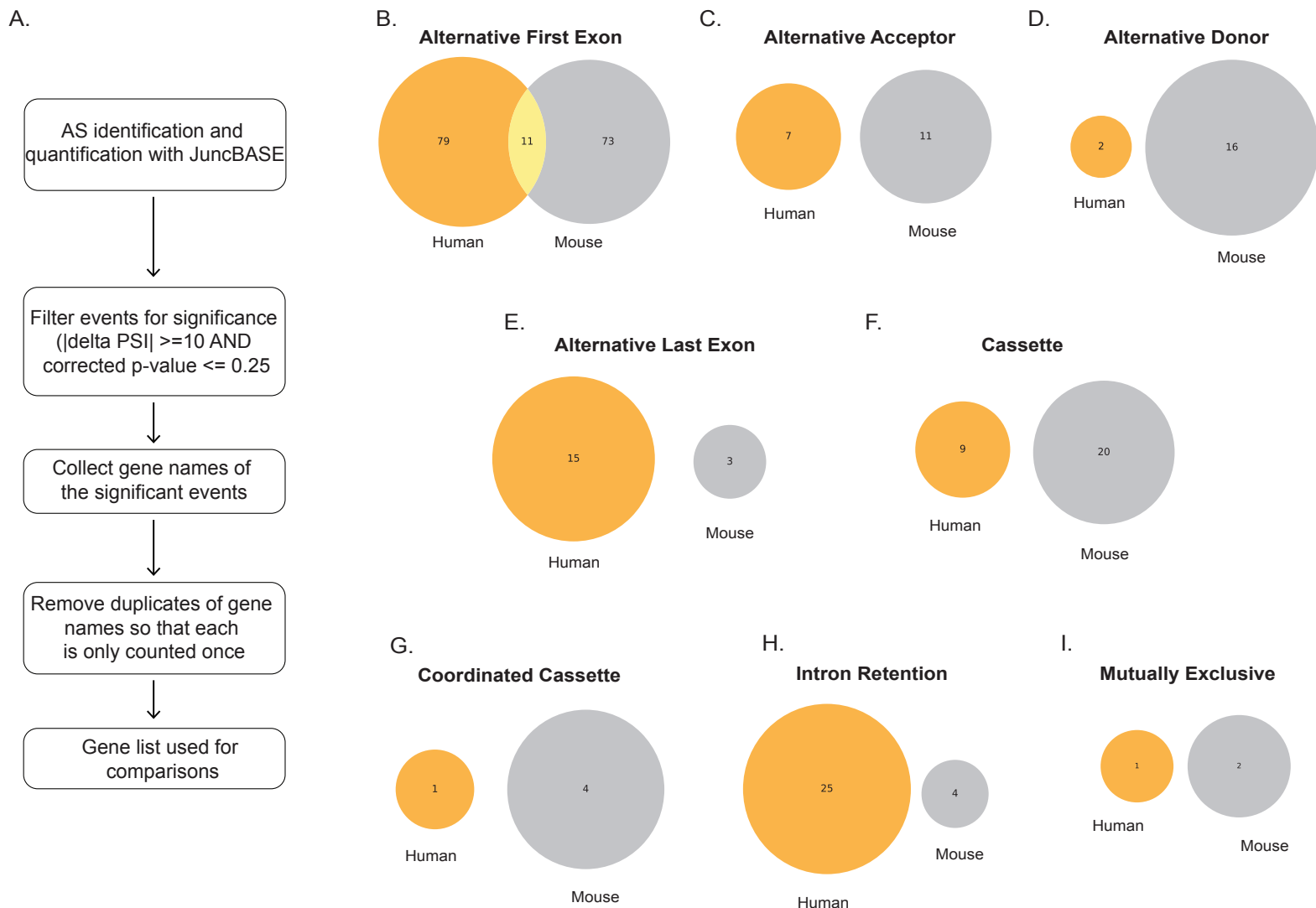


Figure 1 - Supplemental Figure 5: Conserved Genes identified through alternative splicing events between human and mouse using gencode transcriptome. (A) Schematic depicting how gene lists were generated from exon events. Venn digrams denoting unique and overlapping genes with significant splicing events in human (orange) and mouse (grey) for (B) Alternative First Exon, (C) Alternative Acceptor, (D) Alternative Donor, (E) Alternative Last Exon, (F) Casette, (G) Coordinated Casette, (H) Intron Retention and (I) Mutually Exclusive all have conserved splicing events when analyzing LPS stimulated primary macrophage.

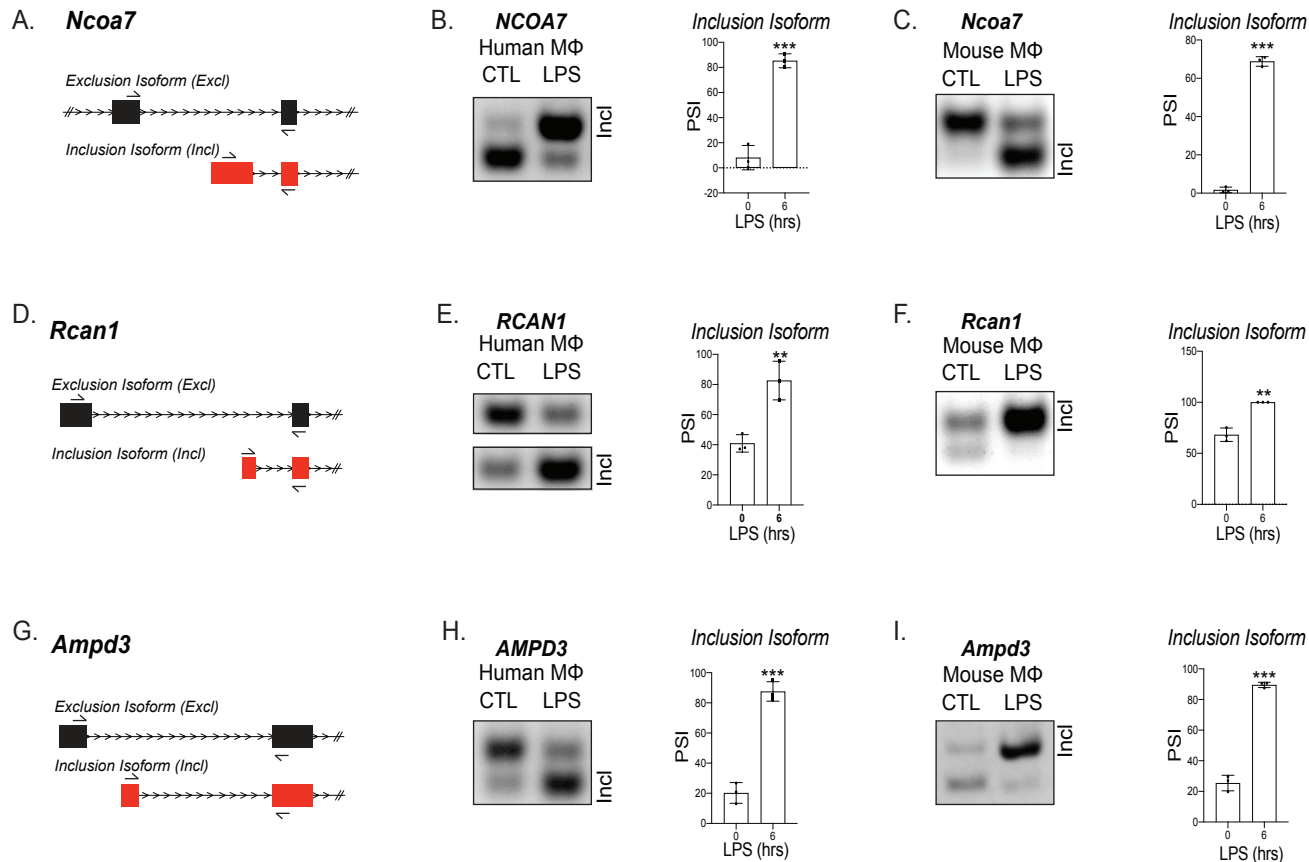


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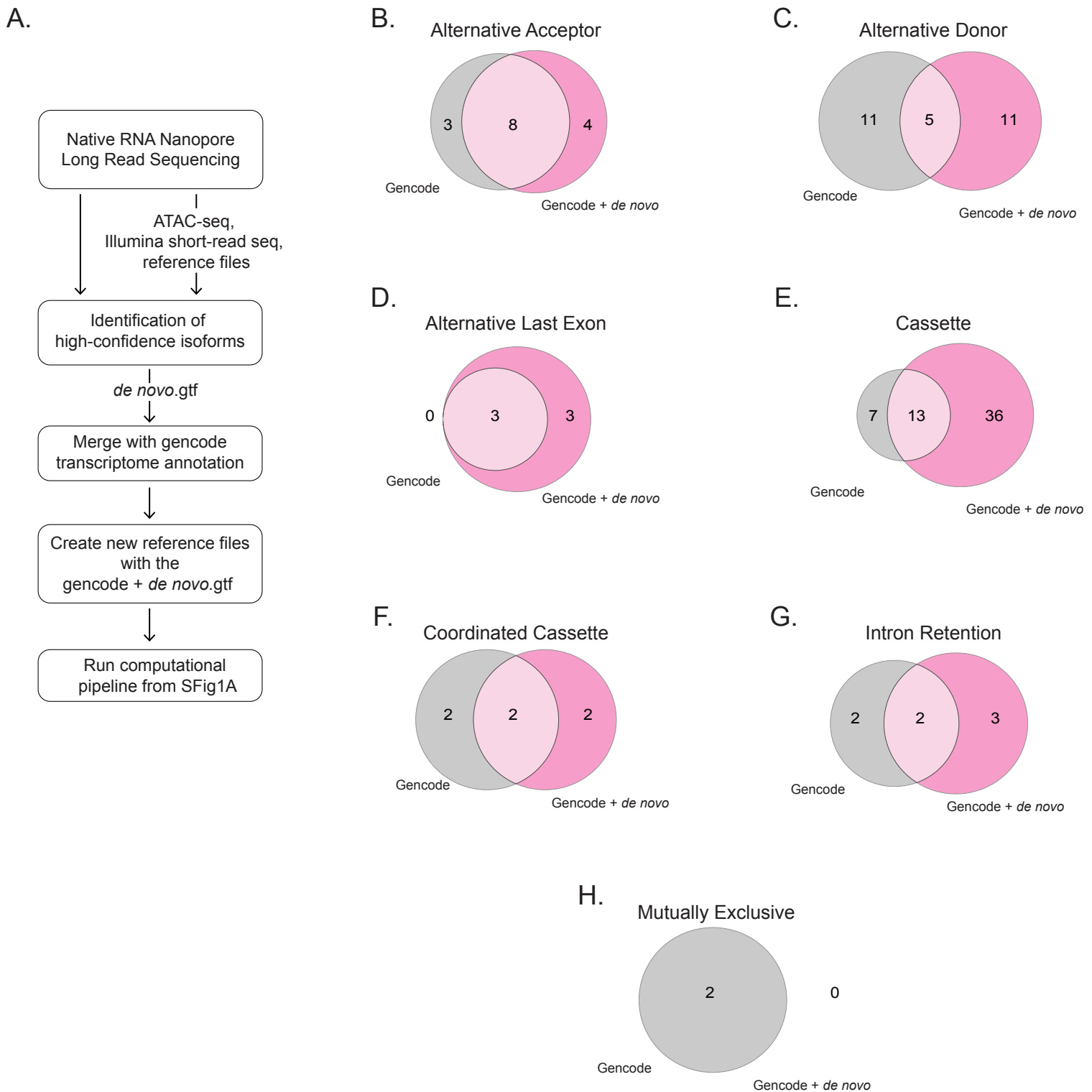


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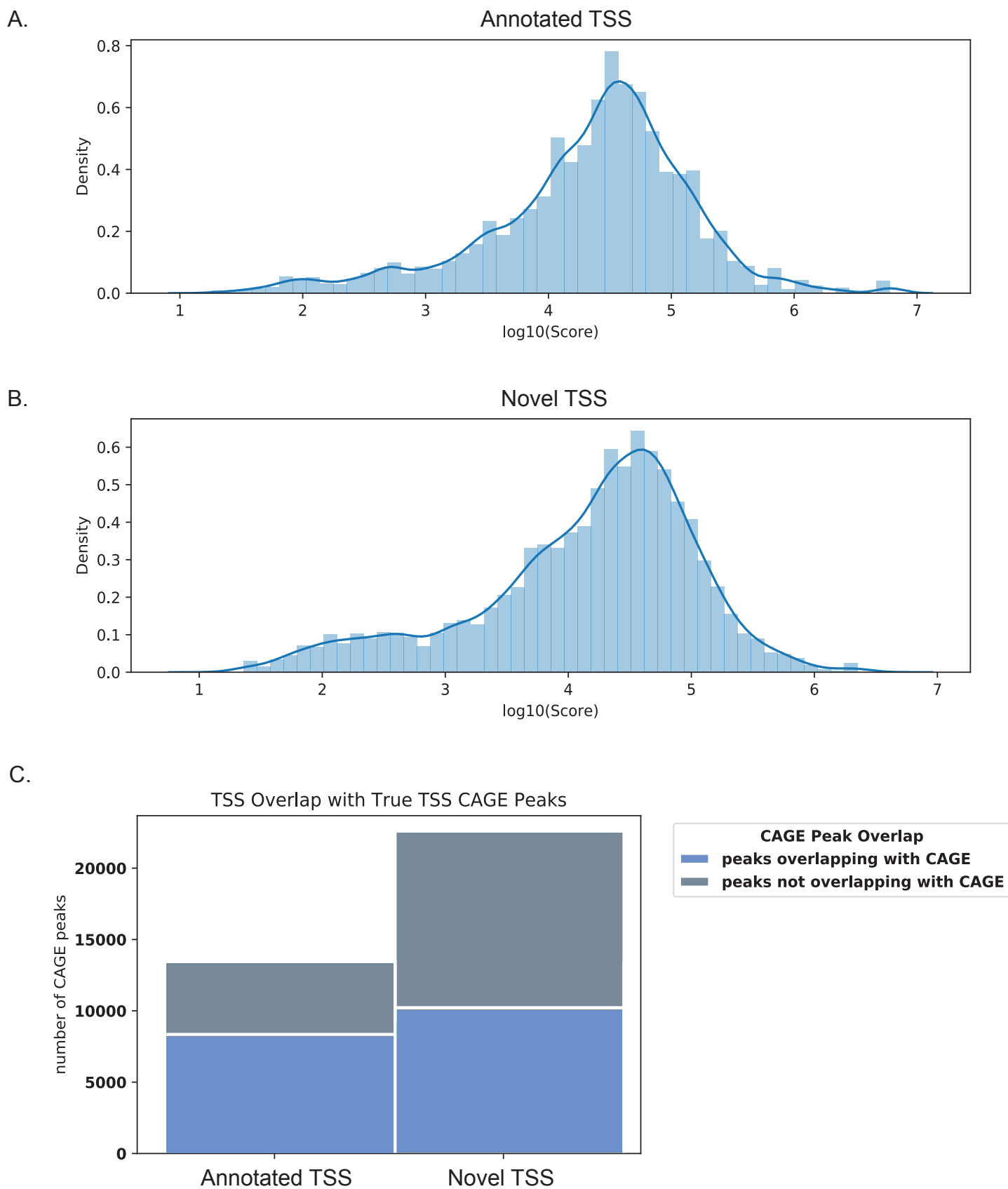


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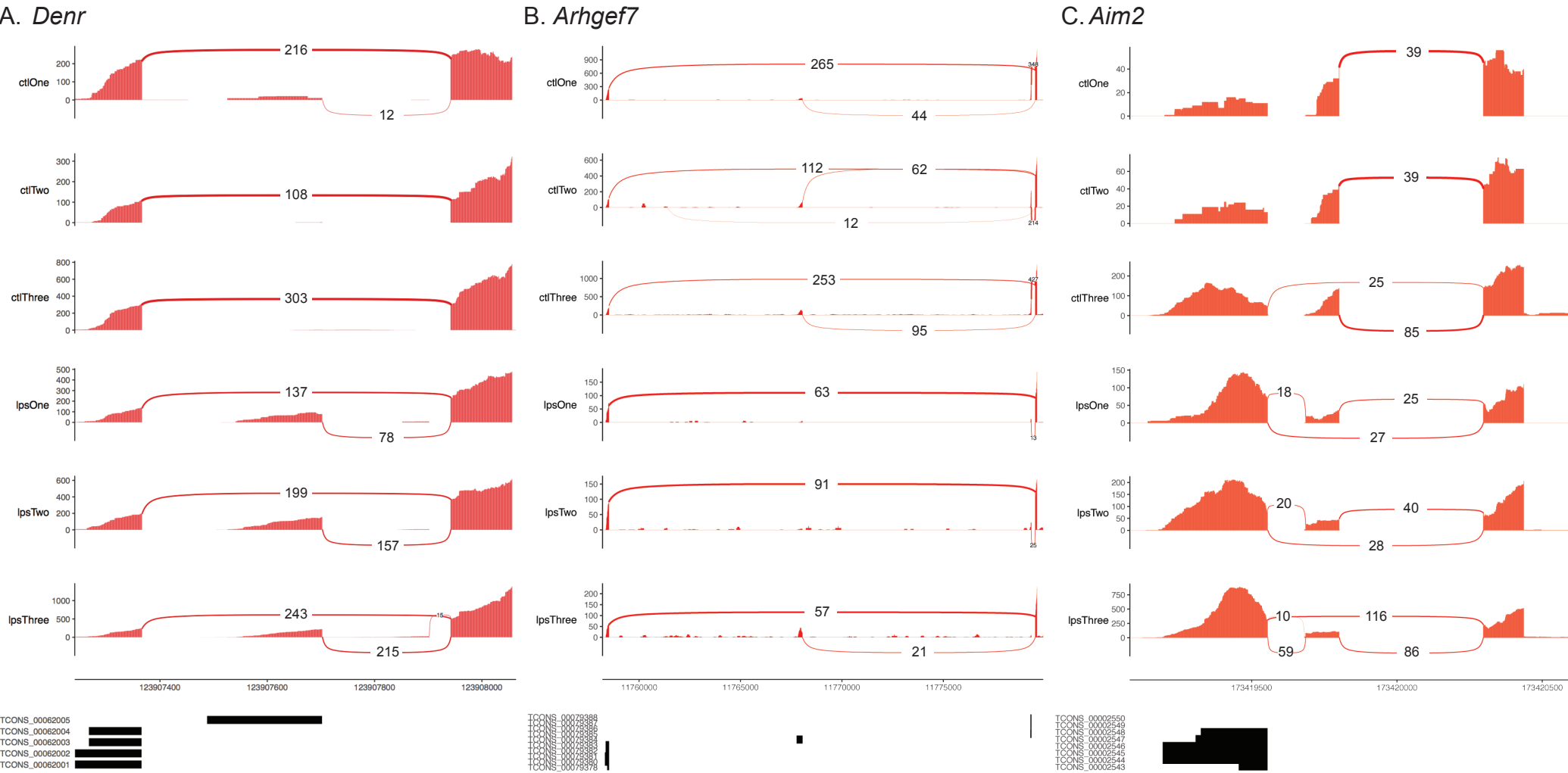
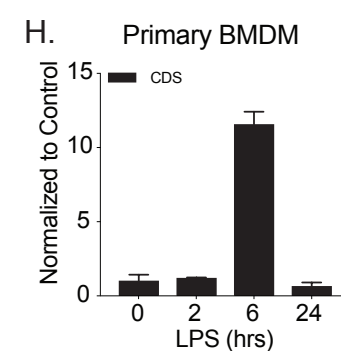
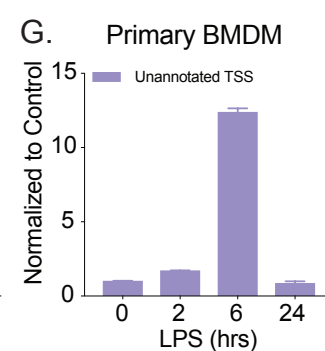
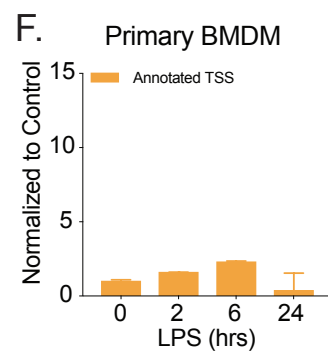
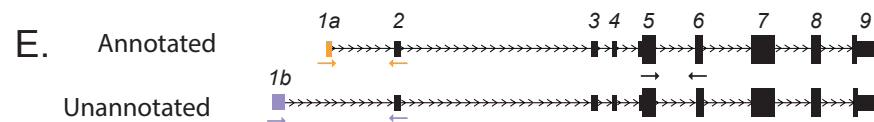
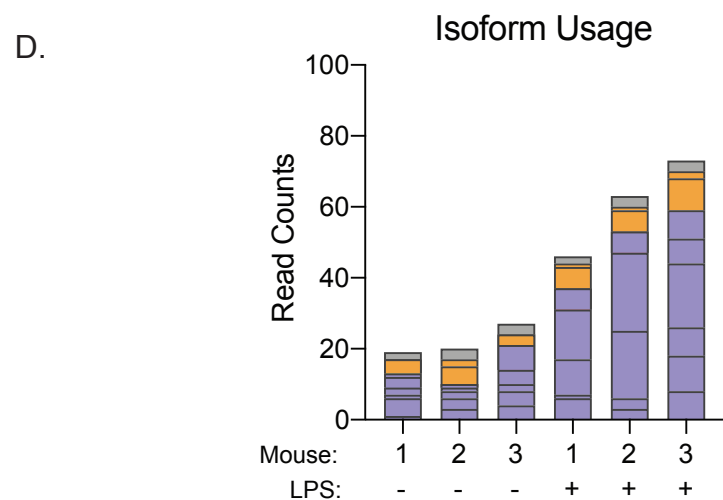
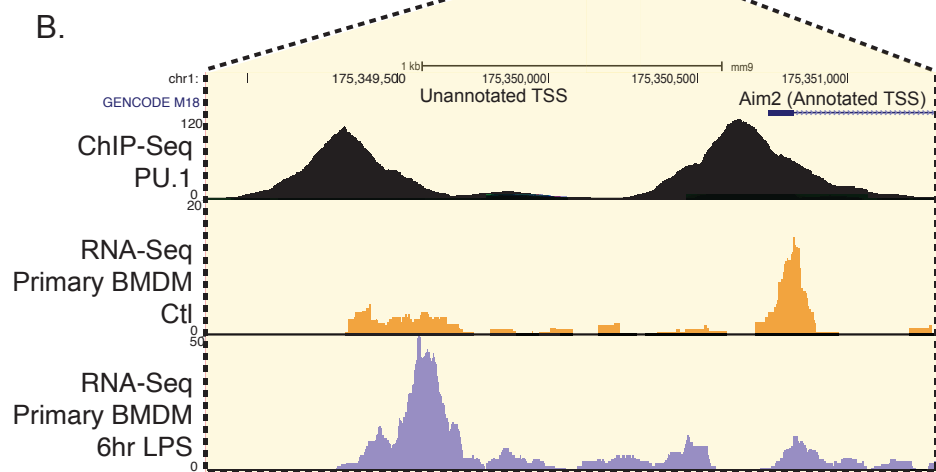
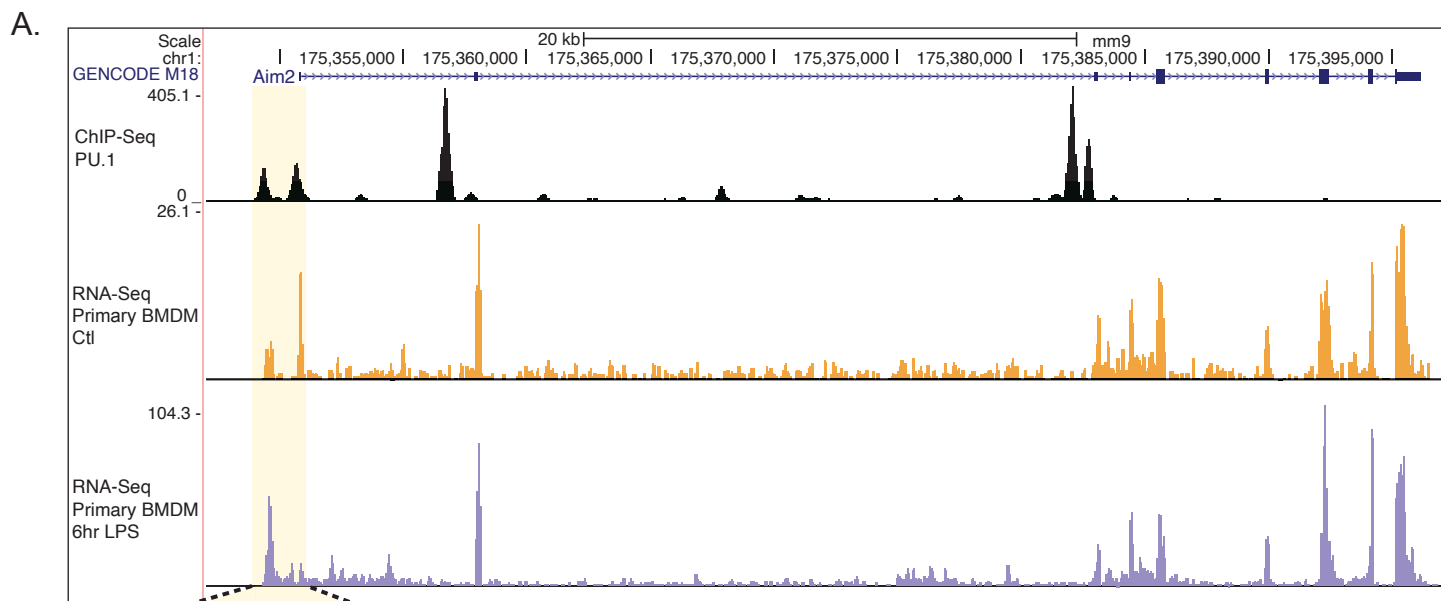
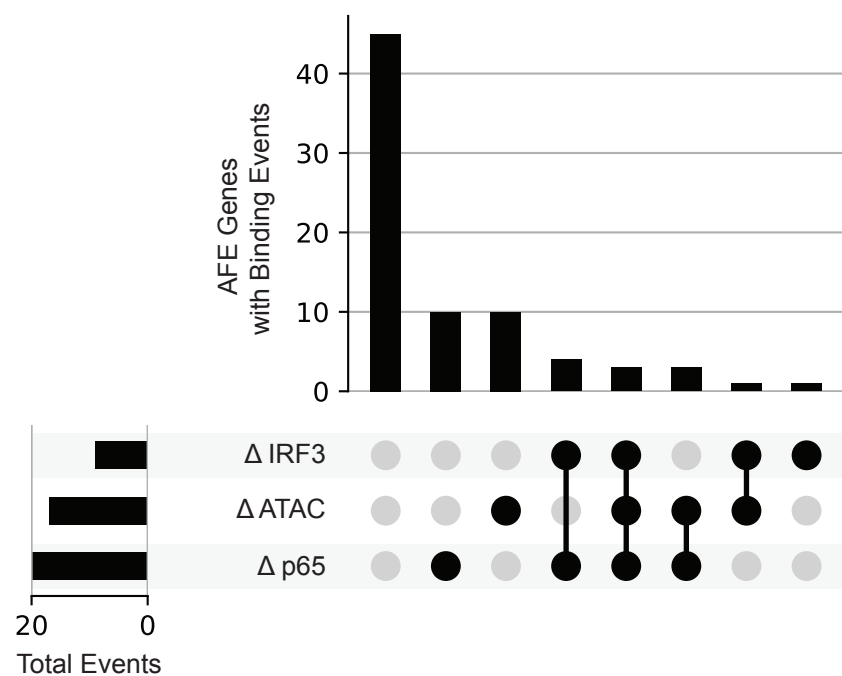


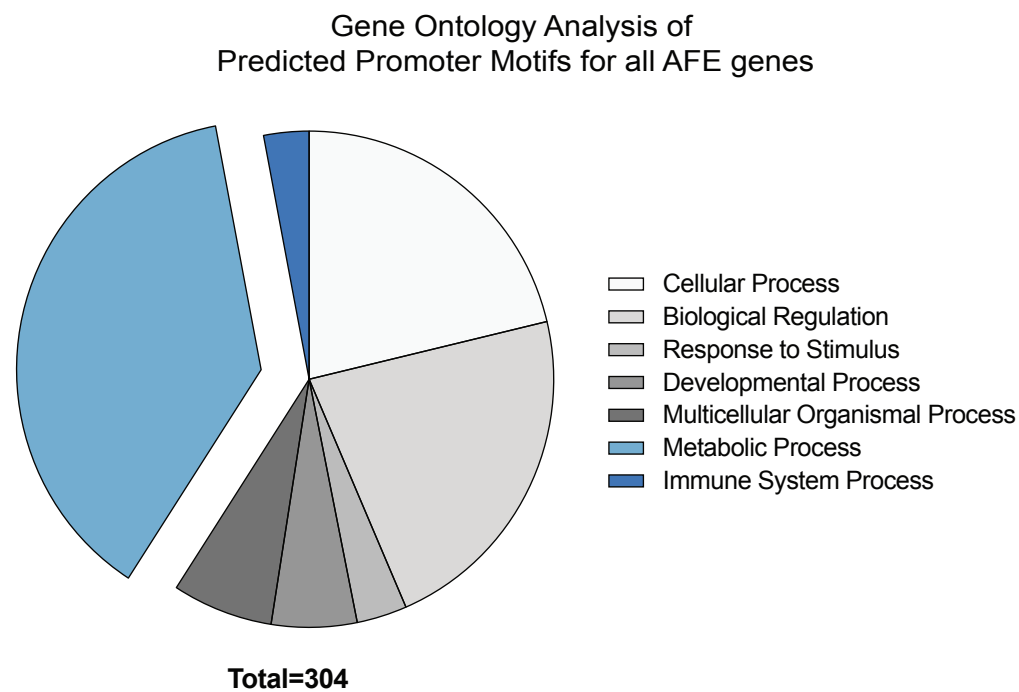
Figure 1 - Supplemental Figure 9: Validated unannotated alternative first exon isoforms. Sashimi plots showing AFE usage in (A) *Denr*, (B) *Arhgef7*, and (C) *Aim2* involving novel isoforms identified using nanopore sequencing.



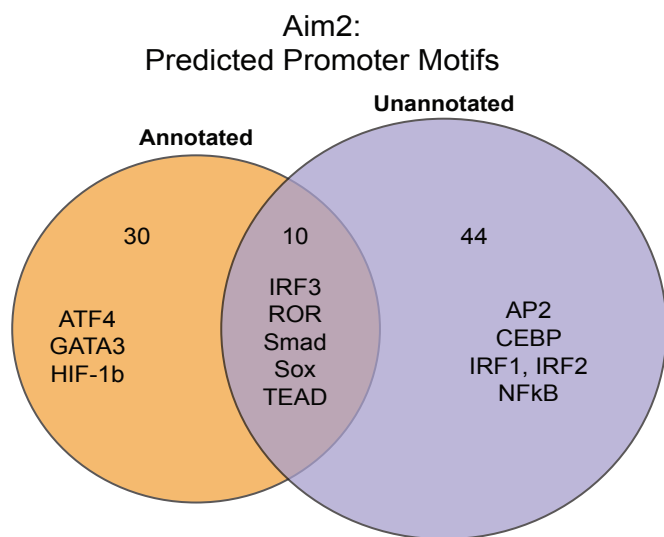
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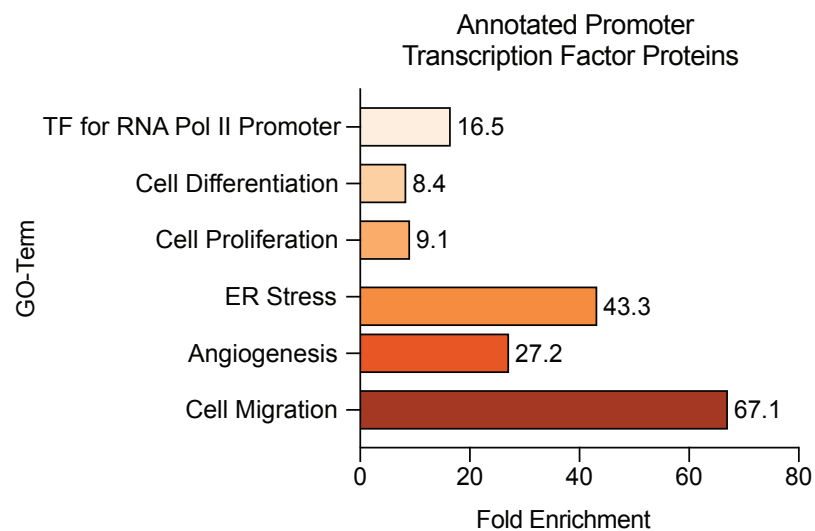
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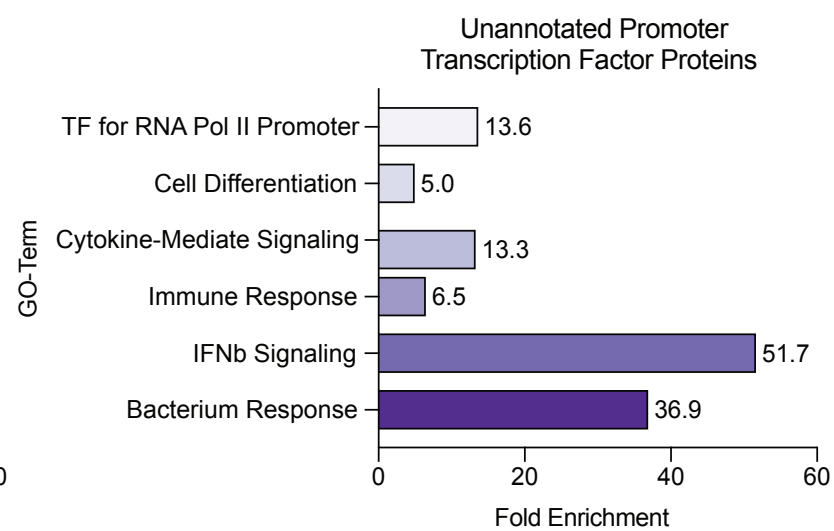
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E.



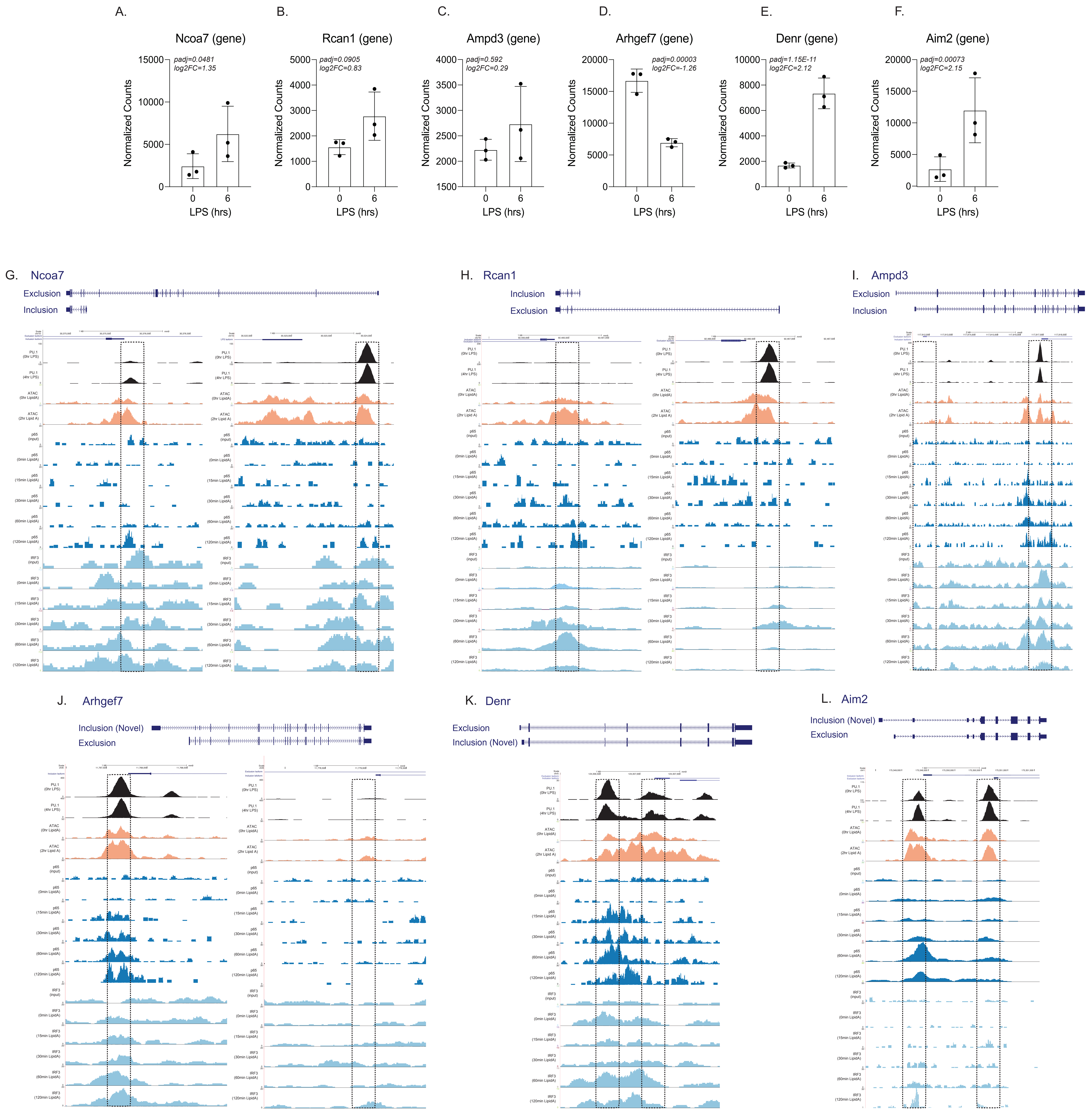
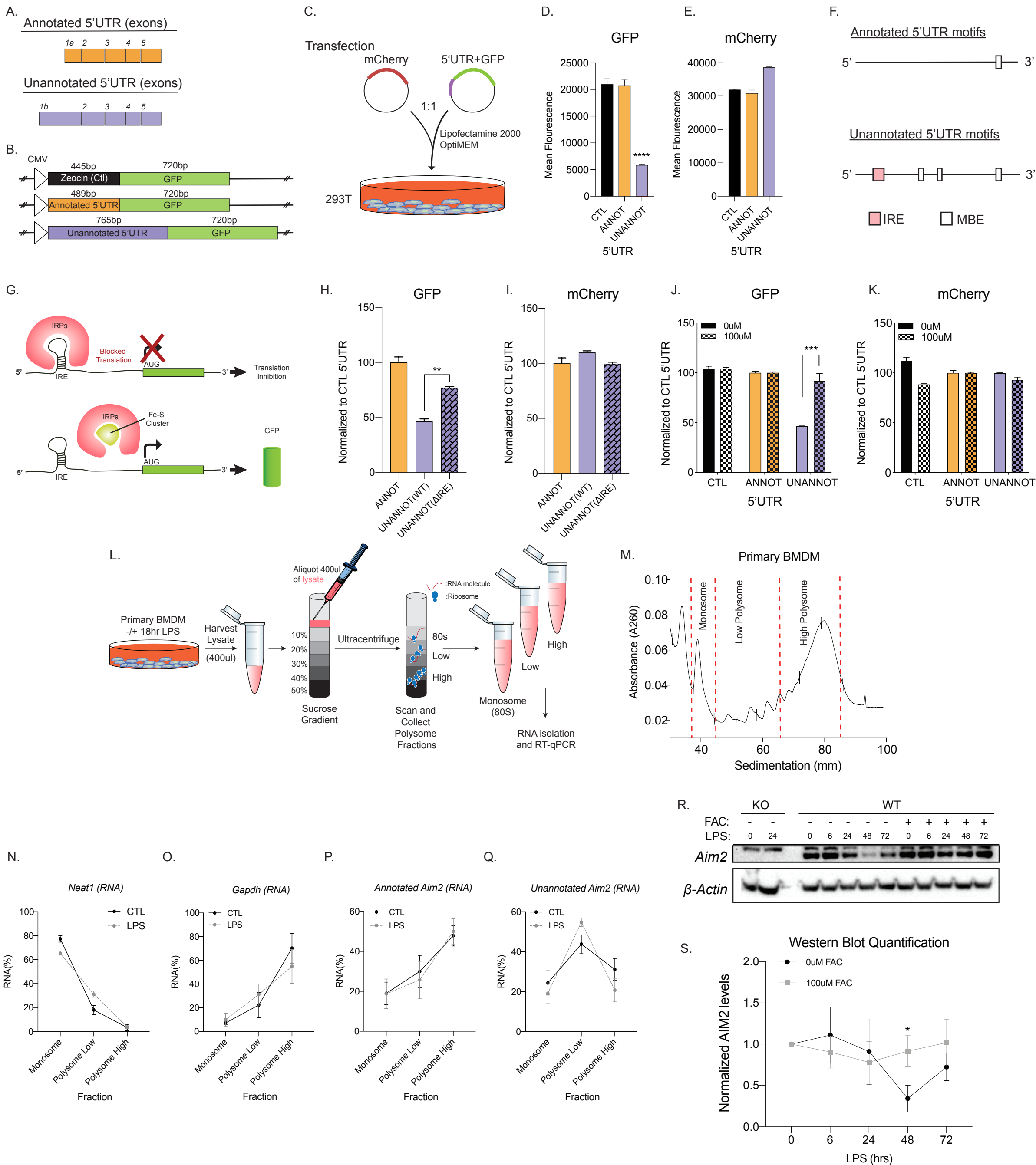


Figure 3 - Supplemental Figure 11: Genome Browser images of ChIP-seq and ATAC-seq for both the promoters of CTL and LPS specific AFE isoforms. The normalized counts and DESeq2 analysis of of Ncoa7 (A), Rcan1 (B), Ampd3 (C), Arhgef7 (D), Denr (E) and Aim2 (F) are represented in a bar graph in triplicate. Genome browser images are taken for the CTL and LPS promoters highlighted in yellow of Ncoa7 (G), Rcan1 (H), Ampd3 (I), Arhgef7 (J), Denr (K), and Aim2 (L). All tracks are next-generation sequencing data from BMDMs. The top track (in black) is ChIP-sequencing data of PU.1 from unstimulated cells, while the second track is PU.1 ChIP-seq data from LPS stimulated cells (GSE109965). The next two tracks (in orange) are of ATAC sequencing data from either unstimulated or LPS stimulated cells (GSE74191). The next five tracks (dark blue) are p65 ChIP-sequencing data from the input, 0min KLA, 15min KLA, 30min KLA, 60min KLA or 120min KLA stimulation (GSE67343). Finally the last five tracks (in light blue) are IRF3 ChIP-sequencing data from the input, 0min KLA, 15min KLA, 30min KLA, 60min KLA or 120min KLA stimulation (GSE67343).



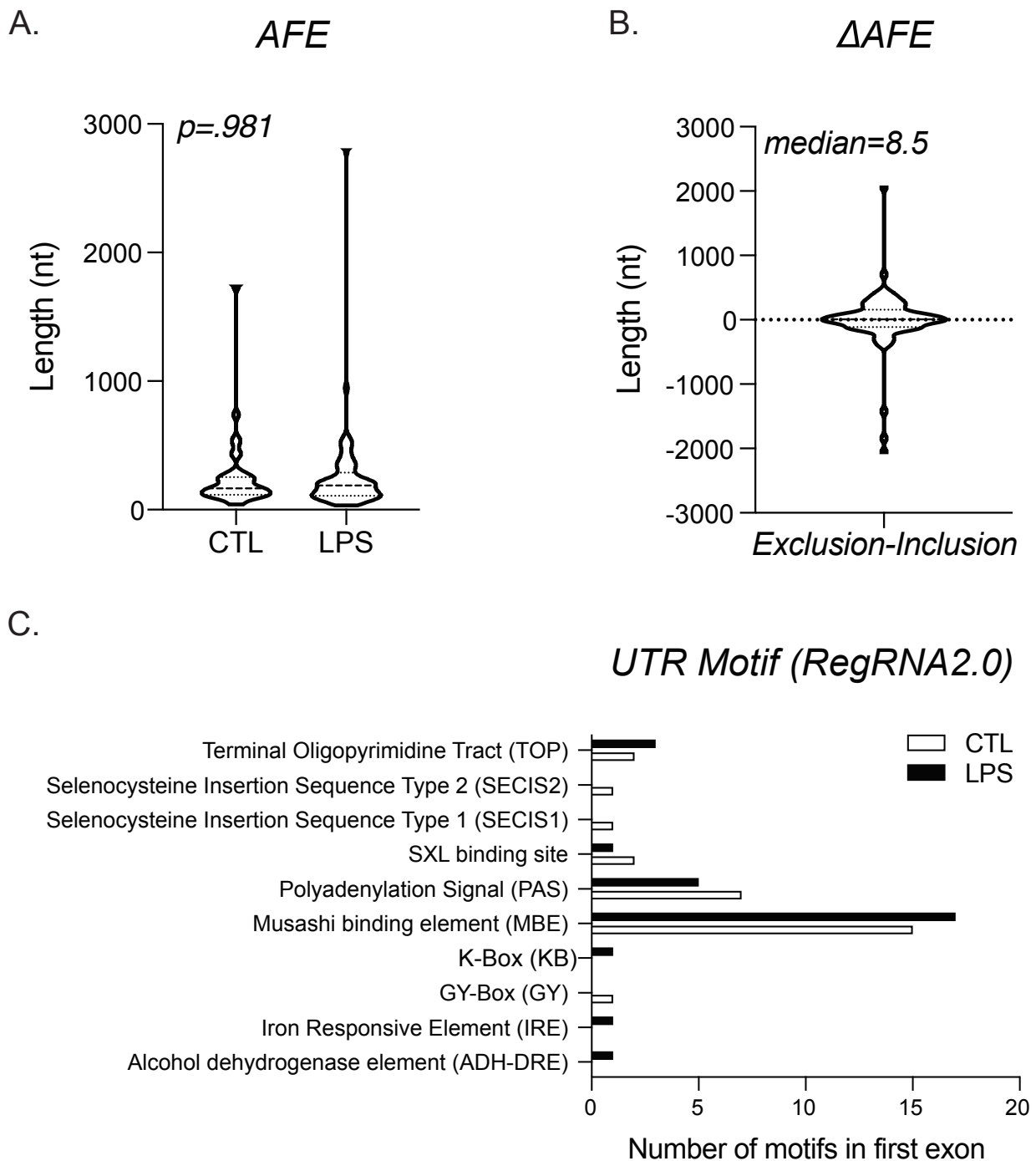


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Mouse IRE sequence
Vienna RNAFold

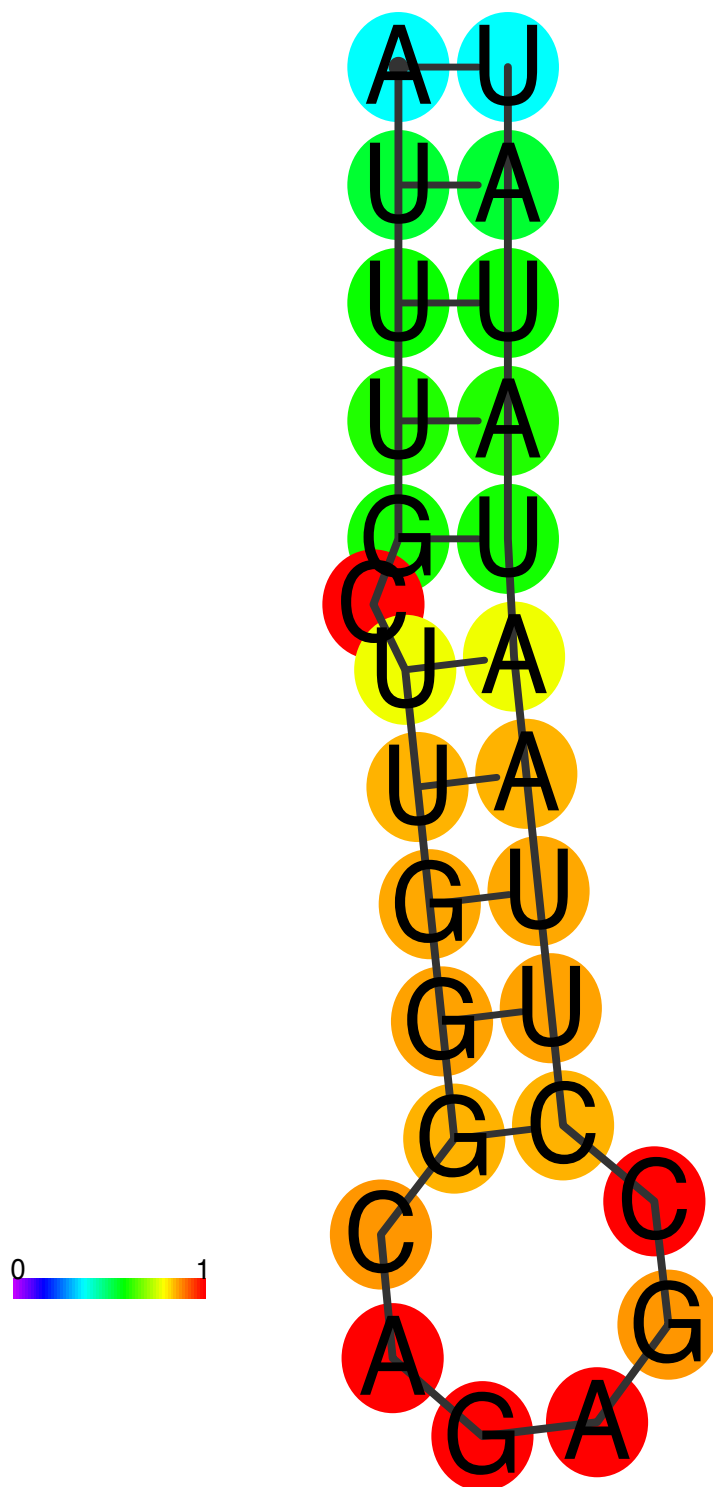


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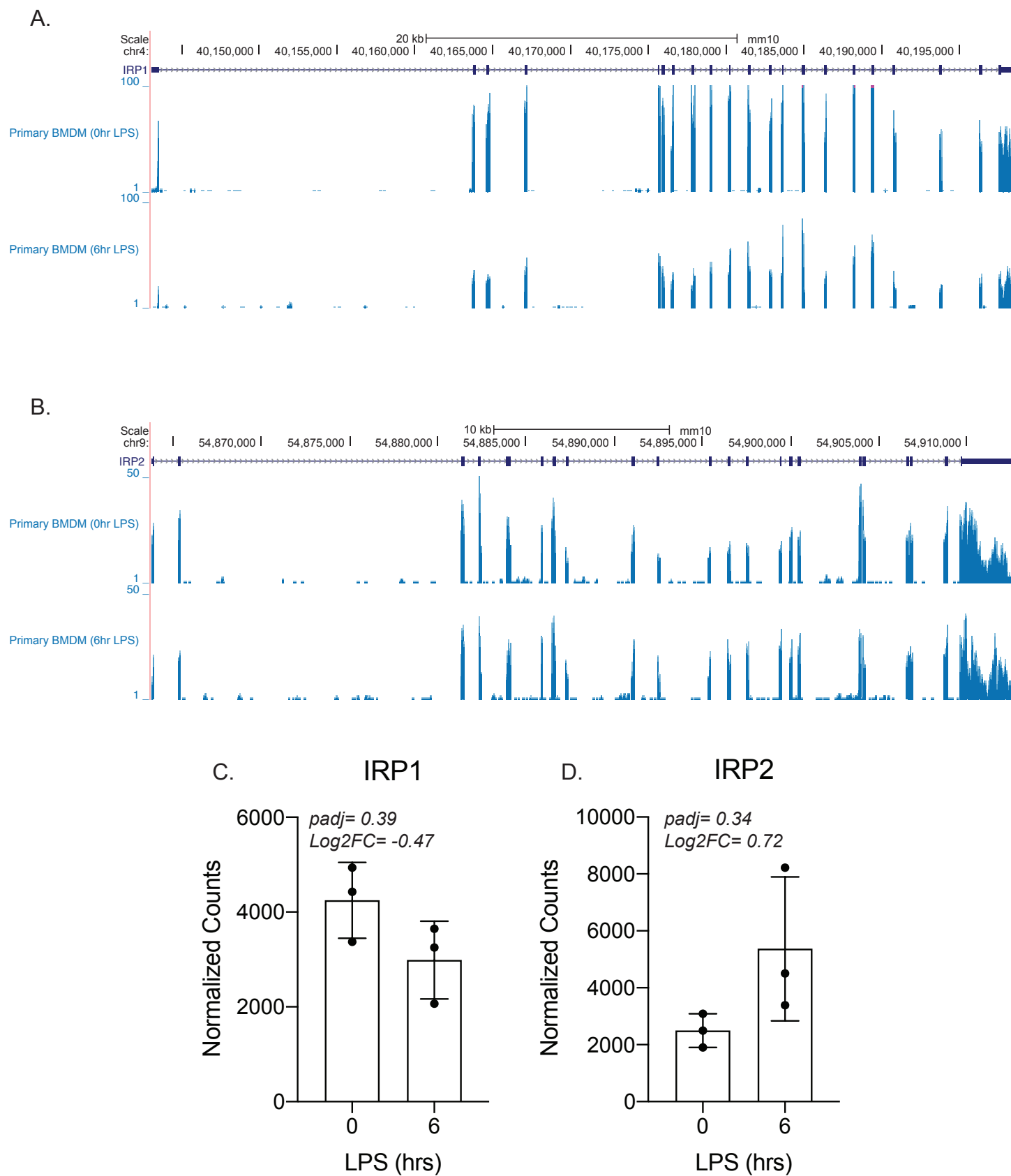


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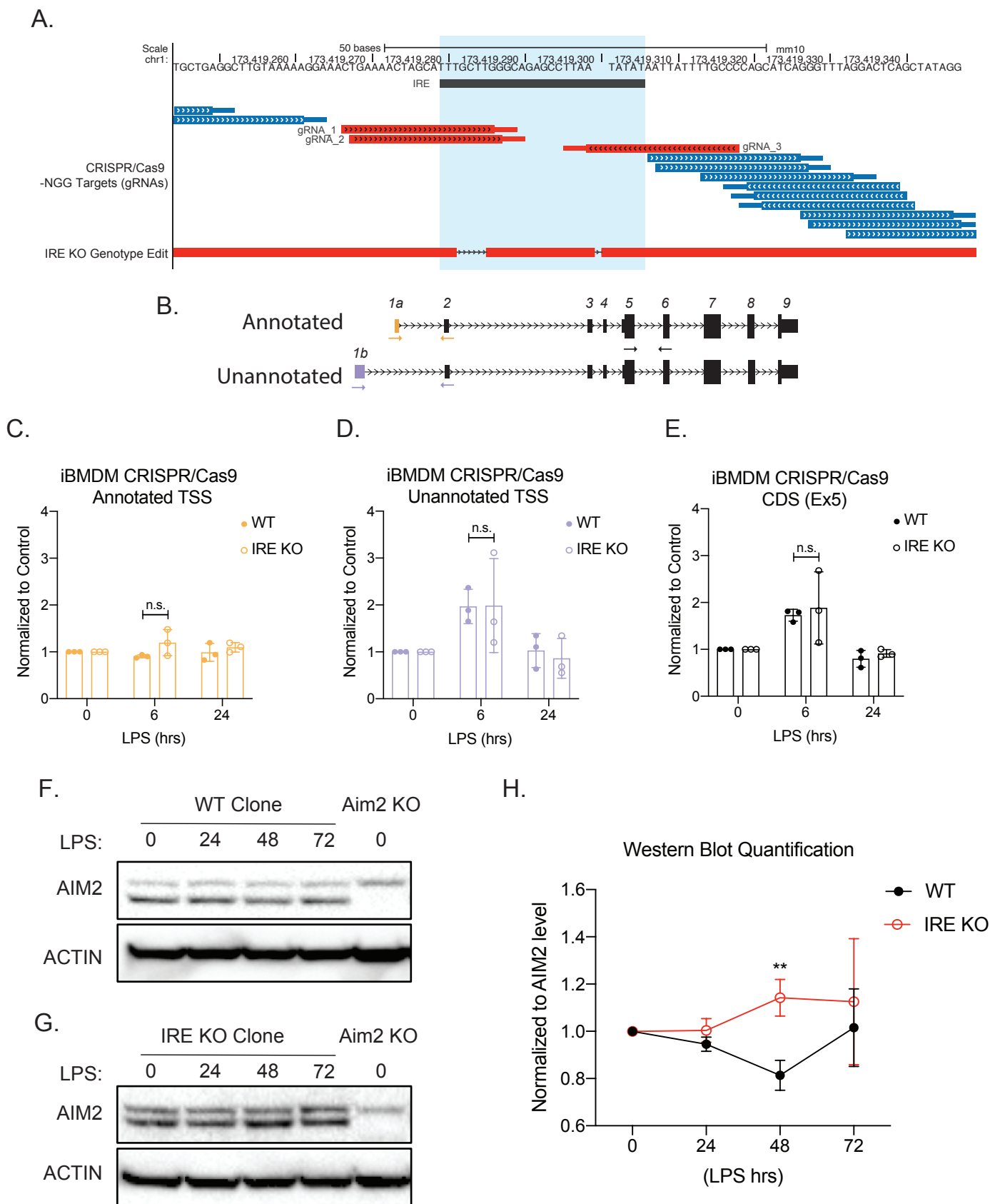


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