

## RESEARCH ARTICLE

# Transcriptomic analyses of gastrulation-stage mouse embryos with differential susceptibility to alcohol

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## ABSTRACT

Genetics are a known contributor to differences in alcohol sensitivity in humans with fetal alcohol spectrum disorders (FASDs) and in animal models. Our study profiled gene expression in gastrulation-stage embryos from two commonly used, genetically similar mouse substrains, C57BL/6J (6J) and C57BL/6NHsd (6N), that differ in alcohol sensitivity. First, we established normal gene expression patterns at three finely resolved time points during gastrulation and developed a web-based interactive tool. Baseline transcriptional differences across strains were associated with immune signaling. Second, we examined the gene networks impacted by alcohol in each strain. Alcohol caused a more pronounced transcriptional effect in the 6J versus 6N mice, matching the increased susceptibility of the 6J mice. The 6J strain exhibited dysregulation of pathways related to cell death, proliferation, morphogenic signaling and craniofacial defects, while the 6N strain showed enrichment of hypoxia and cellular metabolism pathways. These datasets provide insight into the changing transcriptional landscape across mouse gastrulation, establish a valuable resource that enables the discovery of candidate genes that may modify alcohol susceptibility that can be validated in humans, and identify novel pathogenic mechanisms of alcohol.

This article has an associated First Person interview with the first author of the paper.

**KEY WORDS:** Fetal alcohol spectrum disorders, Apoptosis, Inflammation, Embryo, Brain development

## INTRODUCTION

Alcohol exposure during the first weeks of pregnancy is associated with significant birth defects involving the craniofacial region

and central nervous system (Cook et al., 1987). Specifically, prenatal alcohol exposure (PAE) during gastrulation [third week of human pregnancy; embryonic day (E)7 in mice] results in the craniofacial malformations characteristic of fetal alcohol syndrome (FAS), including a thin upper lip, smooth philtrum, reduced head circumference and small eyes (Cook et al., 1987). In addition, gastrulation-stage PAE is associated with loss of midline brain tissue, including agenesis of the corpus callosum and holoprosencephaly (Higashiyama et al., 2007; Godin et al., 2010), disrupted morphogenic signaling (Zhang et al., 2014; Kietzman et al., 2014; Aoto et al., 2008) and widespread apoptosis (Dunty et al., 2001).

An ongoing question in the field of prenatal alcohol research is why some children exposed to alcohol *in utero* develop significant physical and cognitive deficits whereas others are relatively unaffected. While the dose and timing of alcohol exposure are certainly factors, it is known that environmental factors, such as stress or nutrition, and genetics can predispose an embryo to alcohol sensitivity or resistance. Studies using twins exposed to heavy prenatal alcohol revealed that dizygotic twins were less likely to both be diagnosed with FAS compared to monozygotic twins (Streissguth and Dehaene, 1993; Abel, 1988). Of the monozygotic twins examined, if one twin was diagnosed with FAS then the other was also diagnosed in 100% of cases, compared with only 64% concordance in the dizygotic twin sets. In addition, experiments in animal models of fetal alcohol spectrum disorder (FASD) have demonstrated that strains of mice and chicken exhibit different degrees of incidence and severity of PAE-related birth defects (Downing et al., 2009; Su et al., 2001). These data clearly suggest that there is a genetic component to FAS. Although the genetic differences that alter susceptibility to PAE between these strains are not yet clear, it is known that the deletion of certain genes can alter susceptibility to PAE (Eberhart and Parnell, 2016). For example, deleting one copy of either sonic hedgehog (*Shh*) (Kietzman et al., 2014), the *Shh* co-receptor cell adhesion associated oncogene associated (*Cdon*) (Hong and Krauss, 2012, 2013) or downstream transcriptional activator Gli family zinc finger 2 (*Gli2*) (Fish et al., 2017) increases susceptibility to PAE in the brain, face and limbs. Likewise, deletion of one or both copies of the ciliary-related gene *Mns1* exacerbates the effects of PAE on the brain and face in a gene dose-dependent manner (Boschen et al., 2018). However, the identification of further genes that may alter susceptibility to PAE remains elusive.

In order to identify candidate genes that alter susceptibility to early developmental alcohol exposure, our current study identifies PAE-induced transcriptomic changes in the gastrulation-stage embryo using two closely related mouse strains: the C57BL/6J (referred to as 6J) strain obtained from The Jackson Laboratory and the C57BL/6NHsd (referred to as 6N) strain obtained from Envigo (formerly Harlan). Previous work has demonstrated that the 6J strain has a higher incidence of eye defects after prenatal alcohol

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compared to the 6N strain (Dou et al., 2013; Green et al., 2007). These strains were both derived from the original C57BL/6J mice bred by The Jackson Laboratory but were separated when the 6J strain was given to the National Institutes of Health (NIH) in 1951 and given from the NIH to Harlan in 1974. Now, over 200 generations separate the 6J and 6N strains. Notably, two known genetic mutations have emerged over the years. First, the 6J strain has a mutation in the *Nnt* gene, which encodes nicotinamide nucleotide transhydrogenase, an enzyme important for production of NADPH and removal of reactive oxygen species (ROS) from the mitochondria (Ronchi et al., 2013). The mutation in the 6J mice is comprised of two separate mutations: a missense (M35T) mutation in the mitochondrial leader sequence and a multi-exonic deletion of exons 7-11, resulting in a non-functional protein. 6J mice have been shown to have five- to sevenfold lower levels of Nnt in the islets and liver (Toye et al., 2005), impaired insulin secretion and mitochondrial redox abnormalities (Ronchi et al., 2013). Mutations in the *Nnt* gene could cause reduced NADPH and glutathione stores and impaired oxidative stress responses in the 6J embryos, possibly priming these embryos to be more likely to undergo cell death following alcohol exposure. Second, the 6N strain carries a single nucleotide deletion in the *Crb1* gene, called the *Rd8* mutation (Mattapallil et al., 2012). This mutation is associated with retinal degeneration, lesions and folding.

While the *Nnt* and *Rd8* mutation are two well-studied differences between the 6J and 6N strains, it is possible that other genetic variation is present during development that could modulate strain differences in risk and resilience to alcohol damage. In addition, it is unknown what effect these mutations have on gene expression during early embryonic development. The goals of this experiment were two pronged. First, we used the gathered transcriptome data to provide information about gene expression across gastrulation during normal mouse development. To this end, a web-based tool was created to allow gene-by-gene exploration of expression patterns across the first 12 h of gastrulation in both the 6J and 6N strains. Second, we examined PAE-induced gene expression changes 6 h and 12 h after exposure (E7.25 and E7.5, respectively), adding valuable information about the molecular targets of this mouse model of FASD.

## RESULTS

### Web-based tool as a resource for data visualization and exploration

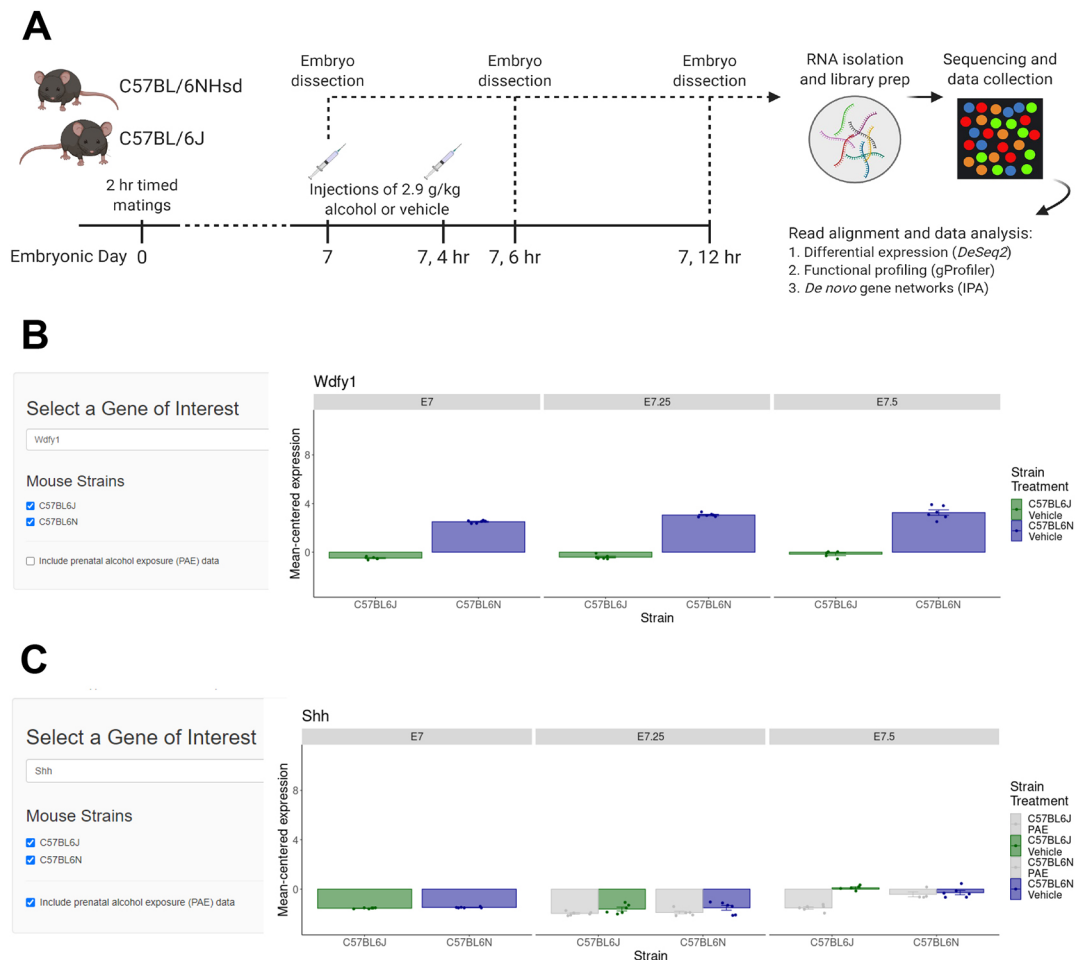
We performed whole transcriptomic analyses of 6J and 6N mouse embryos at three time points (E7.0, E7.25 and E7.5) using RNA sequencing (RNA-seq) (Fig. 1A). We assembled a transcriptomic database of normal embryonic development, as well as characterized how strain differences and PAE treatment governs these processes. A web-based visualization tool (<http://parnell-lab.med.unc.edu/Embryo-Transcriptomics/>) was created for a gene-by-gene query of the transcriptomic data from both strains and from both control and PAE-treated embryos at each time point. Strain and prenatal treatment options can be toggled on or off to compare relative expression of a gene of interest in a single strain across time points, or between the 6J and 6N strains across time. For example, expression of *Wdfy1* significantly differs between the strains across all time points but is not affected by PAE (Fig. 1B). Conversely, *Shh* increases in expression in both strains over time, but PAE significantly reduces expression in the 6J strain (Fig. 1C). The gene expression data generated in this study provide a valuable resource for developmental biologists, toxicologists, mouse geneticists and researchers interested in models of FASD.

### Transcriptional differences between 6J and 6N mouse embryos during gastrulation

Gene expression across the first 12 h of normal mouse gastrulation was compared between the 6J and 6N strains (a representative image of a gastrulation-stage mouse embryo is shown in Fig. 2A). Heat maps showing hierarchical clustering of gene expression of all significant genes for all replicates are in Figs S1-S7, and VST-normalized values for all significant genes are in Dataset 1. We first focused on how 6J and 6N embryonic gene expression differs at E7.0 to establish a baseline and explore strain-dependent transcriptional differences prior to alcohol exposure. Eighty genes were identified as differentially expressed between the 6J and 6N strains at E7.0. Of these, 67 showed higher expression (83.8%) and 13 showed lower expression (16.2%) in the 6J relative to 6N strain (Fig. 2B). Functional profiling revealed upregulation of pathways related to inflammation and cytokine production, cell migration and intracellular signaling (Fig. 2C; Table S1).

Multiple genes that encode cytokines/chemokines and immune signaling molecules had higher expression in the 6J strain, including *Ccl4*, *Il1r1*, *Il1rn* and *Tnfrsf9*. The most upregulated gene [largest positive  $\log_2$  fold-change ( $\text{Log}_2\text{FC}$ )], *Ide*, encodes an insulin-degrading enzyme that is known to degrade the B chain of insulin and amyloid beta (Bennett et al., 2000), suggesting a role in Alzheimer's disease. Expression of *Ide* has been found to be relatively low in embryonic *Drosophila* (Stoppelli et al., 1988) and neonatal rat (Kuo et al., 1993) compared to their adult counterparts, suggesting a more prevalent role of this protein during adulthood. There were no significantly overenriched pathways among the downregulated genes; however, the *Nnt* gene was significantly downregulated in the 6J strain, corroborating the well-known mutation in the 6J mouse strain (Ronchi et al., 2013). The gene most downregulated in the 6J relative to 6N strain was *Wdfy1*, which encodes an adaptor protein involved in protein-protein and protein-DNA interactions. *Wdfy1* also acts as an adaptor protein for Toll-like receptors 3 and 4 (Hu et al., 2015), implicating this protein in the immune signaling response. *Efcab7* was also downregulated in the 6J relative to 6N strain; this gene is associated with primary cilia function and, in particular, *Shh* signaling via smoothed (Smo) (Pusapati et al., 2014).

Because the majority of pathways and genes were related to the cellular immune signaling response, we hypothesized that 6J mice have heightened immune signaling activity that influences the stress response to a stimulus such as alcohol. We therefore sought to more comprehensively characterize the disrupted *de novo* gene networks using the Ingenuity Pathway Analysis (IPA) database of known protein-protein interactions. IPA allows insight into the functional relationships between differentially expressed genes that are not captured in the canonical terms and pathways used in the gene set enrichment analysis above. Six networks were dysregulated in the 6J relative to 6N (Table 1A; Table S2A) related to immune signaling ('Inflammatory disease', 'Immune cell trafficking', 'Inflammatory response') and cell proliferation ('Cell cycle', 'Cell movement', 'Cellular assembly and organization'), supporting that baseline immune signaling differs between the strains. Differences in cell movement are likely to be linked to immune cell migration, although the source, type and function of these immune cells and related signaling molecules in the gastrulation-stage embryo is not yet clear. Overall, these genetic differences set the stage for the disparate responses to PAE observed in these two strains both hours (Figs 4 and 6) and days (Dou et al., 2013; Green et al., 2007) later in development.



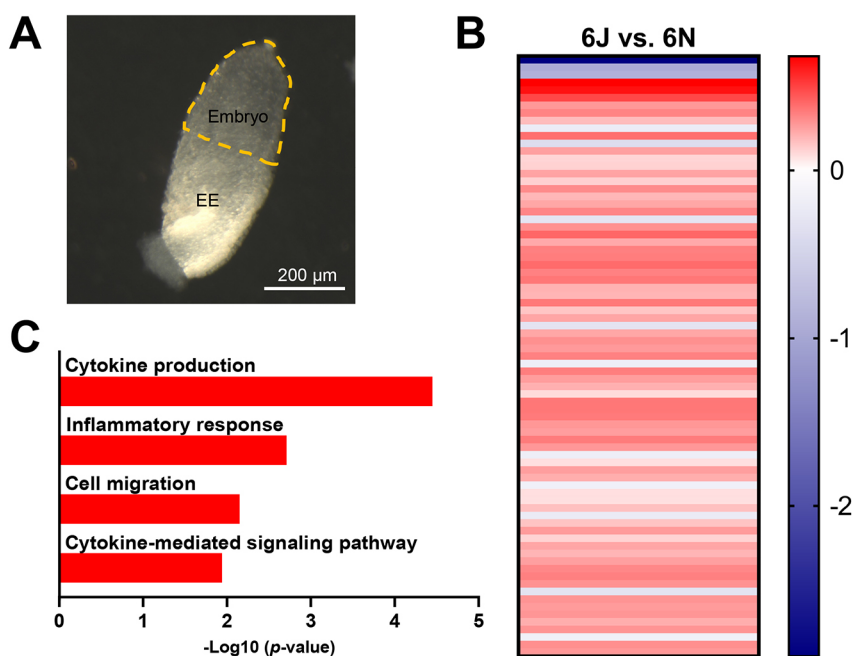
**Fig. 1. Experimental timeline and example of web-based visualization tool.** A web tool was created as a resource to allow gene-by-gene exploration of expression patterns across the first 12 h of normal mouse gastrulation in the 6J and 6N strains. (A) Experimental timeline. (B) Comparison of expression of *Wdfy1*, a gene that significantly differed between the 6J and 6N strains, across time. Single strains can be selected for viewing using the toggles on the left. (C) PAE data can be toggled on and off using the options. *Shh* expression was impacted by prenatal alcohol exposure (PAE) in the 6J, but not 6N, mice. Figure created with Biorender.com.

We next compared the 6J and 6N strains either 6 h or 12 h after E7.0 following two injections of vehicle solution to investigate strain differences at later developmental time points in the absence of alcohol. Gastrulation is a critical time of embryonic development, involving cell proliferation and fate decisions that establish the embryonic germ layers, with developmental events relying on temporally and spatially specific gene expression (Pijuan-Sala et al., 2019). At E7.25, 6 h post-vehicle injection, 315 genes were differentially expressed between the two strains. Of these genes, 128 genes were upregulated (40.6%) and 187 were downregulated (59.4%) in the 6J relative to the 6N strain. Twelve hours after vehicle treatment, at E7.5, there were 304 differentially expressed genes between the 6J and 6N strains. Of these, similar to the E7.25 time point, 120 genes were upregulated (39.5%) and 184 genes were downregulated (60.5%) in the 6J strain.

Functional profiling of genes from the E7.25 time point revealed only a small number of biological pathways that differed between the two strains, including altered hydrolase and endopeptidase activity and pathways related to cAMP signaling and apoptosis related to the downregulated genes (Table S3). The top ten *de novo* networks were related to cell death, intercellular signaling, nutrient metabolism and embryonic development (Table S2B). At E7.5, functional profiling of the upregulated genes indicated increased

prostaglandin signaling and GPCR signaling (Table S4). The downregulated pathways were again related to hydrolase and endopeptidase activity, consistent with the E7.25. *De novo* network analysis identified functions related to organ development, drug metabolism, protein processing and the cell cycle (Table S2C).

Overall, there was little change in which genes were strongly up- or downregulated across these 12 h of development, consistent with other studies showing that most genes expressed during gastrulation show relatively stable expression prior to the onset of organogenesis (Mitiku and Baker, 2007). *Wdfy1* showed the largest downregulation by  $\text{Log}_2\text{FC}$  at both time points in the 6J relative to 6N strain ( $-3.94$  and  $-3.67$   $\text{Log}_2\text{FC}$ , respectively), consistent with what was observed in these two strains at E7.0 prior to any injection. *Efcab7*, which had lower baseline expression in the 6J strain, exhibited the same effect at E7.25 ( $-1.78$   $\text{Log}_2\text{FC}$ ), but not at E7.5. The most upregulated gene in the 6J relative to 6N strain at E7.25 was *Hist1h4m* (*H4c17*), or histone cluster 1, H4m, a gene related to nucleosome assembly. This gene also showed a large upregulation at E7.5 and a small but statistically significant upregulation at E7.0. Interestingly, this gene was found to be downregulated in the hippocampus of fetal 6J mice (purchased from Orient Bio) following alcohol exposure from E8 to E12 (Mandal et al., 2015). The fact that expression of *Hist1h4m* differs between alcohol-



**Fig. 2. Immune signaling gene pathways are upregulated in the 6J compared to the 6N strain.** (A) Representative image of E7.0 mouse embryo. Embryo highlighted in yellow was dissected from the extraembryonic tissue (EE) for sequencing. (B) Heat map of genes altered in the 6J versus 6N strain at baseline (prior to alcohol administration) on E7.0. Data are expressed as  $\log_2$  fold change ( $\text{Log}_2\text{FC}$ ). Blue, downregulated genes; red, upregulated genes.  $n=6/\text{group}$ . (C) Functional profiling of genes differentially expressed in the 6J versus 6N strain at E7.0.  $n=6/\text{group}$ .

sensitive and -resistant strains and expression is affected in certain models of PAE is strongly suggestive that this gene is a possible target of alcohol and mediator of alcohol sensitivity.

#### Strain-specific differences in transcriptional response to PAE are evident as early as 6 h after exposure

We next compared the effect of PAE on embryonic gene expression in each strain at E7.25 to explore how strain differences modulate the initial transcriptional response to alcohol. At E7.25 (6 h post-PAE), 810 genes were significantly differentially expressed between PAE and vehicle in the 6J strain, and 702 genes were differentially expressed between PAE and vehicle in the 6N strain. In the 6J strain, 355 genes were upregulated (43.8%) and 455 were downregulated (56.2%) (Fig. 3A). In the 6N strain, 372 genes were upregulated (52.9%) and 330 were downregulated (47.1%) (Fig. 3B). Of the differentially expressed genes, 228 were altered in both strains (Fig. 3C). In most cases, the directionality (up- or downregulated) was the same between strains, indicating that although there is a substantial subset of genes that are similarly affected in both the 6J and 6N strains, the majority of genes with significantly altered expression in each strain are unique.

Functional profiling of the genes upregulated following PAE in the 6J strain at E7.25 revealed that pathways related to catalytic activity (specifically, hydrolases and endopeptidases) were dysregulated (Fig. 4A; Table S5). Interestingly, we identified ‘Activation, myristoylation of BID and translocation to mitochondria’ as upregulated by PAE. BH3-interacting domain death agonist (BID) is a pro-apoptotic protein of the Bcl-2 family that is activated by the post-translational modification N-myristoylation. Activation of BID causes the insertion of Bax into the mitochondrial membrane and release of cytochrome C (Eskes et al., 2000). This pathway, in combination with others related to cytolysis and apoptotic signaling, indicates that cell death pathways have begun to be activated in the 6J strain as early as 6 h post-PAE (E7.25). Analysis of downregulated genes in the 6J strain found that cellular metabolism and binding activity were reduced. ‘Binding activity’ included enzymatic, DNA and protein binding, and likely indicates an overall reduction in cellular activity that coincides with

decreased metabolism. Multiple terms related to cell cycle regulation were also identified in the downregulated genes, suggesting that cell proliferation is slowed or paused while the embryo responds to the alcohol insult. *De novo* network analysis revealed multiple associations with organ health and development, cancer/cell cycle, drug metabolism and cell death (Table 1B; Table S2D).

The top two genes downregulated following PAE in the 6J strain at E7.25 were *Srsf2*, which encodes a protein that regulates constitutive and alternative splicing of pre-mRNA that has been linked to cell death through the p53 pathway (Comiskey et al., 2020), and *Alyref*, which encodes the molecular chaperone Aly/REF export factor, which is involved in RNA processing and nuclear export. The most upregulated gene in the 6J strain was *Chac1*, which encodes glutathione-specific gamma-glutamylcyclotransferase-1 (Gamma-GCG1), a protein involved in glutathione cleavage, induction of oxidative stress-related apoptosis, and a negative regulator of Notch signaling (Chi et al., 2012). *Trib3* was also significantly upregulated and encodes Tribbles pseudokinase 3 (Trb-3), which is induced by NF- $\kappa$ B signaling, creates a negative feedback loop controlling Atf4 activity in response to cellular stress and prevents apoptosis. Interestingly, Trb-3 has also been shown to block expression of Gamma-GCG (Örd et al., 2016), thus limiting apoptosis through another pathway. Multiple types of cellular stress upregulated Trb-3, including nutritional deprivation (Liu et al., 2012) and endoplasmic reticulum (ER) stress (Örd et al., 2014). In sum, alcohol-induced reductions in the expression of cellular metabolism and gene transcription pathways, as well as upregulation of genes related to oxidative stress and apoptosis, could lead to perturbed cell proliferation and embryonic growth in PAE 6J embryos.

We next compared gene expression patterns following PAE in 6N mice. Although 372 genes were upregulated, there were no significantly enriched pathways among them. However, analysis of the 330 downregulated genes revealed a reduction in cellular metabolism and methyltransferase activity (Fig. 4B; Table S6). Similarly to in the 6J strain, PAE seemingly caused a reduction in cellular activity in the 6N strain. Alteration of methylation could have effects on gene expression and protein function; some of the

**Table 1. *De novo* gene networks altered in the sensitive 6J mice compared to the resistant 6N mice at baseline (E7.0), 6 h after alcohol or 12 h after alcohol**

	Diseases and functions	$-\log_{10}$ (Fisher's exact <i>P</i> -value)	Molecules in network	Molecules
A	E7.0	6J versus 6N		
1	Cell cycle Drug metabolism Molecular transport	31	15	<i>Aak1, Acad11, Adck2, Ahnak, App, Arfgap3, Arglu1, Atp8b1, Ccdc88c, Clpb, Dnah14, Dnajc28, Dynlrb2, Dynlt1, Hspa9, Hspb9, Igsf6, Klif12, Lira5, Mbnl3, Mmp3, Myc, Myo1d, Neat1, Nnt, Nr1h4, Rxr, Sez6l, Sirt5, Tex2, Tktl2, Tmem267, Tnf, tretinoin, Vgll3</i>
2	Cancer Immunological disease Inflammatory disease	28	14	<i>26s proteasome, Anxa11, Btaf1, Cd3, Creb, Cx3cr1, Efcab7, Entpd4, Erk, Fsh, Fxyd5, Gpcr, histone H3, Hsp70, Htr2b, Ide, insulin, Mapk, Nfkb (complex), Pdgf Bb (Pdgfb), Pi3k (complex), Pka (family), Pkc(S), Plc (Hspg2), proinsulin, Ras homolog, Sct, Slc25a12, Src (family), Tiam1, Tpm4, ubiquitin, Uqcc2, Vegf, Wt1</i>
3	Cellular movement Immune cell trafficking Inflammatory response	25	13	<i>Ap1, Ccl4, collagen alpha 1, collagen type I (complex), collagen type II, collagen type IV, collagen(S), Csf3r, Ddx58, Erk1/2, Fcer1, fibrinogen, Hsd11b1, Ifn (family), interferon beta, Il1r1, Il1rn, Ldl, Litaf, Mmp7, Mmp8, Olr1, Pkc alpha/beta (Prkcalb), pro-inflammatory cytokine, Rhob, Saa, Sftpd, Sod, Tgf beta, Tlr, Tnf (family), Tnfrsf9, trypsin</i>
B	E7.25	6J PAE versus vehicle		
1	Cancer Gastrointestinal disease Hepatic system disease	99	64	<i>Aasdh, Abcb8, Aebp2, Aldh3b1, Amfr, Ammecer1, Anxa10, Apbb2, Arhgap23, Atp2a2, Atpase, Bag3, Banp, Bod1, Btbd1, Caap1, Cacybp, Carm1, Cammt1, Crebrf, Dars1, Eed, Entpd2, Faf1, Fxr2, G3bp1, Glud1, H1-0, Hdac, Hemgn, Hsp70, Hspa5, Hspa8, Hspd1, Hsph1, Klhdc2, Lrrc47, Map1lc3, Mapre1, Mettl17, Mpp6, Mrps27, Noa1, Nr2e1, Ogt, Pcbp1, Pex6, Ppp1r10, Pto1, Rabl3, Rbm26, Rbpms, Rif1, Rpa, Sfpq, Slc35e1, Slco2b1, Smc4, Spg7, Tmem165, Trim29, Trmt12, Trmt44, Tssk1b, Ube2m, Ubxn2b, Ugt2b17, Vegf, Ywhah, Znf746</i>
2	Cell morphology Drug metabolism Endocrine system development and function	89	60	<i>Alkbh5, Asb13, B4galt4, Bbc3, Bbs4, Bmt2, Brd7, Brpf3, Cables2, Capns2, Ccnt1, Ccny, Cct3, Cct4, Cdc73, Cnppd1, cofilin, Ctbp1, Ctdp1, Dazap2, Dnaja1, Eif4a2, Ercc8, Fam91a1, Fkbp5, Gcn5l, Gmcl1, Gtf2e2, histone, histone H3, Holo RNA polymerase II, Inpp5k, Ipmk, Kdm1a, Kiaa2013, Mark3, Mbd3, Mcmbp, Mettl3, Mi2, Mlc1, Nars1, Nurd, Oma1, Orc3, P-Tefb, P38 Mapk, P4ha1, Pard3, Pard6b, Parp16, Paxbp1, Per1, Pmpcb, Ppp2r2d, Rhof, Rsrc2, Slc6a12, Snx5, Spata20, Spata3, Srsf2, Srsf9, Stip1, Strm3, Suv39h2, Tada3, Taf10, Taf5, Tip60</i>
3	Cancer Cellular development Tissue development	84	58	<i>5730488b01rik, Ahcy11, Alp, Alpg, Asb15, Bmp1, Cbx6, Chac1, Cirbp, collagen alpha 1, Commd3-Bmi1, Coq2, Ctbp, Erk, Exog, Exosc6, farnesyl transferase, Fgf, Gata6, Get4, hedgehog (family), Hes1, Hoxa9, Hoxd10, Leng8, Loxl1, Luc7l, Mafg, Mllt1, Mocs3, Mrps30, Napsa, Nog, Nop56, P3h2, Pip4k2a, Plcd3, Prc2, Prl2c2 (includes others), Prmt2, Psca, Ptch1, Rangap1, Riox1, Riox2, Rnr, Rrs1, Sdr39u1, Slfn12l, Smarca5, Sox, Sox17, Sox18, Sox2, Sox4, Spout1, Tcf, Tigd5, Tle1, Trmt10c, Trmt11, Tsc22d1, Ugcg, Wars2, Wdr77, Wnt, Yars2, Ypel5, Zc3h14, Zmynd19</i>
C	E7.25	6N PAE versus vehicle		
1	Cancer Gastrointestinal disease Organismal injury and abnormalities	94	60	<i>26s proteasome, Ankrd13a, Apc (complex), Arrdc1, Aven, Azin1, Bmt2, Btbd1, C1ql4, Caap1, Cab39, Cab39l, Cammt1, casein, Ccar1, Ccdc149, Cd200r1, Cdyll, Ctcf, Cxhc1, Exog, Gmcl1, Haus4, histone, Hivep3, Hoxc10, Kdm4b, Krt75, Leng8, Lypd3, Map4k2, Mark3, Mex3c, Mfhas1, Micu2, Mif4gd, Nfkb (complex), Nr2e1, Parp, Parp16, Pdk2, Pip4k2a, Pip4k2b, Pkhd1, Plcd3, Prickle3, Prkaa, Pxx, Rbm26, Rcbtb2, Rnf25, Serpina12, Smurf1, Sult2b1, Tbrg4, Traf, Trib3, Trim13, Trim44, Trim69, Trmt11, Ube2, Ube2g2, Ube2q2, ubiquitin, Utp4, Znf263, Znf414, Znf761, Zranb1</i>
2	Cell cycle Cellular assembly and organization DNA replication, recombination and repair	94	60	<i>Actr5, alpha catenin, Anapc4, Atpase, Bbs4, Bcor, CbplP300, Ccdc117, Ccnl2, Cdc73, Cdipt, Ceacam, Ceacam20, Colec12, Dars1, Daxx, Fam161a, Fam91a1, Fbxo17, Fbxw21 (includes others), Figla, Fsd2, histone H3, Hnmph1, Hspa8, Igdcc3, Il1bos, Ilf2, Kdm1a, Klhl15, Lman2l, Mapk, Mapre1, Mettl3, Mi2, Myo19, Nlrp4b, Nsun4, Orc3, P-Tefb, Padi6, Pex6, Phkg2, Polr3h, Ppp2ca, Prkab1, Rbbp4, Rbpj, Rgs13, RNA polymerase II, Rnr, Samd7, Smarca5, Sohlh1, Sp9, Spata19, Spice1, Spout1, Srsf2, Supt16h, Tcf19, Tmem25, Troap, Tssk1b, Tuba4a, Ythdc1, Ythdf1, Zbtb1, Znf639, Znf804a</i>
3	Gastrointestinal disease Neurological disease Organismal injury and abnormalities	59	44	<i>Akr1c4, amylase, Ankrd39, Anxa10, Arrb2, Bag3, Bdkrb1, calmodulin, Card19, Cg, Chm3, Ck2, Cpa3, Csnk1g3, Cyp4a11, Dazap2, Efcab5, Exoc3, Ffar2, focal adhesion kinase, Fsh, Gbp6, Gnrh, Gpcr, Gpr160, Gpr4, Gpr50, Gpr88, GTPase, hemoglobin, Hsp90, Htr1d, Htr2b, Ice2, Ikk (complex), Insulin, Klif1, Mtorc1, Ncbp2, Pka (family), Plc, Prmt3, Pstpip2, Ptpzr1, Rab5c, Rac1, Ras homolog, Rnf208, Rxfp1, secretase gamma, Sfk, Shc1, Slc8b1, Slitrk1, Smarcal1, Src (family), Sstr1, Stat, Stxbp4, Tas1r2, Tcf, Tcr, Tmem17, Tnk1, Tpcn2, Tsc22d1, Tsc22d2, tubulin, Vamp2, Vegf</i>

Continued

Table 1. Continued

	Diseases and functions	$-\log_{10}$ (Fisher's exact <i>P</i> -value)	Molecules in network	Molecules
D	E7.5	6J PAE versus vehicle		
1	Dermatological diseases and conditions Lipid metabolism Organismal injury and abnormalities	75	70	<i>A4galt, Abhd17b, Agrtrap, Arl14ep, Aunip, Carmil2, Casq1, Casq2, Ccdc184, Ccdc89, Ctnnbip1, Daglb, Dolpp1, Eaf1, Ensa, Epb4115, Eri2, Extl1, Faf2, Fam114a1, Fam234a, Fbxo28, Gbx2, Gdf5, Gon7, Gpank1, Grb2, Grina, Hcn2, Hip1r, Ick, Inca1, Jsrp1, Kbtbd2, Klhdc3, Klhl36, Klk14, Lrrc8a, Lrrc8e, Lyg2, Map3k6, Ntf4, Pard6g, Pkp3, Plcd1, Plcd3, Plekho2, Pnma2, Pnma8a, Pop7, Ppp2r2d, Prap1, Prickle3, Rp1l1, Selenbp1, Snapc1, Snapc3, Spata2l, Stambpl1, Syt16, Tmem102, Ttyh2, Tulp1, Ubxn2a, Ubxn2b, Ubxn7, Ulk2, Vezt, Zfand2b, Zmym6</i>
2	Cell death and survival Cell morphology Cellular compromise	75	70	<i>Adat1, Anln, Anxa6, Anxa7, Atxn7l3b, C18orf54, C19orf44, Canx, Casd1, Ccdc127, Cdv3, Cipc, Dctd, Def6, Dpep3, Dynll1, Eed, Elavl1, Eml2, Fam76a, Fam83f, Gipc1, Gk5, Gpm6b, Gsn, Gvin1 (includes others), Hm13, Hpd1, Inpp1f, Kcng3, Lgals3bp, Lmf1, Lrrc1, Lrtomt, Mfsd13a, Mkm2os, Mob3a, Myo1c, Osgin2, Pafah1b1, Pcmtd1, Pink1, Plekhg6, Proca1, Prcc1, Pskh1, Pwp2, Rbmxl1, Rbmxl2, Rdh13, Sfn, Slc50a1, Slc66a2, Smim14, Surf6, Thap3, Tmem189, Tmem53, Tmem68, Trim14, Trim25, Ttc7a, Uros, Vim, Wdr41, Ywhah, Zbtb41, Zfx, Znf354b, Znf91</i>
3	Cancer Connective tissue disorders Organismal injury and abnormalities	72	69	<i>Arl8a, Arpc1b, Cd101, Cenpb, Clrn1, Cmb1, Cntn2, Col11a1, Col4a5, Col8a1, collagen, Ctsf, Dhrr7b, Dnlz, Errf1, Esyt1, Fam13b, Fbxl2, Foxl1, Hells, Hydin, Jagn1, Kiaa0930, Klhl21, Klhl26, Me1, Metap1, Mllt10, Mxd3, Mxi1, Naa16, Naa40, Nars1, Ndufaf4, Nfxl1, Nol9, Nrp1, Pdrgr1, Pef1, Pogk, Polr2m, Ppp1r16a, Puf60, Pxn, Rabgggb, Rbm22, Rcbtb2, Rfwd3, Ripk4, Rmnd5b, Rnf146, Rnf19b, Rpsud1, Samhd1, Sema6c, Smarcd1, Tmpo, Topors, Tram2, Trim31, Tspan11, Ube2d4, Ubox5, Ubtd1, Vwa1, Wars2, Ypel5, Zfp42, Zg16, Zmynd19</i>
E	E7.5	6N PAE versus vehicle		
1	Cell morphology Embryonic development Hair and skin development and function	70	49	26s proteasome, <i>Adgrg6, Azin2, calmodulin, Col4a6, complement, Cxcr6, Dmc1, Dnajc6, Etv1, Fam222a, Fbn2, Fbxo6, Fpr1, Hemgn, Hnrnpk, Hspd1, Igg1, Igm, Il12 (complex), Il12 (family), Il1bos, Iqcb1, Kcnn1, Kcnn2, keratin, Klhl22, Kpnb1, Krt16, Krt26, Krt33a, Krt35, Krt37, Krt73, Krt75, Krt86, Kyat1, Lgals3bp, Mapk, Mkm3, Ndufa4l2, Noa1, Notch2, Notch3, ornithine decarboxylase, P-Tefb, Pbx1, proinsulin, Pspc1, Ras, Rbm14, RbmX, RNA polymerase II, Sdr39u1, Sfpq, Sil1, Six2, Slc25a20, Speg, Sstr4, Stat, Syt2, Tbx19, Tcea2, Trim28, Trim31, Tuba4a, tubulin, Ubr1, Znf462</i>
2	Cardiovascular disease Cell death and survival Cellular assembly and organization	70	49	<i>Adss1, Ak1, Ankrd37, Aven, Ccdc155, Ccnjl, Cga, Cldn13, Coq8b, Dio2, Dnpep, Eglh, Fcn1, Fhl2, Gata2, Gmppa, Gpr158, Gpr25, Gpr37l1, Hebp2, Hmx1, Hnrnp1, Hnrmpu, Htr3b, Igsf9, Krtcap3, Kyat3, Lhfp15, Lrrc57, Lyg2, Maml2, Marchf2, Mkm2os, Morn1, Mtnr1b, Nanos2, Nr3c1, Nsg1, Nudt13, Odf4, Pdzhp1, Pitx2, Prss56, Ptgr1, Retnlg, Rhag, Rhcel/Rhd, Rhox3a (includes others), Rnf122, Rtn4r11, Scube1, Selenbp1, Serp2, Serpinb9f (includes others), Serpini2, Slc44a4, Smim14, Sox13, Spire2, Stag3, Sifa2l/Stfa2l1, Syng1, Terb1, Tgfb1, Trim25, Trim58, Ubc, Upk3b, Wdpcp, Wdr34</i>
3	Lipid metabolism Nucleic acid metabolism Small molecule biochemistry	58	43	<i>Acss2, Acvr2b, Alp, Alpl, Ampk, Bcl11a, Bmp15, Bspry, calcineurin protein(S), caspase 3/7, Cbpl/P300, Ccnd2, collagen alpha 1, Ctlp, Cuedc2, cyclin A, Cyp11a1, Cyp2c40 (includes others), Cyp2c8, Cyp2e1, Cyp4f11, Dkk1, Elov16, Emid1, Etv6, Fasn, Frizzled, Fsh, Gzmh, Hdac, histone H3, histone H4, Hsp70, Jnk, Kat2b, Khk, Lmcd1, Mef2, Mef2c, Mgmt, Mlycd, N-Cor, Nadh2 or Nadph2, oxygen oxidoreductase, Ncs1, Nr1h, P70 S6k, Prc2, Prkag2, Proc, Qki, Rab29, Rb, Rbm15, Sat2, Scd2, Six3, Smad2l3, Sp7, Tcf, Tgf beta, Tgm2, Tnfrsf14, Tnn, Tp63, Trerf1, unspecific monoxygenase, Vgll2, Vill, Wnt1</i>

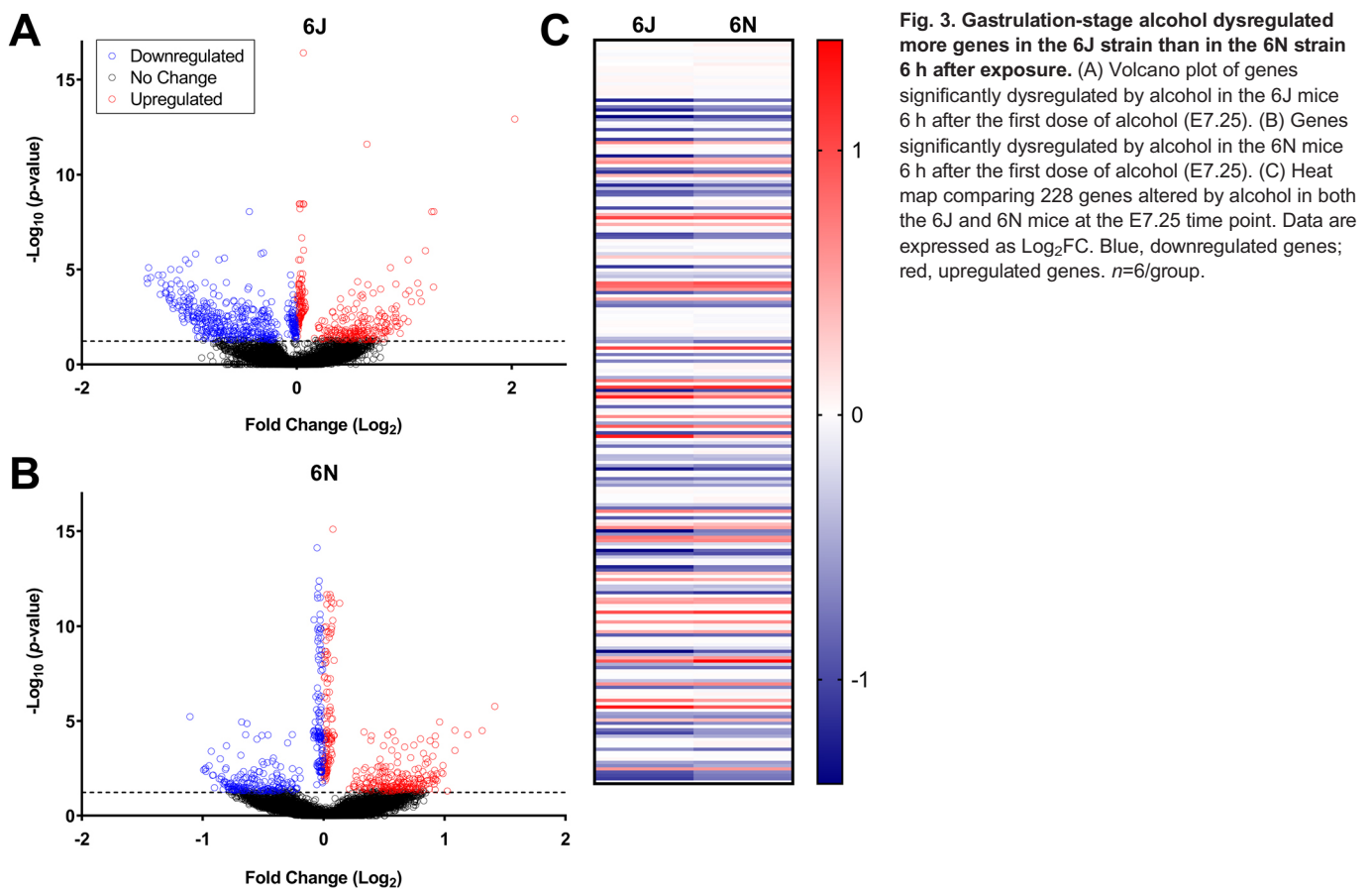
(A) Baseline (E7.0). (B,C) 6 h after alcohol in 6J (B) and 6N (C) mice. (C,D) 12 h after alcohol in 6J (D) and 6N (E) mice.

specific methyltransferases targeted by PAE included *Kdm1a*, *Kdm4b*, *Mettl3*, *Mettl4*, *Mettl16* and *Prdm5*, among others. Gene network analyses also revealed pathways related to organ and tissue disease, cell cycle/DNA replication and repair, and cell and tissue morphology (Table 1C; Table S2E). The two most downregulated genes were *Rsrp1*, which encodes the relatively unknown protein arginine/serine rich protein 1, a target of heat shock protein 1 under certain conditions (Korfanty et al., 2014), and *Alyref*, described above. *Tap2*, a transporter protein involved in multi-drug resistance and antigen presentation through localization of peptides to the ER, where they are then transported to the cell surface, and *Sox15*, a member of the Sox family, were the two most upregulated genes. The Sox family is comprised of transcription factors that play vital roles in embryonic development and specification of cell fate.

*Sox15* expression is highest in undifferentiated embryonic stem cells (Maruyama et al., 2005), suggesting that PAE may disrupt cell differentiation in 6N mice, resulting in increased expression of *Sox15*. Overall, although PAE causes a reduction in cellular activity that could disrupt proliferation and cell fate decisions, there is no evidence that cell death pathways are activated at this point in the 6N strain, a notable difference from the 6J strain.

#### Large-scale strain-specific differences in transcriptional response to PAE apparent 12 h after exposure

To explore how strain differences continue to modulate the transcriptional landscape 12 h after alcohol exposure, we next compared the effect of PAE on embryonic gene expression in each strain at E7.5. At E7.5 (12 h post-PAE), the 6J strain continued to

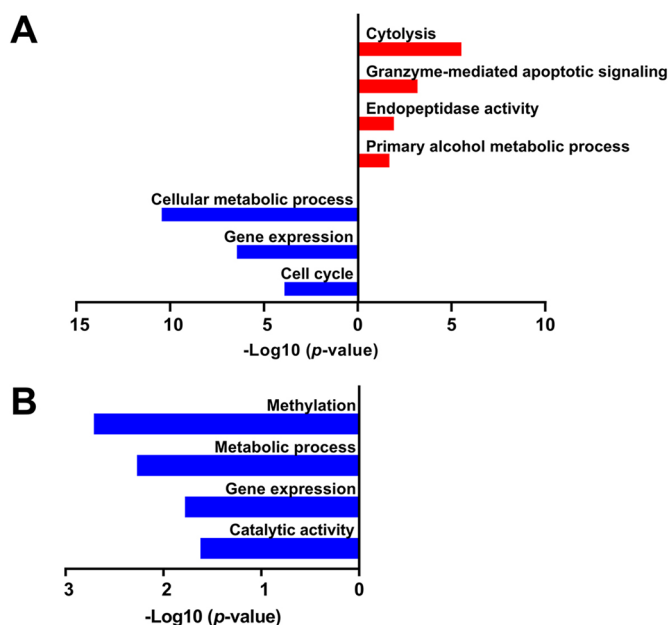


have more pronounced gene expression changes relative to the 6N strain; in fact, the number of differentially expressed genes increased over threefold in the 6J strain, whereas it remained relatively stable in the 6N strain. In the 6J strain, 2987 genes were differentially expressed 12 h after PAE. Of these, 1641 were upregulated (54.9%) and 1346 were downregulated (45.1%) (Fig. 5A). Conversely, only 641 genes were altered by PAE in the 6N strain at this time point, with 366 upregulated (57.1%) and 275 downregulated (42.9%) (Fig. 5B). The significant increase in the number of differentially expressed genes in the 6J but not the 6N strain provides further evidence that 6J mice are more sensitive than 6N mice to PAE; 291 genes in total overlapped between the two strains (Fig. 5C). While most genes significantly altered by PAE in both strains showed the same direction of change, most of the overlapping genes were upregulated at E7.5, compared to most overlapping genes being downregulated in both strains at E7.25.

Functional profiling of the genes upregulated 12 h following PAE in 6J mice revealed pathways related to intracellular signaling, protein transport and localization, and cell death (Fig. 6A; Table S7). One of the upregulated pathways we identified in 6J mice was ‘Formation of xylulose-5-phosphate’. Xylulose-5-phosphate is a ketose sugar known to promote gene transcription through the ChREBP transcription factor (encoded by *Mlxipl*). This was interesting because *Mlxipl* was itself significantly upregulated in the 6J mice at this time point. ChREBP is part of the Myc superfamily and has been found to affect cell proliferation through regulation of transcription of cyclins in certain cell types (Filhoulaud et al., 2013; Tong et al., 2009), although its exact function in early gestational embryos is not known. In addition, ChREBP has multiple isoforms and can be stored in an inactive

form. The downregulated genes in the 6J mice were enriched for embryonic organogenesis and skeletal development, including the head, palate and circulatory system. Notably, holoprosencephaly, cleft palate, and abnormal lip, ear and face shape were identified using the Human Phenotype Ontology (HPO) database as phenotypes associated with PAE. These craniofacial malformations have been associated with heavy alcohol exposure during early gestation in the human population (DeRoo et al., 2008; Romitti et al., 2007; Johnson and Rasmussen, 2010; Jones et al., 2010). Analysis of *de novo* gene networks found differences in pathways related to organismal injury and abnormalities, cell death, organ disease, embryonic development, and protein and RNA post-translational modifications of RNA and proteins (Table 1D; Table S2F).

The most downregulated gene in the 6J strain at E7.5 was *Shh*. Multiple other members of the Shh pathway were also downregulated by PAE in the 6J strain at this time point, including *Ptch1*, *Smo* and *Gli3*. In contrast, *Gli3* was the only member of the pathway affected by alcohol in the 6N mice at either time point. Dysregulation of the Shh pathway is linked to craniofacial malformations such as holoprosencephaly in genetic ciliopathies (Brugmann et al., 2015; Chang et al., 2016), and PAE both downregulates Shh expression (Higashiyama et al., 2007) and causes more severe craniofacial and limb defects in transgenic mice lacking genes in the Shh pathway (Fish et al., 2017; Kietzman et al., 2014). If alcohol is more likely to impact Shh signaling in the 6J than the 6N strain, this presents one way in which 6J may be more likely to develop craniofacial and eye defects. Whether there is an association between the higher baseline expression of immune genes in the 6J strain and differences in Shh signaling after alcohol exposure is not yet clear, but these findings warrant further



**Fig. 4. Functional profiling of biological pathways enriched in the 6J and 6N strains 6 h after alcohol exposure (E7.25).** (A) 6J strain. (B) 6N strain.  $n=6/\text{group}$ .

exploration. *Efcab7*, a gene linked to Smo transduction in the primary cilia (Pusapati et al., 2014), was upregulated by PAE at E7.5 (+0.81 Log<sub>2</sub>FC). This gene had lower expression in the 6J strain relative to the 6N strain at E7.0 and E7.25, suggesting possible pre-existing differences between the strains; however, more work needs to be done to determine the exact role of *Efcab7* during gastrulation and, in particular, in relation to Shh signaling. Another downregulated gene, *Tcf21*, encodes transcription factor 21, a protein with varied and important functions during lung, kidney, heart and gonadal development (Braitsch et al., 2012; Quaggin et al., 1999; Tamura et al., 2001). Downregulation of the *Shh* pathway and *Tcf21* suggest serious and widespread defects in organogenesis in the 6J mice just 12 h following PAE, an effect that does not seem to occur in the 6N mice.

The top two upregulated genes in the 6J strain were *Tap2* and *Fam46b* (*Tent5b*), the first of which was also one of the top upregulated genes in the 6N strain at E7.25. *Fam46b* (*TENT5B* in humans) has recently been shown to be highly expressed in undifferentiated embryonic stem cells, with a sharp drop in expression following cell differentiation (Hu et al., 2020). While its biological functions remain to be fully elucidated, particularly in the embryo, *Fam46b* could play a role in cell cycle regulation as it inhibits cell proliferation in *in vitro* models of prostate cancer (Liang et al., 2018). Overall, these data indicate that PAE has a profoundly damaging effect in the 6J strain that is apparent within 12 h of exposure. In addition to the downregulation of Shh pathway genes, multiple genes regulating the p53 pathway were also dysregulated, including *Hif1a*, *Mdm2*, *Sirt1* and *Sco2*, indicating that cell proliferation, DNA damage repair mechanisms, cell cycle regulation and apoptosis are among the primary targets modulated by PAE in this strain. These data establish an association between baseline genetic variations between strains that lead to more deleterious outcomes in response to alcohol exposure.

Analysis of upregulated genes in the 6N strain indicated that PAE caused an increased inflammatory signaling response in these

embryos compared to controls, as well as catalytic activity and RAGE (AGER) receptor binding (Fig. 6B; Table S8). Increased Il17 signaling was also identified as an upregulated pathway in this dataset, further supporting that PAE is causing immune signaling activation, which could have downstream effects on cell survival and tissue growth. Multiple phenotypes related to hypoxemia were found to be upregulated in the 6N strain using the HPO database, indicating that PAE could be affecting cellular oxygen levels up to 12 h later. Analysis of downregulated genes in the 6N strain revealed pathways related to overall cellular activity, DNA binding, and skeletal and neuronal development. Network analysis revealed that pathways related to cell morphology, embryonic development, cell death, cellular metabolism and inflammation were also altered by PAE in the 6N strain at E7.5 (Table 1E; Table S2G).

The top downregulated genes in the 6N strain at E7.5 were *Mef2c* and *Nkx2-5*. *Mef2c* encodes the transcription factor myocyte enhancer factor 2C (Mef2c) important for skeletal muscle and central nervous system (CNS) development. Humans with mutations in *MEF2C* exhibit severe intellectual disabilities, loss of muscle tone, mild craniofacial dysmorphologies and severe seizures. Transgenic mice with knockout of *Mef2c* display disorganized vasculature and cardiovascular defects. *Nkx2-5* encodes NK2 homeobox 5, known to be involved in heart development and highly expressed in the cardiac crescent cells at E7.5. Knockdown of this gene is embryonically lethal at ~E9-E10 and causes growth retardation and heart defects.

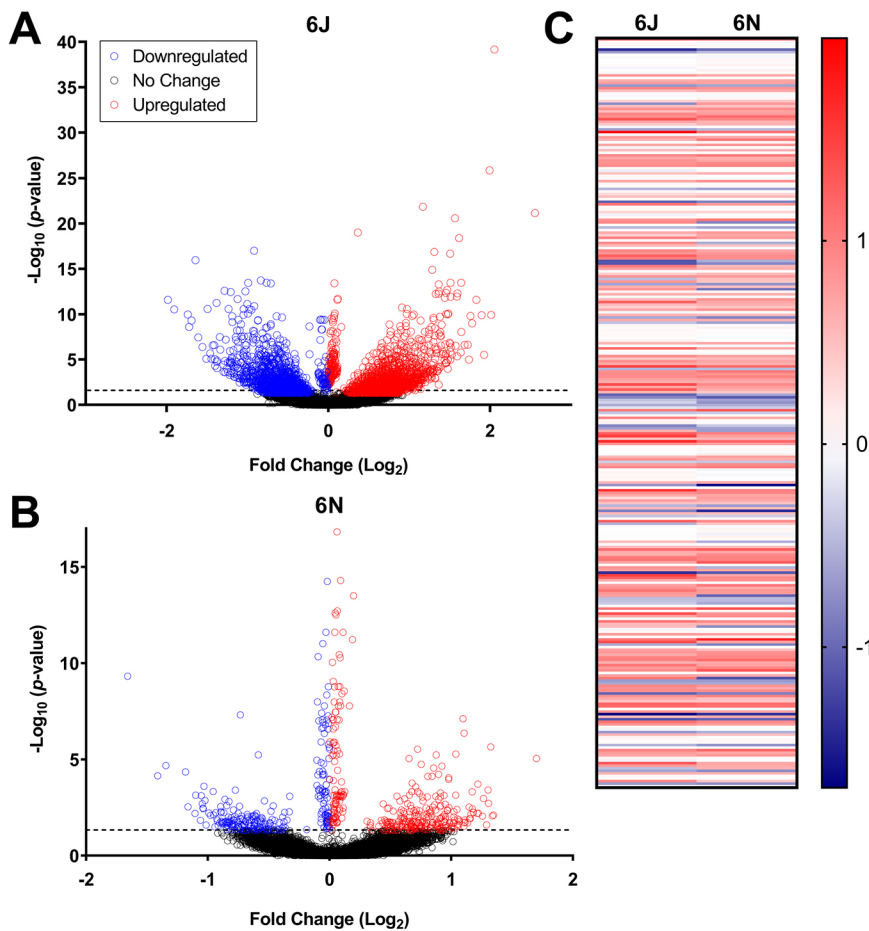
The top two upregulated genes were *S100a9* and resistin-like gamma (*Retnlg*). *S100a9* is a damage-associated molecular pattern molecule (DAMP) that makes a heterodimer with *S100a8* to create calprotectin, a protein complex that produces pro-inflammatory activity when secreted from neutrophils, although cells from a neutrophil lineage are not known to be present in the embryo during gastrulation (McGrath et al., 2014). Increased concentrations of extracellular *S100a9* and *S100a8* induce apoptosis and stimulate ROS production in certain cell types (Lim et al., 2011). *S100a9* is also known to interact with the RAGE receptor pathway, a part of the innate immune system and a primary receptor for Hmgb1, a protein previously shown to be part of the inflammatory response to alcohol in the adolescent and adult brain (Coleman et al., 2018; Vetreno and Crews, 2012). The function of *Retnlg* is largely unexplored, although it shares some similarity with human resistin (*RETN*), a hormone released by adipose tissue.

In summary, while PAE affects pathways related to embryonic development in the 6N strain, these pathways do not seem to be as clearly linked to craniofacial development as those identified in the 6J strain, possibly contributing to the phenotypic differences observed between these strains following PAE.

#### Limited overlap in PAE-induced transcriptional differences between the 6J and 6N strains 6-12 h after exposure

Only seven genes were differentially expressed following PAE in both strains at both time points (Fig. S8). Three of these genes – *Aven*, *Hist3h2a* and *Tbx1* – were strongly downregulated in both strains at both time points. *Aven* encodes the cell death regulator *Aven* protein, which inhibits apoptosis through suppression of pro-apoptotic Apaf1 and augmentation of anti-apoptotic BCL-X<sub>L</sub> activity and regulates the G2/M DNA damage checkpoint during cell cycle progression (Gross, 2008). Interestingly, this gene was also downregulated in the rostroventral neural tube of 6J mice 6 h after neurulation-stage alcohol exposure in a previous study (Boschen et al., 2020), revealing this gene as a marker of PAE across multiple models of FASD. The next gene, *Hist3h2a* (*H2aw*),





**Fig. 5. Gastrulation-stage alcohol dysregulated more genes in the 6J strain than in the 6N strain 12 h after exposure.** (A) Volcano plot of genes significantly dysregulated by alcohol in the 6J mice 12 h after the first dose of alcohol (E7.5).  $n=5$  vehicle-treated,  $n=6$  PAE. (B) Genes significantly dysregulated by alcohol in the 6N mice 12 h after the first dose of alcohol (E7.5).  $n=6$  vehicle-treated,  $n=4$  PAE. (C) Heat map comparing 228 genes altered by alcohol in both the 6J and 6N mice at the E7.5 time point. Data are expressed as  $\text{Log}_2\text{FC}$ . Blue, downregulated genes; red, upregulated genes.  $n=6/\text{group}$ .

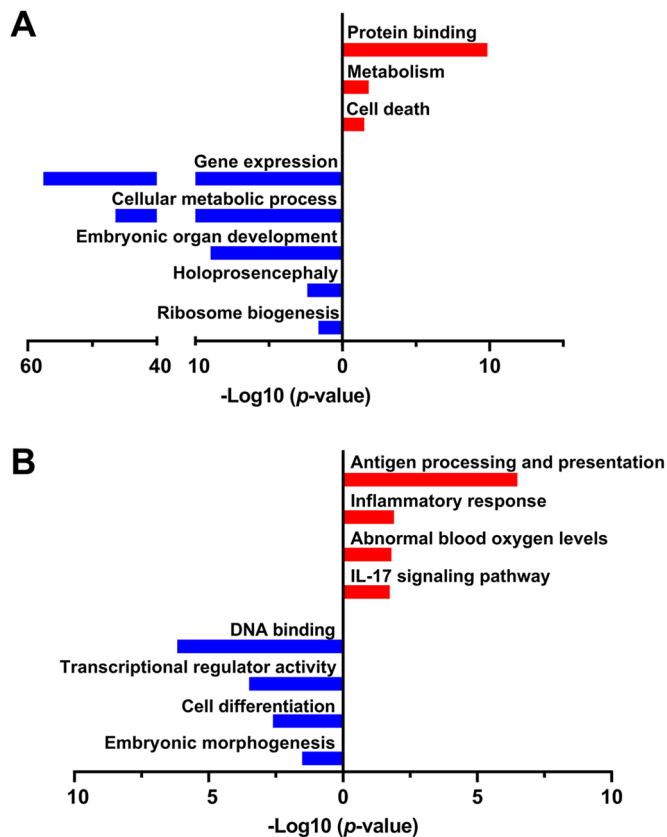
is translated into a core component of chromatin, histone H2A cluster 3. Chromatin dynamics regulate access of transcription factors to the DNA and control processes such as cell proliferation and differentiation. *Hist3h2a* was also found to be downregulated by neurulation-stage alcohol in a whole-embryo culture model derived from C57BL/6J mice (Zhou et al., 2011). The third downregulated gene, *Tbx1*, encodes Tbox-1, a well-studied transcription factor important for cell proliferation during embryonic development. Loss of *Tbx1* function is associated with 22q11 deletion/DiGeorge syndrome phenotypes, including heart defects, craniofacial abnormalities and cleft palates (Jerome and Papaioannou, 2001; Verdelli et al., 2017; Yagi et al., 2003). One of the genes that was upregulated in both strains at both time points was *Sdr39u1*, which encodes a short-chain dehydrogenase with oxidoreductase activity localized to the mitochondria and is thought to have a binding site on NADP. This protein has been identified as a possible biomarker candidate for neurodegenerative diseases such as Alzheimer's disease (Rahman et al., 2020), although its exact function is still under scrutiny. While there is little evidence directly linking *Sdr39u1* to the oxidative stress response, production of NADP is a key player in cellular antioxidation.

## DISCUSSION

Understanding variables that modulate prenatal alcohol sensitivity has been an important area of research given the well-known variability of outcomes in children exposed to alcohol *in utero* and in animal models of FASD. The wide range of signs and symptoms of PAE present problems not only for the diagnosis and treatment of individuals with FASDs, but for a complete understanding of the

pathogenic mechanisms of alcohol. The current study adds valuable information regarding the contribution of genetics to prenatal alcohol susceptibility by demonstrating that baseline genetic differences between two closely related mouse substrains can result in significantly different molecular responses to a teratogen such as alcohol. While only 80 genes differed between the alcohol-sensitive 6J strain compared to the 6N strain at E7.0, the 6J strain had significantly more genes dysregulated by alcohol 6–12 h later. Functional profiling also revealed that the biological functions affected by alcohol in the 6J mice differed from those identified in the 6N mice. Gene expression pathways related to cell proliferation, apoptosis, and those controlling craniofacial and brain development were affected in the 6J embryos. In contrast, cellular metabolism, hypoxemia, and inflammation pathways were altered in the 6N embryos. Overall, these data indicate that gastrulation-stage alcohol exposure might alter cell proliferation in both strains, but apoptosis pathways are more strongly enriched in the 6J strain, likely contributing to the increased incidence of eye defects following PAE in the 6J compared to the 6N fetuses (Dou et al., 2013).

The most well-studied difference between the 6J and 6N strains is the *Nnt* mutation. *Nnt* is a component of the mitochondrial inner membrane that passes hydrogen atoms that are then used in the conversion of  $\text{NADP}^+$  to  $\text{NADPH}$ , an important co-enzyme that regulates metabolism along with  $\text{NADH}$ . A primary function of  $\text{NADPH}$  is the removal of ROS from the mitochondria (Fig. 7).  $\text{NADH}$  is the reduced form of  $\text{NAD}^+$ , and these co-factors are important for redox metabolism, cellular respiration and ATP production. In addition,  $\text{NADPH}$  converts glutathione from the oxidized (GSSG) to the reduced (GSH) state via glutathione



**Fig. 6. Functional profiling of biological pathways enriched in the 6J and 6N strains 12 h after alcohol exposure (E7.5).** (A) 6J strain. (B) 6N strain.  $n=6$ /group.

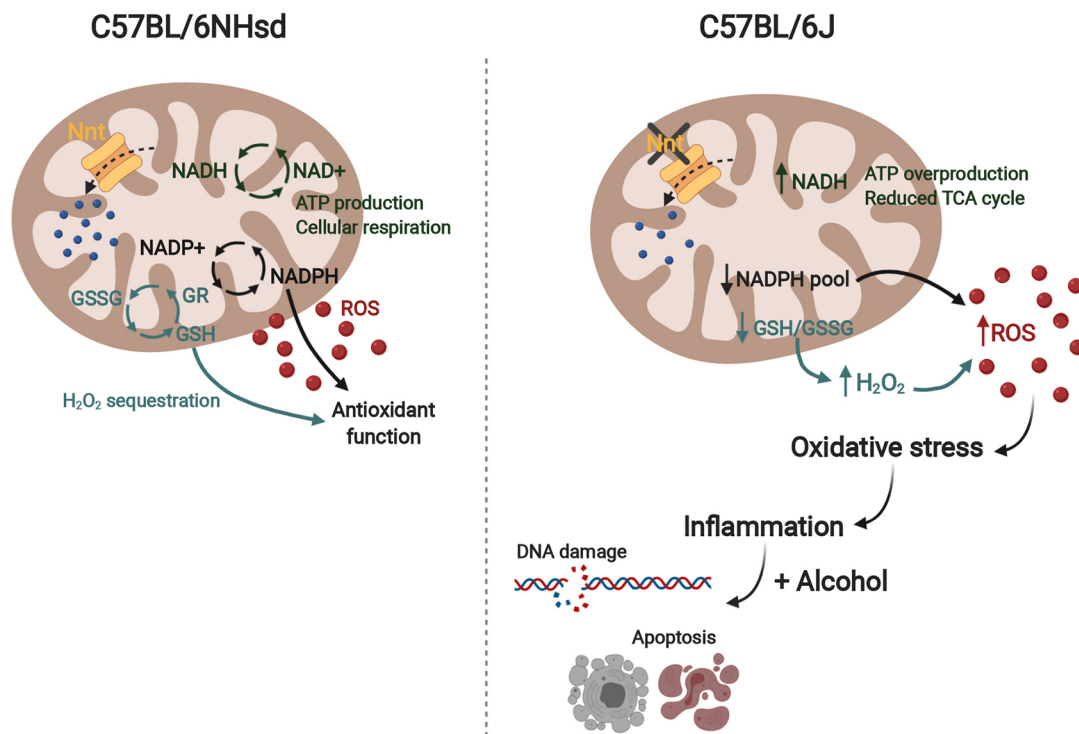
reductase (GR). GSH neutralizes ROS and sequesters and eliminates  $H_2O_2$ . Reductions in *Nnt* disrupt the NADPH/NADH balance, causing smaller NADPH pools and lower GSSG/GSH conversion, with less capacity for ROS removal, as well as increased NADH, leading to an overproduction of ATP and dysregulation of glycolysis and the tricarboxylic acid cycle. Although little research has been done on the effect of the *Nnt* mutation on ROS levels in the 6J embryo, endothelial cells from 6J mice exhibit increased superoxide production after angiotensin II stimulation and reduced glutathione peroxidase activity compared to 6N mice (both substrains obtained from The Jackson Laboratory), indicating altered mitochondrial function as a result of the *Nnt* mutation (Leskov et al., 2017). In addition, the *Nnt* mutation has been shown to be a modifier of other genetic mutations, such as *Bcl2l2* (Navarro et al., 2012) and mitochondrial superoxide dismutase (Huang et al., 2006). Increased DNA damage and altered immune signaling have been observed in the 6J strain compared to others in response to other chemical and environmental stressors, including in the lung after exposure to 1,3-Butadiene, a carcinogenic inhalant (Chappell et al., 2017), and in the brain following postnatal hypoxic ischemia (Wolf et al., 2016), although the specific effects seem to be exposure, organ and age dependent. Aberrant *Nnt* function has also been implicated in cancer, indicating a possible role in cell growth (Ho et al., 2017). A build-up of ROS as a result of the *Nnt* mutation could predispose the embryo to be sensitive to external stressors such as alcohol exposure, which produces oxidative stress on its own (Brocardo et al., 2011; Henderson et al., 1995, 1999).

Oxidative stress can induce inflammation and expression of pro-apoptotic molecules NF- $\kappa$ B and p53 and oxidative stress proteins

HIF-1 $\alpha$  and PPAR- $\gamma$  (Reuter et al., 2010). The *Nnt* mutation has also been directly linked to increased expression of HIF-1 $\alpha$  in the mouse liver. This molecule is critical for cellular response to hypoxia and can protect against oxidative stress. While *Hif1a* expression did not differ between the strains at baseline, it was downregulated by PAE in the 6J strain at the E7.5 time point. The current study found that the 6J strain had increased expression of genes related to inflammation at baseline. The 6N mice showed an upregulation of inflammation-related genes at E7.5, whereas the 6J mice did not show many alcohol-induced changes in inflammatory pathways at either time point, possibly due to the fact that immune signaling genes were already comparatively activated in the 6J mice at baseline. Interpretation of the upregulation of inflammatory signaling in the 6J relative to 6N strain at E7.0 is limited because the exact function of immune molecules during gastrulation remains under investigation. Early macrophages are detected in the yolk sac during neurulation (~E9 in mice) (Naito, 2008), whereas cells from a neutrophil lineage do not emerge in the embryo until E11.5 (McGrath et al., 2014), far after the time points under observation here. However, cytokines and chemokines have been suggested to play a role in cell migration (Nair and Schilling, 2008; Katsumoto and Kume, 2011), cell adhesion and tissue remodeling during gastrulation (Aller et al., 2014). In addition, it is possible that some of the pro-inflammatory signals are due to transfer from maternal circulation or the placenta. A direct link between the timing of immune signaling activity (higher at baseline in the 6J mice versus PAE-induced activation in the 6N mice) and differences in alcohol sensitivity between the strains remains to be determined.

Motile and immotile cilia play important roles throughout embryonic development. Previous work from our laboratory has demonstrated that alcohol administered during neurulation alters over 100 cilia genes in the neural tube within the first 6 h after exposure (Boschen et al., 2020). During gastrulation, motile cilia in the primitive node beat to create a morphogenic gradient that regulates left–right asymmetry. Previously, we have shown that knockdown of the cilia gene *Mns1* results in increased incidence and severity of ocular and craniofacial defects following gastrulation-stage alcohol exposure (Boschen et al., 2018), indicating a possible role for cilia dysfunction in the development of prenatal alcohol-related birth defects. Cilia-related genes in the current dataset were identified through comparison of each gene list with the CiliaCarta compendium (Van Dam et al., 2019). Gastrulation-stage alcohol exposure altered cilia-related genes in both strains but to a greater degree in the 6J strain (25 cilia genes in the 6J mice versus 21 genes in the 6N mice at E7.25; 101 cilia genes in the 6J mice versus 24 genes in the 6N mice at E7.5; Table S9). Immotile cilia, called primary cilia, are responsible for transduction of the Shh pathway, as Smo is trafficked into the cilia following binding of Shh to Ptch1, and the Gli transcription factors are processed within the cilia axoneme. In the 6J mice, multiple genes within the Shh pathway (*Shh*, *Ptch1*, *Smo*, *Gli3*) were downregulated 12 h after alcohol. This time point also coincided with a relatively large increase in the number of cilia genes dysregulated by alcohol in this strain compared to the 6N strain. Further investigation is needed to determine whether the cilia genes altered by alcohol exposure in the 6J strain are directly related to the downregulation of Shh pathway genes or are indicative of any significant motile or immotile cilia dysfunction.

The 6J and 6N strains are widely used to study the effects of prenatal drug exposure. Factors such as timing of alcohol exposure (gastrulation versus neurulation), time elapsed between alcohol administration and tissue collection [e.g. 3 h in Green et al. (2007),



**Fig. 7. Schematic representing a hypothetical mechanism contributing to differences in alcohol sensitivity between the 6J and 6N strains.** The *Nnt* mutation in the 6J strain could affect reactive oxygen species (ROS) breakdown in the mitochondria, leading to higher baseline oxidative stress and inflammation. In the presence of alcohol, 6J mice would undergo increased apoptosis and DNA damage, ultimately resulting in more severe craniofacial and CNS anomalies. GR, glutathione reductase; GSSG, glutathione disulfide; GSH, glutathione; NAD<sup>+</sup>/NADH, nicotinamide adenine dinucleotide (+ hydrogen); NADP<sup>+</sup>/NADPH, nicotinamide adenine dinucleotide phosphate; Nnt, nicotinamide nucleotide transhydrogenase.

6–12 h in our study], and specific tissue type assessed (head fold tissue versus whole embryo) contribute to the differences between previously published gene expression profiles and those reported here. Our previous work (Boschen et al., 2020) sequenced RNA collected from rostroventral neural tube tissue 12 h or 24 h after alcohol in the 6J strain only. Despite methodological differences between these experiments, common targets of alcohol are apparent when the studies are compared. Mitochondrial function and ribosome biogenesis have been reported to be disrupted in multiple models of FASD (Green et al., 2007; Garic et al., 2014; Boschen et al., 2020; Berres et al., 2017) and identified as downregulated pathways in the 6J PAE-treated embryos at E7.5 in the current study. Compromised ribosome biogenesis and mitochondrial function could be indicative of impaired cell growth as synthesis of ribosomes is necessary for cell cycle progression. Cell motility and adhesion have also been determined to be targets of alcohol during early gestation (Dou et al., 2013; Boschen et al., 2020; Green et al., 2007). Cell motility was upregulated in the 6J versus 6N mice at E7.0 in the current study; however, pathways related to cell movement were not enriched by PAE at either time point. Finally, competition between alcohol and retinoic acid (RA) as a mechanism of prenatal alcohol pathogenesis has been a long-standing hypothesis in the field (Deltour et al., 1996; Johnson et al., 2007). Although the current study did not find statistical enrichment of any RA pathways, three genes related to RA signaling were dysregulated after PAE: lecithin retinol acyltransferase (*Lrat*; +0.42 Log<sub>2</sub>FC in 6J mice at E7.25), retinoic acid receptor- $\alpha$  (*Rara*; +0.46 Log<sub>2</sub>FC in 6N mice at E7.25) and cellular retinoic acid binding protein 2 (*Crabp2*; -1.04 and -0.87 Log<sub>2</sub>FC in 6J and 6N mice, respectively, at E7.5). However, interpretation of these single genes is difficult in the absence of

other changes to the pathway. RA has been shown to be a regulator of *Shh* signaling (Ribes et al., 2006; Helms et al., 1997), which was significantly downregulated in the 6J strain 12 h after PAE, although it is beyond the scope of this study to determine whether this change was related to RA signaling.

These data provide information about gene expression patterns in two widely used strains of mice across normal gastrulation and in response to a teratogen. The web tool created to allow for exploration of the dataset visually demonstrates the dynamic nature of certain genes across gastrulation (e.g. *Shh* increases expression over time, *Fgf5* shows reduced expression). The tool will also provide a valuable resource during experimental design, as there are significant differences in gene expression between the two strains that might support the use of one over the other for certain paradigms. The future directions of this study will explore the nuances of gene expression profiles in these two strains, including whether biological sex contributes to prenatal alcohol sensitivity. While all time points used in this study occur prior to gonadal sexual differentiation, differences in gene expression and growth rates have been reported between male and female pre-implantation embryos (Deegan et al., 2019; Werner et al., 2017). Although no sex differences were apparent in the differentially expressed genes in this study, as determined by the consistency between samples (Figs S1–S7), this question needs to be fully explored. In addition, our study used whole embryo tissue, whereas newer sequencing technologies such as single-cell and spatial transcriptomics will allow for investigation of localized mRNA expression patterns, spatiotemporal cell–cell interactions, and a direct link between gene expression and tissue morphology in the gastrulating embryo.

In conclusion, our study demonstrates that a pre-existing genetic susceptibility can mediate sensitivity to teratogens such as alcohol

in mice. Not only did the sensitive 6J mice show a larger response to PAE in sheer number of genes/biological pathways affected, but pathways regulating cell death, proliferation, and craniofacial and CNS development were altered to a greater degree in this strain. We hypothesize that the known mutation in *Nnt* in the 6J strain predisposes these embryos to have increased expression of inflammatory signaling genes than make them more sensitive to the addition of an external stressor such as PAE (Fig. 7). Understanding how genetic variability can mediate risk and resiliency to PAE can help elucidate how alcohol acts on the embryo at the cellular level and, ultimately, assist in identifying candidate genes as biomarkers of PAE in the human population.

## MATERIALS AND METHODS

### Animals

Male and female adult C57BL/6J (The Jackson Laboratory, Bar Harbor, ME, USA; Stock #000664) and C57BL/6NHsd (Envigo, Indianapolis, IN, USA) mice (*Mus musculus*) were obtained. Males were housed singly and females were housed in groups up to five in standard polycarbonate cages with cob bedding, shelter and nesting material. Mice had *ad libitum* access to food (Prolab Isopro RMH 3000, LabDiet, St Louis, MO, USA) and water and were maintained on a 12:12 h light/dark cycle. Up to two female mice were placed into the cage of a male for each 2 h mating session. Upon discovery of a vaginal plug, E0 was defined as the beginning of the mating session (Fig. 1). All experimental procedures were approved by the Animal Care and Use Committee at The University of North Carolina at Chapel Hill (UNC) and were performed in accordance with NIH Guidelines (Approval #18-203). On E7.0, dams were weighed and pregnant dams were either dissected immediately or assigned to one of the experimental treatment groups.

### Alcohol exposure paradigm (PAE)

On E7.0, dams were administered two doses of 2.9 g/kg ethanol (25% vol/vol; Pharmaco-Aaper, Brookfield, CT, USA) in Lactated Ringer's solution 4 h apart via intraperitoneal injection (Fig. 1). This dose and pattern of alcohol exposure results in maternal blood alcohol concentrations of ~400 mg/dl (O'Leary-Moore et al., 2010). Control mice were administered an equal volume of Lactated Ringer's solution (1.5 ml/100 g body weight).

### RNA isolation

RNA was collected from embryos either before alcohol administration (E7.0) or 6 h or 12 h after the first alcohol injection (E7.25 or E7.5) (Fig. 1). Dams were sacrificed via CO<sub>2</sub> followed by cervical dislocation, and embryos were dissected from the placenta. All extraembryonic tissue was removed and embryos were stage matched based on morphological assessment (Theiler Stages 10-11; representative image in Fig. 2A). A total of six embryos per group were used, with no more than two embryos collected per litter to minimize litter effects. Sex was not considered as a biological variable as all time points occur prior to gonadal sexual differentiation. RNA was isolated using an RNeasy Plus Micro Kit (Qiagen, Germantown, MD, USA), and RNA concentrations and purity were assessed using a NanoDrop 2000 and Qubit 3.0 Fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). A separate group of samples was collected at the E7.0 time point and isolated for validation of gene expression using quantitative reverse transcription PCR (qRT-PCR). Expression of *Wdfy1*, *Entpd4* and *Efcab7* was analyzed in each strain and found to validate the RNA-seq results from this time point (Fig. S9). All samples were run in triplicate ( $n=6$ /strain).

### RNA-seq

A total of six samples per group were submitted for sequencing. Libraries for RNA-seq were prepared using the SMARTer Ultra Low Input RNA (Clontech, Mountainview, CA, USA) and Nextera XT DNA (Illumina, San Diego, CA, USA) kits by the UNC High-Throughput Sequencing Facility. Samples were pooled only for sequencing, after RNA extraction and library preparation. For E7.0 samples (12 embryos total), there were four samples per pool (two/group), three pools total, one pool per lane. For E7.25 and E7.5 samples (24 samples/time point), there were four samples per pool (one/

group), six pools total, one pool per lane. Paired-end (50 bp) sequencing was performed (Illumina HiSeq 4000).

### RNA-seq and qRT-PCR data analysis and display

Reads were filtered and aligned as described previously (Boschen et al., 2020). Transcript abundance was measured using Salmon (Patro et al., 2017), and differential expression tests were performed using DESeq2 1.22.2 (Anders and Huber, 2010). Gene expression differences were considered significant at an adjusted *P*-value threshold of 0.05. At the E7.5 time point, three outliers were detected and removed from the analysis: one from the 6J vehicle-treated group and two from the 6N PAE group. Final sample sizes are noted in the figure captions. We used gProfiler 0.1.6 (Raudvere et al., 2019) to detect significantly enriched pathways among differentially expressed genes, primarily using Gene Ontology (GO) (Ashburner et al., 2000; The Gene Ontology Consortium, 2018), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2019; Kanehisa and Goto, 2000), Reactome (Jassal et al., 2020; Fabregat et al., 2018) and HPO (Köhler et al., 2019). In addition, differentially expressed genes were assayed in a *de novo* network analysis using Ingenuity Software (Qiagen). For the E7.0 time point, network analysis was limited to 35 molecules (genes/proteins/protein complexes) per network due to the small number of input genes. For the E7.25 and E7.5 time points, analysis was limited to 70 focus molecules per network. Networks were ranked by the  $-\log_{10}$  Fisher's exact *P*-value testing the likelihood of a similar network being formed by the same number (35 or 70) random molecules. Gene lists were also compared to the CiliaCarta compendium (Van Dam et al., 2019) to analyze the number of cilia-related genes disrupted by alcohol at each time point and in each strain.

qRT-PCR data were analyzed with unpaired Student's *t*-tests corrected for multiple comparisons and  $P < 0.05$  was designated as statistically significant.

The gene expression data browser web tool was developed using the R shiny framework hosted through the Apache HTTP webserver. Several packages are used to process and display the gene expression data, including the tidyverse, here, ggplot2, reactable, dqshiny and shinylogs packages. The computer code for the data browser is available through github (<https://github.com/mbergins/Embryo-Genes>).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: K.E.B., S.E.P.; Methodology: K.E.B., S.E.P.; Software: T.S.P., M.E.B., J.M.S.; Validation: K.E.B.; Formal analysis: T.S.P., J.M.S.; Investigation: K.E.B.; Resources: K.E.B., T.S.P., M.E.B., J.M.S., S.E.P.; Data curation: T.S.P., J.M.S.; Writing - original draft: K.E.B.; Writing - review & editing: M.E.B., J.M.S., S.E.P.; Visualization: K.E.B., T.S.P., M.E.B., J.M.S.; Supervision: S.E.P.; Project administration: S.E.P.; Funding acquisition: K.E.B., T.S.P., J.M.S., S.E.P.

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### Data availability

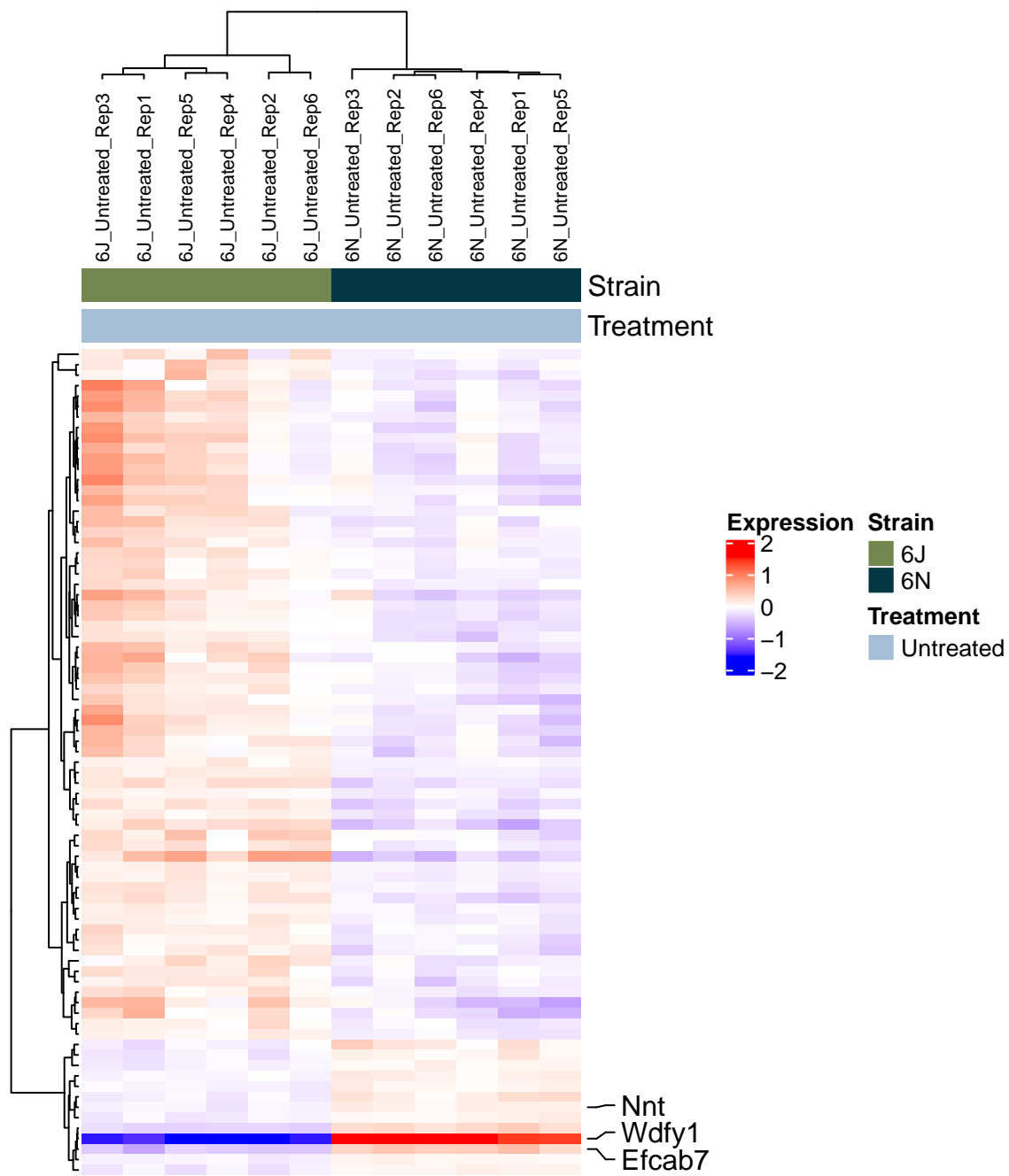
RNA-seq data are available at Gene Expression Omnibus (GEO) under accession number GSE163796.

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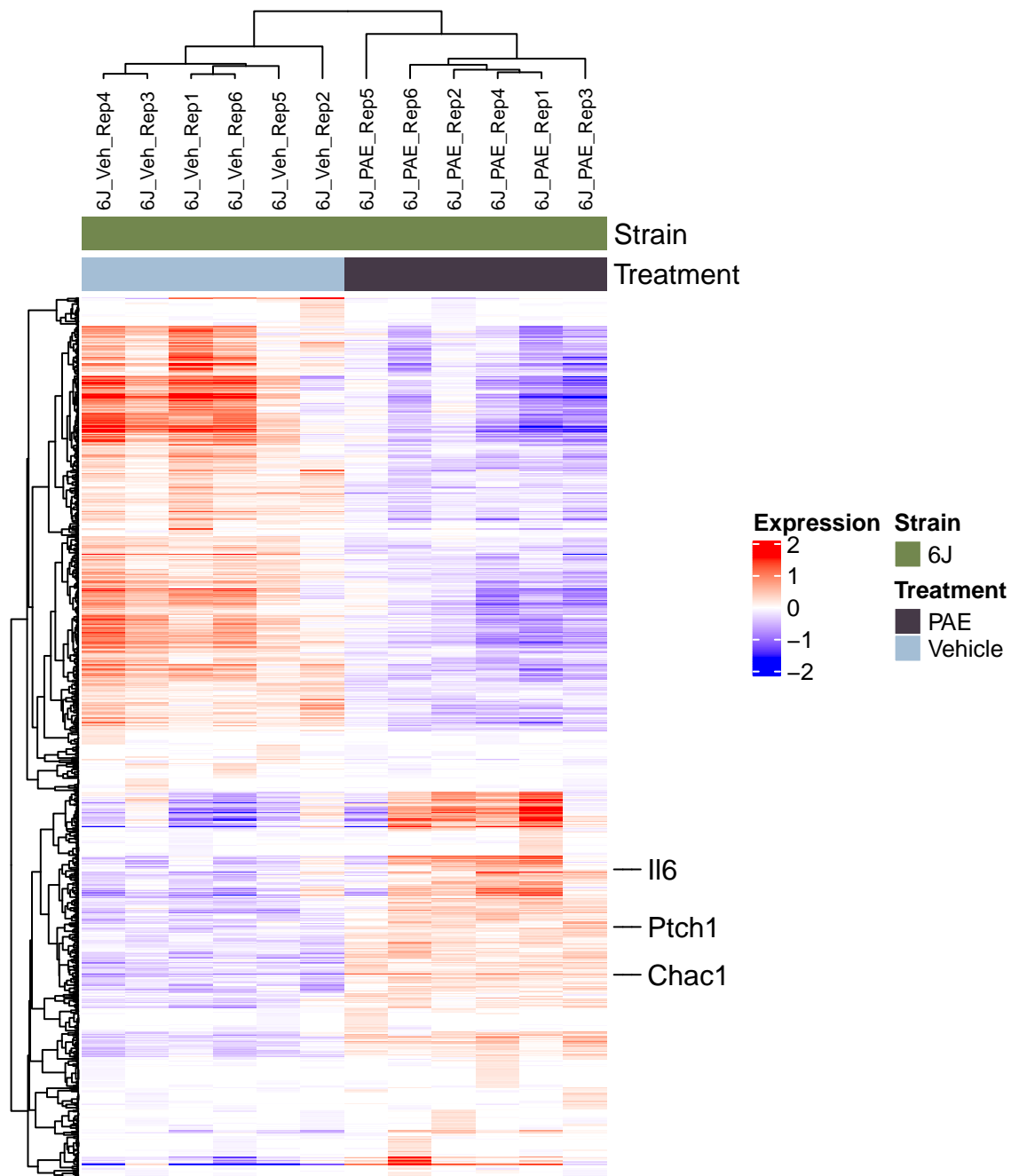
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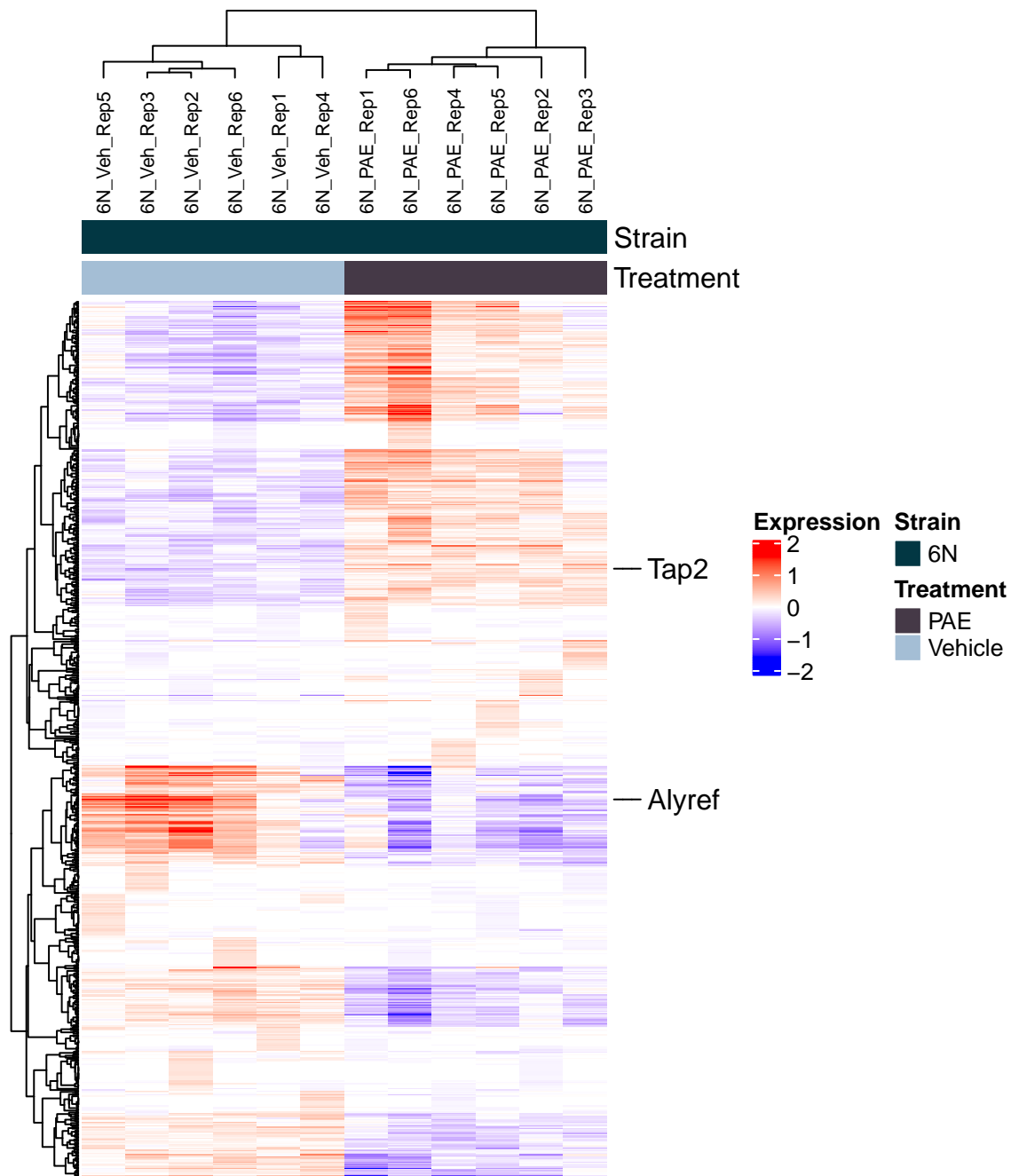


**Fig. S1. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in the E7.0 baseline analysis.** n = 6 replicates/group. Gene expression was median centered and clustered hierarchically using 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.

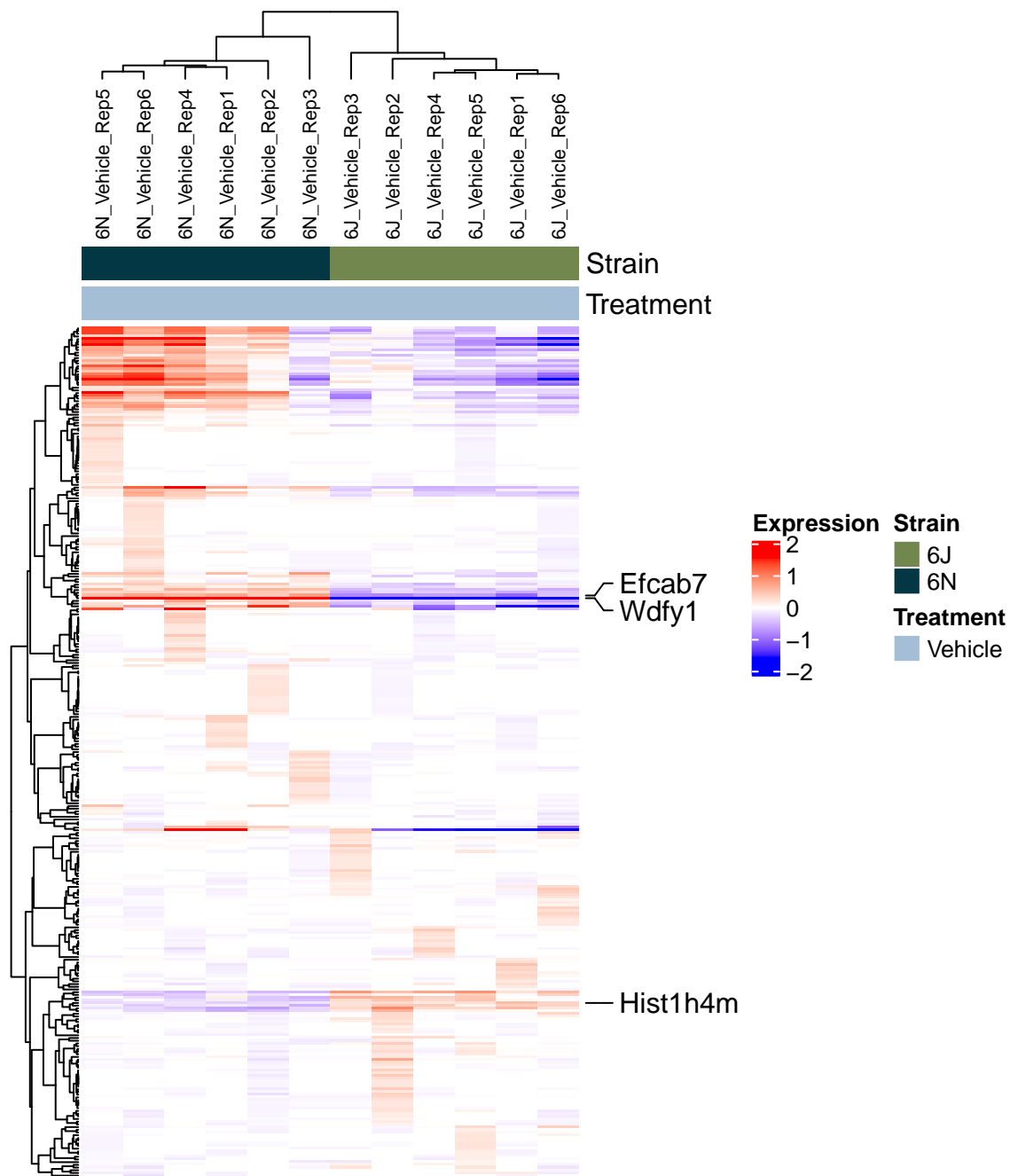




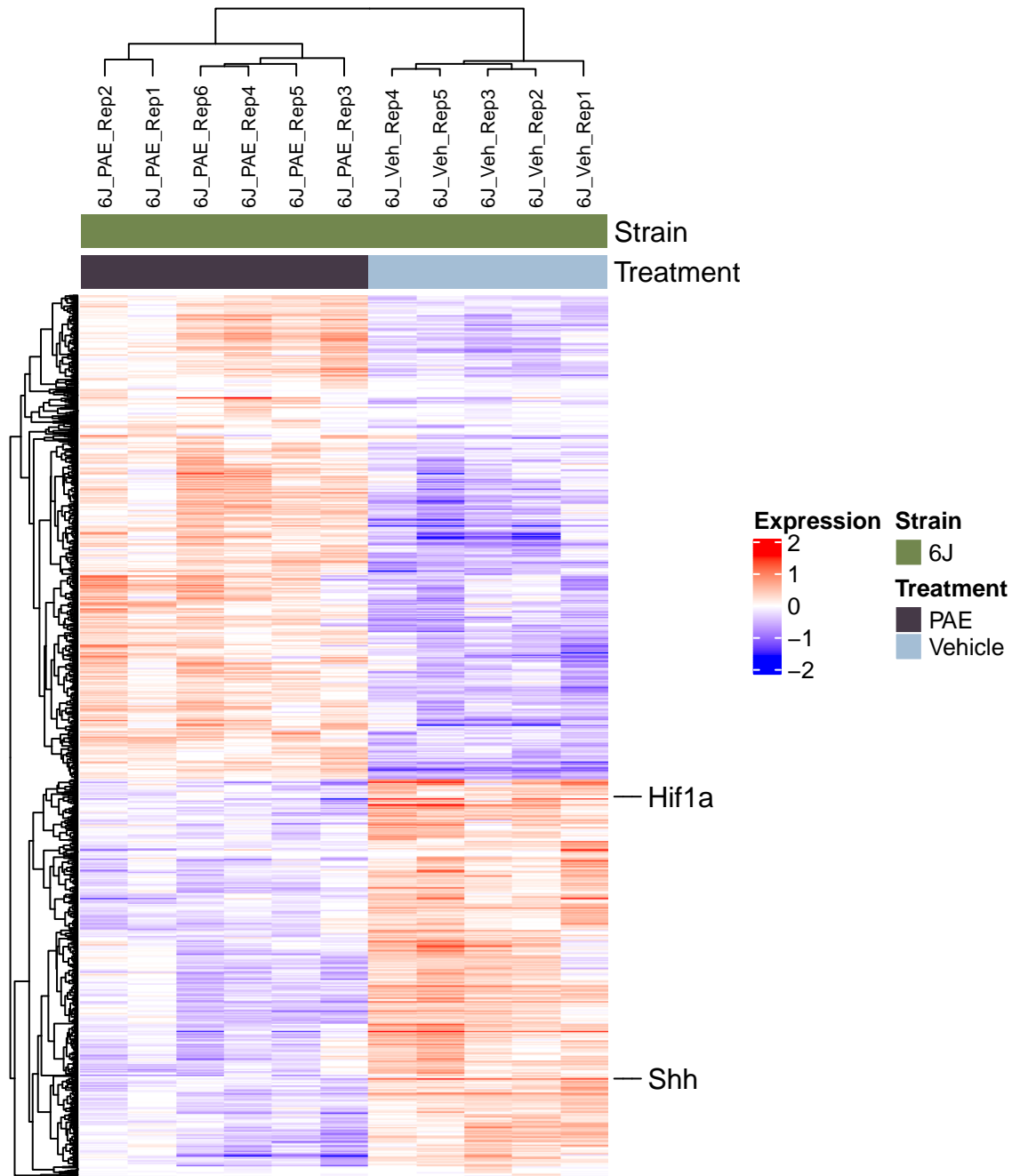
**Fig. S2. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in the vehicle-treated 6N and 6J analyses at E7.25.** Significant genes from the E7.25 time point.  $n = 6$  replicates/group. Gene expression was median centered and clustered hierarchically using 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.



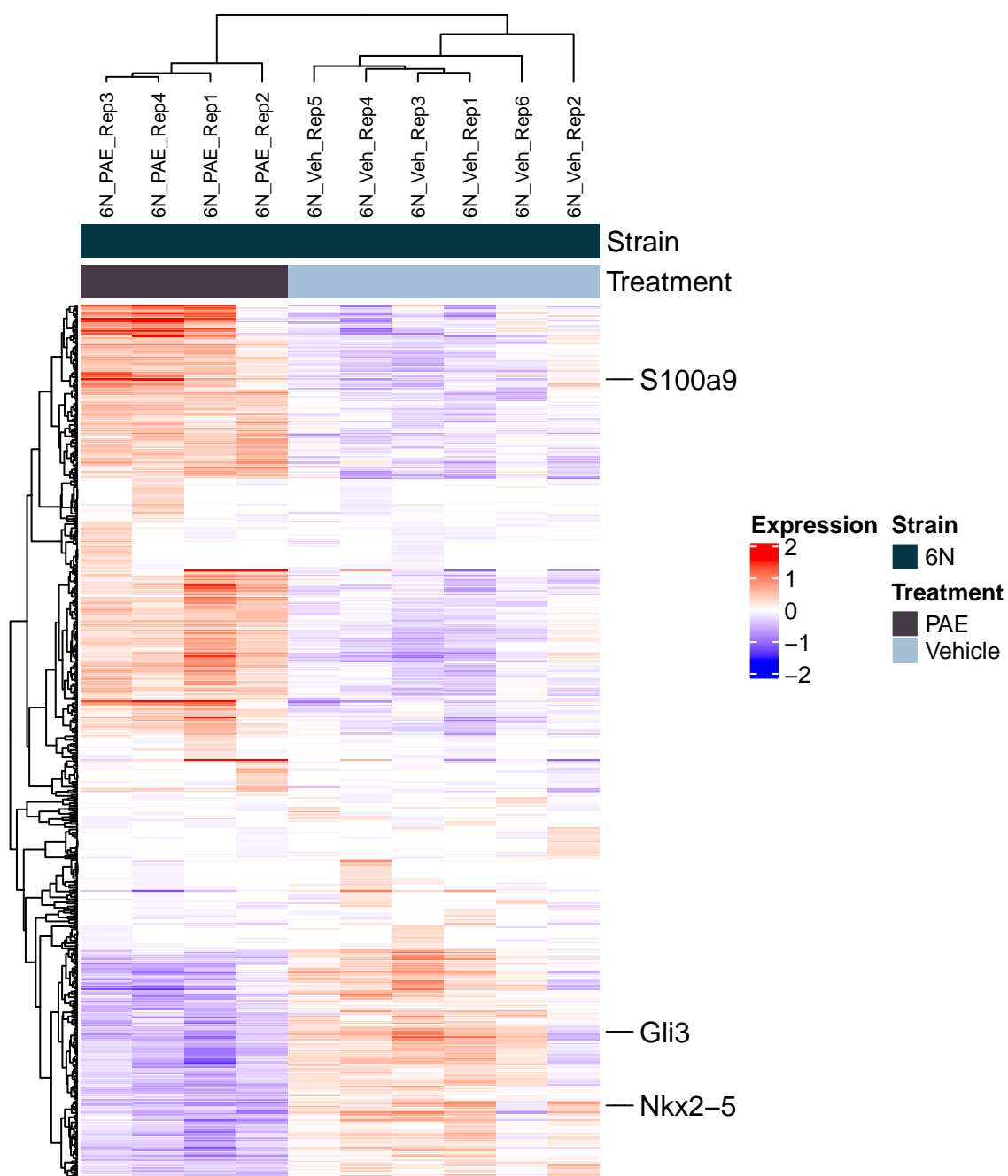
**Fig. S3. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in the vehicle-treated 6N and 6J analyses at E7.5.** Significant genes from the E7.5 time point. n = 6 replicates in the 6N group, 5 replicates for the 6J group. Gene expression was median centered and clustered hierarchically using 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.



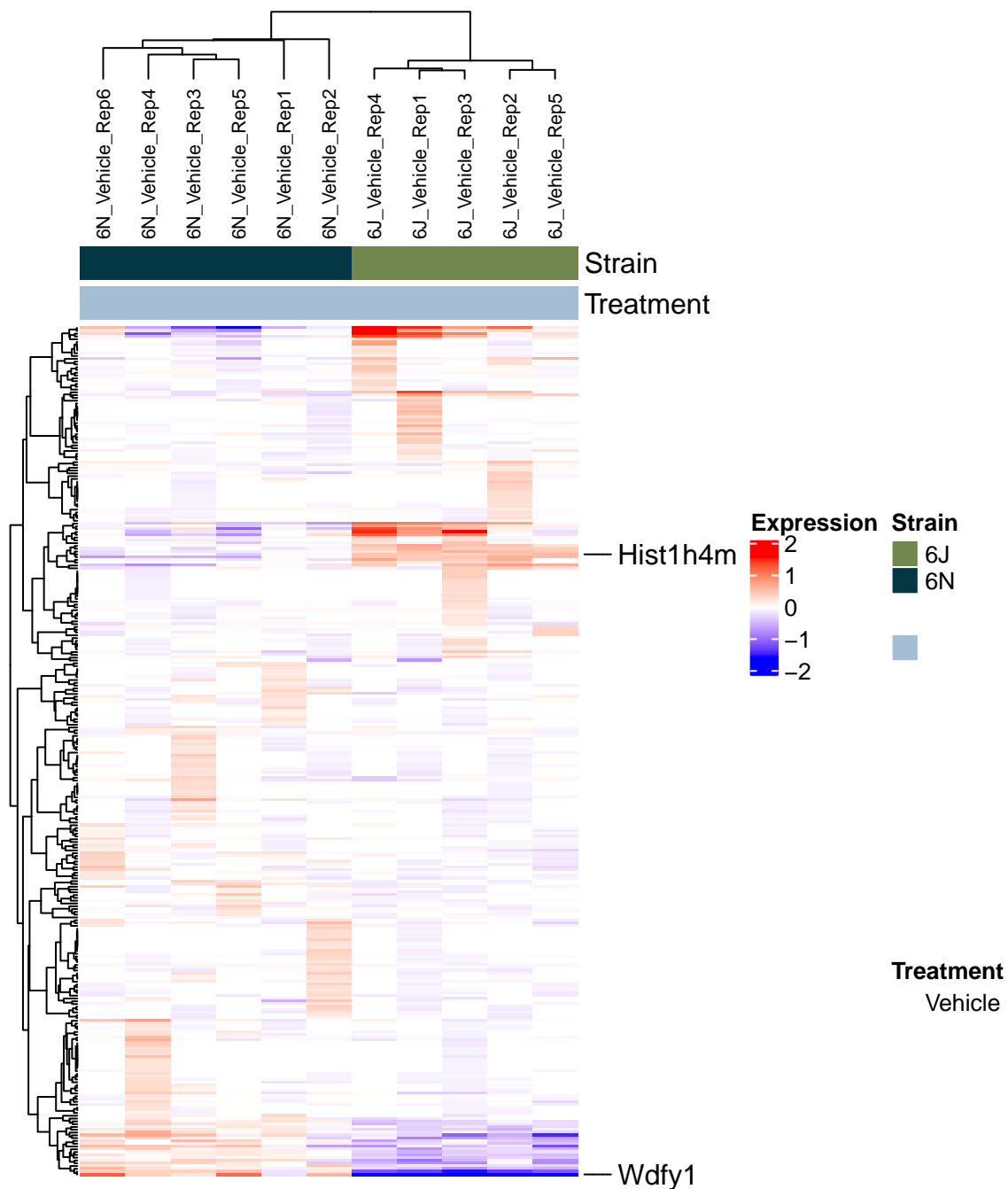
**Fig. S4. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in PAE vs. Vehicle-treated 6J's at the E7.25 time point.** Significant genes from PAE vs. vehicle-treated 6J's. n = 6 replicates/group. Gene expression was median centered and clustered hierarchically using 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.



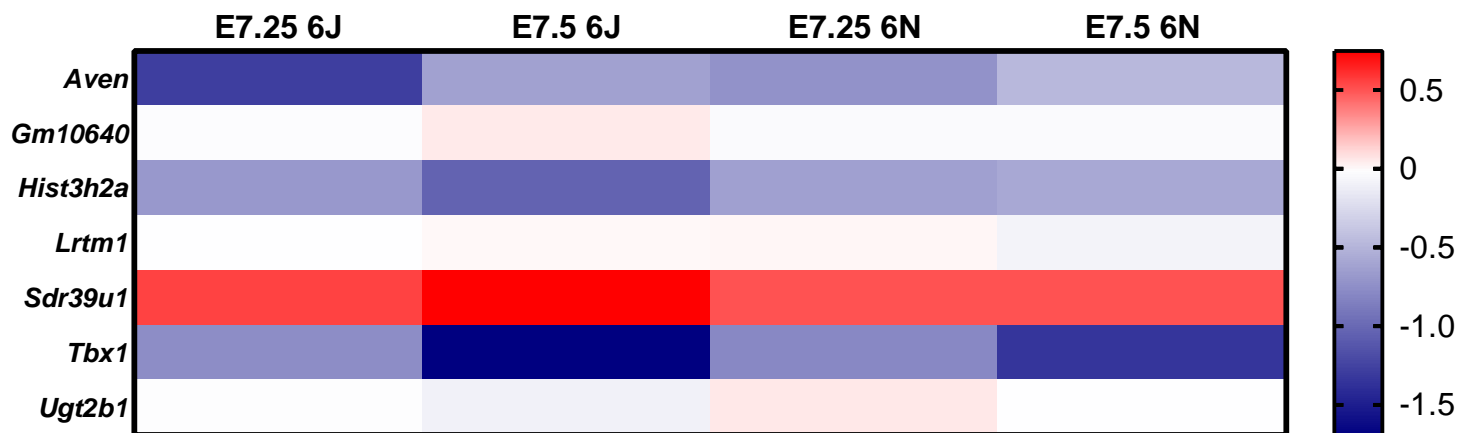
**Fig. S5. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in PAE vs. Vehicle-treated 6N's at the E7.25 time point.** A) Significant genes from PAE vs. vehicle-treated 6N's. n = 6 replicates/group. Gene expression was median centered and clustered hierarchically using 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.



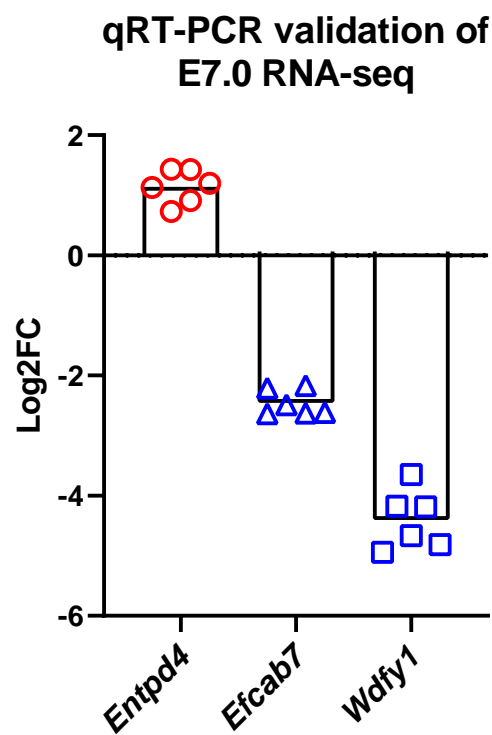
**Fig. S6. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in PAE vs. Vehicle-treated 6J's at the E7.5 time point.** Significant genes from PAE vs. vehicle-treated 6J's. n = 5 replicates for the vehicle-treated group, 6 replicates for the PAE group. Genes and samples were hierarchically clustered by 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.



**Fig. S7. Median-centered VST-normalized expression values for all differentially expressed genes for each replicate in PAE vs. Vehicle-treated 6N's at the E7.5 time point.** Significant genes from PAE vs. vehicle-treated 6N's. n = 6 replicates in the 6N group, 4 replicates for the 6J group. Genes and samples were hierarchically clustered by 1-Pearson correlation distance. Red = higher median-centered expression, blue = lower median-centered expression.



**Fig. S8. Seven genes differentially regulated by PAE in both strains at both time points.** Data are shown as the Log<sub>2</sub>FC for each PAE group vs. the appropriate vehicle-treated group from that strain and time point. Red = up-regulated genes, blue = downregulated genes.



**Fig. S9. Validation of three differentially expressed genes at the E7.0 time point.** qRT-PCR was performed on different samples than those used for RNA-seq (n = 6/strain) to assess expression of *Entpd4*, *Efcab7*, and *Wdfy1*. All genes were expressed at significantly different levels in the 6J vs. 6N in the direction expected based on the RNA-seq output: *Entpd4*: +1.14 Log<sub>2</sub>FC in 6J vs. 6N,  $t(10) = 4.473$ ,  $p = 0.0012$ ; *Efcab7*: -2.46 Log<sub>2</sub>FC in 6J vs. 6N,  $t(10) = 14.36$ ,  $p < 0.0001$ ; *Wdfy1*: -4.41 Log<sub>2</sub>FC in 6J vs. 6N,  $t(10) = 20.05$ ,  $p < 0.0001$ . Error bars indicate standard error of the mean.

**Table S1.** Selected gProfiler results from differentially regulated genes between the 6J and 6N strain at baseline (E7.0). Pathways associated with down-and up-regulated genes are listed separately.

**Up-regulated Genes**

**Gene Ontology: Biological Process**

Term name	Term ID	# of genes	Log10 p-value
Myeloid leukocyte migration	GO:0097529	8	3.68
Cytokine production	GO:0001816	12	2.71
Defense response	GO:0006952	17	2.58
Response to external stimulus	GO:0009605	22	2.22
Inflammatory response	GO:0006954	11	2.15
Cell migration	GO:0016477	15	1.94
Regulation of immune system process	GO:0002682	15	1.94
Cytokine-mediated signaling pathway	GO:0019221	8	1.49
Cell motility	GO:0048870	15	1.44
Localization of cell	GO:0051674	15.00	1.44

**Gene Ontology: Molecular Function**

Term name	Term ID	# of genes	Log10 p-value
Cytokine binding	GO:0019955	7.00	4.45
ATPase activity, coupled	GO:0042623	6.00	1.32

**KEGG: Biological Process**

Term name	Term ID	# of genes	Log10 p-value
Cytokine-cytokine receptor interaction	KEGG:04060	7.00	2.05

**Table S2.** Top ten de novo gene network lists for all time points. A) E 7.06J vs. 6N (before alcohol), B) E7.25 vehicle-treated 6J vs. 6N, C) E7.5 vehicle-treated 6J vs. 6N, D) E7.25 6J Alcohol vs. Vehicle, E) E7.25 6N Alcohol vs. Vehicle, F) E7.5 6J Alcohol vs. Vehicle, G) E7.5 6N Alcohol vs. Vehicle. Gene networks created with Ingenuity Pathway Analysis.

<b>A</b>	<b>E 7.0</b>	<b>6J vs. 6N</b>		
	<b>Diseases and Functions</b>	<b>-log(Fisher's exact p-value)</b>	<b>Molecules in Network</b>	<b>Molecules</b>
1	– Cell Cycle – Drug Metabolism – Molecular Transport	31	15	Aak1, Acad11, Adck2, Ahnak, App, Arfgap3, Arglu1, Atp8b1, Ccdc88c, Clpb, Dnah14, Dnajc28, Dynlrb2, Dynlt1, Hspa9, Hspb9, Igsf6, Klf12, Lira5, Mbnl3, Mmp3, Myc, Myo1d, Neat1, Nnt, Nr1h4, Rxr, Sez6l, Sirt5, Tex2, Tktl2, Tmem267, Tnf, Tretinoin, Vgl3
2	– Cancer – Immunological Disease – Inflammatory Disease	28	14	26s Proteasome, Anxa11, Btaf1, Cd3, Creb, Cx3cr1, Efcab7, Entpd4, Erk, Fsh, Fxyd5, Gpcr, Histone H3, Hsp70, Htr2b, Ide, Insulin, Mapk, Nfkb (Complex), Pdgf Bb, Pi3k (Complex), Pka, Pkc(S), Plc, Proinsulin, Ras Homolog, Sct, Slc25a12, Src (Family), Tiam1, Tpm4, Ubiquitin, Uqcc2, Vegf, Wt1
3	– Cellular Movement – Immune Cell Trafficking – Inflammatory Response	25	13	Ap1, Ccl4, Collagen Alpha1, Collagen Type I (Complex), Collagen Type Ii, Collagen Type Iv, Collagen(S), Csf3r, Ddx58, Erk1/2, Fcer1, Fibrinogen, Hsd11b1, Ifn, Ifn Beta, Il-1r, Il1r1, Il1rn, Ldl, Litaf, Mmp, Mmp7, Mmp8, Olr1, Pkc Alpha/Beta, Pro-Inflammatory Cytokine, Rhob, Saa, Sftpd, Sod, Tgf Beta, Tlr, Tnf (Family), Tnfrsf9, Trypsin
4	– Cancer – Endocrine System Disorders – Organismal Injury and Abnormalities	25	13	Akt, Ampk, C1qtnf5, Chemokine, Cytokine, Hp1bp3, Ifi16, Ifn Alpha/Beta, Ifn Type 1, Ifnar, Ige, Igg, Igg1, Igm, Il1, Il12 (Complex), Il12 (Family), Immunoglobulin, Interferon Alpha, Irf7, Kit, Lh, Map2k1/2, Mtdh, N-Cor, Nfkbiz, Nos, Osbp15, P70 S6k, P85 (Pik3r), Pglyrp1, Pthlh, S100a9, Top2b, Wdfy1
5	– Cellular Movement – Hematological System Development and Function – Immune Cell Trafficking	18	10	Adm2, Atp11b, Ccr1, Cd200, Cdx2, Cx3cr1, D1pas1, Defa1 (Includes Others), Defb104a/Defb104b, Gjb4, Gpr132, Gstp1, Hsh2d, Jnk, Lgals8, Mannan, Mip1, Mir-127, Mrc1, Mrc2, Nipal1, P38 Mapk, Pla2g7, Prr9, Rara, Retnlg, S1pr2, Sparc, Stra6, Tcr, Tlr10, Tmcc3, Tmem184a, Tnfrsf18, Traf5
6	– Cell Morphology – Cellular Assembly and Organization – Post-Translational Modification	16	9	1, 3, 4-Ip3, B4galt4, Brd8, Ceacam10, Ck2, Defb8, E2f8, Egf, Ep400, Epc1, H2az1, H4c4, Hdac2, Hoxc9, Ing2, Ing3, Irs1, Itpkc, Mier2, Mis18a, Nes, Oip5-As1, Papss1, Pcgf2, Pold2, Rbbp4, Rffl, Sap130, Sp3, Spr2h, St5, Tcf21, Tip60, Tp53, Ube2q2

<b>B</b>	<b>E 7.25</b>	<b>6J vs. 6N Vehicle-treated</b>		
	<b>Diseases and Functions</b>	<b>-log(Fisher's exact p-value)</b>	<b>Molecules in Network</b>	<b>Molecules</b>
1	– Antimicrobial Response – Cell Death and Survival – DNA Replication, Recombination, and Repair	39	22	Asb9, Bglap, Casp12, Caspase, Cd200r1, Ces, Ces1b/Ces1c, Ces4a, Cngb3, Cytochrome C, Ecm, Fap, Granzyme, Gzma, Gzmb, Gzmc, Gzmh, Hsp27, Hsp70, Il12 (Family), Il6, Jun/Junb/Jund, Mir124a-1hg, Mt3, Nfkb (Complex), Padi2, Rxr, Serine Protease, Serpinb12, Spink5, Tcr, Tmprss11b, Tmprss7, Trim69, Usp17la (Includes Others)



2	<ul style="list-style-type: none"> <li>Cell-To-Cell Signaling and Interaction</li> <li>Drug Metabolism</li> <li>Nervous System Development and Function</li> </ul>	34	20	Alpha Catenin, Ampk, Atp1b4, Calpain, Capn11, Cd3, Cd4, Cg, Cpa3, Creb, Efcab7, Fmo3, Fsh, Gabrg2, Glucuronosyltransferase, Hbg2, Histone H3, Hla-A, Hoxd10, Il1, Il1bos, Kcnj1, Mek, Nr2e1, P38 Mapk, Pi3k (Complex), Pkc(S), Ppp1r1b, Psca, Slco2b1, Tm4sf1, Ugt2a3, Ugt2b17, Ugt2b28, Vegf
3	<ul style="list-style-type: none"> <li>Cell Signaling</li> <li>Molecular Transport</li> <li>Vitamin and Mineral Metabolism</li> </ul>	32	19	Amylase, Ccl1, Col28a1, Collagen, Collagen Type Ii, Cxcl13, Elastase, Erk1/2, Fcna, Fgf7, Fibrin, Gp6, Hcrt, Hla-Dr, Ifn Alpha/Beta, Il36b, Irak3, Klra7 (Includes Others), Lum, Mhc Class I (Complex), Mhc Ii, Mmp, Ppp1r3a, Pro-Inflammatory Cytokine, Reg3g, Rgs13, Serpina1, Syk/Zap, Tlr, Tnf (Family), Tnfsf15, Trem1, Trypsin, Ucn2, Xcl1
4	<ul style="list-style-type: none"> <li>Hematological System Development and Function</li> <li>Hematopoiesis</li> <li>Humoral Immune Response</li> </ul>	30	18	Aim2, Akt, Bcr (Complex), Cd180, Cd36, Ctnna3, Dbh, Fcamr, Gcsam, Gm9573, Gpnmb, Hemoglobin, Ifn Beta, Ifnar, Iga, Igg, Igg1, Igg2a, Igg2b, Igg3, Igm, Il12 (Complex), Insrr, Ly9, Lyg2, Medag, Mhc Class Ii (Complex), Pax5, Plc Gamma, Stat5a/B, Tfap2b, Tnfrsf17, Trim29, Vav, Wap
5	<ul style="list-style-type: none"> <li>Cell Signaling</li> <li>Molecular Transport</li> <li>Vitamin and Mineral Metabolism</li> </ul>	25	16	Adcy, Adgrf1, Bdkrb1, Calcr1, Calmodulin, Ccr6, Chemokine, Chemokine Receptor, Collagen Type I (Complex), Cxcr1, Cyp2c9, Dynlt1, Ffar2, Fshr, G Protein, G Protein Alpha, Ghrhr, Gpcr, Grm1, Growth Hormone, Gsk3, Jnk, Lh, Mapk, Myoc, Npy, Pka, Plc, Rac, Ras, Serpina3g (Includes Others), Tacr1, Tsh, Tshr, Voltage-Gated Calcium Channel
6	<ul style="list-style-type: none"> <li>Cell-To-Cell Signaling and Interaction</li> <li>Skeletal and Muscular System Development and Function</li> <li>Visual System Development and Function</li> </ul>	23	15	Akr1c3, Aldh1a2, Aldose Reductase, Alp, Ap1, Calml5, Ces1, Collagen(S), Erk, Fcer1, Fgf, Fgf6, Ghrl, Gk, Hdl, Ige, Il23, Krt75, Ldl, Liltrb3, Myh4, Mylk2, Myosin, Nacph Oxidase, Nr1h, Pdgf Bb, Pi3k P85, Prkaa, Prl2c2 (Includes Others), Rap1gds1, Rock, Slfn12l, Src (Family), Tgf Beta, Txk
7	<ul style="list-style-type: none"> <li>Cardiovascular System Development and Function</li> <li>Cellular Function and Maintenance</li> <li>Tissue Morphology</li> </ul>	23	15	60s Ribosomal Subunit, Abca13, Adamts13, Add1, Alas2, Aldh3b1, Arnt2, Bbox1, Cldn13, Clk1, Ddx54, Ess2, Fbxl18, Gata2, Gdf2, Grm6, Gypa, Hemgn, Krt40, Ly6a (Includes Others), Mrgprx3, Myc, Nat3, Ncaph, Noc2l, Nsf11c, Prrg4, Rad2111, Rfli, Rhag, Rpl39l, Tmem200c, Tsga10ip, Vcp, Wapl
8	<ul style="list-style-type: none"> <li>Cell Morphology</li> <li>Cellular Function and Maintenance</li> <li>Developmental Disorder</li> </ul>	21	14	26s Proteasome, 4930417o13rik, Acyl-Coenzyme A, Aim2 Inflammasome, Alas2, Cd209d, Ceacam20, Ctcf, Cyp1a2, Cytokine, Defb8, Ep400, H4c4, Histone, Histone H4, Hsp90, Ide, Immunoglobulin, Insulin, Interferon Alpha, Klhl38, Midn, Mylip, Paf1, Phosphatidylinositol 3, 4-Diphosphate, Phosphatidylinositol-3-Phosphate, Pla2g2a, Pold2, Ppm1k, Prap1, Proinsulin, Rna Polymerase Ii, Tip60, Tssk1b, Wdfy1
9	<ul style="list-style-type: none"> <li>Cell-To-Cell Signaling and Interaction</li> <li>Nervous System Development and Function</li> <li>Organismal Injury and Abnormalities</li> </ul>	21	14	Acot5, Bdnf, Cntnap5, Ctcf, Drd1, Endocannabinoid, Fabp2, Foxa1, Ftsj3, Gpx4, Grb2, H1-1, Kri1, Ly6g6f, Nkx2-5, Olfr503, Or4n2, Pcdhb3, Pcdhb7, Pcdhb8, Plod1, Ppara, Ppp1r1b, Rp1l1, Rps27, S100a10, Scn10a, Scn11a, Sec14l5, Set, Set Complex, Slc17a8, Tespa1, Vgf, Xrn2
10	<ul style="list-style-type: none"> <li>Cell-To-Cell Signaling and Interaction</li> <li>Cellular Function and Maintenance</li> <li>Nervous System Development and Function</li> </ul>	21	14	9-Cis-Retinal, Akap5, Apol11b (Includes Others), Apol9a/Apol9b, App, Arglu1, Asic1, Asic2, Asic3, Cdr1, Chrnb3, Crx, Dhx33, Dlg4, Dmrt1, Dmrtc2, Dopamine, Endocannabinoid, Etd, Girk, Gpr37, Gpr78, Hcrt1, Ifn, Ifnar1, Irak, Myot, Opn5, Rho, Rnu12, Sectm1, Snrmp, Spata16, Tlr7, Trpv3

**C E 7.5 6J vs. 6N Vehicle-treated**

	Diseases and Functions	-log(Fisher's exact p-value)	Molecules in Network	Molecules
1	<ul style="list-style-type: none"> <li>Drug Metabolism</li> <li>Lipid Metabolism</li> <li>Small Molecule Biochemistry</li> </ul>	50	26	Aim2, Amy2a, Amylase, Anxa13, Bglap, Cd200r1, Cdh16, Cebpe, Ces, Ces1, Ces3, Ces4a, Collagen Type Ii, Ctrb2, Cyp7a1, Erk1/2, Fgf7, Growth Hormone, Hcrt, Il36g, Klrc1, Magea3 (Includes Others), Mug1/Mug2, Mycs, Nxph2, Prex1, Proinsulin, Pxr Ligand-Pxr-Retinoic Acid-Rxra, Rxr, Slco1b3, Syt2, T3-Tr-Rxr, Tph2, Ubash3a, Ucn3
2	<ul style="list-style-type: none"> <li>Connective Tissue Development and Function</li> <li>Digestive System Development and Function</li> <li>Skeletal and Muscular System Development and Function</li> </ul>	42	23	Ackr2, Afm, Akt, C3ar1, Cd207, Cd55, Collagen Type I (Complex), Collagen Type Iv, Collagen(S), Csta, Ctsk, F9, Fhit, Fmod, Gm9573, Heph11, Iga, Igg2a, Igg2b, Il23, Itga11, Ldl, Lyg2, Mucin, Myoc, Or51e2, Pdgf Bb, Prg3, Reg3a, Scgb3a1, Serpinb7, Siglec15, Slc13a1, Tcl1a, Tgf Beta
3	<ul style="list-style-type: none"> <li>Post-Translational Modification</li> <li>Protein Degradation</li> <li>Protein Synthesis</li> </ul>	42	23	Ano3, Ccl1, Ccl24, Ccl7, Cd209c, Cd8b, Cnga3, Fcer1, Gngt1, Ifn Alpha/Beta, Ifn Beta, Ifnar, Ifnz (Includes Others), Interferon Alpha, Klk12, Klk5, Klra7 (Includes Others), Mhc Class I (Complex), Mhc Class I (Family), Mhc Class Ii (Complex), Mx2, Nfkb (Complex), Pmaip1, Prss42p, Serine Protease, Serpinb10, Slc46a2, Tap2, Tmprss11e, Tmprss15, Tmprss7, Tnf (Family), Tnfrsf17, Trypsin, Usp17la (Includes Others)
4	<ul style="list-style-type: none"> <li>Cancer</li> <li>Dermatological Diseases and Conditions</li> <li>Organismal Injury and Abnormalities</li> </ul>	35	20	Adamts14, Alb, Atp6v1g3, Bcr (Complex), Chst4, Col8a1, Cox6a2, Creb, Ctnna3, Cxcl3, Cyclin A, Erk, Fhl5, Gabrg2, Gcsam, Gm10408 (Includes Others), Ige, Igg, Igg1, Il1, Il12 (Complex), Il13ra2, Immunoglobulin, Keratin, Krt16, Krt20, Krt73, Lh, Map2k1/2, Pax8, Pck2, Pou2af1, Ppy, Pro-Inflammatory Cytokine, Sos
5	<ul style="list-style-type: none"> <li>Developmental Disorder</li> <li>Endocrine System Disorders</li> <li>Hematological Disease</li> </ul>	28	17	26s Proteasome, Asb9, Calmodulin, Cd3, Chemokine, Cxcr6, Cytokine, Efcab7, Gpcr, Gpr119, Gpr22, Gpr37, Histone H3, Histone H4, Hsp70, Hsp90, Itgad, Jnk, Kcnj1, Kcnj16, Micb, Pi3k (Complex), Pka, Pkc(S), Rb, Rho, Slc12a1, Tcr, Tesk2, Tnn, Tp63, Ube2d4, Ubiquitin, Vegf, Wdfy1

6	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Endocrine System Disorders</li> <li>– Gastrointestinal Disease</li> </ul>	26	16	Abr, Ahctf1, Alms1, Alpk2, Atxn7, Bbox1, Ccdc27, Ccdc88c, Cnm2, Cntnap3, Ctf, Ctnnb1, Cyb5b, Fhit, Gabrq, Gjd4, Hepacam, Itprid2, Lhx6, Lmna, Lnx1, Lvrn, Mastl, Nes, Pcdhb8, Pcdhb8, Plec, Rasi11b, Rnf133, Ropn1, Sall1, Spag11b, Ube2i, Ube2q1, Virma
7	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Organismal Injury and Abnormalities</li> <li>– Tissue Morphology</li> </ul>	23	15	Abcb8, Adam30, Ankrd34c, Atf3, Bpifc, Cpn2, Creb1, Cst9l, Dio2, Dmrt1, Dnm3os, Fbxw21 (Includes Others), Glra1, Hmnr, Ifi202b, Kcnb1, Mapk1, Metalloprotease, Myl6b, Nr1h5, Pdcl2, Prkar2a, Prl3c1, Rest, Rhox3a (Includes Others), Robo1, S6k1, Scn10a, Scn3b, Tp53, Traf6, Trank1, Triiodothyronine, Reverse, Vnn1, Vsig1
8	<ul style="list-style-type: none"> <li>– Nutritional Disease</li> <li>– Psychological Disorders</li> <li>– Small Molecule Biochemistry</li> </ul>	21	14	14, 15-Epoxyeicosatrienoic Acid, Adamdec1, Ar, Beta-Estradiol, Cilp, Cldn18, Cldn6, Drd4, Ear2 (Includes Others), Elovl2, Endothelin, Eppin, Gata2, Gchfr, Gp2, Gpr37, Grk4, Hemgn, Htr6, Ifi202b, Mak, Mhc Class Ii (Complex), Mir-24-3p (And Other Mirnas W/Seed Ggcucag), Msmo1, Myc, Ntf4, Parp8, Pmm2, Pomc, Sprr2g, Sprr2i, Tbx19, Tgm6, Transglutaminase, Zg16
9	<ul style="list-style-type: none"> <li>– Cell Cycle</li> <li>– Cell Morphology</li> <li>– Cell-To-Cell Signaling and Interaction</li> </ul>	19	13	Actl7b, App, Brms1, Ccnb2, Ccnb3, Clec2e/Clec2h, Crebbp, Crybb2, Endothelin, Gjb1, Gng2, Hmgb4, Hspa2, Iqcf5, Kcnn4, Kctd16, Lsmem1, Mac, Mastl, Ms4a15, Nanog, Prkar2a, Psg18 (Includes Others), Ptger2, Rnasel, Sik2, Slc25a13, Stfa2/Stfa211, Sycp2l, Tbx22, Tigd4, Tik, Usp8, Utp, Zdhhc21
10	<ul style="list-style-type: none"> <li>– Cell Cycle</li> <li>– Cell-To-Cell Signaling and Interaction</li> <li>– Cellular Growth and Proliferation</li> </ul>	17	12	9830107b12rik/A530064d06rik, Abcb9, Aim2, Apol11b (Includes Others), Bc048679, Chil3/Chil4, Cntnap5, Crabbp1, Ctsk, Cyp4f11, Dyrk2, Eln, Ern2, Esr1, Fmr1, Gchfr, Gvin1 (Includes Others), Hla Class I, Hla-G, Ifi202b, Ifng, Muc2, Nr1d1, Pcdhb14, Pcdhb4, Pla2g7, Rcvrn, Tgfr2, Th2 Cytokine, Ticam2, Tlr3, Tnfsf9, Tretinoin, Ttc22, Uba6

**D** **E 7.25 6J Alcohol vs. Vehicle**

	Diseases and Functions	-log(Fisher's exact <i>p</i> -value)	Molecules in Network	Molecules
1	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Gastrointestinal Disease</li> <li>– Hepatic System Disease</li> </ul>	99	64	Aasdh, Abcb8, Aebp2, Aldh3b1, Amfr, Ammrec1, Anxa10, Apbb2, Arhgap23, Atp2a2, Atpase, Bag3, Banp, Bod1, Btdb1, Caap1, Cacybp, Carm1, Carnmt1, Crebrf, Dars1, Eed, Entpd2, Faf1, Fxr2, G3bp1, Glud1, H1-0, Hdac, Hemgn, Hsp70, Hspa5, Hspa8, Hspd1, Hsph1, Klhdc2, Lrrc47, Map1lc3, Mapre1, Mettl17, Mpp6, Mrps27, Noa1, Nr2e1, Ogt, Pcbp1, Pex6, Ppp1r10, Pto1, Rabl3, Rbm26, Rbpms, Rif1, Rpa, Sfpq, Slc35e1, Slco2b1, Smc4, Spg7, Tmem165, Trim29, Trmt12, Trmt44, Tssk1b, Ube2m, Ubxn2b, Ugt2b17, Vegf, Ywhah, Znf746
2	<ul style="list-style-type: none"> <li>– Cell Morphology</li> <li>– Drug Metabolism</li> <li>– Endocrine System Development and Function</li> </ul>	89	60	Alkbh5, Asb13, B4galt4, Bbc3, Bbs4, Bmt2, Brd7, Brpf3, Cables2, Capns2, Ccnt1, Ccny, Cct3, Cct4, Cdc73, Cnppd1, Cofilin, Ctbp1, Ctdp1, Dazap2, Dnaja1, Eif4a2, Ercc8, Fam91a1, Fkbp5, Gcn5l, Gmcl1, Gtf2e2, Histone, Histone H3, Holo Rna Polymerase Ii, Inpp5k, Ipmk, Kdm1a, Kiaa2013, Mark3, Mbd3, Mcmbp, Mettl3, Mi2, Mlc1, Nars1, Nurd, Oma1, Orc3, P-Tefb, P38 Mapk, P4ha1, Pard3, Pard6b, Parp16, Paxbp1, Per1, Pmpcb, Ppp2r2d, Rhof, Rsrc2, Slc6a12, Snx5, Spata20, Spata3, Srsf2, Srsf9, Stip1, Strn3, Suv39h2, Tada3, Taf10, Taf5, Tip60
3	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Cellular Development</li> <li>– Tissue Development</li> </ul>	84	58	5730488b01rik, Ahcy1l, Alp, Alpg, Asb15, Bmp1, Cbx6, Chac1, Cirbp, Collagen Alpha1, Commd3-Bmi1, Coq2, Ctbp, Erk, Exog, Exosc6, Farnesyl Transferase, Fgf, Gata6, Get4, Hedgehog, Hes1, Hoxa9, Hoxd10, Leng8, Loxl1, Luc7l, Mafg, Mllt1, Mocs3, Mrps30, Napsa, Nog, Nop56, P3h2, Pip4k2a, Plcd3, Prc2, Prl2c2 (Includes Others), Prmt2, Pscs, Ptch1, Rangap1, Riox1, Riox2, Rnr, Rrs1, Sdr39u1, Sifn12l, Smarca5, Sox, Sox17, Sox18, Sox2, Sox4, Spout1, Tcf, Tigd5, Tle1, Trmt10c, Trmt1l, Tsc22d1, Ugcg, Wars2, Wdr77, Wnt, Yars2, Ypel5, Zc3h14, Zmynd19
4	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Connective Tissue Disorders</li> <li>– Organismal Injury and Abnormalities</li> </ul>	73	53	26s Proteasome, Alyref, Ankrd13a, Apc/Apc2, Arrdc3, Arrdc4, Atp6v0d2, B3gnt5, Barx1, Bmp, Bok, Ccdc155, Cldn5, Clec4g, Cnpy3, Csnk1g3, Ctnna3, Eotaxin, Fam161a, Fgfbp1, Galnt1, Gas1, Gml, Got, Hoxd9, Hsd3b1, Igg, Igg2a, Igg2b, Igl1/Igl5, Il25, Irak3, Lman2l, Lrig1, Lrrtm1, Mhc Class I (Family), Mhc Ii, Mir101, Mrm3, Msl2, Mto1, Nfkb (Complex), Noct, Otulin, P Glycoprotein, Rab6b, Rbm15b, Rnf11, Rnf149, Slc22a16, Srxn1, Tespa1, Tmem245, Tnfrsf17, Tnfsf15, Tpcn2, Trim10, Trim44, Trim8, Tspan33, Tysnd1, Ube2, Ube2q2, Ube2r2, Ubiquitin, Ubiquitin Ligase, Vacuolar H Atpase, Vash1, Wipi1, Zfand5
5	<ul style="list-style-type: none"> <li>– Cardiac Necrosis/Cell Death</li> <li>– Cardiovascular Disease</li> <li>– Cell Death and Survival</li> </ul>	60	47	Adrb, Alcohol Group Acceptor Phosphotransferase, Alpha Actin, Asb9, Becn1, Btg3, Cab39, Calcineurin A, Cdc25b, Cg, Chrna6, Chrb3, Cited2, Creatine Kinase, Dapk3, Dil3, Dusp10, E2f1, Eno2, Ephb, Etfbkmt, Fjx1, Fsh, Gamma Tubulin, Gna11, Grk6, Hsd17b1, Klfl1, Lh, Lmcd1, Mapk1, Mapk1ip1, Mapkapk2, Mcl1, Mef2, Mef2b, Mlc, Mlcp, Myh4, Mylk2, Myosin-Light-Chain Kinase, Neurod4, Ngf, Notch, Nrg (Family), Oxsr1, Pawr, Pi3k (Family), Pkn1, Plat, Ppp6r1, Ptpase, Ptpa, Pyy, Rasal2, Rnf130, Secreted Mmp, Slx1a/Slx1b, Smad6, Snx18, Sod, Spata5l1, Src (Family), Stk39, Tcaf2, Timp2, Tm4sf1, Tmem158, Tmsb4, Tsc22d2

6	<ul style="list-style-type: none"> <li>– Energy Production</li> <li>– Lipid Metabolism</li> <li>– Small Molecule Biochemistry</li> </ul>	49	41	Adh1c, Agfg1, Akirin2, Akr1c3, Ap5z1, Bag1, Brms1l, C/Ebp, Calc, Cbp/P300, Cd207, Coup-Tf, Crebzf, Cuzd1, Cyp11b2, Dgk, Dgkz, Emcn, Erk1/2, Estrogen Receptor, Gc-Gcr Dimer, Gm9573, Hdac7, Histone Deacetylase, Histone H4, Hnf3, Hnf4g, Iga, Jink1/2, Jun/Junb/Jund, Klf9, Mettl16, Mknk2, Mrrf, Muc5b, Mucin, N-Cor, Ncor1, Nr0b2, Nr1h, Nr2f6, Pepck, Phf23, Pki, Pnrc1, Polr2m, Ppp1r3a, Rar, Rdh, Reg3g, Rxr, Sbsn, Sdr16c5, Sema3f, Serpinf1, Slc44a1, Spink5, Suds3, Swi-Snf, T3-Tr-Rxr, Tbx1, Tfiia, Thymidine Kinase, Thyroid Hormone Receptor, Tlr7/8, Tph2, Ube2j2, Ucp3, Vitamd3-Vdr-Rxr, Wdr92
7	<ul style="list-style-type: none"> <li>– Connective Tissue Disorders</li> <li>– Inflammatory Disease</li> <li>– Organismal Injury and Abnormalities</li> </ul>	44	38	Abhd17b, Ago2, Ago2-Mirlet7, Ankrd10, Ap1ar, Arl5a, Atp1b4, Cnot6, Cpd, Ddx56, Eif2, Eif2s1, Elavl1, Fam135a, Fam83f, Fbxo27, Gpr180, Gtpase, Hdhd2, Hras, Hsp90, Ifi27, Iigp1, Interferon Alpha, Ints11, Lamp1, Lmf1, Mex3d, Mir-323-3p (And Other Mirnas W/Seed Acauuag), Mir-330, Mir-363, Mir-423, Mir-423-5p (And Other Mirnas W/Seed Gagggggc), Mob3b, Nadk2, Nfic, Oaf, Oas1b, Pank1, Pank3, Pdc7, Pdia4, Pp1/Pp2a, Ppfia1, Ppfibp2, Ppp2ca, Ppp2r3d, Pptc7, Proinsulin, Prom2, Rasa4, Rbpj, Rna Polymerase Ii, Rnase A, Sfta2, Sh3d19, Smad2/3, Smco3, Spast, Tarbp2, Tbc1d20, Tbc1d30, Tmem14a, Tmem65, Tnf (Family), Txnip, Ubp1, Ulk2, Xxylt1, Zcchc7
8	<ul style="list-style-type: none"> <li>– Cell Death and Survival</li> <li>– Cellular Compromise</li> <li>– DNA Replication, Recombination, and Repair</li> </ul>	40	36	7s Ngf, Alpha 1 Antitrypsin, Alpha Tubulin, Beta Tubulin, Ces, Ces1b/Ces1c, Collagen Type Ii, Ctl2a/Ctl2b, Cytokine Receptor, Ecm, Enac, Fam171a2, Fbxo31, Fbxw8, Fc Gamma Receptor, Fcer1, Fgd1, Granzyme, Gzma, Gzmb, Gzmc, Gzmh, Hsp, Ifit1, Ifn, Ifn Alpha/Beta, Ifn Beta, Ifn Type 1, Ifnar, Il12 (Complex), Il1r1, Il20rb, Il6, Jak, Kallikrein, Kik3, Kik7, Klrb1, Liltr3, Map6, Mapkap2/3, Mbp, Mep1a, Mhc, Mhc Class I (Complex), Mhc Class Ii (Complex), Mir122a, B, Mir124, Mirlet7, Msln, Mus81, Pdk2, Polg2, Prss3, Rgmb, Rnasel, Scarb2, Serine Protease, Serpina1, Serpina9, Syk/Zap, Tap2, Tec/Btk/Itk/Txk/Bmx, Th2 Cytokine, Tlr, Tmem189, Tmprss7, Trypsin, Txk, Vbp1
9	<ul style="list-style-type: none"> <li>– Nervous System Development and Function</li> <li>– Organ Morphology</li> <li>– Tissue Morphology</li> </ul>	35	33	Adam19, Adamts13, Agfg2, App, Arl5c, Arl8a, Arnt2, Brwd1, Cd200r1l, Cpn2, Csnk1g1, Ddx43, Dnajb14, Dnajc30, Entpd2, Fbxl20, Fcho1, Filip1, Gjd4, Gucd1, Gypa, H2-K2/H2-Q9, H2-M2, Hspb7, Il20rb, Il4, Jtb, Kcnq1, Kctd16, Krt40, Laptm4a, Lnx1, Ly6a (Includes Others), Ly6d, Mobp, Mturn, Myot, Nat3, Nudt11, Pcyt1b, Pdxp, Pkib, Purg, Rnf144b, Rnf38, Rwd2b, Serpinb6b, Slc25a38, St6galnac6, Stac2, Tcpl0/Tcp10l2, Tmem176b, Tmem59, Tp73, Traf6, Tshz2, Ubc, Ubl3, Ulk2, Usp6, Wars2, Wfdc8, Zadh2, Zdhhc14, Zfand5, Znf202, Znf706, Znr1, Zscan16, Zxdc

10	<ul style="list-style-type: none"> <li>– Amino Acid Metabolism</li> <li>– Cardiovascular Disease</li> <li>– Organismal Injury and Abnormalities</li> </ul>	34	32	Actin, Agpat5, Alpha Actinin, Alpha Catenin, Angel2, Angpt2, Calpain, Camp-Dependent Protein Kinase, Casein, Chordc1, Cnga2, Collagen, Collagen Type I (Complex), Collagen Type I (Family), Collagen Type Iv, Collagen(S), Creb, Eif4ebp2, Eif4g, Eva1c, Exoc3, Exoc8, Fam136a, Fgfr, Focal Adhesion Kinase, G Protein Alphas, Gaba-A Receptor, Gabrg2, Gclc, Gclm, Gp6, Gria2, Growth Hormone, Gαq, Histone H1, Hrg, Hsp27, Importin Beta, Integrin, Itpka, Jak1/2, Laminin (Complex), Lfa-1, Lrp, Lum, Mapk, Mmp, Nup35, Pi3k (Complex), Pkc(S), Plc Beta, Plc Gamma, Pom121/Pom121c, Pp2a, Prap1, Prkcg, Ptk, Rab5, Secretase Gamma, Si, Spp1, Tars2, Tgf Beta, Ubqln1, Uros, Vps50, Vps51, Ythdc1, Ythdf1, Znf804a
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<b>E</b>		<b>E 7.25 6N Alcohol vs. Vehicle</b>		
	<b>Diseases and Functions</b>	<b>-log(Fisher's exact p-value)</b>	<b>Molecules in Network</b>	<b>Molecules</b>
1	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Gastrointestinal Disease</li> <li>– Organismal Injury and Abnormalities</li> </ul>	94	60	26s Proteasome, Ankrd13a, Apc (Complex), Arrdc1, Aven, Azin1, Bmt2, Btd1, C1ql4, Caap1, Cab39, Cab39l, Carnmt1, Casein, Ccar1, Ccdc149, Cd200r1, Cdbl, Ctcf, Cxxc1, Exog, Gmcl1, Haus4, Histone, Hivp3, Hoxc10, Kdm4b, Krt75, Leng8, Lypd3, Map4k2, Mark3, Mex3c, Mfh1, Micu2, Mif4gd, Nfkb (Complex), Nr2e1, Parp, Parp16, Pdk2, Pip4k2a, Pip4k2b, Pkh1, Plcd3, Prickle3, Prkaa, Pxx, Rbm26, Rcbtb2, Rnf25, Serpina12, Smurf1, Sult2b1, Tbrg4, Traf, Trib3, Trim13, Trim44, Trim69, Trmt11, Ube2, Ube2g2, Ube2q2, Ubiquitin, Utp4, Znf263, Znf414, Znf761, Zranb1
2	<ul style="list-style-type: none"> <li>– Cell Cycle</li> <li>– Cellular Assembly and Organization</li> <li>– DNA Replication, Recombination, and Repair</li> </ul>	94	60	Actr5, Alpha Catenin, Anapc4, Atpase, Bbs4, Bcor, Cbp/P300, Ccdc117, Ccnl2, Cdc73, Cdipt, Ceacam, Ceacam20, Colec12, Dars1, Daxx, Fam161a, Fam91a1, Fbxo17, Fbxw21 (Includes Others), Figla, Fsd2, Histone H3, Hnrnp1, Hspa8, Igdcc3, Il1bos, Ilf2, Kdm1a, Kih15, Lman2l, Mapk, Mapre1, Meitl3, Mi2, Myo19, Nlrp4b, Nsun4, Orc3, P-Tefb, Padi6, Pex6, Phkg2, Polr3h, Ppp2ca, Prkab1, Rbbp4, Rbpj, Rgs13, Rna Polymerase Ii, Rnr, Samd7, Smarca5, Sohlh1, Sp9, Spata19, Spice1, Spout1, Srsf2, Supt16h, Tcf19, Tmem25, Troap, Tssk1b, Tuba4a, Ythdc1, Ythdf1, Zbtb1, Znf639, Znf804a
3	<ul style="list-style-type: none"> <li>– Gastrointestinal Disease</li> <li>– Neurological Disease</li> <li>– Organismal Injury and Abnormalities</li> </ul>	59	44	Akr1c4, Amylase, Ankrd39, Anxa10, Arrb2, Bag3, Bdkrb1, Calmodulin, Card19, Cg, Chrm3, Ck2, Cpa3, Csnk1g3, Cyp4a11, Dazap2, Efcab5, Exoc3, Ffar2, Focal Adhesion Kinase, Fsh, Gbp6, Gnrh, Gpcr, Gpr160, Gpr4, Gpr50, Gpr88, Gtpase, Hemoglobin, Hsp90, Htr1d, Htr2b, Ice2, Ikk (Complex), Insulin, Klf1, Mtorc1, Ncbp2, Pka, Plc, Prmt3, Pstpip2, Ptprrz1, Rab5c, Rac1, Ras Homolog, Rnf208, Rxfp1, Secretase Gamma, Sfk, Shc, Slc8b1, Slitrk1, Smarcal1, Src (Family), Sstr1, Stat, Stxbp4, Tas1r2, Tcf, Tcr, Tmem17, Tnk1, Tpcn2, Tsc22d1, Tsc22d2, Tubulin, Vamp2, Vegf

4	<ul style="list-style-type: none"> <li>Cell Morphology</li> <li>Cellular Development</li> <li>Cellular Movement</li> </ul>	49	39	Acac, Actin, Afmid, Akirin2, Alp, Arp2/3, Arrdc4, Bcar1, Collagen, Collagen Alpha1, Collagen Type I (Complex), Collagen Type I (Family), Collagen Type Iv, Collagen(S), Commd3-Bmi1, Crebzf, Cuzd1, Cxcl13, Dynll2, Ecm, Erk1/2, Fam102a, Fap, Fcer1, Fermt1, Fgf, Fgf13, Fgf16, Fgf6, Fgf7, Fgfr, Gas1, Gp6, Gpiib-iiia, Hcrt, Hedgehog, Hhat, Histone Deacetylase, Igsf3, Igsf8, Integrin, Itga3, Jnk1/2, Laminin (Complex), Lfa-1, Loxl1, Lrig1, Mep1a, Myoc, Plc Gamma, Ppp1r15a, Rab5, Rap1, Rasgrp3, Rsk, Sh2d3c, Sharnin, Skap1, Slfn12l, Sox15, Srsf5, Talin, Tgf Beta, Tlr7/8, Ubiquitin Ligase, Ucn2, Wasf2, Wnt, Wnt8b, Zdhhc2
5	<ul style="list-style-type: none"> <li>Cell Cycle</li> <li>Developmental Disorder</li> <li>Endocrine System Disorders</li> </ul>	47	38	Adaptor Protein, Adaptor Protein 2, Ap1s2, Ap2, Ap2 Alpha, Atn1, Beta Arrestin, Bmp15, Bok, Brms1l, Carboxylic Ester Hydrolase, Caspase, Caspase 3/7, Ccne2, Cdc2, Cdk, Cebpg, Ces, Ces1b/Ces1c, Ces1e, Ces4a, Cidec, Clathrin, Cpt1, Cyclin A, Cyclin D, Cyclin E, Dio1, Dynamamin, Dzip3, E2f, E2f1, Hdac, Histone H4, Jnk, Kcnab3, Map1lc3, Mxd1, N-Cor, Ncor1, Nr1h, Pctp, Pepck, Polr2m, Ptgds, Rar, Rb, Rhobtb3, Rnf165, Rprd1a, Rxr, Shisa5, Siah2, Slc29a2, Smad1/5/9, Smad2/3, Ston2, Swi-Snf, Tars2, Tbx1, Tespa1, Tfp2b, Thymidine Kinase, Tnfrsf14, Uck2, Ulk2, Unc13d, Vps50, Wars2, Wdr62
6	<ul style="list-style-type: none"> <li>Hematological System Development and Function</li> <li>Lymphoid Tissue Structure and Development</li> <li>Tissue Morphology</li> </ul>	45	37	Akt, Arfp2, C/Ebp, Cd180, Cd4, Chemokine, Chka, Cirbp, Cxcl16, Cytokine, Dsg1, Eif2ak2, Fcamr, Galnt1, Gm9573, Got, Grk6, Hk1, Ifn, Ifn Alpha/Beta, Ifn Beta, Ifnar, Iga, Ige, Igg, Igg1, Igg2a, Igg2b, Igg3, Igl1/Igl15, Igm, Ikb, Il1, Il12 (Complex), Il12 (Family), Il17d, Il36b, Immunoglobulin, Insrr, Interferon Alpha, Ly9, Mhc Class I (Complex), Mhc Class I (Family), Mhc Class II (Complex), Mhc II, Mliip, Mucin, Notch, Npas4, Pax5, Pdcd1lg2, Pro-Inflammatory Cytokine, Prr5, Rbm38, Reg3g, Saa3, Sema4b, Stat5a/B, Tlr, Tnf (Family), Tnfrsf17, Tnfsf12-Tnfsf13, Tnfsf13, Tnfsf15, Trem1, Trim59, Trpm4, Ugt2b17, Unc93b1, Vip
7	<ul style="list-style-type: none"> <li>Carbohydrate Metabolism</li> <li>Cell-To-Cell Signaling and Interaction</li> <li>Small Molecule Biochemistry</li> </ul>	45	37	Abra, Adarb1, Adrb, Aldose Reductase, Alpha Tubulin, Ampa Receptor, Ampk, Ap1, Arf, Arhgap8/Prr5-Arhgap8, Bcr (Complex), C6, Calcineurin Protein(S), Camkii, Cd3, Clnk, Cofilin, Dock3, Epb4111, Erk, Ern2, Flrt3, Gadd45a, Gcsam, Gk, Gria2, Histone H1, Ica1, Inpp5, Inpp5a, Inpp5k, Isl1, Kiaa1522, Lrrc10, Map2k1/2, Mgst2, Miip, Mlc, Mtch1, Mthfd2, Nck, Nck1, Nfat (Complex), P70 S6k, Pak, Pak6, Pde, Pdgf (Complex), Pdgf Bb, Pfkfb3, Pp1 Protein Complex Group, Pp2a, Proinsulin, Rac, Rhov, Rock, Sapk, Scarb2, Scd, Slc6a3, Sos, Srsf7, Ssbp4, Synj1, Tra2b, Trmt6, Tyrosine Kinase, Ubp1, Unc5b, Vav
8	<ul style="list-style-type: none"> <li>Energy Production</li> <li>Lipid Metabolism</li> <li>Small Molecule Biochemistry</li> </ul>	42	35	14-3-3, Adcy, Adcy8, Alkbh3, Asb9, Atypical Protein Kinase C, Bbox1, Casp2, Creb, Cyct, Cyp2c8, Cytochrome C, Enac, Estrogen Receptor, F2r11, G Protein, G Protein Alpha1, G Protein Beta Gamma, Gnai1, Growth Hormone, Gsk3, Gαq, Hdl-Cholesterol, Helt, Hsp27, Hsp70, Irs1, Kcnj1, Kik3, Klra7 (Includes Others), Knndc1, Ldl, Ldl-Cholesterol, Lh, Mek, Muc5b, Neurog2, Nfat (Family), Nqo1, Nr0b2, Nr1d1, Nr2e3, Nr4a2, P38 Mapk, P85 (Pik3r), Paqr7, Pde3b, Pex14, Pi3k (Complex), Pi3k (Family), Pi3k P85, Pkc(S), Pnpla2, Prkd1, Prss29, Prss50, Ptpase, Rara, Ras, Serine Protease, Sod, T3-Tr-Rxr, Tmem54, Tmprss11b, Tmprss7, Trypsin, Tsh, Ucp2, Ucp3, Voltage-Gated Calcium Channel
9	<ul style="list-style-type: none"> <li>Cancer</li> <li>Cell Cycle</li> <li>Cellular Development</li> </ul>	36	32	Acetyl-L-Carnitine, Acox, Acox1, Adss1, Ankar, Bco1, Bpifb6, C1orf159, Catsperg, Ces3, Cholesterol, Chtop, Dec1, Egr2, Fabp9, Fgf13, Fuca1, Fyn, Hcn2, Heparin, Hnrpl, Hpd, Impg2, Inpp5a, Jpt2, Katnal1, Lrrc29, Lrtm1, Mir-100-5p (And Other Mirnas W/Seed Acccgua), Msl2, Mtmr11, Mup1 (Includes Others), Ncmap, Nfib, Nxph4, Obp2b, Ogdhl, Osbp15, Pak6, Pctp, Pex2, Plekhg5, Pmm1, Pparg, Ppfia2, Ppp1ca, Ppp1r27, Ppp2r3d, Pstpip2, Ralgapa1, Rassf6, Rdh11, Rnf144b, Rorc, Shisa5, Shroom2, Slc16a14, Slc22a22, Slc22a25, Stard5, Tep1, Tex19.1, Tmem242, Tmem47, Tmem63a, Tmprss5, Tp53, Tpra1, Ubxn2b, Ywhaz
10	<ul style="list-style-type: none"> <li>Cell Cycle</li> <li>Cellular Assembly and Organization</li> <li>DNA Replication, Recombination, and Repair</li> </ul>	35	31	Adal, Adamtsl3, Ammocr1, Amy2b, Arl5a, Asb15, Aspn, Aven, B4galnt3, C15orf39, Ccdc28a, Ccdc97, Cdc42se1, Cdh19, Cdr1, Ces2g, Cyp3a7, Ddx19a, Depdc7, Drmtn, Fam83g, Fermt1, Il10ra, Iws1, Kiaa0408, Klhdc3, Krtap10-3, Meikin, Meox2, Mex3d, Mobp, N4bp3, Npm1, Nr3c1, Nxf1, P3h2, Pax1, Paxbp1, Pnoc, Rad21, Rad21l1, Rdh11, Rec8, Reg4, Rgs18, Rnf144b, Rpp25, Rpp40, Sectm1, Selenop, Slc16a11, Slc22a16, Stag3, Sycp3, Tbccd1, Tcf3, Terb1, Tgfb1, Tlr7, Tmem104, Tomm34, Tomm40l, Tpst2, Tsen54, Ubl4b, Vgll2, Vim, Xpo1, Znf544, Znf780a

**F** **E 7.5** **6J Alcohol vs. Vehicle**

	Diseases and Functions	-log(Fisher's exact p-value)	Molecules in Network	Molecules
1	<ul style="list-style-type: none"> <li>Dermatological Diseases and Conditions</li> <li>Lipid Metabolism</li> <li>Organismal Injury and Abnormalities</li> </ul>	75	70	A4galt, Abhd17b, Agtrap, Arl14ep, Aunip, Carmil2, Casq1, Casq2, Ccdc184, Ccdc89, Ctnnbip1, Daglb, Dolpp1, Eaf1, Ensa, Epb4115, Eri2, Extl1, Faf2, Fam114a1, Fam234a, Fbxo28, Gbx2, Gdf5, Gon7, Gpank1, Grb2, Grina, Hcn2, Hip1r, Ick, Inca1, Jsrp1, Kbtbd2, Klhdc3, Klhl36, Klk14, Lrrc8a, Lrrc8e, Lyg2, Map3k6, Ntf4, Pard6g, Pkp3, Plcd1, Plcd3, Plekho2, Pnma2, Pnma8a, Pop7, Ppp2r2d, Prap1, Prickle3, Rp111, Selenbp1, Snapc1, Snapc3, Spata2l, Stambpl1, Syt16, Tmem102, Ttyh2, Tulp1, Ubxn2a, Ubxn2b, Ubxn7, Ulk2, Vezt, Zfand2b, Zmym6

2	<ul style="list-style-type: none"> <li>– Cell Death and Survival</li> <li>– Cell Morphology</li> <li>– Cellular Compromise</li> </ul>	75	70	Adat1, Anln, Anxa6, Anxa7, Atxn7l3b, C18orf54, C19orf44, Canx, Casd1, Ccdc127, Cdv3, Cipc, Dctd, Def6, Dpep3, Dynl1, Eed, Elavl1, Eml2, Fam76a, Fam83f, Gipc1, Gk5, Gpm6b, Gsn, Gvin1 (Includes Others), Hm13, Hpd1, Inpp1, Kcng3, Lgals3bp, Lmf1, Lrrc1, Lrtomt, Mfsd13a, Mkrn2os, Mob3a, Myo1c, Osgin2, Pafah1b1, Pcmt1d1, Pink1, Plekhg6, Proca1, Prrc1, Pskh1, Pwp2, Rbmxl1, Rbmxl2, Rdh13, Sfn, Slc50a1, Slc66a2, Smim14, Surf6, Thap3, Tmem189, Tmem53, Tmem68, Trim14, Trim25, Ttc7a, Uros, Vim, Wdr41, Ywhah, Zbtb41, Zfx, Znf354b, Znf91
3	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Connective Tissue Disorders</li> <li>– Organismal Injury and Abnormalities</li> </ul>	72	69	Arl8a, Arpc1b, Cd101, Cenpb, Clrn1, Cmb1, Cntn2, Col11a1, Col4a5, Col8a1, Collagen, Ctsf, Dhrr7b, Dnlz, Errf1, Esyt1, Fam13b, Fbxl2, Foxl1, Hells, Hydin, Jagn1, Kiaa0930, Klhl21, Klhl26, Me1, Metap1, Mllt10, Mxd3, Mxi1, Naa16, Naa40, Nars1, Ndurf4, Nfxl1, Nol9, Nrp1, Pdrgr1, Pef1, Pogk, Polr2m, Ppp1r16a, Puf60, Pxn, Rabggtb, Rbm22, Rcbtb2, Rfwd3, Ripk4, Rmnd5b, Rnf146, Rnf19b, Rpusd1, Samhd1, Sema6c, Smarcd1, Tmpo, Topors, Tram2, Trim31, Tspan11, Ube2d4, Ubox5, Ubtld1, Vwa1, Wars2, Ypel5, Zfp42, Zg16, Zmynd19
4	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Organismal Injury and Abnormalities</li> <li>– RNA Post-Transcriptional Modification</li> </ul>	67	67	Abi2, Adal, Ai987944 (Includes Others), Atg16l1, Atp11b, Bricd5, Cbx1, Cbx5, Cebp, Coil, Cxnc1, Dazap1, Dedd2, Dhx15, Efh2, Fgf17, Grwd1, Hebp2, Histone H3, Hnrnpk, Hoxa9, Hp1bp3, Igsf8, Ilf2, Itga4, Kank3, Krt7, Lmna, Lsm2, Lsm6, Magea3 (Includes Others), Maged2, Mmd2, Pcid2, Pou2f3, Ppp4r2, Prpf38b, Ptgr1, Ptrhd1, Rbbp5, Rbm18, Riox2, Rnpc3, Rpl27a, Shmt2, Slc35a4, Smndc1, Snrnp, Snrpd1, Syng1, Tada1, Tcpl111, Thoc1, Tmem14a, Tmem203, Tmem267, Tmem42, Tmem54, Tmem86a, Trappc13, Tuba4a, U1 Snrnp, Ubn1, Wsb1, Yipf6, Zfp1, Znf22, Znf280d, Znf623, Znf688
5	<ul style="list-style-type: none"> <li>– Cellular Assembly and Organization</li> <li>– Cellular Function and Maintenance</li> <li>– Infectious Diseases</li> </ul>	65	66	Alg2, Ano10, Arfgap2, Arrdc1, Atf1, Bc048679, Cdc42ep2, Cenpn, Crim1, Dbr1, Escrt1, Esr1, Fam118a, Fam136a, Fdxacb1, Fibp, Fkbp1b, Gatb, Gc-Gcr Dimer, Gjd3, Hacd1, Hnf4g, Ica1, Kcnq4, Kctd17, Lor, Micu3, Mier1, Miip, Mkks, Mob3c, Ncoa7, Npw, Nsd1, Nup35, Paqr4, Pcdhb14, Pcdhb4, Peptidylprolyl Isomerase, Plscr3, Plxdc1, Pnrc1, Pnrc2, Ppwd1, Prr15l, Pus1, Qrs1, Rar, Rarg, Rnf138, Rnf38, Sec22c, Shroom1, Slc44a2, Slco1c1, Stk16, Tfp2c, Them6, Tjp3, Trim47, Tsg101, Ttc9, Vps37a, Vps37b, Vps37d, Wdr4, Xpnpep1, Ypel3, Zdhhc21, Zfand4
6	<ul style="list-style-type: none"> <li>– Embryonic Development</li> <li>– Organ Development</li> <li>– Organismal Development</li> </ul>	65	66	Actc1, Actr1a, Aipl1, Ankrd34c, Atf7, Atpase, Borcs6, Borcs8, Ccdc8, Dctn2, Dtnbp1, Eif3j, Entpd1, Fbxo25, Fgfr1op, Foxc1, Gemin2, Gins1, Haus8, Hnrnp1, Jdp2, Kcnab3, Kiaa0753, Kiz, Krt16, Kti12, Mab21l3, Mb21d2, Med4, Mettl3, Mphosph9, Myh14, Myo18b, N4bp3, Ncbp2, Nlrp10, Nop2, Odf2l, P Glycoprotein, Panx1, Par, Patz1, Pfkp, Phax, Prickle2, Psmc5, Rhof, Sec31b, Shtn1, Snw1, Sorbs1, Spats2, Spdl1, Swsap1, Tcf15, Tcp1, Tcp11l2, Thg1l, Tnni3k, Tnnt2, Tor1aip1, Tor1b, Trap/Media, Txndc9, Usp9x, Vill, Wtap, Yap1, Znf326, Znf524
7	<ul style="list-style-type: none"> <li>– Developmental Disorder</li> <li>– Hereditary Disorder</li> <li>– Post-Translational Modification</li> </ul>	63	65	Amigo1, Asl, Atp8b2, Azin2, B3gnt3, B4galnt1, B4gat1, Btdb10, Btd, C1ql1, Ccdc106, Cd160, Cers4, Chodl, Cntnap3, Cryl1, Dtwd1, Ethe1, Evx1, Ext1, Fbxo2, Fev, Fktn, Fos, Galnt12, Gpha2, Gpt2, Has, Hus1, Interleukin, Kiaa1841, Klrg2, Lamb2, Lonrf2, Lrif1, Mad2l2, Metr, Mob4, Mto1, Nagpa, Nkiras2, Ornithine Decarboxylase, Pcdh19, Pdf, Ppp2c, Prrx1, Ptcd2, Pxx, Rmnd1, Rtn2, Scf, Sgtb, Sike1, Slc10a6, Slc25a20, Smg7, Smg8, Snai3, Snx25, St6galnac4, St8sia3, St8sia5, Stk25, Strn3, Tbp1, Tmem143, Tmem30b, Tpgs1, Xylt1, Znf146
8	<ul style="list-style-type: none"> <li>– Cell Morphology</li> <li>– Post-Translational Modification</li> <li>– Small Molecule Biochemistry</li> </ul>	63	65	Agfg2, Angel1, Aplp1, Atic, Atxn7l2, C1orf35, Ccdc122, Cdy12, Chst13, Clock, Col9a2, Cspp1, Dennd2d, Dis3l, Epc1, Epc2, Eps15, Etv2, Exosc6, Exosc7, Exosome, Fez1, Fgf16, Foxn2, Foxo6, G2e3, Gcdh, Gnl1, Hap1, Hid1, Hmgcs2, Hoxb8, Hs3st3a1, Ing3, Ing5, Katnbl1, Meaf6, Med23, Mipol1, Mis12, Mphosph6, Nuf2, Paps2, Pdcd7, Pkdcc, Pkn3, Plvap, Pnp, Pxr Ligand-Pxr-Retinoic Acid-Rxr, Rbm48, Rcn3, Reep3, Sec62, Sgf29, Slc25a41, Spc24, Specc1l, Srcap, Stx18, Sulfoltransferase, Sult1d1, Sult1e1, Thap11, Tip60, Vegf, Vps51, Yeats4, Znf24, Znf324, Znf394
9	<ul style="list-style-type: none"> <li>– Digestive System Development and Function</li> <li>– Embryonic Development</li> <li>– Post-Translational Modification</li> </ul>	63	65	Abhd8, Ahcyl2, Ammecn1, Anapc16, Arhgap45, Armc5, Banp, Bcap29, Bend3, Bmt2, Ccng1, Ccnl2, Cdx4, Cfap97, Chp1, Cog8, Dip2a, Fam117a, Fam124a, Fam161a, Fam214b, Farnesyl Transferase, Fnta, Gabp, Gabpb1, Gata, Gem, Ggtase I, Gmip, Hectd1, Hmbox1, Homer2, Iffo1, Irf2bp2, Irx5, Isl1, Itfg2, Lhx6, Lmo1, Lmo2, Lmo4, Loc728392, Lrnf4, Mbnl1, Meis2, Mnx1, Necap2, Nova1, Nudt22, Ooep, Pars2, Pgggt1b, Pitx1, Proser2, Purg, Ras, Rundc3a, Ssbp4, Suox, Tal2, Tle6, Trim54, Uba6, Uck1, Vmac, Wdr33, Zbtb43, Zdhhc1, Znf513, Zzz3
10	<ul style="list-style-type: none"> <li>– Cell Morphology</li> <li>– Cellular Assembly and Organization</li> <li>– Protein Synthesis</li> </ul>	63	65	Adck5, Adss1, Appl2, Atm/Atr, Azin1, Chdh, Cpne7, Crif3, Cxcl12, Dcaf15, Dtwd2, Eme2, Etaa1, Fa, Faap24, Fanc, Fanc, Fance, Gls, Grpel2, Grsf1, Gsap, Hoga1, Iba57, Id4, Lman2l, Mapre3, Mepce, Mfap3, Mgme1, Mmadhc, Mrm3, Mrn, Mrpl19, Mrpl21, Mrpl32, Mrpl39, Mrpl44, Mrpl45, Mrpl49, Mrpl9, Mrps25, Mrps27, Mrps30, Mrps7, Nit1, Oxld1, Pagr1, Psrc1, Rab31, Rmi2, Rpa, Rpusd3, Rpusd4, Sertad3, Slx1a/Slx1b, Slx4ip, Susd1, Tmc4, Tmco4, Tmem231, Tmem245, Top3a, Tra2a, Trmt1, Wfdc1, Xrcc2, Yars2, Ybey, Znf503

**G** E 7.5 6N Alcohol vs. Vehicle

Diseases and Functions	-log(Fisher's exact <i>p</i> -value)	Molecules in Network	Molecules
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1	<ul style="list-style-type: none"> <li>– Cell Morphology</li> <li>– Embryonic Development</li> <li>– Hair and Skin Development and Function</li> </ul>	70	49	26s Proteasome, Adgrg6, Azin2, Calmodulin, Col4a6, Complement, Cxcr6, Dmc1, Dnajc6, Etv1, Fam222a, Fbn2, Fbxo6, Fpr1, Hemgn, Hnrnpk, Hspd1, Igg1, Igm, Il12 (Complex), Il12 (Family), Il1bos, Iqcb1, Kcnn1, Kcnn2, Keratin, Klhl22, Kpnb1, Krt16, Krt26, Krt33a, Krt35, Krt37, Krt73, Krt75, Krt86, Kyat1, Lgals3bp, Mapk, Mkrn3, Ndufa4i2, Noa1, Notch2, Notch3, Ornithine Decarboxylase, P-Tefb, Pbx1, Proinsulin, Pspc1, Ras, Rbm14, RbmX, Rna Polymerase Ii, Sdr39u1, Sfpq, Sil1, Six2, Slc25a20, Speg, Sstr4, Stat, Syt2, Tbx19, Tcea2, Trim28, Trim31, Tuba4a, Tubulin, Ubr1, Znf462
2	<ul style="list-style-type: none"> <li>– Cardiovascular Disease</li> <li>– Cell Death and Survival</li> <li>– Cellular Assembly and Organization</li> </ul>	70	49	Adss1, Ak1, Ankrd37, Aven, Ccdc155, Ccnjl, Cga, Cldn13, Coq8b, Dio2, Dnpep, EglN, Fcn1, Fhl2, Gata2, Gmppa, Gpr158, Gpr25, Gpr37i1, Hebp2, Hmx1, Hnrnp1, Hnrmpu, Htr3b, Igsf9, Krtcap3, Kyat3, Lhfp15, Lrrc57, Lyg2, Maml2, Marchf2, Mkrn2os, Morn1, Mtnr1b, Nanos2, Nr3c1, Nsg1, Nudt13, Odf4, Pdzph1, Pitx2, Prss56, Ptgr1, RetnlG, Rhag, Rhce/Rhd, RhoX3a (Includes Others), Rnf122, Rtn4r1, Scube1, Selenbp1, Serp2, Serpinb9f (Includes Others), Serpin2, Slc44a4, Smim14, Sox13, Spire2, Stag3, Stfa2/Stfa2i1, Syng1, Terb1, Tgfb1, Trim25, Trim58, Ubc, Upk3b, Wdpcp, Wdr34
3	<ul style="list-style-type: none"> <li>– Lipid Metabolism</li> <li>– Nucleic Acid Metabolism</li> <li>– Small Molecule Biochemistry</li> </ul>	58	43	Accs2, Acvr2b, Alp, Alpl, Ampk, Bcl11a, Bmp15, Bspry, Calcineurin Protein(S), Caspase 3/7, Cbp/P300, Ccnd2, Collagen Alpha1, Ctbp, Cuedc2, Cyclin A, Cyp11a1, Cyp2c40 (Includes Others), Cyp2c8, Cyp2e1, Cyp4f11, Dkk1, Elovl6, Emid1, Etv6, Fasn, Frizzled, Fsh, Gzmh, Hdac, Histone H3, Histone H4, Hsp70, Jnk, Kat2b, Khk, Lmcd1, Mef2, Mef2c, Mgmt, Mlycd, N-Cor, Nadh2 Or Nadph2 1 Atom Incorporation:Oxygen Oxidoreductase, Ncs1, Nr1h, P70 S6k, Prc2, Prkag2, Proc, Qki, Rab29, Rb, Rbm15, Sat2, Scd2, Six3, Smad, Smad2/3, Sp7, Tcf, Tgf Beta, Tgm2, Tnfrsf14, Tnn, Tp63, Trerf1, Unspecific Monooxygenase, Vgl12, Vill, Wnt1
4	<ul style="list-style-type: none"> <li>– Cellular Movement</li> <li>– Hematological System Development and Function</li> <li>– Post-Translational Modification</li> </ul>	58	43	2210010c04rik, Abl1, Adam22, Alpha 1 Antitrypsin, Ap1, Arrdc1, Atp6v1g3, Blvrb, Calpain, Caspase, Cd44, Cilp, Ck2, Cog8, Crabp2, Cstf3, Ctla2a/Ctla2b, Cytochrome C, Cytokine, Dsg1, Ecm, Efna4, Entpd1, F9, Focal Adhesion Kinase, Gbe1, Gcdh, Gsk3, H2ax, Histone, Hnrmpa1, Hnrmpdl, Hsp90, Il1r2, Immunoglobulin, Interleukin, Klk12, Lanc12, Ldb1, Ldl, Macrod1, Mdn1, Metalloprotease, Micb, Mmp, Mmp2, Necap2, P Glycoprotein, P38 Mapk, Parp, Phosphatase, Pink1, Pkc(S), Plppr3, Prap1, Prickle3, Prkch, Prss50, Prss56, Serine Protease, Sgca, Shp, St3gal2, Stx4, Tet1, Trypsin, Ubiquitin, Usp17le (Includes Others), Utp20, Vegf
5	<ul style="list-style-type: none"> <li>– Developmental Disorder</li> <li>– Organismal Injury and Abnormalities</li> <li>– Skeletal and Muscular Disorders</li> </ul>	54	41	Alpha Actinin, Amylase, Anxa4, Bmp, C3, Camk1, Cd55, Cd68, Cdh11, Cer1, Chst3, Chymotrypsin, Col10a1, Collagen Type I (Complex), Collagen Type Ii, Collagen Type Iii, Collagen Type Iv, Collagen Type V, Collagen Type X, Collagen(S), Complement Component 1, Cpa1, Cr3, Elastase, Erk1/2, Etv2, Fbx12, Fgf, Fgf13, Fibrin, Fibrinogen, Gli, Gli3, Glipr2, Hcrt, Hgh1, Hopx, Hs6st2, Inpp1, Integrin, Klf13, Klrc1, Laminin (Complex), Lrg1, Mmp8, Nkx2-5, Pdgf (Complex), Pla2, Pla2g5, Pld, Pld2, Prrx1, Pxr Ligand-Pxr-Retinoic Acid-Rxra, Rab26, Rab3d, Rgs5, Rps6ka, Rsph6a, Serpinb7, Ski, Slco1b3, Smooth Muscle Actin, Sox17, St6galnac3, Sulfotransferase, Sult1e1, Tbx1, Ubash3a, Ucn3, Wnt
6	<ul style="list-style-type: none"> <li>– Cellular Movement</li> <li>– Hematological System Development and Function</li> <li>– Immune Cell Trafficking</li> </ul>	52	40	Ackr2, Acox, Acy1, Akt, Asb9, Atrial Natriuretic Peptide, Ccl1, Ccl22, Ccl24, Ccl7, Ccng1, Collagen Type I (Family), Cox6a2, Creatine Kinase, Creb3l3, Ctla4, Cxcl3, Cxcl3, Cyp7a1, Dbh, Dynammin, Fcer1, Fgd2, Foxp1, Gdf15, Guca1a, Hfe, Ige, Il-2r, Il17r, Il17rc, Il23, Il2rb, Jak, Jink1/2, Jun/Junb/Jund, Laminin1, Loc102724788/Prodh, Lpin1, Map2k1/2, Marco, Mcam, Mek, Mir122a, B, Nfatc4, Or51e2, Pi3k (Family), Pi3k P85, Pik3c2g, Plac8, Plk3, Ppp2c, Prcp, Raf, Rap1, Rar, Reg3a, Rsk, Rxr, Rxrg, S100, S100a4, S100a8, S100a9, Scgb3a1, Serpinb6b, Slc6a12, T3-Tr-Rxr, Thyroid Hormone Receptor, Vla-4
7	<ul style="list-style-type: none"> <li>– Endocrine System Development and Function</li> <li>– Inflammatory Response</li> <li>– Organismal Injury and Abnormalities</li> </ul>	52	40	2' 5' Oas, 9830107b12rik/A530064d06rik, Alb, Aldh, Apyrase, Aqp5, C/Ebp, Capg, Cd200r1, Cd209c, Cd276, Chemokine, Collagen, Cspg4, Cyp1a1, Cytokine Receptor, Elf3, Filamin, Fuca1, Gadd45b, Nggt1, Growth Hormone, Hat, Hdl, Hif1, Hp, Ifn, Ifn Beta, Ifna4, Ifnar, Il1, Il36g, Il36m, Interferon Alpha, Krtap11-1, Lcn2, Ldl-Cholesterol, Mapk13, Mhc Class Ii (Complex), Mus81, Nap1l2, Nfkb (Complex), Nlr, Nlrp4, Nlrp6, Notch, Oas, Oas1, Oas1b, Oas1d (Includes Others), Pepck, Polr3gl, Pro-Inflammatory Cytokine, Prtn3, Rras, Sapk, Setbp1, Slc46a2, Ssbp4, Stap2, Tgfbr, Tlr, Tnf (Family), Tnfrsf17, Tnfsf8, Ugt, Ugt2a1, Ugt2b17, Unc93b1, Vcan
8	<ul style="list-style-type: none"> <li>– Cancer</li> <li>– Organismal Injury and Abnormalities</li> <li>– Psychological Disorders</li> </ul>	50	39	Adcy, Adrb, Akr1b7, Anxa9, Ap2, Bace1, Bcas1, Bco1, Beta Arrestin, Blk, Cadm4, Cg, Creb, Estrogen Receptor, Fam3d, Fhl5, Flot1, Foxo6, G Protein AlphaI, Gabrg2, Gimap8, Gipc1, Gnao1, Gnat3, Got, Gpcr, Hnrnp1, Htr1a, Igf2bp3, Insulin, Kcnj1, Kdr, Kiss1r, Ldh (Complex), Lh, Mediator, Nefm, Neurod2, Ntrk3, P85 (Pik3r), Pcyt1b, Pdgr, Pi3k (Complex), Pka, Pka Catalytic Subunit, Plc, Pp2a, Ppm1j, Ppy, Ptpase, Ptpn1, Ptpn18, Rassf2, Ros1, Sfk, Shc, Sod, Src (Family), Srxn1, Stat5a/B, Trh, Trk Receptor, Tro, Tsh, Tspo, Tubb4a, Tubulin (Family), Voltage-Gated Calcium Channel, Ypel3, Znf326

9	<ul style="list-style-type: none"> <li>– Cellular Function and Maintenance</li> <li>– Cellular Growth and Proliferation</li> <li>– Hematological System Development and Function</li> </ul>	43	35	<p>Acr, Actin, Alpha Catenin, Alpha Tubulin, Arhgap9, Arhgef2, Bach2, Bcr (Complex), Camkii, Cap2, Cblc, Cd207, Cd3, Cofilin, Ctnna3, Eps8l2, Erk, Erm, F Actin, Frs3, Gcsam, Gm9573, Gmpr, Grik5, Gsn, H2-L, H2-M10.1 (Includes Others), Hla-A, Hla-G, Iga, Igg, Igg2a, Igg3, Iqub, Itgad, Jph4, Kcna5, Klra7 (Includes Others), Mapk15, Mhc, Mhc Class I (Complex), Mhc Class I (Family), Mlc, Mucin, Myosin, NADPH Oxidase, Nfat (Complex), Nfat (Family), Pak, Pdgf Bb, Plc Gamma, Ptk, Rab11, Rac, Rac3, Ras Homolog, Rhoc, Rnd2, Rock, Sec14l1, Serpinb10, Sos, Sptbn4, St8sia3, Tap, Tappb, Tcr, Tesk1, Trio, Tyrosine Kinase</p>
10	<ul style="list-style-type: none"> <li>– Cell Death and Survival</li> <li>– DNA Replication, Recombination, and Repair</li> <li>– Nervous System Development and Function</li> </ul>	41	34	<p>Acss2, Acy3, Adra2b, Aoc1, Apoc3, Asl, Bmper, Bpifb2, C11orf71, Calcl, Ccer1, Ccr5, Ces2e, Cst9l, Cxcr6, Cyp2a12/Cyp2a22, Depp1, Dhx29, Dmc1, Fgf13, Fxyd6, Fxyd7, Fzd3, Fzd6, Gchfr, Gdpd4, Gpr37, Gpr37l1, Hdl-Cholesterol, Hipk1, Hnf4a, Ikzf2, Il10ra, Impg1, Kcnk3, Krt8, Loc102724788/Prodh, Map3k8, Mutyh, Mycn, Ndr4, Npffr2, Npm1, Npnt, Nrarp, Nynrin, Plscr1, Prkcz, Rhox6/Rhox9, Rnase4, Sdr42e1, Sh3bgrl2, Slc10a5, Slc39a2, Sox2, Speer4a (Includes Others), Sprr2h, Sprr2i, Sstr4, Sycn, Tada1, Tapppl, Tet1, Tex15, Tm6sf1, Tp53, Unc5a, Urah, Yy2, Zic3</p>

**Table S3. Selected pathways significantly enriched among differentially expressed genes between the vehicle-treated 6J and 6N embryos 6 hr after injection (E7.25).****Down-regulated Genes****Gene Ontology: Biological Process**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Cytolysis	GO:0019835	7	5.12
Granzyme-mediated apoptotic signaling	GO:0008626	5	4.85

**Gene Ontology: Molecular Function**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Serine-type endopeptidase activity	GO:0004252	11	4.30
Hydrolase activity, acting on acid phosphorus-nitrogen bonds	GO:0016825	11	3.80
Serine hydrolase activity	GO:0017171	11	3.80
Peptidase activity	GO:0008233	16	2.18
Peptidase activity, acting on L-amino acid peptides	GO:0070011	15	1.79

**KEGG: Biological Process**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Graft-versus-host disease	KEGG:05332	5	2.71
cAMP signaling	KEGG:04024	9	2.68
Neuroactive ligand-receptor interaction	KEGG:04080	10	1.84

**Reactome**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Activation, myristoylation of BID and translocation to mitochondria	REAC:R-MMU-75108	5	5.59
Intrinsic pathway for apoptosis	REAC:R-MMU-109606	5	2.63

**Up-regulated Genes****KEGG: Biological Process**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Caffeine metabolism	KEGG:00232	2	1.94



**Table S4. Selected pathways significantly enriched among differentially expressed genes between the 6J and 6N embryos 12 hr after vehicle treatment (E7.5).****Down-regulated Genes****Gene Ontology: Molecular Function**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Serine-type endopeptidase activity	GO:0004252	9	2.62
Serine-type peptidase activity	GO:0008236	9	2.30
Hydrolase activity, acting on acid phosphorus-nitrogen bonds	GO:0016825	9	2.22
Serine hydrolase activity	GO:0017171	9	2.22
Endopeptidase activity	GO:0004175	12	1.60

**Up-regulated Genes****Reactome**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Peptide ligand-binding receptors	R-MMU-375276	7	2.74
GPCR ligand binding	R-MMU-500792	9	2.58
Class A/1 (Rhodopsin-like receptors)	R-MMU-373076	8	2.42

**Human Phenotype Ontology**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Hyperprostaglandinuria	HP:0003527	2	1.93
Increased serum prostaglandin E2	HP:0003566	2	1.93
Renal juxtaglomerular cell hypertrophy/hyperplasia	HP:0000111	2	1.93
Abnormal circulating prostaglandin circulation	HP:0011023	2	1.93

**Table S5. Selected pathways significantly enriched among differentially expressed genes between the PAE and vehicle-treated 6J embryos 6 hr after exposure (E7.25).****Down-regulated Genes****Gene Ontology: Biological Process**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Cellular metabolic process	GO:0044237	275	10.45
Gene expression	GO:0010467	162	6.44
Methylation	GO:0032259	27	5.47
Protein modification process	GO:0036211	119	4.32
Cell cycle	GO:0007049	66	3.91
Cellular biosynthetic process	GO:0044249	154	2.96
RNA modification	GO:0009451	14	2.69
Chromosome organization	GO:0051276	45	1.73
Programmed cell death	GO:0012501	65	1.43
Transcription by RNA polymerase II	GO:0006366	66	1.39

**Gene Ontology: Molecular Function**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Heterocyclic compound binding	GO:1901363	172	7.10
Methyltransferase activity	GO:0008168	19	4.81
Catalytic activity	GO:0003824	159	3.24
Nucleic acid binding	GO:0003676	110	3.05
Enzyme binding	GO:0019899	76	2.66
ATP binding	GO:0005524	55	2.57
Protein binding	GO:0005515	241	2.51
miRNA binding	GO:0035198	6	1.83
Ribonucleotide binding	GO:0032553	64	1.82
transcription regulator activity	GO:0140110	49	1.31

**Human Phenotype Ontology**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Broad forehead	HP:0000337	13	2.57

### **Up-regulated Genes**

#### **Gene Ontology: Biological Process**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Cytolysis	GO:0019835	9	5.53
Granzyme-mediated apoptotic signaling	GO:0008626	5	3.19
Primary alcohol metabolic process	GO:0034308	8	1.69

#### **Gene Ontology: Molecular Function**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Serine-type endopeptidase activity	GO:0004252	12	2.14
Endopeptidase activity	GO:0004175	19	1.93
Hydrolase activity, acting on acid phosphorus-nitrogen bonds	GO:0016825	12	1.66
Serine hydrolase activity	GO:0017171	12	1.66

#### **KEGG: Biological Process**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Graft-versus-host disease	KEGG:05332	5	1.38

#### **Reactome**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Activation, myristoylation of BID and translocation to mitochondria	REAC:R-MMU-75108	5	4.08

**Table S6. Selected pathways significantly enriched among differentially expressed genes between the PAE and vehicle-treated 6N embryos 6 hr after exposure (E7.25).****Down-regulated Genes****Gene Ontology: Biological Process**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Organic substance metabolic process	GO:0071704	179	3.38
Macromolecule metabolic process	GO:0043170	159	3.01
Primary metabolic process	GO:0044238	171	2.98
Methylation	GO:0032259	18	2.71
Metabolic process	GO:0008152	182	2.27
Macromolecule methylation	GO:0043414	16	2.17
Regulation of metabolic process	GO:0019222	119	1.71
Nitrogen compound metabolic process	GO:0006807	159	1.68
RNA metabolic process	GO:0016070	87	1.67
Regulation of cellular metabolic process	GO:0031323	111	1.44

**Gene Ontology: Molecular Function**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
rRNA (adenine) methyltransferase activity	GO:0016433	3	1.74
Catalytic activity	GO:0003824	107	1.62
Sterol esterase activity	GO:0004771	4	1.50
Methyltransferase activity	GO:0008168	11	1.35

**Reactome**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Gene expression (transcription)	R-MMU-74160	29	1.78
RNA polymerase II transcription	R-MMU-73857	27	1.73

**Table S7. Selected pathways significantly enriched among differentially expressed genes between the PAE and vehicle-treated 6J embryos 12 hr after exposure (E7.5).****Down-regulated Genes****Gene Ontology: Biological Process**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Nucleic acid metabolic process	GO:0090304	552	69.99
Gene expression	GO:0010467	559	57.57
Primary metabolic process	GO:0044238	819	45.95
Embryo development	GO:0009790	136	13.2
Heart development	GO:0007507	77	7.33
Skeletal system development	GO:0001501	66	6.40
Cellular response to DNA damage stimulus	GO:0006974	82	4.75
Regulation of cell cycle	GO:0051726	100	4.02
Circulatory system development	GO:0072359	108	3.84
Brain development	GO:0007420	76	3.40

**Gene Ontology: Molecular Function**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Nucleic acid binding	GO:0003676	432	54.31
Organic cyclic compound binding	GO:0097159	550	40.57
DNA binding	GO:0003677	266	33.72
RNA binding	GO:0003723	156	18.46
Transcription factor binding	GO:0008134	88	9.27
Catalytic activity, acting on RNA	GO:0140098	55	7.99
Histone acetyltransferase activity	GO:0004402	14	2.62
Protein binding	GO:0005515	636	1.86
miRNA binding	GO:0035198	9	1.68
Histone binding	GO:0042393	30	1.50

**KEGG: Biological Process**

<b>Term name</b>	<b>Term ID</b>	<b># of genes</b>	<b>Log10 p-value</b>
Spliceosome	KEGG:03040	22	3.67
Signaling pathways regulating pluripotency of stem cells	KEGG:04550	21	2.67
RNA transport	KEGG:03013	22	2.06
Ribosome biogenesis in eukaryotes	KEGG:03008	13	1.64

**Reactome**

Term name	Term ID	# of genes	Log10 p-value
Gene expression (transcription)	R-MMU-74160	112	6.92
Metabolism of RNA	R-MMU-8953854	72	6.67
Processing of capped intron-containing pre-mRNA	R-MMU-72203	38	4.93
RNA polymerase II transcription	R-MMU-73857	94	3.84
mRNA splicing	R-MMU-72172	29	3.04
mRNA splicing - major pathway	R-MMU-72163	28	2.86
RNA polymerase II transcribes snRNA genes	R-MMU-6807505	15	2.34

### Human Phenotype Ontology

Term name	Term ID	# of genes	Log10 p-value
Abnormal lip morphology	HP:0000159	81	3.41
Abnormal palate morphology	HP:0000174	84	3.07
Abnormality of the outer ear	HP:0000356	89	2.93
Holoprosencephaly	HP:0001360	15	2.39
Abnormal facial shape	HP:0001999	80	1.73
Anal atresia	HP:0002023	21	1.65
Atrioventricular canal defect	HP:0006695	11	1.53
Oral cleft	HP:0000202	53	1.42

### Up-regulated Genes

#### Gene Ontology: Biological Process

Term name	Term ID	# of genes	Log10 p-value
Localization	GO:0051179	529	9.04
Transport	GO:0006810	411	7.64
Transmembrane transport	GO:0055085	160	4.75
Protein phosphorylation	GO:0006468	189	4.25
Intracellular signal transduction	GO:0035556	236	4.11
Cellular response to chemical stimulus	GO:0070887	272	3.12
Anatomical structure development	GO:0048856	466	2.18
Response to stress	GO:0006950	316	1.71
Cell death	GO:0008219	191	1.48
Positive regulation of cell differentiation	GO:0045597	109	1.42

### Gene Ontology: Molecular Function

Term name	Term ID	# of genes	Log10 p-value
Protein binding	GO:0005515	821	9.85
TAP binding	GO:0046977	8	4.52
GTP binding	GO:0005525	54	3.42
Nucleotide binding	GO:0000166	204	2.82
Enzyme binding	GO:0019899	207	1.79
Beta-2-microglobulin binding	GO:0030881	6	1.78
Cytoskeletal protein binding	GO:0008092	98	1.57
GTPase activity	GO:0003924	40	1.48
Protein kinase binding	GO:0019901	76	1.38

### Reactome

Term name	Term ID	# of genes	Log10 p-value
Metabolism	R-MMU-1430728	158	1.78
Ion channel transport	R-MMU-983712	27	1.68
Stimuli-sensing channels	R-MMU-2672351	18	1.35
Formation of xylulose-5-phosphate	R-MMU-5661270	4	1.31

**Table S8. Selected pathways significantly enriched among differentially expressed genes between the PAE and vehicle-treated 6N embryos 12 hr after exposure (E7.5).****Down-regulated Genes****Gene Ontology: Biological Process**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Positive regulation of transcription by RNA polymerase II	GO:0045944	37	5.02
Positive regulation of cellular metabolic process	GO:0031325	69	4.56
Transcription by RNA polymerase II	GO:0006366	47	3.27
Regulation of cell differentiation	GO:0045595	44	2.61
Cell fate commitment	GO:0045165	13	1.85
Skeletal system development	GO:0001501	18	1.72
Primary metabolic process	GO:0044238	140	1.53
Embryonic morphogenesis	GO:0048598	20	1.52
Generation of neurons	GO:0048699	36	1.47
Regulation of nervous system development	GO:0051960	27	1.46

**Gene Ontology: Molecular Function**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Sequence-specific DNA binding	GO:0043565	38	6.17
RNA polymerase II regulatory region sequence-specific DNA binding	GO:0000977	29	5.17
Transcription regulatory region DNA binding	GO:0044212	32	5.15
Cis-regulatory region binding	GO:0035326	25	4.87
Transcription regulator activity	GO:0140110	37	3.50
DNA-binding transcription activator activity	GO:0001216	17	2.77
DNA binding	GO:0003677	43	1.65

**KEGG: Biological Process**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Steroid hormone biosynthesis	KEGG:00140	6	1.77

**Human Phenotype Ontology**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Hypoplasia of the epiglottis	HP:0005349	3	1.31



**Up-regulated Genes****Gene Ontology: Biological Process**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Antigen processing and presentation of endogenous antigen	GO:0019883	10	6.37
Antigen processing and presentation via MHC class Ib	GO:0002475	9	5.31
Antigen processing and presentation of peptide antigen	GO:0048002	10	4.18
Defense response	GO:0006952	52	3.78
Response to stress	GO:0006950	91	3.16
Antigen processing and presentation of peptide antigen via MHC class I	GO:0002474	6	3.07
Antigen processing and presentation	GO:0019882	11	2.87
Inflammatory response	GO:0006954	27	1.90

**Gene Ontology: Molecular Function**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
TAP binding	GO:0046977	6	5.78
Beta-2-microglobulin binding	GO:0030881	6	5.78
Peptide antigen binding	GO:0042605	6	4.06
Catalytic activity	GO:0003824	121	2.68
RAGE receptor binding	GO:0050786	4	2.64

**KEGG: Biological Function**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
IL-17 signaling pathway	KEGG:04657	8	1.74

**Reactome**

Term name	Term ID	# of genes	Log10 <i>p</i> -value
ER-phagosome pathway	R-MMU-1236974	8	4.39
Neutrophil degranulation	R-MMU-6798695	25	3.47
Endosomal/vacuolar pathway	R-MMU-1236977	6	2.66
Antigen presentation: Folding, assembly and peptide loading of class I MHC	R-MMU-983170	7	2.57
Metal sequestration by antimicrobial proteins	R-MMU-6799990	3	2.23
Peptide ligand-binding receptors	R-MMU-375276	12	1.60

### Human Phenotype Ontology

Term name	Term ID	# of genes	Log10 <i>p</i> -value
Crazy paving pattern on pulmonary HRCT	HP:0025391	5	3.27
Elevated carcinoembryonic antigen level	HP:0031029	5	3.05
Recurrent acute respiratory tract infection	HP:0011948	6	2.94
Acute infectious pneumonia	HP:0011949	5	2.48
Foam cells	HP:0003651	5	2.18
Respiratory failure requiring assisted ventilation	HP:0004887	5	1.92
Abnormal blood oxygen level	HP:0500165	5	1.80
Hypoxemia	HP:0012418	5	1.80
Abnormal blood gas level	HP:0012415	5	1.48
Abnormal pulmonary thoracic imaging finding	HP:0031983	5	1.48

**Table S9. Cilia-related genes significantly different between the strains at baseline or within the strains after alcohol.**

GD7.0		GD7.25				GD7.5			
6J's vs. 6N's		6J's		6N's		6J's		6N's	
Baseline		PAE vs. Vehicle		PAE vs. Vehicle		PAE vs. Vehicle		PAE vs. Vehicle	
Gene	Log2 FC	Gene	Log2 FC	Gene	Log2 FC	Gene	Log2 FC	Gene	Log2 FC
<i>Fam65b</i>	0.30	<i>Fam161a</i>	0.86	<i>Fam161a</i>	0.98	<i>Mchr1</i>	1.28	<i>Iqub</i>	0.95
<i>Efcab7</i>	-0.89	<i>Tbc1d30</i>	0.75	<i>Tex40</i>	0.94	<i>Fam161a</i>	1.17	<i>Tekt1</i>	0.82
		<i>Hydin</i>	0.65	<i>Hk1</i>	0.81	<i>Hydin</i>	0.88	<i>Tulp1</i>	0.80
		<i>Map6</i>	0.61	<i>Ak1</i>	0.80	<i>Rab29</i>	0.85	<i>Rab29</i>	0.68
		<i>Bbs4</i>	0.61	<i>Tuba4a</i>	0.61	<i>Efcab7</i>	0.81	<i>Tuba4a</i>	0.67
		<i>Ptch1</i>	0.46	<i>Bbs4</i>	0.52	<i>Tmem231</i>	0.81	<i>Wdpcp</i>	0.62
		<i>Arl2bp</i>	0.38	<i>Tmem17</i>	0.43	<i>Mxi1</i>	0.80	<i>Cep72</i>	0.49
		<i>Till1</i>	0.28	<i>Haus4</i>	0.35	<i>Tuba4a</i>	0.78	<i>Wdr34</i>	0.48
		<i>Cnga2</i>	0.041	<i>Pkhd1</i>	0.34	<i>Rilpl2</i>	0.74	<i>Pkd2l1</i>	0.33
		<i>Myoc</i>	0.02	<i>Tekt3</i>	0.03	<i>Ccno</i>	0.72	<i>Ropn1</i>	-0.06
		<i>Ccr6</i>	-0.03	<i>Till8</i>	0.01	<i>Tulp1</i>	0.71	<i>Iqcb1</i>	-0.35
		<i>Mapre1</i>	-0.17	<i>Myoc</i>	-0.06	<i>Hspb11</i>	0.68	<i>Rsg1</i>	-0.80
		<i>Cct3</i>	-0.21	<i>Cngb3</i>	-0.08	<i>Rilpl1</i>	0.68	<i>Gli3</i>	-0.83
		<i>Exoc3</i>	-0.23	<i>Mapre1</i>	-0.19	<i>Mok</i>	0.60		
		<i>Dnaja1</i>	-0.30	<i>Exoc3</i>	-0.24	<i>Kiz</i>	0.56		
		<i>Nup35</i>	-0.44	<i>Hspa8</i>	-0.36	<i>Kif3b</i>	0.52		
		<i>Hspa8</i>	-0.45	<i>Ralgapa1</i>	-0.51	<i>Mdm1</i>	0.51		
		<i>Gna11</i>	-0.48	<i>Trim59</i>	-0.56	<i>Snx10</i>	0.44		
		<i>Pard3</i>	-0.58	<i>Katnal1</i>	-0.58	<i>Pde6d</i>	0.44		
		<i>Tapt1</i>	-0.59	<i>Pex6</i>	-0.74	<i>Usp9x</i>	0.44		
		<i>Gnb1</i>	-0.71	<i>Wdr78</i>	-0.78	<i>Prkar2a</i>	0.43		
		<i>Dyx1c1</i>	-0.72			<i>Ick</i>	0.40		
		<i>Atp2a2</i>	-1.02			<i>Tubb2a</i>	0.34		
		<i>Prkaca</i>	-1.19			<i>Unc119</i>	0.33		
		<i>Pex6</i>	-1.38			<i>Rp111</i>	0.07		
						<i>Dpcd</i>	-0.35		
						<i>Plk4</i>	-0.39		
						<i>Aurka</i>	-0.42		
						<i>Fgfr1op</i>	-0.48		
						<i>Tmem80</i>	-0.51		
						<i>Topors</i>	-0.56		
						<i>Smo</i>	-0.59		
						<i>Gli3</i>	-0.59		
						<i>Pafah1b1</i>	-0.59		
						<i>Pifo</i>	-0.61		
						<i>Dnajb13</i>	-0.65		
						<i>Cspp1</i>	-0.66		
						<i>Rttm</i>	-0.66		
						<i>Flcn</i>	-0.67		
						<i>Noto</i>	-0.68		
						<i>Pkd2</i>	-0.69		
						<i>Rab11b</i>	-0.69		
						<i>Morn3</i>	-0.72		
						<i>Cfap161</i>	-0.77		
						<i>Ptch1</i>	-0.79		
						<i>Spef2</i>	-0.83		
						<i>Tapt1</i>	-0.90		
						<i>Mkks</i>	-1.03		
						<i>Osr1</i>	-1.43		

**Dataset 1.** Excel file containing VST-normalized values for all replicates, group means, Log2FC, and adjusted p-values for all differentially expressed genes from each comparison.

[Click here to download Dataset1](#)