Transcriptomic analyses of gastrulation-stage C57BL/6J and C57BL/6NHsd mouse embryos with differential susceptibility to alcohol

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SUMMARY STATEMENT

RNA-seq in gastrulation-stage mouse embryos provides information about gene expression patterns during normal mouse development and evidence that pre-existing genetic variability mediates risk to prenatal alcohol-induced birth defects.

ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) are a serious public health concern, affecting approximately 5% of live births in the US. The more severe craniofacial and central nervous system malformations characteristic of FASD are caused by alcohol exposure during gastrulation (embryonic day 7 in mice; 3rd week of human pregnancy). Genetics are a known contributor to differences in alcohol sensitivity in humans and in animal models of FASD. Our study profiled gene expression in gastrulation-stage embryos from two commonly used, genetically similar mouse substrains, C57BL/6J and C57BL/6NHsd, that differ in alcohol sensitivity. First, we established normal gene expression patterns at three finely resolved timepoints during gastrulation and developed a web-based interactive tool. Baseline transcriptional differences across strains were associated with immune signaling, indicative of their molecular divergence. Second, we examined the gene networks impacted by alcohol in each strain. Alcohol was associated with a more pronounced transcriptional effect in the 6J’s vs. 6N’s, matching the 6J’s increased susceptibility. The 6J strain exhibited down-regulation of cell proliferation and morphogenic signaling pathways and up-regulation of pathways related to cell death and craniofacial defects, while 6N’s show enrichment of hypoxia (up) and cellular metabolism (down) pathways. Collectively, these datasets 1) provide insight into the changing transcriptional landscape across gastrulation in two commonly used mouse strains, 2) establish a valuable resource that enables the discovery of candidate genes that may modify susceptibility to prenatal alcohol exposure that can be validated in humans, and 3) identify novel pathogenic mechanisms potentially involved in alcohol’s impact on development.

Keywords: 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 (3rd 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 as 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 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. While 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, Hong and Krauss, 2013), and 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 may 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 obtained from Jackson Laboratories and the C57BL/6NHsd obtained from Envigo (formerly Harlan, referred to henceforth as 6N). Previous work
has demonstrated that the 6J strain has a higher incidence of eye defects after prenatal alcohol compared to the 6N strain (Dou et al., 2013, Green et al., 2007b). These strains were both derived from the original C57BL/6J mice bred by Jackson Laboratories but were separated when the 6J strain was given to NIH in 1951 and given from 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 seven-fold 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 this 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 hr of gastrulation in both the 6J and 6N strains. Second, we examined PAE-induced gene expression changes 6 and 12 hr after exposure (E7.25 and 7.5, 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-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 timepoint. 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 across time. For example, expression of \textit{Wdfy1} significantly differs between the strains across all time points but is not affected by PAE (Fig 1B). Conversely, \textit{Shh} increases in expression in both strains over time, but PAE significantly reduces expression in the 6J (Fig 1C). The gene expression data generated in this study comprise a valuable resource for developmental biologists, toxicologists, mouse geneticists, and researchers interested in models of FASD.

**Transcriptional differences at between 6J and 6N mouse embryos during gastrulation**

Gene expression across the first 12 hr normal mouse gastrulation was compared between the 6J and 6N strains (representative image of gastrulation-stage mouse embryo in Fig 2A). Heatmaps showing hierarchical clustering of gene expression of all significant genes for all replicates are in Fig S1- S7 and VST-normalized values for all significant genes are in Dataset S1. 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 the 6N (Fig 2B). Functional profiling revealed up-regulation 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, including \textit{Ccl4, Il1r1, Il1rn}, and \textit{Tntrsf9}. The most up-regulated gene (largest positive log$_2$ fold-change [Log$_2$FC]), \textit{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 \textit{Ide} has been found to be relatively low in embryonic \textit{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 over-enriched pathways among the down-regulated genes, however the \textit{Nnt} gene was significantly down-regulated in the 6J strain, corroborating the well-known mutation in the 6J mouse strain (Ronchi et al., 2013). The gene most down-regulated in the 6J relative to 6N was \textit{Wdfy1}, which encodes an adaptor protein involved in protein-protein and protein-DNA interactions. \textit{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. \textit{Efcab7}
was also down-regulated in the 6J relative to 6N; this gene is associated with primary cilia function and, in particular, Shh signaling via Smoothened (Smo) (Pusapati et al., 2014).

Since 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 1, 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 linked to immune cell migration, though 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 (Fig 4, Fig 6) and days (Dou et al., 2013, Green et al., 2007a) later in development.

We next compared the 6J and 6N strains either 6 or 12 hr 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 hr post-vehicle injection, 315 genes were differentially expressed between the two strains. Of these genes, 128 genes were up-regulated (40.6%) and 187 were down-regulated (59.4%) in the 6J relative to the 6N. Twelve hours after vehicle treatment, E7.5, there were 304 differentially expressed genes between 6J and 6N. Of these, similar to the E7.25 time point, 120 genes were up-regulated (39.5%) and 184 genes were downregulated (60.5%) in the 6J.

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 down-regulated 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 up-regulated genes indicated increased prostaglandin signaling and GPCR signaling (Table S4). The down-regulated 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 down-regulated across these 12 hours of development, consistent with other studies showing most genes expressed during gastrulation show relatively stable expression prior to the onset of organogenesis (Mitiku and Baker, 2007). *Wdfy1* showed the largest down-regulation by Log2FC at both time points in the 6J relative to 6N (-3.94 and -3.67 Log2FC, respectively), consistent with what was observed in these two strains at E7.0 prior to any injection. *Efcab7*, which had lower baseline expression in 6J, exhibited the same effect at E7.25 (-1.78 Log2FC), but not at E7.5. The most up-regulated gene in the 6J relative to 6N at E7.25 was *Hist1h4m*, or histone cluster 1, H4m, a gene related to nucleosome assembly. This gene also showed a large up-regulation at E7.5 and a small but statistically significant up-regulation at E7.0. Interestingly, this gene was found to be down-regulated in the hippocampus of fetal 6J mice (purchased from Orient Bio Inc) following alcohol exposure from E8-12 (Mandal et al., 2015). The fact that expression of *Hist1h4m* differs between alcohol 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 hours 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 hr 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, 355 genes were upregulated (43.8%) and 455 were down-regulated (56.2%) (Fig 3A). In the 6N, 372 genes were upregulated (52.9%) and 330 were down-regulated (47.1%) (Fig 3B). Of the differentially expressed genes, 228 were altered in both strains (Fig 3C). In most cases, the directionality (up- or down-regulated) was the same between strains, indicating that while there is a substantial subset of genes that are similarly affected in both the 6J and 6N, the majority of genes with significantly altered expression in each strain are unique.

Functional profiling of the genes up-regulated following PAE in the 6J at E7.25 revealed 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 up-regulated by PAE. BID (BH3 interacting-domain death agonist) is a pro-apoptotic protein of the Bcl-2 family that is activated by the posttranslational modification N-myristoylation.
Activation of BID causes 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, indicate that cell death pathways have begun to be activated in the 6J strain as early as 6 hr post-PAE (E7.25). Analysis of down-regulated genes in the 6J 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 down-regulated genes, suggesting that cell proliferation is slowed or paused while the embryo responds to the alcohol insult. \textit{De novo} network analysis revealed multiple associations with organ health and development, cancer/cell cycle, drug metabolism, and cell death (Table 2A; Table S2D).

The top two genes down-regulated following PAE in the 6J at E7.25 were \textit{Srsf2}, 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 \textit{Alyref}, which encodes the molecular chaperone Aly/REF export factor which is involved in RNA processing and nuclear export. The most upregulated gene in 6J was \textit{Chac1}, which encodes Glutathione-specific gamma-glutamylcyclotransferase-1 (Gamma-GCG 1), a protein involved in glutathione cleavage, induction of oxidative stress-related apoptosis, and a negative regulator of Notch signaling (Chi et al., 2012). \textit{Trib3} was also significantly upregulated and encodes Tribbles pseudokinase 3 (Trb-3), which is induced by NF-kB signaling and 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 1 (encoded by \textit{Chac1}) (Örd et al., 2016), thus limiting apoptosis through another pathway. Multiple types of cellular stress up-regulated Trb-3, including nutritional deprivation (Liu et al., 2012) and endoplasmic reticulum (ER) stress (Örd et al., 2014). In sum, alcohol-induced reductions in expression of cellular metabolism and gene transcription pathways, as well as up-regulation 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 down-regulated genes revealed a reduction in cellular metabolism and methyltransferase activity (Fig 4B; Table S6). Similar to the 6J, PAE seemingly caused a reduction in cellular activity in the 6N. Alteration of methylation could have effects on gene expression and protein function; some of the specific methyltransferases targeted by PAE included \textit{Kdm1a, Kdm4b, Mettl3, Mettl4, Mettl16, 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 2B; Table S2E). The two most down-regulated genes were \textit{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 up-regulated 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, while 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, a notable difference from the 6J.

Large-scale strain-specific differences in transcriptional response to PAE apparent 12 hours following exposure

To explore how strain differences continue to modulate the transcriptional landscape 12 hours after alcohol exposure, we next compared the effect of PAE on embryonic gene expression in each strain at E7.5. At E7.5 (12 hr post-PAE), the 6J strain continued to have more pronounced gene expression changes relative to the 6N; in fact, the number of differentially expressed genes increased over three-fold in the 6J while it remained relatively stable in the 6N. In the 6J, 2,987 genes were differentially expressed 12 hr after PAE. Of these, 1,641 were up-regulated (54.9%) and 1,346 were down-regulated (45.1%) (Fig 5A). Conversely, only 641 genes were altered by PAE in the 6N at this time point, with 366 up-regulated (57.1%) and 275 down-regulated (42.9%) (Fig 5B). The significant increase in the number of differentially expressed genes in the 6J but not the 6N provides further evidence that 6J mice are more sensitive 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 up-regulated at E7.5, as compared to most overlapping genes being down-regulated in both strains at E7.25.

Functional profiling of the genes up-regulated 12 hr 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), though 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 down-regulated 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 3A; Table S2F).

The most down-regulated gene in the 6J at E7.5 was Shh. Multiple other members of the Shh pathway were also down-regulated by PAE in the 6J 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 down-regulates 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, 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 and differences in Shh signaling after alcohol exposure is not yet clear, but these findings warrant further exploration. Efcab7, a gene linked to Smo transduction in the primary cilia (Pusapati et al., 2014), was up-regulated by PAE at E7.5 (+0.81 Log2FC). This gene had lower expression in the 6J relative to the 6N 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 down-regulated 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). Down-regulation of the Shh pathway and Tcf21 suggest serious and widespread defects in organogenesis within the 6J just 12 hours following PAE, an effect that does seem to occur in the 6N mice.

The top two up-regulated genes in the 6J were Tap2 and Fam46b, the first of which was also one of the top up-regulated genes in the 6N at E7.25. Fam46b (also known as 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 that is apparent within 12 hours following exposure. In addition to the down-regulation 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 up-regulated genes in the 6N indicated that PAE caused an increased inflammatory signaling response in these embryos compared to controls, as well as catalytic activity and RAGE receptor binding (Fig 6B; Table S8). Increased IL-17 signaling was also identified as an up-regulated pathway in this data set, 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 up-regulated in the 6N using the HPO database, indicating that PAE could be affecting cellular oxygen levels up to 12 hr later. Analysis of down-regulated genes in the 6N 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 at E7.5 (Table 3B; Table S2G).

The top down-regulated genes in the 6N 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 this gene exhibit severe intellectual disabilities, loss of muscle tone, mild craniofacial dysmorphologies, and severe seizures. Transgenic mice with a 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 ~E9-10 and causes growth retardation and heart defects.

The top two up-regulated genes were *S100a9* and *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, though 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., 2010). *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 Jr et al., 2018, Vetreno and Crews, 2012). The function of resistin-like gamma (Retnlg) is largely unexplored, though 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, these pathways do not seem to be as clearly linked to craniofacial development as those identified in the 6J, possibly contributing to the phenotypic differences observed between these strains following PAE.

**Limited overlap in PAE-induced transcriptional differences between 6J and 6N strains 6–12 hours following 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 down-regulated 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-XL activity and regulates the G2/M DNA damage checkpoint during cell cycle progression (Gross, 2008). Interestingly, this gene was also down-regulated in the rostroventral neural tube of 6J mice 6 hr after neurulation-stage alcohol exposure in a previous study (Boschen et al., 2020), revealing this gene as a marker of prenatal alcohol exposure across multiple models of FASD. The next gene, Hist3h2a (also called H2aw), 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 down-regulated by neurulation-stage alcohol in a whole embryo culture model derived from C57BL/6J mice (Zhou et al., 2011). The third down-regulated 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, Verderelli et al., 2017, Yagi et al., 2003). One of the genes that was up-regulated 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. While this protein has been identified as a possible biomarker candidate for neurodegenerative diseases such as Alzheimer's disease (Rahman et al., 2019), 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 both children exposed to alcohol in utero and in animal models of FASD. The wide range of signs and symptoms of prenatal alcohol exposure present problems not only for diagnosis and treatment of individuals with FASD, 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 sub-strains can result in significantly different molecular responses to a teratogen such as alcohol. While only 80 genes differed between the alcohol-sensitive 6J compared to the 6N at E7.0, the 6J had significantly more genes dysregulated by alcohol 6–12 hr later. Functional profiling also revealed that the biological functions affected by alcohol in the 6J differed from those identified in the 6N. 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. 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, likely contributing to the increased incidence of eye defects following PAE in the 6J fetuses compared to the 6N (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 NADP+ to NADPH, an important co-enzyme that regulates metabolism along with NADH. A primary function of NADPH is removal of ROS from the mitochondria (Fig 7). NADH is the reduced form of NAD+, and these co-factors are important for redox metabolism, cellular respiration, and ATP production. In addition, NADPH converts glutathione from the oxidized (GSSG) to the reduced state (GSH) via glutathione reductase (GR). GSH neutralizes ROS and sequesters and eliminates H2O2. 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 TCA cycle. While 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 sub-strains obtained from Jackson Labs), 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), though 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 buildup 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, Henderson et al., 1999).

Oxidative stress can induce inflammation and expression of pro-apoptotic molecules NF-κB and p53 and oxidative stress proteins HIF-1α and PPAR-γ (Reuter et al., 2010). The Nnt mutation has also been directly linked to increased expression of HIF-1α 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 down-regulated 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, while the 6J 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 at baseline. Interpretation of the up-regulation of inflammatory signaling in the 6J relative to 6N at E7.0 is limited since 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), while 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’s vs. PAE-induced activation in 6N’s) 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 lab has demonstrated that alcohol administered during neurulation alters over 100 cilia genes in the neural tube within the first 6 hr 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 data set 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 (25 cilia genes in the 6J vs. 21 genes in the 6N at E7.25; 101 cilia genes in the 6J vs. 24 genes in the 6N 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 down-regulated 12 hr 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. Further investigation is needed to determine if the cilia genes altered by alcohol exposure in the 6J are directly related to the down-regulation 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 vs. neurulation), time elapsed between alcohol administration and tissue collection (e.g. 3 hr in Green and colleagues (Green et al., 2007a), 6 – 12 hr in our study), and specific tissue type assessed (head fold tissue vs. 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 or 24 hr after alcohol 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., 2007a, Garic et al., 2014, Boschen et al., 2020, Berres et al., 2017) and identified as down-regulated 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., 2007a). While cell motility was up-regulated in the 6J’s vs. 6N’s at E7.0 in the current study, 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). While the current study did not find statistical enrichment of any RA pathways, three genes related to RA signaling were dysregulated after PAE: Lrat (Lecithin retinol acyltransferase, +0.42 Log2FC in 6J’s at E7.25), Rara (Retinoic acid receptor-α, +0.46 Log2FC in 6N’s at E7.25), and Crabp2 (Cellular retinoic acid binding protein 2, -1.04 and -0.87 Log2FC in 6J’s and 6N’s, 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 down-regulated in the 6J strain 12 hr after PAE, though it is beyond the scope of this study to determine if 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). Though no sex differences were apparent in the differentially expressed genes in this study, as determined by the consistency between samples (Fig 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 prenatal alcohol exposure in the human population.
MATERIALS AND METHODS

Animals

Male and female adult C57BL/6J (Jackson Labs, Bar Harbor, ME; Stock #: 000664) and C57BL/6NHsd (Envigo, Indianapolis, IN) 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) and water and were maintained on a 12:12 hr light/dark cycle. Up to two female mice were placed into the cage of a male for each 2 hr 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 UNC-Chapel Hill 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) in Lactated Ringer’s solution 4 hr apart via i.p. 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 or 12 hr after the first alcohol injection (E7.25 or E7.5) (Fig 1). Dams were sacrificed via CO₂ 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 6 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 the RNeasy Plus Micro Kit (Qiagen, Germantown, MD) and RNA concentrations and purity were assessed using a NanoDrop 2000 and Qubit 3.0 Fluorimeter (Thermofisher Scientific, Waltham, MA). A separate group of samples was collected at the E7.0 time point and isolated for validation of gene expression using 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 6 samples per group were submitted for sequencing. Libraries for RNA sequencing (RNA-seq) were prepared using the SMARTtr Ultra Low Input RNA (Clontech, Mountainview, CA) and Nextera XT DNA (Illumina, San Diego, CA) 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 4 samples per pool (2/group), 3 pools total, 1 pool per lane. For E7.25 and E7.5 samples (24 samples/time point), there were 4 samples per poll (1/group), 6 pool total, 1 pool per lane. Paired-end (50bp) 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, 3 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 Human Phenotype Ontology (HPO) (Kohler et al., 2019). In addition, differentially expressed genes were assayed in a de novo network analysis using Ingenuity Software (Qiagen, Germantown, MD). 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 7.5 time points, analysis was limited to 70 focus molecules per network. Networks were ranked by the negative 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 number of cilia-related genes disrupted by alcohol at each time point and in each strain.

qRT-PCR data was analyzed with unpaired t-tests corrected for multiple comparisons and a $p$-value of $< 0.05$ designated as statistically significant.
The gene expression data browser web-tool was developed using the R shiny framework and is 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).

DATA AVAILABILITY

RNA-seq data have been deposited in GEO under accession GSE163796. The accession is currently private, but reviewers can access with the following password: uhydcsyknzyxhmv.

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COMPETING INTERESTS

The authors have no conflicts of interest to disclose.
AUTHOR CONTRIBUTIONS

S.E.P and K.E.B. conceptualized and designed the experiment. K.E.B. performed the investigation and validation studies. T.S.P. and J.M.S. performed the formal analysis. T.S.P., M.E.B., and J.M.S. performed data curation and visualized the findings along with K.E.B. All authors were involved in provision of resources for their specific contributions to the manuscript. K.E.B. wrote the original draft, M.E.B., J.M.S. and S.E.P. reviewed and edited the manuscript. S.E.P. supervised and acted as project administrator. Financial support was acquired by K.E.B., T.S.P., J.M.S., and S.E.P.

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Figure 1. Experimental timeline (A) 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 hr of normal mouse gastrulation in the 6J and 6N strains. 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 PAE in the 6J, but not 6N mice.
Figure 2. Immune signaling gene pathways are up-regulated 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 vs. 6N at baseline (prior to alcohol administration) on E7.0. Data are expressed as Log₂ fold change (Log₂FC). Blue = down-regulated genes, red = up-regulated genes. n = 6/group. C) Functional profiling of genes differentially expressed in the 6J vs. 6N at E7.0. n = 6/group.
Figure 3. Gastrulation-stage alcohol dysregulated more genes in the 6J strain than the 6N strain 6 hr after exposure. A) Volcano plot of genes significantly dysregulated by alcohol in the 6J mice 6 hr after the first dose of alcohol (E7.25). B) Genes significantly dysregulated by alcohol in the 6N mice 6 hr after the first dose of alcohol (E7.25). C) Heat map comparing 228 genes altered by alcohol in both the 6J and 6N at the E7.25 time point. Data are expressed as Log$_2$FC. Blue = down-regulated genes, red = up-regulated genes. n = 6/group.
Figure 4. Functional profiling of biological pathways enriched in the (A) 6J and (B) 6N strains 6 hr after alcohol exposure (E7.25). n = 6/group.
Figure 5. Gastrulation-stage alcohol dysregulated more genes in the 6J strain than the 6N strain 12 hr after exposure. A) Volcano plot of genes significantly dysregulated by alcohol in the 6J mice 12 hr after the first dose of alcohol (E7.5). n = 5 vehicle-treated, 6 PAE. B) Genes significantly dysregulated by alcohol in the 6N mice 12 hr after the first dose of alcohol (E7.5). n = 6 vehicle-treated, 4 PAE. C) Heat map comparing 228 genes altered by alcohol in both the 6J and 6N at the E7.5 time point. Data are expressed as Log₂FC. Blue = down-regulated genes, red = up-regulated genes. n = 6/group.
Figure 6. Functional profiling of biological pathways enriched in the (A) 6J and (B) 6N strains 12 hr after alcohol exposure (E7.5). n = 6/group.
Figure 7. Schematic representing a hypothetical mechanism contributing to the difference in alcohol sensitivity between the 6J and 6N strains. The Nnt mutation in the 6J could affect reactive oxygen species (ROS) breakdown in the mitochondria, leading to higher baseline oxidative stress and inflammation. In the presence of alcohol, 6J would undergo increased apoptosis and DNA damage, ultimately resulting in more severe craniofacial and CNS anomalies. Nnt: Nicotinamide nucleotide transhydrogenase, NAD+/NADH: nicotinamide adenine dinucleotide (+ hydrogen), NADP+/NADPH: nicotinamide adenine dinucleotide phosphate, GR: glutathione reductase, GSSG: glutathione disulfide, GSH: glutathione.
Table 1. *De novo* gene networks altered in the sensitive 6J mice compared to the resistant 6N mice at baseline (E 7.0) (A), 6 hr after alcohol in 6J (B) and 6N (C) strains, or 12 hr after alcohol in 6J (D) and 6N (E) mice.
Figure S1.
Figure S2.
Figure S3.
Figure S4.
Figure S5.
Figure S6.
Figure S7.
Figure S8.

qRT-PCR validation of E7.0 RNA-seq

Figure S9.
| 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           |           | 8          | 1.49          |
| Cell motility                                 | GO:0048870| 15         | 1.44          |
| Localization of cell                          | GO:0051674|            | 15.00         |

**Gene Ontology: Molecular Function**

| Term name                                      | Term ID   | # of genes | Log10 p-value |
|-----------------------------------------------|-----------|------------|---------------|
| Cytokine binding                              | GO:0019955| 7          | 4.45          |
| ATPase activity, coupled                      | GO:0042623| 6          | 1.32          |

**KEGG: Biological Process**

| Term name                                      | Term ID   | # of genes | Log10 p-value |
|-----------------------------------------------|-----------|------------|---------------|
| Cytokine-cytokine receptor interaction        | KEGG:04060| 7          | 2.05          |

A  E 7.0  6J vs. 6N

| Diseases and Functions                          | -log(Fisher’s exact p-value) | Molecules in Network | Molecules |
|------------------------------------------------|-----------------------------|----------------------|-----------|
| 1 - Cell Cycle                                 | -                            | Aak1, Acaat1, A2k2, Ahnuk, App, Artgap3, Arv1gu1, Abp1f1, Coto8lf, Clp, Dna1h4, Drcg28l, Dymr12b, Dynr1h, Hap52, Hapdr, Iyf12, Klf15, Lka6, Mtb13, Mmp3, Myc, Myf12, Naa11, Ncr, Nhr14, Ror, Sze8l, Srr5, Te2c, Tk32, Tmnen27, Tnf, Tel6don, Vglf3 |
| 2 - Cancer                                     | 31                          | 15                   |
| 2 - Immunological Disease                      | 28                          | 14                   |
| 3 - Cellular Movement                          | 25                          | 13                   |
| 4 - Cancer                                     | 25                          | 13                   |
| 5 - Cellular Movement                          | 18                          | 10                   |
| 6 - Cell Morphology                            | 16                          | 9                    |

B  E 7.25  6J vs. 6N Vehicle-treated

| Diseases and Functions                          | -log(Fisher’s exact p-value) | Molecules in Network | Molecules |
|------------------------------------------------|-----------------------------|----------------------|-----------|
| 1 - Antimicrobial Response                      | 39                          | 22                   |

Degeneracy Models & Mechanisms: doi:10.1242/dmm.049012: Supplementary information
| Section                                                                 | Gene Names                                                                 | Rows | Columns |
|------------------------------------------------------------------------|---------------------------------------------------------------------------|------|---------|
| 2 - Cell-To-Cell Signaling and Interaction                              | Alpha Catenin, Ampk, Atg10b4, Calpain, Capn11, Cdt3, Cd4, Cg, Cpa3,       | 34   | 20      |
| 2 - Drug Metabolism                                                      | Creb,乙酰化, Fmn3, Fsh, Gagbg2, Glucuronoyltransferase, Hdp2,                |      |         |
| 2 - Nervous System Development and Function                             | Histone H3, Hla-A, Hoxf10, S1, S100a, Klf1, Mek, Nfat, P38,                |      |         |
|                                                                       | Mapk, P33 (Complex), Pkc3, Ppp1r1b, Pска, Sко2b1, Tnf4f1, Uг2g3,            |      |         |
|                                                                       | Uг2d17, Uг2d28, Vegf                                                        |      |         |
| 3 - Cell Signaling                                                       | Amylase, Ccf1, Cд2b1, Collagen, Collagen Type II, Cцц13, Elastase,        | 32   | 19      |
| 3 - Molecular Transport                                                  | Erk1/2, Fona, Fgf7, Fibrin, Gpl, Hct, Hla-Dr, Ifh1 Alpha Beta,           |      |         |
|                                                                       | Iк3b, Iк3e7 (Includes Others), Lum, Mhc Class I (Complex), Mhc II,         |      |         |
|                                                                       | Mnp, Pp1r1c3a, Pro-Inflammatory Cytokine, Rα3g3, Rα3g13, Serpon1,       |      |         |
|                                                                       | Sly3zap, Tr, Trf (Family), Trnf15, Trm1, Tyrosin, U02c, Xcl1               |      |         |
| 4 - Hematological System Development and Function                       | Aim2, Akt, Bcr (Complex), Cд180, Cд36, Cнna3, Dб, Fоамr, Gосаm,         | 30   | 18      |
|                                                                       | Gm9573, Gпmоs, Hemoglobin, Ifh Beta, Ifnar, Ipа, Ipаg, Ipp1, Ipp2b,       |      |         |
|                                                                       | Igp3, Igm, If12 (Complex), Insr, Ls9, Lсg2, Medag, Mhc Class II (Complex),|      |         |
|                                                                       | Pаx5, Pи3k Gamma, Stа5a8, Tlp2b, Trnf117, Trm29, Vаv, Wар                 |      |         |
| 5 - Cell Signaling                                                       | Adcy, Adgф1, Bдбh1, Cалдр1, Cалмодulin, Cесф, Chemokine, Chemokine       | 25   | 16      |
|                                                                       | Receptor, Collagen Type I (Complex), Cесr1, Cвp2d2, Cшт1, Pшa2, Fатr,   |      |         |
|                                                                       | G Protein, G Protein Alpha, Gнhr, Gор, Gм1, Growth Hormone, Gк3,          |      |         |
|                                                                       | Jnk, Lh, Masp, Mysc, Npy, Pka, Pkc, Rac, Ras, Serpinсh2 (Includes         |      |         |
|                                                                       | Others), Tacr1, Тшr, Voltage-Gated Calcium Channel                         |      |         |
| 6 - Cell-To-Cell Signaling and Interaction                              | Akr1c3, Аldоf1a2, Аldоse Reduced, Аlp, Ap1, Caiм5, Cε1,                  | 23   | 15      |
|                                                                       | Collagen(3), Erk, Fсаr1, Fgf, Fgf6, Ghf, Gl, Hд8, Ige, Iis3, Kт75, Ldt,   |      |         |
|                                                                       | Lшβ3, Myx4, Myk2, Myosin, Nаdпh Oxidase, Nф1, Ptof1 В6, P3k-P65,          |      |         |
|                                                                       | Pкaa, Pкд2 (Includes Others), Rα1гд1б1, Rock, Sнf123, Sно (Family),      |      |         |
|                                                                       | Tgf Beta, Tкh                                                          |      |         |
| 7 - Cardiovascular System Development and Function                      | 60s Ribosomal Subunit, Аbcα13, Адам13, Адад1, Адад2, Аldо3b1, Аnt2,       | 23   | 15      |
|                                                                       | Bсо1, Cдβ313, Cк1, Doles5, Еss, Fос11b, Gα2, Gδ2, Gмн6, Gшγa,           |      |         |
|                                                                       | Hепm, Kнδ0, Lβя (Includes Others), Mго3, Mы, Nαδ, Nαдр, Nаср, Нс1,     |      |         |
|                                                                       | Ншф1в, Нрг4, Нαд2б1т1, Rтч, Rаг, Rшγ3β, Tмн120с, Тгα1β10, Vоp, Вαп        |      |         |
|                                                                       | Wαп                                                    |      |         |
| 8 - Cell Morphology                                                      | 26s Proteasome, 4930417о13,mark, Asп-Содумzyme A, Aм2 Inflammationmeone,   | 21   | 14      |
|                                                                       | Алα2, Cдβ9, Gαсам13, Cдβ13, Cйт1ra, Cвyt1ra, Cytokinе, Def80, Ep400,     |      |         |
|                                                                       | H4с4, Histone, Histone H4, Hсп90, Hд, Immunoglobulin, Insulin, Interferon|      |         |
|                                                                       | Alpha, Kнδ18, Mδδ, Мшγ, Paф1, Phosphatidylinositol 3, 4-Diphosphate,     |      |         |
|                                                                       | Phosphatidylinositol 3-Phosphate, Рα3g2а, Pоd2, Pоp1k1k, Pпр1,            |      |         |
|                                                                       | Prolinуlin, Rина Polymerase I, Тp50, Тар1k, Wδf1                           |      |         |
| 9 - Cell-To-Cell Signaling and Interaction                              | Ace6, Bш1, Cрmап6, Cшф, Эдна, Endocannabinoid, Fatp2, Foxa1, Fтп3,       | 21   | 14      |
|                                                                       | Glu4, Gls2, H1-1, Krr1, Lyg6кf, Нц2-5, Oкш93, Ош1,2, Pчh63, Pчh67,     |      |         |
|                                                                       | Pчh88, Pшd1, Pгαα, Pπ1p1р, Rш111, Rп527, Sлш10a1, Sон10а,                |      |         |
|                                                                       | Sон11а, Се14б5, Се1, Сет Complex, Скт17а8, Teос1, Vгf, Xн2               |      |         |
| Diseases and Functions | E 7.5 | 6J vs. 6N Vehicle-treated |
|------------------------|-------|--------------------------|
| Drug Metabolism         | 50    | 26                       |
| Small Molecule Biochemistry |     |                           |
| Connective Tissue Development and Function | 42    | 23                       |
| Digestive System Development and Function |     |                           |
| Skeletal and Muscular System Development and Function |     |                           |
| Post-Translational Modification | 42    | 23                       |
| Protein Degradation     |       |                           |
| Protein Synthesis       |       |                           |
| Cancer                  | 35    | 20                       |
| Dermatological Diseases and Conditions |     |                           |
| Organismal Injury and Abnormalities |     |                           |
| Developmental Disorder  | 28    | 17                       |
| Endocrine System Disorders |     |                           |
| Hematological Disease   |       |                           |

Molecules in Network:

- Aim2, Amy2a, Amylase, Anxa13, Bglap, Cd2001, Cdh16, Cebpe, Cas, Ces1, Ces3, Ces4a, Collagen Type II, Ctb2, Cyprf1, Erk1/2, Fgf7, Growth Hormone, Htr1, Il36g, Kcnq1, Magf/Mag2, Myos, Nph7, Prx1, Prnsinulin, Prx Ligand-Prratinoc-Acid-Rara, Rv, S0c10/03, Sry2, T3-Rx/Rx, Tfr2, Ubra3/3a, Uroc
- Akir2, Alm, Akt, C3ar1, Cd207, Cd55, Collagen Type I (Complex), Collagen Type IV, Collagen(V), Cts, Ctk, Fg, Fhi, Fmd, Gmft673, Hefp1, Iga, Igk2a, Igk2b, Igf1, Ld, Lyrz2, Muscle, Myoc, Dh51e2, Pdgf Bb, Pgr3, Ral3a, Sgbl3a1, Sertpinb7, Siglec15, Sllc13a1, Tct1a, Tgfbeta
- Ano3, Cc11, Cd24, Cc57, Ccd9, Ccd10, Cct, Cd55, Cdg2, Foer1, Gnst1, Ihn AlphaBeta, Ih Beta, Ihrr, Il36r (Includes Others), Interferon-Apha, Ik1k12, Iki8, Il6r (Includes Others), Mhc Class I (Complex), Mhc Class II (Complex), Mhcl (Family), Mhc Class II (Complex), Mhc2, Mhc (Complex), Mpiap1, Pms42p, Sertine Protease, Sertpinb10, Sllc10a2, Tapp2, Tmpras15, Tmpras7, Tfr (Family), Tmpstat17, Tpsin, Usp17a (Includes Others)
- Adamstik, Alk, Alpiv-lgp3, Bcr (Complex), Chd4, Colo6f1, Cnx2, Cnb, Chnn3, Cx31, Cx91, Eh2, Fh5, Gabr2, Gmft, Gmft6408 (Includes Others), Igf, Igk, Igk1, Iil1, Iil2, Iil3 (Complex), Il1fr2, Immunoglobulin, Keratin, Krt15, Krt20, Krt5-3, Krt7, Lp, Maphk112, Padd8, Poc2, Poc2at1, Ppy, Pro-Inflammatory Cytokine, Sox

Molecules:

- 9-Cis-Retinal, Akap5, Aplt14, Apol9a/Apol9b, App, Argfl1, Ascl1, Ascl2, Ascl3, Cd117, Chrm3, Crk, Dn33, Dpak, Dmrt1, Dmrt2, Dopamine, Endocannabinoid, Ftd, Girk, Gpr37, Gpr78, Htr1, Ihn1, Il6r, Inh, Mklk3, Myct, Oci16, Rho, Rnu12, Sestr1, Sestr3, Sps3a16, Trpv3, Trpv
| Disease Models & Mechanisms: doi:10.1242/dmm.049012: Supplementary information |
|----------------------------------------------------------------------------------|
| **D** E 7.25 6J Alcohol vs. Vehicle |
| Diseases and Functions | -log(Fisher’s exact p-value) | Molecules in Network | Molecules |
| --- | --- | --- | --- |
| **1** | Cancer |  |  |
| | Gastrointestinal Disease |  |  |
| **6** | Cancer | 26 | 16 |
| | Endocrine System Disorders | Abr, Abch11, Alms1, Alpk2, Anpk7, Bbox1, Cob27, Cock8b, Cnmm2, Cntnap3, Ctx1, Cxcl1, Cxcl14, Cxcl8, Dnmt1, Dnmt3a, Fis1, Fkbp14, Gpr6, Hepacam, Iprn2, Lhx6, Lmna, Lmx1, Lym, Mao, Npc1, Pchbl, Pchtlb, Pec, Rasl11b, Rnf133, Rop11, Sael, Spag11b, Ubc23, Ubc24, Ubp1, Vima |
| | Gastrointestinal Disease |  |  |
| **7** | Cancer | 23 | 15 |
| | Organismal Injury and Abnormalities | Abcb8, Adam30, Ankrd34c, Ato3, Bpifc, Cnp, Creb1, Cstd, Ddx2, Dnmt1, Dnmt3a, Fis1 (Includes Others), Gprtaf1, Hmnr, Itprid2, Kcnq1, Mapk1, Metalloprotease, Myl6, Nrf1, Pim2, Prkar2a, Prkar3, Ralr, Rhox3a (Includes Others), Robo1, Sik1, Soric1a, Scn3b, Tgf3, Traf8, Trank1, Tridiothryonine, Vap, Vn1 |
| | Tissue Morphology |  |  |
| **8** | Nutritional Disease | 21 | 14 |
| | Psychological Disorders | Abcb8, Adam30, Ankrd34c, Ato3, Bpifc, Cnp, Creb1, Cstd, Ddx2, Dnmt1, Dnmt3a, Fis1 (Includes Others), Elov2, Endothelin, Eppin, Gata2, Gclt, Gclt2, Gmgn, Hmnr, Itprid2, Kcnq1, Mapk1, Metalloprotease, Myl6, Nrf1, Pim2, Prkar2a, Prkar3, Ralr, Rhox3a (Includes Others), Robo1, Sik1, Soric1a, Scn3b, Tgf3, Traf8, Trank1, Tridiothryonine, Vap, Vn1 |
| | Small Molecule Biochemistry |  |  |
| **9** | Cell Cycle | 19 | 13 |
| | Cell Morphology | Acd7b, App, Bm1, Ccne2, Ccne3, Clec2e/Clec2h, Crebb, Crybb2, Endothelin, Gqbt1, Gpg, Hmph, Hspa, Iotcf, Konu4, Koc7, Lsmem1, Mac, Mass, Mk4, Nanog, Pim2, Polm, Sprt2g, Sprt2i, Tbx19, Tgm6, Transglutaminase, Zg16 |
| | Cell-To-Cell Signaling and Interaction |  |  |
| **10** | Cell Cycle | 17 | 12 |
| | Cell-To-Cell Signaling and Interaction | 9830107b12rik/A530064d06rik, Abcb9, Aim2, Apol11b (Includes Others), Bc066779, Chil3/Chil4, Cntnap5, Cro, Cyprf11, Cyptk2, E8n, Em2, Esl, Fm1t, Gchfr, Ginvl (Includes Others), Hia Class i, Hia-G, Irs2, Irs6, Lmna, Mkd4, Ms4a15, Nanog, Prkar2a, Psg18 (Includes Others), Phgr2, Rnas, Slik2, Slic25a13, Slic25a21, Slop2, Tbx22, Tgid4, Tnk, Usp8, Up3, Zbq2b |
| | Cellular Growth and Proliferation |  |  |
| **DE** |  |  |  |
DNA Replication, Recombination, and Repair

Cytokine, Tlr, Tmem189, Tmprss7, Trypsin, Txk, Vbp1

Cytokine, Tlr, Tmem189, Tmprss7, Trypsin, Txk, Vbp1
| E 7.25 6N Alcohol vs. Vehicle |
|-----------------------------|
| **Diseases and Functions** | -log(Fisher's exact p-value) |
| **Molecules in Network**    | **Molecules** |
| **Supplementary information** |
| **Disease Models & Mechanisms** | doi:10.1242/dmm.049012: Supplementary information |
| **Organismal Injury and Abnormalities** | Cardiovascular Disease |
| **Amino Acid Metabolism** | Gastrointestinal Disease |
| **Organismal Injury and Abnormalities** | Cancer |
| **Small Molecule Biochemistry** | Developmental Disorder |
| **Cell-To-Cell Signaling and Interaction** | Cell Cycle |
| **Carbohydrate Metabolism** | DNA Replication, Recombination, and Repair |
| **Tissue Morphology** | Gastrointestinal Disease |
| **Immune System and Function** | Hematological System Development |
| **Lymphoid Tissue Structure and Development** | Cancer |
| **Neurological Disease** | Developmental Disorder |
| **Cell-to-Cell Signaling and Interaction** | DNA Replication, Recombination, and Repair |
| **Gastrointestinal Disease** | Neurological Disease |
| **Cancer** | Developmental Disorder |
| **Cancer** | DNA Replication, Recombination, and Repair |
| **Cell Cycle** | Small Molecule Biochemistry |
| **Amino Acid Metabolism** | Cell-to-Cell Signaling and Interaction |
| **Amino Acid Metabolism** | Carbohydrate Metabolism |
| **Amino Acid Metabolism** | Gastrointestinal Disease |
| **Amino Acid Metabolism** | Hematological System Development |
| **Carbohydrate Metabolism** | Developmental Disorder |
| **Cell-To-Cell Signaling and Interaction** | Gastrointestinal Disease |
| **Cancer** | Hematological System Development |
| **Cell Cycle** | Developmental Disorder |
| **Cell Cycle** | DNA Replication, Recombination, and Repair |
| **Cell Cycle** | Small Molecule Biochemistry |
| **Cell Cycle** | Cell-to-Cell Signaling and Interaction |
| **Cell Cycle** | Carbohydrate Metabolism |
| **Cell Cycle** | Neurological Disease |
| **Gastrointestinal Disease** | Developmental Disorder |
| **Gastrointestinal Disease** | DNA Replication, Recombination, and Repair |
| **Gastrointestinal Disease** | Small Molecule Biochemistry |
| **Gastrointestinal Disease** | Cell-to-Cell Signaling and Interaction |
| **Gastrointestinal Disease** | Carbohydrate Metabolism |
| **Gastrointestinal Disease** | Hematological System Development |
| **Gastrointestinal Disease** | Developmental Disorder |
| **Neurological Disease** | DNA Replication, Recombination, and Repair |
| **Neurological Disease** | Small Molecule Biochemistry |
| **Neurological Disease** | Cell-to-Cell Signaling and Interaction |
| **Neurological Disease** | Carbohydrate Metabolism |
| **Neurological Disease** | Hematological System Development |
| **Neurological Disease** | Developmental Disorder |
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| **Cell Cycle** | Small Molecule Biochemistry |
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| **Cell Cycle** | Carbohydrate Metabolism |
| **Cell Cycle** | Neurological Disease |
| **Gastrointestinal Disease** | DNA Replication, Recombination, and Repair |
| **Gastrointestinal Disease** | Small Molecule Biochemistry |
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| **Gastrointestinal Disease** | Hematological System Development |
| **Gastrointestinal Disease** | Developmental Disorder |
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| **Neurological Disease** | Developmental Disorder |
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| **Cell Cycle** | Carbohydrate Metabolism |
| **Cell Cycle** | Neurological Disease |
| **Gastrointestinal Disease** | DNA Replication, Recombination, and Repair |
| **Gastrointestinal Disease** | Small Molecule Biochemistry |
| **Gastrointestinal Disease** | Cell-to-Cell Signaling and Interaction |
| **Gastrointestinal Disease** | Carbohydrate Metabolism |
| **Gastrointestinal Disease** | Hematological System Development |
| **Gastrointestinal Disease** | Developmental Disorder |
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| **Cell Cycle** | Neurological Disease |
| **Gastrointestinal Disease** | DNA Replication, Recombination, and Repair |
| **Gastrointestinal Disease** | Small Molecule Biochemistry |
| **Gastrointestinal Disease** | Cell-to-Cell Signaling and Interaction |
| **Gastrointestinal Disease** | Carbohydrate Metabolism |
| **Gastrointestinal Disease** | Hematological System Development |
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| **Neurological Disease** | Carbohydrate Metabolism |
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| **Cell Cycle** | Cell-to-Cell Signaling and Interaction |
| **Cell Cycle** | Carbohydrate Metabolism |
| **Cell Cycle** | Neurological Disease |
| **Gastrointestinal Disease** | DNA Replication, Recombination, and Repair |
| **Gastrointestinal Disease** | Small Molecule Biochemistry |
| **Gastrointestinal Disease** | Cell-to-Cell Signaling and Interaction |
| **Gastrointestinal Disease** | Carbohydrate Metabolism |
| **Gastrointestinal Disease** | Hematological System Development |
| **Gastrointestinal Disease** | Developmental Disorder |
| **Neurological Disease** | DNA Replication, Recombination, and Repair |
| **Neurological Disease** | Small Molecule Biochemistry |
| **Neurological Disease** | Cell-to-Cell Signaling and Interaction |
| **Neurological Disease** | Carbohydrate Metabolism |
| **Neurological Disease** | Hematological System Development |
| **Neurological Disease** | Developmental Disorder |
- Developmental Disorder
- Hereditary Disorder
- Post-Translational Modification

Cell Morphology
- Post-Translational Modification
- Small Molecule Biochemistry

Hematological System Development

Digestive System Development and Function

Small Molecule Biochemistry

Post-Translational Modification

Cellular Movement
- Hematological System Development and Function
- Post-Translational Modification

| Diseases and Functions | E 7.5 | 6N Alcohol vs. Vehicle |
|------------------------|-------|-----------------------|
| - Molecules in Network |       | - Molecules           |

| 1 - Cell Morphology | 70 | 49 |
|                    |    |    |
| 2 - Cardiovascular Disease | 70 | 49 |

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Disease Models & Mechanisms: doi:10.1242/dmm.049012: Supplementary information
| Disease Models & Mechanisms: doi:10.1242/dmm.049012: Supplementary information |
|-----------------|------------------------|
| **Developmental Disorder** | **Organismal Injury and Abnormalities** |
| **5** | **54** |
| **Organismal Injury and Abnormalities** | **Skeletal and Muscular Disorders** |
| **6** | **41** |
| **Cellular Movement** | **Hematological System Development and Function** |
| **6** | **52** |
| **Immune Cell Trafficking** | **Cellular Growth and Proliferation** |
| **6** | **35** |
| **Endocrine System Development and Function** | **Cell Death and Survival** |
| **7** | **50** |
| **Inflammatory Response** | **DNA Replication, Recombination, and Repair** |
| **7** | **34** |
| **Organismal Injury and Abnormalities** | **Nervous System Development and Function** |
| **8** | **41** |
| **Cancer** | **Cellular Function and Maintenance** |
| **8** | **43** |
| **Organizational Injury and Abnormalities** | **Hematological System Development and Function** |
| **9** | **35** |
| **Psychological Disorders** | **Cell Death and Survival** |
| **9** | **43** |

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The table above lists various categories of diseases and the genes associated with them. Each category is further divided into subcategories, and the numbers indicate the number of genes associated with each subcategory. The genes listed are related to different biological processes and disorders, highlighting the complexity of disease models and mechanisms.
### Down-regulated Genes

#### Gene Ontology: Biological Process

| Term name                               | Term ID     | # of genes | Log10 p-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 p-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 p-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 p-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 p-value |
|-------------------------------------------------------|-------------|------------|---------------|
| Caffeine metabolism                                   | KEGG:00232  | 2          | 1.94          |
### Down-regulated Genes

#### Gene Ontology: Molecular Function

| Term name                                                                 | Term ID    | # of genes | Log10 p-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 p-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 p-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          |
### Down-regulated Genes

#### Gene Ontology: Biological Process

| Term name                        | Term ID   | # of genes | Log10 p-value |
|----------------------------------|-----------|------------|---------------|
| 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

| Term name                        | Term ID   | # of genes | Log10 p-value |
|----------------------------------|-----------|------------|---------------|
| 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

| Term name                        | Term ID   | # of genes | Log10 p-value |
|----------------------------------|-----------|------------|---------------|
| Broad forehead                   | HP:0000337| 13         | 2.57          |
### Up-regulated Genes

#### Gene Ontology: Biological Process

| Term name                              | Term ID   | # of genes | Log10 p-value |
|----------------------------------------|-----------|------------|---------------|
| 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

| Term name                              | Term ID   | # of genes | Log10 p-value |
|----------------------------------------|-----------|------------|---------------|
| 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

| Term name                              | Term ID   | # of genes | Log10 p-value |
|----------------------------------------|-----------|------------|---------------|
| Graft-versus-host disease              | KEGG:05332| 5          | 1.38          |

#### Reactome

| Term name                              | Term ID   | # of genes | Log10 p-value |
|----------------------------------------|-----------|------------|---------------|
| Activation, myristolyation of Bid and translocation to mitochondria | REAC:R-MMU-75108| 5          | 4.08          |
### Down-regulated Genes

**Gene Ontology: Biological Process**

| Term name                                               | Term ID    | # of genes | Log10 p-value |
|---------------------------------------------------------|------------|------------|---------------|
| 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**

| Term name                                               | Term ID    | # of genes | Log10 p-value |
|---------------------------------------------------------|------------|------------|---------------|
| 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**

| Term name                                               | Term ID    | # of genes | Log10 p-value |
|---------------------------------------------------------|------------|------------|---------------|
| Gene expression (transcription)                        | R-MMU-74160 | 29        | 1.78          |
| RNA polymerase II transcription                        | R-MMU-73857 | 27        | 1.73          |
### Down-regulated Genes
#### Gene Ontology: Biological Process

| Term name                              | Term ID    | # of genes | Log10 p-value |
|----------------------------------------|------------|------------|---------------|
| 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

| Term name                              | Term ID    | # of genes | Log10 p-value |
|----------------------------------------|------------|------------|---------------|
| 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

| Term name                              | Term ID    | # of genes | Log10 p-value |
|----------------------------------------|------------|------------|---------------|
| 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 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          |
### Down-regulated Genes

#### Gene Ontology: Biological Process

| Term name                                           | Term ID      | # of genes | Log10 p-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 p-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 p-value |
|-----------------------------------------------------|--------------|------------|---------------|
| Steroid hormone biosynthesis                        | KEGG:00140   | 6          | 1.77          |

#### Human Phenotype Ontology

| Term name                                           | Term ID      | # of genes | Log10 p-value |
|-----------------------------------------------------|--------------|------------|---------------|
| Hypoplasia of the epiglottis                        | HP:0005349   | 3          | 1.31          |
### Up-regulated Genes

#### Gene Ontology: Biological Process

| Term name                                                                 | Term ID   | # of genes | Log10 p-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 of peptide antigen                     | GO:0019882| 11         | 2.87          |
| Inflammatory response                                                     | GO:0006954| 27         | 1.90          |

#### Gene Ontology: Molecular Function

| Term name                                                                 | Term ID   | # of genes | Log10 p-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 p-value |
|---------------------------------------------------------------------------|-----------|------------|---------------|
| IL-17 signaling pathway                                                   | KEGG:04657| 8          | 1.74          |

#### Reactome

| Term name                                                                 | Term ID   | # of genes | Log10 p-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          |
| Term name                                                   | Term ID    | # of genes | Log10 p-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 S2

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| Gene | Log2 FC | Gene | Log2 FC | Gene | Log2 FC | Gene | Log2 FC | Gene | Log2 FC |
|------|---------|------|---------|------|---------|------|---------|------|---------|
| Fam65b | 0.30 | Fam161a | 0.86 | Fam161a | 0.98 | Mchr1 | 1.28 | Igub | 0.95 |
| Efcab7 | -0.89 | Tbc1d30 | 0.75 | Tex40 | 0.94 | Fam161a | 1.17 | Tekt1 | 0.82 |
| Hydin | 0.65 | Hk1 | 0.81 | Hydin | 0.88 | Tulp1 | 0.80 | | |
| Map6 | 0.61 | Ak1 | 0.80 | Rab29 | 0.85 | Rab29 | 0.88 | | |
| Bbs4 | 0.61 | Tuba4a | 0.61 | Tuba4a | 0.67 | Efcab7 | 0.81 | Tupa4a | 0.67 |
| Ptc1 | 0.46 | Bbs4 | 0.52 | Tmen231 | 0.81 | Wtipcp | 0.62 | | |
| Arf2bp | 0.38 | Tmem17 | 0.43 | Mxl1 | 0.90 | Cep72 | 0.49 | | |
| Tbl1 | 0.28 | Haus4 | 0.35 | Tuba4a | 0.78 | Wdr34 | 0.48 | | |
| Cnga2 | 0.041 | Pkh61 | 0.34 | Rlip2 | 0.74 | Pkd21 | 0.33 | | |
| Myoc | 0.02 | Tek3 | 0.03 | Ccno | 0.72 | Ropn1 | -0.06 | | |
| Ccr6 | -0.03 | Tbl8 | 0.01 | Tulp1 | 0.71 | Icb1 | -0.35 | | |
| Mapre1 | -0.17 | Myoc | -0.06 | Hasp11 | 0.68 | Reg1 | -0.80 | | |
| Cct3 | -0.21 | Cngb3 | -0.08 | Rlip1 | 0.68 | Gili | -0.83 | | |
| Exoc3 | -0.23 | Mapre1 | -0.19 | Mok | 0.60 | | | | |
| Dnaj1 | -0.30 | Exoc3 | -0.24 | Kiz | 0.56 | | | | |
| Nup35 | -0.44 | Hspa8 | -0.36 | Kif3b | 0.52 | | | | |
| Hspa8 | -0.45 | Relpa1 | -0.51 | Md1 | 0.51 | | | | |
| Gna11 | -0.48 | Tmem59 | -0.56 | Snx10 | 0.44 | | | | |
| Pard3 | -0.58 | Katna1 | -0.58 | Pde6d | 0.44 | | | | |
| Tapt1 | -0.59 | Pex6 | -0.74 | Usp9x | 0.44 | | | | |
| Gnb1 | -0.71 | Wdr78 | -0.78 | Pkar2a | 0.43 | | | | |
| Dyx1c1 | -0.72 | Ick | 0.40 | | | | | | |
| Atx2a2 | -1.02 | Tubb2a | 0.34 | | | | | | |
| Prkaca | -1.19 | Unc119 | 0.33 | | | | | | |
| Pex6 | -1.38 | | | | | | | | |
| Dpocd | -0.35 | Pik4 | -0.39 | | | | | | |
| Aurka | -0.42 | Fgrtop | -0.48 | | | | | | |
| Tmem80 | -0.51 | Topors | -0.56 | | | | | | |
| Sino | -0.59 | Gli3 | -0.59 | | | | | | |
| Pafanb1 | -0.59 | Pif0 | -0.61 | | | | | | |
| Dnajb13 | -0.65 | Capp1 | -0.66 | | | | | | |
| Rltn | -0.66 | Plcn | -0.67 | | | | | | |
| Noto | -0.68 | | | | | | | | |
| Pdk2 | -0.69 | Rab11b | -0.69 | | | | | | |
| Morn3 | -0.72 | Ctap161 | -0.77 | | | | | | |
| Ptc1 | -0.79 | Spef2 | -0.83 | | | | | | |
| Tapt1 | -0.99 | Mkm | -1.03 | | | | | | |
| Orr1 | -1.43 | | | | | | | |