Global transcriptome study of Dip2B-deficient mouse embryonic lung fibroblast reveals its important roles in cell proliferation and development

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1. Introduction

Disco gene was identified as a transcription factor with two C2H2 type zinc finger domains that involve neuronal connection in visual system of Drosophila melanogaster [1–2]. By using yeast two-hybrid system, Mukhopadhyay et al. [3] identified a protein that interacts with Disco and named disco-interacting protein 2 (Dip2). Dip2 is highly conserved from insects to mammals and evolved into three different proteins, Dip2A, Dip2B and Dip2C in mammals [3]. Based on amino acid sequences, Dip2B has three putative functional domains, a binding domain for the transcriptional regulator DMAP1 (DNMT1-associated protein 1), an AMP-binding domain and an adenylate-forming domain [4]. Study suggests that Dip2B may play important roles in DNA methylation and metabolism. Dip2B may epigenetically control cell proliferation and differentiation through DNA methylation [4]. Dip2B deficiency has been associated with mental retardation and developmental delay [4,5]. A small RNA miR-133b-3p can down-regulate Dip2B and epigenetically repress genes for KIT+K5 progenitor cell expansion [6]. A correlation in methylation status between colorectal cancer and aberrant miR-133b expression was also reported [7].

Abstract

Disco-interacting protein 2 homolog B (Dip2B) is a member of Dip2 family encoded by Dip2b gene. Dip2B has been reported to regulate murine epithelial KIT+ progenitor cell expansion and differentiation epigenetically via exosomal miRNA targeting during salivary gland organogenesis. However, its molecular functions, cellular activities and biological process remain unstudied. Here, we investigated the transcriptome of Dip2B-deficient mouse embryonic lung fibroblasts (MELFs) isolated from E14.5 embryos by RNA-Seq. Expression profiling identified 1369 and 1104 differentially expressed genes (DEGs) from Dip2b−/− and Dip2b+/− MELFs in comparisons to wild-type (Dip2b+/+). Functional clustering of DEGs revealed that many gene ontology terms belong to membrane activities such as ‘integral component of plasma membrane’, and ‘ion channel activity’, suggesting possible roles of Dip2B in membrane integrity and membrane function. KEGG pathway analysis revealed that multiple metabolic pathways are affected in Dip2b−/− and Dip2b+/− when compared to Dip2b+/+ MELFs. These include ‘protein digestion and absorption’, ‘pancreatic secretion’ and ‘steroid hormone synthesis pathway’. These results suggest that Dip2B may play important roles in metabolism. Molecular function analysis shows transcription factors including Hox-genes, bHLH-genes, and Forkhead-genes are significantly down-regulated in Dip2b−/− MELFs. These genes are critical in embryo development and cell differentiation. In addition, Dip2B-deficient MELFs demonstrated a reduction in cell proliferation and migration, and an increase in apoptosis. All results indicate that Dip2B plays multiple roles in cell proliferation, migration and apoptosis during embryogenesis and may participate in control of metabolism. This study provides valuable information for further understanding of the function and regulatory mechanisms of Dip2B.

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However, the mechanism underlining most of Dip2B’s roles including on cell proliferation, cell differentiation are still not clear. RNA sequencing (RNA-Seq) takes advantage of the second-generation sequencing technology and is a powerful approach for studying global gene expression profile in a particular cell or tissue. It helps us to understand the regulatory network of genes and pathways [8,9]. Fibroblasts cells were identified as ubiquitous mesenchymal cells that play many essential roles including tissue repair and wound healing [10]. Embryonic fibroblasts are highly diversified and have potential to differentiate into different cell types. Mouse embryonic lung fibroblasts (MELFs) cells from genetically manipulated mouse models have been used to study the molecular mechanism of genes and their regulatory networks, especially those genes that their ablations resulted in mouse prenatal lethality. To explore the potential role of Dip2B, MELFs were isolated from embryos of homozygous knockout (Dip2b−/−), heterozygous (Dip2b+/−) and wild type (Dip2b+/+) at E14.5. Genes and pathways under Dip2B regulation were investigated.

2. Materials and methods

2.1. Animals

Mouse study has been approved by Institutional Animal Care and Use Committee for Animal Experimental Ethics Committee of Northeast Normal University with approval number of (ENU/ IACUC, AP2018011) and carried out in accordance with the Guide for Care and Use of Laboratory Animals of National Institutes of Health as well. Mice were housed in a pathogen-free facility in Northeast Normal University with temperature at 21 ± 1 °C, humidity 30–60%, 12:12 light/dark cycles and free access to water and food.

2.2. Isolation and culture of lung MELFs

MELFs were isolated from mouse embryos obtained by intercrossing of Dip2b+/− transgenic mice. Dip2b+/− pregnant mouse at E14.5 day post-coitus (p.c.) was euthanized by cervical dislocation and soaked in 70% ethanol for 5 min, then the uterine horns were dissected out and rinsed in 70% EtOH. Uterine horns were then transferred into petri dish and the embryos were separated individually. Visceral organs of each embryo were removed except lungs. Lungs were washed in 1× PBS and placed in a new Petri dish. Lung tissues were gently minced using a sterile razor blade till possible to pipette. The tissues were then incubated with 0.25% trypsin (Sigma Aldrich) at 37 °C. Trypsin was inactivated 15 min later by adding Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Shanghai, China) containing 10% fetal bovine serum (Sigma, St Louis, USA) and 1% penicillin/streptomycin (Invitrogen Life Technologies, USA). Plates were coated with 0.2% gelatin (Gelatin from bovine skin, Type B, Sigma) for 2 h. The cells were then cultured at 37 °C in a humidified incubator with 5% CO2.

2.3. Hematoxylin and eosin staining (H&E)

Mice embryos from E19.5 were dissected out from uterus and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich; EMD Millipore) for overnight at 4 °C. Fixed tissues were then washed three times in 1x PBS and processed in 70% ethanol. Tissues were dehydrated with a series of graded alcohols, cleared in xylene, and embedded in paraffin. 6-μm thick sections were obtained, mounted on glass slides and stained with Hematoxylin and eosin using standard protocol. Images were taken using microscope (Olympus, Tokyo, Japan).

2.4. Cell proliferation assay by MTT

Methylthiazolyldiphenyl-tetrazolium (MTT, Sigma) test was performed on Dip2b−/−, Dip2b+/− and Dip2b+/+ MELFs to measure the proliferation and cell viability. MELFs were detached in 0.25% trypsin/EDTA and seeded into 96-well plates with 5 × 103 cells per well in 200 μL DMEM containing 10% FBS. Cells were incubated at 37 °C and 5% CO2 for 24 h and allowed to grow to 70–80% confluence. Then 20 μL of MTT (5 mg/ml in 1× PBS) was added to each well and incubated for 4hrs. The medium was discarded and 100 μl of dimethyl sulfoxide (DMSO) added to each well. Optical density was analyzed at 490 nm using (BioTek Instruments, Winoski, VT). A total of 4 replicate for each sample was prepared and experiment repeated 3 times.

2.5. Cell apoptosis analysis by FITC-Annexin-V and PI

Apoptosis was detected using FITC-Annexin-V and PI staining kit (Cat #630109, Takara, Japan) according to manufacturer’s instructions. Briefly, Dip2b−/−, Dip2b+/+ and WT MELFs were seeded in 6-well plates at a concentration of 3 × 105 cells/well. MELFs were trypsinized and washed twice with 1X PBS. A 5 μL Annexin V-FITC and 10 μl propidium iodide (PI) at 50 μg/ml in 1× binding buffer (10 mM HEPES/pH 7.4, 140 mM NaOH, 2.5 mM CaCl2) were added for 15 min at room temperature in the dark. Apoptotic cells were analyzed using a Becton-Dickinson FACSscan cytofluorometer (Mansfield, MA). Both early (Annexin V-positive, PI negative) and late (Annexin V-positive and PI-positive) were considered as apoptotic cells.

2.6. Cell cycle analysis

Dip2b−/−, Dip2b+/− and Dip2b+/+ MELFs were seeded in 6-well plates at a density of 2 × 105 cells/well. Cells were trypsinized and washed twice with 1× PBS. Then cells were fixed with 70% ice-cold EtOH overnight. Fixed cells were treated with RNase (200 μg/ml) for 2hrs at RT, followed by staining with 500 μl PI (50 μg/ml) (Cat#630109, Takara, Japan). Cells were transferred to FACS tubes and incubated for 30 min at RT in dark. Afterwards, cells were subjected to FACS analysis using Caliber flow cytometer (Becton Dickinson, USA). Dead cells and cell debris were excluded.

2.7. Cell migration assay

Cells were grown in 6-well culture plates. Confluent monolayer was scratched using a 200 μl pipette tips and medium replaced with fresh medium to remove cell debris. Cells were allowed to grow for 48 h. Photographs were taken with an inverted microscope (Olympus, Tokyo, Japan). Digital straight lines were drawn on the borders of scratches and distance of cell growth with time was recorded.

2.8. Preparation of RNA-Seq libraries

Total RNA was purified from MELFs of Dip2b+/+, Dip2b+/− and Dip2b−/− using RNAiso plus reagent (Takara, Dalian, China) according to manufacturer’s protocol. RNA concentration and quality were determined using NanoDrop 2000 (Thermo Fisher Scientific, USA) and Bioanalyzer (Agilent Technologies, USA). Messenger RNA was purified using oligo (dT)-attached magnetic beads, fragmented before cDNA Synthesis.
2.9. RNA-Seq data processing and identification of differentially expressed genes (DEGs)

Low quality reads, adaptor-only and reads with more than one unknown base (N) were removed through SOAPnuke software [11] to obtain clean reads. Q20 (%), Q30 (%) and GC content (%) were calculated. More than 1.14 Gigabyte clean reads were obtained as FASTQ format from each library. Reads were assembled into longer transcripts and mapped to reference genome using HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) [12] and Bowtie2 tool [13].

The level of transcripts was quantified and presented as paired-end RNA-Seq FPKM (Fragments Per Kilobase per Million mapped reads) normalized reads. Differentially expressed genes (DEGs) were identified by comparison of two different libraries using Poisson distribution [14] and Expectation-Maximization (RSEM) softwares [15].

2.10. Gene ontology and Kyoto Encyclopedia of genes and genome pathways analysis

DEGs with FDR of ≤0.01 and absolute value of FC ≥ 2 (Two-fold change) were considered significant for further analysis. Gene ontology (GO) annotation enrichment analysis of DEGs was implemented using GOseq R package software and estimated by hyper-geometric test. DEGs were also used to identify the enriched Kyoto Encyclopedia of Genes and Genome (KEGG) pathways [16].

2.11. Validation of RNA-Seq results by quantitative real-time PCR (qPCR)

One µg of total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) with Prime Script RT Reagent Kit (Perfect Real Time, TaKaRa, Dalian, China) according to the manufacturer’s instructions. Quantitative real-time PCR (qPCR) was performed with 50 ng of cDNA using One-Step SYBR PrimeScript™ RT-PCR kit (Takara, Dalian, China). All reactions were performed in triplicate. All primers were initially evaluated for efficiency using relative standard curve and electrophoresis on gel.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software Inc). Significant differences between groups were evaluated using Student’s t test. P-values were two-sided and P-values <0.05 was considered statistically significant.

3. Results and discussion

3.1. Dip2b mRNA expression level of Dip2B-deficient MELFs

Since Dip2b−/− mice dies postnatally and lung development seems the major cause (Supplementary Fig. 1), intercrosses of Dip2b+/- mice were used to prepare MELFs and total RNAs from all three genotypes, Dip2b−/−, Dip2b+/− and Dip2b+/+ at E14.5 (Fig. 1A). Dip2b mRNA expression in MELFs was confirmed by quantitative real-time PCR (qPCR) (Fig. 1B, C). Results show expected decrease of Dip2b mRNA expression. The mRNAs from MELFs were prepared for RNA-Seq.

3.2. Gene expression profiling of MELFs under Dip2B

To study the potential biological role of Dip2B, three libraries were generated from Dip2b−/−, Dip2b+/− and Dip2b+/+MELFs and analyzed on BGISEQ-500 platform. The raw data of RNA-Seq is accessible at the Sequence Read Archive (SRA) database in NCBI with the following link (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA647133/) under the accession number PRJNA647133. Clean reads were mapped to Mus musculus reference genome (GRCm38. p6/NCBI, GCF_000001635.26). As shown in Table 1, ~97% of total mapping was acquired and >80% was uniquely mapped, indicating the reliability of sequencing data. Table 2 is summarizing the sequencing reads among samples. Normalized FPKM was calculated and expression level distribution shown in (Supplementary Fig. 1.

![Fig. 1. MELF cell isolation and Dip2b mRNA expression analysis by qPCR (A) Cell images of MELF cultures at low (Top panel) and high (Bottom panel) density. (B) Relative expression levels of Dip2b mRNA in MELFs by qPCR. (C) Gel electrophoresis image showing PCR products.]

| Sample | Total Mapping (%) | Uniquely Mapping (%) |
|--------|------------------|---------------------|
| Dip2b+/+ | 97.06 | 79.67 |
| Dip2b+/− | 97.08 | 80.82 |
| Dip2b−/− | 96.98 | 81.42 |

| Sample  | Total Raw Reads (M) | Total Clean Reads (M) | Total Clean Bases (Gb) | Clean Reads Q20 | Clean Reads Q30 | Clean Reads Ratio |
|---------|---------------------|-----------------------|------------------------|-----------------|----------------|------------------|
| Dip2b+/+ | 23.38 | 22.7 | 1.14 | 99.12 | 96.3 | 97.1 |
| Dip2b+/− | 23.51 | 22.92 | 1.15 | 99.19 | 96.75 | 97.5 |
| Dip2b−/− | 23.44 | 22.81 | 1.14 | 99.1 | 96.55 | 97.3 |

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Fig. 2A). The overlapped genes were identified 1677 and 118 genes differentially expressed in both homozygous and heterozygous MELFs. 269 unique DEGs were expressed in Dip2b+/−, 740 unique DEGs were expressed in Dip2b−/− vs to Dip2b+/+ and 73 unique DEGs were expressed in Dip2b+/− vs to Dip2b−/− MELFs respectively (Fig. 2A).

Comparisons between Dip2b−/− vs Dip2b+/+ and Dip2b+/− vs Dip2b+/+ identified 1369 and 1104 differentially expressed genes (DEGs) based on fold change ≥2 and adjusted FDR < 0.01. Among them, 839 and 549 are significantly up- and 530 and 555 significantly down-regulated (Fig. 2B). Volcano plot and heatmap are used to show the significant DEGs (Fig. C2 and Supplementary Fig. 2B). Fifty of the most up- and down-regulated DEGs are listed in Table 3 and Supplementary Table 1. Among them, six genes Enrp2, Entpd4, Fpr2, Hist2h3c1, Zfp967 and Gm14296 are the most significantly up-regulated while 6 genes Ear2, Ear2, Fpr2, Hist2h3c1, Zfp967 and Gm14296 are the most significantly down-regulated.

3.3. Gene ontology (GO) analysis of Dip2b-regulated DEGs.

In order to identify the potential biological roles of Dip2B in embryonic stages, DEGs identified from comparison of Dip2B-deficient and WT MELFs were used for GO analysis. GO is classified into three independent categories, biological process (BP), molecular function (MF) and cellular component (CC). Each DEG could be assigned to one or more GO terms. A total of 1369 and 1104 DEGs with FC ≥2 and FDR < 0.01 were identified from comparisons of Dip2b−/− vs Dip2b+/+ and Dip2b+/− vs Dip2b+/+ MELFs. GO analysis found 28 BP, 17 MF and 12 CC significantly annotated (Fig. 3, Supplementary Fig. 3 and Supplementary Fig. 4). Annotated BP categories include ‘cell adhesion’, ‘cell differentiation’, ‘regulation of signaling receptor activity’, ‘multicellular organism development’. MF categories include ‘ion channel activity’, ‘ligand-gated sodium channel activity’, ‘voltage-gated potassium channel activity’, ‘extracellular matrix structural constituent’, ‘calcium ion binding’ and ‘DNA binding transcription activity’. DEGs-annotated CC categories include ‘ion channel activity’, ‘ligand-gated sodium channel activity’, ‘voltage-gated potassium channel activity’, ‘extracellular matrix structural constituent’, ‘calcium ion binding’ and ‘apical plasma membrane’ (Supplementary Table 2). Based on DAVID (Database for Annotation, Visualization and Integrated Discovery [17,18]), the functional annotation clustering tool revealed that a list of cluster terms from BP, MF, and CC categories were mainly enriched with DEGs related to membrane structure and its activities (Supplementary Table 3). All the results suggest that Dip2B may play important roles in cell to cell interactions, membrane integrity and membrane activities which are critical for cell differentiation and function.

Dip2B-deficient MELFs resulted in upregulation of 60 (Dip2b−/− vs Dip2b+/+) and 52 (Dip2b+/− vs Dip2b+/+) DEGs that are involved in immune system and inflammatory responses respectively. In immune system process, 43 genes were enriched in innate immune response, 14 genes in defense response to virus and 11 genes in adaptive immune response (Supplementary Table 4, Supplementary Table 5). In inflammatory response category, most of the genes are chemotaxis genes including (Fpr2, Fpr1, Ccr2, Cxcl15, Ccl9, Ccl6, Ccl11, Cxcl2, Cxcl12, S100a8, Ccl12, Ccl7, Plk3cg, Ccl2, Cxcl3), 12 genes in neutrophil chemotaxis, 7 genes in Eosinophil chemotaxis, 8 genes in monocyte chemotaxis and 8 genes in lymphocyte chemotaxis (Supplementary Table 6), indicating the importance of Dip2B in regulation of cell migration in immune responses.

3.4. KEGG pathway analysis on Dip2B-regulated DEGs

To elucidate the potential biological pathways under Dip2B regulation, KEGG pathway analysis was performed for Dip2B-regulated DEGs. KEGG classification revealed that ‘signal transduction’ and ‘immune system’ terms have the highest number of DEGs (Supplementary Fig. 5). The Q value <0.05 was considered significantly represented and KEGG pathways were enriched based on FC ≥2 and FDR < 0.01. Top 20 most enriched pathways between Dip2b−/− vs Dip2b+/+ and Dip2b+/− vs Dip2b+/+ are shown in Fig. 4.
and Supplementary Fig. 6. Among them, 'ko04974 protein digestion and absorption', 'ko04924 renin secretion', 'ko04972 pancreatic secretion', 'ko04060 cytokines-cytokines receptor interaction' and 'ko00140 steroid hormone synthesis' metabolism pathways are the most significantly down-regulated pathways, whereas 'ko04380 Osteoclast differentiation', 'ko04621 NOD-like receptor signaling pathway' and 'ko04145 Phagosome' were the most up-regulated pathways (Supplementary Table 7a and 7b). Results demonstrate that multiple pathways are under Dip2B regulation and confirms that Dip2B is important in regulating membrane activities and be involved in regulation of metabolism.

3.5. Hox gene family are regulated by Dip2B

Homeodomain proteins encoded by Hox genes are responsible for regulating expression of many target genes involved in cell proliferation and differentiation [19–22]. These genes are also known to be involved in cancers and controlled by DNA methylation [23,24]. Among differentially expressed transcription factors (TFs), homeobox (Hox) gene family was the most dysregulated genes in Dip2B-deficient MEFLs (38 down- and 6 up-regulated genes in both heterozygous and homozygous MEFLs). Results strongly suggest that Dip2B may regulate embryo development and differentiation through Hox gene expression. Dip2B contains DMAP1 binding domain and may regulate Hox gene transcription by DNA methylation [4]. Several TFs that belong to zinc finger (Zf-C2H2), basic helix-loop-helix (bHLH) and Fox proteins are also significantly dysregulated. TFs with fold change ≥ 2 and FDR < 0.01 are listed in Table 4 and Supplementary Tables 8 and 9.

3.6. Dip2B regulates cell proliferation

Cell proliferation is an important process during development and tissue maintenance [25]. GO analysis shows that DEGs were significantly enriched in 'GO:0008285 –negative regulation of cell proliferation' and 'GO:0030308 –negative regulation of cell proliferation'.
growth', indicating Dip2B knockout reduces cell proliferation and growth (Fig. 5A, B). Genes including Adora3, Il1b, Slfn1 and Nrk [26–30] responsible for inhibition of cell proliferation were significantly up-regulated. To validate the role of Dip2B in cell proliferation, isolated MELFs were subjected to MTT viability assay. As shown in Fig. 5C, growth rates of MELFs from Dip2b−/−/− are significantly slower than cells from Dip2b+/+ (p < 0.01), and more obvious in Dip2b−/− MELFs (p < 0.001). Result suggests that Dip2B may be a critical factor in regulation of cell proliferation.

3.7. Dip2B regulates apoptosis and cell cycle

GO analysis shows that apoptosis and cell cycle are regulated by Dip2B. Several DEGs were found significantly enriched in 'positive

Fig. 3. GO analysis. Top seven GO terms of up- and down-regulated DEGs of Dip2b+/+ vs Dip2b−/− (FC ≥ 2, FDR < 0.01).

Fig. 4. KEGG pathway analysis of DEGs from Dip2b+/+ vs Dip2b−/− MELFs. Adjusted p-values (Q-values) depicting significant enrichment (q-value < 0.05).
regulation of apoptotic process', such as up-regulation of Wnt11, Pycard, Il1b, Trp73, Clu and Fas [27,31–35]. To verify whether Dip2B affects apoptosis, cells were analyzed using Annexin V and PI staining and cell cycle by flow cytometry (Fig. 6A). Results indicate that percentage of later apoptotic MELFs is significantly increased in Dip2b/C0/C0 in comparison to Dip2b+/+ heterozygous or WT MELFs. The early apoptotic proportions in Dip2b/C0/+ MELFs are similar to Dip2b+/+ MELFs but increased later (Fig. 6B). Dip2b/C0/C0 MELFs exhibited an increase in G2/M population in cell-cycle profiles (Fig. 6C). Results confirm that Dip2B can significantly inhibit MELF proliferation and induce apoptosis and cell cycle arrest.

3.8. Dip2B regulates cell migration

Transcriptomic data revealed that Dip2B is involved in promotion of cell migration. Knockout of Dip2B results in significantly
down-regulation of Cdh2, Podxl, L1cam, Bcar1, Tdgf1 and Hbegf [36–41] that are known for promoting cell migration (Table 5). Genes including Podn, Ptprf and Adora3 [26,42,43] responsible for inhibition of cell migration were up-regulated. To confirm the effect of Dip2B in cell migration, MELFs were allowed to grow to confluence and scratch wounds were made. Gap closing were recorded at different time points. Results show that Dip2b+/− MELFs was significantly slower in migration than that of Dip2b+/+ and Dip2b+/0 MELFs (Fig. 7).

3.9. Verification of RNA-Seq results

Ten important DEGs were validated by qPCR. Overall, qPCR results are highly consistent with the RNA-Seq results (Fig. 8). Primer sequences are listed in Supplementary Table 10.

Table 5
Differentially expressed genes involved in cell migration.

| Gene symbol | Dip2b+/+ vs Dip2b+/0 | | Dip2b+/+ vs Dip2b+/0 | |
|-------------|---------------------|----------------|---------------------|----------------|
|             | FC ≥ 2              | FDR            | P value             | FC ≥ 2              | FDR            | P value             |
| Cdh2        | 2.21226596          | 0              | 0                   | 2.974162444        | 0              | 0                   |
| Podxl       | 1.835315003         | 5.00E−51       | 2.81E−59            | 2.258655831        | 1.74E−115      | 6.12E−117           |
| L1cam       | 1.975442896         | 4.16E−99       | 0                   | 2.22105744         | 2.68E−05       | 3.28E−06            |
| Bcar1       | 1.233105744         | 0              | 0                   | 1.223105744        | 2.68E−05       | 3.28E−06            |
| Tdgf1       | 6.794415866         | 2.73E−12       | 4.78E−13            | 6.794415866        | 2.73E−12       | 5.94E−13            |
| Vil1        | 7.658211483         | 7.92E−33       | 2.30E−37            | 6.073248982        | 7.92E−33       | 8.27E−34            |
| Adra2a      | 3.195256291         | 3.99E−85       | 1.38E−97            | 4.457997953        | 3.47E−107      | 1.29E−109           |
| Erbb4       | 3.841302254         | 5.32E−24       | 3.09E−11            | 4.426247955        | 7.99E−24       | 9.68E−25            |
| Tgfa2a      | 3.14567455          | 3.12E−21       | 4.16E−99            | 3.660250628        | 6.38E−24       | 8.68E−25            |
| Lgr6        | 2.220729372         | 9.02E−09       | 1.16E−13            | 3.42003818         | 1.16E−13       | 3.33E−14            |
| Sema5b      | 1.247972513         | 0.0794798      | 5.76E−04            | 3.247927513        | 5.76E−04       | 2.35E−04            |
| Hbegf       | 2.796893512         | 0              | 0                   | 2.713977039        | 0              | 0                   |
| Ctnn1       | 2.302638712         | 0              | 0                   | 2.604238401        | 0              | 0                   |
| Gper1       | 4.812498225         | 1.93E−18       | 1.58E−34            | 2.379538818        | 1.93E−18       | 3.20E−19            |
| Sema3g      | 2.12993723          | 2.63E−06       | 1.97E−06            | 2.350497247        | 2.63E−06       | 8.44E−07            |
| Podxl       | 1.835315003         | 2.81E−52       | 2.81E−59            | 2.297219108        | 2.88E−67       | 6.12E−117           |
| Pdgfd       | 2.460097222         | 4.99E−93       | 3.83E−57            | 2.242945253        | 6.32E−90       | 2.74E−91            |
| Sema6a      | 1.808903709         | 2.14E−71       | 3.83E−57            | 2.19176803         | 2.14E−71       | 1.16E−72            |
| Col18a1     | 1.61403128          | 0              | 0                   | 2.074086318        | 0              | 0                   |
| Sema7a      | 1.018378529         | 4.99E−47       | 1.49E−18            | 2.025796001        | 4.99E−47       | 3.92E−48            |
| Fzr1        | 2.152928449         | 8.34E−270      | 1.25E−299           | 2.003572503        | 8.34E−270      | 1.23E−271           |
| Fam107a     | 3              | 4.89E−05       | 4.89E−05            | 1.19265078        | 0.03853376     | 0.0208544           |
| Ntrk3       | 2.237039197         | 2.03E−04       | 2.03E−04            | 1.137503524        | 0.025004585    | 0.01329822           |
| Podn        | 2.162271429         | 5.72E−13       | 5.72E−13            | 3.402084444        | 3.81E−44       | 3.14E−45            |
| Ptprf       | 0.567122422         | 1.55E−55       | 1.55E−55            | 1.018699606        | 1.21E−204      | 2.37E−206           |
| Adora3      | 6.906890596         | 6.58E−17       | 6.58E−17            | 6.845940051        | 2.05E−15       | 3.83E−16            |

Fig. 6. Apoptosis and cell cycle analysis. (A) Heatmap for DEGs associated with GO terms ‘positive regulation of apoptotic process’. (B) Apoptotic cells measured using a Becton-Dickinson FACScan cytofluorometer. (C) Histogram of flow cytometric analysis of cell cycle showing the distribution of cell phase.
4. Conclusions

In this study, MELFs were isolated from Dip2b knockout mouse embryos at E14.5. Dip2b+/+, Dip2b+-/ and Dip2b--/ MELFs were examined for genome-wide gene expression. DEGs were identified and analyzed by GO and KEGG for Dip2B-regulated bioprocesses and pathways. The most enriched bioprocesses and pathways were confirmed that include cell proliferation, cell cycle, cell apoptosis and cell migration.

GO analysis showed that DEGs were mostly annotated to membrane-related GO terms including ‘integral component of plasma membrane’, ‘anchored component of plasma membrane’, ‘basolateral plasma membrane’, ‘ion channel activity’, and ‘voltage-gated potassium channel activity’, whereas KEGG pathways enriched DEGs are related to metabolism including protein digestion and absorption’, ‘renin secretion’, ‘pancreatic secretion’, and ‘steroid hormone synthesis’. Results demonstrate that Dip2B promotes cell proliferation, increases cell migration and inhibits cell apoptosis. Knockout Dip2B leads to cell arrest at G2/M phase. Results indicate that Dip2B is involved in multiple biological processes and pathways. Dip2B may regulate many important biologic functions that are highly correlated with development, differentiation and morphogenesis. Dip2B caused upregulation of multiple genes involves in innate and adaptive immune response and inflammatory responses. A number of these genes are related to leukocyte chemotaxis, such as Ccl9, Ccl6, Ccl11, Ccl22, Ccr5, Ccl7, Ccl2, Cxcl2, Cxcl13, and Cxcl15, highlighting the potential role of Dip2B in regulation of the immune cell mobilization and inflammatory responses. A number of these genes are related to leukocyte chemotaxis, such as Ccl9, Ccl6, Ccl11, Ccl22, Ccr5, Ccl7, Ccl2, Cxcl2, Cxcl13, and Cxcl15, highlighting the potential role of Dip2B in regulation of the immune cell mobilization and inflammatory responses.

![Fig. 7. Scratch assay for cell migration. Images were taken at 0, 24, 48hrs post-scratching.](image1)

![Fig. 8. Gene expression validation of DEGs from RNA-Seq by qPCR.](image2)
of transcription factors such as homeobox (Hox) gene family, zinc finger (zf-C2H2), basic helix-loop-helix (bHLH) and Fox proteins were found to be significantly dysregulated. The most dysregulated TFs was Homeobox (Hox) genes suggesting that Dip2B may regulate cell differentiation and embryo development. Together, these information are valuable for further deciphering Dip2B roles in development and disease.

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Conflict of Interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2020.08.030.

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