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ORIGINAL RESEARCH

Murine Model Insights: Identifying Dusp15 as a Novel Biomarker for Diabetic Cardiomyopathy Uncovered Through Integrated Omics Analysis and Experimental Validation

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Background: Diabetic Cardiomyopathy (DCM) is a heart condition that arises specifically from diabetes mellitus, characterized by cardiac dysfunction in the absence of coronary artery disease or hypertension. The prevalence of DCM is rising in tandem with the global increase in diabetes, necessitating the development of early diagnostic markers and therapeutic targets. This study integrates bioinformatics analysis with experimental validation to identify potential biomarkers for DCM.

Methods: We performed gene expression data mining from the Gene Expression Omnibus (GEO) database. We employed Weighted Gene Co-expression Network Analysis (WGCNA) coupled with machine learning techniques to sift through hub differentially expressed genes (DEGs). Functional enrichment and protein-protein interaction (PPI) network analysis were also conducted to pinpoint key genes functions. Subsequent in vitro and in vivo experiments were performed to validate the findings.

Results: Our analysis revealed six core genes significantly associated with DCM. The expression of Dusp15 was notably down-regulated and validated in both high-glucose cultured cardiomyocytes and DCM animal models, suggesting its potential role in DCM pathogenesis.

Conclusion: The integration of bioinformatics with experimental approaches has identified Dusp15 as a promising candidate for a DCM biomarker, offering valuable insights for early diagnosis and potential therapeutic development.

Keywords: diabetic cardiomyopathy, biomarker, WGCNA, machine learning, Dusp15

Introduction

Diabetic Cardiomyopathy (DCM) is a distinct form of cardiac disease intricately linked to the chronic sequelae of diabetes mellitus (DM). It is characterized by alterations in cardiac structure and function in the absence of other identifiable cardiac pathologies.[1] The incidence of DCM is rising in line with diabetes, highlighting the need for enhanced awareness, earlier detection, and more comprehensive management strategies to reduce the impact of diabetes on cardiac health.[2]

Early diagnosis of DCM remains a significant challenge, as its subtle clinical presentation that often resemble other cardiac disorders. The definitive diagnostic procedure for DCM, endomyocardial biopsy, requires an exceptionally high level of technical precision and is usually accompanied by considerable, even often unpredictable risks and complications.[3] Therefore, developing bio-diagnostic markers for DCM is an essential research priority.

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The evolution of next-generation sequencing has demystified the genetic underpinnings of clinical diseases, providing novel perspectives on their etiological mechanisms.[4,5] Bioinformatics plays a pivotal role in dissecting the gene expression profiles within tissues or cells sourced from patients and experimental models, thereby pinpointing genetic targets crucial for disease diagnosis and therapeutic intervention.[6] Weighted Gene Co-expression Network Analysis (WGCNA) technique stands as a key technique in biology, delineating intricate patterns of gene associations across diverse samples. It adeptly uncovers gene modules with significant synergistic changes, and identifying potential biomarkers or therapeutic targets by examining their interplays with phenotypic manifestations.[7] Machine Learning (ML) has become an indispensable tool in data analytics and clinical diagnostics, with algorithms like the Least Absolute Shrinkage and Selection Operator (LASSO) and Support Vector Machine Recursive Feature Elimination (SVM-RFE) enhancing model performance by selecting the most relevant features.[8]

This study applied a comprehensive bioinformatics approach to scrutinize the differential gene expression patterns in DCM animal models, as recorded in the Gene Expression Omnibus (GEO) database. By integrating WGCNA with machine learning algorithms, we aimed to identify biological diagnostic markers for DCM and validate these findings using cellular and animal models.

Methods and Materials

Data Mining and Screening of Differentially Expressed Genes (DEGs) of DCM

The schematic representation of the workflow is outlined in Figure 1. The GSE5606 dataset from the GEO database provides comprehensive gene expression profiles from 14 left ventricular cardiac tissues of Wistar rats, which were equally distributed between the control cohort and the DCM group induced by streptozotocin (STZ, Sigma-Aldrich, USA).[9] Upon acquisition of the raw data, a thorough normalization process was conducted. Subsequently, the identification of DEGs was screened employing the "limma" package in R software. The stringent criteria for DEGs were set at log2 fold change (log_2FC) > 1 and a false detection rate (FDR) of 0.05. Then, the visually informative volcano plots and heatmaps was conducted with "ggplot2" package and "heatmap" package, respectively.

Functional Enrichment Analysis and Protein–Protein Interaction (PPI) Network Construction

The R package "clusterProfiler" was utilized to perform the functional enrichment analysis on DEGs in DCM. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used to explore potential biological processes, cellular components, molecular functions, and important signaling pathways. $P < 0.05$ and $FDR < 0.1$ were considered statistically significant. Subsequently, the interrelationships among DEGs in DCM were analyzed using the STRING database.[10] Cytoscape software (version 3.10.2) was employed for the construction and visualization of PPI networks.[11] Hub genes were identified using the plug-in Cytohubba based on the gene's Mean Connectivity Centrality (MCC) in the PPI network.

WGCNA

The R package "WGCNA" was employed to construct a gene co-expression network aimed at elucidating modules highly correlated with diabetic cardiomyopathy. The "PickSoftThreshold" function was used to select and confirm an optimal soft threshold. An adjacency matrix was then constructed and transformed into a Topological Overlap Matrix (TOM). A hierarchical cluster dendrogram was generated to classify genes with similar expression profiles into gene modules. The correlation coefficient between modules and phenotypes was calculated to identify modules closely associated with DCM. Genes within the most significant modules were further analyzed, based on criteria where the absolute value of Gene Significance (GS) exceeded 0.20 and Module Membership (MM) was greater than 0.80.

Machine Learning

Herein, LASSO regression and SVM-RFE were applied to pinpoint hub genes from a pool of candidate DEGs by utilizing the "glmnet" and "e1071" R packages. LASSO, known for its ability to select variables and regularize models, was tuned with a penalty parameter (λ) to handle high-dimensional data effectively. The optimal λ was determined using

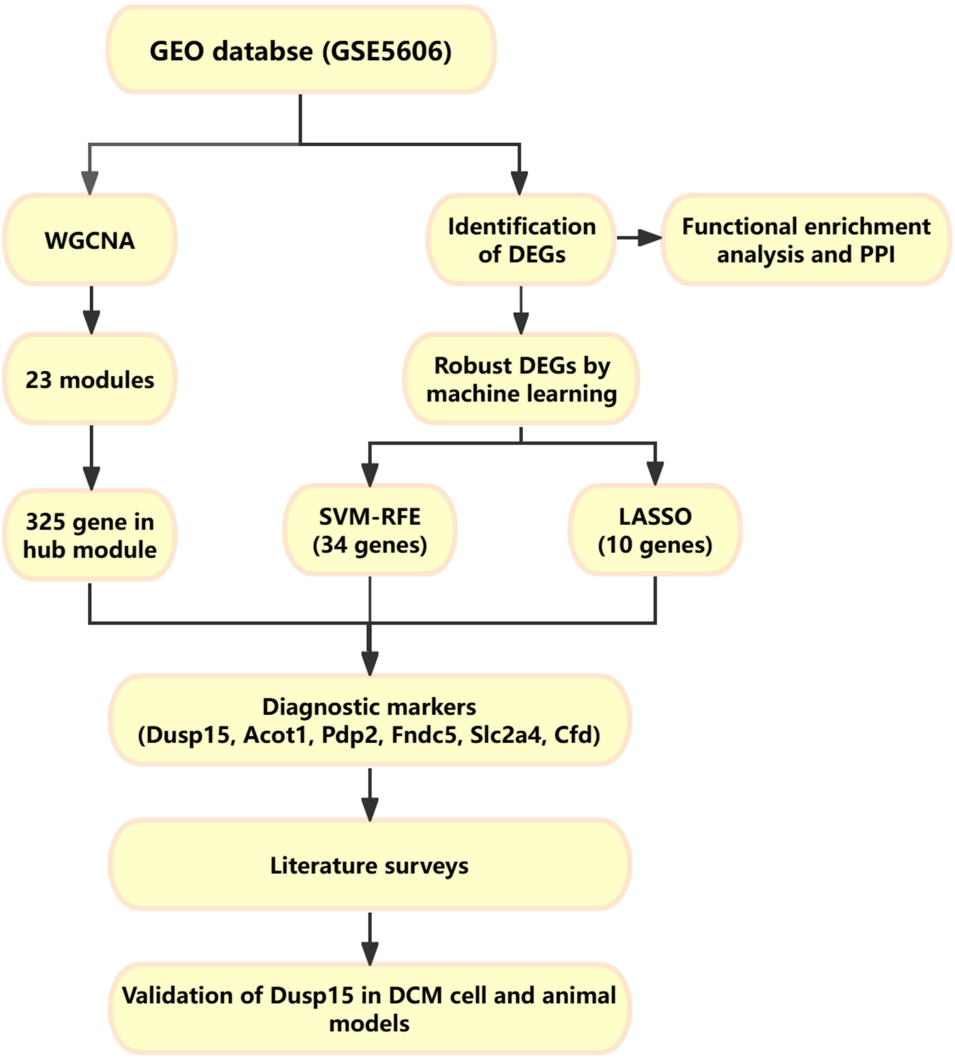

Figure 1 Workflow Diagram.

cross-validation to minimize the error and retain the most relevant genes. SVM-RFE was also adopted to classify the candidate biomarkers of DCM, with k = 10 set for k-fold cross-validation.

Identification of Hub Genes of DCM

To find the key DCM biomarkers, we used an online Venn diagram tool (bioinformatics.psb.ugent.be/webtools/Venn/) to overlap the genes identified by WGCNA analysis, LASSO and SVM-RFE machine learning algorithms based on the candidate DEGs.

Cell Culture

HL-1 murine cardiomyocytes, procured from the Chinese Academy of Sciences' Cell Bank, were cultured in MEM medium containing 5.5 mmol/l glucose, supplemented with 10% FBS and 1% penicillin/streptomycin, at 37°C under 5% CO2. The DCM cellular model was induced in HL-1 cells by exposure to medium with a high glucose concentration of 33 mmol/l for 24 hours.

Animals

The present study was approved by the Medical Animal Care and Welfare Committee of Tianjin Medical University Chu Hsien-I Memorial Hospital (DXBYY-IACUC-2023021). All procedures conformed to the Institutional Animal Care and Use Committee (IACUC) guidelines. Twenty male 6-week-old C57BL/6J mice weighted 22–25g were obtained from GemPharmatech Company

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(Nanjing, China) and housed in a specific pathogen-free (SPF) setting. All animals were maintained under controlled conditions with a constant temperature of 20 ± 2°C and humidity of 45–55%, on a 12:12-hour light-dark cycle, with access to standard diet and water ad libitum. Following a 1-week adaptive feeding period, mice were randomized into DCM (n=10) and control (n=10) groups. The DCM group animals received intraperitoneal injections of 50 mg/kg STZ after 16-h fasting for 5 consecutive days. Control group animals were administered an equivalent volume of citrate buffer (pH 4.2–4.5). Fasting blood glucose levels were measured once every two weeks following 6 h of fasting via the tail vein using an Accu-Chek Go glucometer (Roche Diagnostic, Mannheim, Germany) starting on Day 14 to confirm the reliability of diabetic model. Mice with fasting glucose ≥16.7 mmol/L at 72 h and 7 days post-STZ injection were classified as diabetic mice. All mice were sacrificed at 12 weeks, and the hearts were harvested, partial heart tissue was placed in formaldehyde fixative, while the remaining tissue was rapidly frozen in a liquid nitrogen bucket and stored in a −80 °C freezer for subsequent experiments. The DCM mice were identified according to the extent of cardiac histopathological pathological damage.

Hematoxylin and Eosin (H&E) and Masson Staining

The heart tissues of the mice were fixed in 4% paraformaldehyde and embedded in paraffin to generate 5μm serial sections. These sections were deparaffinized, and subjected to hematoxylin and eosin staining. For the assessment of collagen deposition in cardiac tissues, paraffin sections were stained with Masson trichrome, followed by counterstaining with Mayers Hematoxylin. Anonymized samples were carefully examined, and the pathological characteristics of the tissues were evaluated via light microscopy.

RT-qPCR

The total RNA was isolated from HL-1 cells and animal heart tissues with TRIzol (Solarbio, China) and subjected to by RT-qPCR analysis on QuantStudio 6 Flex instrument (Applied Biosystems, USA), following the manufacturer's protocol. Primers for Dusp15 and GAPDH were as follows: forward 5′-CCTGGACTCTACCTTGGAAAC-3′, reverse 5′-GTGGATAAAGT GGACGCATTC-3′; GAPDH: forward 5′- TCTCCTGCGACTTCAACA-3′, reverse 5′- TGTAGCCGTATTCATTGTCA-3′.

Western Blot

Proteins from animal heart tissues and cells were extracted by RIPA buffer (Solarbio, Beijing, China) supplemented with 1% protease inhibitors and phosphatase inhibitors (Solarbio, Beijing, China). Equivalent protein samples were loaded on a 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, USA). Following a 1-hour blocking step, the membranes were probed with anti-Dusp15 (1:500, Cat. #A16322, Abcolonal) or anti-tubulin (1:1000, Cat. #2146s, Cell Signaling Technology, USA) overnight at 4 °C. Then, the membranes were washed three times with TBST and incubated with a secondary antibody for 1 hours at room temperature. ECL kit (Advansta, USA) was used to visualize the bands and ImageJ software was performed to quantify the band intensity.

Statistical Data Analysis

The raw data was sourced from the GEO database and analyzed with R software (version 4.1.1). Experimental results are expressed as mean ± SD. GraphPad Prism (version 9.0) was utilized for statistical processing, employing Student's *t*-test for comparisons. Statistical significance was set at the 95% confidence level ($P < 0.05$).

Results

Screening of Differential Genes Related to DCM

The GSE5606 dataset revealed extensive gene expression alterations in the left ventricle of rodent models with diabetic cardiomyopathy. A total of 104 DEGs were identified in myocardial tissue samples from DCM animals compared to the control group, with 56 genes upregulated and 48 downregulated (adjusted P value < 0.05). A volcano plot (Figure 2A) graphically represents the gene expression distribution, where red and blue spots denote significantly upregulated and downregulated genes, respectively ($|\log_2 FC|>1$). DEGs with $|\log_2 FC|>2$ were labeled with gene names. The heatmap in Figure 2B illustrates the top 50 DEGs, providing a visual summary of their expression patterns.

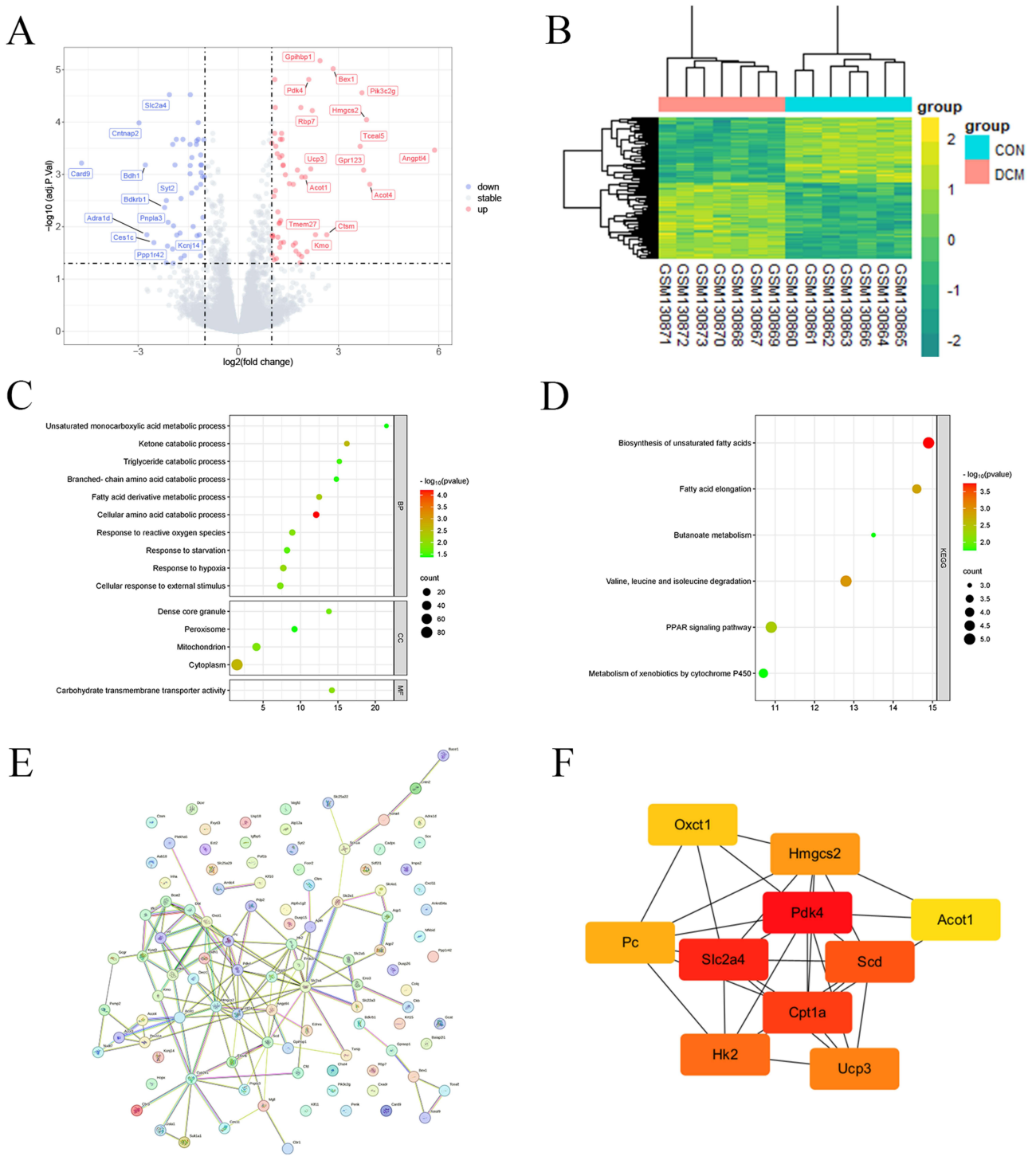

Figure 2 Identification of DEGs and functional analysis. (**A**) volcano plot that represents the distribution of gene expression changes between DCM and control groups. Genes with |log2FC| greater than 2 are labeled with their gene names. (**B**) Heatmap displays the expression patterns of the top 50 significant DEGs, with color intensity reflecting the level of gene expression. (**C** and **D**) GO and KEGG analysis of DCM DEGs. (**E** and **F**) PPI network of the DEGs using STRING database and Cytoscape.

Functional Enrichment Analysis of DEGs

To delve into the biological implications of the DEGs, GO and KEGG analysis were conducted. These genes were predominantly linked to biological processes such as amino acid metabolism, fatty acid derivative metabolism, and responses to various stimuli, including reactive oxygen species, starvation, hypoxia, and external stimuli. In terms of cellular

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components, the genes were associated with the cytoplasm, mitochondria, peroxisomes, and dense core granules. Molecular function analysis highlighted the significance of carbohydrate transmembrane transporter activity (Figure 2C). KEGG pathway analysis revealed associations with pathways like the biosynthesis of unsaturated fatty acids, fatty acid elongation, and the PPAR signaling pathway, among others (Figure 2D). The PPI network was displayed in Figure 2E, illustrating the interaction between DEGs. Then, the optimized candidate genes were identified using the plug-in Cytohubba function, and the gene interaction diagram (Figure 2F) visually map the connectivity and centrality of DEGs, with colors indicating the MCC values.

WGCNA

Utilizing the GSE5606 dataset, hierarchical clustering with a soft threshold of β = 8 was instrumental in constructing a scale-free network (Figure 3A). WGCNA analysis identified 23 co-expression modules with potential contributions to DCM development, where each module is represented by a distinct color (Figure 3B and C). The magenta module containing 325 genes showed the strongest correlation with DCM (Figure 3C). The scatter plot indicates the significant genes in the magenta module (Figure 3D).

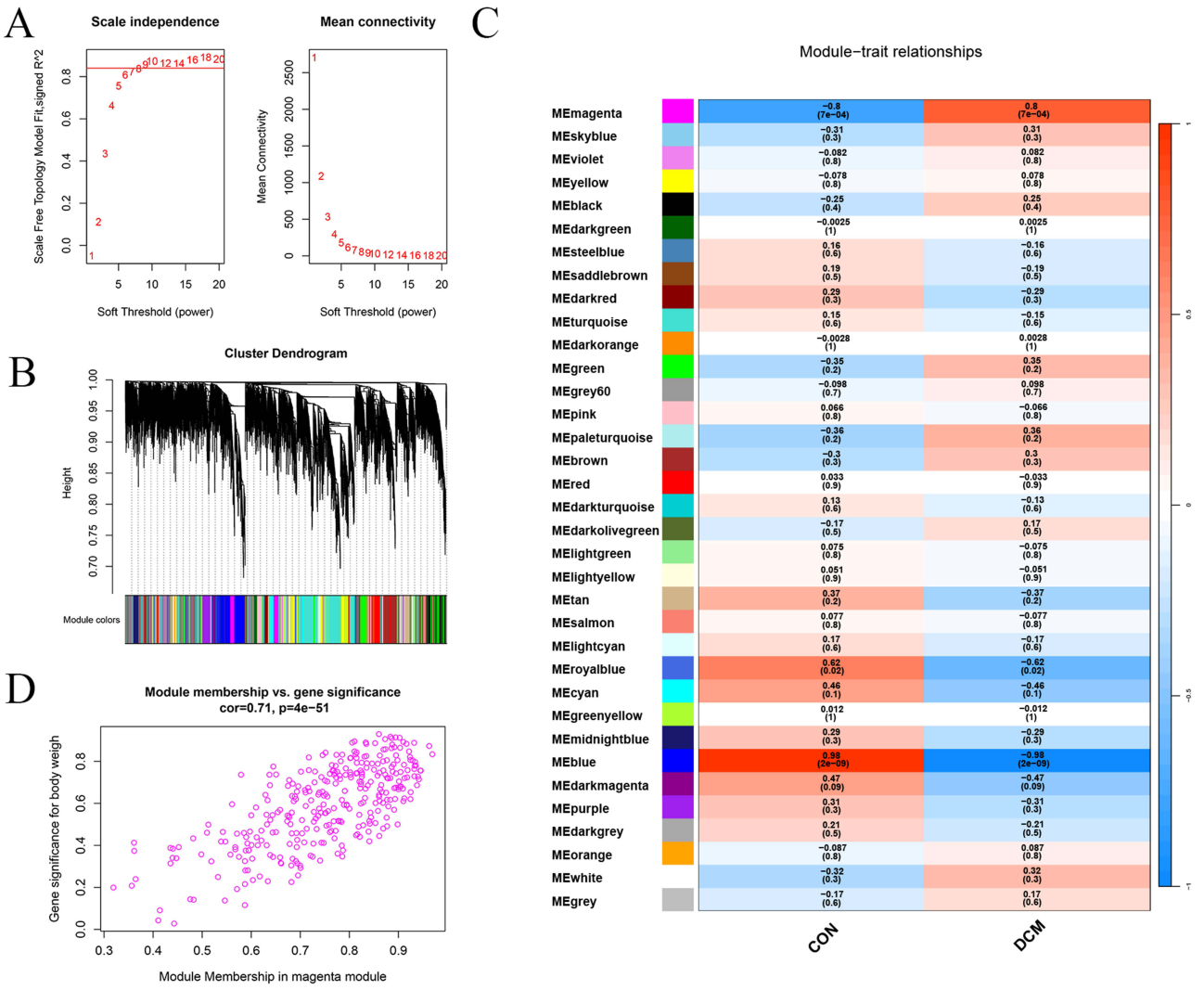

Figure 3 WGCNA Analysis. (**A**) shows hierarchical clustering with a soft threshold of β=8, constructing a scale-free network. (**B** and **C**) presents 23 co-expression modules, each represented by a distinct color, with the magenta module showing the strongest correlation with DCM. (**D**) A scatter plot indicating significant genes within the magenta module.

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Machine Learning

LASSO logistic regression and SVM-RFE algorithms were employed to identify the DCM-related feature variables from the DEGs. LASSO logistic regression identified 10 signature genes with relative importance scores greater than 1 (Figure 4A and B). SVM-RFE specifically selected 34 signature genes, as illustrated in Figure 4C. A Venn diagram (Figure 4D) illustrates the intersection of genes identified by WGCNA, LASSO, and SVM-RFE, leading to the identification of six signature genes (Dusp15, Acot1, Pdp2, Fndc5, Slc2a4, Cfd) for further analysis. The expression patterns of these genes in DCM relative to normal controls are displayed in Figure 4E.

Validation of Dusp15 in vitro and in vivo

A literature search on the six genes related to DCM was conducted in the PubMed database. Existed studies have underscored the importance of Acot1, Pdp2, Fndc5, Slc2a4, and Cfd in the context of DCM. However, the specific role of Dusp15 in DCM has not been elucidated. Thus, we subsequently verified Dusp15 expression in HL-1 murine cardiomyocytes and DCM animal models.

Utilizing HL-1 mouse myocardial cells, we developed an in vitro diabetic cardiomyopathy model, and detected the Dusp15 gene expression under high and low glucose conditions through RT-qPCR. Our findings revealed a significant downregulation of Dusp15 in the high-glucose incubated cell model compared to the low-glucose group (Figure 5A), corroborating bioinformatic predictions. Moreover, Western blot analysis confirmed these findings at the protein level (Figure 5B and C), further validating our observations.

Subsequently, we proceeded to induce diabetic cardiomyopathy in mice using STZ. Timeline for the establishment of DCM animal model was summarized in Figure 6A. The blood glucose data in both group animals was shown in Figure 6C, indicating the reliability of DCM model. After three months of STZ treatment, HE staining of cardiac tissues extracted from the experimental and control groups revealed the characteristic structural alterations of diabetic cardiomyopathy. Briefly, the

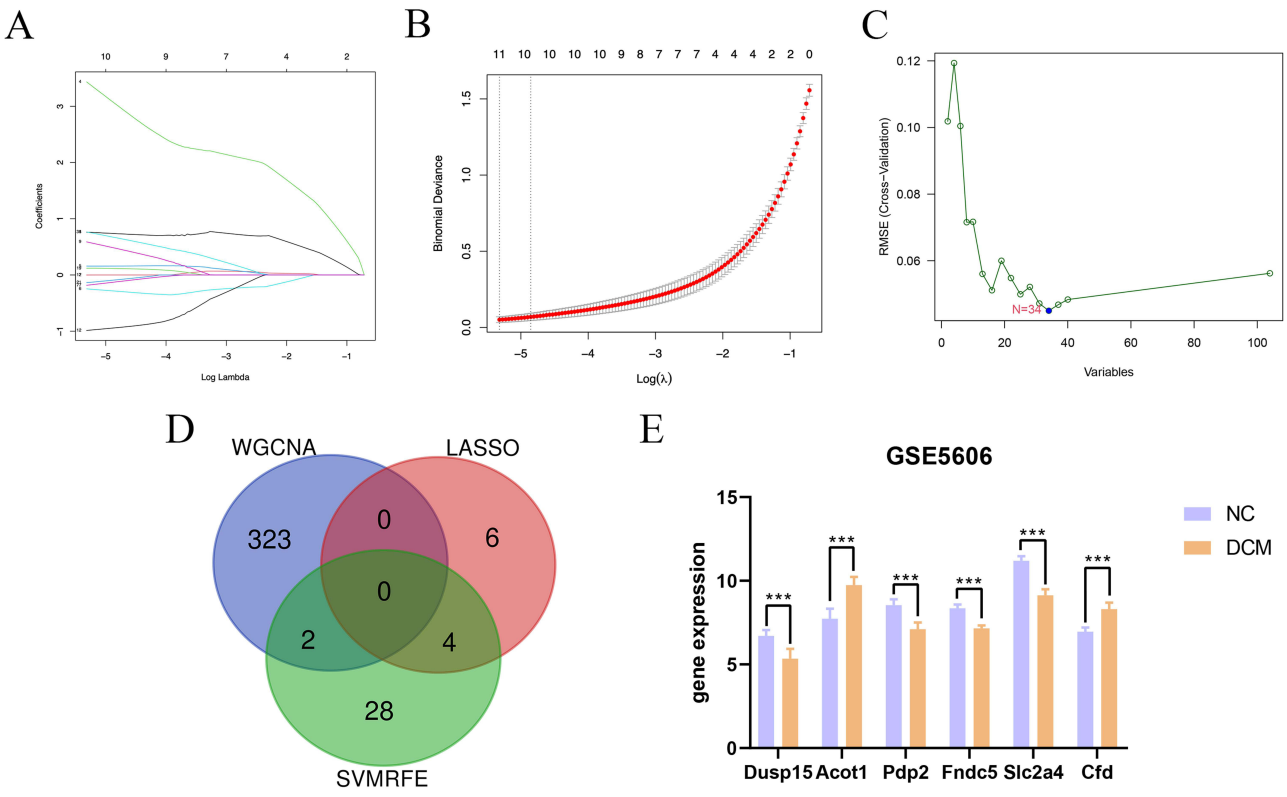

Figure 4 Machine Learning Analysis. (**A** and **B**) LASSO logistic regression identified 10 signature genes. (**C**) SVM-RFE algorithm selected 34 signature genes for DCM. (**D**) A Venn diagram showing the intersection of genes identified by WGCNA, LASSO, and SVM-RFE, leading to the identification of six signature genes. (**E**) displays the expression patterns of six signature genes in DCM relative to normal controls. ***P<0.001.

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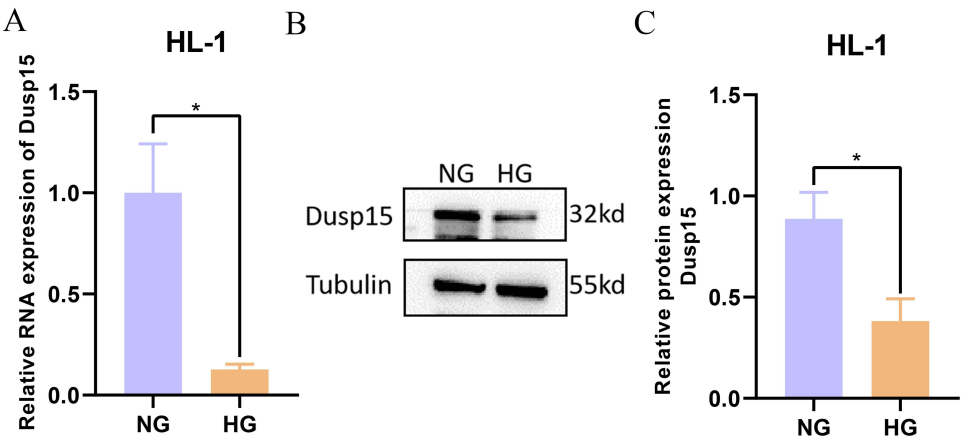

Figure 5 In Vitro and In Vivo Validation of Dusp15. (**A**) RT-qPCR analysis of Dusp15 gene expression under high and low glucose conditions in HL-1 murine cardiomyocytes. (**B** and **C**) Western blot analysis confirming the expression levels of Dusp15 at the protein level in the high-glucose incubated cell model. *P<0.05.

myocardial cells of the control mice exhibited a normal morphology, with orderly arranged bundles, tightly packed intercellular spaces, and clearly defined cross-striations. The deposition of collagen between the cells was minimal, and no significant inflammatory infiltration was observed (Figure 6B). In contrast, the myocardial tissue of the DCM mice displayed a disarrayed structure, including enlarged and distorted myocardial cells, separation of between cytoplasm and nucleus, blurred and enlarged intercellular spaces. There was a significant increase in collagen content (Figure 6B).

We then assessed the expression of Dusp15 at both the mRNA and protein levels in myocardial tissues. Notably, Dusp15 expression at both the mRNA and protein levels was significantly reduced in the myocardial tissues of diabetic mice, reinforcing its potential as a biomarker for DCM (Figure 6D–F).

Predictive Functions of Dusp15 in DCM

The potential functions of Dusp15 were investigated using the GeneMANIA database,[12] which predicted its involvement in critical biological processes such as the MAPK cascade, JNK cascade, and stress-regulated protein kinase signaling pathways (Figure 7). These pathways are well-established in the context of cellular responses to stress and are implicated in the pathophysiology of various diseases, including DCM.

Discussion

Diabetic cardiomyopathy is a prevalent complication, and poses enduring challenges for clinical diagnosis and therapeutic intervention. The application of gene sequencing technologies and bioinformatics methods presents unprecedented opportunities for the clinical diagnosis of diabetic cardiomyopathy,[13] heralding a future of more personalized and efficacious treatments.

The present study leverages a comprehensive bioinformatics approach coupled with rigorous in vitro and in vivo experiments to identify Dusp15 as a potential biomarker for diabetic cardiomyopathy. The Dual-specificity phosphatases (DUSPs) family encompasses a class of protein phosphatases capable of dephosphorylating both serine/threonine and tyrosine residues. These enzymes play a vital role in the regulation of cellular signal transduction,[14] immune responses,[15,16] and the progression of cancer,[17] serving as integral regulatory factors in both physiological and pathological processes. Few published studies showed that DUSP family could protects against stress-induced insulin resistance[18] and recuses diabetic nephropathy via repressing mitochondrial fission pathways.[19] The Dusp15 involvement in the MAPK and JNK signaling pathways, established in our functional analysis, indicates a connection to the cellular stress responses that are heightened under diabetic conditions. These pathways are integral to the regulation of cell survival, growth, and apoptosis, and their dysregulation has been implicated in various cardiac diseases, including DCM.[20]

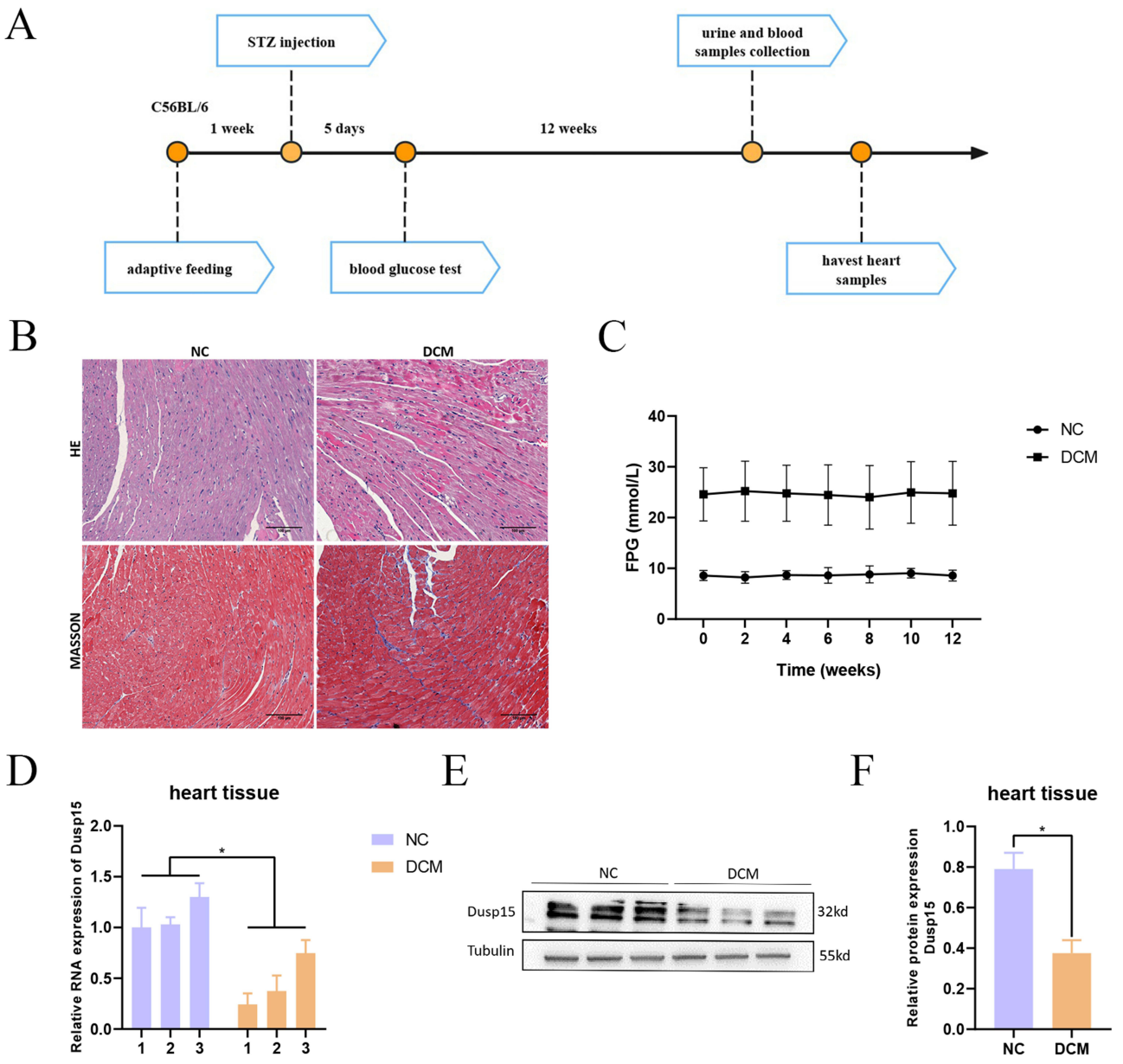

Figure 6 Establishment of STZ-Induced DCM Animal Model and in vivo validation of Dusp15 expression. (**A**) summarizes the timeline for the establishment of the DCM animal model. (**B**) HE and Masson staining of cardiac tissues from experimental and control groups, revealing characteristic structural alterations of diabetic cardiomyopathy. (**C**) Blood glucose data of both groups indicating the reliability of the DCM model. (**D–F**) Assessment of Dusp15 expression at both mRNA and protein levels in myocardial tissues, showing significant reduction in diabetic mice. *P<0.05.

The metabolic dysregulation evident in our study, characterized by the altered expression of genes linked to fatty acid metabolism and glucose transport, may point to a shift in the cardiac energy substrate preference. Under physiological conditions, fatty acids serve as the primary energy source, contributing approximately 70% of ATP for the working heart, with the remainder derived from glucose, ketones, and branched-chain amino acids (BCAAs).[21] In diabetes, however, there's a marked increase in fatty acid and ketone uptake alongside a decrease in glucose and BCAA utilization. This metabolic shift results in lower ATP generation and higher oxygen demand, thereby implicating metabolic inefficiency in the progression of DCM.[22]

Acot1 is an enzyme involved in lipid metabolism, known for its role in the hydrolysis of acyl-CoA esters to generate free fatty acids and coenzyme A. Studies[23,24] have suggested that Acot1 expression is markedly increased in the

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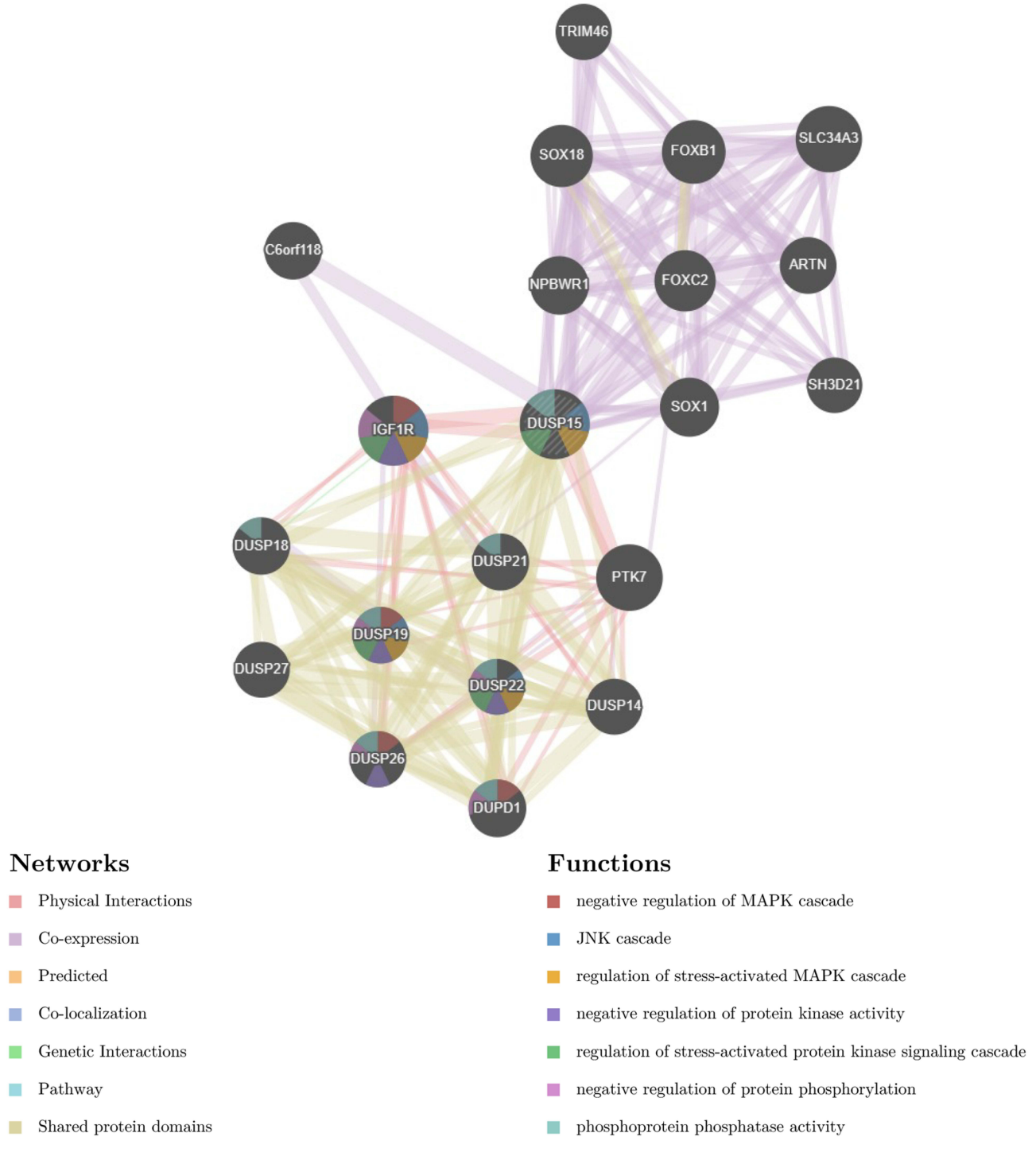

Figure 7 Investigation of the potential functions of Dusp15 using the GeneMANIA database.

myocardial tissue of diabetic mice, which aligns with our results. It may contribute to the mitigation of myocardial dysfunction in diabetes by reducing lipid accumulation and improving mitochondrial function.[25]

Slc2a4, also known as GLUT4, is a key glucose transporter in adipose and muscle tissues. Its dysregulation in diabetic cardiomyopathy could impair the heart's glucose uptake, leading to reduced energy efficiency and contributing to cardiac dysfunction.[26]

Pdp2 is a phosphatase that activates the pyruvate dehydrogenase complex, which is pivotal for glucose metabolism. Its deregulation can result in a diminished ability to metabolize glucose, which further affects the heart's energy production.[27] A previous study demonstrated that streptozotocin-induced diabetes caused decreases in Pdp2 abundance in rat heart and kidney,[28] which is consistent with our data.

The GO analysis revealing the enrichment of DEGs in mitochondrial and peroxisomal components in DCM. Similarly, previous studies have elucidated the intricate relationship between mitochondrial metabolism and DCM by integrating DEGs screening with the analysis of mitochondrial-related gene sets.[29] What distinguishes this article is that our study has gone a step further by incorporating WGCNA and two machine learning algorithms to identify more precise markers. Electron microscopy findings suggested that mitochondria in diabetic patients are smaller and have a reduced capacity to retain Ca^{2+}, which is associated with decreased expression of mitochondrial fission proteins.[30] The identified mitochondrial alterations may be linked to the development of cardiomyopathy, indicating the potential of mitochondria-focused therapies.[31–33]

Fndc5 has been a subject of interest in the study of DCM. Fndc5 is known for its role in the secretion of irisin, a protein associated with the regulation of energy metabolism. A previous study found that the peripheral blood expression level of Fndc5 was significantly lower in patients with subclinical DCM compared to a control group of diabetic patients without heart dysfunction.[34] In the context of diabetic cardiomyopathy, alterations in Fndc5 expression could impact the heart's metabolic flexibility and mitochondrial function, which are critical for maintaining cardiac health under metabolic stress. Study indicated that FNDC5/irisin protects against cardiac diastolic dysfunction by attenuation of oxidative/ nitrosative stress and mitochondria-dependent apoptosis.[35]

Cfd, also known as adipsin, is implicated in the activation of complement system.[36] It's released into the blood and can be used as a sign to help predict heart disease. In mechanism, Cfd/Adipsin was demonstrated to alleviate diabetic cardiomyopathy by regulating mitochondrial dysfunction and improving fatty acid β-oxidation.[37,38]

In addition, our KEGG pathway analysis highlighted the enrichment of pathways involved in BCAA degradation, and the Peroxisome proliferator-activated receptors (PPARs) signaling pathway. PPARs act as ligand-dependent transcriptional regulators. They are involved not only in the regulation of lipid and glucose metabolism but in the modulation of inflammation, and oxidative stress in the heart.[39] Modulating cardiac PPAR to prevent metabolic disruptions may offer a new avenue for managing dilated cardiomyopathy. BCAAs, composed of valine, leucine, and isoleucine, are regulated by a series of BCAA catabolic enzymes. Impaired BCAA oxidation, reflected by reduced expression of BCAA catabolic enzymes, has been confirmed in DCM patients.[40]

Through comprehensive data mining and bioinformatics analysis, we identified vital DCM-specific gene markers, which were subsequently validated using both in vitro and in vivo DCM models. These genes were closely associated with mitochondrial energy metabolism, which is intimately linked to the pathogenesis of DCM. Thus, our findings may offer valuable insights for the precise diagnosis of DCM, contributing to its clinical relevance. However, there are several limitations in this study that warrant acknowledgment: First, our study is primarily based on bioinformatics analyses, future work should focus on experimental investigation to elucidate the biological functions. Second, due to the inherent limited access of human heart samples, understanding regarding the mechanisms of dilated cardiomyopathy has been gleaned from the application of rodent models This study also utilized cardiac expression profile data from DCM animal models, with validations at cellular and animal levels. However, further validation of our findings in clinical practice is still necessary in the future.

Conclusion

In summary, our integration of diverse bioinformatics approaches using DCM murine models has identified six specific core genes, with Dusp15 showing promise as an early diagnostic biomarker. Nonetheless, Further investigation is warranted to substantiate our findings presented and to ascertain the therapeutic potential of Dusp15 as a target for DCM intervention.

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Disclosure

The authors declare no competing interests.

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