

DR. LINGJIE TANG (Orcid ID : 0000-0002-4312-5810)

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Title page

Down-regulated SHARPIN may accelerate the development of atopic dermatitis through activating interleukin-33/ST2 signaling

Running title: The potential role of SHARPIN in the pathogenesis of AD

Authors: Lingjie Tang*, Jiaman Wang*, Jingna Zhu, Yanhua Liang

Affiliations: Department of Dermatology, Cosmetology & Venereology, Shenzhen Hospital, Southern Medical University, Shenzhen, Guangdong 518101, P.R. China

* Lingjie Tang and Jiaman Wang contribute equally to this work.

Corresponding author : Yanhua Liang, Professor, M.D., Ph.D, Department of Dermatology, Cosmetology & Venereology, Shenzhen Hospital, Southern Medical University

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Address: 1333 Xinhua Road, Shenzhen, Guangdong 518101, P.R. China

Phone and FAX: +86-755-23360448

E-mail: liangdoctor@163.com

Abstract

SHARPIN is an important component of the linear ubiquitin chain assembly complex (LUBAC). Loss of function of SHARPIN results in eosinophilic inflammation in multiple organs including skin with Th₂-dominant cytokines and dysregulated development of lymphoid tissues in mice. The clinicopathological features are similar to atopic dermatitis (AD) in humans. In order to investigate the potential role of *SHARPIN* in the pathogenesis of AD, we performed genetic association study of the genotypes and haplotypes as well as SHARPIN's expression between AD cases and controls. We found 3 mutations (g.480G>A, g.4576A>G and g.5070C>T) in patient group, and significantly decreased expression in AD lesions, suggesting a primary role of SHARPIN during AD development.

Lentivirus-mediated *in vitro* assays identified that knockdown of SHARPIN can induce elevated expression of IL-33 and its orphan receptor ST2, FLG and STAT3, and NF-κB inactivation in HaCaT keratinocytes, which has been widely evidenced in regulating AD development. ST2 expression was highly induced in SHARPIN-silenced HaCaT keratinocytes after the combined stimulation of IL-4 and IL-13. Our *in vivo* and *in vitro* findings implicated that SHARPIN may be a novel participant in the pathogenesis and/or new therapeutic target of AD.

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Keywords: SHARPIN, atopic dermatitis, IL-33, ST2, FLG

Introduction

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disease, characterized by skin barrier disruption and imbalance of Th₁/Th₂ inflammation. The filaggrin protein is a key protein in the structure of the stratum corneum, ensuring a resilient and tight skin barrier, and filaggrin gene (*FLG*) mutations are the identified genetic risk factor for the development of AD^{1,2,3}. Classical Th₂ cytokines such as IL-4, IL-5, and IL-13 are predominantly expressed in the acute lesions of AD, whereas in chronic AD skin Th₁ cells that can produce IFN- γ and IL-12 are dominant^[1]. Except for classical Th₂ cytokines, new tissue-derived cytokines including TSLP and IL-33 have been suggested to have an important role in AD^[2-3].

IL-33 is a recently described cytokine that belongs to the IL-1 superfamily and is related to Th₂ immune responses. IL-33 is constitutively expressed by cells of barrier tissues such as skin, lung, and gastrointestinal tract and so on^[4]. It is known to activate mast cells, Th₂ lymphocytes, and eosinophils to produce Th₂ cytokines^[5]. However, its signaling pathway is different from classical Th₂ cytokines. Extracellular IL-33 can activate various immune cells through a membrane-bound IL-33-specific receptor ST2, which leads to the production of various molecules including Th₂-associated cytokines^[6-7]. ST2, as the orphan receptor for IL-33 ligand, has two major isoforms by differential splicing of ST2 mRNA, including a transmembrane form (ST2) and a secreted soluble form (sST2). The sST2 form can act as a decoy receptor for IL-33 and then increase blocking of IL-33 signaling^[8]. ST2 associates with

IL-1R accessory protein (IL-1RAcP) to form a complex. IL-33 binding to this heterodimer leads to the recruitment of MyD88 adapter protein, IL-1R-associated kinase1,4 (IRAK1/4) and TNFR-associated factor 6 (TRAF6) in mast cells^[4]. This signaling cascade further induces activation of signaling proteins such as transcription factor NF- κ B, the mitogen-activated protein kinase (MAPK) pathway and the production of inflammatory mediators such as Th₂-type cytokines^[9]. IL-33 and ST2 both are found to be increased in the skin of patients with AD and play an important role for pathological development of this disease^[10].

SHARPIN combines with heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1) and HOIL-1-interacting protein (HOIP) to structure the linear ubiquitin chain assembly complex (LUBAC), which are required for the activation of NF- κ B signaling pathway^[11]. Previous researches usually linked SHARPIN to inflammatory diseases via activation of NF- κ B pathway^[12]. SHARPIN is widely expressed in multiple tissues in human and mice. Mice with spontaneous mutations in the *Sharpin* gene can develop chronic proliferative dermatitis mice (*cpdm*) that is characterized by multiorgan inflammation and dysregulated development of lymphoid tissues, including chronic dermatitis, arthritis and immune disorders^[13]. The skin lesions in *cpdm* mice are characterized by increasing of type 2 cytokines and IgE, eosinophilic inflammation, which similarities to AD clinically and histologically. Furthermore, among eleven IL1 members, IL-1 β , IL-18, and IL-33 are mostly up-regulated in the skin of *cpdm* mice^{[14],[15]}. Transgenic animal study by Liang *et al* has demonstrated that knockout of IL1RAcP can primarily rescue the phenotypes of *cpdm/cpdm* mice^[15], implicating that SHARPIN may play role on the upstream of IL1 activating pathways.

Taking the clinical phenotypes, the pathological changes, and the immunologic features into consideration, the cutaneous phenotypes of *cpdm* mice with loss of function of SHARPIN highly resemble AD changes in human, suggesting *SHARPIN* could be further investigated as the candidate gene of AD.

Methods

PCR and Genetic Association Analysis

The peripheral blood samples from 65 AD patients including 29 females and 36 males, aged from 18 months to 62 years, were collected for genotyping, compared to that of 100 healthy controls composed of 46 females and 54 males. Genomic DNA was isolated using a commercial kit (SIMGEN Biotechnology Co. Ltd, Hangzhou, China). PCR primers for *SHARPIN* gene were designed using the Primer3 software (**Supplementary Table 1**). To screen for genomic variants in the human *SHARPIN* gene, all exons and adjunct splicing sites were PCR-amplified from genomic DNAs. The PCR products were analyzed on an ABI PRISMTM 3730XL DNA Analyzer. The sequencing results were analyzed respectively by two experimenters with the Chromas software. The variant sites were finally determined and compared with the NCBI SNP database.

Immunohistochemical staining

The skin samples were obtained from 5 female and 3 male AD cases, aged from 6 to 25 years, with the disease duration of 2 to 20 years, and 3 healthy controls including 2 females and 1 male, aged from 21 to 31 years. The skin tissues were processed for H&E staining and immunohistochemical analysis. Primary antibody of polyclonal rabbit anti-human antibody SHARPIN (G-18) was purchased from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA). Secondary antibody of goat anti-rabbit antibody IHC kit was commercially available from Neobioscience Technology Company (Shenzhen, Guangdong, China). Skin samples were collected and fixed by immersion in 10% formaldehyde solution. Fixed tissues were embedded routinely in paraffin and serially sectioned at 3 μ m. Each section was placed on an adhesion microscope slide from Citotest Labware Manufacturing Co., LTD (Haimen, Jiangsu, China). For immunocytochemical staining, sections were deparaffinized, rehydrated, antigen retrieved, incubated by dilution of SHARPIN antibody (@1:100) and secondary antibody, colorated by DAB color reagent, counterstained by hematoxylin, differentiated by hydrochloric acid alcohol, and then dehydrated in an alcohol series and cleared in Xylene.

For each section, 8~10 randomly selected microscopic fields of epidermis were photographed (including blank areas for level adjustment). The non-uniform background color/brightness of each image was eliminated by the Levels instruction of the software Photoshop by taking the blank area mentioned above as background. Then the average gray value of epidermis of each image was calculated by the software Image J (excluding stratum corneum and dermis). The average gray value of cases or controls was expressed as mean $\pm$ SE, and the statistical comparison of gray value between cases and controls was performed

by the Independent-Samples T Test by SPSS. Statistical significance was determined as $p < 0.05$.

Cell cultures

Human immortalized HaCaT keratinocytes were cultured in Dulbecco's minimum essential medium (DMEM) (Gibco, USA) including L-glutamine with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Hyclone, SV30010) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Cell transfection with lentivirus vectors

For HaCaT cells transfection, the cells were re-suspended in DMEM (Gibco, USA) with 10% fetal and antibiotics at a density of 2.5×10^5 cells per 6-well plate, then cultured for 24 hours.

The lentiviruses were purchased from Cyagen Bioscience (GuangZhou, China). The recombinant lentiviral vectors (**Supplementary Figure S1**) were diluted respectively at a multiplicity of infection (MOI) of 100 in DMEM supplemented with 10% FBS and in the presence of 5 µg/ml polybrene (5 µg/ml; VectorBuilder). Transfection was performed in round-bottomed 6-well plates for 48 hours. After puromycin (1 µg/ml) for cells transfection with sh-SHARPIN and sh-Ctrl selection and five consecutive days of culturing, stable transfection of these cells was obtained.

Stimulation procedure

Transfected HaCaT cells were grown to about 80% confluence, stimulated respectively with 50 ng/mL of combination of IL-4 and IL-13 (both PeproTech, Rocky Hill, NJ) for 24 hours for mRNA isolation and protein extraction. Transfected cells were stimulated with 50 ng/mL of TNF- α (PeproTech, Rocky Hill, NJ) for 30 minutes for phosphorylated protein extraction. Transfected cells were treated with Janus kinase (JAK)/signal transducer and activator of transcription (STAT) inhibitor ruxolitinib (5 μ M) (HY-50856) for 24 hours for protein extraction.

Quantitative RT-PCR

Transfected HaCaT cells were seeded in 6-well plates. Total RNA from the cells was isolated with TRIzol reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Reverse transcription was performed using the HiScript® II Reverse Transcriptase (R223) (Vazyme, Nanjing, China). Quantitative real-time PCR was run on 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) with ChamQ SYBR qPCR Master Mix (Q331) (Vazyme, Nanjing, China) for SHARPIN, IL-33, ST2, IL-5, FLG, KLK5, KLK7, CAP1, Caspase14, Matriptase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used in this study were designed as shown in **Supplementary Table 2**. The measured expression level of each target gene was normalized to that of GAPDH.

Western blotting

The expression of SHARPIN, FLG, IL-33, ST2, IL-5, STAT3, I κ B- α , p65, and the phosphorylation of I κ B- α in transfected HaCaT cells and stimulating factors-treated transfected HaCaT cells were analyzed by means of Western blotting. Protein was extracted from cells with RIPA lysis buffer (Merck Millipore). Protein concentrations were determined with the BCA Protein Assay Kit (Beyotime) by using BSA as the standard. Protein lysates were fractionated by means of 10% SDS-PAGE. Western blot assays were carried out on polyvinylidene difluoride membranes(Millipore) using the specific antibodies for SHARPIN (Abcam, ab197853, 1:2000), FLG (GeneTex, GTX23137, 1:500), IL-33 (R&D Systems, AF3625, 0.1 μ g/ml), ST2 (R&D Systems, MAB523-100, 1 μ g/ml), IL-5(Abcam, ab25034, 1:1000), STAT3(Wanleibio, WL01731, 1:500), I κ B- α (Santa Cruz sc-371, 1:500), p-I κ B- α (Cell Signaling 2859, 1:1000), p65(Wanleibio, WL01980, 1:500) and GAPDH (Santa Cruz sc-25778, 1:500). The immunoreactive bands were visualized by means of an enhanced chemiluminescence reaction.

Statistic analysis

All *in vitro* experiments were conducted in triplicate and independently repeated at least 3 times. The data were expressed as means ($\pm$ SEM), and differences between means were analyzed with the Graph Pad Prism software using a Student's t-test with a two-tailed test of significance. Differences at a p value of less than 0.05 were considered statistically significant.

Results

Genetic variations of *SHARPIN* in AD

All exons with splice junctions of *SHARPIN* were successfully PCR-amplified. A total of 13 variations were found in 65 cases, including 3 mutations, designated as Mut1 (g.480G>A) at exon 1, Mut2 (g.4576A>G) at intron 4, and Mut3 (g.5070C>T) at exon 7, and 10 SNPs including 5 novel SNPs and 5 reported ones (rs200698932, rs769935596, rs535666509, rs760934092 and rs1280524235) (**Table 1, Supplementary Figure S2**).

Decreased *SHARPIN* Expression in AD

SHARPIN was highly expressed in the epidermis and hair follicle of healthy controls, compared to the decreased *SHARPIN* staining in AD lesions (**Figure 1**). The average grey value of controls (129.929) is significantly lower than that of AD lesions (145.996) ($P<0.0001$), demonstrating that the expression of *SHARPIN* is significantly decreased in the skin lesions of AD patients, and supporting that *SHARPIN* may play an inhibitory role in the development of AD.

HaCaT-*SHARPIN* model was successfully constructed

After transfection of HaCaT cells, immunofluorescence, qRT-PCR and Western blot analyses were performed to analyze the expression levels of both *SHARPIN* mRNA and protein. Cells were respectively transfected with sh-*SHARPIN* (*SHARPIN* knockdown group), sh-Ctrl

(SHARPIN knockdown control group). As shown in **Supplementary Figure S3**, the expression levels of SHARPIN mRNA and protein were significantly decreased in sh-SHARPIN groups compared with sh-Ctrl groups, suggesting successful construction of lentivirus-mediated SHARPIN recombinants ($P < 0.05$).

Knockdown of SHARPIN can inactivate NF- κ B pathway

SHARPIN is an important protein to assemble the LUBAC, which are required for the activation of NF- κ B pathway^[11]. To better understand the effect of SHARPIN on NF- κ B pathway in HaCaT cells, we analyzed the expression of various molecules of NF- κ B signaling by using Western blot. As shown in **Supplementary Figure S4**, sh-SHARPIN groups displayed a significant reduction of p-I κ B- α , I κ B- α and p65 compared with sh-Ctrl groups ($P < 0.05$). These results indicated that although knockdown of SHARPIN may inhibit the I κ B- α phosphorylation, it ultimately inactivates the NF- κ B signaling pathway.

SHARPIN silencing can stimulate the expression of IL-33 and ST2

Regarding the important role of IL33/ST2 signaling in AD pathogenesis, we investigated the expression of IL-33 and ST2 in transfected HaCaT cell. We found an approximate 2-fold increasing of IL-33 mRNA ($P < 0.01$) and 1.5-fold increase of ST2 mRNA ($P < 0.01$) in sh-SHARPIN groups that *SHARPIN* gene was silenced in HaCaT cells compared with its

controls (**Figure 2A, 2B**). Furthermore, the pattern of protein expression of IL-33 and ST2 were consistent with the findings of their mRNA level (**Figure 2C**).

IL-4/13 can synergistically stimulate ST2 expression in SHARPIN-knockdown HaCaT cells

ST2 and IL5 expression were investigated in SHARPIN-knockdown HaCaT cells after stimulation by IL-4 and IL-13. We found that both mRNA and protein expression of ST2 was significantly upregulated after stimulation in sh-SHARPIN groups as compared with its controls (**Figure 3A, 3C**). However, the mRNA and protein expression of IL-5 showed a bit increasing (**Figure 3B, 3C**) without big differences. ST2 protein expression was also significantly increased in sh-SHARPIN groups (**Figure 3C**).

SHARPIN silencing can stimulate the expression of FLG and STAT3

IL-33 was found to be increased in the skin of patients with AD and impact on the skin barrier by down-regulating the expression of FLG^[16]. Lee and colleagues found that pSTAT3 was highly expressed in AD lesional skin^[17], suggesting that STAT3 may be involved in the pathogenesis of AD. As a transcription factor, STAT3 not only has DNA binding sequences on the FLG promoter^[16], but also promotes ST2 production^[18]. Proteases, such as KLK5 and 7, CAP1, matriptase, and caspase-14 can promote FLG degradation process^[19-22]. We found both mRNA and protein of FLG was significantly up-regulated in sh-SHARPIN groups as

compared with its controls (**Figure 4A, 4G**). KLK5 and KLK7 mRNA expression were significantly decreased in sh-SHARPIN groups (**Figure 4B, 4C**). Nevertheless, CAP1, matriptase, and caspase-14 mRNA expression had no different in sh-SHARPIN groups (**Figure 4D, 4E, 4F**). Furthermore, we found STAT3 protein expression was increased in SHARPIN-knockdown HaCaT cells (**Figure 4G**). To clarify how the STAT signaling pathway was involved in FLG and ST2 expression, we used specific inhibitor of JAK/STAT ruxolitinib. We found that SHARPIN-induced FLG and ST2 protein expression were both inhibited by ruxolitinib (**Figure 4H**), implicating a cross-link role of SHARPIN-mediated STAT signaling in HaCaT cells.

Discussion

Our study is proposed by the discovery that mutation of *Sharpin* is the genetic basis of chronic proliferative dermatitis in mice^[23]. Based on the similarities of clinical manifestation, pathological changes and immunological mechanism of *cpdm/cpdm* mice to that of human AD patients, SHARPIN was regarded as one of AD candidate genes. We found 3 mutations and 10 SNPs in 65 AD cases and not in 100 healthy controls. Although these 3 mutations were predicted not to change the amino acid sequence of SHARPIN, they may be regulatory sequences controlling SHARPIN transcription and/or expression. When explaining the genetic factor of complex disease, the “common disease/common variant (CD/CV)” hypothesis is most prevailing, which argues that variations with high frequency (MAF>1%) but relatively low penetrance are the main causes of genetic susceptibility to complex disease^[24]. However, our results suggest that the 3 variations with low frequency

(MAF=0.008%) may relate to AD phenotype, supporting another hypothesis of “common disease/rare variant (CD/RV)”, which argues that multiple rare (MAF<1%) DNA sequence variations with relatively high penetrance are the major contributors to genetic susceptibility^[24].

Genetic association study mentioned above suggests variations of human *SHARPIN* gene may be associated with the occurrence and/or development of AD. We observed decreased SHARPIN expression in AD lesions than that in healthy skins by immunochemical staining. Genetic changes and decreased SHARPIN expression may play an important role in the pathogenesis of some AD patients. However, the larger sample size was needed to present more convincing evidences for association studies.

To better understand the role of SHARPIN in AD, we performed *in vitro* functional assays to investigate whether SHARPIN interacts with key molecules of AD such as IL-33/ST2 signaling, FLG, and NF- κ B.

IL-33 is a cytokine that promotes Th₂-type immune responses by binding to its heterodimeric receptor complex consisted of membrane-bound ST2 and IL-1RAcP. Recent data suggests an important role of IL-33 and ST2 during the development of AD.

Furthermore, genetic polymorphism within the *ST2* gene region had been reported to have relations with AD^[25]. To better understand the relation of IL-33/ST2 and SHARPIN in AD, we sought to examine expression profiles of IL-33 and its receptor ST2 in SHARPIN-knockdown HaCaT cells. Our results show elevated expression levels of IL-33 and ST2 when SHARPIN was knocked down in HaCaT cells. These results suggest that

SHARPIN gene silencing could promote the generation of intracellular IL-33 and ST2. IL-33 is stored in the nucleus and secreted upon necrosis or damage^[26]. It can be released after cell necrosis or tissue damage, which may be caused by allergen proteins, enzymes, and physical stress or trauma^[27 28]. Therefore, IL-33 has been classified as an alarmin concerned with alerting the immune system. AD is clinically characterized by intense pruritus, which causes scratching of the skin. Thus, mechanical trauma to the skin, produced by the repeated scratching in AD patients, leads to the release of IL-33^[29]. Extracellular IL-33 together with the ST2 promotes the production of Th₂ cytokines.

Th₂ type cytokines such as IL-4, IL-5 and IL-13 are known primary cytokines in acute AD skin lesions. We demonstrate significantly increased expression of ST2 and IL-5 in *SHARPIN* down knocked HaCaT cells exposed to IL-4/13. This is in accordance with the finding by Savinko T *et al.* who found that ST2 expression was up-regulated during the sensitization in parallel with expression of Th₂ cytokines^[10]. This study confirms decreasing of *SHARPIN* expression can enhance the Th₂ immune response by up-regulated ST2 in acute AD environment. Taken together, IL-33-ST2 interaction can be regulated by down-regulated *SHARPIN* in AD, which may be critical in the development of the disease.

AD is also characterized as skin barrier dysfunction, resulting in transepidermal water loss, allergen penetration, and skin colonization with microbes^[30]. FLG is the key structural protein for maintaining normal epidermal barrier integrity and stratum corneum hydration, and that Seltmann, J *et al.* found a direct action of IL-33 on down-regulation of FLG expression^[31]. So we also detected the expression of FLG in HaCaT cells at decreasing *SHARPIN* expression models. We found silencing of *SHARPIN* can increase FLG expression

in HaCaT cells. In line with our results, a synergistic increase of FLG was demonstrated in areas of hypergranulosis in *cpdm/cpdm* mice, but they thought increased expression of FLG may contribute to the irregular thickness of the granular cell layer of the epidermis^[32]. However, our study indicated that *SHARPIN* itself can influence the expression of FLG in keratinocytes. FLG has several site-specific functions dominated by the program of epidermal terminal differentiation in which several proteases are involved, such as corneum Trypsin-like enzymes and chymotrypsin-like enzymes (kallikrein 5 [KLK5] and KLK7), channel-activating serine protease1 (CAP1), matriptase (coded by ST14), and cysteine protease caspase-14. All of the proteases can enhance FLG degradation processing. Our results showed that silencing of *SHARPIN* can decrease KLK5 and KLK7 expression in HaCaT cells. These results suggest that decreasing of SHARPIN expression could prohibit FLG degradation through down-regulated KLK5 and KLK7.

On the other hand, FLG expression was shown to be regulated by the activator protein 1 (AP1) family of transcriptional regulators that consists of heterodimers between individual members of the Jun and Fos families of transactivators or of homodimers between different Jun proteins^[33]. Inhibition of NF- κ B reduced AP-1 activity and AP-1-dependent gene expression^[34]. Our experiments showed that NF- κ B pathway was inactivated in SHARPIN-knockdown HaCaT cells, but FLG expression was increased. Hence, there may be another pathway to regulate FLG expression.

Furthermore, we found STAT3 was highly expressed in SHARPIN-knockdown HaCaT cells. STAT3 is a transcription factor that regulates a variety of cellular events including proliferation, differentiation and apoptosis. Several studies suggest that STAT3 transmits

inflammatory signals^[35]. There is evidence that enforced expression of constitutively active STAT3 enabled activation of the proximal ST2 promoter and induced the expression of ST2 gene products^[18]. Sequence based analysis also identified that, the FLG promoter has also an STAT3 binding sequence^[16]. Interestingly, we found JAK/STAT inhibitor suppressed the induction of FLG and ST2 in SHARPIN-knockdown HaCaT cells, supporting that STAT is positively involved in their induction. So overexpression of FLG and ST2 may be partly due to STAT3 regulation in SHARPIN-knockdown HaCaT cells. Besides, previous reports showed STAT3 also participate in the development of AD through various ways. STAT3 protein has emerged as an important determinant of whether the naïve T cell differentiates into regulatory or an inflammatory T cell lineage^[36]. Thymic stromal lymphopoietin (TSLP) which is highly expressed by keratinocytes in AD lesions inhibits FLG expression via STAT3 in keratinocytes^[37]. STAT3 might be a major regulator of antimicrobial peptides (AMPs) such as human β -defensin2 (hBD2) expression which is host defense molecules in epidermis^[17]. Therefore how STAT3 regulates the development of AD remains to be further explore using SHARPIN-knockdown HaCaT cells.

In conclusion, we found decreased expression of SHARPIN in AD lesions, and *in vitro* functional studies demonstrated silencing of *SHARPIN* can upregulate IL-33/ST2 and FLG in HaCaT cells. Furthermore, we report significant overexpression of ST2 upon stimulation with IL-4/13, which may turn out to be the positive feedback loop of Th₂ immune response system in the intraepidermal pathophysiology of AD. We speculate that STAT3 has primary regulatory effects on the transcription of ST2 and FLG in HaCaT cells and could be a major

target for future research into barrier dysfunction in AD. These results recommend an important role for SHARPIN in the pathogenesis of AD.

Acknowledgments

LJT and JMW performed the experiments, analyzed the data and prepared the manuscript draft. JNZ managed the lab materials and financial work. YHL designed the research study, revised the manuscript, and provided funding support. All authors worked on and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest

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Table 1. Locus and allelotype of genetic variants of *SHARPIN* gene

SNP Name	Locus	Genotype
Mut1	g.480	A/C
rs200698932	g.4319	A/C/G
SNP1	g.4320	A/C/G
rs769935596	g.4328	A/G
SNP2	g.4334	A/G
rs535666509	g.4341	A/G
rs760934092	g.4342	A/G
SNP3	g.4343	A/G
SNP4	g.4344	A/G
Mut2	g.4576	A/G
rs1280524235	g.4791	A/G
Mut3	g.5070	C/T
SNP5	g.5363	A/G

Mut1, Mut2, and Mut3 represent the sequence changes we identified only present in AD patients but not in controls.

SNP1, SNP2, SNP3, SNP4, and SNP5 means the DNA sequence changes we identified in both AD patients and controls.

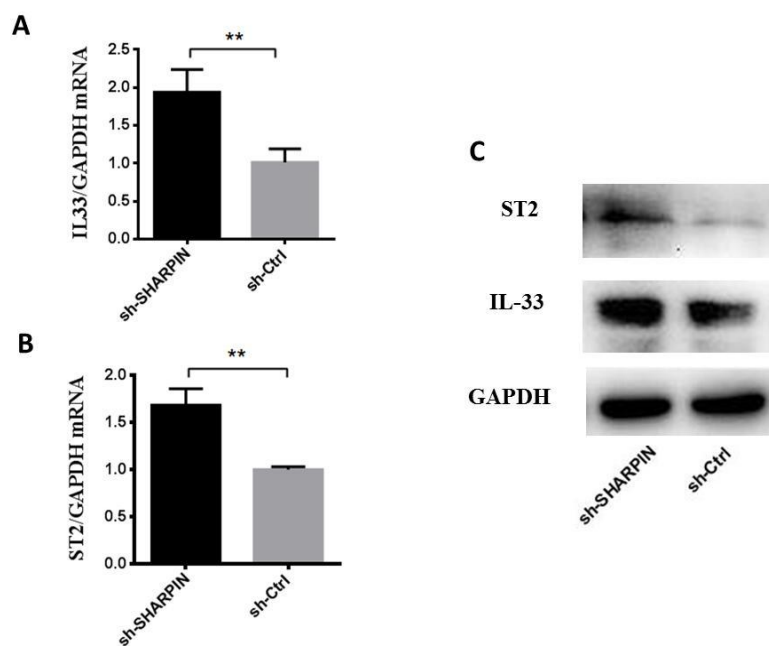
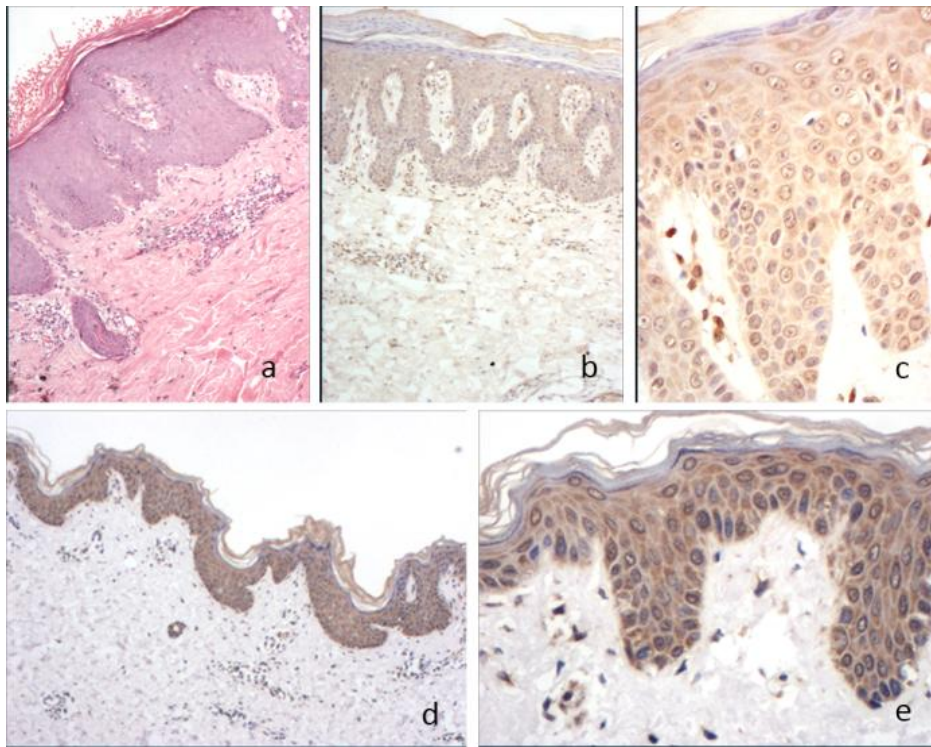
rs200698932, rs769935596, rs535666509, rs760934092 and rs1280524235 are the DNA sequence changes reported by NCBI database, and were also present in our experimental samples.

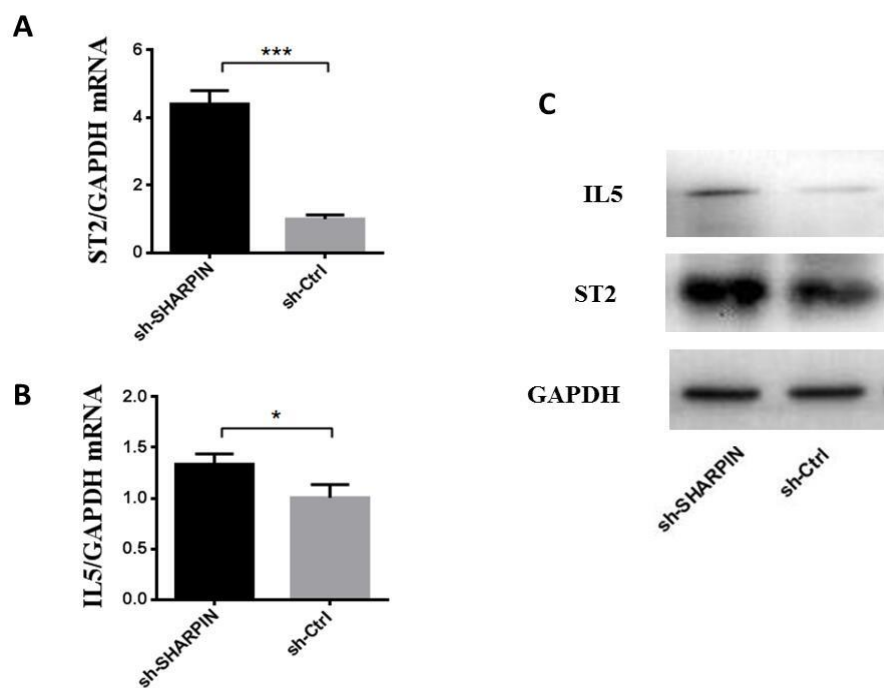
Figure 1. SHARPIN immunohistochemical staining in atopic dermatitis skin compared with normal controls (a-c: AD lesions, a: 10 x HE, b: 10 x SHARPIN, c: 40 x SHARPIN; d-e: normal skins, d: 10 x SHARPIN, e: 40 x SHARPIN), visible b and c staining was significantly lower than d and e.

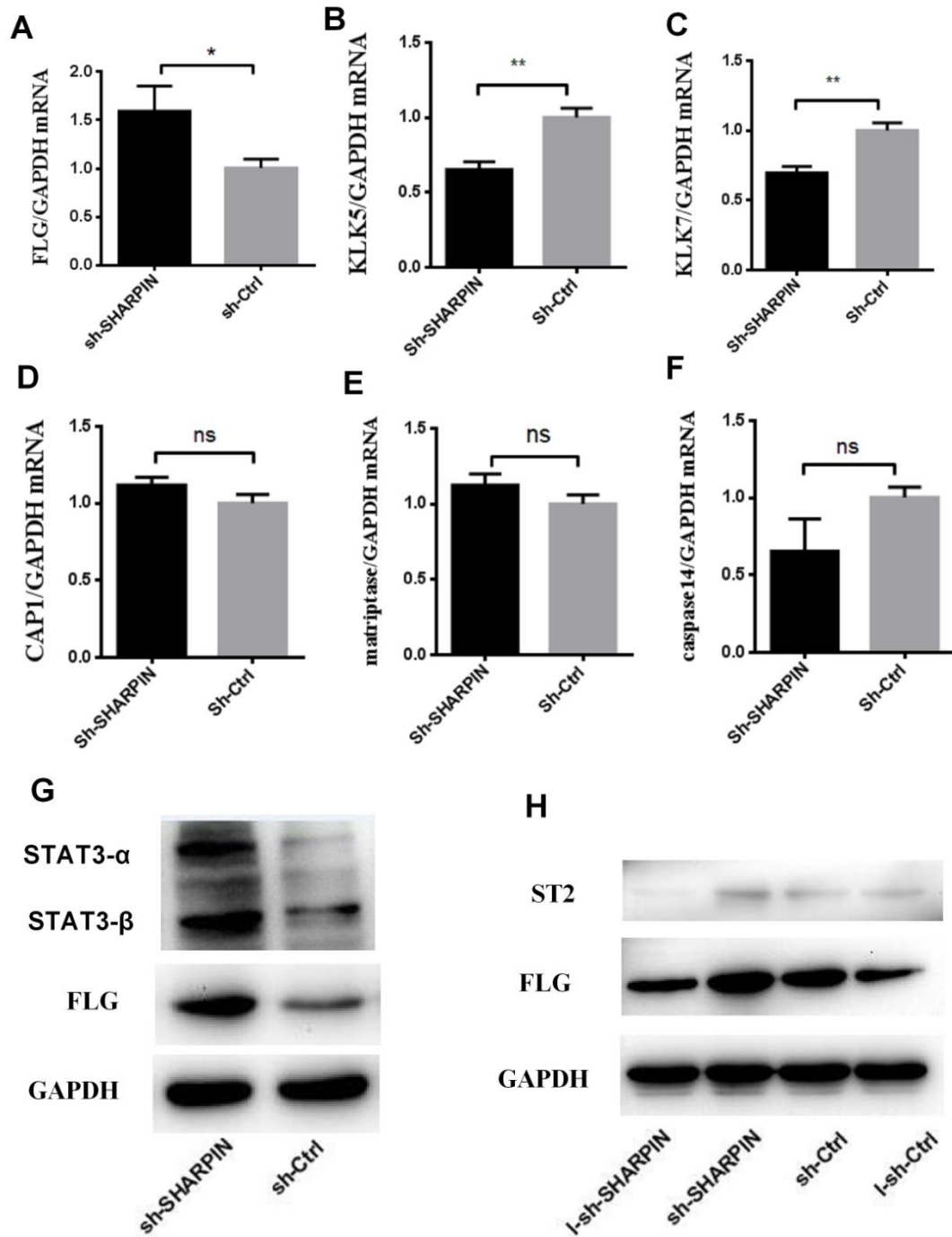
Figure 2. Down-regulated SHARPIN boosted expression of IL33 and ST2. (A) IL33 mRNA and (B) ST2 mRNA were increased in sh-SHARPIN groups than that in sh-Ctrl groups (**P<0.01; **P<0.01). (C) IL33 and ST2 protein expression was evaluated, similar to that of qRT-PCR.

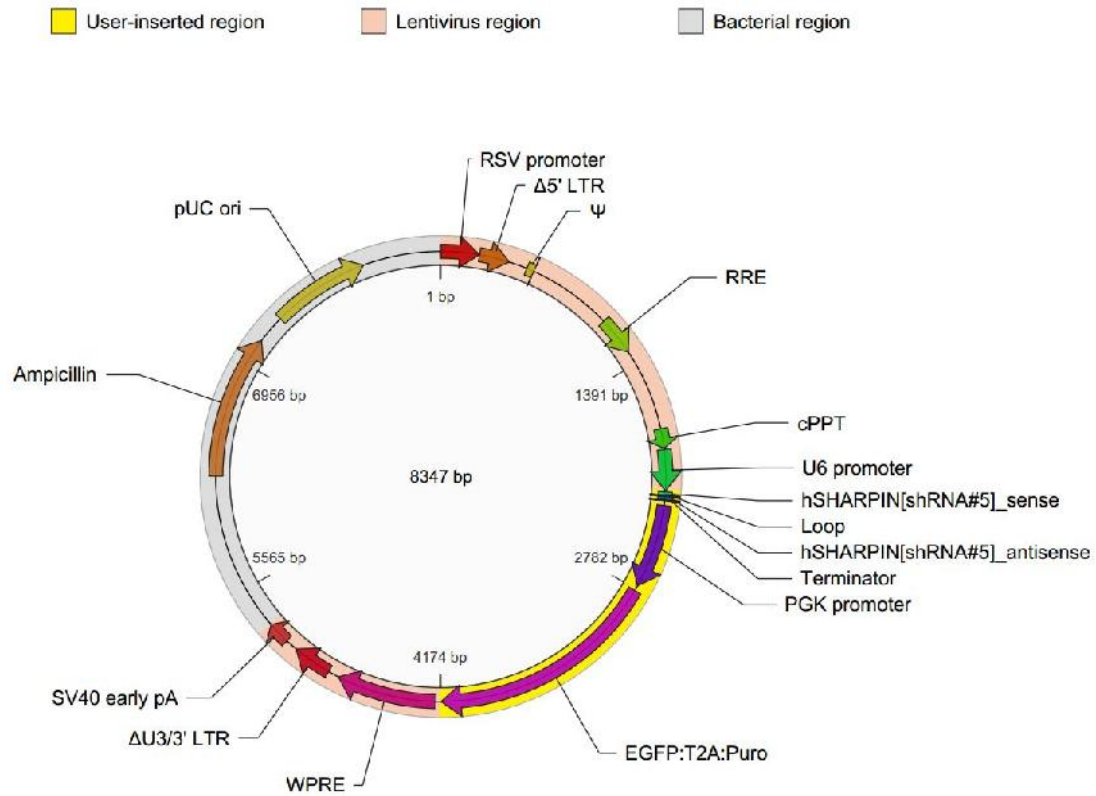
Figure 3. ST2 expression was significantly increased in HaCaT cells of down-regulated SHARPIN expression after IL-4/13 stimulation. (A) ST2 mRNA and (B) IL-5 mRNA were increased in sh-SHARPIN groups than that in sh-Ctrl groups (** $P < 0.001$; * $P < 0.05$). (C) ST2 and IL-5 protein expression was evaluated by Western blotting, the results similar to that of qRT-PCR.

Figure 4. FLG expression was increased in HaCaT cells after down-regulated SHARPIN expression. (A) FLG mRNA were increased in sh-SHARPIN groups than that in sh-Ctrl groups (* $P < 0.05$). (B) KLK5 and (C) KLK7 mRNA were decreased in sh-SHARPIN groups (** $P < 0.01$). However, (D) CAP1, (E) matriptase and (F) caspase 14 mRNA had no different in sh-SHARPIN groups. (G) Protein expression was evaluated by Western blotting, FLG and STAT3 protein expression were increased in sh-SHARPIN groups than control groups. (H) Transfected cells were treated with inhibitor of JAK/STAT for 24h for Western blotting. FLG and ST2 protein expression were decreased than that in inhibitor-free groups. I-sh-SHARPIN and I-sh-Ctrl were inhibited groups. KLK5: Kllikrein 5; KLK7: Kllikrein 7; CAP1: Channel-activating serine protease 1, STAT3: signal transducers and activators of transcription 3.



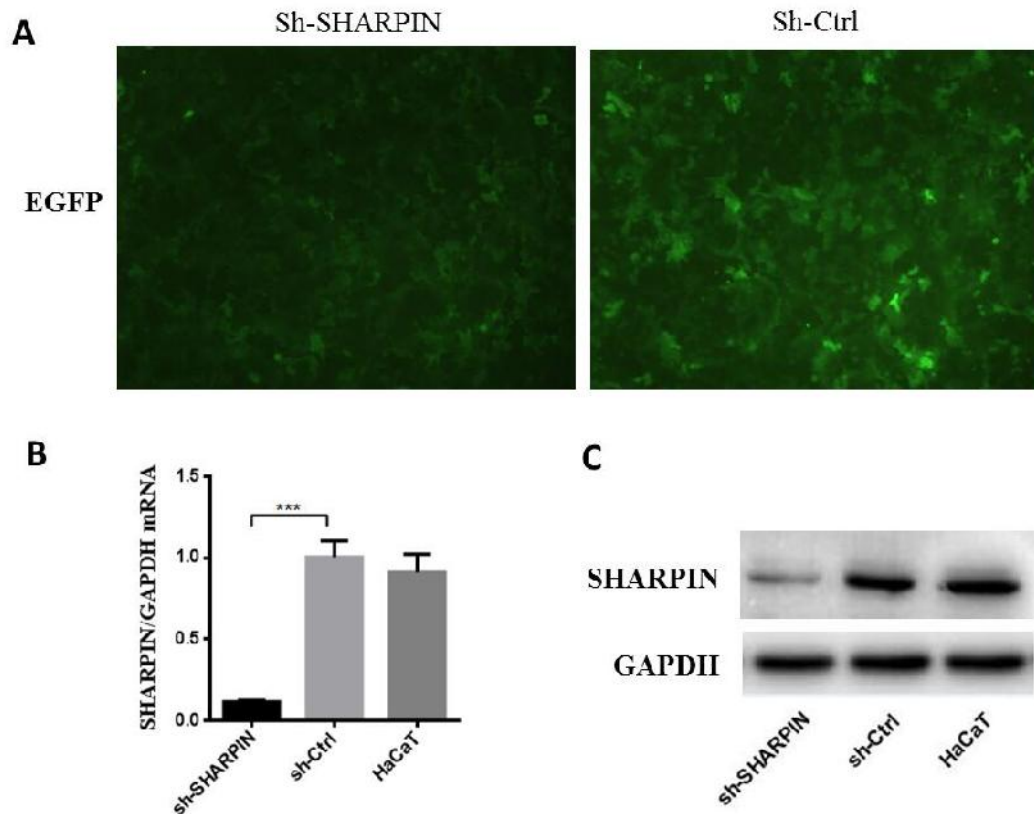




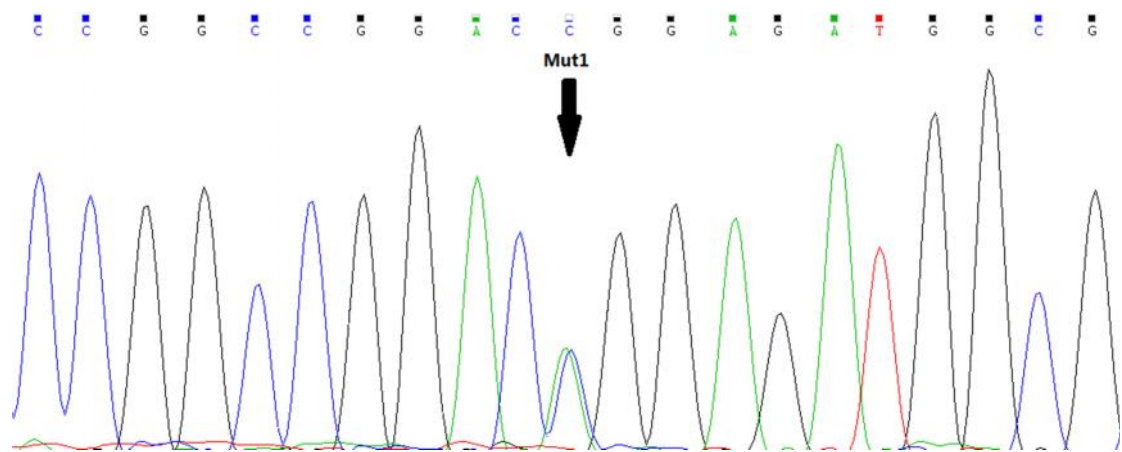


Supplementary Figure S1. The molecular structure of lentiviral vectors.

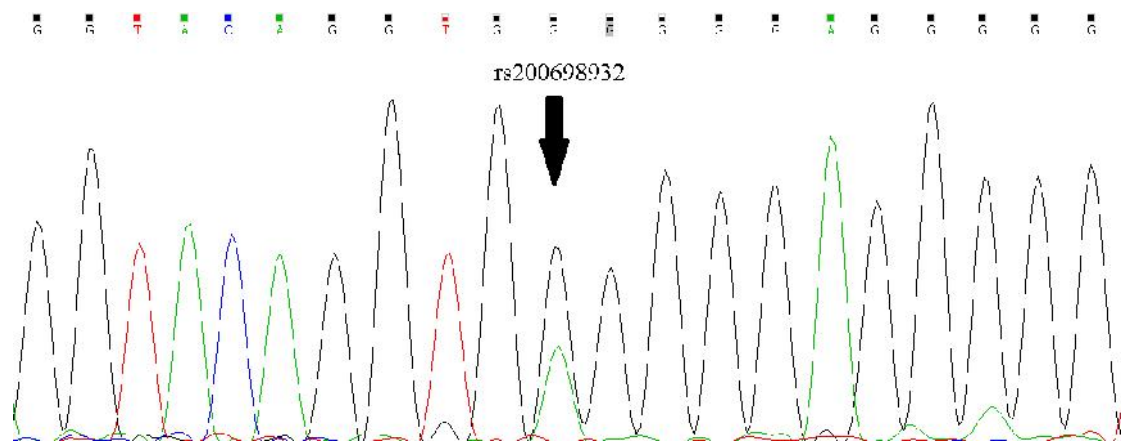
pLV[shRNA]-EGFP:T2A:Puro-U6>hSHARPIN[shRNA#5] encoding for knockdown of SHARPIN. The Size is 8347 bp. The target Sequence is GGGAAGTTGGACGCTTGTTTC.



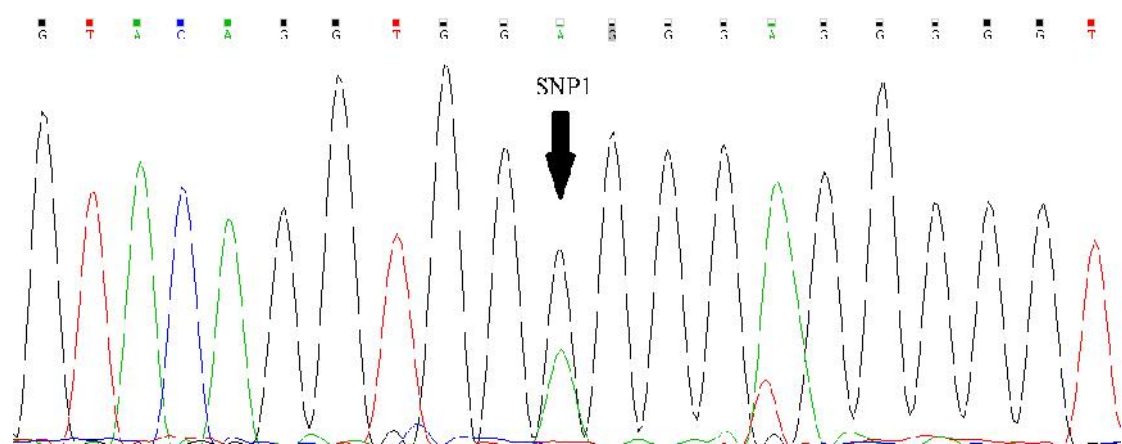
Supplementary Figure S3. sh-SHARPIN and its control vectors was successfully transfected into HaCaT cells and confirmed by fluorescence observation, qRT-PCR and Western blot. (A) sh-SHARPIN and sh-Ctrl were found to be green fluorescence. **(B)** qRT-PCR and **(C)** Western Blot analysis validated that SHARPIN expression level in HaCaT cells of down-regulated SHARPIN groups were decreased significantly than that in control groups ($***P < 0.001$). The SHARPIN expression of sh-Ctrl groups (Experimental Control infected by blank vectors) was similar to that of HaCaT groups (Blank Control infected without any vector).



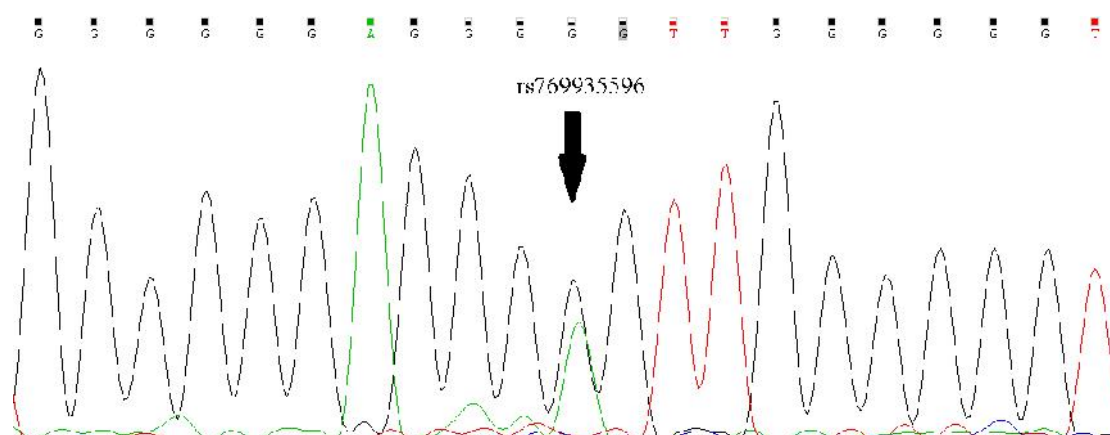
Forward sequencing, the arrow refers to the Mut1 locus



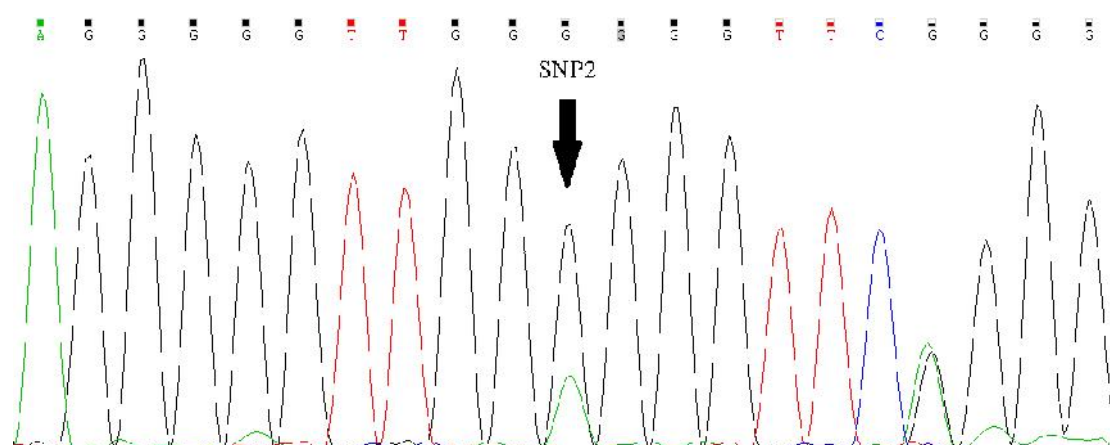
Forward sequencing, the arrow refers to the rs200698932 locus



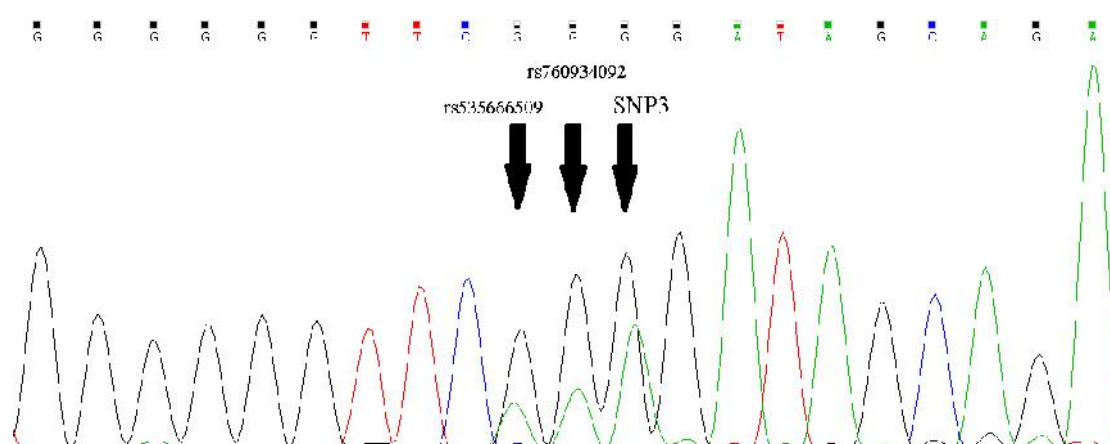
Forward sequencing, the arrow refers to the SNP1 locus



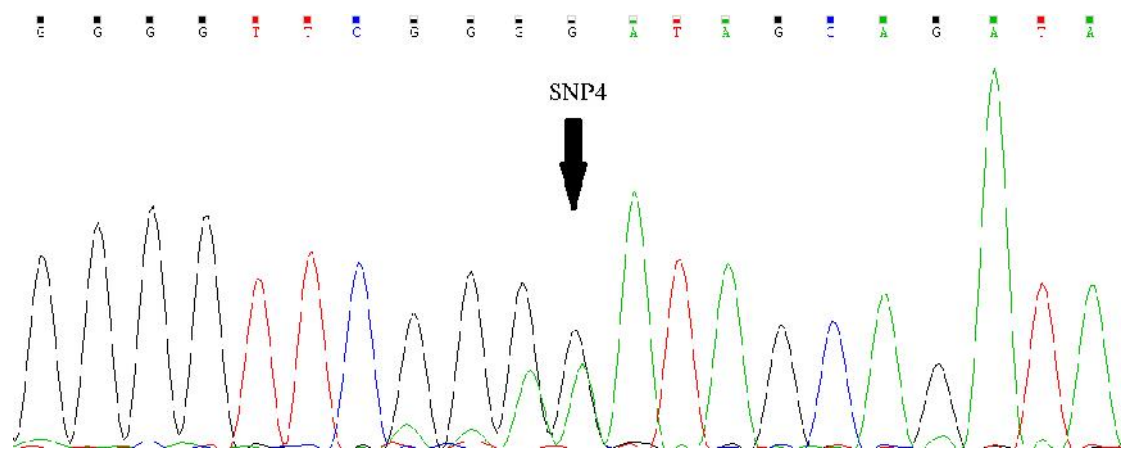
Forward sequencing, the arrow refers to the rs769935596 locus



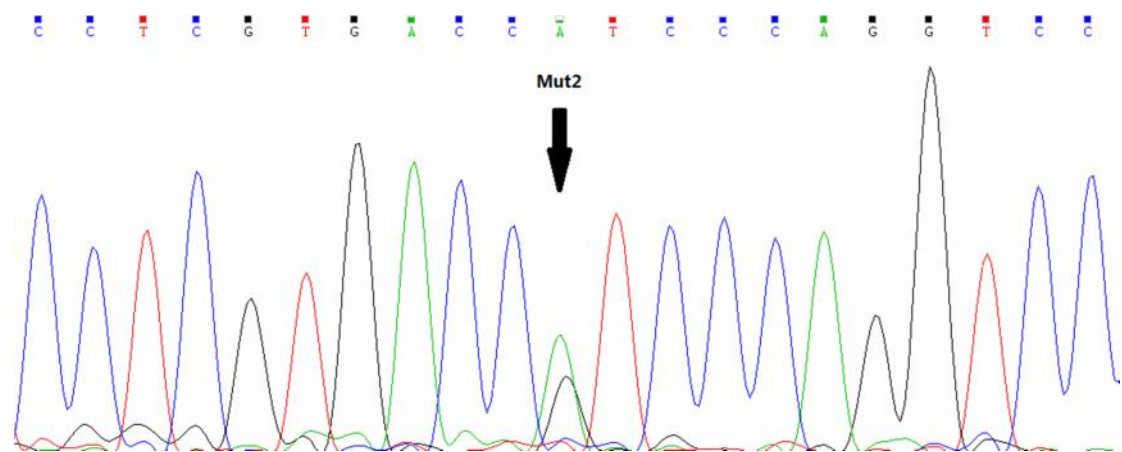
Forward sequencing, the arrow refers to the SNP2 locus



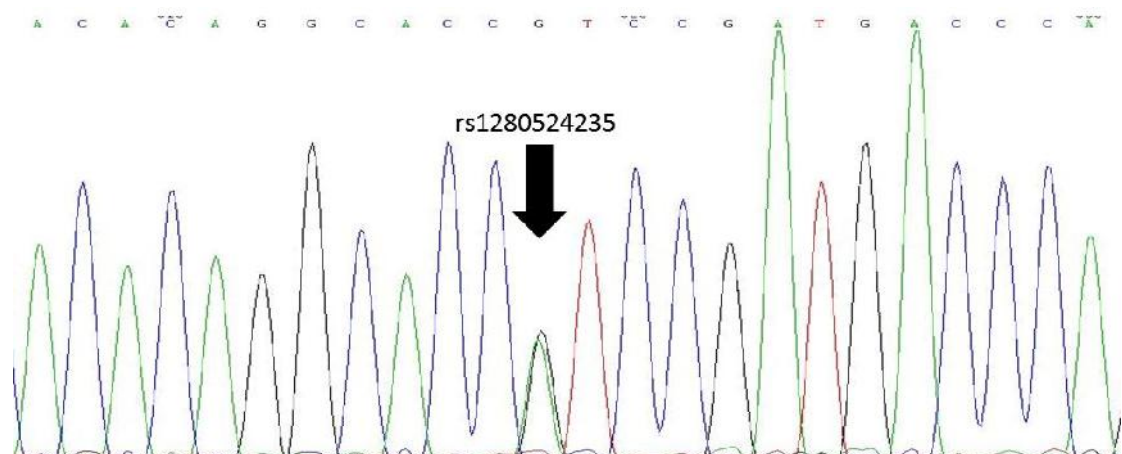
Forward sequencing, the arrows point to rs535666509, rs760934092 and SNP3 sites respectively



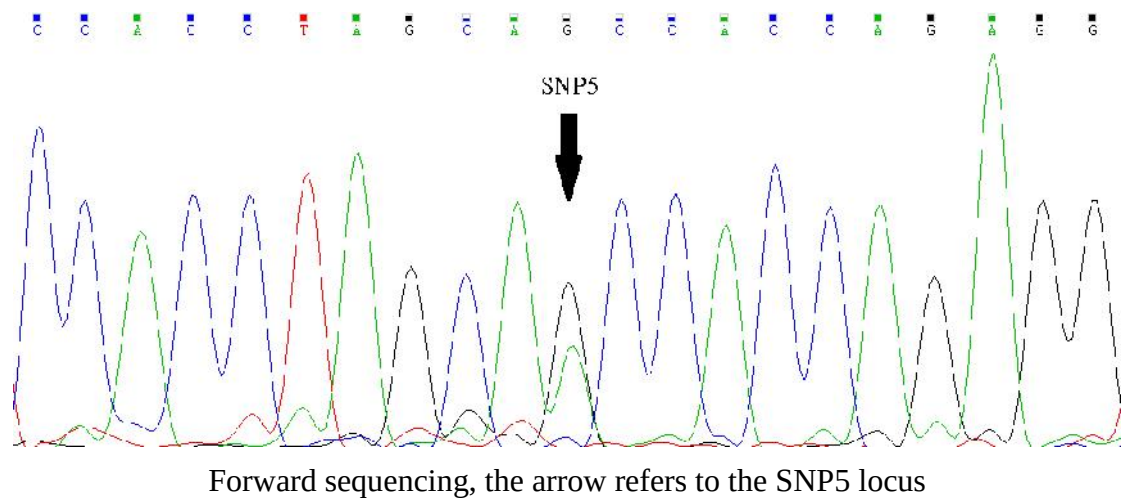
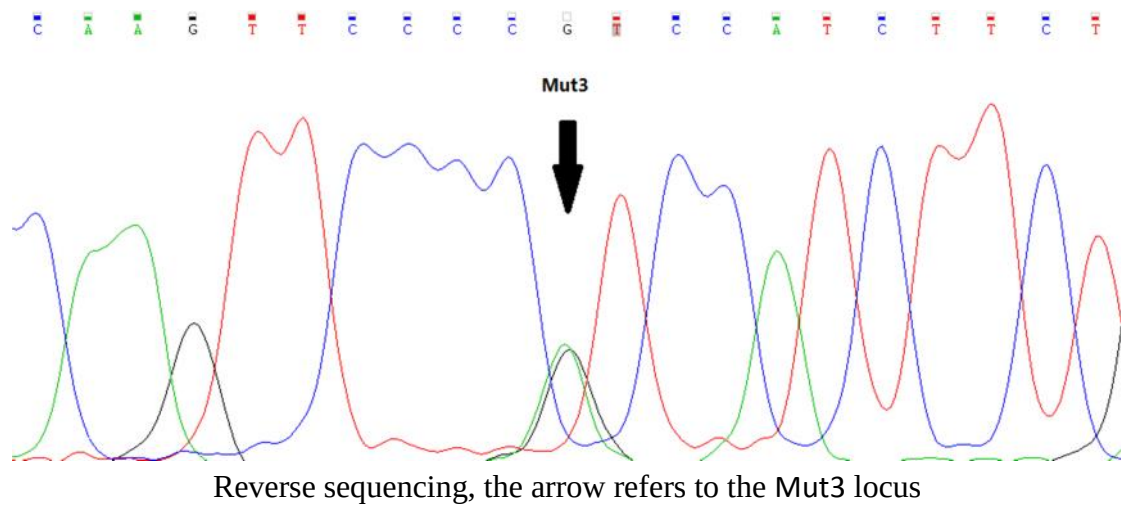
Forward sequencing, the arrow refers to the SNP4 locus

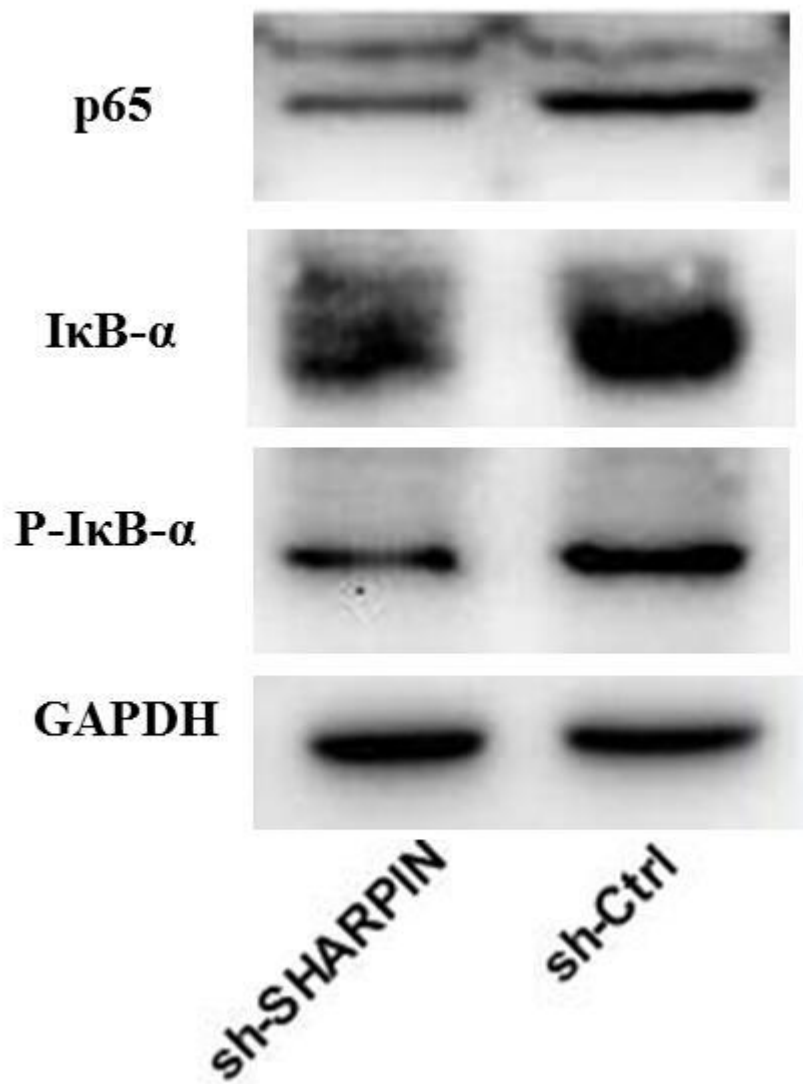


Forward sequencing, the arrow refers to the Mut2 locus



Reverse sequencing, the arrow refers to the rs1280524235 locus





Supplementary Figure S4. SHARPIN regulates NF- κ B activation in HaCaT cells.

Decreased SHARPIN expression by shRNA interference resulted in NF- κ B inactivation. The expression level of p-I κ B- α , I κ B- α and p65 were decreased in sh-SHARPIN groups than that in sh-Ctrl groups.

Supplementary Table 1. PCR primers of *SHRAPIN* gene

Exon	Forward primer(5' → 3')	Reverse primer(5' → 3')	Size(bp)
E1	CAGGTTCGCGGCCCGTGTTT	AAGAGGACTGACCGCGCGCC	333
E2	ATTTCTTTGCTCCTCGTGCG	CTTCCCAGACATCCAGCAGT	348
E3	CAGCACAGCACACCCATATC	GGGACTATCTGCTATCCCCG	221
E4E5	GTCCCCAGTGGTGACAGCTG	ACCTAGGCTCCTCTGCCCCT	422
E6E7	TCACCTGCCCTGATGCTCTG	TGCTCTTTCCAATGCCCATG	433
E8	CAGGGAAGCAACAACACTGTCT	ATTCCTGTGGATTCTGCCCT	217

Supplementary Table 2: The primer sequences of target genes and housekeeping genes for quantitative RT-PCR

Gene	Sequences of primer	Size of amplification fragment (bp)
GAPDH	FW: 5'-GAGTCAACGGATTGGTCGT-3' Rev: 5'-TTGATTTTGGAGGGATCTCG-3'	238
IL-33	FW: 5'-GCCTGTCAACAGCAGTCTACTG-3' Rev: 5'-TGTGCTTAGAGAAGCAAGATACTC-3'	135
ST2	FW: 5'-TGGTTTAAGAATTGTCAGGCTCTTC-3' Rev: 5'-GCGTCCTCAGTCATCACATTATCA-3'	89
IL5	FW: 5'-GGAATAGGCACACTGGAGAGTC-3' Rev: 5'-CTCTCCGCTTTTCTTCTCCACAC-3'	127
FLG	FW: 5'-ACTTCACTGAGTTTCTTCTGATGGTATT-3' Rev: 5'-TCCAGACTTGAGGGTCTTTTCTG-3'	178
KLK5	FW: 5'-TCGTCCCCTAAAGATGTCA-3' Rev: 5'-ATCTATCTGTCTCGGGTAAGC-3'	190
KLK7	FW: 5'-TCATGTGCGTGGATGTCAAG-3' Rev: 5'-TGTGGCGTTAGCGATGCT-3'	282
CAP1	FW: 5'-CATTTGACTCGCTGCTTG-3' Rev: 5'-CCTGGATACTTTTCGCTGA-3'	282
Caspase14	FW: 5'-CACGGTAGAGGGATACATCG-3' Rev: 5'-GCTTTTCCTTCTTGAACCAG-3'	162
Matriptase	FW: 5'-TGCCAGTCAACAACGTCAAG-3' Rev: 5'-ACGTCCCGGTACTGCAAAT-3'	136

FW: Forward; Rev: Reverse; *FLG*: Filaggrin; *CAP1*: Channel-activating serine protease 1; *KLK*: Kallikrein; RT-PCR: Real-time polymerase chain reaction