Transcriptome profiling of mouse brain and lung under Dip2a regulation using RNA-sequencing

Rajiv Kumar Sah¹, Analn Yang¹, Fatoumata Binta Bah¹, Salah Adlat¹, Ameer Ali Bohio², Zin Mar Oo¹, Chenhao Wang¹, May Zun Zaw Myint¹, Noor Bahadar¹, Luqing Zhang¹,²*, Xuechao Feng¹,²*, Yaowu Zheng¹,²*

¹ Transgenic Research Center, School of Life Sciences, Northeast Normal University, Changchun, China, ² Key Laboratory of Molecular Epigenetics of Ministry of Education, Northeast Normal University, Changchun, China

* Zhanglq479@nenu.edu.cn (LQZ); fengxc997@nenu.edu.cn (XCF); zhengyw442@nenu.edu.cn (YWZ)

Abstract

Disconnected interacting protein 2 homolog A (DIP2A) is highly expressed in nervous system and respiratory system of developing embryos. However, genes regulated by Dip2a in developing brain and lung have not been systematically studied. Transcriptome of brain and lung in embryonic 19.5 day (E19.5) were compared between wild type and Dip2a⁻/⁻ mice. An average of 50 million reads per sample was mapped to the reference sequence. A total of 214 DEGs were detected in brain (82 up and 132 down) and 1900 DEGs in lung (1259 up and 641 down). GO enrichment analysis indicated that DEGs in both Brain and Lung were mainly enriched in biological processes ‘DNA-templated transcription and Transcription from RNA polymerase II promoter’, ‘multicellular organism development’, ‘cell differentiation’ and ‘apoptotic process’. In addition, COG classification showed that both were mostly involved in ‘Replication, Recombination, and Repair’, ‘Signal transduction and mechanism’, ‘Translation, Ribosomal structure and Biogenesis’ and ‘Transcription’. KEGG enrichment analysis showed that brain was mainly enriched in ‘Thyroid cancer’ pathway whereas lung in ‘Complement and Coagulation Cascades’ pathway. Transcription factor (TF) annotation analysis identified Zinc finger domain containing (ZF) proteins were mostly regulated in lung and brain. Interestingly, study identified genes Skor2, Gpr3711, Runx1, Erbb3, Frmd7, Fut10, Sox11, Hapln1, Tlap2c and Plxb3 from brain that play important roles in neuronal cell maturation, differentiation, and survival; genes Hoxa5, Eya1, Erfl1, Sox11, Shh, IGF1, Ccbe1, Cth, Fgf9, Lama5, Pdgfra, Prn, Rbp4 and Wnt7a from lung are important in lung development. Expression levels of the candidate genes were validated by qRT-PCR.

Genome wide transcriptional analysis using wild type and Dip2a knockout mice in brain and lung at embryonic day 19.5 (E19.5) provided a genetic basis of molecular function of these genes.
Introduction

DIP2A is a member of disconnected (disco)-interacting 2 (DIP2) protein family whose molecular anatomical function remains to be clarified. Dip2a was firstly identified in Drosophila as a novel transcription factor that interacts with disconnected (disco) gene needed for proper neural connection during visual system development in Drosophila [1–3]. Previous studies have shown that Dip2a is highly expressed in human brain and may play a role in axon patterning in Central Nervous System (CNS) [4]. Bioinformatics analysis using Homologene suggests that DIP2A is a receptor molecule with DMAP, AMP and CAIC binding domains [5]. At DNA replication site, DIP2A, in a complex with DNA methyltransferase 1-associated protein 1 (DMAP1)—DNA (cytosine-5) -methyltransferase 1 (DNMT1)—Histone deacetylases (HDAC), regulates neurite outgrowth and synaptic plasticity [6]. Moreover, Dip2a has been previously identified as a risk gene associated with neurodevelopment diseases like autism spectrum disorder, development dyslexia and Alzheimer diseases [7–9]. All of these evidences strongly support the role of Dip2a gene in both vertebrate and invertebrate nervous system development. However, which biological process or molecular function is regulated by Dip2a gene during embryonic brain development is not known.

Earlier, using Dip2a−/−-LacZ knockin mice [10], we notice that Dip2a is highly expressed in brain neurons, retinal ganglion cell, reproductive, vascular and Lung tissue in adult and ectodermal tissue in developing embryos. RNA sequencing (RNA-Seq) has rapidly emerged as a favorite approach for high throughput gene expression and function studies. Through RNA-Seq, gene expression and gene interactions at any time point or in a particular tissue can be investigated [11]. In present study, Transcriptome (RNA-seq) analysis of E19.5 brain and lung of WT and Dip2a−−/− embryo was performed.

Dip2a role in brain and lung development has not been studied before. A global Transcriptome analysis of brain and lung will help us in understanding of Dip2a function in regulating brain and lung development. A total of 214 genes in brain and 1900 genes in lung were identified differentially expressed under Dip2a, suggesting that these genes are potentially relevant to brain and lung development and function. Those genes are further explicated and discussed in this study.

Materials and methods

Animals

Dip2a specific knockout transgenic mice (Dip2a−−) was generated in the lab using CRISPR-Cas9 technology as previously described [12]. All mice were genotyped by PCR from tail DNA. All procedures were conducted following guidelines recommended in the guide for Care and Use of Laboratory Animals of National Institutes of Health with approval of Institutional Animal Care and Use Committee of Northeast Normal University (NENU/IACUC, AP2013011). Mice were housed in clean facility in individual IVC cages under a normal 12:12h light:dark cycles in a temperature of 20˚C and humidity 50 ± 20% in Northeast Normal University. All mice were anesthetized before euthanasia with 1% pentobarbital at a dose of 10mg/kg and all effort was made to minimize suffering.

RNA isolation and library preparation for RNA-Seq

Total RNA from brain and lung of E19.5 Dip2a−− and wild type embryos was isolated by using RNAiso plus reagent (Takara, Dalian) in accordance with the manufacturer’s instruction and followed by additional step of DNase I digestion to eliminate genomic DNA contamination.
The quality and purity of RNA was checked by Nano drop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bio analyzer (Santa Clara, CA, USA).

A total amount of 1 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq™ 2500 platform (Biomarker, Beijing, China) and paired-end reads were generated.

Sequence Mapping, assembly and gene functional annotation

Raw data (raw reads) of fastaq format were firstly processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were also calculated. The clean reads were then mapped to mouse reference genome using Bowtie2 and Tophat 2 that allows up to two mismatches. Reads were assembled into transcript with Cufflink. Isolated and annotated based on the reference genome. The mRNA-Seq raw data are available at the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA540099/) under the accession number PRJNA540099. Gene function of the mapped reads (unique transcripts) was annotated based upon the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); KOG/COG (Clusters of Orthologous Groups of proteins); EggNOG; KO (KEGG Ortholog database) and GO (Gene Ontology).

Gene expression quantification and analysis of differentially expressed genes (DEG)

Quantification of transcript expression levels was presented by FPKM (fragments per kilo base of exon per million fragments mapped) that minimize the reads output variations between samples. In order to identify DEGs between WT and Dip2a<sup>-/-</sup> embryos in brain and lungs, we used DESeq software from R package. Resulting P values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate (FDR). DEGs with a threshold FDR adjusted, p value < 0.001 and fold change ≥ 2 (log2 > ±1) were selected for further analysis. Gene Ontology (GO) enrichment analysis of DEGs was implemented by GOseq R packages. KOBAS was used to test the statistical enrichment of DEGs in KEGG pathways. For transcription factor analysis, Genes were subjected under Animal TFDB database (Zhang et al., 2012).

Quantitative real time PCR (qPCR) validation of RNAseq

One microgram of total RNA from brain and lung tissue of E19.5 WT and Dip2a<sup>-/-</sup> embryos was reverse transcribed using primescript™<sup>TM</sup> cDNA synthesis kit (Takara, Dalian, China). QPCR was performed using Thermo cycler (Analytik Jena AG, Jena, Germany) and SYBR II premix (Takara, Dalian, China). All results were normalized to housekeeping gene 18S ribosomal RNA and relative quantification was calculated using comparative threshold cycle (2<sup>-ΔΔCt</sup>) values for 3 biological replicates.
Results and discussion
Gene expression profiling of brain and lung from WT and Dip2a<sup>−/−</sup> mice

Four cDNA libraries were prepared from brain and lung of WT and Dip2a<sup>−/−</sup> E19.5 embryos (n = 3; biological replicates per sample) and sequenced using Illumina Hiseq™ 2500. After filtering out adaptors sequence and low quality reads, 24.08 GB of Clean Data were obtained, or 6.02 GB per sample, with a Q30 base percentage above 92.49%. The clean reads from each sample were then mapped to mouse reference genome (ftp://ftp.ensembl.org/pub/release-78/fasta/mus_musculus/) and quantification of transcripts expression levels were calculated and presented by FPKM. As shown in Table 1, the matching efficiency between the clean read and the reference genome of each sample ranged from 89.25% to 91.95%. On an average, about 6000 genes were expressed in each sample. Genes comparison between WT and Dip2a<sup>−/−</sup> identified 5787 genes overlap in all sample and only 2 and 4 genes were unique in WT brain and WT lung respectively (Fig 1).

Identification of differentially expressed genes and functional annotation

To identify differentially expressed genes, unigenes from WT brain vs. Dip2a<sup>−/−</sup> brain and WT lung vs. Dip2a<sup>−/−</sup> lung were compared. DESeq identified 214 genes in brain and 1900 genes in lung to be differentially expressed, with Fold Change ≥2 and FDR < 0.01. In Dip2a<sup>−/−</sup> brain, 82 genes were up-regulated and 132 genes were down-regulated whereas in Dip2a<sup>−/−</sup> lung, 1259 genes were up-regulated and 641 genes were down-regulated when compared to WT (Fig 2). In Dip2a<sup>−/−</sup> brain, Rpsa-ps10, Tpm3-rs7, Amd2 and Gm8730, Gm10709, Gm6768 and Gm9825 genes were highly over expressed whereas Acp5, Ifi204, Coll10a1, Ibsp and Mmp13 genes were highly under expressed. Similarly, in Dip2a<sup>−/−</sup> lung, genes like Rps2-ps6, Gm10709, Bhmt and Gm8730 were highly increased whereas genes like Il1r2, Nr4a3, Cela1 and Dlk2 were significantly decreased (Table 2). Functional annotation of brain and lung DEGs shows that more than 90% of DEGs from brain and lung had significant matches in Nr, EggNOG, GO, COG, KEGG and Swiss-Prot database respectively (S1 Table).

GO enrichment analysis and COG classification of Dip2a-regulated DEGs

For gene ontology (GO) analysis, 185 DEGs from brain and 1709 DEGs from lung were classified into three GO categories and 51 terms (Fig 3). In biological process category, most of the DEGs in brain and lung were assigned to ‘cellular process’, ‘single-organism process’ and ‘metabolic process’. In molecular function category, most DEGs were annotated under ‘binding’, ‘catalytic activity’ and ‘signal transducer activity’. Within cellular component, ‘cell’, ‘cell part’ and ‘organelle’ was annotated with most DEGs. To further clarify the biological process, DEGs from both groups were enriched in 84 terms and the 10 most significant terms from each groups are summarized in Fig 3(c) and 3(d). In lung, the most significant biological terms include ‘regulation of transcription, DNA-templated’ and ‘positive-negative regulation of Transcription from RNA polymerase II promoter’ and ‘apoptotic process’. In brain, the most
Fig 1. Venn diagrams showing overlap and unique unigenes identified within Wild type (WT) brain, Wild type (WT) lung, *Dip2a*<sup>-/-</sup> brain and *Dip2a*<sup>-/-</sup> lung. (a) In WT group, total 5884 genes were expressed in both samples, 314 genes unique to brain and 257 genes unique to lung. (b) In *Dip2a*<sup>-/-</sup> group, total 5908 genes were expressed in both samples, 319 genes were unique to brain and 233 unigenes were unique to lung. (C) 5787 genes were expression in all samples, 2 genes were unique to WT brain and 4 genes were unique to WT lung.

https://doi.org/10.1371/journal.pone.0213702.g001

Fig 2. Differentially expressed genes volcano map. (a) WT brain vs. *Dip2a*<sup>-/-</sup> brain. (b) WT lung vs. *Dip2a*<sup>-/-</sup> lung. The red and green dots in the figure represent up-regulated and down-regulated differentially expressed genes respectively.

https://doi.org/10.1371/journal.pone.0213702.g002
significant terms were ‘multicellular organism development’, ‘positive-negative regulation of Transcription from RNA polymerase II promoter’ and ‘cell differentiation’. In addition, 34 DEGs from lung and 12 DEGs from brain were annotated under GO term ‘in utero embryonic development’ (Fig 4). These DEGs are important in progression of embryo in uterus over time.

To further clarify the molecular function of Dip2a, total 54 and 677 DEGs from brain and lung were assigned to COG classification and divided into 26 specific categories (Fig 5). In both groups, the top hits include ‘Replication, Recombination and repair (7.25% & 10.86%)’, ‘Signal transduction and mechanism (5.8% and 8.69%)’, ‘Translation, Ribosomal structure and Biogenesis (2.61% &13.04%)’ and ‘Transcription (7.6% & 8.7%)’.

### KEGG pathway annotation of brain and lung DEGs

In the process of pathways annotation for Dip2a regulated DEGs, 70 DEGs from brain and 625 DEGs from lung were annotated to 112 and 264 pathways respectively in KEGG pathway

---

**Table 2.** Highly significant differentially expressed genes in Dip2a−/− group compared to Wild type group (FDR < 0.01, FC > 20).

| Gene ID          | Gene symbol | WT Brain FPKM | Dip2a−/− Brain FPKM | FDR | log2FC |
|------------------|-------------|---------------|---------------------|-----|--------|
| ENSMUSG00000047676 | Rpsa-ps10   | 0.91          | 294.08              | 0   | 8.28   |
| ENSMUSG00000058126 | Tpm3-rs7    | 0.20          | 44.44               | 0   | 7.66   |
| ENSMUSG00000063953 | Amd2        | 0             | 5.41                | 0   | 7.60   |
| ENSMUSG00000063696 | Gm8730      | 0.07          | 22.26               | 0   | 7.51   |
| ENSMUSG00000074516 | Gm10709     | 0.66          | 90.26               | 0   | 7.01   |
| ENSMUSG00000021908 | Gm6768      | 0.02          | 4.32                | 0   | 6.70   |
| ENSMUSG00000096403 | Gm9825      | 0.57          | 55.52               | 0   | 6.59   |
| ENSMUSG00000013148 | Acp5        | 4.33          | 0.15                | 0   | -4.70  |
| ENSMUSG00000073489 | Ifi204      | 0.97          | 0                   | 2.63E-08 | -5.02 |
| ENSMUSG00000029307 | Dmp1        | 1.54          | 0.039               | 1.11E-16 | -5.09 |
| ENSMUSG00000039462 | Col10a1     | 0.75          | 0                   | 2.10E-09 | -5.16 |
| ENSMUSG00000029306 | Ibsp        | 5.75          | 0.13                | 0   | -5.38  |
| ENSMUSG00000050578 | Mmp13       | 3.90          | 0.04                | 0   | -6.16  |

| Gene ID          | Gene Name   | WT Lung FPKM | Dip2a−/− Lung FPKM | FDR | log2FC |
|------------------|-------------|--------------|-------------------|-----|--------|
| ENSMUSG00000096403 | Gm9825      | 0.005        | 80.34             | 0   | 10.62  |
| ENSMUSG00000095427 | Rps2-ps6    | 0.17         | 30.4              | 0   | 7.12   |
| ENSMUSG00000074516 | Gm10709     | 0.25         | 34.49             | 0   | 6.65   |
| ENSMUSG00000074768 | Bhnt        | 2.9          | 242.2             | 0   | 6.53   |
| ENSMUSG00000063696 | Gm8730      | 1.7          | 148.4             | 0   | 6.41   |
| ENSMUSG00000047676 | Rpsa-ps10   | 2.63         | 144.52            | 0   | 5.83   |
| ENSMUSG00000032315 | Cyp1a1      | 0.28         | 13.25             | 0   | 5.55   |
| ENSMUSG00000045027 | Prss22      | 0.55         | 23.05             | 0   | 5.32   |
| ENSMUSG00000078956 | Gm14221     | 5.32         | 1.12              | 1.01E-14 | 5.2   |
| ENSMUSG00000026073 | Ilr12       | 14.53        | 0.64              | 0   | -4.27  |
| ENSMUSG00000028341 | Nraa3       | 24.47        | 0.99              | 0   | -4.48  |
| ENSMUSG00000023031 | Cela1       | 10.47        | 0.23              | 7.77E-16 | -4.68 |
| ENSMUSG00000047428 | Dlk2        | 3.84         | 0.086             | 1.01E-12 | -4.76 |
| ENSMUSG00000049796 | Crh         | 6.74         | 0.13              | 1.11E-16 | -4.79 |
| ENSMUSG00000027313 | Chac1       | 10.42        | 0.2               | 0   | -5.23  |
| ENSMUSG00000020591 | Ntsr2       | 10.57        | 0.08              | 0   | -5.56  |

https://doi.org/10.1371/journal.pone.0213702.t002
database (S1 Fig). In order to analyze whether DEGs are over-presented on a pathway, the pathway enrichment analysis was performed (Fig 6). The top 5 enriched pathways in brain with the least significant Q value < 0.05 and enrichment factor greater than 2 were 'ko04610 Complement and coagulation cascades', 'ko05150 Staphylococcus aureus infection', 'ko01230 Biosynthesis of amino acids', 'ko04066 HIF-1 signaling pathway' and 'ko04151 PI3K-Akt

https://doi.org/10.1371/journal.pone.0213702.g003

Fig 3. Gene Ontology (GO) classification of DEGs from WT brain vs. Dip2a<sup>−/−</sup> brain (a, c) and WT lung vs. Dip2a<sup>−/−</sup> lung (b, d). (a,b) Histogram of GO annotation was generated by KOBAS (kobas.cbi.pku.edu.cn). The X-axis indicates GO classification, the Y-axis on the left indicates the percentage of genes, and the Y-axis on right indicates the number of genes. One gene could be assigned with more than one GO term. (c,d) Most significant enriched biological terms in brain and lung.

https://doi.org/10.1371/journal.pone.0213702.g003

Fig 4. List of DEGs annotated to GO term 'In-utero embryonic development'.

https://doi.org/10.1371/journal.pone.0213702.g004
signaling pathway', whereas in lung, the most enriched pathways with the least Q value <1 and enrichment factor >2 are 'ko05216 Thyroid cancer', 'ko00740 Riboflavin metabolism', 'ECM-receptor interaction', 'ko05202 Transcriptional misregulation in cancer' and 'ko05200 Pathways in cancer'.

**Transcription factor annotation of DEGs**

Zinc finger domain containing transcription factor are the most abundant proteins whose function are extraordinarily diverse and include epithelium development, neo-cortex development, transcription activation, regulation of apoptosis, protein folding and assembly [13–14]. Dip2a is thought to be a transcription factor due to its zinc finger motif [2]. To extend these findings, 14 DEGs (9 up & 5 down) from brain and 203 DEGs (163 up & 40 down) from lung were annotated with transcription factor (animal TFDB) database. In both group, the most of up-regulated genes belong to Zinc finger Cys2His2-like class group (ZF-C2H2) [124 & 2], Homeobox (5 & 2), High-mobility group (HMG) [4 & 1], Zinc finger and BTB domain-containing protein (ZBTB) [4 & 1], whereas the most of down-regulated genes accounts to transcription factor basic leucine zipper domain (TF-bZIP) [8 & 1], Thyroid hormone receptor [2&1] and Interferon regulatory factor (IRF) [2,1]. Based upon these evidences, our study

![Fig 5. COG classification of differential expression genes](https://doi.org/10.1371/journal.pone.0213702.g005)

![Fig 6. KEGG pathway enrichment scatter plot of DEGs](https://doi.org/10.1371/journal.pone.0213702.g006)
strongly suggests that DIP2A protein regulate expression of Zinc Finger domain containing proteins during lung and brain development. Transcription factor with the highest fold change (FC>6) from each group is listed in Table 3.

### DEGs validation by quantitative real-time PCR

To evaluate validity of RNA-Seq data, five up-regulated DEGs and five-down regulated DEGs from each group were selected for quantitative real-time RT-PCR (qPCR) (Fig 7). The RNA-Seq results of these genes were similar to those obtained by qPCR. These results confirmed the good quality of RNA-Seq results.

### Roles of Dip2a in neuronal cell maturation, differentiation and survival

Previous studies have suggested that Dip2a is highly expressed in neuronal cells of developing central nervous system such as retinal ganglion cells, Purkinje cell layer and granular cell, and may play important roles in synapse formation and axon guidance [1–4]. In this study, we found 10 genes that are important in neuronal cell maturation and in brain development were differentially expressed in Dip2a−/− brain. Skor2 and Gpr37l1 genes important in Purkinje cell maturation, differentiation and layer formation were down- regulated [15–16]. Runx1 gene is an important in cell fate specification and axonal projections of dorsal root ganglion neurons and Erbb3 gene is required in the control of growth and development of Schwann cell [17–18].
These genes were down-regulated. Similarly, *Frm7* gene which promotes neuronal outgrowth and migration of neural precursor cell was up-regulated [19]. *Fut10* is important in maintenance and differentiation of neuron stem cell and was up-regulated [20]. Extracellular matrix component *Hapln1* gene that plays an important in neo-cortex development and expansion was found over expressed [21]. Transcription factor SRY-box (Sox) family gene *Sox11* is expressed abundantly in all type of embryonic sensory neurons including sensory ganglion and trigeminal ganglion and promotes neuronal maturation was found up-regulated [22]. In addition, transcription factor AP-2 family gene *Tfap2c* important in neural crest induction was under expressed [23]. We also found *Plxnb3* gene was down-regulated. Increasing evidence suggests that Plexin-B3 is axon guidance molecule and promotes synapse formation in
rat hippocampal neurons [24]. Hence, these finding strongly supports the role of Dip2a in all type of neuronal cell maturation, differentiation and survival.

**Roles of Dip2a in lung development**

*Dip2a* gene role in lung development has not been symmetrically studied before. In this study, we found significantly altered expression of multiple genes known to participate in lung development. Among them include genes important in epithelial and mesenchyme cell proliferation and differentiation, vasculogenesis, alveologenesis and branching morphogenesis. *Hoxa5, Sox11, Errf1* and *Eya1* genes important in embryonic respiratory tract morphogenesis/organogenesis, lung epithelial, mesenchymal and vascular development were up-regulated [25–28].

*Ccbe1* gene is required for development of lymphatic vascular network and was found down-regulated [29]. Similarly, *Lama5* gene needed for proper immune system process was down-regulated [30]. *Rbp4* and *Wnt7a* genes play an important role in alveologenesis were also found under expressed [31–32]. *FGF9* gene is expressed in the pulmonary epithelium and is needed for epithelial branching was over expressed [33]. Pleiotrophin (*Ptn*) gene is involved in fibroblast and epithelial cell communication during fetal lung development was up-regulated [34]. *IGF-1* signaling modulates the development and differentiation of many types of lung cells, including airway basal cells, club cells, alveolar epithelial cells, and fibroblasts was over expressed [35]. In addition, *Dhc7* gene plays an important role in lung saccular development was also up-regulated [36]. *Crh* gene required for epithelial and mesenchyme cell proliferation was under expressed [37]. *Pdgfra* is known to regulate cell differentiation, proliferation, migration, actin reorganization and apoptosis was under represented [38].

**Conclusion**

In this report, four Transcriptome, including WT brain and lung, *Dip2a*−/− brain and lung at embryonic E19.5 were analyzed. On an average 6000 unigenes in each sample were generated with the Illumina Hiseq™ 2500 platform. In WT brain vs. *Dip2a*−/− brain comparison, a total of 214 DEGs were detected, including 82 up- and 132 down-regulated genes. These DEGs included genes involved in neuronal cell maturation, differentiation and survival. In WT lung vs. *Dip2a*−/− lung comparison, a total of 1900 DEGs were detected, including 1259 up- and 641 down-regulated genes. These DEGs are important in apoptosis process, lung epithelial development and in morphogenesis. To conclude, we have identified several candidate genes that are regulated by *Dip2a* at E19.5 brain and lung. It would be interesting to further study the biological functions of these genes in brain and lung development.

**Supporting information**

S1 Table. BLAST analysis of the non-redundant DEGs against six public databases. (TIF)

S1 Fig. Annotated diagram of the KEGG pathway of differentially expressed genes; (a) WT lung vs. *Dip2a*−/− lung (b) WT brain vs. *Dip2a*−/− brain. (TIF)

**Acknowledgments**

We are very thankful to Huiyan Wu and Xiu lu for microinjection and mouse colony management.
Author Contributions

Conceptualization: Rajiv Kumar Sah, Luqing Zhang, Yaowu Zheng.

Data curation: Rajiv Kumar Sah, Analn Yang, Zin Mar Oo, Chenhao Wang.

Formal analysis: Rajiv Kumar Sah, Analn Yang, Fatoumata Binta Bah.

Funding acquisition: Luqing Zhang, Xuechao Feng, Yaowu Zheng.

Investigation: Yaowu Zheng.

Methodology: Rajiv Kumar Sah, Ameer Ali Bohio.

Project administration: Luqing Zhang, Xuechao Feng, Yaowu Zheng.

Resources: Luqing Zhang, Yaowu Zheng.

Software: Rajiv Kumar Sah, Salah Adlat, Ameer Ali Bohio, Noor Bahadar.

Supervision: Luqing Zhang, Noor Bahadar.

Visualization: Rajiv Kumar Sah, Zin Mar Oo, May Zun Zaw Myint.

Writing – original draft: Rajiv Kumar Sah.

Writing – review & editing: Rajiv Kumar Sah, Analn Yang, Fatoumata Binta Bah, Salah Adlat, Zin Mar Oo, Yaowu Zheng.

References

1. Steller H, Fischbach K F, Rubin G M. Disconnected: A locus required for neuronal pathway formation in the visual system of drosophila. Cell. 1987; 50(7): 1139–1153. PMID: 3113740
2. Heilig J S, Freeman M, Laverty T, Lee K J, Campos A R, Rubin Gerald M, et al. Isolation and characterization of the disconnected gene of Drosophila melanogaster. The EMBO Journal. 1991; 10(4): 809–815. PMID: 1901262
3. Collewijn H, Holstege G. Establishment of neuronal connectivity during development of the drosophila larval visual system. Journal of Neurobiology. 1995; 28(3): 313–329. https://doi.org/10.1002/neu.480280305 PMID: 8568513
4. Mukhopadhyay M, Pelka P, Desousa D, Kablar B, Schindler A, Rudnicki MA, et al. Cloning, genomic organization and expression pattern of a novel Drosophila gene, the disco-interacting protein 2 (dip2), and its murine homolog. Gene. 2002; 293(1): 59–65.
5. Tanaka M, Murakami K, Ozaki S, Imura Y, Tong XP, Watanabe T, et al. DIP2 disco-interacting protein 2 homolog A (Drosophila) is a candidate receptor for follistatin-related protein/follistatin-like1—analysis of their binding with TGF-beta super family proteins. FEBS J. 2010; 277(20): 4278–89. https://doi.org/10.1111/1742-4658.2010.07816.x PMID: 20860622
6. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, et al. HDAC2 negatively regulates memory formation and synaptic plasticity. Nature. 2009; 459(7243):55–60. https://doi.org/10.1038/nature07925 PMID: 19424149
7. Egger G, Roetzer K M, Noor A, Lionel A C, Mahmood H, Schwarzbraun T, et al. Identification of risk genes for autism spectrum disorder through copy number variation analysis in Austrian families. Neurogenetics. 2014; 15(2):117–127. https://doi.org/10.1007/s10048-014-0394-0 PMID: 24643514
8. Kong R, Shao S, Wang J, Zhang X, Guo S, Zou L, et al. Genetic variant in DIP2A gene is associated with developmental dyslexia in Chinese population. American Journal of Medical Genetics. 2016; 171 (2): 203–206.
9. De Jager P L, Srivastava G, Lunnon K, Burgess J D, Schalkwyk L C, Lei Y, et al. Alzheimer’s disease: early alterations in brain DNA methylation at ANK1, BIN1, RHBDF2 and other loci. Nature Neuroscience. 2014; 17(9): 1156–1163. https://doi.org/10.1038/nn.3786 PMID: 25129075
10. Zhang L, Mabwi H A, Palange N J, Jia R, Ma J, Binta F, et al. Expression Patterns and Potential Biological Roles of Dip2a. PLOS ONE. 2015; 10(11).
11. Ozsolak F, Milos P M. RNA sequencing: advances, challenges and opportunities. Nature Reviews Genetics. 2011; 12(2): 87–98. https://doi.org/10.1038/nrg2934 PMID: 21191423

12. Zhang L, Jia R, Palange N J, Satheka A C, Togo J, An Y, et al. Large genomic fragment deletions and insertions in mouse using CRISPR/Cas9. PLOS ONE. 2015; 10(3).

13. Li Hong, Lu Melfang, Liu X. Zinc-Finger Proteins in Brain Development and Mental Illness. J Transl Neurosci. 2018; 3:4.

14. Laitly John H, Lee Brian M, Wright Peter E. Zinc finger proteins: new insights into structural and functional diversity. Current Opinion in Structural Biology. 2001; 11(1).

15. Wang B, Harrison W R, Overbeek P A, Zheng H. Transposon mutagenesis with coat color genotyping identifies an essential role for Skor2 in sonic hedgehog signaling and cerebellum development. Development. 2011; 138(20): 4487–4497. https://doi.org/10.1242/dev.067264 PMID: 21937600

16. Marazziti D, Pietro C D, Golini E, Mandillo S, Sala G L, Matteoni R, et al Precocious cerebellum development and improved motor functions in mice lacking the astrocyte cilium-, patched 1-associated Gpr37l1 receptor. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(41): 16486–16491. https://doi.org/10.1073/pnas.1314819110 PMID: 24062445

17. Masaaki Y, Kouji S, Tomomasa Y, Satoru O, Takashi S. Runx1 selectively regulates cell fate specification and axonal projections of dorsal root ganglion neurons. Developmental Biology. 2007; 303(2): 663–647. https://doi.org/10.1016/j.ydbio.2006.12.007 PMID: 17208218

18. Britsch S, Li L, Kirchhoff S, Theuring F, Brinkmann V, Birchmeier C. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes & Development. 1998; 12(12): 1825–1836.

19. Bettshenderson J, Bartesaghi S, Crosier M, Lindsay S, Chen H, Paolo S, et al. The nystagmus-associated FRMD7 gene regulates neuronal outgrowth and development. Human Molecular Genetics. 2010; 19(2): 342–351. https://doi.org/10.1038/hmg/ddp500 PMID: 19892780

20. Kumar A, Torii T, Ishino Y, Muraoka D, Yoshimura T, Togayachi A, et al. The Lewis X-related α1,3-fucosyltransferase, Fut10, is required for the maintenance of stem cell populations. Journal of Biological Chemistry. 2013; 288(40): 28859–28866. https://doi.org/10.1074/jbc.M113.469403 PMID: 23986452

21. Long K R, Newland B, Florio M, Kaebeln S, Langen B, Kolterer A, et al. Extracellular Matrix Components HAPLN1, Lumican, and Collagen I Cause Hyaluronic Acid-Dependent Folding of the Developing Human Neocortex. Neuron. 2018; 99(4).

22. Lin L, Lee V. M, Wang Y, Lin J. S, Sock E, Wegner M, et al. Sox11 regulates survival and axonal growth of embryonic sensory neurons. Dev. Dyn. 2011; 240: 52–64. https://doi.org/10.1002/dvdy.22489 PMID: 21117150

23. Coelho D.J., Sims D.J., Ruegg P.J., Minn I., Muench A.R., Mitchell P.J., et al. Cell type-specific and sex-dimorphic expression of transcription factor AP-2 in the adult mouse brain. Neuroscience. 2005; 134(3): 907–919. https://doi.org/10.1016/j.neuroscience.2005.04.060 PMID: 16009501

24. Laht P, Tammuru E, Otsus M, Rohtla J, Tiismus L, Veske A, et al. Plexin-B3 suppresses excitatory and inhibitory synapse formation in rat hippocampal neurons. Experimental Cell Research. 2015; 335(2): 269–278. https://doi.org/10.1016/j.yexcr.2015.05.007 PMID: 25989221

25. Landry truchon K, Fournier S, Houde N, Rousseau J, Jeannotte L, Kinkead R. Respiratory consequences of targeted losses of Hoxa5 gene function in mice. The Journal of Experimental Biology. 2017; 220(24): 4571–4577.

26. Zhu Y, Li Y, Wei J, Liu X. The Role of Sox Genes in Lung Morphogenesis and Cancer. International Journal of Molecular Sciences. 2012; 13(12): 15767–15783. https://doi.org/10.3390/ijms131215767 PMID: 23443092

27. Jin N, Cho S N, Rasof M G, Wistuba I, Smith Y, Yang Y, et al. Mig-6 is required for appropriate lung development and to ensure normal adult lung homeostasis. Development. 2009; 136(19): 3347–3356. https://doi.org/10.1242/dev.032979 PMID: 19710174

28. Elnashash A H, Alam D A, Turcatel G, Bellusci, Warburton D. Eyes absent 1 (Eya1) is a critical coordinator of epithelial, mesenchymal and vascular morphogenesis in the mammalian lung. Developmental Biology. 2011; 350(1): 112–126. https://doi.org/10.1016/j.ydbio.2010.11.022 PMID: 21129374

29. Jakus Z, Gleghorn J P, Enis D R, Sen A, Chia S, Liu X, et al. Lymphatic function is required prematurely for lung inflation at birth. Journal of Experimental Medicine. 2014; 211(5): 815–826. https://doi.org/10.1084/jem.20132308 PMID: 24733830

30. Kikkawa Y, Miner J H. Molecular dissection of laminin α5 in vivo reveals separable domain-specific roles in embryonic development and kidney function. Developmental Biology. 2006; 296(1): 265–277. https://doi.org/10.1016/j.ydbio.2006.04.463 PMID: 16750824

31. Maden M. Retinoids in Lung Development and Regeneration. Current Topics in Developmental Biology, 2004: 153–189.
32. Newman Donna R., Zhang Huiying, Bortoff Katherine, Bonner James C., Sannes Philip L. Alveolar Epithelial Differentiation during Repair Involves FoxA1, Wnt7A, and TGF-β. Proceedings of the american thoracic society. 2010; 7.

33. Yin Y, Wang F, Ornitz DM. Mesothelial- and epithelial-derived FGF9 have distinct functions in the regulation of lung development. Development. 2011; 138. 15: 3169–77. https://doi.org/10.1242/dev.065110 PMID: 21750028

34. Weng T, Gao L, Bhaskaran M, Guo Y, Guo D, Narayanaper J, et al. Pleiotrophin Regulates Lung Epithelial Cell Proliferation and Differentiation during Fetal Lung Development via β-Catenin and Dlk1. Journal of Biological Chemistry. 2009; 284(41): 28021–28032. https://doi.org/10.1074/jbc.M109.052530 PMID: 19661059

35. Wang Z, Li W, Guo Q, Wang Y, Ma L, Zhang X. Insulin-Like Growth Factor-1 Signaling in Lung Development and Inflammatory Lung Diseases. BioMed Research International. 2018; 1–27.

36. Yu H, Wessels A, Chen J, Phelps A L, Oatis J E, Tint G S, et al. Late gestational lung hypoplasia in a mouse model of the Smith-Lemli-Opitz syndrome. BMC Developmental Biology. 2004; 4(1): 1–18.

37. Muglia L J, Bae D S, Brown T T, Vogt S K, Alvarez J G, Sunday M E, et al. Proliferation and Differentiation Defects during Lung Development in Corticotropin-Releasing Hormone–Deficient Mice. American Journal of Respiratory Cell and Molecular Biology. 1999; 20(2): 181–188. https://doi.org/10.1165/ajrcmb.20.2.3381 PMID: 9922208

38. Andrae J., Gallini R. and Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 2008; 22: 1276–1312 https://doi.org/10.1101/gad.1653708 PMID: 18483217