

# Tissue kallikrein promotes survival and $\beta$ -catenin degradation in SH-SY5Y cells under nutrient stress conditions via autophagy

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**Abstract.** Previous studies by our group showed that tissue kallikrein (TK) exerts neuroprotective effects during cerebral ischemia. Autophagy is an important adaptive response to cellular stress during nutrient deprivation, and  $\beta$ -catenin is known to repress autophagy. The present study investigated the possible involvement of autophagy and  $\beta$ -catenin signaling in the protective effects of TK under nutrient deprivation-induced stress conditions. TK was shown to promote the survival and inhibit the death of SH-SY5Y cells under serum starvation and enhanced autophagic activity in a concentration-dependent manner, as indicated by augmented light chain (LC)3-II levels and Beclin-1 expression. The autophagy inhibitors 3-methyladenine and  $\text{NH}_4\text{Cl}$  abolished the protective effects of TK. Of note, although serum starvation alone and TK treatment increased p62 protein levels and mRNA expression, incubation with the lysosome inhibitor  $\text{NH}_4\text{Cl}$  increased the accumulation of LC3-II and p62 protein, indicating normal autophagic flux. It was also observed that  $\beta$ -catenin expression was significantly downregulated by TK treatment. TK stimulated the interaction between LC3 and  $\beta$ -catenin, and  $\text{NH}_4\text{Cl}$  abolished the effects of TK on  $\beta$ -catenin levels in serum-starved cells, suggesting the autophagic degradation of  $\beta$ -catenin, which may have led to the enhancement of autophagy. In conclusion, the findings of the present study demonstrated that TK promoted cell survival and  $\beta$ -catenin degradation in serum-starved SH-SY5Y cells via increasing autophagy, which indicated the therapeutic potential of TK under nutrient deprivation-associated stress conditions.

## Introduction

Autophagy is an adaptive response to cellular stress induced by conditions such as nutrient deprivation. During autophagy, cellular components to be degraded are surrounded by autophagosomes and catabolized after fusion with lysosomes to provide precursors for synthetic processes and energy production, which contributes to cellular survival (1).

Tissue kallikrein (TK), an important component of the kallikrein-kinin system, belongs to a sub-group of serine proteinases and processes low-molecular-weight kininogen to release kinin peptide (2). Previous studies by our group showed that TK exerted neuroprotective effects in oxygen-glucose-deprived cells as well as in the ischemic brain (3-5), in which autophagy has been reported to have an important role in maintaining neuronal survival (6). It has been reported that TK is able to activate the Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway (4) and adenosine 5'-monophosphate-activated protein kinase phosphorylation (7), which participate in autophagy induction (8,9). However, to date, no direct evidence has revealed whether autophagy is involved in TK-mediated protective effects under nutrient deprivation-induced stress conditions.

$\beta$ -catenin is a key downstream effector in the Wnt signaling pathway. The activation of the Wnt/ $\beta$ -catenin axis is known to increase cell survival and proliferation under various conditions (10). A recent study demonstrated that activated  $\beta$ -catenin signaling was able to repress autophagy. However,  $\beta$ -catenin protein contains a light chain (LC)3-interacting motif, which is targeted for autophagic clearance, thus facilitating autophagy induction and cell survival under nutrient deficiency (11).

The present study used serum-starved SH-SY5Y human neuroblastoma cells as a canonical model for investigating autophagy. It was attempted to evaluate the role of TK in modulating autophagy and its crosstalk with  $\beta$ -catenin signaling under nutrient deprivation-induced stress conditions. It was indicated that TK promoted the induction of protective autophagy along with autophagic degradation of  $\beta$ -catenin and maintained normal autophagic flux.

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## Materials and methods

**Reagents.** TK was purchased from Techpool Bio-Pharma Co. (Guangzhou, China). Rabbit anti-human LC3A/B (cat no. 12741), LC3B (cat no. 3868) and Beclin-1 (cat. no. 3495) as well as mouse anti-human  $\alpha$ -tubulin (cat. no. 3873) monoclonal antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-human sequestosome 1 (p62; cat. no. ab56416) and rabbit anti-human  $\beta$ -catenin (cat. no. ab32572) monoclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (cat. no. GGHL-15P) and HRP-conjugated goat anti-mouse IgG (cat. no. GGHL-90P) secondary antibodies were obtained from Immunology Consultants Laboratory (Portland, OR, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-methyladenine (3-MA) and  $\text{NH}_4\text{Cl}$  were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture.** Human SH-SY5Y neuroblastoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Invitrogen) at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . Cells were grown to 80% confluency prior to the experiments.

**Cell viability and apoptosis assays.** SH-SY5Y cells were seeded into 96-well plates at  $2 \times 10^4$  cells per well and allowed to adhere overnight. Cells were treated with indicated concentrations of TK (0, 0.125, 0.25, 0.5 and 1  $\mu\text{M}$ ) followed by serum starvation for 12 or 24 h. Subsequently, 10  $\mu\text{l}$  MTT solution (5 mg/ml) was added to each well, followed by incubation at 37°C for 4 h in the dark. Following removal of the MTT solution and addition of 150  $\mu\text{l}$  dimethyl sulfoxide (Sigma-Aldrich) to dissolve the formazan crystals, the absorbance at 570 nm was measured using a plate reader (Synergy H1, BioTek Instruments, Winooski, VT, USA). The cell viability was determined by calculating the absorbance ratio of treated cells vs. that of unstarved control cells (viability, 100%).

For the apoptosis assay, cells were treated as stated above, harvested and stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions and subsequently analyzed using flow cytometry (BD FACSCanto; BD Pharmingen) with BD FACSDiva software (BD Pharmingen). Quantitative analysis of cell death by flow cytometry Annexin V/PI staining. Dead cells included early and late apoptotic cells (lower and upper right quadrants in dot plot) as well as necrotic cells (upper left quadrant).

**Co-immunoprecipitation.** Co-immunoprecipitation was performed according to the manufacturer's instructions of the protein A/G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Briefly, SH-SY5Y cells were lysed on ice in 1 ml radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for 30 min.

Following pre-clearing with normal IgG (Beyotime Institute of Biotechnology), cell lysates were incubated overnight at 4°C with anti-LC3B (diluted at 1:50), followed by precipitation with 20  $\mu\text{l}$  protein A/G Plus-Agarose (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The precipitated complexes were washed four times with phosphate-buffered saline (PBS) and heated at 100°C for 5 min in 5X sodium dodecyl sulfate (SDS) sample buffer (Beyotime Institute of Biotechnology). The samples were then separated on 10% SDS-polyacrylamide gels and immunoblotted with  $\beta$ -catenin monoclonal antibody overnight at 4°C. Membranes were routinely washed four times in Tris-buffered saline with 0.1% Tween 20 (TBST) for 5 min and then incubated with HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature. The antigen-antibody complexes were visualized using the Immobilon™ western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and images were captured using the ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA).

**Western blot analysis.**  $2 \times 10^7$  SH-SY5Y cells were lysed on ice and washed with ice-cold PBS and lysed on ice for 20 min with RIPA lysis buffer supplemented with protease inhibitors (Roche Diagnostics, Basel, Switzerland). Protein concentrations were determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (20  $\mu\text{g}$ ) were separated by electrophoresis on SDS-polyacrylamide gels [prepared with 30% acrylamide-bisacrylamide, 1 M Tris-HCl (pH 6.8 and pH 8.8), ammonium persulfate and tetramethylethylenediamine; all from Beyotime Institute of Biotechnology) and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore). Immunoblotting was performed with the appropriate primary antibody at 1:1,000 dilution overnight at 4°C, followed by HRP-conjugated secondary antibody at 1:5,000 dilution for 1 h at room temperature. Blotted proteins were visualized using enhanced chemiluminescence (using the Immobilon™ western chemiluminescent HRP substrate) and images were captured using the ChemiDoc XRS+ system (Bio-Rad Laboratories). Densitometric analysis was performed using Image J 1.4.3.67 software (National Institutes of Health, Bethesda, MD, USA) with  $\alpha$ -tubulin as the loading control. Relative density values to  $\alpha$ -tubulin for each sample were normalized to the basal levels in the control group to obtain fold-change values.

**Reverse-transcription quantitative polymerase chain reaction (RT-qPCR).** Following treatment as stated above, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized using the PrimeScript™ RT Master Mix (no. RR036A; Takara Bio Inc., Otsu, Japan). qPCR was performed using a SYBR Green PCR kit (no. DRR041A; Takara Bio Inc.) in an ABI real-time PCR system (Applied Biosystems; Thermo Fisher Scientific) according to the manufacturer's instructions. The following primers (Sangon Biotech, Shanghai, China) were used: p62 forward, 5'-AAATGGGTCCACCAGGAACTGGA-3' and reverse, 5'-TCAACTCAATGCCAGAGGGCTA-3'; GAPDH forward, 5'-CTGGGCTACACTGAGCACC-3' and reverse, 5'AAGTGGTCGTTGAGGGCAATG-3'. The thermocycling conditions were as follows: Polymerase activation/denaturation (95°C for 30 sec) and 45 amplification cycles

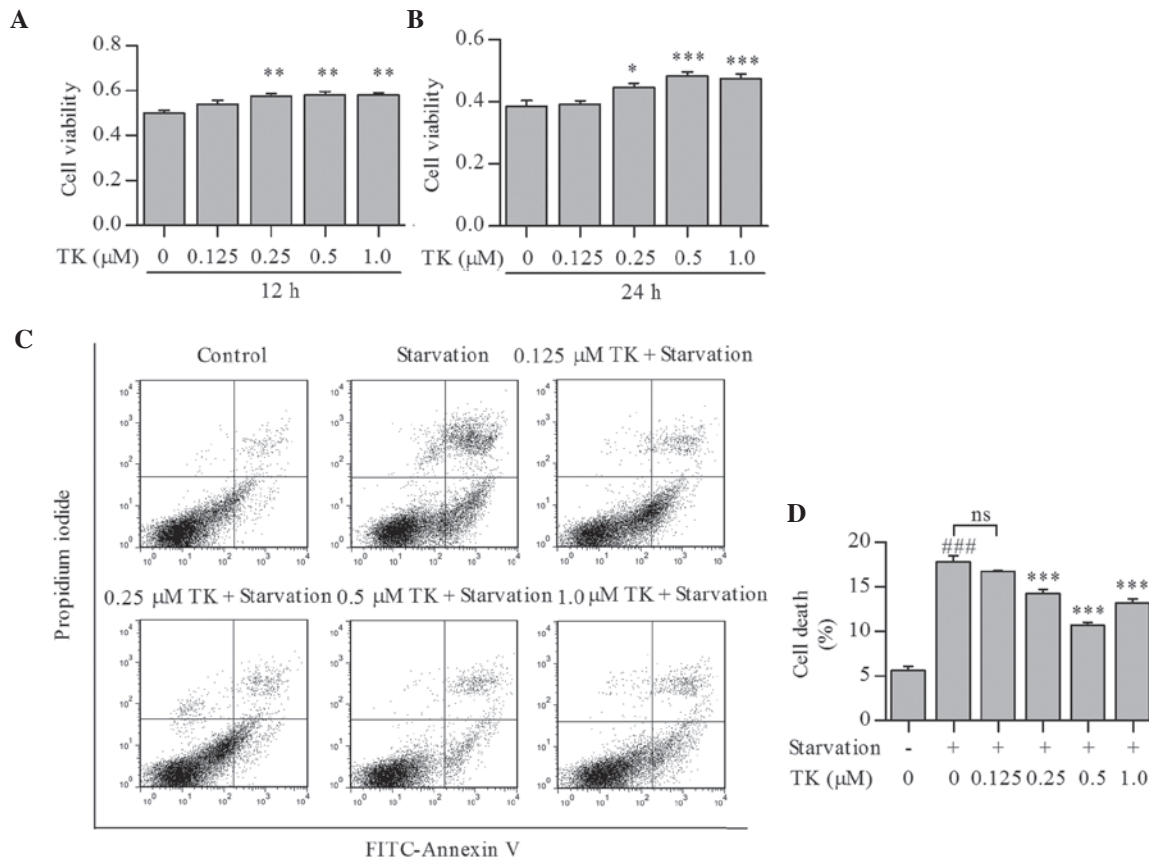


Figure 1. TK promotes survival of SH-SY5Y cells under serum starvation. (A and B) SH-SY5Y cells were pre-treated with 0.125–1.0  $\mu\text{M}$  TK followed by serum starvation for (A) 12 h or (B) 24 h, and the cell viability was examined using an MTT assay. (C) Representative flow cytometry dot plots of Annexin V/propidium iodide-stained SH-SY5Y cells pre-treated with 0.125–1.0  $\mu\text{M}$  TK and serum-starved for 24 h. (D) The apoptotic rate was determined by quantification of Annexin V and propidium iodide staining. Dead cells included early and late apoptotic cells (lower and upper right quadrants in dot plot) as well as necrotic cells (upper left quadrant). Values are expressed as the mean  $\pm$  standard deviation of at least three individual experiments. ns, not significant; ###P<0.001 vs. no starvation; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. serum-starved group not treated with TK. FITC, fluorescein isothiocyanate; TK, tissue kallikrein.

(95°C for 5 sec, 58°C for 30 sec and 72°C for 30 sec). Relative p62 mRNA levels were normalized to GAPDH and calculated using the  $2^{-\Delta\Delta\text{Cq}}$  method.  $\Delta\text{Cq}$  was calculated by subtracting their Cq values from the average Cq value of GAPDH.

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard deviation. Differences were evaluated by one-way analysis of variance followed by Bonferroni's post-hoc test with GraphPad Prism 5 software (GraphPad Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference between values.

## Results

**TK promotes survival of SH-SY5Y cells under serum starvation.** To determine whether TK is able to promote cell survival under nutrient deprivation-induced stress conditions, SH-SY5Y cells were treated with TK at 0.125–1.0  $\mu\text{M}$  and then subjected to serum starvation for 12 or 24 h. As shown in Fig. 1A and B, TK augmented the viability of SH-SY5Y cells in a time- and concentration-dependent manner, with 0.5  $\mu\text{M}$  of TK displaying the most significant protective effects at 24 h. Annexin V and propidium iodide staining also showed that cell death induced by starvation for 24 h was partly prevented by TK at 0.25–1.0  $\mu\text{M}$  (Fig. 1C and D).

*Pro-survival effects of TK are based on the induction of autophagy.* To explore whether autophagic pathways were involved in the pro-survival effects of TK, LC3 conversion and Beclin-1 expression were assessed as autophagic markers in serum-starved SH-SY5Y cells pre-treated with TK. Compared to serum starvation alone, TK concentration-dependently induced a more profound elevation of LC3-II and expression of Beclin-1 (Fig. 2A–C), with the most significant effects at 0.5 and 1.0  $\mu\text{M}$ .

In an additional experiment, SH-SY5Y cells were pre-treated with 0.5  $\mu\text{M}$  TK in combination with the autophagy inhibitors 3-MA or  $\text{NH}_4\text{Cl}$  (12), followed by serum starvation for 24 h and detection of cell viability using an MTT assay. Pre-treatment with 3-MA or  $\text{NH}_4\text{Cl}$  alone diminished the viability of SH-SY5Y cells under serum starvation. Furthermore, the protective effects of TK were abolished by 3-MA as well as  $\text{NH}_4\text{Cl}$  (Fig. 2D). These results suggested that autophagy induction contributed to the pro-survival effects of TK.

*TK upregulates p62 expression while maintaining normal autophagic flux in serum-starved SH-SY5Y cells.* p62 is known to scavenge soluble mis-folded proteins for removal in autophagosomes (1) and can be used as a protein marker of autophagic degradation and autophagic flux in certain

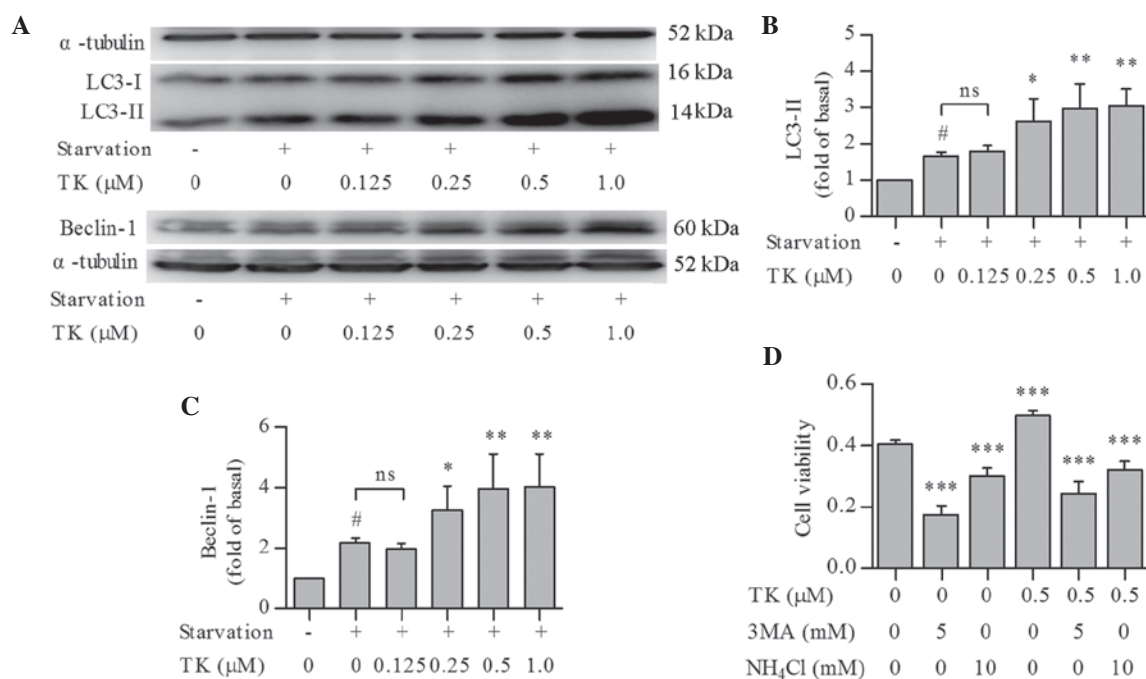


Figure 2. Autophagy contributes to TK-mediated pro-survival effects in serum-starved SH-SY5Y cells. (A-C) SH-SY5Y cells were pre-treated with 0.125-1.0 μM TK and serum-starved for 12 h. LC3 conversion and Beclin-1 levels were examined by western blot analysis with α-tubulin used as the loading control. (D) SH-SY5Y cells were pre-treated with 5 mM 3-MA or 10 mM NH<sub>4</sub>Cl or 0.5 μM TK in combination with 3-MA or NH<sub>4</sub>Cl and serum-starved for 24 h. Cell viability was examined using an MTT assay. Values are expressed as the mean ± standard deviation of at least three individual experiments. ns, not significant; #P<0.05 vs. no starvation; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. serum-starved group not treated with any drugs. TK, tissue kallikrein; LC, light chain; MA, methyladenine.

settings (12). Unexpectedly, p62 protein levels were significantly increased by serum starvation and further increased by TK. TK elevated the protein levels of p62 in a concentration-dependent manner, with the most significant changes at 0.5 μM (Fig. 3A). Similarly to its effect on p62 protein levels, TK at 0.25-1.0 μM also promoted the transcription of p62 as compared to that following serum starvation alone, as indicated by RT-qPCR analysis (Fig. 3B). This result indicated that increases in p62 protein were due to enhanced transcription, and did not reflect autophagic flux in this scenario. To further assess autophagic flux, cells were pre-treated with 0.5 μM TK in combination with NH<sub>4</sub>Cl, an inhibitor of lysosomal acidification that can inhibit autophagic degradation (12), followed by serum starvation. NH<sub>4</sub>Cl significantly raised the protein levels of p62 in serum-starved SH-SY5Y cells at 12 h, irrespective of the presence of TK (Fig. 3C), indicating that p62 was indeed degraded in the autophagic process. In addition, the effects of NH<sub>4</sub>Cl on LC3-II levels were assessed at 6 h (Fig. 3D) and 12 h (Fig. 3E), which revealed that NH<sub>4</sub>Cl led to the accumulation of LC3-II at each time-point with or without pre-treatment with TK. These results confirmed that TK maintained a normal autophagic flux.

*TK stimulates the autophagic degradation of β-catenin.* It was previously reported that β-catenin represses autophagy but is also degraded during the autophagic process (11). Therefore, β-catenin levels in serum-starved SH-SY5Y cells treated in the presence or absence of TK were assessed. β-catenin expression remained unchanged following serum starvation for 12 h as compared to that in the control cells. While 0.125 μM TK led to an upregulation of β-catenin expression, TK at 0.25-1.0 μM significantly decreased the

levels of β-catenin, with 0.5 μM TK causing the most significant reduction of β-catenin (Fig. 4A).

To further investigate whether downregulation of β-catenin by TK was due to autophagic degradation, β-catenin levels were examined in serum-starved SH-SY5Y cells pre-treated with 0.5 μM TK in combination with NH<sub>4</sub>Cl. NH<sub>4</sub>Cl abolished the effects of 0.5 μM TK on β-catenin levels in serum-starved SH-SY5Y cells, while treatment with NH<sub>4</sub>Cl alone had no significant impact on β-catenin levels in serum-starved cells (Fig. 4B). Co-immunoprecipitation analysis then revealed that TK induced an interaction between LC3 and β-catenin in serum-starved SH-SY5Y cells (Fig. 4C), suggesting that TK stimulated the autophagic degradation of β-catenin.

## Discussion

TK has been reported to have important roles in various physiological and pathological processes (13,14). The present study revealed that TK enhanced the survival of human SH-SY5Y cells under serum starvation in a starvation time- and TK concentration-dependent manner. Previous studies demonstrated that TK transactivates multiple downstream signaling pathways, including the phosphoinositide-3 kinase (PI3K)/Akt and epidermal growth factor receptor (EGFR) signaling (3,15,16). PI3K/Akt activation and EGFR are able to activate Wnt/β-catenin signaling but suppress autophagy induction (17-20). However, the present study revealed that β-catenin expression was significantly downregulated by TK treatment, accompanied by increased autophagy, suggesting that the underlying molecular mechanism is independent of the PI3K/Akt and EGFR pathways.



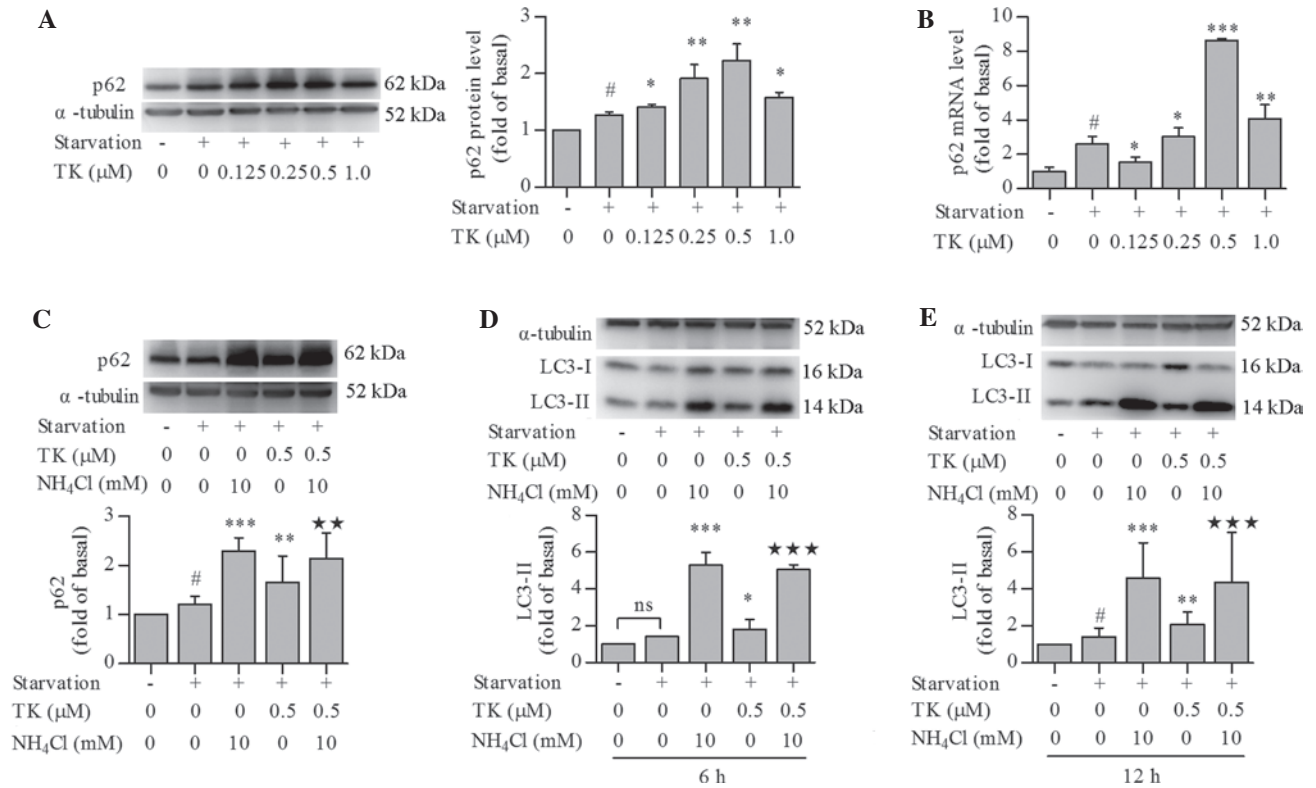


Figure 3. TK upregulates p62 expression, while maintaining normal autophagic flux in serum-starved SH-SY5Y cells. (A) SH-SY5Y cells were pre-treated with 0.125-1.0 μM TK and serum-starved for 12 h. p62 protein levels were examined by western blot analysis. α-tubulin was used as the loading control. (B) SH-SY5Y cells were treated with 0.125-1.0 μM TK and serum-starved for 8 h. p62 mRNA levels were examined by quantitative polymerase chain reaction. (C-E) SH-SY5Y cells were treated with 0.5 μM TK or in combination with 10 mM NH<sub>4</sub>Cl and serum-starved for 6 or 12 h. (C) p62 levels at 12 h, (D) LC3 levels at 6 h and (E) LC3 levels at 12 h were detected by western blot. α-tubulin was used as the loading control. Values are expressed as the mean ± standard deviation of at least three individual experiments. ns, not significant; \*P<0.05 vs. no starvation; \*\*P<0.01, \*\*\*P<0.001 vs. serum-starved group treated with neither TK nor NH<sub>4</sub>Cl; \*\*P<0.01, \*\*\*P<0.001 vs. serum-starved group treated with TK but not NH<sub>4</sub>Cl. TK, tissue kallikrein; LC, light chain.

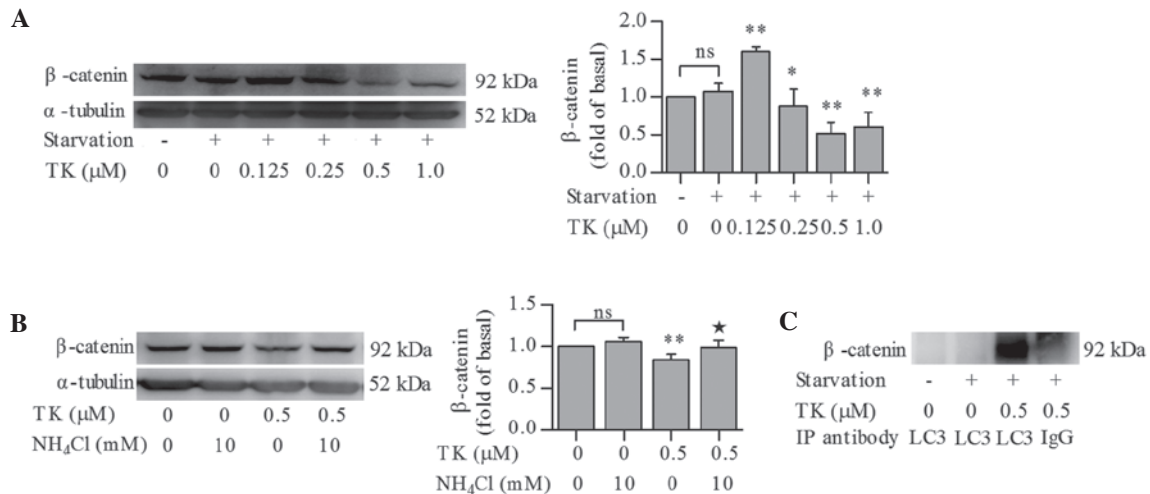


Figure 4. TK promotes autophagic degradation of β-catenin in serum-starved SH-SY5Y cells. (A) SH-SY5Y cells were pre-treated with 0.125-1.0 μM TK and serum-starved for 12 h. (B) SH-SY5Y cells were treated with 0.5 μM TK or in combination with 10 mM NH<sub>4</sub>Cl and serum-starved for 12 h. β-catenin expression was detected by western-blot analysis with α-tubulin used as the loading control. Values are expressed as the mean ± standard deviation of at least three individual experiments. (C) SH-SY5Y cells were left untreated or treated with 0.5 μM TK and serum-starved for 8 h. Co-immunoprecipitation analysis was performed to detect the interaction between LC3 and β-catenin. ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. serum-starved group treated with neither TK nor NH<sub>4</sub>Cl. \*P<0.05 vs. serum-starved group treated with TK but not NH<sub>4</sub>Cl. IP, immunoprecipitation; TK, tissue kallikrein; LC, light chain; IgG, immunoglobulin G.

The findings of the present study are consistent with a previous study, which reported that Wnt/β-catenin represses autophagy and p62 expression, while β-catenin itself is targeted by autophagic clearance in autolysosomes upon induction of

autophagy (11). Indeed, TK was shown to induce β-catenin downregulation, which was abrogated by NH<sub>4</sub>Cl; furthermore, co-immunoprecipitation analysis revealed that TK stimulated the interaction between LC3 and β-catenin. These findings

indicated that TK promoted the autophagic degradation of  $\beta$ -catenin to favor adaptation-promoting autophagy, thereby mitigating the deleterious consequences of continued proliferation and transcription during stress.

In the experimental model used in the present study, serum starvation as well as TK treatment increased the protein levels of p62, which however, is not necessarily indicative of blocked autophagic flux in this context (8,11). Under starvation conditions, depletion of p62 has been reported to inhibit the recruitment of LC3 to autophagosomes and the background level of LC3-II was shown to be elevated in cells overexpressing p62, indicating that autophagic activity is increased with increasing levels of p62 (21). In the present study, RT-qPCR analysis further suggested that the observed elevation of p62 protein was, at least partially, attributed to increased transcription. Furthermore, pre-treatment with the lysosome inhibitor  $\text{NH}_4\text{Cl}$  in the presence as well as absence of TK led to a recovery of the levels of p62 and LC3-II, indicating normal autophagic flux.

In conclusion, the present study provided direct evidence that autophagy contributed to the pro-survival effects of TK in SH-SY5Y cells under serum starvation. TK initiated autophagy while maintaining normal autophagic flux. The present study enhanced the present understanding of key cellular processes affected by TK and revealed the potential use of TK for alleviating nutrient deprivation-induced stress conditions such as ischemic stroke. The underlying molecular mechanisms of the modulation of autophagy by TK require to be elucidated in future studies.

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