STK35L1 regulates Plasmodium infection and expression of cell cycle genes during liver stage of malaria

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Article

**Keywords:** Plasmodium, malaria, STK35L1

**DOI:** https://doi.org/10.21203/rs.3.rs-133230/v1

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Abstract

Protein kinases of both the parasite and host are crucial in parasite invasion and survival and might be drug targets against drug-resistant malaria. STK35L1 was among the top five hits in kinome-wide screening, suggesting a role in malaria’s liver stage. The function of STK35L1 in malaria is not known yet. We found that STK35L1 was highly upregulated during the infection of P. berghei in HepG2 cells and mice liver. Knockdown of STK35L1 remarkably suppressed the sporozoite infection in hepatocytes. STAT3 is upregulated and phosphorylated during P. berghei sporozoites infection. We found that STAT3 activation is required for both STK35L1 and STAT3 upregulation. Furthermore, ten cell cycle genes were upregulated in the sporozoite-infected hepatocytes. Knockdown of STK35L1 completely inhibited the upregulation of these genes. We identified STK35L1 as a host kinase that plays an obligatory role in malaria’s liver stage. It may be a potential drug target against drug-resistant malaria.

Introduction

Malaria caused by Plasmodium sp. infection is endemic and remains one of the predominant infectious diseases in South-East Asia and sub-Saharan African regions. The asymptomatic liver stage of Plasmodium infection is obligatory in the parasite’s lifecycle, and various host factors are critical for invasion and replication of the parasite in hepatocytes. Protein kinases of both the parasite and the host are pivotal players in parasite invasion and survival strategies and might be drug targets against drug-resistant malaria. A high throughput siRNA-based screening of 727 kinases by Prudencio et al. identified five hepatocyte kinases, viz. MET, PKCz, PRKWNK1, SGK2, and STK35 that might regulate the infection of Plasmodium berghei. Among these kinases, STK35 (also known as STK35L1 and CLIK1) was previously shown to interact with CLP-36, a PDZ-LIM protein, and actin stress fibers suggesting its involvement in actin dynamics. The reorganization of the host cell actin cytoskeleton is one of the crucial factors for Plasmodium infection, indicating the role of STK35L1 in malaria. However, the function of STK35L1 is not yet established in Plasmodium infection during the liver stage of malaria.

STK35L1 is a member of the NKF4 (New Kinase Family 4) Ser/Thr kinases family and is classified in the group “Other” of the human kinome and is mainly localized in the nucleus and nucleolus. Previously, we showed that nuclear actin is a binding partner of STK35L1 in endothelial cells signifying that STK35L1 might have a role in gene transcription, cell migration, and chromatin remodeling. Moreover, in endothelial cells, the silencing of the STK35L1 gene drastically impaired cell migration. A study showed that importin-a2 enhanced the expression of the stk35l1 gene, and constitutive overexpression of STK35L1 activates caspase-independent cell death under oxidative stress conditions. STK35 (STK35L1) knockout mice showed defects in eye development and were infertile. STK35L1 transcript was altered in colorectal cancer and cardiomyopathy. All these studies suggest that STK35L1 has diverse roles in the physiology and pathophysiology of various diseases. A cell cycle-specific gene array analysis revealed that three genes (CDKN2A, DDX11, and GADD45A) were strongly downregulated in STK35L1 gene knockdown-endothelial cells. Notably, a study showed that CDKN2A expression was highly upregulated.
during acute malaria infection. It suggests that STK35L1 might be associated with CDKN2A expression in malaria.

A study showed that STAT3 plays a critical role in malaria pathogenesis as Plasmodium berghei (P. berghei) infection leads to the activation of STAT3. Recently, it was shown that STAT3 regulates the expression of STK35L1 in osteosarcoma, suggesting a link of STK35L1 with STAT3 and malaria. The STATs are the transcription factors, and their family is composed of seven members; STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. STAT activation is mediated via phosphorylation of Tyr705 by activation of AK/STAT pathway leading to homodimerization and induce gene transcription by binding to a specific DNA-response element in the promoter region of target genes. Activation of STAT3 plays a central role during viral infection in the liver. In various cell types, STAT3 gets phosphorylated and upregulates the expressions of cell cycle genes, including Cyclin B1 and Cdc2. Infection of mice with Plasmodium yoelii strains (Py17XNL, Py17XL) activated STAT1 and STAT3 via phosphorylation, on the first day after parasite inoculation. It is unknown whether activation of STAT3 regulates the expression of STK35L1 in Plasmodium infection during the liver stage of malaria.

Based on the previous studies from other groups and the report from our lab, we hypothesized that STK35L1 might be a crucial player in the pathogenesis of Plasmodium infection during the liver stage of malaria, and STAT3 might play an essential role in STK35L1 regulation. In this study, we showed for the first time that STAT3 mediates the upregulation of STK35L1 after infection of P. berghei in hepatocytes. STK35L1 inhibition leads to the reduction of P. berghei sporozoite infection in hepatocytes. Additionally, various cell cycle genes were upregulated during P. berghei infection, and STK35L1 regulates their expression.

Results

**STK35L1 is highly upregulated in vitro and in vivo during P. berghei infection**

To gain an insight into the role of STK35L1 in governing susceptibility to parasite infection in the liver stage of malaria, a hepatocyte cell line HepG2 were infected with P. berghei ANKA sporozoites. The expression of STK35L1 was measured by qPCR, and we found that it was significantly upregulated (~5 fold; p<0.01) after sporozoite infection in HepG2 cells (Figure 1A). The upregulation of STK35L1 was further confirmed at the protein level by Western blotting using an anti-STK35L1 antibody. We found an increased expression (~2 fold) of STK35L1 protein in infected HepG2 cells (Figure 1B; black arrow).

We asked whether infection of P. berghei sporozoites in mice liver would also enhance the expression of Stk35l1. Indeed, we observed a significant increase (~2 fold; p<0.03) of Stk35l1 gene expression in mice liver infected with P. berghei sporozoites. (Figure 1C). The parasite load in the mice livers was also determined by the copy number of the P. berghei 18S rRNA in the infected and uninfected liver. The data showed that all the mice were infected, but the infection (parasite load) was highly variable (Figure 1D).
In a nutshell, these data suggest that the STK35L1 upregulation during P. berghei infection might play a crucial role in facilitating the parasite's infection and growth during the liver stage of malaria.

**Inhibition of STK35L1 impairs the infection of host cells by Plasmodium sporozoites.**

We asked whether STK35L1 is essential for P.berghei sporozoite infection in hepatocytes. We knocked down the expression of STK35L1 using three STK35L1 siRNAs and then measured the parasite load to address this question. We procured commercial siRNAs that were designed from different regions of STK35L1. We confirmed the knockdown efficiency of these siRNAs via qPCR and Western blotting. Two of the siRNAs (siRNA1 and siRNA2) showed the highest knockdown efficiency (~82% and ~87%, respectively) for STK35L1 (Figure 2A). The knockdown of the STK35L1 protein was also confirmed by immunoblotting (Figure 2B). These siRNAs(siRNA1 and siRNA2) were chosen for further studies. To investigate the role of STK35L1 in P.berghei infection, HepG2 cells were treated with siRNA1 and siRNA 2 for 72 hours and then infected with P.berghei sporozoites for 48 hours. The scrambled siRNA was used as a negative control. Notably, we found that the sporozoite infection (parasite load) was significantly reduced to ~20% in cells treated with siRNA1 and siRNA 2 (Figure 2C). The infection was decreased slightly but was non-significant in cells treated with scrambled siRNA (Figure 2C).

Similarly, the STK35L1 upregulation was significantly reduced after sporozoites infection in cells treated with siRNA1 and siRNA2 (Figure 2D). Scrambled siRNA could not affect the expression of stk35l1 (Figure 2D). These data showed the physiological relevance of STK35L1 as a vital host factor required for the liver infection with Plasmodium sporozoites.

**STAT3 is highly upregulated during Plasmodium infection in HepG2 cells.**

We hypothesized that the STK35L1 upregulation during P. berghei infection is regulated by the member(s) of the STAT transcription factor family. First, we examined the expression of STAT family transcription factor genes in HepG2 cells via qPCR. We found that STAT1 and STAT3 genes were highly abundant in HepG2 cells (Figure 3A). The STAT4 gene was relatively less expressed (~6 fold lower) in comparison to STAT1. Other family members (STAT2, STAT5A, STAT5B, and STAT6) were expressed at 50 to a 100-fold lesser extent in HepG2 cells (Figure 3A). We further studied the effect of sporozoite infection on STAT1, STAT3, and STAT4 expression in HepG2 cells. Notably, we found that only the expression of STAT3 was significantly upregulated (~7 fold; p<0.01) in infected HepG2 cells (Figure 3B). We could not find any significant changes in the expression levels of STAT1 andSTAT4 (Figure 3B). The upregulation of STAT3 in infected cells was also confirmed by Western blotting using the anti-STAT3 antibody (Figure 3C). Collectively, these data show that only STAT3 is significantly upregulated via Plasmodium sporozoite infection, and STAT3-dependent signaling might be necessary for the liver stage of malaria.

**STAT3 is activated during Plasmodium infection in HepG2 cells and autoregulates its expression**

We investigated whether sporozoite infection activates STAT3 via phosphorylation in addition to enhancing its expression. We could observe that the phosphorylation of STAT3 residue tyrosine-705 was
increased after sporozoite infection in HepG2 cells (Figure 4A). We found a very low STAT3 phosphorylation in non-infected cells (Figure 4A). These data indicate that sporozoite infection leads to STAT3 phosphorylation, which might regulate various host gene expressions.

Furthermore, to examine whether STAT3 activation regulates its expression, we used a specific STAT3 activator ML115 \(^{22}\). Notably, ML115 (4µM) could significantly increase the expression of STAT3 by ~3 fold; \((p<0.002)\) within 6 hours of treating HepG2 cells with ML115 (Figure 4B). To confirm that STAT3 regulates ML115-dependent its upregulation, we used highly specific STAT3 inhibitors Stattic and 5,15-DPP \(^{23,24}\). We observed that both inhibitors could not significantly affect the basal expression of STAT3 (Figure 4B). As expected, both inhibitors successfully inhibit the ML115-dependent upregulation of STAT3 by ~85% and ~80%, respectively (Figure 4B). Together, these data suggest that activation of STAT3 via sporozoite infection leads to the upregulation of its expression in a positive feedback manner.

**Activation of STAT3 regulates the expression of STK35L1 in HepG2 cells.**

We hypothesized that STAT3 activation might regulate the STK35L1 expression. We treated the HepG2 cells with specific STAT3 inhibitors, Stattic, and 5,15-DPP. Interestingly, the basal expression of STK35L1 was significantly down-regulated after the treatment of Stattic (~60%; \(p<0.013\)) and 5,15-DPP (~85%; \(p<0.0025\)) (Figure 5A). This result suggests that STK35L1 expression is tightly regulated via STAT3.

Furthermore, we asked whether activation of STAT3 upregulates the expression of STK35L1. We incubated HepG2 cells with a specific STAT3 activator, ML115(4µM), for 6 hours and found that STK35L1 was significantly upregulated (~2 fold; \(p<0.005\)) after the treatment of cells with ML115 (Figure 5B). Next, we examined whether ML115-dependent upregulation of the STK35L1 gene can be reverted after the inhibition of STAT3 activation. Indeed, Stattic, and 5,15-DPP could completely block the upregulation of STK35L1 (Figure 5B). These data collectively confirm that STAT3 is the major transcription factor that plays a crucial role in regulating the expression of STK35L1. The STAT3/STK35L1 axis might be critical for the liver stage of malaria.

**Various cell cycle genes were upregulated during Plasmodium infection, which were regulated via STK35L1**

Previously, we found that STK35L1 regulates various cell cycle-related genes in endothelial cells \(^9\). To identify the role of STK35L1 upregulation during \(P.\) berghei infection, we checked the expression of 11 genes (Table S2) during sporozoite infection. Interestingly, 10 genes (RAD51, MKI67, CDKN3, CDKN2A, CDK6, CDC20, DDX11, GADD45A, CCNB2, and CDC2) were significantly upregulated after sporozoite infection in HepG2 cells (Figure 6 and Table S2), but GTSE1 was not significantly upregulated (Figure 6; bottom right panel). Interestingly, RAD51 was upregulated ~18 fold \((p<0.0001)\) in infected cells (Figure 6; top left panel). CDK6, DDX11, and CDC2 were ~4-5fold upregulated after sporozoite infections (Figure 6). Moreover, GADD45A, CDC20, and CDKN2A were ~3-4 fold upregulated (Figure 6). CDKN3, MKI67, and CCNB2 were ~2-2.5 fold upregulated (Figure 6). To summarize, these data indicate that these cell cycle-related genes might play a crucial role in hepatocyte growth and, consequently, on malaria’s liver stage.
We asked whether STK35L1 has a role in regulating the infection-dependent upregulation of these genes. Indeed, upregulation of the studied genes except CDC2 and GTSE1 was significantly downregulated after knockdown of the STK35L1 gene (Figure 6). The upregulation of CDC2 after sporozoites infection is independent of STK35L1. These data signify that STK35L1 is a major factor that controls the parasite infection-induced expressions of cell cycle-related genes.

**Discussion**

Protein kinases are emerging as new drug targets for therapeutic intervention against drug-resistant malaria. It is now evident that *Plasmodium* infection during the liver stage of malaria drastically changes the cell signaling within hepatocytes. In this study, we deciphered a new host cell signaling network that regulates the sporozoite infection during the liver stage of malaria. We identified a novel kinase, STK35L1, as one of the significant host kinases that play an obligatory role in malaria’s liver stage.

The knowledge about the role of host kinases in the liver stage of malaria is mostly unknown. Only a few kinases were identified via kinome-wide high throughput screening, which might be crucial in the liver stage infection\(^{25,26}\). In a study, STK35 (STK35L1) was found among the top five hits during hepatocytes infection by *P. berghei* sporozoites\(^{26}\). The information about the functions of STK35L1 in various diseases is minimal, and its role in malaria was utterly unknown. In this study, we confirmed that the knockdown of STK35L1 remarkably suppressed the sporozoite infection in the hepatocyte cell line, HepG2. Interestingly, we found that STK35L1 expression was enhanced in infected hepatocytes during liver stage infection in mice. These findings suggest a new and crucial role of STK35L1 in the liver stage of malaria.

It is evident from previous studies that STAT3 transcriptionally regulates STK35L1 expression in osteosarcoma cells\(^{16}\). Activation of STAT3 has been reported in the pathogenesis of severe malaria and viral infections, including human cytomegalovirus and hepatitis B in hepatocytes\(^{15,27,28,29}\). In line with this, we proposed that the STAT3/STK35L1 axis is active during the liver stage of malaria. First, we checked the basal expression of STAT family members in HepG2 cells and found that STAT1, STAT3, and STAT4 were highly expressed compared to other family members. Notably, we showed the first time that STAT3 is autoregulated during *P. berghei* sporozoites infection. Additionally, we found that STAT3 is upregulated and phosphorylated at the residue, Y-705, which induces dimerization of STAT3 and then activates the expression of STAT3-targeted genes\(^{30}\). Together with previous studies, these data indicate that STAT3 signaling might play a pivotal role in the pathogenesis of the liver stage of malaria via controlling the STK35L1 expression.

The small molecules that selectively inhibit or activate STAT3 can act as useful tools to study the STAT3-specific signaling in host-pathogen interaction during malaria. To further extend our study, we used selective STAT3 inhibitors, Stattic, 5,15-DPP, and ML115, a specific pharmacological activator of STAT3 signaling\(^{22,23,24}\). To support our findings, we mimicked the state of infection in hepatocytes by activating STAT3 signaling using ML115. Indeed, we found the upregulation of STK35L1 and STAT3 in
ML115-treated cells. Further, the upregulation was entirely blocked by Stattic and 5,15-DPP. These data confirm that STAT3 activation is crucial for STK35L1 upregulation during sporozoite infection in hepatocytes.

It is not well understood whether Plasmodium sporozoites alter the cell cycle genes in hepatocytes for their benefit or it requires a specific stage of the host cell cycle for infection. A previous study showed that Plasmodium infection during the liver stage of malaria does not require host cell cycle progression. Interestingly, transcriptional profiling of host cells during the liver stage infection at different time points revealed that various cell cycle linked genes were differentially expressed, suggesting cell cycle genes’ role in survival and expansion of parasite growth and development. Another study showed that Plasmodium introduced circumsporozoite protein in hepatocyte cytoplasm that regulates the expression of various genes including cell cycle-related genes to promote parasite growth. Additionally, various studies indicate that STK35L1 has a role in multiple cellular functions, including cell migration, apoptosis, and cell cycle. Previously, we showed that STK35L1 regulates 11 cell cycle genes in endothelial cells. It indicates that the STAT3/STK35L1 axis might regulate the cell cycle-related gene expression during sporozoite infection in hepatocytes.

We focused on the previously identified cell cycle genes controlled by STK35L1 and found that ten cell cycle genes were differentially expressed in the sporozoite-infected hepatocytes. Earlier, it was reported that cell cycle genes, GTSE1, CDC20, MKI67, CDC2, and CDKN3, were highly upregulated within 6 hours of infection in HepG2 cells. We also found the upregulation of these genes except GTSE1 after 48 hours post-infection. A study identified that cell cycle regulatory genes (58 genes, including GADD45A) were differentially expressed after 18 hours of infection. Another report showed that CDKN2A was highly expressed during acute malaria. Besides these genes, we identified additional genes, viz. RAD51, DDX11, CDK6, and CCNB2 were highly upregulated in liver-stage parasite-infected hepatocytes. Notably, knockdown of STK35L1 completely inhibited the upregulation of studied cell cycle genes except CDC2 gene. These data indicate that cell cycle genes facilitate the infection and growth of Plasmodium in hepatocytes, and the expression of these genes is regulated via STK35L1.

This study established a new signaling axis STAT3/STK35L1-dependent differential gene expression of specific cell cycle genes and the necessity of this axis for Plasmodium infection and proliferation during the liver stage of malaria.

Hepatocyte apoptosis is crucial for Plasmodium survival, and it must be regulated. Interestingly, STK35L1 holds caspase-independent cell death in stress conditions. The role of STK35L1 in hepatocyte cell death during sporozoite infection may be explored in future studies. Furthermore, STK35L1 interacts with nuclear actin and actin stress fibers. Notably, the host cell actin cytoskeletal reorganization is critical for Plasmodium infection during the liver stage of malaria. Nuclear actin plays a significant role in chromatin remodeling, transcriptional regulation, and DNA repair. The highly upregulated genes RAD51 and DDX11 are involved in DNA replication and DNA repair, and might
play role in repair of host damaged DNA, arising due to pathogenic condition. Based on our findings, we propose a functional model of STK35L1 role in malaria's liver stage parasite growth (Figure 7). This study will provide a new opportunity to develop STK35L1 as a potential therapeutic drug target that could be used to treat drug-resistant malaria and improve clinical outcomes while reducing malaria mortality.

**Materials And Methods**

**Materials**

Primers were procured from Eurofins, India, ML115, a specific STAT3 activator, and STAT3 inhibitors, stattic and 5,15-DPP (5,15-Diphenylporphyrin), were procured from Cayman chemicals, USA. All cell culture reagents were purchased from HyClone, GE life sciences, USA. Scrambled siRNA and STK35L1 gene-specific siRNAs were procured from Sigma-Aldrich, USA. STAT3, p-STAT3 (Y-705), and GAPDH antibodies were purchased from Santa Cruz Biotechnology, USA. Anti-mouse IRDye800 coupled secondary antibodies were purchased from LI-COR Biosciences, USA. Anti-STK35L1 antibody (STK35-P1, rabbit) was established in our lab as described in supplementary method.

**Mosquito rearing and sporozoite production**

C57BL/6 mice were bred in institute facilities as per the guidelines of the Institutional Animal Ethics Committee (IAEC) of the National Institute of Immunology (NII), and all the experiments were carried out following the CPCSEA guidelines (Govt. of India). GFP expressing Plasmodium berghei ANKA (P. berghei ANKA) sporozoites strain was obtained from BEI Resources Repository, NIAID, NIH: MRA-671, contributed by M. F. Wiser. P. berghei ANKA parasites were cycled between mice and Anopheles stephensi mosquitoes. Mosquitoes (3-5 days old, female) were starved overnight and fed on six to eight-week-old C57BL/6 mice. After infection, mosquitoes were kept at 19°C, 70% to 80% relative humidity, and 12 hours light cycle for 18 days. The mosquitoes were fed on a 20% sucrose solution-soaked cotton pad during this period. After 18 days, mosquitoes were washed with 50% ethanol and then with PBS (phosphate-buffered saline) three times. These washed mosquitoes were dissected in complete DMEM (Dulbecco’s modified Eagle’s medium) without antibiotics. Salivary glands were isolated and ground gently in complete DMEM to release sporozoites. Mosquito debris was removed by centrifuging at 100 × g for 4 min at 4°C, and the number of sporozoites in the supematant was determined using a hemocytometer.

**Cell culture and HepG2 cell infection**

All in vitro experiments were conducted with the HepG2 cell line, which was routinely maintained in DMEM containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were maintained in a humidified incubator with 5% CO₂ and at 37°C. For infection, cells were grown (1x10⁶ cells/well) in 6-well plates. After 24 hours, sporozoites (1x10⁵ sporozoites/well) were added, and then cells were harvested 48 hours post-infection. Infected cells were sorted via FACS (BD FACS Aria). Control (non-infected) and GFP parasite positive cells were lysed using Trizol reagent, and total RNA was isolated as per manufacturers’ protocol.
**In vivo infectivity assay.**

To perform in vivo infection, $1 \times 10^4$ *P. berghei* ANKA sporozoites were injected intravenously into C57BL/6 mice. After 48 hours of sporozoite infection, livers were isolated from infected and non-infected mice and then homogenized in denaturing solution (4M guanidine thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, and 0.7% β-mercaptoethanol in DEPC treated water). Total RNA was isolated, and the liver parasite load was estimated by employing quantitative real-time PCR (qPCR) using parasite-specific 18SrRNA primers\(^40\).

**Quantitative real-time PCR (qPCR)**

cDNA was synthesized with an iScript cDNA synthesis kit (BioRad, USA) as per manufacturers' protocol. Gene-specific primers (Table S1) were designed using NCBI primer blast tool, and qPCR was performed on Roche Light Cycler 96 or Mastercycler® ep realplex (Eppendorf, Germany) as described previously\(^41,42\). Cycling program: 7 min at 95°C and then 40 cycles with 10 s at 95°C and 20 s at 60°C. The specificity of the amplicons was analyzed by thermal dissociation curve and agarose gel electrophoresis. Data were normalized against the housekeeping gene, β-actin.

**Gene silencing**

The HepG2 cells (5x$10^4$ cells/well) were grown in a 24-well plate. STK35L1 specific siRNA (50pmol) or scrambled siRNA (50pmol) were transfected in duplicates using Lipofectamine 2000 transfection reagent (Invitrogen) as described previously\(^43\). After 48-72 hours of transfection, cells were harvested, and knockdown efficiency was calculated at the gene level by qPCR and at the protein level using Western blotting.

**Western Blot**

Whole protein extracts were prepared in RIPA buffer (20mM HEPES; pH 7.9, 350mM NaCl, 1mM MgCl\(_2\), 0.5mM EDTA, 0.1mM, 1% NP-40, 0.5mM DTT, 50mM NaF, 1mM Na\(_3\)VO\(_4\), 0.2mM PMSF, and 1µg/mL aprotinin) with protease and phosphatase inhibitors. Protein concentration was determined using a BCA protein kit (Thermo Scientific) as per the manufacturers' protocol. Equal amounts of protein were loaded in each well, resolved using SDS PAGE gel electrophoresis, and then transferred to nitrocellulose membranes (200volts for 60 min at 4°C) using the Mini Trans-Blot electrophoresis unit (Bio-Rad). Membranes were blocked with Odyssey blocking buffer for an hour at room temperature and then probed at 4°C overnight with primary antibodies followed by secondary antibodies at room temperature for 1 hour. The dilution of primary antibodies was as follows: anti-STK35L1 (1:200), anti-STAT3 (1:1000), anti-p-STAT3 (Tyr-705; 1:1000) and anti-GAPDH (1:2000), anti-mouse IRDye800 coupled secondary antibodies (1:5000). The membrane was scanned using Odyssey Infrared Imager (LI-COR Biosciences), and densitometric analysis of the proteins was done using ImageJ (1.34s) software (NIH, Bethesda, MD).
**Statistical analysis**

Each experiment was performed three or more times independently with different cell passages. Statistical analysis was performed on GraphPad PRISM 7 software (GraphPad, La Jolla, CA, USA) using one-way ANOVA or two-tailed unpaired t-test. The p-values of <0.05 were considered a statistically significant difference. Results are calculated as mean ± SD, and data are shown as mean + SD.

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