The DNA methylation status of Wnt and **Tgfß signals is a key factor on functional** regulation of skeletal muscle satellite cell development

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

Weiya Zhang conducted the experiments and prepared the materials involved in this study. Saixian Zhang and Yueyuan Xu performed the bioinformatics analysis. Shuhong Zhao and Xinyun Li conceived this study. Shuhong Zhao, Xinyun Li, and Weiya Zhang participated in its design and coordination. Xinyun Li, Weiya Zhang, and Saixian Zhang contributed to the analysis and interpretation of the data. Weiya Zhang drafted the manuscript. Shuhong Zhao, Xinyun Li, Yunlong Ma, and Dingxiao Zhang helped to revise the manuscript. All authors read and approved the final manuscript.

Keywords

MeDIP-Seq, DNA Methylation, Wnt, TGFß, satellite cells, Skeletal muscle development

Abstract

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DNA methylation is an important form of epigenetic regulation that can regulate the expression of genes and the development of tissues. Muscle satellite cells play an important role in skeletal muscle development and regeneration. Therefore, the DNA methylation status of genes in satellite cells is important in the regulation of the development of skeletal muscle. This study systematically investigated the changes of genome-wide DNA methylation in satellite cells during skeletal muscle development. According to the MeDIP-Seq data, 52,809-123,317 peaks were obtained for each sample, covering 0.70%-1.79% of the genome. The number of reads and peaks was highest in the intron regions followed by the CDS regions. A total of 96,609 DMRs were identified between any two time points. Among them 6198 DMRs were annotated into the gene promoter regions, corresponding to 4726 DMGs. By combining the MeDIP-Seq and RNA-Seq data, a total of 202 overlap genes were obtained between DMGs and DEGs. GO and Pathway analysis revealed that the overlap genes were mainly involved in 128 biological processes and 23 pathways. Among the biological processes, terms related to regulation of cell proliferation and Wnt signaling pathway were significantly different. Gene-gene interaction analysis showed that Wnt5a, Wnt9a, and Tgfß1 were the key nodes in the network. These results indicated that the Wnt and Tgfß signaling pathways play an important role in functional regulation of satellite cells, and the DNA methylation status of Wnt and Tgfß signals is a key regulatory factor during skeletal muscle development. This study provided new insights into the effects of genome-wide methylation on the function of satellite cells.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

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Abstract

DNA methylation is an important form of epigenetic regulation that can regulate the expression of genes and the development of tissues. Muscle satellite cells play an important role in skeletal muscle development and regeneration. Therefore, the DNA methylation status of genes in satellite cells is important in the regulation of the development of skeletal muscle. This study systematically investigated the changes of genome-wide DNA methylation in satellite cells during skeletal muscle development. According to the MeDIP-Seq data, 52,809-123,317 peaks were obtained for each sample, covering 0.70%-1.79% of the genome. The number of reads and peaks was highest in the intron regions followed by the CDS regions. A total of 96,609 DMRs were identified between any two time points. Among them 6198 DMRs were annotated into the gene promoter regions, corresponding to 4726 DMGs. By combining the MeDIP-Seq and RNA-Seq data, a total of 202 overlap genes were obtained between DMGs and DEGs. GO and Pathway analysis revealed that the overlap genes were mainly involved in 128 biological processes and 23 pathways. Among the biological processes, terms related to regulation of cell proliferation and Wnt signaling pathway were significantly different. Gene-gene interaction analysis showed that *Wnt5a*, *Wnt9a*, and *Tgfβ1* were the key nodes in the network. These results indicated that the Wnt and Tgfβ signaling pathways play an important role in functional regulation of satellite cells, and the DNA methylation status of Wnt and Tgfβ signals is a key regulatory factor during skeletal muscle development. This study provided new insights into the effects of genome-wide methylation on the function of satellite cells.

Key words: MeDIP-Seq, DNA methylation, Wnt, Tgfβ, satellite cells, skeletal muscle development

Introduction

Skeletal muscle satellite cells are a kind of mononuclear cells with a flattened projection, which locate between the muscle fiber basement membrane and the muscle cell membrane in skeletal muscle (*1*). Satellite cells play an important role in skeletal muscle development and regeneration (*2-5*). Satellite cells can undergo myogenic differentiation, thereby providing the nucleus for the attached muscle fibers and participating in the development of muscle fibers (*6*). In adult skeletal muscle, satellite cells can be activated, and then undergo myogenic differentiation to form new muscle fibers or to fuse with myotubes to repair damaged areas, when muscle fibers are damaged (*2, 7*).

Methylation is a common form of epigenetic regulation affecting the expression of related genes and the development of tissue (*8*). The hypermethylation of promoter regions can lead to gene silencing, but the hypomethylated regions are usually an open area (*9, 10*). In recent years, many studies have shown that methylation plays an important role in skeletal muscle development, in which satellite cells are involved.

Histone methylation and DNA methylation are the two most common forms of methylation modification, and these two forms of methylation modification could effectively affect the function of satellite cells in skeletal muscle development (*11*-*13*). Methylated *Pax7* could directly bind to MLL1/2 protein and then recruited histone H3K4 methyltransferase complex to regulate the expression of Myf5 (*14*, *15*). The expression level of *Myogenin* was up-regulated during the differentiation of satellite cells on account of the decrease of the 5'-flanking methylation level (*16*). Methyl-CpG-binding protein, named CIBZ, could affect the methylation status of the promoter-proximal region of *Myogenin* gene and then inhibited its transcription level (*17*). Although many studies have shown that methylation plays an important role in regulating the functions of satellite cells, the systematic exposition of the methylation profile of satellite cells in the process of skeletal muscle development is still very limited.

Our group studied the transcriptome and differentiation characteristics of satellite cells at different developmental stages. We confirmed that the Wnt, Tgfβ, and Fgfr signals were responsible for the attenuation of satellite cell differentiation with development, but the mechanisms of expression change of key factors were unknown (*18*). Hence, in this study, mouse satellite cells from four time points were obtained. Furthermore, MeDIP-Seq analysis was performed to investigate the genome-wide temporal dynamic changes of DNA methylation during skeletal muscle development. This study systematically analyzed the DNA methylation profile of satellite cells in skeletal muscle development, and identified the potential effects of DNA methylation on skeletal muscle development.

Results

Global mapping of DNA methylation of satellite cells with the postnatal development

In our previous study, the skeletal muscle development of mice postnatal can be divided into four stages, namely, Week 2, Week 4&Week 6, Week 8, and Week 10&Week 12 (*18*). Therefore, the leg muscle of four different time points (Week 2, Week 6, Week 8, and Week 12) was obtained to isolate satellite cells. Then the MeDIP-Seq analysis was performed. Firstly, we investigated the global DNA methylation status at different developmental stages. According to our data, 21 million clean reads per sample were obtained through filtered the raw reads. Furthermore, the clean reads were mapped to the mouse reference genome, and the mapping rates ranged from 95.74% to 96.34% with uniquely mapped rates ranging from 36.05% to 45.36% (Table S1). The uniquely mapped reads were considered during further analysis. The global distribution of uniquely mapped reads (chromosome 1-19 and X) was investigated for each sample (Figure S1). Furthermore, the coverage of different methylated models CG, CHG, and CHH were analyzed, demonstrating that the higher coverage area had lower sequencing depth (Figure 1,

Figure S2, S3, S4).

Usually, methylation peak is an important index for the study of differential methylation sites genome-wide. In our study, 52,809-123,317 peaks were obtained for each sample, covering 0.70%-1.79% of the genome (Table S2).To further investigate the genome-wide distribution of methylation data, the proportion of reads and the number of peaks in different genome components was calculated in seven different regions. The result showed that the distribution of reads and peaks in CDS regions was second only to intron regions (Figure 2A, 2B). Furthermore, the specific pattern of gene body was investigated. We divided each gene into 60 equal windows, and the upstream and downstream 2 kb regions were split into 20 non-overlapping windows. The result showed that the reads at the transcription start site obviously decreased (TSS) in all samples but gradually increased in the intragenic region (Figure 2C).

Differential methylation regions and differential methylation gene identification

The methylation data was further explored to compare the differential methylation regions (DMRs) between any two time points (Week 2 versus Week 6, Week 2 versus Week 8, Week 2 versus Week 12, Week 6 versus Week 8, Week 6 versus Week 12, and Week 8 versus Week 12). According to the data, a total of 96,609 DMRs were identified. Among them, 6198 DMRs were annotated into the gene promoter regions (Table S3). In this study, we considered the genes with methylation peaks in the promoter regions as the methylated genes. A total of 4726 differential methylation genes (DMGs) were identified (Table S4), and then clustering analysis was performed. The result showed that the methylation status of genes in satellite cells was a dynamic process with the development of skeletal muscle (Figure S5).

Functional clustering analysis of overlap genes between DMGs and DEGs

Studies showed that the methylation of promoter regions could affect gene expression (*19, 20*). To further understand the effects of methylation on gene function and expression, the differentially expressed genes (DEGs) were compared between any two time points. A total of 1717 DEGs were identified (Table S4). Furthermore, overlap analysis was performed between DEGs and DMGs, and a total of 202 genes were obtained (Figure 3A, Table S4). The result indicated that changes in the expression levels of these genes might be influenced by methylation. Furthermore, the cluster analysis result showed that most genes had a lower methylation level at Week 2, and the methylation level of overlap genes was most significantly different between Week 2 and Week 12 (Figure 3B).

In addition, Gene Ontology (GO) analysis was performed using the DAVID program. The result showed that these overlap genes were mainly involved in 128 biological processes ($p<0.05$). The top 30 GO terms were showed in Figure 4A, including positive regulation of cell migration, positive regulation of smooth muscle

cell proliferation, Wnt signaling pathway, calcium modulating pathway, and regulation of cell proliferation (Figure 4A). To further understand the role of DMGs in the functional regulation of satellite cells, signaling pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes database (KEGG). The result showed that the overlap genes were enriched in 23 signaling pathways ($p<0.05$), some of which were related to muscle development, including Calcium signaling pathway, Wnt signaling pathway, and Insulin secretion (Figure 4B). These results indicated that the DNA methylation status in satellite cells plays an important role in the development of skeletal muscle.

***Wnt5a, Wnt9a,* and *Tgfβ1* were the key nodes in the gene interaction net-work**

To identify the potential key genes that can regulate the function of satellite cells with skeletal muscle development, overlap genes between DMGs and DEGs were selected to study the interaction using String software. A total of 52 genes were considered in the net-work after filtering genes with weak interaction strength (confidence>0.7) (Figure 5). The network revealed that *Wnt5a*, *Wnt9a*, *Tgfβ1*, *Cxcl12*, *Mmp14*, *Cdh5*, and *Egr1* were the core nodes. In addition, *Tgfβ1* had the largest number of interactive genes in the net-work. Moreover, Wnt signaling pathway, including *Wnt5a*, *Wnt9a*, *Fzd2*, and *Ror2* genes, was located in the upstream of the interaction network and could regulate *Mmp4*, *Cdh5*, and *Egr1* indirectly through the *Cxcl12* gene. This result indicated that *Wnt5a*, *Wnt9a*, and *Tgfβ1* were the key nodes in the gene interaction network and that the methylation status of Wnt and Tgfβ signaling pathways may play an important role in functional regulation of satellite cells during skeletal muscle development.

Discussion

Skeletal muscle satellite cells are a class of stem cells located between the myofibril membrane and the myofibril basement membrane, and they can participate in the development and regeneration of muscle fibers (*21*, *22*). In our previous studies, the capacity of the differentiation of satellite cells decreased with age, and Week 4 and Week 6 were two similar stages of development, as well as Week 10 and Week 12. Moreover, we confirmed that many genes and pathways related to growth and development were involved in this process through transcriptome analysis (*18*). Therefore, we isolated skeletal muscle satellite cells from mice of different ages for MeDIP-Seq analysis to study the expression change mechanism of related genes during skeletal muscle development. In this study, only four time points were selected to represent the development of skeletal muscle postnatal for the law of skeletal muscle development shown in our previous article. In addition, from Day1 to Week 2 are the early stages of growth and development after birth. Considering the operability, satellite cells of Week 2 mice were selected as the representative of early stages.

According to the MeDIP-Seq data, 21 million clean reads per sample were

obtained by filtering the raw reads. The rate of uniquely mapped ranged from 36.05% to 45.36% (Table S1). Studies have shown that methylation peak was an important index of the methylation level (*23, 24*). In our study, the genome-wide coverage of peaks was 0.70%-1.79%. Moreover, the coverage of peaks in the CDS regions was second only to that in the intron regions and has a lower coverage ratio in CpG islands. This result indicated that hypermethylated regions may occur more frequently in non-CpG islands in our samples.

Researches showed that DNA methylation in promoter regions could inhibit the gene expression, namely high methylation levels correspond to low expression levels (*25, 26*). In the present study, a total of 96,609 DMRs were annotated into the gene promoter regions, and these corresponded to 4726 DMGs. Moreover, the 202 overlap genes were identified by comparing DMGs and DEGs. Most of the genes were related to satellite cell function and muscle development, including positive regulation of cell migration, positive regulation of smooth muscle cell proliferation, Wnt signaling pathway, calcium modulating pathway, and regulation of cell proliferation. Cluster analysis revealed that many of the overlap genes had a lower methylation levels at Week 2 (Figure 3B). This result indicated that these genes were transcriptionally activated at early stages due to related to the growth and development processes of tissues. Interestingly, the changes of methylation levels of the overlap genes was not linear in these four developmental stages, indicating that these genes may be involved in different biological processes due to the developmental characteristics at the different stages of skeletal muscle development.

Furthermore, signaling pathway analysis was performed. As expected, we found that Wnt signaling pathway was significantly enriched. Researches have shown that Wnt and Tgfβ signaling pathway play important roles in skeletal muscle development regulation (*18, 27-29*). Gene-gene interaction analysis showed that *Wnt5a*, *Wnt9a*, and *Tgfβ1* were the key nodes in the net-work. Previous study showed that WNT5a could promote the proliferation of skeletal muscle satellite cells (*30*). Our previous study has shown that Wnt and Tgfβ signals could regulate the differentiation of satellite cells, and the inhibition of WNT9a, TGFβ2, and TGFβ3 could significantly suppress the differentiation of satellite cells (*18*). In addition, studies showed that TGFβ1 could inhibit the proliferation of myoblasts and the regeneration of myofiber (*31, 32*). Moreover, we also found that *Acvrl1* and *Mstn*, which are members of Tgfβ superfamily (*33, 34*), also occurred in the interaction net-work (Figure 5). Studies showed that MSTN could inhibit the differentiation of myoblasts (*35, 36*). Combining results from our study and those in these researches, we concluded that DNA methylation could affect the function of satellite cells during skeletal muscle development process in mice, and Wnt and Tgfβ signaling pathways may be the key regulators during skeletal muscle development.

In conclusion, the DNA methylation in satellite cells dynamically changed during skeletal muscle development, which may affect the expression of genes and

regulate the function of satellite cells. The DNA methylation status of Wnt and Tgfβ signaling pathways may be the key regulatory factor during skeletal muscle development. This study provides evidence for epigenetic studies of skeletal muscle development. Such information is potentially useful for improving the muscle growth of livestock.

Materials and Methods

Mice. All C57BL/6 mice used in this study were obtained from Hubei Center for Disease Control and Prevention (Wuhan, China). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). The protocols were approved by the Hubei Province Committee on Laboratory Animal Care (HZAUMU2013-0005).

Tissue and Cell. Satellite cells were isolated from the hind-limb muscle tissues at four different time points (postnatal Week 2, Week 6, Week 8, and Week 12) (n=8~12). Satellite cell isolation method referred that described in previous study (*18, 37*).

MeDIP-Seq. DNA was isolated from cultured satellite cells using OMEGA TISSUE DNA Kit（200）. The concentration and quality of DNA were assessed by NanoDrop® ND-1000 and agarose gel electrophoresis. The library was prepared according to MeDIP library development flow. Sequencing was performed by SHANGHAI BIOTECHNOLOGY CORPORATION. The sequencing process included the following. (1) Library construction was performed and the integrity were verified with Agilent 2100 (Agilent technologies, Santa Clara, CA, USA). (2) Cluster generation and primer hybridization were completed on the cBot equipped with the Illumina HiSeq sequencing instrument according to the corresponding process shown in the cBot User Guide. (3) The sequencing process was controlled by data collection software provided by Illumina, and real-time data analysis was carried out.

Analysis of MeDIP-Seq data. Raw sequencing data were first filtered with the following five steps: (a) Reads with > 50% bases having phred quality < 20 were removed; (b) Removal of the bases with phred quality < 20 in 3’ end; (c) Removal of adapter contained in reads; (d) Removal of reads with length < 20; (e)Removal of reads that contain ‘N’ base.

Clean reads were aligned to the Ensembl mouse reference genome (GRCm38, ftp://ftp.ensembl.org/pub/release-94/fasta/mus_musculus/dna/) using BWA (Version 0.7.17) with default parameters (*38*). To investigate the general model of methylation, we analyzed the distribution of aligned reads on the whole reference genome, CGIs, and gene regions, as well as the genome coverage of CG, CHG and CHH regions with different sequencing depths in each sample and group. The peak distribution was analyzed using MACS (version 2.1.1) software in different genome components,

including upstream 2 kb, 5' UTR, CDS, intron, 3' UTR, downstream 2 kb, and CpG islands (*39*).

DMRs and DMGs identify. EdgeR integrated in the R packages MEDIPS with 200 bp window size was used to identify DMRs between different groups (*40*). MEDIPS identified DMRs between groups by calculating Wilcoxon rank tests for the reads per million (rpm) values of each window (cite MEDIPS). DMRs were filtered for windows with $p < 0.01$. Further, ChIPseeker was performed to annotate the DMRs in gene level, and only the genes with promoter annotated by DMRs were defined as differential methylated genes (DMGs) (*41*). All DMGs were subsequently analyzed with GO and KEGG pathway of the DAVID web server (*42*).

RNA-Seq and data analysis. The sequencing process and analysis methods used were in accordance with those used in our previous research (*18*).

Availability of supporting data. The data sets supporting the results of this article were included within the article and additional files.

Acknowledgments

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Conflict of Interest

The authors declare that they have no competing or financial interests.

Author contributions

Weiya Zhang conducted the experiments and prepared the materials involved in this study. Saixian Zhang and Yueyuan Xu performed the bioinformatics analysis. Shuhong Zhao and Xinyun Li conceived this study. Shuhong Zhao, Xinyun Li, and Weiya Zhang participated in its design and coordination. Xinyun Li, Weiya Zhang, and Saixian Zhang contributed to the analysis and interpretation of the data. Weiya Zhang drafted the manuscript. Shuhong Zhao, Xinyun Li, Yunlong Ma, and Dingxiao Zhang helped to revise the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. Genome coverage of the CG, CHG, and CHH sites under different sequencing depths in different samples. The average value of each stage was considered.

Figure 2. Distribution of unique mapped reads and peaks in different genomic regions. (A) The percentage of unique mapped reads in different genomic regions. (B) The number of peaks in different genomic regions. (C) The distribution of reads in 2 kb region upstream of the transcription start site (TSS), the gene body from the TSS to the transcription termination site (TTS), and a 2 kb region downstream of the TTS.

Figure 3. (A) The number of overlap genes between DMGs and DEGs is shown by a Venn diagram. (B) Cluster analysis of overlap genes between DMGs and DEGs according to the methylation levels of genes.

Figure 4. (A) Significantly enriched GO terms (top 30) of the overlap genes. (B) Significantly enriched signaling pathways of the overlap genes.

Figure 5. Analysis of the interaction between overlap genes using String software according to interplay index (confidence>0.7). Interplay index between genes was represented by width and transparency of edges. Dark and wide edge indicated high confidence.

Figure 1.TIF

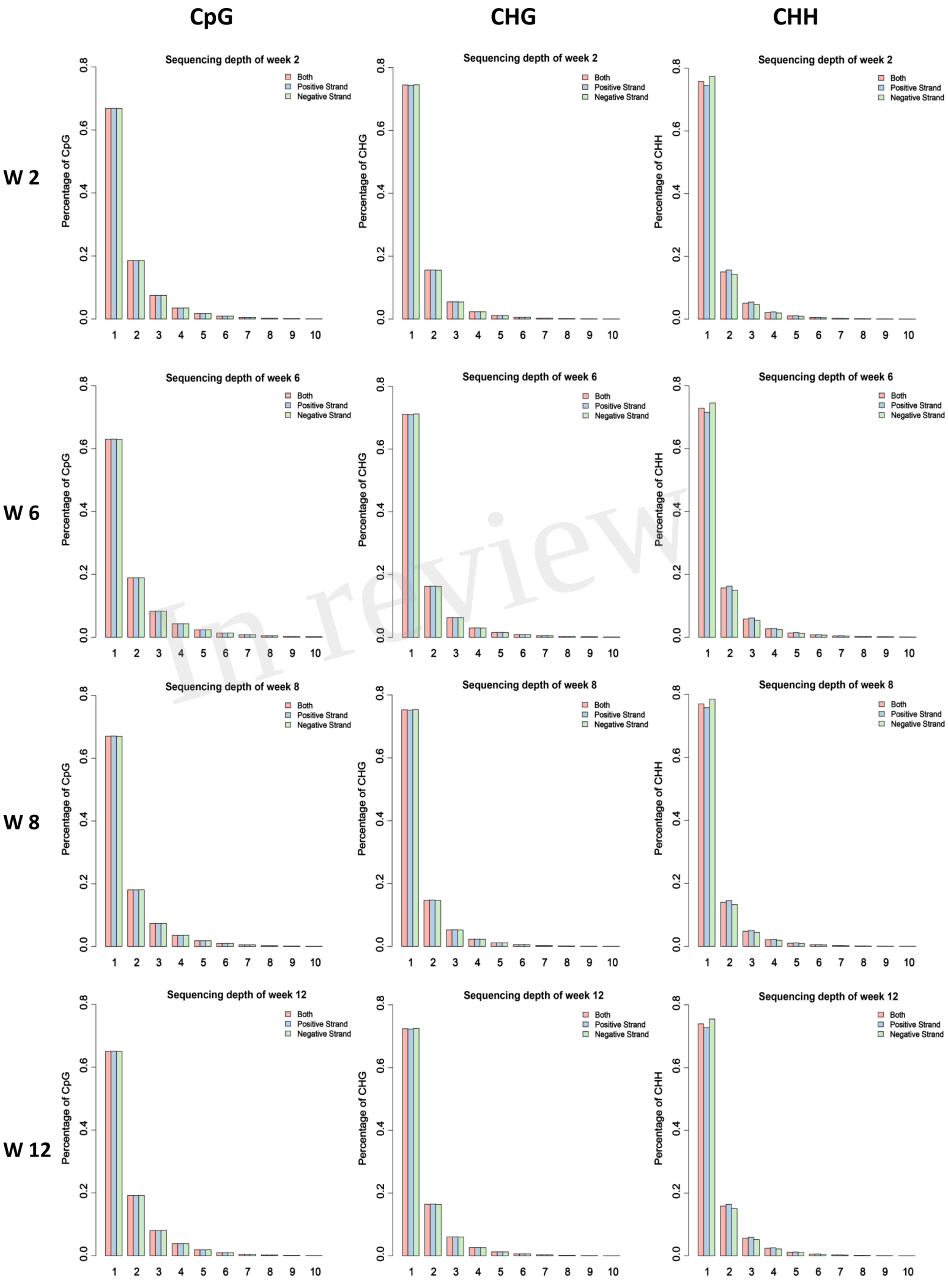


Figure 2.TIF

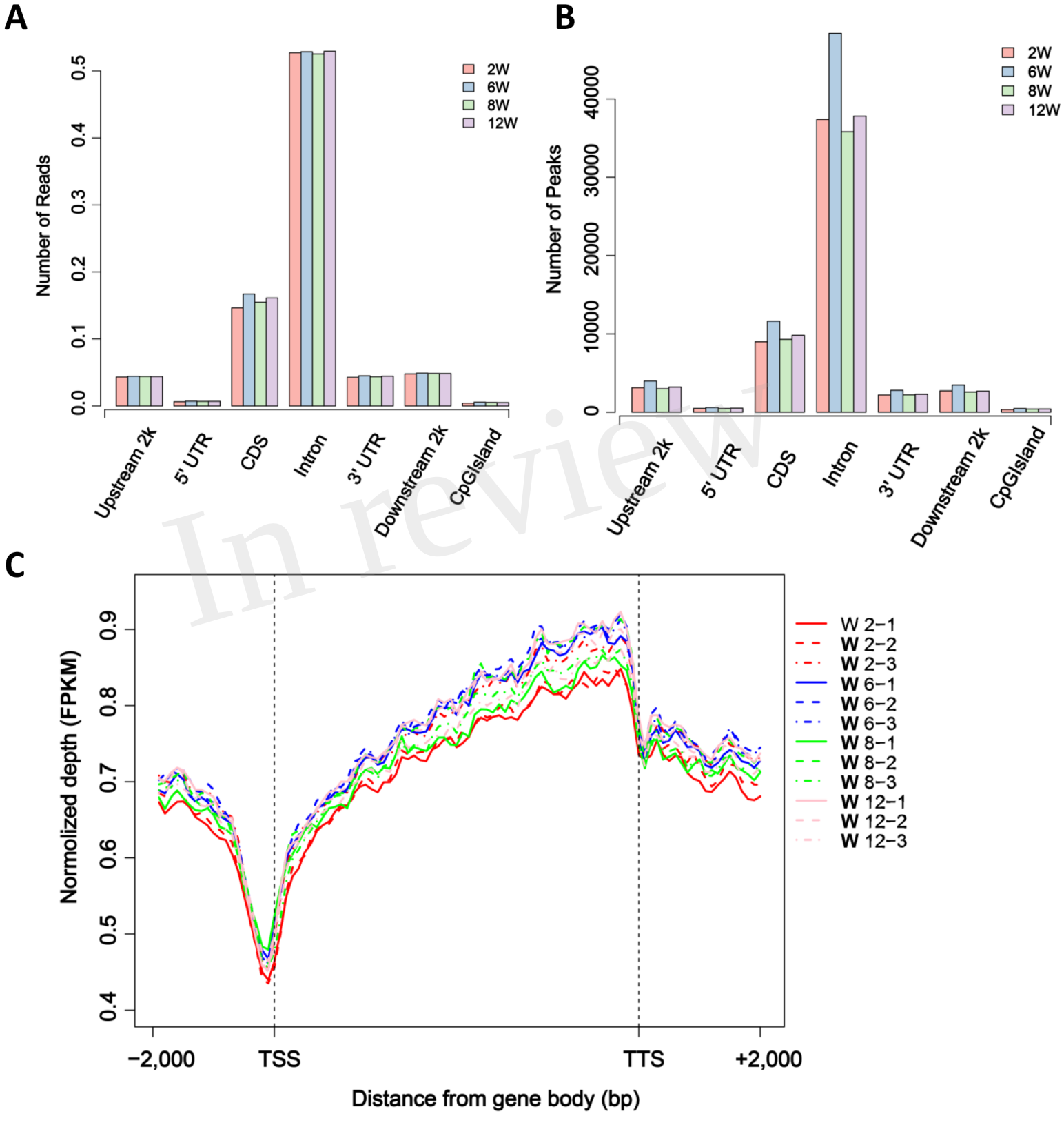


Figure 3.TIF

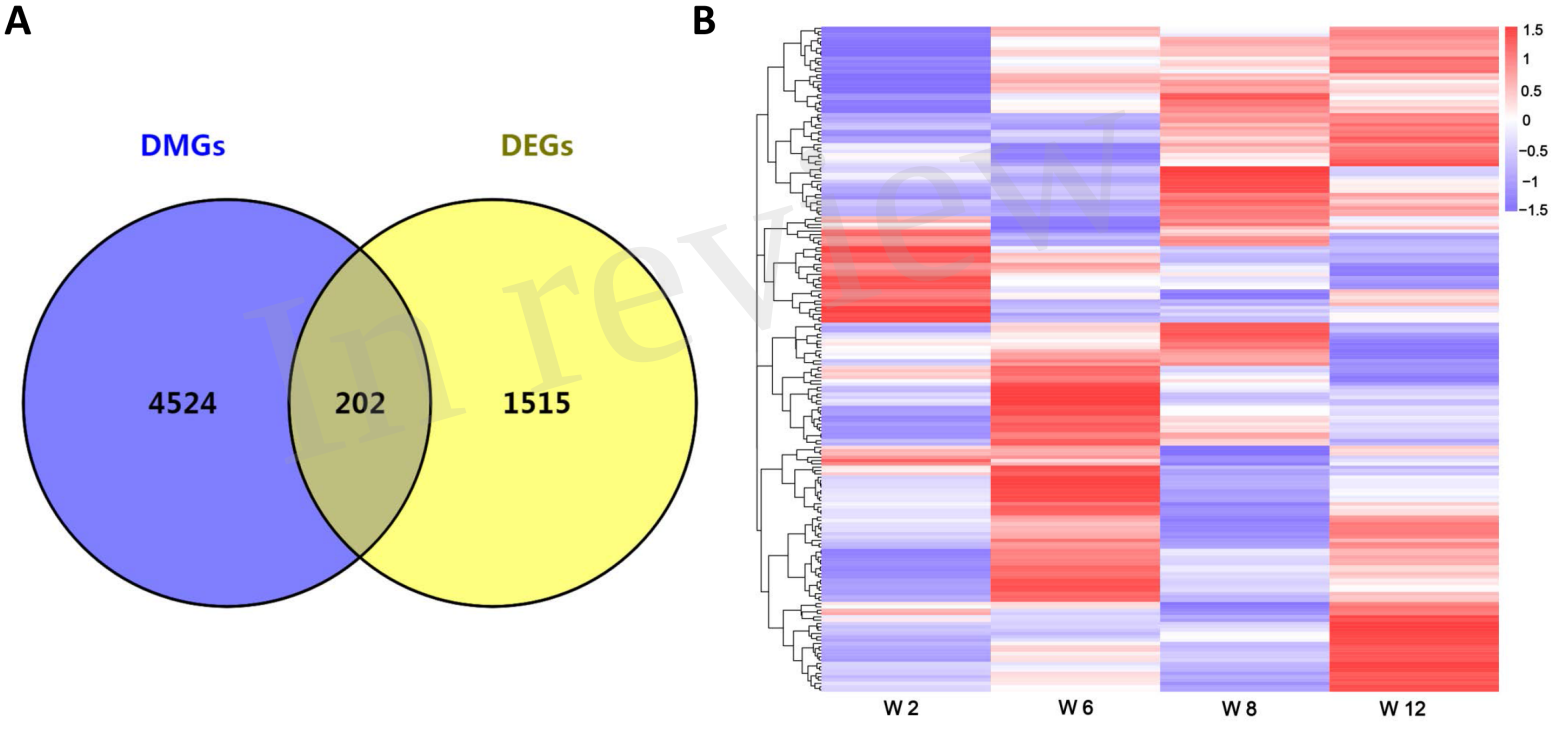


Figure 4.TIF

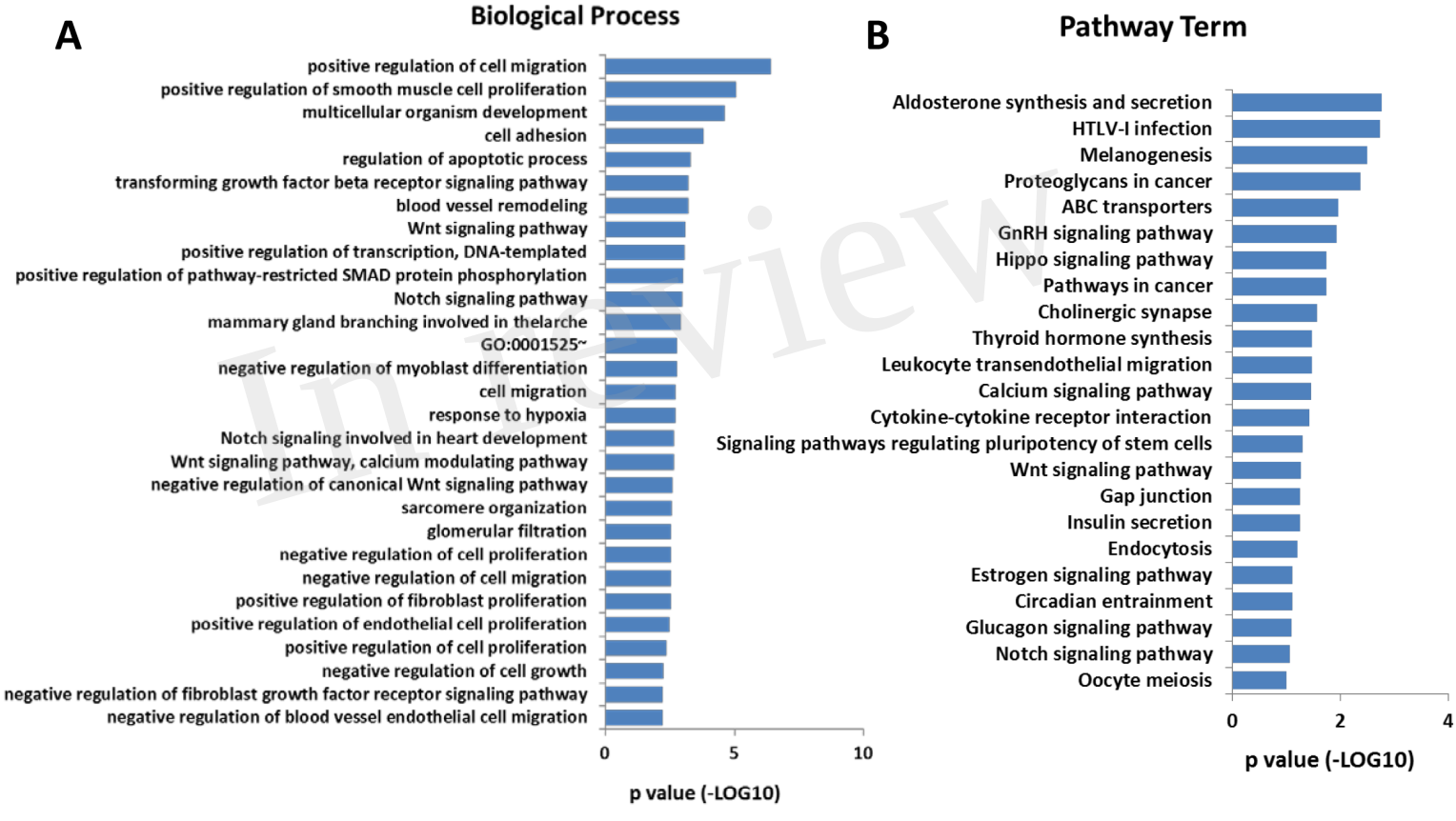
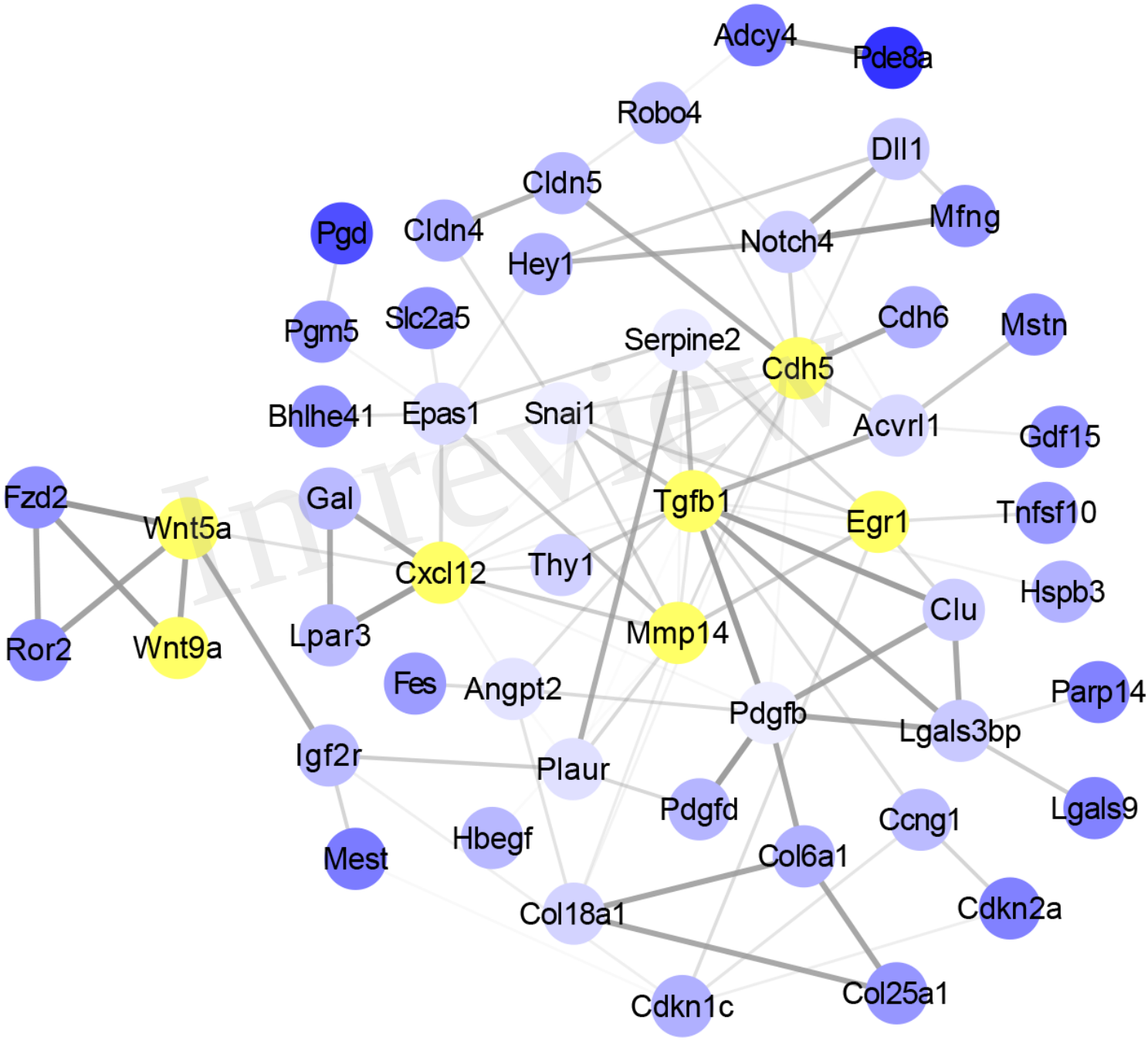


Figure 5.TIF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Pde8a
Robo4
Dll1
Cldn5
Pgd
Cldn4
Mfng
Hey1
Notch4
Pgm5
Slc2a5
Cdh6
Mstn
Serpine2
Cdh5
Bhlhe41
Epas1
Snai1
Acvrl1
Gdf15
Fzd2
Gal
Tgfb1
Tnfsf10
Wnt5a
Egr1
Cxcl12
Thy1
Hspb3
Clu
Ror2
Wnt9a
Lpar3
Mmp14
Fes
Angpt2
Parp14
Pdgfb
Lgals3bp
Igf2r
Plaur
Pdgfd
Ccng1
Lgals9
Hbegf
Mest
Col6a1
Col18a1
Cdkn2a
Cdkn1c
Col25a1