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AFF1-DEPENDENT REGULATION OF GENE EXPRESSION IN EPIDERMAL CELL DIFFERENTIATION

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Abstract: The differentiation of epidermal cells is a complex and tightly regulated process that is crucial for maintaining skin barrier function and homeosta. AFF1/FMR2 family member 1 (AFF1) has been shown to be involved in various cellular processes, but its role in epidermal cell differentiation has not been fully elucidated. In this study, we investigated the role of AFF1 in epidermal cell differentiation by analyzing transcriptome data. We found that AFF1 undergoes dynamic regulation of expression during the differentiation of human keratinocytes. AFF1 knockdown significantly upregulates the expression of key transcription factors such as GRHL3, OVOL1, and PRDM1, thereby promoting epidermal cell differentiation. Our findings provide new insights into the mechanisms by which AFF1 regulates epidermal cell differentiation and highlight its potential as a therapeutic target for skin diseases.
Keywords: AFF1; Transcriptome analysis; Differentiation

1 INTRODUCTION

In living organisms, self-renewal cellular tissues such as the skin epidermis continuously maintain their homeostasis and function through finely regulated cell fate selection processes. This process includes two primary outcomes for progenitor cells one portion proliferates to sustain the population size, while the other undergoes progressive terminal differentiation to perform specific tissue functions. Although this complex regulatory network is crucial for maintaining tissue homeostasis, the gene regulatory mechanisms that control these two different fate choices, especially the early events that initiate terminal differentiation, are still not fully elucidated [1].

The skin epidermis, as a highly accessible self-renewal somatic tissue, is mainly composed of keratinocytes, which occupy 90% of the epidermis. The differentiation of keratinocytes is a tightly regulated process involving multiple signaling pathways， with key roles played by signaling molecules such as calcium and protein kinase C (PKC) in activating this differentiation [1]. Recent studies have further revealed a set of key keratinocyte differentiation activating factors, including transcription factors ZNF750, OVOL1, GRHL3, and PRDM1[2-6]. These factors are significantly upregulated in the early stages of differentiation and subsequently activate a series of target genes related to epidermal barrier function. However, how these differentiation activating factors are upregulated in the early differentiation process and how their inhibitory state in progenitor cells is released remains a current research hotspot.

RNA polymerase II (Pol II) pause release plays an important role in cell fate determination and differentiation as an evolutionarily conserved gene expression regulatory mechanism. In this mechanism, Pol II accumulates and pauses near the promoter of the gene, waiting for specific signaling events to trigger its productive RNA synthesis along the genome. The phenomenon of Pol II pause release has been observed in multiple developmental processes, including early events in embryonic development, hematopoiesis, and progenitor cell fate selection. Although this phenomenon has been confirmed in multiple systems, further in-depth research is needed on the specific molecular events that cause the pause and elongation of Pol II.

CDK9 kinase functions as a crucial regulator of Pol II release pausing and is integral to the extension of gene transcription by forming a complex known as " positive transcription elongation factor b" (PTEFb) with Cyclin T. CDK9 phosphorylates the C-terminal domain (CTD) and negative elongation factor (NELF) of Pol II，helping to release it from the transcription initiation complex and facilitating its transition into the productive stage of RNA synthesis. The kinase activity of CDK9 is tightly controlled to avoid non-specific gene activation. In its inactive state, CDK9 is bound by the 7SK snRNP complex (containing HEXIM1 as a basic component), thereby inhibiting its kinase activity. When cells receive differentiation signals, CDK9 is released from this complex, transforming into its active form and binding to the super elongation complex (SEC)[7] or bromodomain protein 4 (BRD4), thereby promoting gene transcription [1].

In this study, we found that SEC containing AFF1 binds to inactive CDK9 containing HEXIM1, directly inhibiting a set of rapid response genes in keratinocytes in the progenitor cell state. These SEC controlled rapid response genes exhibit a highly enriched and paused state of Pol II in the progenitor cell state, while displaying strong Pol II extensibility during differentiation. By using SEC peptide inhibitors such as KL1 or KL2, we observed that these rapid response genes were rapidly suppressed and revealed ATF3 as a key trigger factor in the differentiation process. The upregulation of ATF3 is sufficient to promote the expression of various differentiation driven transcription factors，including ZNF750，OVOL1, GRHL3, and PRDM1.

2 METHODS

This study adopted sequencing data from samples GSM5543704, GSM5543705, GSM5543506, and GSM5543707 in the NCBI GEO dataset series GSE182959. To induce differentiation, keratinocytes were seeded in confluence and cultured with the addition of 1.2mM of calcium to growth medium. 10 uM of siRNA for AFF1 were nucleofected. Each experimental condition was replicated twice.

To ensure the accuracy of the data, we have adopted FastQC (version 0.12.1) and Cutadapt (version 2.6) to conduct quality control evaluations on the raw read data, aiming to eliminate low-quality read data and adapters. Afterwards, we used STAR (version 2.7.11b) to map the cleaned read data to the human reference genome (based on GENCODE Release 21, version GRCh38). Next, Samtools (version 1.6) was used to filter and sort the uniquely mapped read data. To evaluate the correlation distance between samples, we used principal component analysis (PCA) method. For the differentially expressed genes (DEGs) between the control (NC) and AFF1 knockdown (AFF1-KD), we identified them using the DESeq2 package in R language. The screening criteria were set as an adjusted p-value (padj) less than 0.05 and an absolute log2 fold change value of 0.58 or more. In order to gain a deeper understanding of key biological functions, we use the R package clusterProfiler to perform ontological (GO) and genomic (KEGG) enrichment analysis on genes.

3 RESULTS

3.1 The Knockdown of AFF1

AFF1 is a key element within the SEC complex， which is essential for regulating transcriptional elongation. Transcription extension is an important step in gene expression, which involves the synthesis of mRNA by RNA polymerase II (Pol II) on DNA templates [8][9]. Knockdown of AFF1 may affect the function of SEC, thereby interfering with the transcription extension process and causing changes in the expression levels of specific genes. These genes may include genes related to epidermal cell differentiation. The differentiation of epidermal cells is a complex process involving the synergistic action of multiple signaling pathways and transcription factors. These transcription factors guide the gradual differentiation of epidermal cells from the basal cell layer to the outer layer of the epidermis by regulating the expression of specific genes. If knocking down AFF1 can affect the expression or activity of these transcription factors, then it may indirectly affect the differentiation of epidermal cells [10].

To determine the full spectrum of gene expression influenced by AFF1 knockdown, we performed RNA-seq data analyses. First, we performed principal component analysis (PCA) on the two replicates of both the control (NC) and AFF1 knockdown (KD) groups to assess the consistency and quality of the RNA-seq data. The PCA plot clearly demonstrates the distinct clustering of the NC and KD groups, indicating a high degree of reproducibility between the replicates within each group. The first principal component (PC1) explains 46.7% of the variance and effectively separates the NC and KD samples, highlighting the significant impact of HEXIM1 knockdown on the global gene expression profile of the keratinocytes. The second principal component (PC2) accounts for 28.3% of the variance, further supporting the robustness of the data and providing additional insight into the variability within the samples [11] (**Figure 1**).

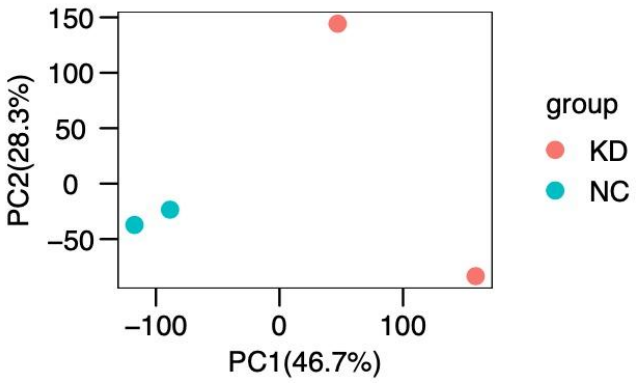

Figure 1 Analysis of Pricipal Componnent Anlysis (PCA)

Following the PCA, we conducted differential expression analysis to identify genes that were significantly upregulated or downregulated in the AFF1 knockdown (KD) group compared to the control (NC) group. Using the DESeq2 R package (version 1.44.0), we identified a total of 1967 differentially expressed genes (DEGs) with an adjusted p-value (padj) < 0.05 and a log2 fold change (FC) of 0.58 or higher (**Figure 2**). Specifically, 1046 genes were upregulated, and 921 genes were downregulated in the KD group. Among the upregulated genes, several key differentiation-activating transcription factors were identified, including GRHL3, OVOL1 and PRDM1[12]. These transcription factors are known to play critical roles in the regulation of keratinocyte differentiation and epidermal development. The upregulation of these genes suggests that AFF1 knockdown may promote the differentiation of keratinocytes by activating these transcription factors.

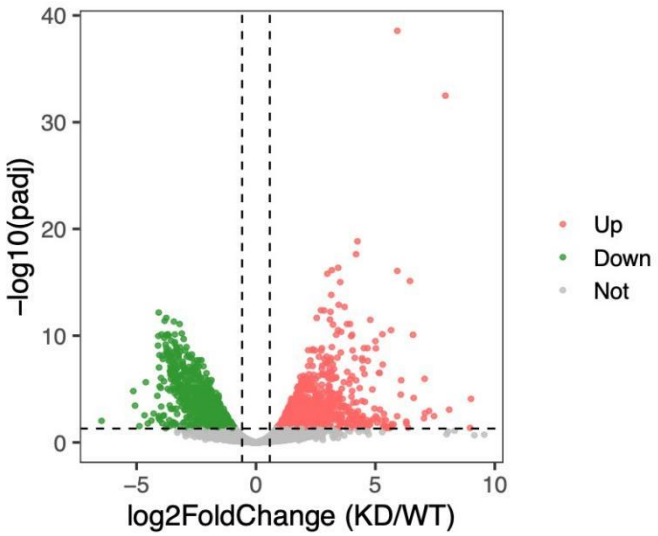

Figure 2 Volcano Plot Show the Upregulated and Downregulated Genes in the NC and AFF1 Knockdown Groups from RNA-Seq Data

3.2 The effect of AFF1 on Epidermal Cell Differentiation

We demonstrated the statistical significance levels of different types of DNA replication processes after p-adjustment. These processes include DNA replication, DNA template replication, chromosome segregation, nuclear division, mitochondrial region, plasma membrane region related DNA replication activities, as well as condensed chromosomes, central regions, nuclear chromosomes, extracellular replication, and ATP dependent activity to investigate the effects of AFF1 knockdown on BP, MF, and CC (**Figure 3**).

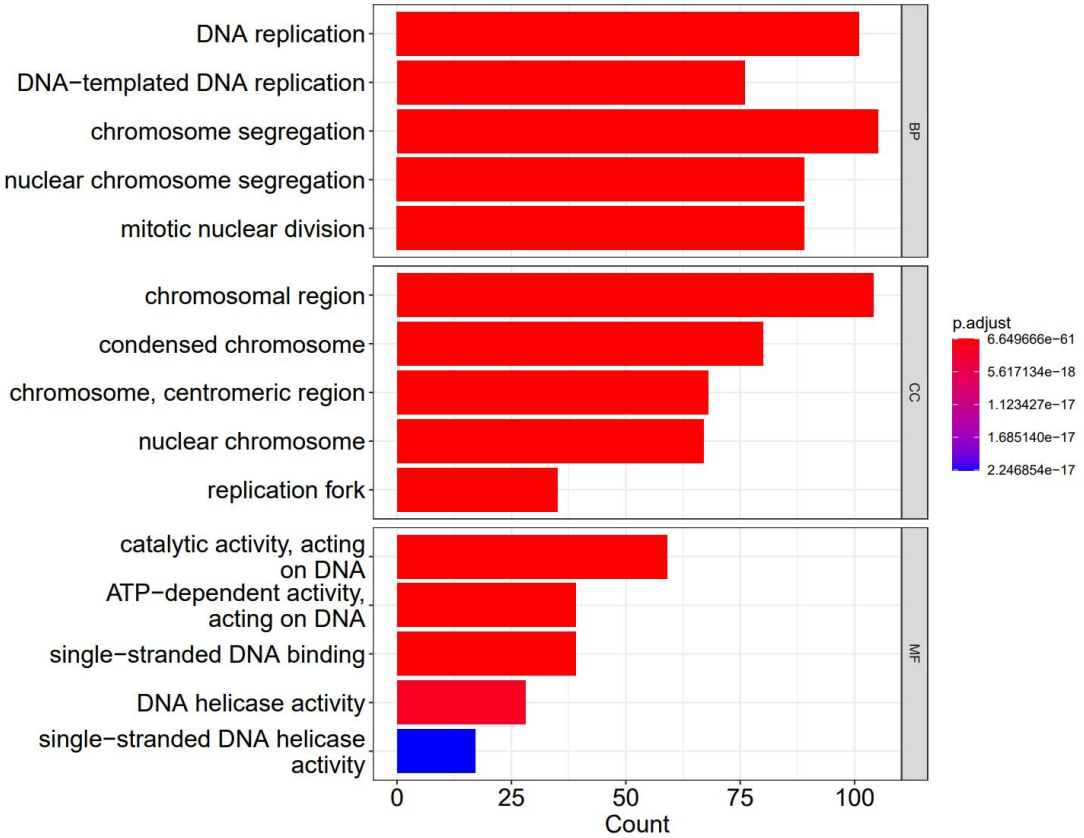

Figure 3 Top GO Terms of Downregulated Genes in AFF1 Knockdown RNA-seq

Through Figure2, we found that certain processes such as DNA replication and DNA templated, as well as DNA replication, have extremely high significance, with p-values reaching extremely low 6.649666e-61 and 5.617134e-18, respectively. This indicates that these processes are very significant and critical in our study. Meanwhile， the segregation of chromosomes，mitotic nuclear division，and related activities in specific chromosomal regions such as condensed chromosomes and centromeric regions exhibited significant statistical significance，with adjusted p-values of

1.123427e-17 and 1.685140e-17. Furthermore, we noted that the activities of replication fork and ATP dependence are statistically significant， providing further evidence of AFF1's crucial role in regulating epidermal cell differentiation [13].

3.3 The Correlation between Different Biological Processes

We examined the link between biological processes such as the cell cycle， DNA replication， genome repair(including the FANCI pathway，base excision repair，homologous recombination，nucleotide excision repair，and mismatch repair)，and the allocation of a carbon pool to chloroplasts and the gene ratio (GeneRatio). The results showed that the cell cycle and DNA replication processes occupy significant positions in the graph, indicating their core roles in cell growth and division. At the same time, the genome repair process has also shown high importance, although its GeneRatio value is relatively low, it is crucial in maintaining genome stability. In addition, the allocation of a carbon pool to chloroplasts appears at a lower GeneRatio value in the figure, indicating its specific role in a particular metabolic pathway (**Figure 4**). These findings provide valuable references for understanding the mechanism by which AFF1 inhibits epidermal cell differentiation [11].

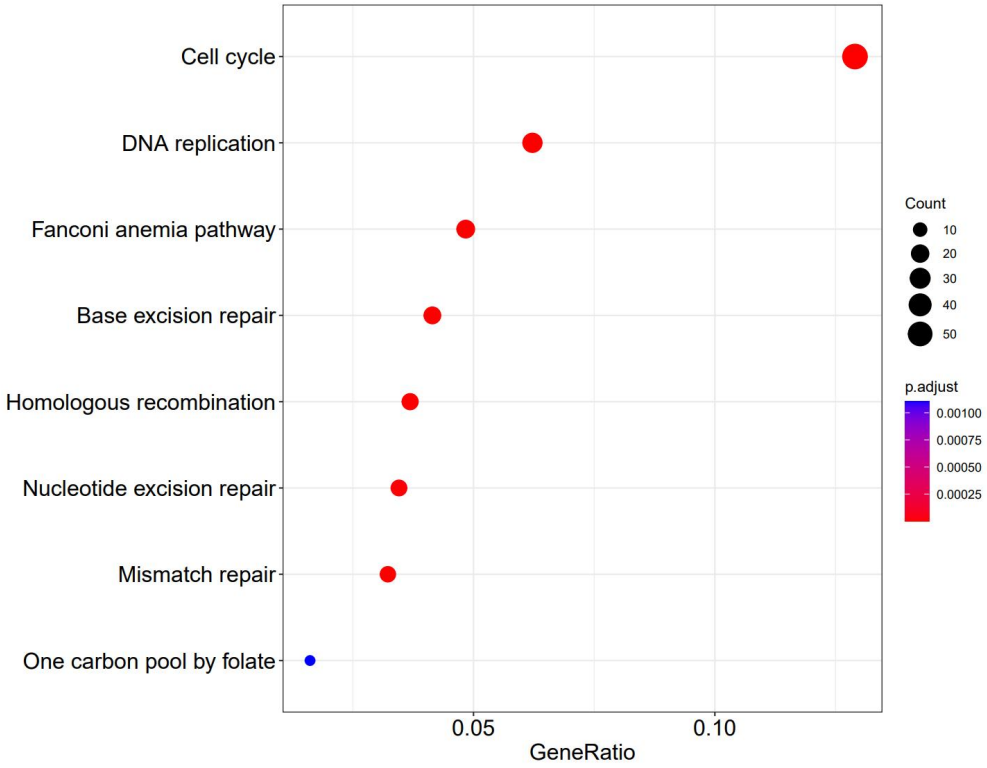

Figure 4 Top KEGG Terms of Downregulated Genes in AFF1 Knockdown RNA-seq

In conclusion，this study highlights the varying significance of distinct biological processes in cellular function. The cell cycle and DNA replication processes occupy a central position, while the genome repair process serves as a key backup mechanism, playing an important role in maintaining genome stability. In addition, the allocation of a carbon pool to chloroplasts provides us with new insights into intracellular metabolic balance. These findings provide valuable references for further exploring cellular biology mechanisms and optimizing biotechnology applications [14].

We analyzed the relationship between gene expression levels and adjusted p-values (p.adjust) in different KEGG terms. By visualizing data on a range of genomics topics and biological processes， we found that gene expression levels cover a wide range from low to high，indicating differences in the activity levels of different genes or processes in cells. Some genes or processes，like the Hippo signaling pathway，Mitophagy animal and TNF signaling pathway，exhibit p-values of less than 0.01， suggesting that these genes or processes have demonstrated significant statistical importance in the experiment (**Figure 5**).

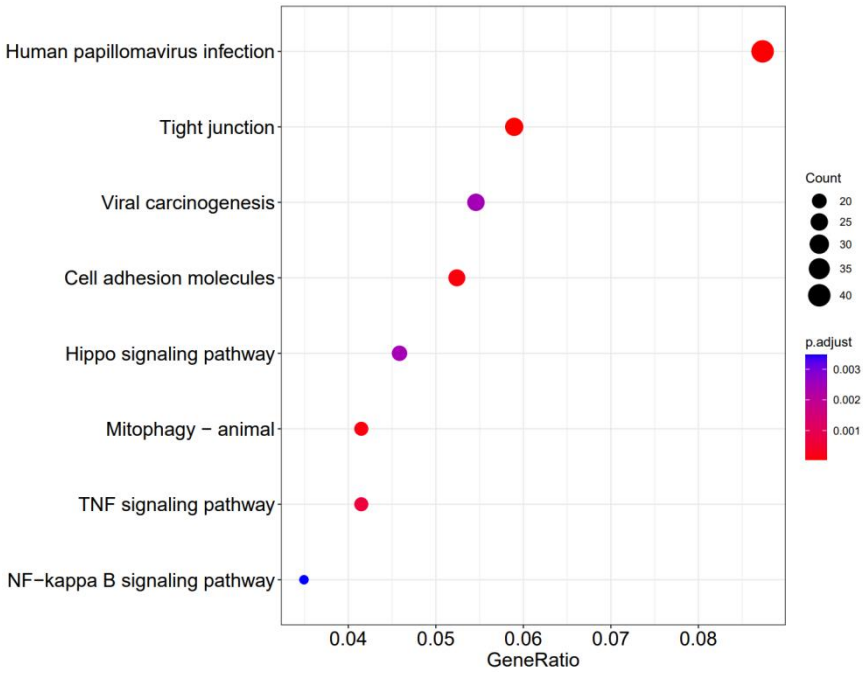

Figure 5 Top KEGG Terms of Upregulated Genes in AFF1 Knockdown RNA-seq

Specifically, genes or processes related to human papillomavirus infection, tight junctions, and cell adhesion molecules are widely distributed in the figure, demonstrating their diversity and complexity in cell biology. Although genes or processes related to viral carcinogenesis are not directly labeled in the figure, given their association with other genomic topics, it can be inferred that they may have important effects on cell transformation and tumorigenesis under certain conditions. The Hippo signaling pathway stands out with extremely low p-values and high gene expression levels, indicating that this signaling pathway may play a key role in regulating cell proliferation, apoptosis, and organ size. Mitophagy (Mitophagy animal) in mammalian autophagy also showed high statistical significance, suggesting its importance in cellular autophagy and mitochondrial quality control. The TNF signaling pathway and NF kappa B signaling pathway，essential inflammatory and stress response pathways，show moderate to low p-values and extensive gene expression in the graph， suggesting their significant involvement across various physiological and pathological conditions [15].

3.4 Genes Controlled by AFF1 that Affect Epidermal Cell Differentiation

Just as Table 1, we found that knocking down AFF1 has an impact on the expression of some other genes that affect epidermal cell differentiation, such as GRHL3 is a transcription factor that plays a critical regulatory role in the differentiation process of epidermal cells and regulates the expression of various genes related to epidermal cell differentiation, thereby affecting the fate selection of epidermal cells. During the normal differentiation process of the epidermis, basal layer cells gradually mature from their previous undifferentiated state. During this process, GRHL3 can promote cell migration and contribute to the formation and remodeling of the epidermal layer [16]. PRDM1, as a transcription factor, has the ability to regulate gene expression. It contains a PR domain and five C2H2 zinc finger domains, which can bind to DNA and regulate the expression of target genes [17]. Also, TP63 promotes normal differentiation of epidermal cells by regulating gene expression related to epidermal cell differentiation. These genes may include keratin, intercellular adhesion molecules, etc, which play a key role in the differentiation process of epidermal cells. In addition, keratinocytes are one of the main cell types in the epidermis, and TP63 can regulate their proliferation and differentiation abilities, thereby maintaining the normal structure and function of the epidermal layer [18].

Table 1 Expression Changes of Differentiation-Activating TFs upon AFF1 Knockdown

Gene Name	Gene Id	log2FoldChange	pvalue	padj	baseMean
GRHL3	ENSG00000158055.13	2.307718051	9.69E-05	0.001738396	807.1217575
PRDM1	ENSG00000057657.12	2.92814099	4.88E-09	4.02E-07	1316.947795
TP63	ENSG00000073282.10	-1.364430932	0.000414447	0.005828631	2261.068155

4 DISCUSSION

Our study reveals the key role of AFF1 in regulating the biphasic determination between self-renewal and differentiation of progenitor cells in human skin epidermal tissue. We found that knocking down AFF1 has an impact on

the expression of some other genes such as GRHL3, OVOL1 and PRDM1， which affect epidermal cell differentiation, thereby promoting terminal differentiation. The significant changes observed during DNA replication and cell cycle processes after AFF1 knockout highlight the critical role of this complex in cellular function and differentiation.

Furthermore， examination of gene expression levels and adjusted p-values across different genomic studies highlighted the significance of pathways like the Hippo signaling pathway，mitochondrial phagocytosis，and TNF signaling pathway in orchestrating cellular processes. These findings provide valuable insights into the complex interactions between signaling pathways and transcription factors in controlling cell fate decisions.

In conclusion，our research has made important advancements in comprehending the molecular mechanisms that govern the fate selection and differentiation of progenitor cells. Future research should aim to further clarify the detailed molecular events to explore the potential therapeutic significance of regulating this process in skin and other self-renew tissues.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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