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Targeted disruption of TAK1 in HEK293 cells via CRISPR/Cas 9: A tool for functional dissection of inflammatory and autophagic signaling

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Abstract: **Transforming growth factor-β activated kinase1 (TAK1) is a serine threonine kinase stimulated by TGF-β, in addition to other stimuli including inflammatory cytokines, microbial products and environmental stress. TAK1 regulates multiple cellular events notably developmental processes, differentiation, autophagy, apoptosis and cell survival through extensive cross-talk with crucial signalling pathways like, MAP kinases, IKK and JNK. On one hand it engages with energy sensor mechanisms via AMPK and on the other with growth regulation processes dominated by mTOR signaling. By creating a stable TAK1 knockout cell line, we can dissect how loss of TAK1 affects mTORC1 activation, autophagy induction, and cell survival under conditions of nutrient restriction or metabolic stress, thereby clarifying whether TAK1 functions as a pro-survival, pro-death, or context-dependent modulator in hyperproliferative and aging-like states. For this, three sgRNAs were designed for TAK1 gene. The sgRNA1 and sgRNA2 with the higher activity were respectively used to construct pSpCas9(BB)-2A-Puro-sgRNA1 and pSpCas9(BB)-2A-Puro-sgRNA2. The knockout plasmid vectors were transfected into HEK-293 cells. Through drug screening, the TAK1 gene-knockout HEK293 (TAK1$^{-/-}$) cell lines was obtained which was subsequently confirmed with Western blotting and sequencing. This isogenic CRISPR-derived knockout line will also provide a clean genetic background for rescue experiments and pharmacological studies, improving mechanistic rigour and enabling us to identify TAK1-dependent nodes that could be targeted for therapeutic intervention in cancer or age-related pathologies.**

Keywords: **CRISPR/Cas, TAK1, mTOR, Puromycin.**

I. INTRODUCTION

As a member of MAP3K subfamily TAK1, (Transforming growth factor-β activated kinase) designated frequently as MAP3K7 functions as a pivotal serine/threonine protein kinase [1-2]. Upon activation, it serves as a central orchestrator to integrate downstream signals emanating from various signalling pathways like mitogen activated kinase (MAPK), I κB kinase (IKK)/NF-κ-B, c-Jun N-terminal kinase to activate diverse stimuli, including pro-inflammatory cytokines, environmental stress and microbial products in addition to TGF-β [3-9]. Through these signalling pathways TAK-1 regulates fundamental cellular processes in particular development, differentiation, survival, apoptosis, autophagy and immune responses [10-17]. Beyond its well-established role in inflammatory and stress regulated signalling, TAK-1 has also been shown to mediate crosstalk between MAP kinase and mTORC1 pathway, a central cell growth and proliferation regulator which suggests a broader role for TAK1 to integrate metabolic control with stress response signalling [18]. mTORC1, a central cellular growth and metabolism regulator, senses the energy signals which include sufficiency of

nutrients, in particular the amino acids as well as the growth factors to subsequently drive the biosynthetic processes such as biogenesis of ribosomes, synthesis of proteins and lipid production.

A principal downstream effector of mTORC1 is S6K1 (ribosomal protein S6 Kinase1) [19]. The activation of S6K1 requires phosphorylation of various threonine residues, critically the threonine 412 (Thr- 412) with in its hydrophobic motif. Phosphorylation of S6K1 at Thr-412 residue is widely regarded as a hallmark of mTORC1 activity and is essential for full activation of S6K1 [20]. Conversely energy stress in the form of nutrient scarcity inhibits mTORC1 signalling, and triggers the onset of autophagy, a process favouring catabolic pathway subsequently enabling cells to sustain energy homeostasis and hence their survival [21-22].

The rationale for generating a TAK1 knockout cell line using CRISPR lies in understanding the precise role of TAK1 in nutrient-sensing, stress-signaling, and autophagy pathways that are central to our project. TAK1 (TGF-β-activated kinase-1) acts as a key upstream regulator of NF-κB, MAPK, and autophagy-related signaling cascades, which intersect with mTORC1 activity and cellular responses to nutrient availability and stress.

II. MATERIALS AND METHODS

A. Cell culture

Human Embryonic Kidney cells (HEK-293) were cultured/grown in the (DMEM) medium; Dulbecco's modified Eagles medium (Gibco USA), supplemented by 10% (v/v) FBS fetal bovine serum (Gibco USA origin). The complete media was then treated with the antibiotic: penicillin (50µg/ml) and streptomycin (50µg/ml) purchased from Gibco. HEK cells were grown in an incubator, under humidified conditions with 5% CO2, 95% air and the temperature of 37°C

The cell population was regularly maintained through regular passaging as and when required by trypsinization using trypsin (0.25%) purchased from Gibco. The cells were stocked properly using freeze down media (80% FBS, 10% DMSO and 10% DMEM) and stored at -80°C initially and finally consigned to the liquid nitrogen storage for longevity and future usages.

Post 48-72 hours of transfection, the harvesting process of the cells was conducted in PBS (chilled) for 5 minutes with the speed of 5000rpm at 4 °C. The cells were then subjected to lysis, using a buffer of the following composition: MgCl2 10 milli molar, EDTA 5 milli molar, Tris-Cl (pH 7.5) 50 milli molar, DTT 2 mM, β-Glycero- phosphate 50 milli molar and Triton X-100 0.5%, the whole process was carried out on ice. Lysis buffer was then used to resuspend the cells followed by the incubation on ice for thirty minutes. Post incubation, cells were pelleted down at 14000 rpm for 30-minute (centrifuged at 4°C). The resulting clarified supernatant was finally collected and processed as per our experimental requirements.

B. Transfections

Transfection of the desired DNAs was performed by chemical reagent; PEI (Polyethylene Imine). HEK cells were maintained until they achieved near-total confluence (about 80% in 25 cm^2 or 75 cm^2 vented cap flasks (as per our requirements). The cells were then subjected to proteolytic cleavage/trypsinization and subcultured in 6cm dishes. The culture was there after allowed to grow overnight in the humidified incubator (37°C temperature, 5% CO2, and 95% air) until the confluency of 60-70% was attained. Transfection mixture was made by adding the DNA (4µg) to the antibiotic and serum free media of 400µL of volume (with the final ratio of 1:3 ratio of DNA to PEI). The mixture by was then incubated at an ambient temperature, for five minutes and then left it undisturbed for 5 minutes. PEI with working concentration of 1mg/mL and the volume of 15µL was incorporated into the above mixture and then further subjected to incubation of 20 minutes. Following the removal of initial culture medium, media of the cells was then replenished with complete growth media, with the subsequent incorporation of the transfection reagent mixture. The cells were there after placed in the humidified incubator and allowed to grow.

C. *Western blotting*

The SDS gel of different percentages were prepared depending upon the size of the proteins to be analyzed. For TAK1, 12% SDS gel was used (for resolving gel). Separated proteins from SDS-PAGE were then electroblotted on to the PVDF membrane pore size of 1.5 um (procured from MERK Millipore USA) via wet blotting procedure. Proteins were then probed with specific antibodies (TAK1 antibody purchased from Abcam USA). For the visualization of our target proteins, the signal detection was facilitated by the use of secondary antibodies: goat anti-mouse antibody conjugated to an IR dye (680CW) and goat anti-rabbit (CST USA) conjugated to the IR dye (800 CW) on odyssey infrared imager (LI-COR)

D. Cloning of TAK1 sgRNA in pSpCas9 (BB)-2A-Puro (PX459) knockout plasmid

Guide RNAs specific to our gene of interest (TAK1/MAP3K7) were designed using a web tool known as Benchling. Using Benchling we selected the sgRNAs with minimum off-target score and hence the maximum efficiency. The oligonucleotides used were synthesized from IDT (Integrated DNA Technology) and annealed using hybridization buffer (discussed in detail in the following table). The reverse compliments were also generated by PCR. Adapter sequence CACC and AAAC were ligated in forward and reverse primer guide RNAs respectively. This addition was done to ensure the proper insertion of the guides in the vector backbone. The addition of the G nucleotide at 5'end of the guide RNAs, (whose sequence did not start with guanine nucleotide) was done to initiate the transcription through U6 promoter. The Following guide sequences were subsequently synthesized through Integrated DNA Technology (IDT).

TAK1 sgRNA1 F.P 5'CACCGGAGGAGGAGGAGGAGCAGA3'

TAK1 sgRNA1 RP. 5'AAACTCTGCTCCTCCTCCTCCTCC3'

TAK1 sgRNA2 F.P 5'CACCGCTCCTCCTCCTCCTCGTCTT3'

TAK1 sgRNA2 R.P 5'AAACAAGACGAGGAGGAGGAGGAGC3'

E. ***Reaction mixture for the hybridization of oligos***

The hybridization reaction mixture was prepared with the following composition: Forward oligo /FP (100 µM) 5µl, Reverse oligo/RP (100 µM) 5 µl, 2X Hybridization Buffer (2nmEDTA, 20mM tris and 100mM Nacl (pH 8.0) 10 µl. The final volume of the above mixture was finally adjusted to 20 µL. The reaction mixture (forward and reverse primers) was placed in the thermocycler for the incubation at the temperature of 90 °C for 7 minutes. This was followed by the gradual cooling to the temperature of 30°C. The annealed oligos were then phosphorylated with an incubation period of 30 minutes at the temperature of 37 ˚C with the following reaction composition mixture: T4 Poly nucleotide kinase 1µL, T4 DNA ligase buffer (10x) 1µL, annealed oligos1µL, ddH2O 7µL. The final volume was adjusted to 10 µL.

F. Digestion of the vector (PX459 pSpCas9 BB-2A-Puro).

The reaction mixture prepared for the digestion of the vector constituted of the following components: Px459 pSpCas9vector (10µg) 30µL, enzyme Bbs1 2µL, 10x NEB Buffer 4µL, Milli Q water 4µl. After the completion of the digestion reaction, CIP (Calf Intestinal Phosphatase) stock solution (1 µl 10,000 U/ ml) treatment was given to the vector at 37 C° for 3 hours to remove 5′ phosphate groups from the digested plasmid. The above reaction was incubated at 37 C°. The digested 1% agarose gel gel extraction kit hybridized oligos were then ligated to the digested vector for 3 hours.

G. Ligation of oligonucleotides with purified digested vector: pSpCas9 (BB)-2A-Puro (PX459).

Reaction mixture for the ligation of the digested vector with the oligonucleotides was performed according to the following composition: digested purified pSpCas9(BB)-2Puro (PX459) vector 6µL, T4 DNA ligase 1µL, Ligase Buffer 1.5µL, Milli Q 3.5µL, annealed oligos 3 µL. To facilitate the proper enzymatic ligation, the ligation reaction mixture was equilibrated at constant temperature of 16°C overnight. The vector-only-religation reaction was also set up as a control to ensure that any observed ligation was due to the insertion of the target DNA and not the self-ligation of the vector.

H. Transformation of sgRNA- ligated vector.

The ligated sgRNA and vector were then transformed into competent DH5-α cells. Recombinant plasmid vector was extracted from these transformed cells by plasmid DNA extraction kit (Qiagen). The validation of the recombinant clones was assessed by colony PCR using U6 promoter as forward primer and sgRNA1/sgRNA2 as reverse oligo primers (respectively for the sgRNA1 and sgRNA2 recombinant clones), and finally run on 1% agarose gel. The following conditions were used to set up a PCR reaction:

I. PCR confirmation of sgRNA ligated vector.

The PCR reaction mixture was made with the following composition: Plasmid 0.5µL, Forward primer (U6) (20 mM) 1.25µL, Reverse primer SgRNA 1/sgRNA2, specific (20 mM) 1.25µL, dNTP's (2mM) (Sigma Aldrich) 5µL, PCR buffer (10X) 5µL, Taq polymerase (Sigma Aldrich) 0.2µL, Milli Q 28.8 µL, Mgcl2 8 µL. The total volume of the reaction was adjusted to 50µL. The thermal cycler was set at the following pararmeters: Hot start 94°C for 5 minutes Denaturation at 94°C for 60 seconds, annealing at 55°C for 45 seconds, Extension of 72°C for 1 minute and last extension was done at 72°C for 7 minutes. The total no of cycles performed were 25. PCR products were confirmed on 1% agarose gel.

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III. RESULTS AND DISCUSSION

A. Generation of TAK1 knockout in HEK 293 cells: Target sequence of our desired gene (MAP3K7/TAK1) was pasted it in a web tool called Benchling as shown in (Fig 1A). Guide RNA with lowest off target score & maximum efficiency were selected based on the score of guides (Fig 1B, C). An extra G was added to the 5' end of those guides whose sequence did not start with G in order to allow transcription from the U6 promoter. Adapters with sequence CACC in F.P and AAAC in R.P was added for proper insertion of the guides in the vector backbone. The following guides were accordingly synthesized through IDT.

TAK1 sgRNA1 F.P 5'**CACC**GGAGGAGGAGGAGGAGCAGA 3'

TAK1 sgRNA1 R.P 5'**AAAC**TCTGCTCCTCCTCCTCCTCC 3'

TAK1 sgRNA2 F.P 5'**CACCG**CTCCTCCTCCTCCTCGTCTT 3'

TAK1 sgRNA2 R.P 5'**AAAC**AAGACGAGGAGGAGGAGGAG**C**3'

The guide RNAs were annealed and phosphorylated (as described in Materials and Methods section above), and ligated in Bbs-I digested vector. Successful ligation of sgRNA oligonucleotides into the CRISPR/Cas9 vector-backbone (PX459) was validated using colony PCR method. Competent cells made from E.coli DH5- α were utilized for transformation of recombinant plasmids. Cells (competent cells) were selected on LB agar plates containing ampicillin antibiotic. Five colonies were randomly picked from each transformed plate and inoculated in LB broth containing ampicillin followed by plasmid extraction using miniprep kit following manufacturers protocol. PCR was done for confirmation of positive knockout clones using U6 F.P and respective sgRNA R.P to amplify 240bp region in the positive clones. The products of PCR were finally run on 1% agarose gel. Three colonies from sgRNA1 and all five from sgRNA2 turned out to be positive by showing band at 240 bp (Expected size of our PCR amplified region) (as shown in Fig 1D).

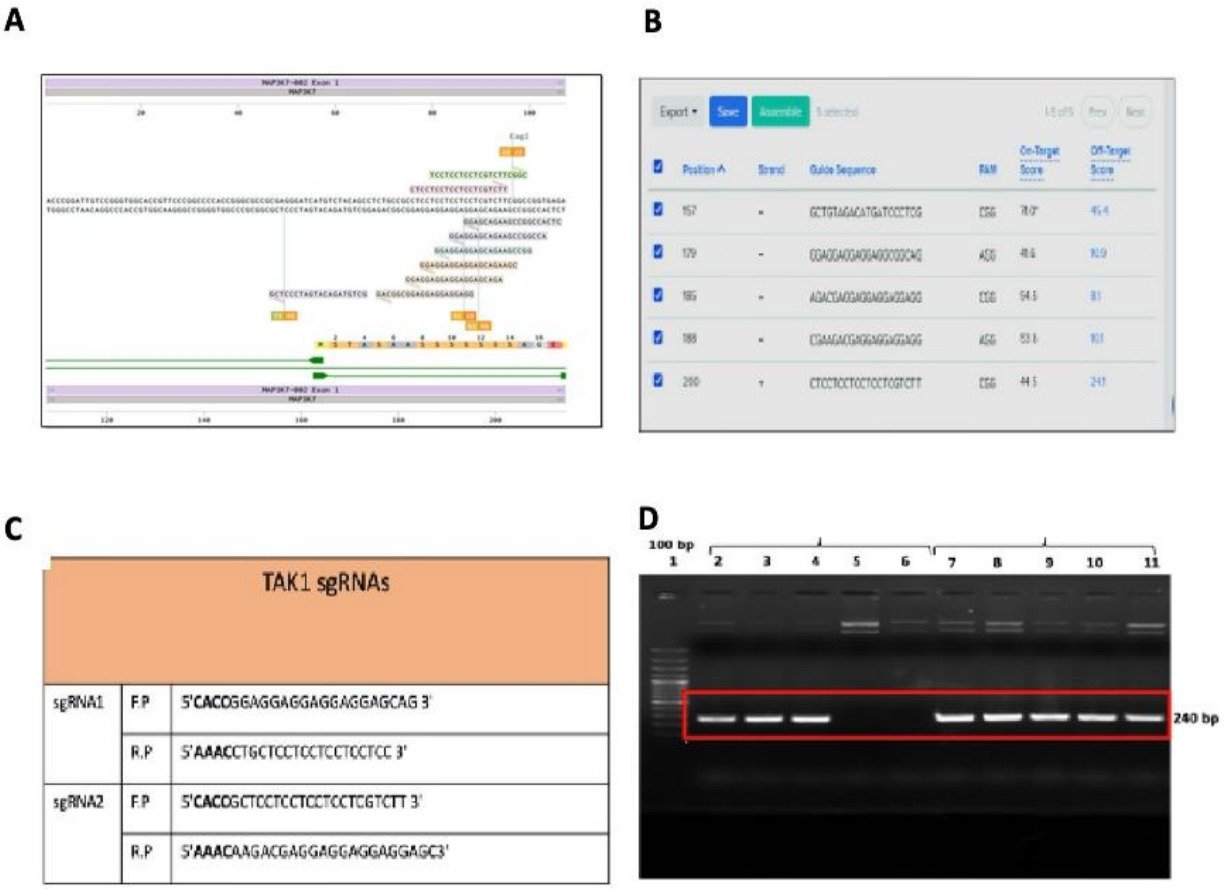

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Fig 1: (A) Representative picture of detailed exon map of the target gene MAP3K7/TAK1. (B) Representative image developed from benchling online tool highlighting the candidate single guide RNAs for TAK1 gene with different on score and off score targets. (C) Selected sgRNA1 and sgRNA2 sequences from the online tool benchling. (D) Colony PCR confirming the ligation of sgRNA1 and sgRNA2 withPX-459 plasmid**.** (Agarose gel picture (0.1%) validating the succesful sgRNA1amplification of sgRNA- 1 ligated- PX459 plasmid recovered from 5 different colonies, lane 2,3,4,5,6, whereas lane 7,8,9,10,11 shows amplification of sgRNA-2 ligated- PX459 plasmid recovered from 5 different colonies.

B. ***Screening of TAK1 knock out HEK-293- cell line using antibiotic selection.*** The recombinant vector was transfected into HEK-293-T cells. The transfected cells containing the CRISPR/Cas construct were enriched by the selection pressure using puromycin. Puromycin kill curve was used to work out the effective concentration of puromycin, which killed the HEK-293- cells (Fig2). For obtaining the most efficient lethal concentration of puromycin for selection of cells, HEK-293 cells were seeded in 24-well transfection plate and cells were grown up to 95% -100% confluency. Concentrations of puromycin ranging from (0.2 to 3ug /mL) were tested against cells over a span of 3 days. Cell death was noted and the most efficient concentration (1.2ug/ml) was chosen for selection of transfected cells (Fig 2A, B2, and 2C).

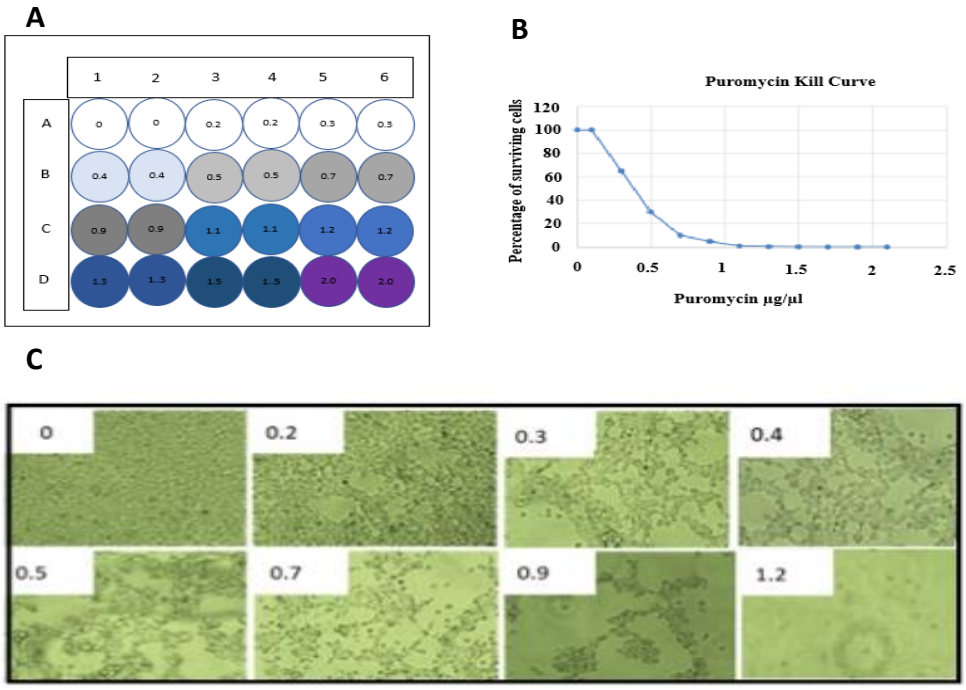

Fig 2 Representative picture of HEK-293 cells illustrating the effective concentration of puromycin which resulted in the cell death. (A) HEK-293 cells were seeded into a 24-well plate and treated with the different concentrations of puromycin 0-1-3ug. (B) Puromycin kill curve plotted against percentage of viable cells and the concentration of puromycin (ug/ml). (C) Representative image generated through the microscope effectively illustrating the lethal concentration of puromycin (1.2ug).

C. Validation of TAK1 knock out HEK-293-T cell lines through western blotting and sequencing analysis.

The recombinant vector was then transfected into HEK293 cells. The transfected cells containing the CRISPR/Cas construct were enriched by the selection pressure using puromycin. Puromycin kill curve was used to work out the effective concentration of puromycin, which killed the HEK-293 cells (as discussed above). The puromycin resistant cells were subsequently subjected to protein level confirmation by western blotting. While as the wild type HEK-293 cells robustly expressed TAK1 protein, the significant absence of TAK1 proteins from the lysates extracted from candidate knockout cells validated the successful gene disruption at the protein level (Fig 3B). Finally, the sequencing analysis also confirmed the precise alterations in the form of frame shift mutations in the TAK1 exon compared to the wild type cells (Fig 3A). Taken together the above-mentioned evidences clearly confirm the generation of TAK1 knockout cell lines, thus providing a reliable platform for subsequent functional studies.

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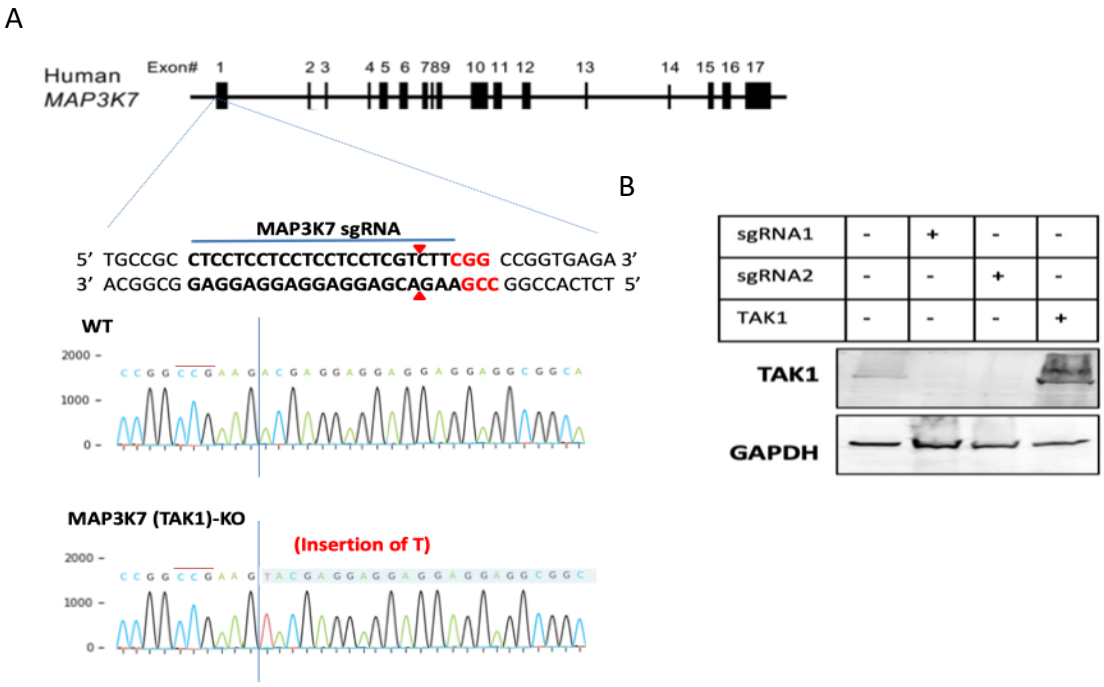

Fig 3. Validation of CRISPR-Cas9 mediated TAK1 knockout in HEK293T cells using sequencing and western blotting. (A) Sequencing analysis validating the successful knockout TAK1 gene confirmed by the insertion of T leading to the frame shift mutations in the MAP3K7 coding region. (B) Immunoblot depicting the clear presence of TAK1 proteins in wild type HEK cells compared to HEK-293 cells transfected with TAK1 sgRNA1 and sTAK1 gRNA2. HEK-293 cells were transfected with TAK1-sgRNA1 and TAK1-sgRNA2. Post 72 hours of transfection, cells were lysed in ice cold lysis buffer. Cell lysates were subjected to SDS-PAGE followed by the western blotting, using antibodies as indicated in the panel. GAPDH was taken as a loading control.

IV. CONCLUSION

We report the succesful knock-out of TAK1 gene employing CRISPR/Cas gene editing technique in HEK-293 cell line. The successful knock out of TAK1 gene in HEK cells was confirmed by PCR, immunoblotting and sequencing analysis.

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Conflict of interest:

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

[1] Ju, Q., Sheng, W., Zhang, M., Chen, J., Wu, L., Liu, X., ... & Sun, C. (2025). TAK1-mediated phosphorylation of PLCE1 represses PIP2 hydrolysis to impede esophageal squamous cancer metastasis. *Elife*, *13*, RP97373

[2] Le Goff, Carine, Curtis Rogers, Wilfried Le Goff, Graziella Pinto, Damien Bonnet, Maya Chrabieh, Olivier Alibeu et al. "Heterozygous mutations in MAP3K7, encoding TGF-β-activated kinase 1, cause cardiospondylocarpofacial syndrome." *The American Journal of Human Genetics* 99, no. 2 (2016): 407-413.

[3] Wang, W., Gao, W., Zhu, Q., Alasbahi, A., Seki, E., & Yang, L. (2021). TAK1: a molecular link between liver inflammation, fibrosis, steatosis, and carcinogenesis. *Frontiers in cell and developmental biology*, *9*, 734749.

[4] Ouyang, Chuan, et al. "Transforming growth factor (TGF)-β-activated kinase 1 (TAK1) activation requires phosphorylation of serine 412 by protein kinase A catalytic subunit α (PKACα) and X-linked protein kinase (PRKX)." *Journal of Biological Chemistry* 289.35 (2014): 24226-24237.

[5] Adhikari, Anindya, Ming Xu, and Zhijian J. Chen. "Ubiquitin-mediated activation of TAK1 and IKK." *Oncogene* 26, no. 22 (2007): 3214-3226.

[6] Totzke, Juliane, et al. "TAK1: a potent tumour necrosis factor inhibitor for the treatment of inflammatory diseases." *Open biology* 10.9 (2020).

[7] Takatsu, Yoshihiro, et al. "TAK1 participates in c-Jun N-terminal kinase signaling during Drosophila development." *Molecular and cellular biology* 20.9 (2000): 3015-3026.

[8] Song, Zifang, et al. "Roles of the kinase TAK1 in CD40-mediated effects on vascular oxidative stress and neointima formation after vascular injury." *PloS one* 9.7 (2014): e101671

[9] Irie, Takashi, Tatsushi Muta, and Koichiro Takeshige. "TAK1 mediates an activation signal from toll-like receptor (s) to nuclear factor-κB in lipopolysaccharide-stimulated macrophages." *FEBS letters* 467.2-3 (2000): 160-164.

[10] K. Yumoto, Kenji, et al. "TGF-β-activated kinase 1 (Tak1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells." *Journal of Biological Chemistry* 288.19 (2013): 13467-13480.

[11] Hunter, Andrew, et al. "TAK1/Map3k7 enhances differentiation of cardiogenic endoderm from mouse embryonic stem cells." *Journal of molecular and cellular cardiology* 137 (2019): 132-142.

[12] Takaesu, Giichi, et al. "TAK1 (MAP3K7) signaling regulates hematopoietic stem cells through TNF-dependent and-independent mechanisms." *PLoS One* 7.11 (2012): e51073.

[13] Zhang, Yongchun, Regis J. O'Keefe, and Jennifer H. Jonason. "BMP-TAK1 (MAP3K7) Induces Adipocyte Differentiation Through PPARγ Signaling." *Journal of cellular biochemistry* 118.1 (2017): 204-210.

[14] Li, Lei, et al. "Transforming growth factor β–activated kinase 1 signaling pathway critically regulates myocardial survival and remodeling." *Circulation* 130.24 (2014): 2162-2172.

[15] Damhofer, Helene, et al. "TAK1 inhibition leads to RIPK1-dependent apoptosis in immune-activated cancers." *Cell Death & Disease* 15.4 (2024): 273.

[16] Shin, Ju Hyun, et al. "TAK1 regulates autophagic cell death by suppressing the phosphorylation of p70 S6 kinase 1." *Scientific reports* 3.1 (2013): 1561.

[17] Sato, Shintaro, et al. "Essential function for the kinase TAK1 in innate and adaptive immune responses." *Nature immunology* 6.11 (2005): 1087-1095.

[18] Inokuchi-Shimizu, Sayaka, et al. "TAK1-mediated autophagy and fatty acid oxidation prevent hepatosteatosis and tumorigenesis." *The Journal of clinical investigation* 124.8 (2014): 3566-3578.

[19] Showkat, Mehvish, Mushtaq A. Beigh, and Khurshid I. Andrabi. "mTOR signaling in protein translation regulation: implications in cancer genesis and therapeutic interventions." *Molecular biology international* 2014.1 (2014): 686984.

[20] Magnuson, Brian, Bilgen Ekim, and Diane C. Fingar. "Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks." *Biochemical Journal* 441.1 (2012): 1-21.

[21] Deleyto-Seldas, Nerea, and Alejo Efeyan. "The mTOR–autophagy axis and the control of metabolism." *Frontiers in cell and developmental biology* 9 (2021): 655731.

[22] Kim, Joungmok, et al. "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1." *Nature cell biology* 13.2 (2011): 132-141