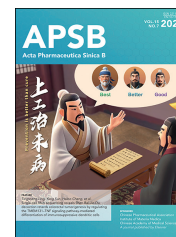




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ORIGINAL ARTICLE

Melatonin receptor 1a alleviates sleep fragmentation-aggravated testicular injury in T2DM by suppression of TAB1/TAK1 complex through FGFR1



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Abstract A major obstacle in type 2 diabetes mellitus (T2DM) is sleep fragmentation (SF), which negatively affects testicular function. However, the underlying mechanisms remain to be elucidated. In this study, we demonstrate that SF induces testicular damage through a mechanism involving lipid metabolism, specifically mediated by melatonin (MEL) receptor 1a (MT1). T2DM mice with SF intervention displayed several deleterious phenotypes such as apoptosis, deregulated lipid metabolism, and impaired testicular function. Unexpectedly, sleep recovery (SR) for 2 consecutive weeks could not completely abrogate SF's detrimental effects on lipid deposition and testicular function. Interestingly, MEL and MT1 agonist 2-iodomelatonin (2IM) effectively improved lipid homeostasis, highlighting MEL/2IM as a promising therapeutic drug for SF-triggered testicular damage. Mechanistically, MEL and 2IM activated FGFR1 and sequentially restrained the crosstalk and physical interaction between TAB1 and TAK1, which ultimately suppressed the phosphorylation of TAK1 to block lipid deposition and cell apoptosis caused by SF. The ameliorating effect of MEL/2IM was overtly nullified in *Fgfr1* knockout (*Fgfr1-KO*^{+/−}) diabetic mice. Meanwhile, testicular-specific overexpression of *Tak1* abolished the

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protective effect of FGF1^{mut} on diabetic mouse testis. Our findings offer valuable insights into the molecular mechanisms underlying the testicular pathogenesis associated with SF and propose a novel therapeutic approach for addressing male infertility in T2DM.

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1. Introduction

Male infertility accounts for half of all cases of infertility that affects approximately 15% of couples worldwide¹. The underlying etiologies of infertility are complex and may be partly attributed to the rising prevalence of type 2 diabetes mellitus (T2DM) among men of reproductive age². Projections suggest that by 2045, the number of adults living with T2DM could reach approximately 700 million, with nearly one-third of male T2DM patients likely to experience infertility, underscoring the urgent need for intervention^{3,4}. The mechanisms underlying diabetes-related infertility are intricate, involving both micro- and macrovascular disorders, as well as metabolic complications that are closely associated with sleep disturbances⁵. Importantly, sleep disorders represent a significant risk factor for reproductive health, with a higher prevalence observed among T2DM patients⁶. These disturbances further exacerbate clinical manifestations of T2DM by increasing the risk of insulin resistance and obesity^{6,7}. However, to date, no studies have investigated whether sleep disturbances exacerbate male reproductive function in T2DM, and the underlying mechanisms remain inadequately understood.

Regulation of lipid metabolism is crucial for alleviating damage to multiple organs. For instance, podocyte-specific deletion of junction adhesion molecule like (*Jaml*) could regulate lipid accumulation in podocytes by modulating sterol regulatory element binding transcription factor 1 (SREBP1) signaling, thereby attenuating renal injury in diabetic mice⁸. As the central organ of the male reproductive system, the testis primarily functions in sperm production and the secretion of testosterone. Cholesterol, being the precursor of testosterone, plays a vital role in regulating spermatogenesis and semen quality, underscoring its close association with reproductive function⁹. Moreover, the down-regulation of genes related to fatty acid metabolism leads to spermatogenic arrest^{10,11}. Reportedly, fibroblast growth factor receptor 1 (FGFR1) is a key lipid metabolism regulator that controls lipogenesis and fatty acid oxidation upon metabolic stress¹². However, to date, studies have yet to establish a definitive connection between FGFR1 and testicular development and function. Moreover, mutations in the *Fgfr1* gene have been implicated as a genetic etiology of the rare condition known as congenital hypogonadotropic hypogonadism¹³, highlighting the essential role and potential therapeutic implications of FGFR1 in male reproductive health. However, whether FGFR1 plays a role in SF-induced testicular injury is not yet known, and the specific mechanisms underlying its action need to be further elucidated.

Melatonin (MEL) is a pleiotropic molecule that displays a range of physiological functions. As an endogenous indoleamine, it is synthesized and secreted primarily by the pineal gland, but it is also produced in various peripheral tissues and organs, including the gastrointestinal tract, biliary tract, and testis^{14–16}. Furthermore, melatonin has been widely employed as a dietary supplement in clinical trials¹⁷. There are two main subtypes of

melatonin receptors, melatonin receptor 1a (MT1) and melatonin receptor 1b (MT2), which are widely distributed in living organisms, including the brain as well as in peripheral organs such as the kidney, pancreas, testis, intestine, and uterus¹⁸. By regulating circadian rhythms, MEL plays a crucial physiological role in modulating the sleep–wake cycle. Additionally, it exhibits a wide array of biological activities, including antioxidant, anticancer, anti-apoptotic, and anti-aging effects¹⁹. Furthermore, MEL has been demonstrated to possess protective effects on the testis, mitigating testicular damage triggered by heat stress, heavy metal exposure, and chemotherapeutic agents^{20–22}. However, the role of MEL in SF-mediated reproductive dysfunction has not been investigated yet.

In this study, we aimed to investigate the role and potential mechanisms of SF in T2DM testicular injury, and the promoter role of MEL in modulating SF-aggravated imbalance of lipid metabolism *via* FGFR1 activation and downstream signaling pathways was subsequently elucidated by using a combination of multiple functional and pathological analysis, RNA-sequencing (RNA-seq), molecular biology, genetically modified mice and *in vitro* approaches. Considering the non-negligible impact of sleep on reproductive health, exploring its intrinsic mechanisms might provide a new potential drug target for the future treatment of male infertility in patients with T2DM.

2. Materials and methods

Detailed descriptions of our animal models and methodologies are available in the Supporting Information

2.1. Animals and treatment

Male mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal experiments were conducted in accordance with the guidelines set by the Ethics Committee of the School of Nursing and Rehabilitation, Shandong University (permit number: 2021-D-008).

In this study, we performed four distinct sets of animal experiments: (1) to evaluate the effects of SF on testicular pathology and function in diabetic mice; (2) to investigate the role of MT1 in the exacerbation of testicular injury by SF in the context of T2DM; (3) to determine whether FGFR1 mediates the protective effects of MEL against SF-induced exacerbation of testicular injury associated with T2DM; and (4) to elucidate the involvement of TGF- β activated kinase 1/MAP3K7 binding protein 1/TGF- β activated kinase 1 (TAB1/TAK1) in the exacerbation of testicular injury by SF in diabetic conditions.

Generally, after a 1-week adaptation period on a normal diet (ND), mice were induced to develop T2DM through a high-fat diet (HFD; 60% energy derived from fat, Cat# D12492, Research Diets, NJ, USA) for 4 weeks. This was followed by daily

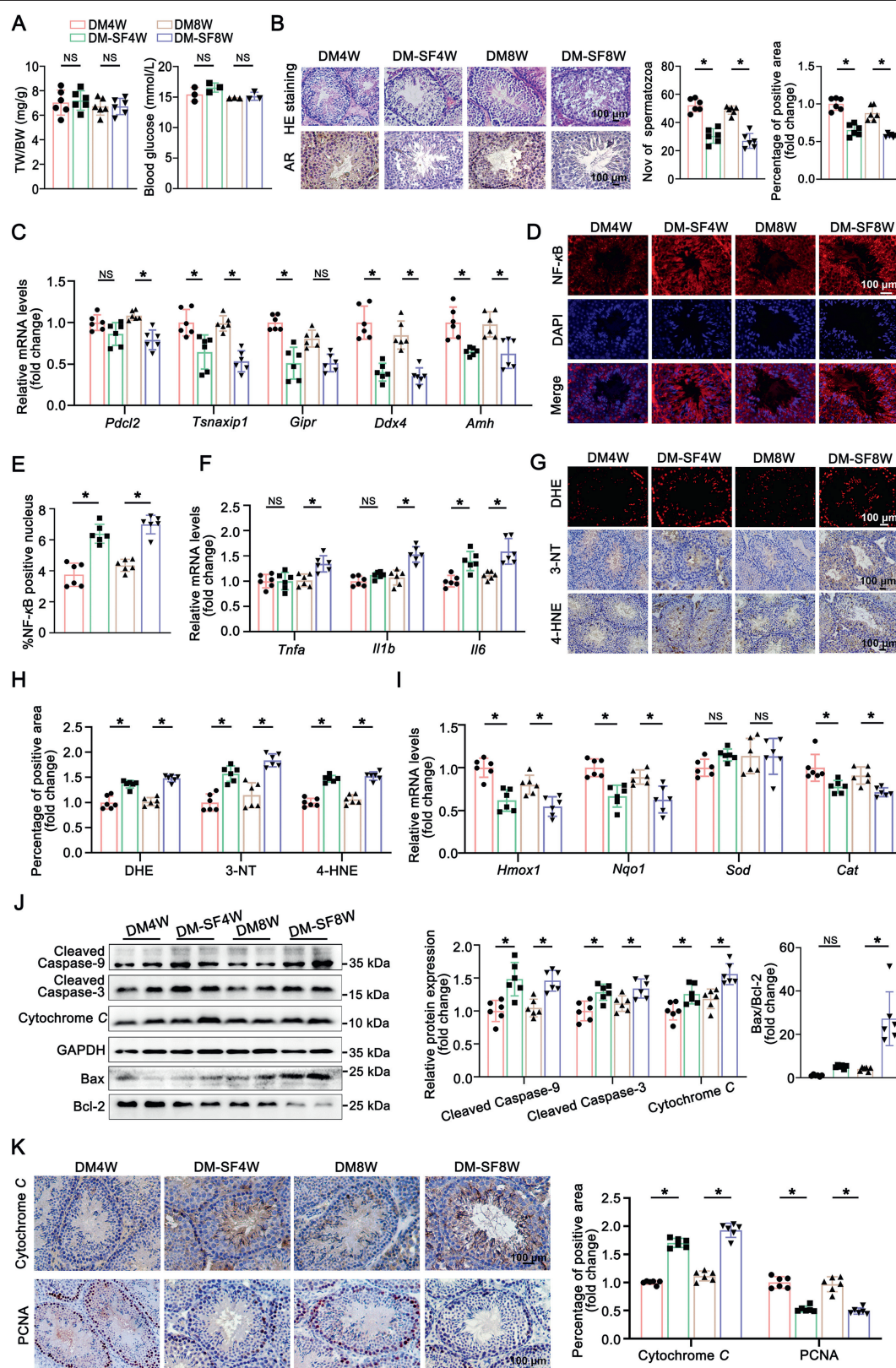


Figure 1 SF exacerbated testicular damage in mice. (A) The testis-to-body weight ratio (mg/g) and blood glucose levels (mmol/L). (B) Representative images of H&E, the number of spermatozoa in the tubule cross sections, and the representative images and quantitative analysis of AR ($n = 6$). (C) Relative mRNA levels of *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4*, and *Amh* in testis ($n = 6$). (D, E) Nuclear accumulation of NF-κB determined by IF staining with anti-NF-κB p65 antibody (red) in testis tissues ($n = 6$). (F) *Tnfa*, *Il1b*, and *Il6* mRNA levels in the testis of T2DM and SF mice ($n = 6$). (G, H) Representative microphotographs and quantification of fluorescence intensity of DHE staining (red) in testis sections; IHC staining using 4-HNE and 3-NT antibody in testis tissues and related quantitative analysis ($n = 6$). (I) Relative mRNA levels of *Hmox1*, *Nqo1*, *Sod*, and *Cat* in testis ($n = 6$). (J) The protein expression levels of Cleaved Caspase-9, Cleaved Caspase-3, Cytochrome c, Bax, and Bcl-2 were assessed using Western blot analysis, with quantification of relative protein levels performed ($n = 6$). (K) IHC staining of testis apoptosis detected with Cytochrome c and proliferation detected with PCNA and related quantitative analysis ($n = 6$). Data shown in graphs represent the means $\pm$ SD; GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

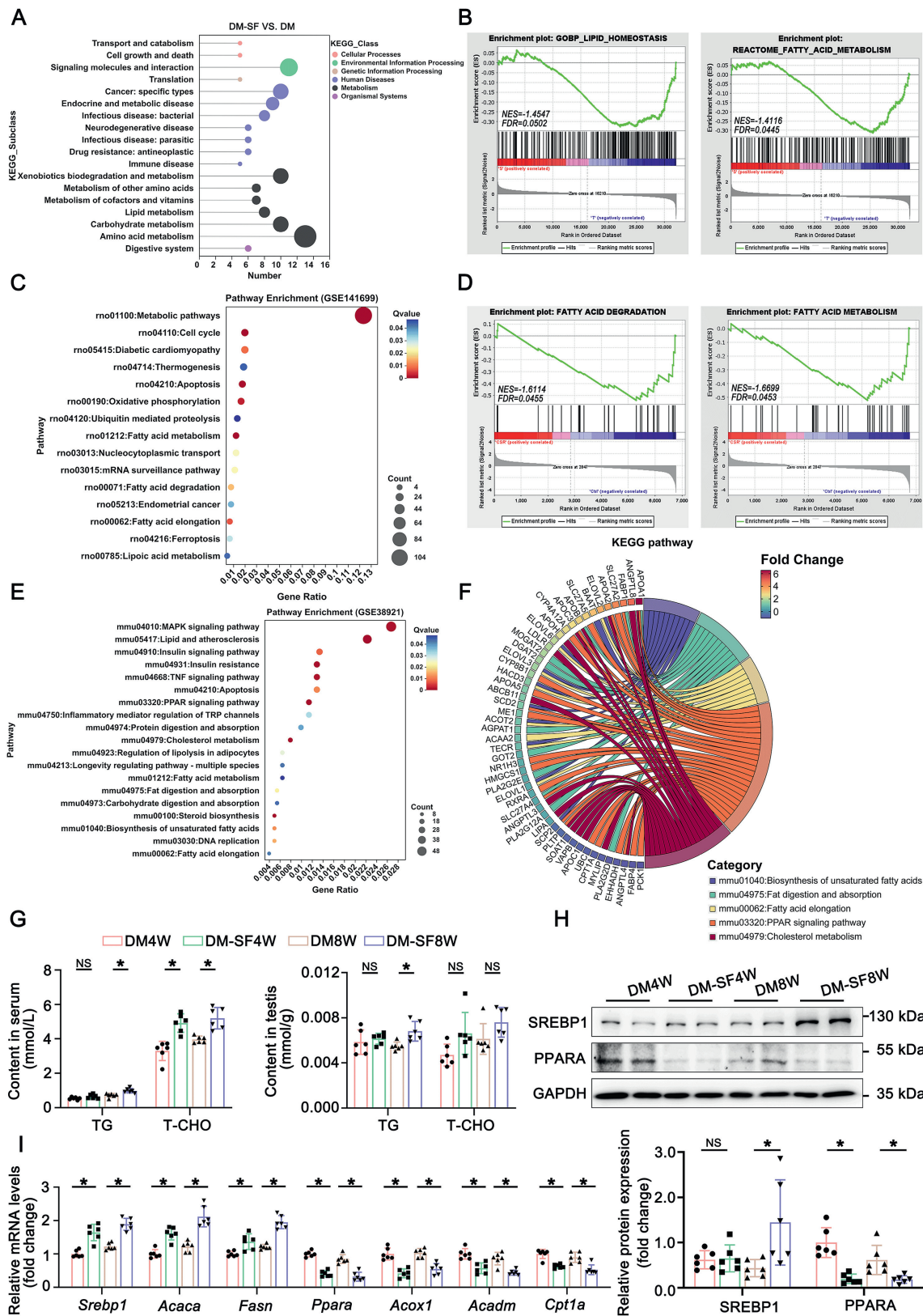


Figure 2 SF exacerbated lipid metabolism disorders in mice. (A) KO annotation for the DEGs in testis between DM-SF vs. DM mice. (B) Effect of SF on characteristic genes indicative of lipid homeostasis and fatty acid metabolism pathway shown by GSEA analysis. (C) KEGG pathway enrichment analysis of DEGs in CSR rat (GSE141699). (D) GSEA analysis showed that CSR was negatively associated with fatty acid metabolism and fatty acid degradation. (E) KEGG pathway enrichment analysis of DEGs in timed sleep restriction mice (GSE38921). (F) A chordal graph of enriched KEGG terms showed the relationship between DEGs and KEGG pathways related to lipid metabolism. (G) TG and

intraperitoneal injections of 50 mg/kg streptozotocin (STZ; Cat# S8050, Solarbio, Beijing, China) for 5 consecutive days. Subsequently, the T2DM mice were subjected to intermittent tactile stimulation during the light phase (7 am–7 pm) using a near-silent sleep deprivation apparatus, with stimulation occurring every 2 min.

Melatonin (Cat# HY-B0075) was obtained from MCE (NJ, USA) and administered to mice *via* intraperitoneal injection at 10 mg/kg 2-iodomelanin (Cat# SC-203463) from Santa Cruz Biotechnology (CA, USA) was given at doses of 0.1 mg/kg, 1 mg/kg, and 10 mg/kg. FGF1^{mut}, kindly provided by Dr. Jianlou Niu from Wenzhou Medical University, was diluted from 2.6 to 0.05 mg/mL with saline and injected intraperitoneally at 0.5 mg/kg.

2.2. Histopathology and cellular staining

Testicular tissues were processed to obtain paraffin-embedded sections, which were subsequently analyzed using hematoxylin and eosin (H&E) staining according to the manufacturer's protocol. The resultant images were captured with a light microscope (Nikon, Tokyo, Japan). Additionally, oxidative stress markers, specifically 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE), alongside the apoptotic protein Cytochrome *c*, the androgen receptor (AR) and the proliferative marker proliferating cell nuclear antigen (PCNA), were examined through immunohistochemical (IHC) staining. Testis sections were subsequently incubated with primary antibodies specific to each target: anti-3-NT (1:300, Cat# AB5411, Millipore, Billerica, MA, USA), anti-4-HNE (1:300, Cat# ab46545, Abcam, Cambridge, UK), anti-Cytochrome *c* (1:300, Cat# 10993-1-AP, Proteintech, Wuhan, China), anti-AR (1:200, Cat# 22089-1-AP, Proteintech), and anti-PCNA (1:300, Cat# 2586, Cell Signaling Technology, MA, USA). After removing primary antibodies, the sections were incubated with HRP-conjugated secondary antibodies. Afterward, the peroxidase substrate 3,3'-diaminobenzidine (DAB; Servicebio Technology, Wuhan, China) was introduced to facilitate the development of the staining reaction, which was subsequently counterstained with hematoxylin. All sections were thoroughly examined using a light microscope, and the staining intensity was quantified using ImageJ software.

For immunofluorescent staining (IF) of nuclear factor κ B (NF- κ B), MT1, MT2 and DDX4 testis sections were incubated with primary antibodies: anti-NF- κ B (1:300, Cat# 8242, Cell Signaling Technology), anti-MT1 (1:200, Cat# bs-0027R, Bioss Biological Technology, Beijing, China), anti-MT2 (1:200, Cat# bs-0963R, Bioss Biological Technology) and anti-DDX4 (1:500, Cat# 67147-2-Ig, Proteintech). Following this, sections were treated with either Alexa Fluor 647 goat anti-rabbit (Cat# ab150079, Abcam) or Alexa Fluor 488 goat anti-mouse (Cat# ab150113, Abcam) secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Cat# ab104139, Abcam). The stained slides were then examined under a fluorescence microscope (Nikon), and the fluorescence signals were quantified using ImageJ software.

Testis sections were stained with DHE (Dihydroethidium, Cat# S0063, Beyotime Biotechnology, Shanghai, China) probe to assess intracellular reactive oxygen species (ROS) production.

Additionally, testicular cell apoptosis was evaluated using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, performed with the *In Situ* Cell Death Detection Kit TMR Red (12156792910, Roche, Meylan, France).

Lipid accumulation was performed according to the manufacturer's protocol. Oil Red O was purchased from Sigma–Aldrich, MO, USA. After different treatments, GC-1 spg cells were fixed with 4% paraformaldehyde and stained with 0.5% Oil Red O staining solution. For Nile Red staining, GC-1 spg cells were incubated with 0.05 mg/mL Nile Red staining solution (Solarbio). DAPI staining was used for cell nuclei. Images were acquired using a fluorescence microscope (Olympus, Tokyo, Japan).

2.3. Plasmid transfection and adenovirus infection

Plasmid transfection of GC-1 spermatogonia (GC-1 spg) cells was performed using Lipofectamine 3000 transfection reagent (Lipo3000, Heyuan Biotechnology, Shanghai, China) following the manufacturer's instructions. The plasmid and Lipo3000 transfection reagents were diluted and mixed with Opti-DMEM, then allowed to stand for 20 min before being added to the well plates. Additionally, 293T cells were transfected using a polyethyleneimine (PEI) reagent. Once the cells reached 80%–90% confluence, the PEI was combined with the plasmid and incubated for 15 min. This mixture was then added to the well plates and incubated with the cells for 24–48 h.

2.4. Colocalization analysis

To elucidate the subcellular localization of FGFR1, TAK1, and TAB1, GC-1 spg cells were cultured in six-well plates and transfected with Flag-FGFR1 and HA-TAK1 or HA-TAB1 for 48 h. Following two washes with PBS, cells were fixed in 4% formaldehyde for 10 min and permeabilized for 20 min using 0.5% Triton X-100. Subsequently, the cells were blocked with 3% BSA for 1 h. Cells were then incubated with rabbit anti-HA antibodies (Cat# 51064-2-AP, Proteintech) or mouse anti-Flag antibodies (Cat# 66008-4-Ig, Proteintech), followed by labeling with Alexa Fluor 647 goat anti-rabbit (Cat# ab150079, Abcam) or Alexa Fluor 488 goat anti-mouse (Cat# ab150113, Abcam) secondary antibodies. DAPI was used to stain the nuclei. Images were obtained using a fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Protein–protein docking

The three-dimensional structures of MT1, FGFR1, TAK1, and TAB1 were obtained from UniProt (<https://www.uniprot.org/>). The widely used rigid-body protein–protein docking program ZDOCK (<https://zdock.umassmed.edu/>) was employed to predict the interactions among MT1 and FGFR1, FGFR1 and TAK1, as well as FGFR1 and TAB1. Each docking procedure produced the ten most probable predictions from thousands of candidates, assessed based on the geometric, hydrophobic, and electrostatic complementarity of the molecular surfaces. The resulting docking complexes from ZDOCK were uploaded to PDBePISA (https://www.ebi.ac.uk/msd-srv/prot_int/) for analysis of protein–protein

T-CHO content in serum and testis homogenates ($n = 6$). (H) SREBP1 and PPARA protein levels in the testis and densitometric quantification ($n = 6$). (I) Relative mRNA levels of *Srebp1*, *Acaca*, *Fasn*, *Ppara*, *Acox1*, *Acadm*, and *Cpt1a* in the testis ($n = 6$). Data shown in graphs represent the means $\pm$ SD; GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

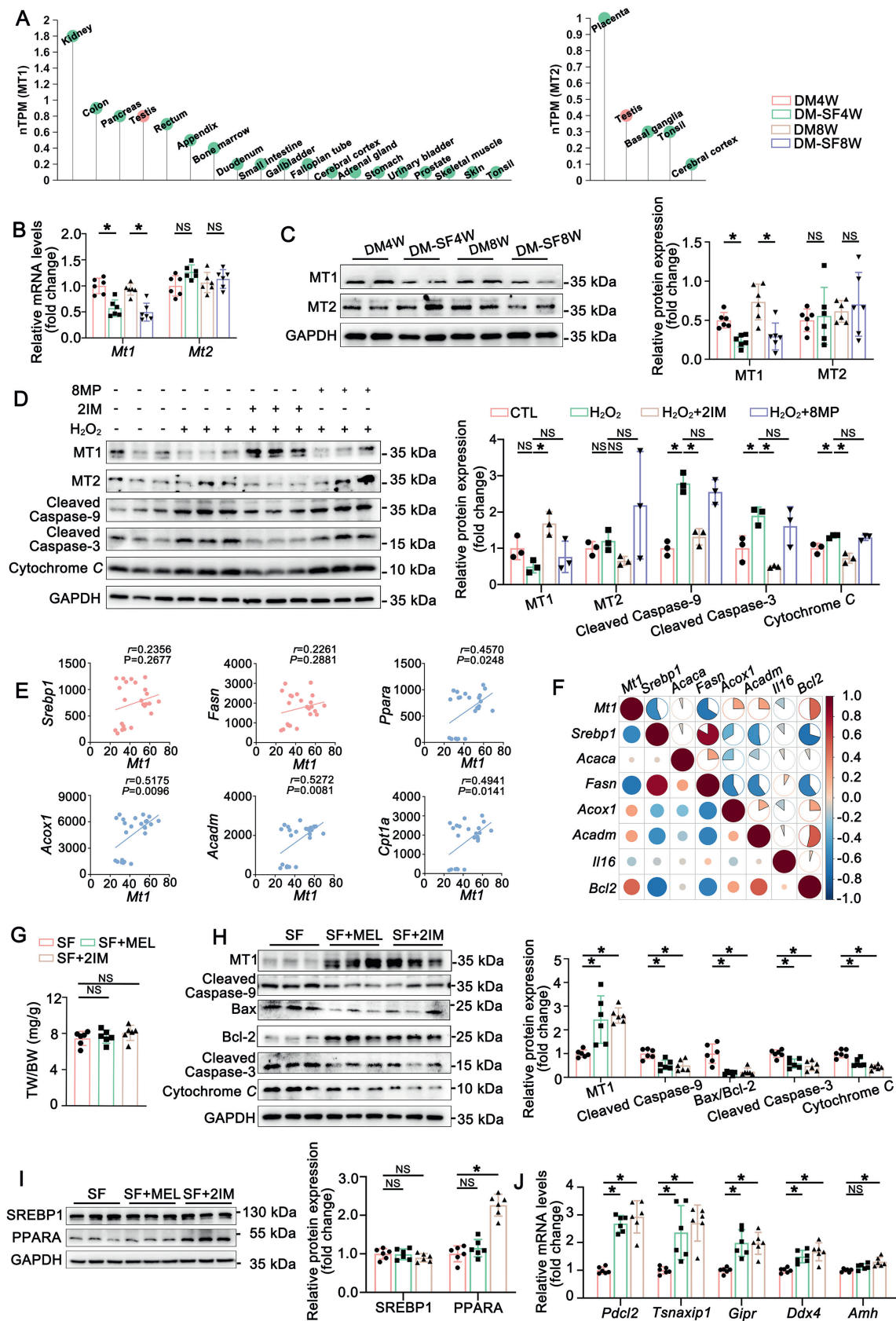


Figure 3 MT1 alleviated SF-induced testicular injury and dysregulated lipid metabolism (A) Enrichment of the melatonin receptors in different tissue-specific cell types. (B) Relative mRNA levels of *Mt1* and *Mt2* in testis ($n = 6$). (C) The protein expression of MT1 and MT2 in testis quantification of the relative protein levels ($n = 6$). (D) Western blot analysis of MT1, MT2, Cleaved Caspase-9, Cleaved Caspase-3, and

interactions, which included the identification of hydrogen bonds, interacting interfaces, non-bonded contacts, salt bridges, and the Gibbs free energy of binding (Δ^1G , kcal/mol). The presence of pores and tunnels within the protein complexes was also evaluated. Visualization and editing of the docking complexes were performed using PYMOL software.

2.6. Co-immunoprecipitation assays (Co-IP)

Co-IP assays were performed as described previously²³. Briefly, 293T cells or GC-1 spg cells were lysed using IP lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease and phosphatase inhibitors. Total cell lysates were immunoprecipitated with anti-HA antibodies or anti-Flag antibodies. Immunoglobulin G (IgG) from rabbit sources (ThermoFisher Scientific, MA, USA) or mouse sources (Santa Cruz Biotechnology, Dallas, TX, USA) were used as the negative control. Protein G magnetic beads (Santa Cruz Biotechnology) were used to capture the primary antibodies according to the supplier's instructions. After washing three times with IP lysis buffer, the immunopurified proteins were denaturation by boiling the beads in 15 μ L of 5 $\times$ loading buffer. Finally, the samples were subjected to Western blot analysis.

2.7. Recombinant adeno-associated virus serotype 9 (AAV9) vector delivery

AAV9 vectors expressing green fluorescent protein (AAV9-*Ctrl*) and full-length *Tak1* (AAV9-*Tak1*) were generated by Obio Technology (Shanghai, China) according to the manufacturer's instructions. Targeted injections were administered into both testes using 30G insulin needles for testis-specific overexpression.

2.8. Western blotting analysis

Testis tissues and GC-1 spg cells were harvested, prepared, and subjected to Western blotting as previously described^{23,24}. The primary antibodies utilized in this study were as follows: anti-MT1 (1:1000, Bioss Biological Technology), anti-MT2 (1:1000, Bioss Biological Technology), anti-Cleaved Caspase-3 (1:1000, Cat# 9664, Cell Signaling Technology), anti-Cleaved Caspase-9 (1:1000, Cat# 10380-1-AP, Proteintech), anti-Cytochrome *c* (1:1000, Cat# 10993-1-AP, Proteintech), anti-Bcl-2 (1:1000, Cat# BF9103, Affinity Biosciences, China), anti-Bax (1: 5000, Cat# 60267-1-Ig, Proteintech), anti-FGFR1 (1:1000, Cat# 9740S, Cell Signaling Technology), anti-SREBP1 (1:1000, Cat# AF6283, Affinity Biosciences, China), anti-PPARA (1:1000, Cat# AF5301, Affinity Biosciences, China), anti-TAK1 (1:1000, Cat# 12330-2-AP, Proteintech), anti-p-TAK1 (1:1000, Cat# bs-5435R, Bioss Biological Technology), anti-TAB1 (1:1000, Cat# 27566-1-AP, Proteintech), anti-Flag (1:5000, Cat# 66008-4-

Ig, Proteintech), anti-HA (1:5000, Cat# 51064-2-AP, Proteintech) and anti-GAPDH (1:1000, Cat# GB11002-100, Servicebio Technology). Western blotting was developed and qualified by ImageQuant 800 (GE Healthcare, Chicago, USA) and Image J, respectively.

2.9. Statistical analyses

All data are presented as the mean $\pm$ standard deviation (SD). The Student's *t*-test was employed to assess differences between the two groups. For comparisons involving three or more groups, a one-way analysis of variance (ANOVA) followed by either the Tukey or Bonferroni *post hoc* test was utilized. Statistical analyses were conducted using GraphPad Prism v8.0.2.263. Differences were deemed statistically significant at $P < 0.05$. All experiments were performed in triplicate unless otherwise specified.

3. Results

3.1. SF aggravated pathological and functional abnormalities of the testis in T2DM

Considering the potential adverse effects of sleep disorders, particularly SF, on testicular pathology and function in diabetic mice, we subjected T2DM mice to mechanical SF, tactilely rousing diabetic mice every 2 min during their rest period (Zeitgeber [ZT] 0–12 h) for 4 or 8 weeks. Fig. 1A illustrates that SF did not have a significant impact on the testis-to-body weight ratio or fasting blood glucose levels in diabetic mice. However, following 4 and 8 weeks of SF (DM-SF4W and DM-SF8W), we observed a marked reduction in the number of spermatozoa, androgen receptor (AR) expression as well as histological evidence of vacuolization and necrosis within the seminiferous tubules, when compared to the T2DM groups at 4 and 8 weeks (DM4W and DM8W) (Fig. 1B). Genes such as phosducin-like 2 (*Pdcl2*, acrosome formation of sperm), translin-associated factor X interacting protein 1 (*Tsnaxip1*, sperm motility), gastric inhibitory polypeptide receptor (*Gipr*, fertilization capacity), DEAD box helicase 4 (*Ddx4*, spermatogenesis and the differentiation of embryonic stem cells into primordial germ cells and spermatogonium stem cells) and anti-Mullerian hormone (*Amh*, sertoli cells marker) are specifically expressed in the testis, and their aberrant expression has been shown to impact fertility in male mice²⁵⁻³⁰. Notably, in both the DM-SF4W and DM-SF8W groups, the expression levels of most of these genes were significantly suppressed, indicating an impairment of reproductive function (Fig. 1C). To determine if SF mediated an aberrant inflammatory response in the testis, we monitored the activation of NF- κ B by IF staining and Western blot, which is well recognized to regulate pro-inflammatory cytokines/chemokines³¹. The results demonstrated an increase in nuclear translocation/accumulation of NF-

Cytochrome *c* in GC-1 spg cells stimulated with H₂O₂ and treated with 25 μ mol/L 2IM or 100 μ mol/L 8 MP for 24 h ($n = 3$). (E) Pearson correlation analysis between *Mt1* and lipid metabolism-related genes in GSE33381. (F) Correlation heatmap of hub genes with *Mt1* in GSE33302. The size of the colored sector area in the upper right and the size of the dots in the lower left are positively related to the strength of correlations. (G) The testis-to-body weight ratio in the different groups ($n = 6$). (H) Western blot analysis and densitometric quantification of MT1, Cleaved Caspase-9, Bax, Bcl-2, Cleaved Caspase-3, and Cytochrome *c* in the testis of SF mice treated with MEL or 2IM for 2 weeks ($n = 6$). (I) Western blot analysis of SREBP1 and PPARA and densitometric quantification in the testis of SF mice treated with MEL or 2IM for 2 weeks ($n = 6$). (J) RT-qPCR analysis of indicated gene expression of *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4*, and *Amh* in testis ($n = 6$). Data shown in the graphs represent the means $\pm$ SD; GAPDH was used as an internal control ($n = 6$); * $P < 0.05$; NS, not significant.

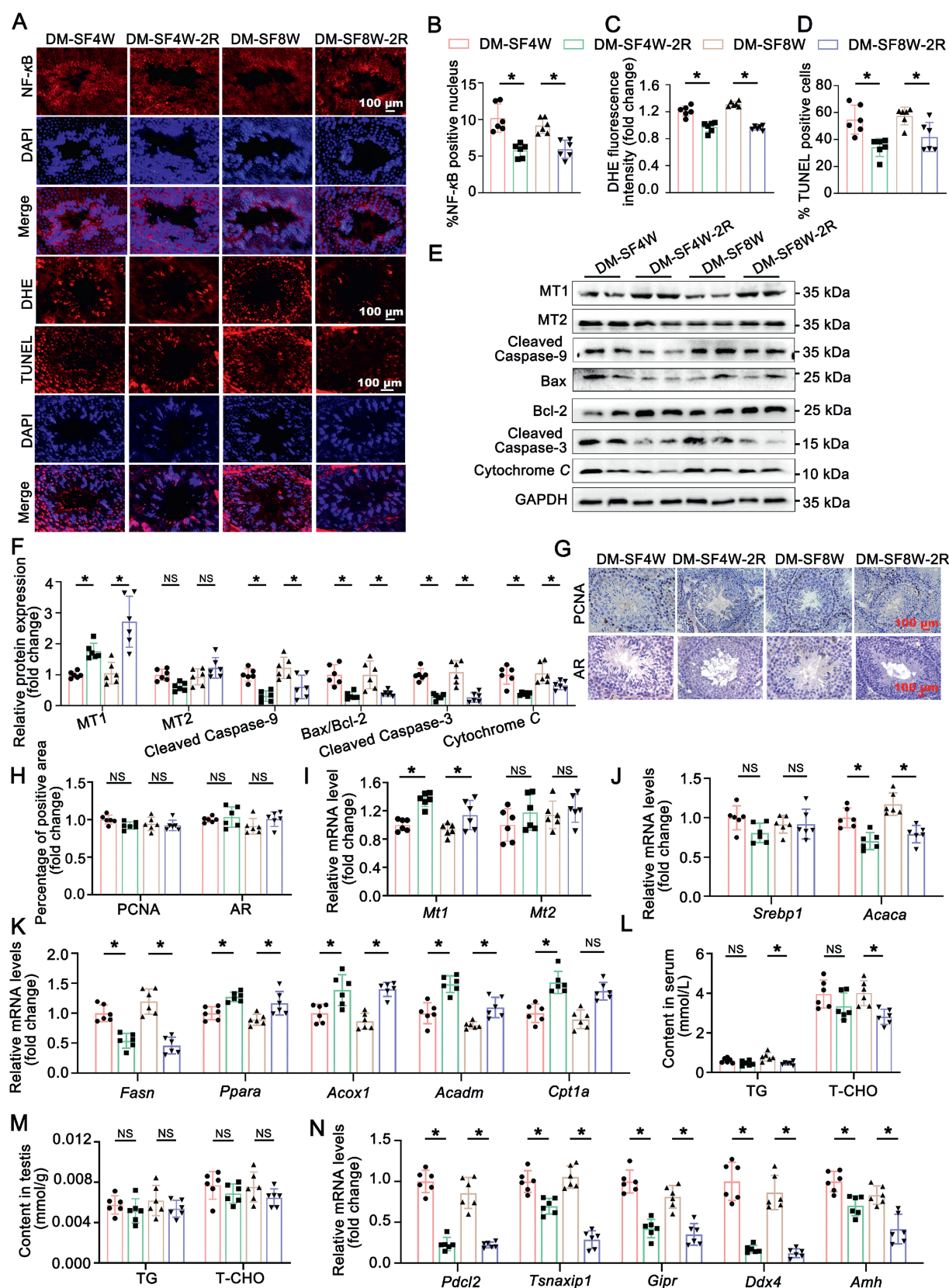


Figure 4 SR partially reversed testicular damage and MT1 expression in diabetic mice. (A–D) NF- κ B's nuclear accumulation, oxidative stress, and apoptotic cells were detected by IF staining with anti-NF- κ B p65 antibody (red), DHE staining (red) and TUNEL (red) assay in the testicular tissues, respectively ($n = 6$). (E, F) Western blot analysis of MT1, MT2, Cleaved Caspase-9, Bax, Bcl-2, Cleaved Caspase-3, and Cytochrome *c* and densitometric quantification in the testis of SF and SR mice ($n = 6$). (G, H) IHC staining of testicular proliferation was assessed using PCNA

κ B (Fig. 1D and E and Supporting Information Fig. S1A), similarly, the mRNA expression levels of pro-inflammatory genes such as tumor necrosis factor α (*Tnfa*), interleukin 1 beta (*Il1b*), and interleukin 6 (*Il6*) in the testis were also elevated after SF (Fig. 1F). Meanwhile, we observed that oxidative stress was remarkably increased after SF challenge, evidence by high levels of superoxide anion that stained by DHE in the DM-SF4W and DM-SF8W groups (Fig. 1G and H). Simultaneously, IHC staining results for SF diabetic mice exhibited significantly higher levels of oxidative stress markers 3-NT and 4-HNE in the testicular tissue (Fig. 1G and H). Consistent results were observed in the alterations of mRNA levels for heme oxygenase 1 (*Hmox1*), NAD(P)H quinone dehydrogenase 1 (*Nqo1*), and Catalase (*Cat*), respectively (Fig. 1I). To evaluate the impact of SF on apoptosis, we employed the TUNEL assay to detect testicular apoptosis. Following 4 and 8 weeks of SF, we observed a significant increase in fluorescence intensity in TUNEL-positive (TUNEL⁺) cells within the testicular tissue (Fig. S1B and S1C). Importantly, TUNEL⁺ cells exhibited significant overlap with DDX4⁺ cells, a marker of germ cells, indicating that SF primarily induces apoptosis within the germ cell population of testicular tissue (Fig. S1D). Concurrently, the apoptosis-related markers, including Cleaved Caspase-9, Cleaved Caspase-3, and Cytochrome *c*, as well as the ratio of the pro-apoptotic protein Bax to the anti-apoptotic protein Bcl-2, were notably elevated (Fig. 1J and K). PCNA is a protein synthesized and localized in the nucleus of cells. IHC staining demonstrated that the number of PCNA-positive cells in the seminiferous tubules was significantly reduced following SF, indicating a severe impairment in cell proliferation (Fig. 1K).

To further substantiate the aforementioned conclusion, we investigated the GC-1 spg cells cultured in high glucose (HG) DMEM, both with and without H₂O₂ stimulation. This methodology is widely acknowledged as an effective cellular model for replicating the *in vivo* conditions associated with SF, as previously reported³², and serves to confirm SF-induced testicular damage *in vitro* experiments. Not surprisingly, the expression levels of Cleaved Caspase-9 and Cytochrome *c* proteins, as well as Bax/Bcl-2 ratio were markedly elevated in the H₂O₂ 48-h group. Notably, the expression of Bcl-2 proteins exhibited a decline in the H₂O₂ 24-h group, followed by an increase in the H₂O₂ 48-h group. This inconsistency appears to be due to its regulatory response at different stages to adaptive H₂O₂ stimuli (Fig. S1E).

As reported, lipid metabolism and its byproducts play a crucial role in modulating testicular inflammation, oxidative stress, and apoptotic cell death. RNA-seq was performed to characterize transcriptome-wide changes with or without SF in diabetic testis. Based on Kyoto Encyclopedia of Genes and Genomes Orthology (KO) annotation, the differentially expressed genes (DEGs) identified between the DM-SF and DM groups were significantly enriched in lipid metabolism pathways (Fig. 2A). Additionally, Gene Set Enrichment Analysis (GSEA) further revealed that SF was negatively associated with both the fatty acid metabolism pathway and the lipid homeostasis pathway (Fig. 2B). Further analysis of DEGs following chronic sleep restriction (CSR) revealed significant enrichment in metabolic pathways, including the fatty acid metabolism pathway, fatty acid degradation pathway, and fatty acid

elongation pathway, within testicular tissues as determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Fig. 2C). GSEA analysis demonstrated CSR had a significant inhibitory effect on both the fatty acid degradation pathway and the fatty acid metabolism pathway (Fig. 2D). In alignment with these results, an analysis of mice subjected to short-term timed sleep restriction (5 consecutive days, GSE38921) also revealed enrichment of DEGs across several key pathways, including the PPAR signaling pathway, cholesterol metabolism pathway, fatty acid metabolism pathway, fat digestion and absorption pathway, and fatty acid elongation pathway (Fig. 2E and F). Together, the results obtained from our study, alongside other RNA-Seq data, reinforce the notion that lipid metabolism is closely regulated by sleep disorders in mice. Following this, the triglyceride (TG) and total cholesterol (T-CHO) concentrations were evaluated in serum and testicular tissue of diabetic mice. The level of TG in serum was increased after SF8W, but T-CHO increased in both DM-SF4W and DM-SF8W groups. Nevertheless, TG was only observed to elevate at DM-SF8W in testicular tissue homogenates (Fig. 2G). SREBP1 serves as the master transcription factor that promotes lipid synthesis, whereas peroxisome proliferator activated receptor alpha (PPARA) is critical for regulating lipolysis. Following SF interruption, the protein expression levels of SREBP1 increased, while those of PPARA decreased, resulting in abnormal lipid accumulation (Fig. 2H). Meanwhile, the increased mRNA levels of genes related to lipid synthesis, including *Srebp1*, acetyl-Coenzyme A carboxylase alpha (*Acaca*), and fatty acid synthase (*Fasn*), and the decreased mRNA levels of genes related to lipid catabolism, such as *Ppara*, acyl-Coenzyme A oxidase 1 (*Acox1*), acyl-Coenzyme A dehydrogenase (*Acadm*), and carnitine palmitoyltransferase 1a (*Cpt1a*), further corroborated the disorders of lipid metabolism in mouse testicular tissues (Fig. 2I). Consistent with these findings, GC-1 spg cells subjected to Oil Red O and Nile Red staining displayed severe lipid droplet accumulation after incubation with HG-DMEM, the effect of which appeared to be significantly increased with prolonged exposure to H₂O₂ (Fig. S1F).

3.2. SF-induced testicular injury and dysregulated lipid metabolism were alleviated by MEL and MT1 agonists

SF typically disrupts MEL secretion and impairs the functionality of melatonin receptors, a condition associated with an increased incidence of future T2DM³³. Indeed, SF significantly inhibited MEL secretion in T2DM mice (Supporting Information Fig. S2A). Furthermore, recognizing that MEL exerts its biological effects through melatonin receptors, we verified the expression of MT1 and MT2 in testicular tissue using the Human Protein Atlas database (Fig. 3A). Subsequently, we aimed to discern which melatonin receptors predominantly contribute to SF-induced testicular damage. As displayed in Fig. 3B and C, the mRNA and protein levels of MT1 in testicular tissue markedly decreased after SF, whereas the expression of MT2 showed no significant between-group differences, which was further confirmed by IF staining (Fig. S2B–S2D). Consistently, upon stimulation with H₂O₂ in GC-1 spg cells, we found the expression of MT1 was reduced at both protein and mRNA levels, yet

and AR ($n = 6$). (I) Relative mRNA levels of *Mt1* and *Mt1* in testis. Gene expression was normalized against *Gapdh* mRNA ($n = 6$). (J, K) Relative mRNA levels of *Srebp1*, *Acaca*, *Fasn*, *Ppara*, *Acox1*, *Acadm*, and *Cpt1a* in the testis of SF and SR mice ($n = 6$). (L, M) TG and T-CHO content in serum and testis homogenates ($n = 6$). (N) Relative mRNA levels of reproduction-related genes in testis ($n = 6$). Data shown in graphs represent the means $\pm$ SD; GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

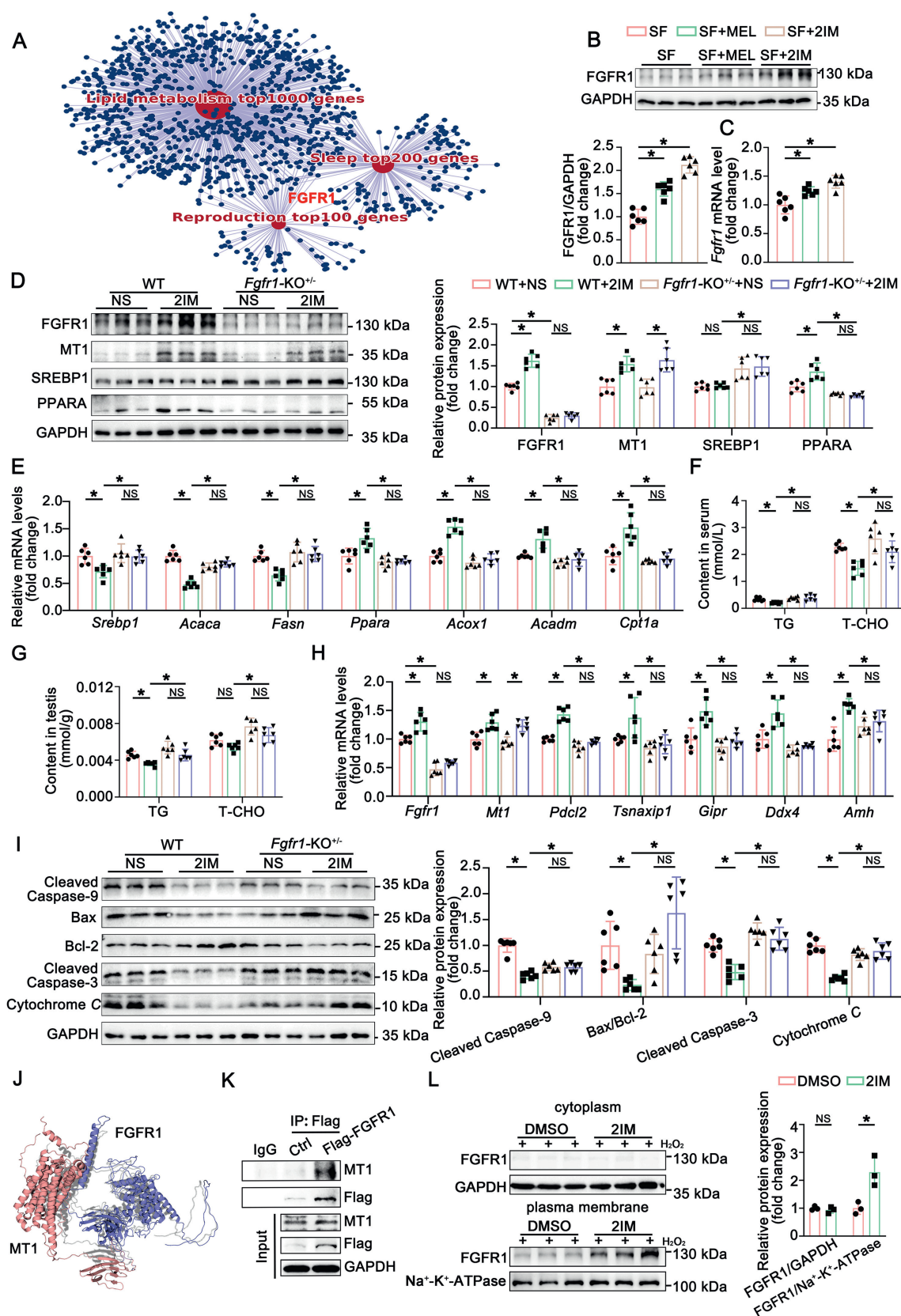


Figure 5 MT1 attenuates SF-induced testicular damage and aberrant lipid metabolism via FGFR1. (A) Network map of genes related to lipid metabolism, sleep, and reproduction obtained from GeneCards. (B) The protein expression of FGFR1 was detected by Western blot in the testis ($n = 6$). (C) Relative mRNA level of *Fgfr1* in the testis of mice treated with MEL or 2IM for 2 weeks ($n = 6$). (D) Western blot analysis of FGFR1, MT1, SREBP1, PPARA in WT mice and *Fgfr1*-KO^{-/-} mice treated with 2IM ($n = 6$). (E) Relative mRNA levels of *Srebp1*, *Acaca*, *Fasn*, *Ppara*, *Acox1*, *Acadm*, and *Cpt1a* in WT and *Fgfr1*-KO^{-/-} mice treated with NS or 2IM ($n = 6$). (F) Content in serum (mmol/L) of TG and T-CHO in WT and *Fgfr1*-KO^{-/-} mice treated with NS or 2IM ($n = 6$). (G) Content in testis (nmol/g) of TG and T-CHO in WT and *Fgfr1*-KO^{-/-} mice treated with NS or 2IM ($n = 6$). (H) Relative mRNA levels of *Fgfr1*, *Mt1*, *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4*, and *Amh* in WT and *Fgfr1*-KO^{-/-} mice treated with NS or 2IM ($n = 6$). (I) Western blot analysis of Cleaved Caspase-9, Bax, Bcl-2, Cleaved Caspase-3, Cytochrome C, and GAPDH in WT and *Fgfr1*-KO^{-/-} mice treated with NS or 2IM ($n = 6$). (J) 3D structure model of the FGFR1-MT1 complex. (K) Co-immunoprecipitation (Co-IP) assay. IP: Flag. Input: IgG, Ctrl, Flag-FGFR1. Western blot analysis of MT1, Flag, and GAPDH. (L) Western blot analysis of FGFR1 and GAPDH in cytoplasm and plasma membrane fractions of cells treated with DMSO or 2IM ($n = 6$). The bottom panel shows Na⁺-K⁺-ATPase (100 kDa) as a marker for the plasma membrane. The right panel shows the relative protein expression of FGFR1/GAPDH and FGFR1/Na⁺-K⁺-ATPase. Statistical significance is indicated by asterisks (*).

the change in MT2 was not statistically significant (Fig. S2E and S2F). To determine the key receptor target for testicular damage phenotype, the GC-1 spg cells were cultured in HG DMEM containing 2-iodomelatonin (2IM), a specific agonist of MT1, and 8-M-PDOT (8 MP), which selectively targets MT2. Pre-experimental results indicated that treatment with 2IM at a concentration of 25 $\mu\text{mol/L}$ for 24 h produced the most pronounced effect (Fig. S2G), while 8 MP was applied at 100 $\mu\text{mol/L}$ for 24 h based on prior studies. As expected, the administration of 2IM and 8 MP significantly increased the expression of MT1 and MT2, respectively. The H_2O_2 -treated group exhibited an increase in apoptosis-related proteins (Cytochrome *c*, Cleaved Caspase-3, and Cleaved Caspase-9). Notably, a significant reduction in the initiator Cleaved Caspase-9 and the executioner Cleaved Caspase-3 was observed in the H_2O_2 -2IM group; however, no significant difference in the anti-apoptotic effect was noted in the H_2O_2 -8 MP group (Fig. 3D). These findings suggest that MT1 may serve as the primary target involved in SF-induced testicular damage. Importantly, several diseases related to *Mt1* and *Mt2* genes were obtained from Disease Gene Network (DisGenet, <https://www.disgenet.org/home/>), among these, 27 diseases are particularly associated with melatonin receptors, including androgen-insensitivity syndrome, gestational diabetes, sleeplessness, hypersomnia, diabetes mellitus, and metabolic diseases (Fig. S2H), indicating that MEL may regulate multiple metabolic syndromes through its receptors. Thus, we further analyzed the relationship between melatonin receptors (mainly focusing on *Mt1*) and lipid metabolism. KO-based annotation showed that DEGs in the SF+2IM and SF groups were enriched in lipid metabolic pathways (Fig. S2I). Correlation analysis of the public dataset GSE33381 indicated a highly positive correlation between *Mt1* and lipid catabolism-related genes after timed sleep restriction for 2 weeks (Fig. 3E). Consistently, eight hub genes, including *Srebp1*, *Fasn*, *Il6*, and B cell leukemia/lymphoma 2 (*Bcl2*), were identified as being closely associated with *Mt1* in GSE33302 (Fig. 3F). Additionally, *in vivo* administration of 2IM at a dosage of 10 mg/kg confirmed its stimulatory effect on MT1 activation (Fig. S2J and S2K). Although no significant difference was observed in the testis-to-body weight ratio of diabetic mice receiving MEL or 2IM treatment following SF intervention, there was a marked improvement in testicular apoptosis (Fig. 3G and H). Notably, 2IM treatment also resulted in a substantial enhancement of lipid metabolism; however, this effect did not correlate with any prominent changes in SREBP1 protein expression (Fig. 3I). Simultaneously, MEL or 2IM treatment exerted a beneficial effect on the expression of testicular reproduction-related genes (Fig. 3J). Collectively, the above findings suggest that MEL, particularly MT1 agonists, can abrogate SF-induced testicular damage and dysregulated lipid metabolism, highlighting the central role of MT1 in the pathogenesis of testicular damage under SF diabetic condition.

3.3. Sleep recovery only partially reversed testicular damage and MT1 expression in diabetic mice

As reported, substantial evidence connects SF to dysfunctions in the immune, neuroendocrine, and inflammatory systems, leading to significant public health concerns^{34,35}. Consequently, the potential impact of SR on these adverse changes requires further investigation. Building on the aforementioned findings, we aimed to clarify whether SR could reverse, either partially or entirely, the testicular injury and diminished melatonin receptor activation observed in our study. To this end, we randomly assigned SF mice to undergo an additional two weeks of recovery sleep, designated as DM-SF4W-2R and DM-SF8W-2R. Our findings demonstrated two weeks of SR had an overtly ameliorative effect on the expression of NF- κ B and decreased the levels of superoxide anion in testicular cells (Fig. 4A–C and Supporting Information Fig. S3A). The TUNEL-positive cell, the ratio of Bax/Bcl-2 as well as the expression of Cytochrome *c*, cleavage of apoptosis-associated proteins Caspase-9, and Caspase-3 were apparently decreased by two weeks SR (Fig. 4A–D, E, and F). Notably, no significant change in PCNA and AR expression was observed following SR, indicating that merely 2 weeks of SR had minimal impact on dysregulated cell proliferation and sex hormone metabolism (Fig. 4G and H). Strikingly, we observed that SR in the testis led to a partial restoration of MT1 at both the mRNA and protein levels, while the expression of MT2 exhibited no significant differences across the various experimental conditions (Fig. 4I). Additionally, with the exception of *Srebp1*, the expression of most genes associated with key lipogenic enzymes, such as *Acaca* and *Fasn*, decreased, whereas genes involved in lipid oxidation, including *Ppara*, *Acox1*, *Acadm*, and *Cpt1a*, showed increased expression. This finding suggests a promotive role for SR in ameliorating dysregulated lipid accumulation (Fig. 4J and K). Furthermore, circulating levels of TG and T-CHO were significantly reduced in the DM-SF8W-2R group; however, SR did not produce any additional improvement in TG and T-CHO levels within the testicular tissue (Fig. 4L and M). Surprisingly, we found that testicular reproduction-related genes, including *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4*, and *Amh*, continued to decline in SR-treated mice, indicating that SR does not ameliorate the impairment of reproductive function (Fig. 4N). Collectively, unlike exogenous MEL supplementation or direct stimulation of MT1 using 2IM, SR alone is insufficient to fully reverse SF-induced reproductive dysfunction, thereby contributing to the progressive decline in testicular function.

3.4. MT1 attenuates SF-induced testicular damage and aberrant lipid metabolism via FGFR1

Based on the observations outlined above, we noted that SF-induced testicular damage is closely linked to dysregulated lipid metabolism. To elucidate the underlying molecular mechanisms

Ppara, *Acox1*, *Acadm*, and *Cpt1a* in testis tissues ($n = 6$). (F, G) TG and T-CHO content in serum and testis homogenate ($n = 6$). (H) Relative mRNA levels of *Fgfr1*, *Mt1*, *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4* and *Amh* in testis ($n = 6$). (I) Western blot analysis of Cleaved Caspase-9, Bax, Bcl-2, Cleaved Caspase-3 and Cytochrome *c* in testis ($n = 6$). (J) Predicted interaction of protein structures of MT1 and FGFR1 was modeled by ZDOCK (<https://zdock.umassmed.edu/>). (K) Co-IP assays performed the interaction between Flag-FGFR1 and endogenous MT1 in GC-1 spg cells. GAPDH was used as an internal control. (L) The protein expression of FGFR1 in the cytoplasm and plasma membrane of the testis was detected by Western blot analysis. GAPDH and $\text{Na}^+\text{-K}^+\text{-ATPase}$ were used as internal controls, respectively ($n = 3$). Data shown in the graphs represent the means $\pm$ SD, GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

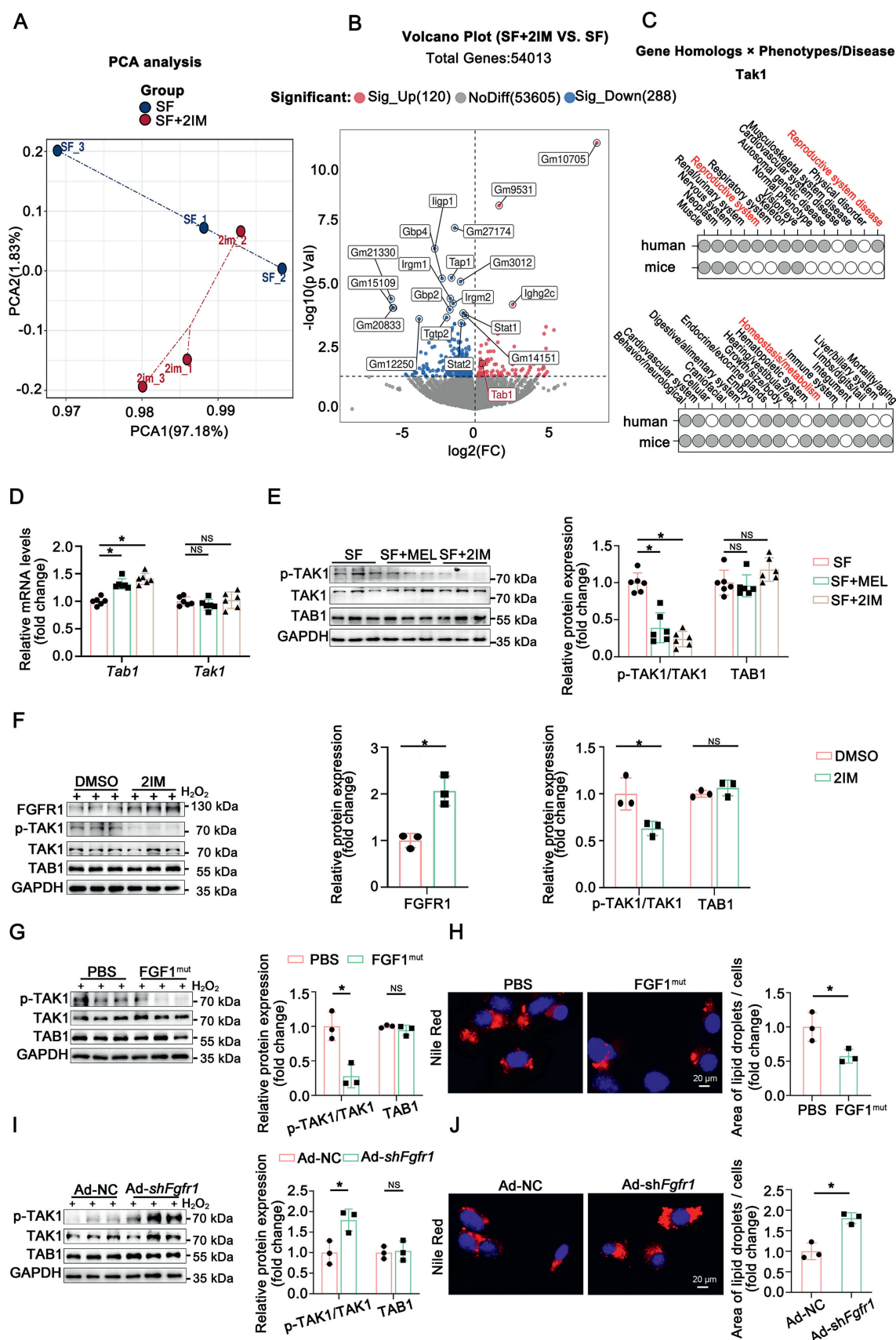


Figure 6 TAK1/TAB1 is a key downstream factor of MT1 and negatively regulated by FGFR1. (A) PCA analysis for SF and SF+2IM mice ($n = 3$). (B) Volcano plot of DEGs. (C) The association between gene homologs and phenotypes/diseases derived from the Human-Mouse

by which the MT1 influences lipid metabolism following SF, we utilized the GeneCards database to identify eight intersection genes that are concurrently associated with reproduction (top 100 genes), sleep (top 200 genes), and lipid metabolism (top 1000 genes). Among these, FGFR1 captured our particular interest (Fig. 5A). The robust association between FGFR1 and lipid metabolism has been extensively documented in the literature³⁶. To determine whether FGFR1 mediated SF-induced testicular lipid metabolism, we analyzed *Fgfr1* expression at the gene level in the testis after SF and SR interruption. Our findings showed that the expression of *Fgfr1* mRNA in the testis was much lower in SF4W and SF8W diabetic mice, although intriguingly there appeared to be a rebound increase in the DM-SF4W-2R group (Fig. S3B). We, therefore, assessed testicular levels of FGF21, which is known to promote lipid β -oxidation in the liver through FGFR1¹², but there were no differences in FGF21 expression in the testis after SF and SR (Fig. S3C), implying that change in the expression level of FGFR1 is independent of its ligand. We further validated the increase in FGFR1 expression after MEL or 2IM administration by RT-qPCR and Western blot, and this effect was more pronounced upon 2IM treatment (Fig. 5B and C). By transfecting GC-1 spg cells with *Fgfr1*-shRNA adenovirus (Ad-sh*Fgfr1*), we confirmed a substantial inhibitory effect on FGFR1 expression at a titer of 2.4×10^7 PFU/mL, compared to the control adenovirus (Supporting Information Fig. S4A). Consistent with the above results, we found that *in vitro*, 2IM significantly promoted FGFR1 expression in GC-1 spg cells, accompanied by an elevation of PPARA protein level. However, the ameliorating effect of 2IM was almost completely nullified when the cells were infected with *Fgfr1*-shRNA adenovirus (Fig. S4B).

Next, *Fgfr1* knockout (*Fgfr1*-KO^{+/-}) mice were utilized to comprehensively investigate the critical role of FGFR1 *in vivo*. Western blot analysis revealed that FGFR1 was barely expressed in the testis, heart, liver, and kidney of *Fgfr1*-KO^{+/-} mice (Fig. S4C). Wild-type (WT) and *Fgfr1*-KO^{+/-} diabetic mice were injected with 2IM or saline for two weeks after SF interruption. 2IM administration in WT mice remarkably promoted the expression of FGFR1, and the expression of PPARA was correspondingly increased, the effect of which was almost completely abrogated in *Fgfr1*-KO^{+/-} mice (Fig. 5D). Likewise, the effect of 2IM on mRNAs of lipid metabolism-related genes (*e.g.*, *Srebp1* and *Ppara*) was overtly abolished in *Fgfr1*-KO^{+/-} diabetic mice (Fig. 5E). However, the inhibition of FGFR1 did not lead to a significant decrease in TG and T-CHO levels in either serum or testicular tissue following 2IM administration (Fig. 5F and G). Furthermore, the SF-induced increase in the expression of apoptosis markers (*e.g.*, Cleaved Caspase-3) and the ratio of Bax/Bcl-2 in testicular tissues was reduced by the administration of 2IM. However, it is noteworthy that 2IM did not exert any effect in *Fgfr1*-KO^{+/-} diabetic mice (Fig. 5I). Accordingly, *Fgfr1*-KO^{+/-} diabetic mice exhibited complex disturbances of reproductive development and function after 2IM treatment, characterized by aberrant gene expression of *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4*, and

Amh (Fig. 5H). To elucidate the mechanisms by which MT1 regulated lipid metabolism and testicular injury through FGFR1, the predicted interaction of protein structures between MT1 and FGFR1 was performed by ZDOCK (<https://zdock.umassmed.edu/>), which showed that the free energy (Δ^1G) for docking was -49 kcal/mol, revealing the possibility of interaction between the two proteins (Fig. 5J). Furthermore, Co-IP studies in GC-1 spg cells demonstrated that MT1 interacts with Flag-FGFR1 (Fig. 5K). Notably, FGFR1 was predominantly absent from the cytoplasm, while its expression at the cell membrane was significantly enhanced following 2IM treatment (Fig. 5L). Collectively, these findings indicate that FGFR1 mediates the regulatory effects of MT1 on testicular lipid metabolism, thereby contributing to the improvement of reproductive function after SF in diabetic testicular tissues.

3.5. TAK1/TAB1 is a key downstream factor of MT1 and negatively regulated by FGFR1

To elucidate the underlying mechanism of MT1/FGFR1 on lipid metabolism after SF, transcriptome analysis of testicular tissues from mice in the SF group and SF+2IM group was conducted to identify the potential key modulator of SF-diabetic mice. Principal component analysis (PCA) was therefore applied to identify the characteristic features between the two groups (Fig. 6A). After analyzing the transcriptome data of SF+2IM-treated mice, we found that *Tab1*, a TAK-associated binding protein, is one of the up-regulated differentially expressed genes after 2IM treatment. As displayed in the Volcano Plot, there was no significant difference in the expression levels of *Tak1* (Fig. 6B). Considering that TAB1 directly regulates the phosphorylation state of TAK1 when it forms a signaling complex with TAK1³⁷, thereby regulating multiple cascading reactions, we further characterized the association of TAK1 in human disease and mouse models using the Human-Mouse: Disease Connection (HMD) of the MGI database, a robust tool that is useful for the discovery of candidate genes in mouse models of human disease. As revealed in Fig. 6C, *Tak1* is associated with metabolism homeostasis and reproductive phenotypes in mouse and human species. Given the evidence that inhibition of TAK1 phosphorylation mitigates obesity or HFD-induced damage to the liver, adipose tissue, and other cell types through various pathways, we aimed to further explore the role of TAK1 in SF-induced testicular damage and its relationship to lipid metabolism³⁸⁻⁴⁰. The mRNA level detection by RT-qPCR showed that *Tab1* was slightly but significantly up-regulated after MEL or 2IM treatment, while *Tak1* had no significant change (Fig. 6D). But the phosphorylation status of TAK1 was specifically reduced, whereas TAB1 expression at the protein level remained still unchanged in SF diabetic mice treated after MEL or 2IM treatment (Fig. 6E).

Consistent with *in vivo* findings, *in vitro* studies with GC-1 spg cells after H₂O₂ stimulation showed that administration of 2IM significantly increased FGFR1 protein levels, and the

database (<https://www.informatics.jax.org/diseasePortal>). The gray color indicates that TAK1 plays a role in the labeled diseases, while the white color indicates that it does not. (D) RT PCR for *Tab1* and *Tak1* in the testis ($n = 6$). (E) The protein expression of p-TAK1, TAK1, and TAB was detected by Western blot in the testis ($n = 6$). (F) Protein expression levels of FGFR1, p-TAK1, TAK1, and TAB1 in H₂O₂ stimulated GC-1 spg cells with 25 μ mol/L 2IM for 24 h ($n = 3$). (G, I) Western blot analysis of p-TAK1, TAK1, and TAB1 in GC-1 spg cells stimulated with H₂O₂ and then treated with FGF1^{mut} or infected with Ad-sh*Fgfr1* ($n = 3$). (H, J) Representative Nile Red staining of GC-1 spg cells stimulated with H₂O₂ and then treated with FGF1^{mut} or infected with Ad-sh*Fgfr1* ($n = 3$). Data shown in graphs represent the means $\pm$ SD; GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

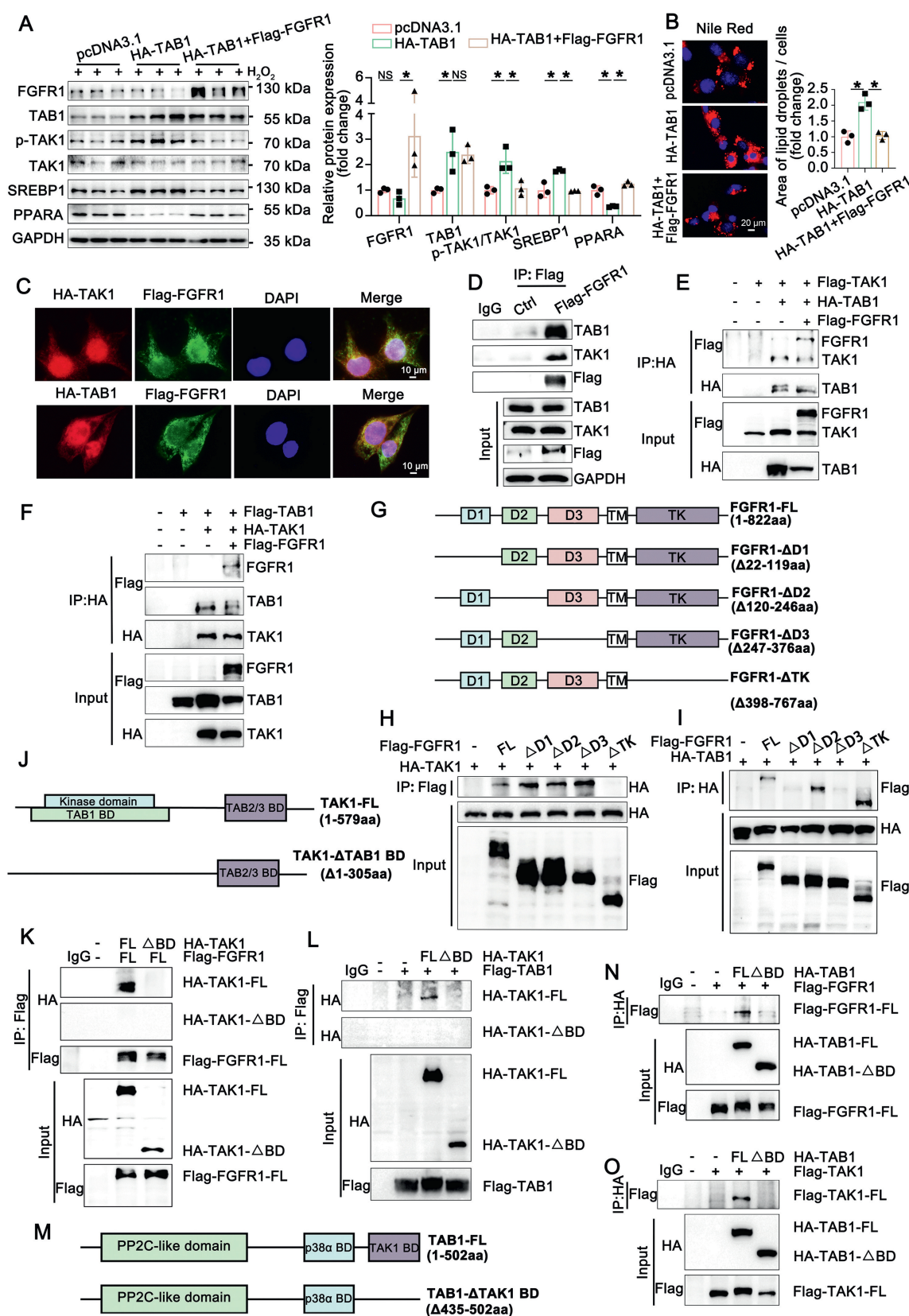


Figure 7 FGFR1 regulated testicular lipid metabolism by competitively inhibiting the interaction between TAB1 and TAK1. (A) GC-1 spg cells were treated with H_2O_2 , and then transfected with pcDNA3.1 plasmid or HA-TAB1 plasmid with or without Flag-FGFR1 plasmid for 48 h. The protein expression of FGFR1, TAB1, p-TAK1, TAK1, SREBP1, and PPARA was detected by Western blot with densitometric quantification of each group ($n = 3$). (B) Representative Nile Red staining of GC-1 spg cells ($n = 3$). (C) Colocalization of FGFR1 (green) and TAK1 or TAB1

phosphorylation level of TAK1 was likewise blocked (Fig. 6F). To investigate the role of FGFR1 on SF-induced TAK1 phosphorylation, pharmacological gain of function and genetic loss-of-function strategies were performed in GC-1 spg cells. FGF1^{mut}, a potent agonist of FGFR1, was found to effectively enhance FGFR1 expression at an optimal concentration of 10 ng/mL. However, as the concentration of FGF1^{mut} increased beyond this optimal level, a significant decrease in FGFR1 expression was observed in GC-1 spg cells. In comparison to the PBS group, FGFR1 expression was significantly elevated following the administration of FGF1^{mut} (S4D). Concurrently, phosphorylation of TAK1 was notably reduced, while the expression of TAB1 remained essentially unchanged. These molecular alterations were accompanied by a marked improvement in lipid accumulation (Fig. 6G and H). In contrast, the knockdown of FGFR1 using Ad-shFgfr1 markedly elevated the phosphorylation levels of TAK1 and exacerbated lipid deposition, while having no significant impact on TAB1 expression (Fig. 6I and J). Collectively, these demonstrations favorably suggested that FGFR1 regulated lipid accumulation by inhibiting TAK1 phosphorylation, while leaving TAB1 expression unaltered.

3.6. FGFR1 regulated testicular lipid metabolism and apoptosis by competitively inhibiting the interaction between TAB1 and TAK1

Given the inhibitory effect of FGFR1 on TAK1 phosphorylation, we constructed a *Tab1* overexpression vector with tagged proteins to further elucidate the role of TAB1 in this process (Supporting Information Fig. S5A), as well as the mechanism by which FGFR1 regulates TAK1 phosphorylation. FGFR1 overexpression with Flag-FGFR1 plasmid eliminated the effect of TAB1 on the phosphorylation level of TAK1, although TAB1 expression was not significantly affected. Then, PPARA expression was correspondingly increased and SREBP1 expression decreased (Fig. 7A). Importantly, overexpression of FGFR1 also mitigated the lipid accumulation induced by TAB1, as evidenced by Nile Red staining (Fig. 7B). These results suggest that FGFR1 does not directly inhibit TAB1 expression; rather, it modulates TAB1's ability to enhance TAK1 activation, ultimately contributing to the improvement of dysregulated lipid metabolism. The functional protein association network illustrates that FGFR1 may interact with both TAK1 and TAB1 (Fig. S5B). ZDOCK rigid-body docking showed that FGFR1 may have additional protein–protein docking or interaction domains with both TAK1 and TAB1 (Fig. S5C). Interestingly, immunofluorescence staining also showed strong co-localization of FGFR1 with TAK1 or TAB1 in GC-1 spg cells after transfecting Flag-FGFR1 and HA-TAK1 plasmids or HA-TAB1 plasmids respectively (Fig. 7C and Fig. S5D). Similarly, both exogenous and endogenous immunoprecipitation assays revealed that FGFR1 reciprocally co-immunoprecipitated with TAB1 and TAK1 in GC-1 spg cells as well as in 293T cells, thereby confirming a physical interaction among FGFR1, TAK1, and TAB1. Notably, FGFR1 displayed the

capability to bind to both TAK1 and TAB1 independently, thereby competitively inhibiting the interaction between TAB1 and TAK1 (Fig. 7D–F, Fig. S5E and S5F).

To explore the structural domains of FGFR1 interacting with TAK1 and TAB1, we used full-length or truncated *Fgfr1* plasmids co-transfected with *Tak1* or *Tab1* and we found that FGFR1 interacts with TAK1 through its tyrosine kinase structural domain (398–767 amino acid (aa)) and with TAB1 through its 22–119 aa and 247–376 aa (Fig. 7G–I). Unexpectedly, additional Co-IP experiments indicated that the 1–305 aa domain in TAK1 is responsible for binding both TAB1 and FGFR1. This finding suggests that FGFR1 exerts a competitive inhibition of the interaction between TAK1 and TAB1 (Fig. 7J–L and Fig. S5G). Similarly, FGFR1 competitively interacted with the domain in TAB1 that binds to TAK1 (435–502 aa), thereby disrupting the interaction between TAK1 and TAB1 (Fig. 7M–O and Fig. S5H). Overall, these results provide further support for our hypothesis that FGFR1 inhibits TAB1 from binding to TAK1 by occupying the structural domains of TAK1. This interaction thereby blocks TAB1's influence on TAK1 phosphorylation, ultimately contributing to improvements in lipid metabolism.

3.7. Testicular-specific overexpression of TAK1 reverses the protective effect of FGF1^{mut} on the testis

To determine whether the role of FGFR1 in testicular lipid accumulation depends on TAK1 activity, GC-1 spg cells were infected with Ad-shFgfr1 adenovirus. Subsequently, these cells were transfected with either *Tak1*-shRNA (sh*Tak1*) plasmid or *Tab1*-shRNA (sh*Tab1*) plasmid, utilizing the most effective knockdown constructs for each factor (Fig. S5I and S5J). The phosphorylation modification of TAK1 was barely detectable after TAK1 and TAB1 silencing. Correspondingly, the decrease in PPARA and increase in SREBP1 expression induced by FGFR1 knockdown were consistently counteracted by silencing TAK1 and TAB1 during H₂O₂ stimulation (Fig. 8A). Oil red O and Nile Red staining suggested that lipid accumulation was exacerbated at low expression profiles of FGFR1, which was reversed after transfection of sh*Tak1* or sh*Tab1* (Fig. 8B). To elucidate the role of TAK1 in FGFR1-mediated testicular injury in SF diabetic mice, we achieved testis-specific *Tak1* overexpression by testis-targeted injection of AAV9. AAV9 vehicle injection or FGF1^{mut} treatment had a more limited effect on testis-to-body weight ratio (Fig. 8C). Green fluorescence intensity was prominently observed in the testicular tissues of mice injected with AAV9-*Tak1*, indicating a greater enrichment of TAK1 in the testis compared to that in WT testis (Fig. 8D). The mRNA and protein levels of TAK1 were detected and we found specifically highly expressed in testicular tissue (Fig. 8E, Fig. S5K and S5L). However, the decrease in SREBP1 and the phosphorylation modification of TAK1, along with the increase in PPARA expression induced by FGF1^{mut}, were consistently counteracted by the overexpression of TAK1 in testicular tissue (Fig. 8F and G). Additionally, the reduction of T-CHO in serum, as well as TG and T-CHO in the testis induced by FGF1^{mut}, was reversed following the overexpression of TAK1 in testicular tissue (Fig. 8H). Similarly, FGF1^{mut} significantly improved key

(red) and in GC-1 spg cells transfected with Flag-FGFR1 and HA-TAK1 or HA-TAB1 plasmid. (D) Co-IP assays performed the interaction between Flag-FGFR1 and endogenous TAK1 and TAB1 in GC-1 spg cells. (E, F) Co-IP assay showed the interactions among FGFR1 and TAK1 or TAB1 in 293T cells transfected with indicated plasmids. (G–I) The binding domains of FGFR1 with TAK1 and TAB1 were explored by transfecting full-length or truncated Flag-FGFR1 plasmid and full-length HA-TAK1 or HA-TAB1 plasmid in 293T cells. (J–L) The binding region of FGFR1 and TAB1 by Co-IP assay in 293T cells. (M–O) The binding region of FGFR1 and TAK1 by Co-IP assay in 293T cells. Data shown in graphs represent the means $\pm$ SD; GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

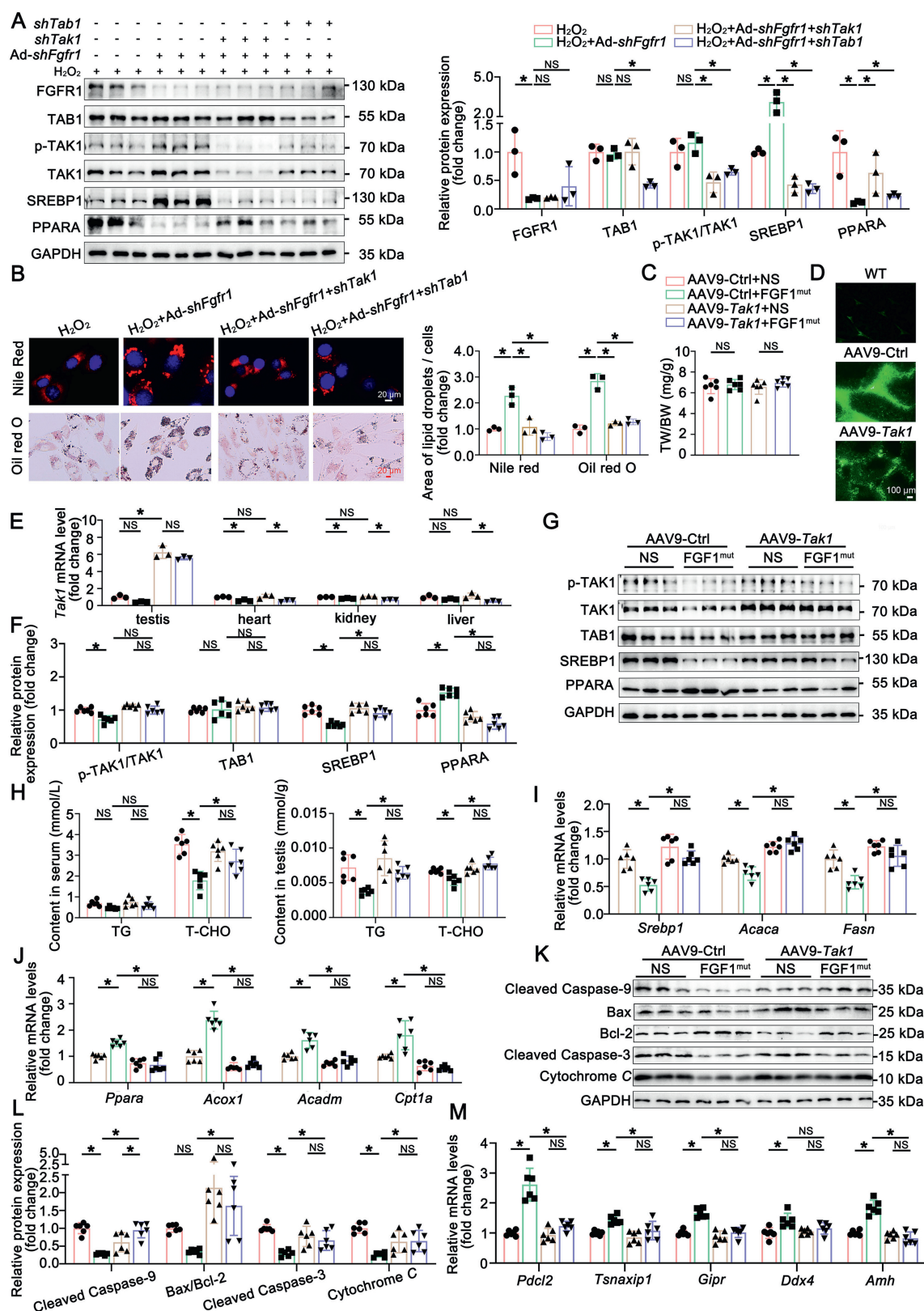


Figure 8 Testicular-specific overexpression of TAK1 reverses the protective effect of FGF1^{mut} on the testis. (A) GC-1 spg cells were treated with H_2O_2 , and then infected with ctrl or *shFgfr1* adenovirus and transfected with pcDNA3.1 plasmid or *shTak1* plasmid or *shTab1* plasmid for 48 h. The protein expression of FGFR1, TAB1, p-TAK1, TAK1, SREBP1, and PPARA was detected by Western blot analysis with densitometric quantification of each group ($n = 3$). (B) Lipid deposition was assessed by Nile Red staining and Oil Red O staining in GC-1 spg cells ($n = 3$). (C) The testis-to-body weight ratio in the different groups ($n = 6$). (D) Immunostaining against GFP (green) in frozen sections of testis 2 weeks after the local administration of 1×10^{10} vg of AAV9 on both sides. (E) The *Tak1* mRNA expression level in different organs from diabetic mice

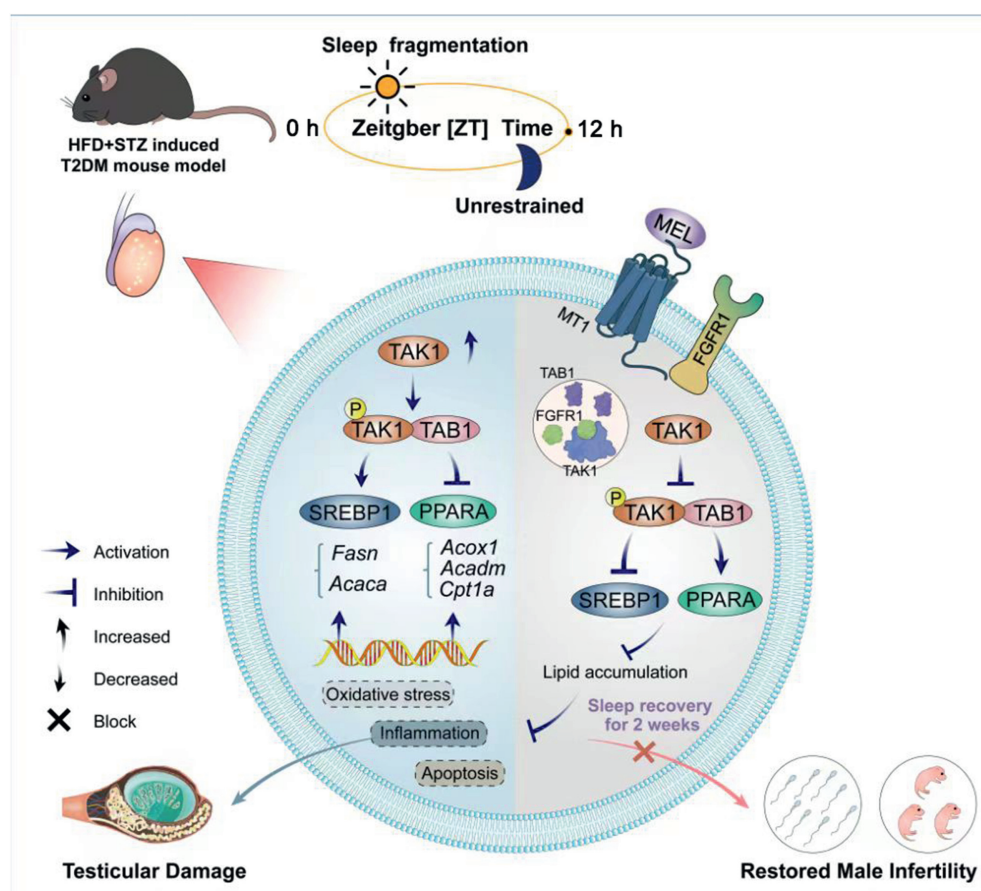


Figure 9 Schematic illustration depicting the role of melatonin and its receptor in alleviating testicular injury via the FGFR1–TAK1 pathway.

lipogenic enzymes including *Serbp1*, *Acaca* and *Fasn*, whereas correcting the aberrant expression of genes involved in lipid oxidation such as *Ppara*, *Acox1*, *Acadm*, and *Cpt1a*, the effect of which was almost completely eliminated after injection with testis-specific TAK1 overexpression (Fig. 8I and J). Similarly, the reduction in testicular apoptosis-related markers, including Cytochrome *c*, Cleaved Caspase-3, Cleaved Caspase-9, and the Bax/Bcl-2 ratio, induced by FGF1^{mut} was largely reversed following TAK1 overexpression in testicular tissue (Fig. 8K and L). Concurrently, the upregulation of reproduction-related genes, such as *Pdcl2* and *Tsnaxip1*, was significantly inhibited by AAV9-*Tak1* in the testis (Fig. 8M). Collectively, these findings suggest that FGFR1 exerts protective effects against dysregulated lipid metabolism and functional impairment in the testis primarily through the modulation of TAK1.

4. Discussion

Sleep is integral to life. Insufficient or disrupted sleep increases the risk of multiple pathological conditions, especially in the male

reproductive system. In the present study, we unveil a previously unrecognized role of MEL and its receptor in SF-induced testis damage. The main findings can be summarized as follows: 1) SF exacerbated lipid metabolism disorders, subsequently contributing to reproductive dysfunction in T2DM mice; however, the impairment of testicular reproductive function was not fully reversed by SR; 2) MEL and its receptor MT1 mitigated testicular damage by stimulating key genes involved in lipid synthesis and fatty acid β -oxidation, while positively regulating the expression of FGFR1 in a ligand-independent manner; and 3) FGFR1 alleviated reproductive dysfunction in SF-affected mice by inhibiting TAK1 phosphorylation. Mechanistic studies demonstrated that FGFR1 dampened the interaction between TAK1 and TAB1 by competitively binding to the structural domain on TAK1 responsible for TAB1 binding, thereby restricting the critical role of TAB1 on TAK1 activation (Fig. 9). This study is the first to elucidate the molecular mechanism underlying SF-induced testicular lipid accumulation and to evaluate the beneficial effect of MEL, offering new insights into the therapeutic management of male infertility in the T2DM population facing SF challenges.

injected with AAV9-Ctrl or AAV9-*Tak1* ($n = 3$). (F, G) The protein expression of TAB1, p-TAK1, TAK1, SREBP1, and PPARA was detected by Western blot analysis with densitometric quantification in AAV9-Ctrl mice or AAV9-*Tak1* mice treated with or without FGF1^{mut} for 2 weeks ($n = 6$). (H) TG and T-CHO content in serum and testis homogenate ($n = 6$). (I, J) Relative mRNA levels of *Srebfl*, *Acaca*, *Fasn*, *Ppara*, *Acox1*, *Acadm*, and *Cpt1a* in testis ($n = 6$). (K, L) Western blot analysis of Cleaved Caspase-9, Bax, Bcl-2, Cleaved Caspase-3 and Cytochrome *c* in testis. (M) Relative mRNA levels of *Pdcl2*, *Tsnaxip1*, *Gipr*, *Ddx4*, and *Amh* in testis ($n = 6$). Data shown in graphs represent the means $\pm$ SD; GAPDH was used as an internal control; * $P < 0.05$; NS, not significant.

Previous studies have established that lipid metabolism serves as a critical mechanism in testicular damage, as the maintenance of spermatogenic function and androgen hormonal secretion is significantly dependent on lipid homeostasis⁴¹. Furthermore, SF directly impacts nocturnal androgen production, which contributes to sperm dysplasia⁴². Additionally, it has been reported that SF can lead to excessive weight gain, elevated blood glucose levels, and an increased risk of insulin resistance, all of which accelerate the progression of T2DM^{6,7}. Collectively, these findings indicate that SF may exacerbate infertility in T2DM patients by disrupting lipid metabolism. However, comprehensive, population-representative data on this issue remain limited. In the present study, we confirmed the exacerbating effect of SF on testicular lipid deposition in T2DM mice and observed that a subsequent two-week period of SR could restore MT1 expression and alleviate testicular injury, although it was insufficient to reverse the reproductive dysfunction induced by SF. Indeed, the persistence of adverse effects of suboptimal sleep after SR has been demonstrated in numerous studies. Zhang et al.⁴³ demonstrated that testicular damage in rats persisted after 96 h of sleep deprivation, with incomplete recovery even after seven days of sleep recovery. Similarly, prolonged sleep deprivation lasting over two weeks was shown to cause varying degrees of testicular damage, which improved but was not completely reversed after five weeks of sleep recovery⁴⁴. Additionally, it is well established that a complete spermatogenic cycle—approximately 35 days in mice—is essential for sperm formation, and any damage incurred during this cycle can adversely affect subsequent sperm quality⁴⁵. Therefore, we speculated that the ineffectiveness of SR in achieving the desired outcomes may be attributed, in part, to the natural recovery period being less than one complete spermatogenic cycle. In contrast, melatonin and MT1 agonists effectively ameliorated the reproductive dysfunction induced by SF, likely due to the potent effects of pharmacological intervention.

The second innovation of our study is to highlight the potential role of MEL in SF-induced testicular injury. MEL is a hormone that is endogenously secreted by the pineal gland and is well recognized for its regulatory roles in a variety of central and peripheral functions related to circadian rhythms⁴⁶, reproduction¹⁵ and immunoregulation⁴⁷. A growing number of studies have shown that MEL can exert beneficial effects on the organism in a receptor-dependent manner^{48,49}. Furthermore, the positive impact of MEL on lipid metabolism is becoming progressively evident. Xu et al.¹⁵ revealed that MEL attenuated adipotoxicity and obesity-associated male infertility *via* upregulating SIRT1 signaling pathway. In contrast, Zhu et al.⁵⁰ found that in testicular Leydig cells of roosters, MEL reduced lipolysis of lipid droplets and inhibited testosterone synthesis by lowering intracellular free cholesterol levels. These findings suggest that MEL may exert divergent effects on male reproduction through its regulation of lipid metabolism. However, the specific role of MEL in SF-induced testicular damage remains poorly understood. Here, we present direct evidence that MEL dramatically prevents SF-induced lipid accumulation through its interaction with the MT1, ultimately reversing the adverse effects of SF on male reproductive function. Notably, the superior therapeutic efficacy of MEL compared to SR underscores its considerable potential in the treatment of male infertility associated with sleep disorders.

Our third significant finding is the exploration of the molecular regulatory mechanism by which MEL and its receptor MT1 modulate lipid accumulation. The FGFR1 signaling pathway has been shown to regulate diverse processes related to stress responses and lipid

metabolism¹². In the present study, we found that *Fgfr1* expression was significantly decreased in mice subjected to SF, while treatment with MEL or 2IM promoted FGFR1 expression and preserved the balance between lipogenesis and fat oxidation in testicular tissue. Considering that FGFR1 has been demonstrated to mediate the function of FGF21 across multiple tissues, we found it intriguing that there was no significant alteration in the expression level of FGF21 in the testis following SF and SR treatment. Similarly, in our other study, we observed a marked reduction in hepatic FGF21 functional changes in mice subjected to SF; however, a subsequent SR period of two weeks or more failed to effectively ameliorate these effects, revealing a “memory” phenomenon. These findings suggest that alterations in FGFR1 expression were not synchronized with FGF21 levels in the testis, indicating that FGFR1 expression in this context may be regulated by a ligand-independent pathway following SF intervention. Additionally, FGFR1 has been shown to be influenced by blood glucose levels^{51,52}. Consequently, alterations in the body’s internal environment induced by SF may exert a direct or indirect impact on the underlying mechanisms at play. Further investigations revealed that MT1 was present in the FGFR1 immunoprecipitation, and likewise, FGFR1 in the MT1 complex, testifying that MT1 and FGFR1 have physically interacted in tests. Although this study did not extensively explore the specific mechanism by which MT1 enhances FGFR1 expression, we speculate that this phenomenon may be closely linked to receptor tyrosine kinase (RTK) activation *via* G protein-coupled receptors (GPCRs) in trans⁵³, or that MT1 may enhance the stabilization of FGFR1 by inhibiting proteasome-mediated degradation or endocytosis⁵⁴.

Further mechanistic studies have elucidated that the TAK1 signaling pathway is a critical downstream target responsible for mediating the lipid metabolism disorders induced by aberrant FGFR1 expression following the SF challenge. TAK1, a member of the mitogen-activated protein kinase family, plays essential roles in several important processes, including inflammation, fibrosis, and lipid metabolism⁵⁵. In particular, its potential role in hepatic tissue lipid metabolism has been extensively studied and well documented, making it an attractive drug target. However, the role of TAK1 in reproduction, especially in testicular lipid metabolism, has not been fully investigated. Here, through RNA-Seq analysis, we observed an elevated expression of *Tab1* following SF and 2IM treatment. Considering that TAB1 is essential for TAK1 phosphorylation, the increased levels of TAB1 may be the result of complex feedback mechanisms stemming from the regulation of low levels of phosphorylated TAK1. Furthermore, we demonstrated that FGFR1 competitively interacts with the same domain of TAB1 that binds to TAK1 (amino acids 435–502), thus disrupting the interaction between TAK1 and TAB1. Unfortunately, considering that FGFR1 interacts with TAB1 through its extracellular structural domain in heterologous cells, the implications of this unexpected interaction in the pathological phenotype triggered by SF still require further investigation. Meanwhile, to further testify an essential role for TAK1 in the present study, we generated genetic-modified mice with testis-specific *Tak1* overexpression, which remarkably abolished the benefits of FGFR1 activation in SF mice, as evidenced by aberrant expression of genes involved in testicular lipid metabolism and a decline in reproductive function. These results indicate that FGFR1 primarily regulates the progression of testicular damage through TAK1. FGFR1 acts as a favorable inhibitor of TAK1 phosphorylation, highlighting its potential as a therapeutic target for the treatment of male infertility.

5. Conclusions

Overall, our study offers new insights into the mechanisms underlying impaired reproductive function in diabetic patients plagued by SF challenge and confirms the crucial role of lipid signaling networks in testicular homeostasis and disease. Importantly, we propose a novel perspective on the intervention strategy, *i.e.*, the exogenous supplementation of MEL and 2IM to trigger a previous-unrecognized regulatory axis *via* MT1/FGFR1/TAK1 signaling pathway, by which cell apoptosis and reproductive dysfunction can be markedly ameliorated in testis. This research offers a strong rationale for the application of MEL and 2IM, emphasizing their potential as effective treatments for male infertility in T2DM patients impacted by SF.

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Author contributions

Xiaohui Zhang: Writing – review & editing, Writing – original draft, Methodology. Xinyu Tang: Writing – review & editing, Methodology. Ting Gao: Investigation. Yuanfang Guo: Methodology. Guangping Lu: Methodology. Qingbo Liu: Validation. Jiahao Li: Formal analysis. Jie Wang: Validation. Mingrui Liu: Investigation. Dongmei Zhang: Investigation. Yufeng Tang: Writing – review & editing, Supervision. Junlian Gu: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.05.018>.

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