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SPNS1 deficiency alleviates the development of atopic dermatitis by suppressing keratinocyte proliferation

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ABSTRACT: This study aimed to investigate the role of the protein SPNS1 in the pathogenesis of atopic dermatitis (AD) and the underlying mechanisms, with a particular focus on keratinocyte proliferation and lysosomal function. Analysis of GEO datasets revealed a significant upregulation of SPNS1 in lesional skin from AD patients and DNCB-induced mouse models. An AD-like mouse model was established by applying DNCB to SPNS1 heterozygous ($SPNS1^{+/-}$) mice, and human keratinocytes (HaCaT) were stimulated with IL-4/IL-13 following SPNS1 knockdown. Epidermal thickness and cell proliferation were assessed via H&E staining and Ki-67 immunofluorescence, while cell viability, apoptosis, and lysosomal morphology were evaluated using the CCK-8 assay, flow cytometry, and immunofluorescence, respectively. The results showed that SPNS1 deficiency alleviated AD-like skin inflammation and epidermal hyperplasia. Both *in vivo* and *in vitro*, SPNS1 knockdown suppressed keratinocyte hyperproliferation, induced G1/S cell cycle arrest, and promoted apoptosis. Moreover, SPNS1 deficiency disrupted lysosomal morphology and function. Clinically, effective AD treatment was associated with downregulated SPNS1 expression. These findings demonstrate that SPNS1 promotes AD progression by driving keratinocyte hyperproliferation, potentially through the disruption of lysosomal homeostasis, thereby positioning SPNS1 as a promising therapeutic target for AD.

KEYWORDS: atopic dermatitis, SPNS1, keratinocytes, cell proliferation, apoptosis

INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin disease characterized by dry skin, chronic eczematous lesions, and intense pruritus [1, 2]. According to the Global Burden of Disease Study, AD affects approximately 15–20% of children and up to 10% of adults worldwide [3]. Unlike physiological acute itch, chronic pruritus in AD represents a pathological process driven by the convergence of skin barrier dysfunction, type 2 immune responses, and neural sensitization [1, 4]. A key feature of AD is the overexpression of type 2 cytokines, particularly interleukin-4 and interleukin-13 (IL-4 and IL-13), which not only amplify skin inflammation and barrier damage but also directly activate sensory neurons, thereby sustaining chronic itch [1, 5–7]. This process involves complex crosstalk among keratinocytes, immune cells, and sensory nerve endings, ultimately establishing an "itch-scratch" cycle that exacerbates skin lesions and inflammation [1, 4]. Elucidating the molecular and cellular mechanisms underlying pruritus and inflammation in AD is essential for developing novel targeted therapies aimed at disrupting this cycle and improving clinical outcomes.

SPNS1 (Protein spinster homolog 1), a ubiquitously expressed lysosomal transmembrane protein belonging to the Major Facilitator Superfamily(MFS) [8] that functions as a proton-dependent transporter of lysoglycerophospholipids [9–11]. These lysoglycerophospholipids are degradation products of lysosomal phospholipid catabolism, and their normal efflux is crucial for maintaining cellular lipid homeostasis [12]. Multiple studies have indicated that SPNS1 plays a key role in development and metabolism: In Drosophila, deletion of Spin/SPNS1 causes abnormal autolysosomes, leading to developmental delay and premature aging [13, 14]; in mice, neural-specific deletion of SPNS1 causes impaired myelination and hypomyelination [15]. Additionally, SPNS1 functions in the late stage of autophagy. Systemic SPNS1 knockout in mice leads to embryonic lethality between E12.5 and E13.5, accompanied by lipid accumulation in lysosomes, indicating that SPNS1 plays an important role in embryogenesis [11]. Recent clinical studies have identified that loss-of-function mutations in SPNS1

cause a multisystem disorder characterized by neurodevelopmental abnormalities, hepatosplenomegaly, and metabolic disturbances [16]. However, the role of SPNS1 in inflammatory skin diseases, particularly in AD, remains unclear.

Given the central role of keratinocyte proliferation in AD-associated skin barrier disruption and considering SPNS1's established function as a critical lysosomal lipid transporter, we hypothesized that SPNS1 might contribute to AD by dysregulating keratinocyte behavior and lysosomal homeostasis. The primary goals of this study were therefore: (1) to characterize SPNS1 expression patterns in skin tissues from AD patients and disease models; (2) to investigate the functional consequences of SPNS1 deficiency for AD-like inflammation and keratinocyte hyperproliferation *in vivo*; and (3) to elucidate the underlying cellular mechanisms, focusing on lysosomal function, cell cycle progression, and apoptosis in keratinocytes. This investigation seeks to define a novel role for SPNS1 in AD pathogenesis and assess its potential as a therapeutic target.

MATERIALS AND METHODS

Mice

Wild-type (C57BL/6) and SPNS1 heterozygous knockout (SPNS1$^{+/-}$, on a C57BL/6 background) mice were housed in an SPF-level animal facility. Male C57BL/6 mice were purchased from Cyagen (Suzhou, China) Biosciences Co., Ltd. SPNS1$^{+/-}$ heterozygous transgenic mice were kindly provided by CAM-SUGRC, Soochow University. The animals were provided with unlimited access to food and water. All animal experiments in this study were approved by the Laboratory Animal Ethics Committee of Guilin Medical University (Guilin, China), with an IACUC approval number of GLMC202303096.

Animal model of atopic dermatitis (AD)

The AD animal model used in our study was a 2,4-dinitrochlorobenzene (DNCB)-induced mouse model, with olive oil applied as the control. One day before modeling, the backs of all mice (approximately 3×4 cm in area) were shaved. Baseline physiological skin measurements were taken before initiating treatments (Day 1), including edema, erythema/bleeding, ulceration/epidermal peeling, scabbing/dryness, and ear thickness. Skin and ear appearances were documented via photographs, and body weight was recorded. To quantify AD-like symptoms, skin appearances were blindly scored independently by one or more investigators using a scale of 0 (none) to 3 (most severe). The cumulative score (range: 0–12) was used to assess the severity of clinical signs. From Day 1 to Day 4, 200 µl and 60 µl of 1% DNCB (Aladdin, Shanghai, China) solution were applied to the dorsal skin and left ear of mice in the DNCB-treated group every other day, respectively; the control group received equal volumes of solvent (acetone:olive = 3:1) on their dorsal skin and left ear. From Day 5 to Day 7, the mice were housed under normal conditions without any treatment. From Day 9 to Day 13, 0.5% DNCB solution was applied to the same sites using the same method. On Day 14, the mice were anesthetized and then sacrificed by cervical dislocation. Skin and ear tissues were collected for subsequent analysis.

Histology and immunofluorescence staining

For histological analysis, skin tissues from euthanized mice were fixed, paraffin-embedded, and sectioned into 4–6 µm slices. Sections were stained with a Hematoxylin and Eosin (H&E) kit (Servicebio, Hubei, China) to evaluate epidermal thickness, and imaged using a Leica light microscope (Leica, Wetzlar, Hesse, Germany). For immunofluorescence, sections were incubated overnight at 4 °C with primary antibodies against Ki67 (Servicebio, China) and SPNS1 (Abcam, Cambridge, Cambridgeshire, UK). After PBS washes, sections were incubated with secondary antibodies, counterstained with DAPI (Solarbio, Beijing, China), and visualized with a Nikon confocal microscope (Nikon Corporation, Tokyo, Japan).

Induction of the *in vitro* atopic dermatitis model

Cells were stimulated with 10 ng/ml recombinant IL-13 (Thermo Fisher Scientific, Waltham, MA, USA) and IL-4 (Thermo Fisher Scientific) in medium supplemented with 2% (v/v) FBS to recapitulate numerous features of AD [6].

RNA interference

The cells were seeded 12 h prior to transfection to reach 30–50% confluence. Then, they were transfected with 40 nM siRNA using siRNA-mate plus (Genepharma, Shanghai, China) and Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific), according to the manufacturers' instructions. SPNS1 siRNA (Genepharma) was utilized, with the sequences as follows: forward: 5′GCUCCAAAGUGAAGGAUAU3′, reverse: 5′AUAUCCUUCACUUUGGAGC3′.

Real-time quantitative PCR

Total RNA was extracted from skin tissues or cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript™ Fast RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Kusatsu, Japan). Real-time PCR was carried out using SYBR Green Master Mix (Bio-Rad, CA, USA) on a CFX Connect system (Bio-Rad). The primer sequences used for PCR amplification were as follows: (human) hSPNS1-F, 5′-CCAAGCAGATGATCCTGATGAC-3′, SPNS1-R, 5′-TGTAGCCCCTCACAGTCTGG-3′. *mCXCL2*-F: 5′-TCCA GGTCAGTTAGCCTTGC-3′, *CXCL2*-R: 5′-CGGTCAAAA

AGTTTGCCTTG-3′; *mTnf*-F: 5′-GGTGCCTATGTCTC AGCCTCTT-3′, *mTnf*-R: 5′-CGATCACCCCGAAGTTC AGTAG-3′; *IL-6*-F: 5′-TAGTCCTTCCTACCCCAATTT CC-3′, *mIL-6*-R: 5′-TTGGTCCTTAGCCACTCCTTC-3′; *mIL-1b*-F: 5′-GCAACTGTTCCTGAACTCAACT-3′, *IL-1b*-R, 5′-ATCTTTTGGGGTCCGTCAACT-3′, m: mouse. The mRNA expression levels of the analyzed genes were normalized and quantified using the $2^{-\Delta\Delta Ct}$ method.

Lyso-tracker staining

Cells were stained with 50 nM LysoTracker Red (Beyotime, Shanghai, China) in medium for 30 min. Hoechst 33342 (1:100) (Solarbio) was then added for the final 10 min of incubation. Live cells were imaged using a Nikon AX series confocal microscope (Nikon Corporation) equipped with NIS-Elements 6.0 imaging software.

Apoptosis assay and cell cycle flow cytometry

The apoptotic rate of HaCaT cells was determined using an Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Analysis was performed on an Agilent NovoCyte Quanteon flow cytometer (Agilent Technologies, CA, USA), and data were processed with FlowJo software. Cell Cycle Analysis: HaCaT cells were treated as indicated, fixed in 70% ethanol overnight at −20 °C, and stained with FxCycle™ PI/RNase Staining Solution (Thermo Fisher Scientific). Cell cycle distribution was analyzed on an Agilent NovoCyte Quanteon flow cytometer (Agilent Technologies), recording at least 10,000 events per sample. Data analysis was performed using FlowJo software (BD Biosciences).

CCK8 and colony formation assay

Cell Counting Kit-8 (CCK-8) Assay: HaCaT cells were seeded in 96-well plates at 1,500 cells per well. Each well received 10 µl of CCK-8 solution (Beyotime) daily for 5 days. After 4 h of incubation on the final day, absorbance at 450 nm was measured using a Varioskan LUX multimode microplate reader (Thermo Scientific). Colony Formation Assay: HaCaT cells were plated in 6-well plates and cultured for 2 weeks. Resulting colonies were fixed with 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet for 30 min at room temperature.

nrichment analysis of KEGG pathway and construction of PPI network

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs was performed with "cluster Profiler" R package [17]. FDR < 0.05 was used in the study. To further select the crucial modules and genes, protein-protein interaction (PPI) analysis of the DEGs was performed by the STRING database (https://cn.string-db.org/) with a medium confidence score more than 0.4. Then a PPI network was created using Cytoscape software and the key modules were constructed by Molecular Complex Detection (MCODE) module based on the cutoff score ⩾ 2 [18].

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 software and are presented as the mean ± SEM. The results were analyzed using two-tailed, unpaired Student's *t*-test (two groups) or one-way ANOVA (more than two groups). Different levels of significance were denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns, not significant.

RESULTS

SPNS1 is upregulated in AD and a DNCB-induced mouse model

To investigate the association between SPNS1 and AD, we collected three Gene Expression Omnibus (GEO) datasets (GSE16161, GSE32924, GSE121212) containing gene expression profiles of lesional and non-lesional skin from AD patients (Fig. 1a). Analysis revealed that SPNS1 mRNA expression was significantly higher in lesional skin compared to non-lesional skin from AD individuals across all three datasets. Furthermore, SPNS1 demonstrated excellent diagnostic performance (AUC > 0.75) (Fig. 1b), suggesting a potential correlation between SPNS1 and AD development. Furthermore, immunofluorescence staining showed elevated levels of SPNS1 in the epidermis of the DNCB-induced AD mouse model, which strongly correlated with the pathological progression of AD (Fig. 1c). Collectively, these data indicate that SPNS1 expression is upregulated in AD lesions and is associated with AD pathogenesis.

SPNS1 deficiency ameliorates the AD-like phenotype

To define the functional role of SPNS1 in AD, we generated SPNS1 heterozygous knockout ($SPNS1^{+/-}$) mice, as previous studies indicated that constitutive SPNS1 knockout results in embryonic lethality at E12.5–E13.5(11).We then established an AD mouse model by daily topical application of DNCB (Fig. 2a). This model recapitulated key features of AD, including elevated levels of the type 2 cytokines IL-4 and IL-13 [19, 20]. Compared to control mice treated with olive oil alone, DNCB-treated mice developed severe skin inflammation with histopathological features reminiscent of human AD, such as epidermal hyperplasia and immune cell infiltration. H&E staining revealed that under steady-state conditions, SPNS1 haploinsufficiency did not significantly affect epidermal thickness (Fig. 2d,e), suggesting that SPNS1 is not essential for skin homeostasis in the absence of external stimuli or pathological challenge. In the AD model, however, $SPNS1^{+/-}$ mice exhibited reduced dermatitis area and severity scores (including assessments of erythema

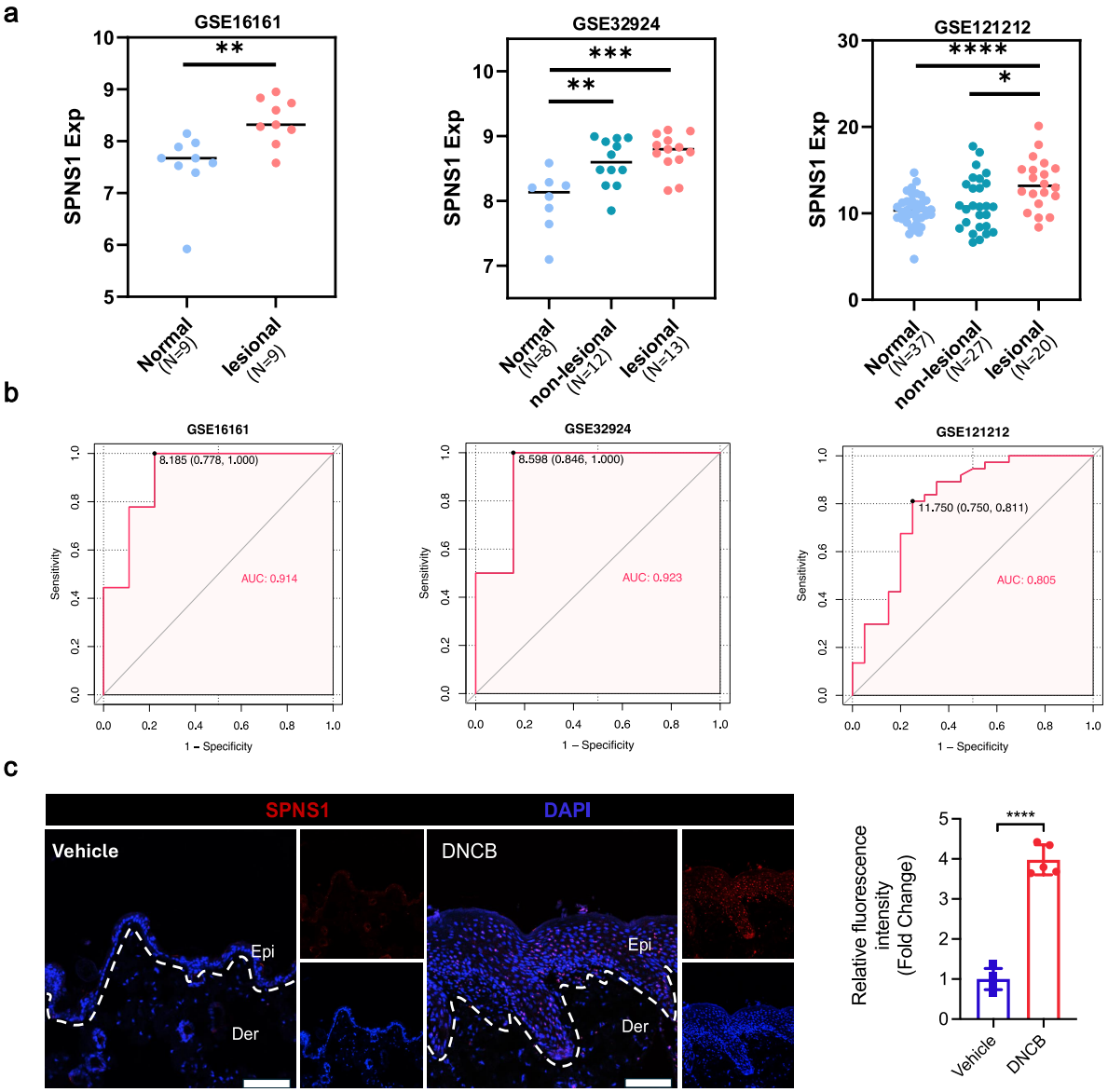

Fig. 1 SPNS1 is upregulated in AD and a DNCB-induced mouse model. (a) The mRNA levels of SPNS1 in the skin from non-lesional skin and lesional skin of AD patients analyzed using the GEO database. (b) ROC curve analysis of SPNS1 mRNA expression in the GEO database. (c) Immunofluorescent and quantitative analysis of SPNS1 in the skin from vehicle-treated mice and DNCB-treated mice. Nuclei were stained with DAPI (blue) ($n = 5$). Scale bars = 100 µm. Error bars represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$. The p value was determined using two-tailed unpaired Student's t-test or one-way ANOVA.

and scaling), and decreased epidermal thickness, compared to wild type (WT) (Fig. 2b,c). To further validate these observations, we employed an independent AD model using DNCB-challenged ear skin [21, 22]. Consistent with the dorsal skin results, no significant phenotypic or histological alterations were observed in the ears of SPNS1$^{+/-}$ mice under unstimulated conditions. In the AD context, both WT and SPNS1$^{+/-}$ mice developed aggravated skin symptoms and marked epidermal thickening (Fig. 2f,g). Notably, SPNS1$^{+/-}$ mice showed significantly attenuated ear redness, scaling, and thickening compared to their WT littermates.

Given that accelerated keratinocyte proliferation is a key driver of AD pathogenesis [23, 24], we further assessed proliferative changes in SPNS1$^{+/-}$ mice via immunohistochemical analysis of Ki-67. Under unstimulated conditions, SPNS1 haploinsufficiency did not alter Ki-67 expression, indicating that keratinocyte

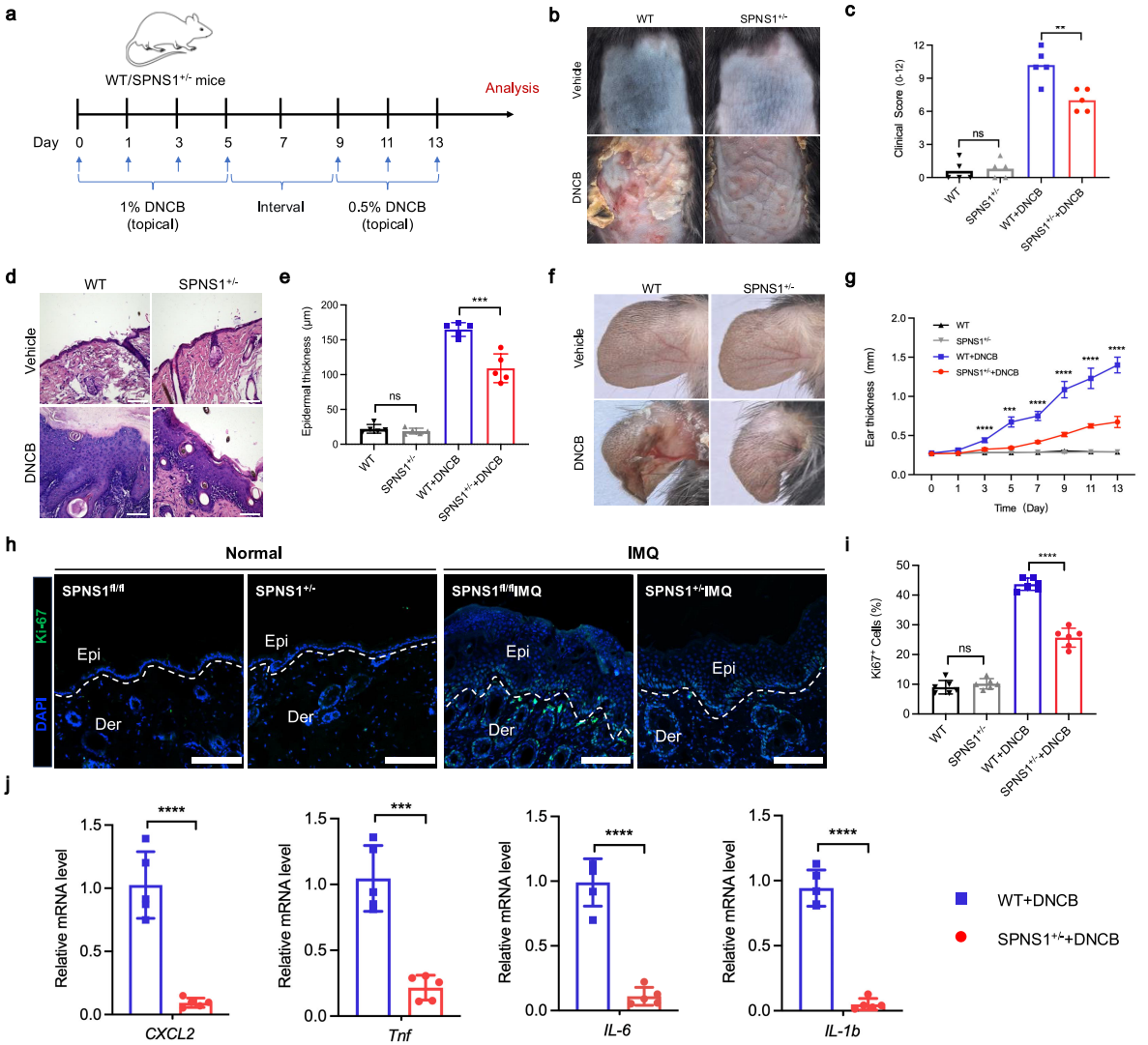

Fig. 2 SPNS1 deficiency ameliorates the AD-like phenotype. (a) Schematic diagram of the experimental timeline and DNCB treatment protocol. (b) Representative images of the dorsal back skin from vehicle- or DNCB-treated mice on day 11. (c) Clinical scores of the vehicle- or DNCB-treated mice on day 9, $n = 5$ mice/group. (d) Representative H&E staining of the dorsal back skin from WT and SPNS1$^{+/-}$ mice treated with vehicle- or DNCB for 11 days, scale bars = 100 μm. (e) Quantification of epidermal thickness. $n = 5$ mice/group. (f) Representative images of vehicle- or DNCB-treated WT and SPNS1$^{+/-}$ mice on day 9. (g) Ear thickness (mean ± SEM) of vehicle- or DNCB-treated WT and SPNS1$^{+/-}$ mice. $n = 5$–6 mice/group. (h–i) Immunofluorescent and quantitative analysis of Ki-67 positive cells in the skin from WT and SPNS1$^{+/-}$ mice treated with vehicle- or DNCB for 9 days. Nuclei were stained with DAPI (blue) ($n = 5$). Scale bars = 100 μm. (k) qRT-PCR analysis of CXCL2, *Tnf*, *IL-6* and *IL-1b* mRNA levels in the skin from WT and SPNS1$^{+/-}$ mice treated with DNCB for 9 days ($n = 5$). Error bars represent the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$. The p value was determined using two-tailed unpaired Student's t-test or one-way ANOVA.

proliferation remains unaffected under homeostasis (Fig. 2h,i). In the AD context, however, immunofluorescent analysis revealed a marked upregulation of Ki-67 in keratinocytes, which was effectively suppressed by SPNS1 haploinsufficiency. To investigate the impact of SPNS1 on the inflammatory response in the development of AD, we quantified the mRNA expression levels of various inflammatory markers by qRT-PCR. The expression of the tumor necrosis factor (Tnf), neutrophil chemotactic factors (CXCL2), and proinflammatory cytokines interleukin-6 (IL-6) and IL-1β was lower in lesional skin from SPNS1$^{+/-}$ mice compared to WT mice (Fig. 2j). These findings provide important insights into the potential role of SPNS1 in modulating keratinocyte proliferation and inflammation in response to DNCB challenge, suggest that

SPNS1 deficiency mitigates DNCB-induced skin inflammation and hyperproliferation in mice. Together, these results offer key evidence supporting the promotive role of SPNS1 in AD pathogenesis.

SPNS1 is required for keratinocyte proliferation

Having observed that SPNS1 expression is upregulated in the lesional skin of AD individuals and the epidermis of a DNCB-induced mice model (Fig. 1a–c), we hypothesized that SPNS1 may play a regulatory role in keratinocyte function during AD pathogenesis. To further investigate the specific function and underlying mechanisms of SPNS1 in AD, we employed small interfering RNA (siRNA) to silence endogenous SPNS1 expression in immortalized human keratinocytes (HaCaT) (Fig. 3a). Quantitative PCR analysis confirmed the efficient knockdown of SPNS1 at the mRNA level. Since the overproduction of type 2 cytokines, such as IL-4 and IL-13, is a hallmark of AD that drives inflammation [25, 26], we treated HaCaT cells with 10 ng/ml IL-4 and IL-13 for 24 h to establish an *in vitro* AD-like inflammation model, with untreated cells serving as controls (Fig. 3b). CCK-8 assays and colony formation experiments revealed that SPNS1 knockdown significantly suppressed keratinocyte proliferative capacity under both basal and IL-4/13-stimulated conditions (Fig. 3c–f). To further validate these findings, we performed Ki-67 immunofluorescence staining to visualize actively proliferating cells. The results demonstrated that the number of Ki-67-positive cells was significantly reduced in the si-SPNS1 group compared with the WT controls, both in the absence and presence of IL-4/13 stimulation (Fig. 3g,h). These *in vitro* findings are consistent with our previous *in vivo* observations from the DNCB-induced AD mouse model, in which SPNS1 deficiency similarly attenuated epidermal proliferation and Ki-67 expression. Taken together, our data from both *in vivo* and *in vitro* experiments collectively supporting the role of SPNS1 in driving keratinocyte hyperproliferation.

SPNS1 promotes keratinocyte proliferation by regulating cell cycle progression

Building on our previous findings that SPNS1 promotes keratinocyte proliferation in both *in vivo* and *in vitro* models, we sought to delineate the underlying molecular mechanisms. We first identified the top 10 protein interactors of SPNS1 using the STRING database and visualized the PPI network with Cytoscape (Fig. 4a). Through Cytoscape analysis, we have identified potential interactions between SPNS1 and proteins. Notably, given the established roles of BCL2 and BCL2L1 as key anti-apoptotic proteins [27], our findings strongly suggest that SPNS1 may influence cell survival by regulating the mitochondrial pathway of apoptosis. Furthermore, the interactions between SPNS1 and other members of the MFS, such as MFSD10 and MFSD2B, indicate the potential existence of a coordinated intracellular network for lipid transport [8]. Subsequent KEGG pathway enrichment analysis indicated significant involvement of SPNS1 in key biological processes, including the cell cycle, apoptosis, and the p53 signaling pathway (Fig. 4b), suggesting its potential role in regulating cell cycle progression and apoptosis during AD pathogenesis. To validate these predictions and elucidate the specific role of SPNS1 in cell cycle regulation in AD, we utilized flow cytometry to analyze the cell cycle distribution. The results demonstrated that SPNS1 knockdown significantly increased the proportion of cells in the G1 phase under both unstimulated conditions and upon IL-4/13 stimulation (Fig. 4c,d), indicating cell cycle arrest at the G1 phase. This finding provides direct evidence that SPNS1 deficiency inhibits keratinocyte proliferation by impeding the G1/S phase transition. Considering that enhanced proliferative capacity is often associated with suppressed apoptosis, we further investigated whether SPNS1 participates in the regulation of keratinocyte apoptosis. As expected, flow cytometric analysis demonstrated that the percentage of Annexin V-FITC+ apoptotic cells was significantly higher in the si-SPNS1-treated group than in the untreated group (Fig. 4e,f), indicating that SPNS1 loss promotes keratinocyte apoptosis in this IL-4/13-induced AD-like model. Collectively, our findings establish a mechanistic role for SPNS1 in promoting keratinocyte proliferation by facilitating cell cycle progression and suppressing apoptosis.

SPNS1 deficiency induces lysosomal dysfunction and represents a therapeutic target in AD

SPNS1 has been implicated in the regulation of lysosomal pH homeostasis and cellular senescence [11, 28]. In adult mice, knockdown or genetic deletion of SPNS1 results in pathologies including liver inflammation, demyelination in the brain, and skeletal muscle atrophy [13, 29]. Such pathological changes are associated with enlarged lysosomes and impaired autophagy, similar to the zebrafish models of SPNS1 deficiency [11]. However, the role of SPNS1-mediated lysosomal dysregulation in AD pathogenesis remains unclear. To investigate the relationship between SPNS1-mediated lysosomal function and AD, we knocked down SPNS1 in HaCaT cells using siRNA and visualized the lysosomal compartments with LysoTracker staining. Our results demonstrated that the number and size of lysosomes increased following the knockdown of SPNS1 under both basal and IL-4/13 conditions (Fig. 5a,b), indicating lysosomal dysfunction. These findings support the notion that SPNS1 deficiency-induced lysosomal dysregulation may contribute to skin barrier impairment in AD.

To further assess the pathological relevance of these findings, we examined the clinical translational value of SPNS1 by analyzing gene expression data from skin specimens of AD patients treated with three

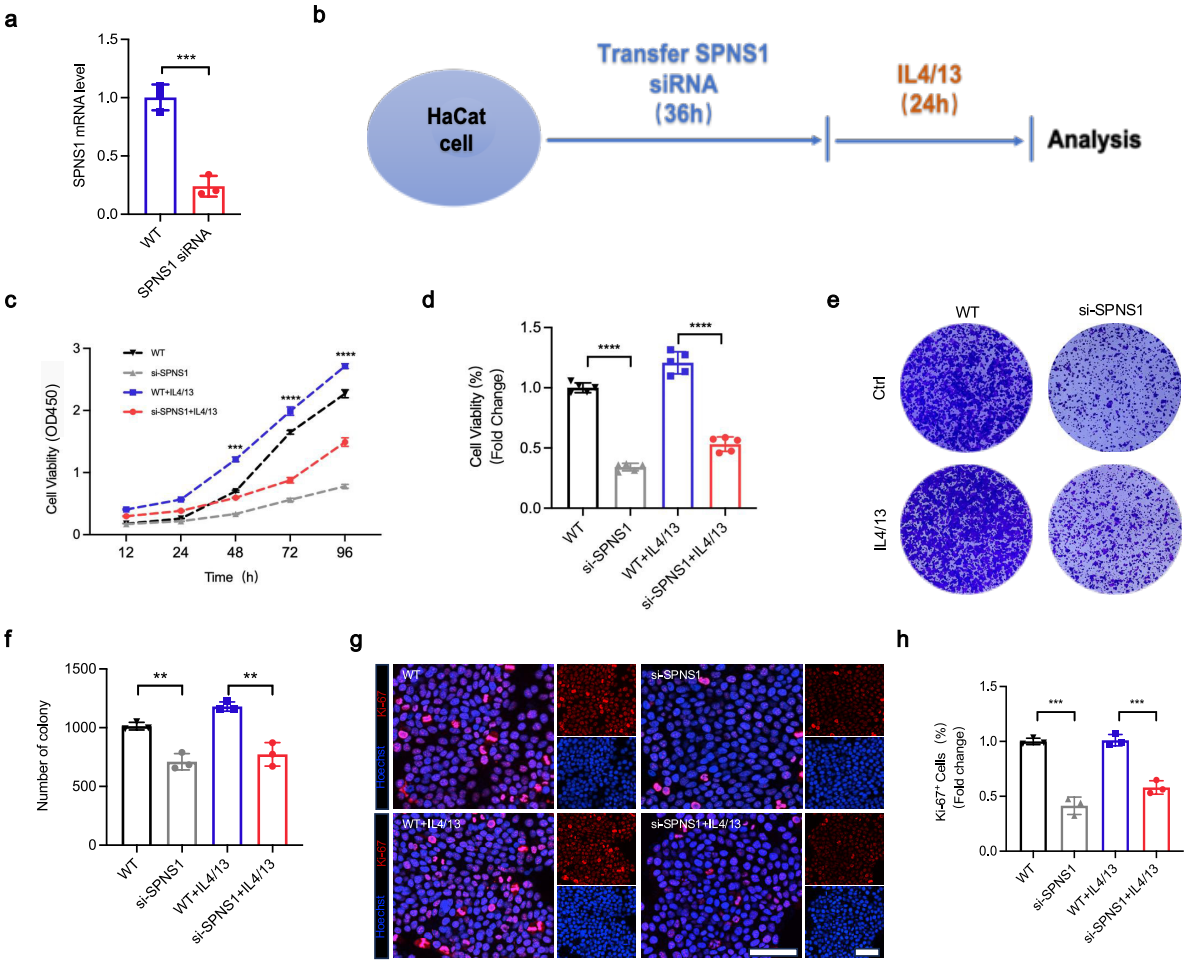

Fig. 3 SPNS1 is required for keratinocyte proliferation. (a) Quantification analysis of SPNS1 mRNA levels in HaCaT cells that were transfected with control or si-SPNS1. β-Actin was used as a loading control ($n = 3$). (b) Schematic diagram of IL-13/4 treatment in SPNS1-knockdown HaCaT cells. (c–d) CCK-8 assay of WT and SPNS1-knockdown HaCaT cells treated with IL4/13 for 24 h. The OD450 values (c) and corresponding cell viability (d) are shown. (e–f) Colony formation assay in WT and si-SPNS1 HaCaT cells treated with IL4/13, with quantitative analysis of the colony numbers shown. (g–h) Immunofluorescent and quantitative analysis of Ki-67-positive WT and si-SPNS1 HaCaT cells treated with or without IL-4/13 for 24 h. Nuclei were stained with Hoechst (blue) ($n = 3$). Scale bars = 100 µm. Error bars represent the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$. The p value was determined using two-tailed unpaired Student's t-test or one-way ANOVA.

therapeutic agents (Fezakinumab, Dupilumab, and Crisaborole). Analysis of post-treatment samples revealed a consistent downward trend in SPNS1 expression across all treatment groups, with Crisaborole treatment achieving statistically significant reduction in SPNS1 levels (Fig. 5c). These clinical observations strengthen the proposition that SPNS1 not only functions as a key regulator in AD pathogenesis but also represents a promising therapeutic target, with its expression levels potentially serving as a responsive biomarker for treatment efficacy.

DISCUSSION

SPNS1 is a lysosomal sphingolipid transporter. Previous studies have predominantly focused on its roles in embryonic development, the nervous system, and hepatic metabolic homeostasis [29–31], while in the pathogenesis of AD has remained elusive. In this study, we aimed to investigate the abnormal expression of SPNS1 in AD keratinocytes and its potential involvement in AD pathogenesis. We found that SPNS1 expression was significantly upregulated in the lesional skin of AD patients and in a DNCB-induced mouse model of AD, correlating with disease severity. Using conditional knockout mice, we demonstrated that SPNS1 deficiency significantly alleviated skin inflammation and suppressed keratinocyte hyperproliferation in DNCB-induced AD mice. These findings indicate that SPNS1 plays a key role in the abnormal proliferation of keratinocytes in AD and represents a potential

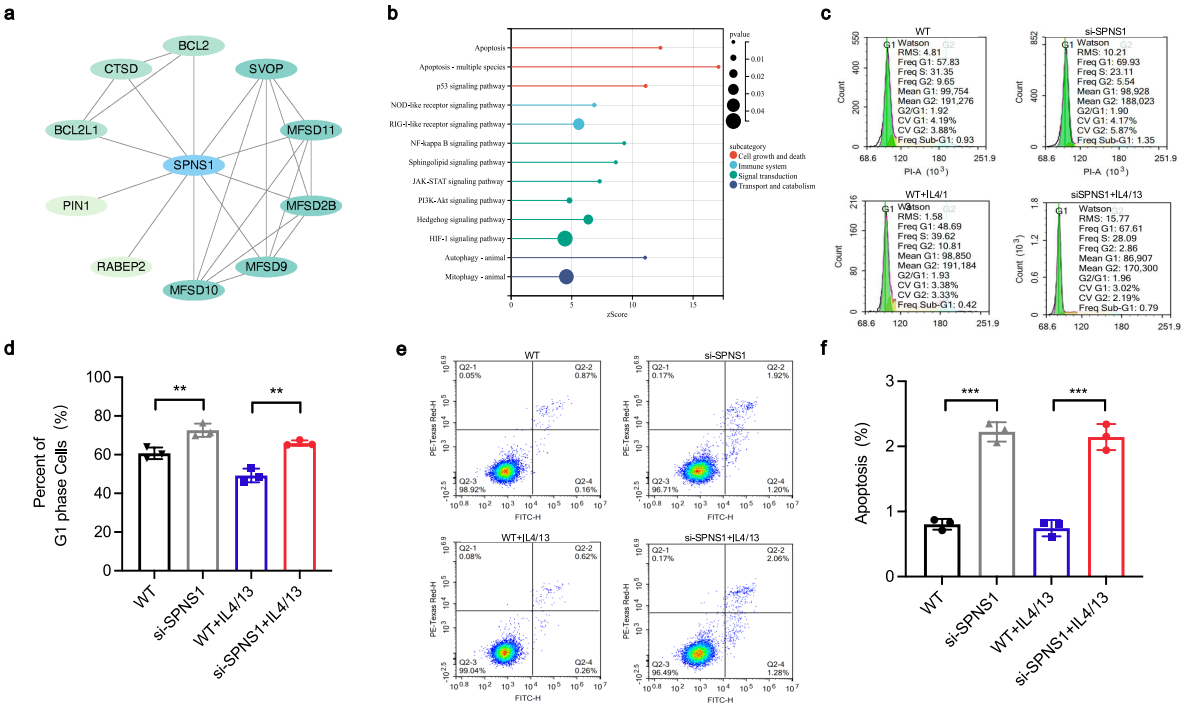

Fig. 4 SPNS1 promotes keratinocyte proliferation by regulating cell cycle progression and inhibiting apoptosis. (a) PPI network of SPNS1. (b) KEGG pathway enrichment analysis of SPNS1-associated pathways. (c) Flow cytometric analysis of the cell cycle performed by PI staining in WT and si-SPNS1 HaCaT cells treated with or without IL-4/13 for 24 h. (d) Quantitative assessment of the percentage of cells in the G1 phase ($n = 3$). Data are presented as mean ± SEM. (e) Apoptosis was monitored by Annexin V-FITC/PI staining and flow cytometry in WT and si-SPNS1 HaCaT cells treated with or without IL-4/13 for 24 h. (f) Quantitative analysis of the apoptotic cell ratio ($n = 3$). Error bars represent the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$. The p value was determined using two-tailed unpaired Student's t-test or one-way ANOVA. All numbers (n) are biologically independent experiments.

therapeutic target for this disease.

AD is a chronic inflammatory skin disease characterized by dysregulated innate immune responses involving various cell types such as keratinocytes, dendritic cells, and T cells [32, 33]. Our results demonstrate that suppressing keratinocyte proliferation effectively alleviates AD symptoms. Consistent with this, we found that SPNS1 deficiency significantly reduced the proportion of Ki-67-positive cells. Functional assays revealed that SPNS1 knockdown increased the proportion of cells in the G1 phase while inhibiting apoptosis. These findings collectively demonstrate that SPNS1 drives keratinocyte proliferation by promoting cell cycle progression and suppressing apoptosis. This aligns with established therapeutic strategies targeting cell cycle-related signaling and protein kinase-mediated activation in inflammatory skin diseases [34–36]. Mechanistically, SPNS1 knockdown led to lysosomal enlargement and dysfunction, suggesting that disrupted lysosomal homeostasis may represent a critical mechanism underlying skin barrier defects in AD. Based on previous studies and our current findings, we further speculate that lysosomal dysfunction may regulate keratinocyte proliferation through the modulation of the autophagic process [37]. As the lysosome serves as the key terminal executor of autophagic flux, its functional impairment may hinder the degradation of autophagic substrates, leading to the accumulation of abnormal proteins and damaged organelles [37, 38]. This, in turn, may disrupt cellular metabolic homeostasis and reduce cellular viability, ultimately affecting the proliferative capacity of keratinocytes.

In addition, our findings have clear translational insights. By analyzing transcriptomic data from AD patient skin following treatment with three clinically effective therapies (Fezakinumab, Dupilumab, and Crisaborole), we observed a consistent downregulation of SPNS1 expression after treatment, with statistical significance reached in the Crisaborole-treated group (Fig. 5c). Although this association does not establish a direct causal relationship between SPNS1 downregulation and therapeutic efficacy, the consistent trend suggests that decreased SPNS1 expression may be closely associated with clinical improvement. Combined with our observation that heterozygous SPNS1 deficiency is sufficient to significantly alleviate skin

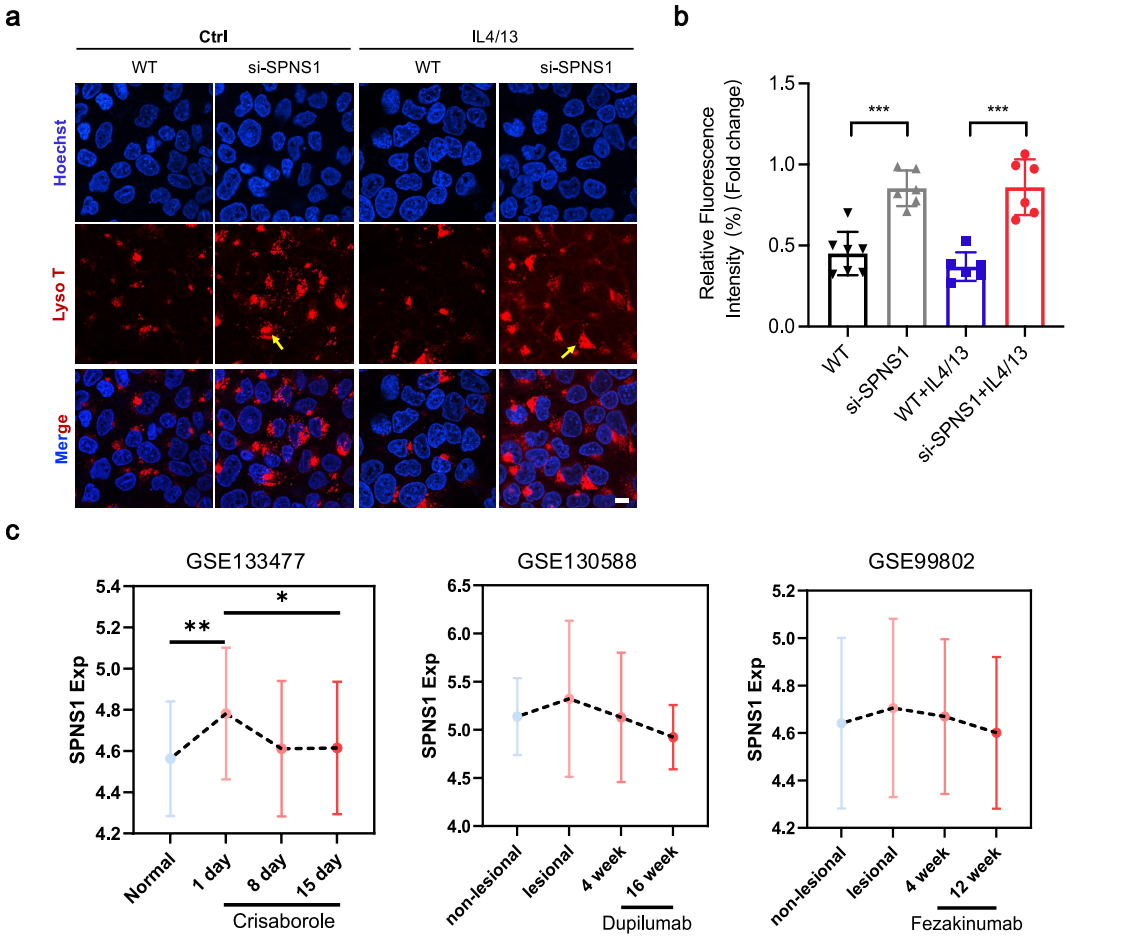

Fig. 5 SPNS1 deficiency induces lysosomal dysfunction and represents a therapeutic target in AD. (a) Live-cell LysoTracker staining of WT and si-SPNS1 HaCaT cells treated with or without IL-4/13 for 24 h. Scale bar, 10 µm. Arrows indicate enlarged lysosomes. (b) Quantification of the LysoTracker (red) fluorescence-positive particles numbers. (c) Analysis of SPNS1 gene expression in skin specimens after treatment with AD therapeutic agents.

inflammation and suppress keratinocyte hyperproliferation, we propose that partial downregulation of SPNS1 may already be sufficient to achieve meaningful therapeutic effects. This finding not only supports the protective phenotype observed in SPNS1$^{+/-}$ mice but also suggests that SPNS1 may be a target more amenable to moderate modulation rather than complete inhibition, providing a rationale for the development of therapeutic strategies that balance efficacy and safety.

It should be noted that a key limitation of this study is that our *in vitro* functional analyses were exclusively performed in immortalized HaCaT keratinocytes. Although HaCaT cells are widely used in epidermal biology and inflammatory research due to their stability and reproducibility, their immortalized nature may limit their ability to fully recapitulate the physiological characteristics of primary keratinocytes. In contrast, primary keratinocytes more closely resemble *in vivo* conditions but are constrained by limited availability, donor variability, and stringent culture requirements, which may hinder their application in systematic mechanistic studies. To enhance the robustness of our conclusions, we integrated multi-level evidence, including human transcriptomic data and *in vivo* mouse models, thereby partially mitigating the limitations of a single *in vitro* system. Nevertheless, we acknowledge the importance of further validation in primary human keratinocytes and patient-derived skin tissues. Future studies incorporating primary cell cultures and organoid or skin-equivalent models will be essential to better define the role of SPNS1 under physiologically relevant conditions and to strengthen its translational potential.

CONCLUSION

In summary, this study systematically characterizes a novel mechanism by which SPNS1 mediates keratinocyte proliferation, apoptosis, and lysosomal dysfunction in AD. This finding not only fills a critical

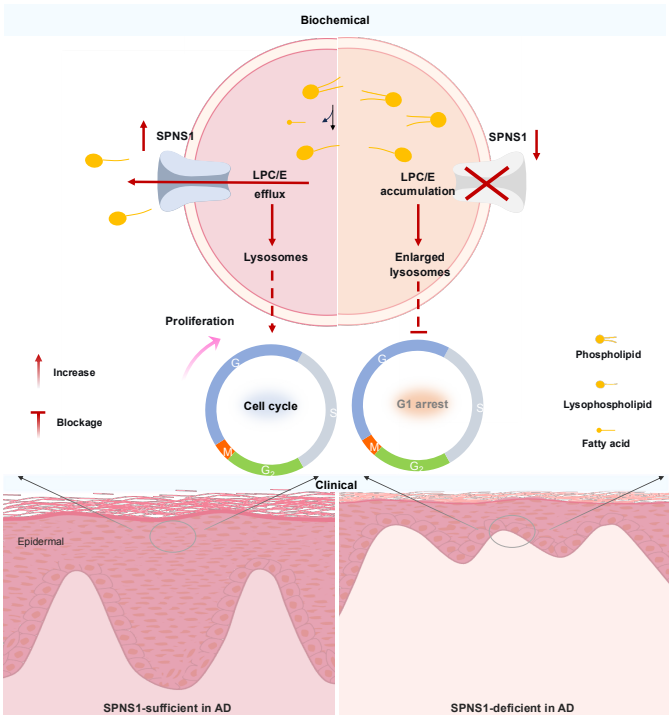

Fig. 6 Working model of SPNS1 regulation of epidermal and cell cycle homeostasis during AD-like skin inflammation. Briefly, in the AD microenvironment, upregulated SPNS1 promotes keratinocyte hyperproliferation and drives disease progression by maintaining lysosomal homeostasis. In contrast, SPNS1 deficiency disrupts lysosomal function, consequently inhibiting proliferation and inducing apoptosis to alleviate AD.

knowledge gap regarding the role of SPNS1 in AD pathogenesis but also deepens the understanding of the "keratinocyte dysfunctionlysosomal impairmentinflammatory exacerbation" pathological network in AD (Fig. 6). Furthermore, it provides solid theoretical support from both basic and clinical perspectives for establishing SPNS1 as a potential therapeutic target in AD. Future studies will focus on deciphering the precise mechanisms of SPNS1 in AD-related lysosomal signaling, keratinocyte lipid metabolism, and inflammatory networks, thereby laying the groundwork for developing novel SPNS1-targeted therapeutic strategies for AD.

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