Effects of Tetrahedral DNA Nanostructures on Autophagy in Chondrocytes

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Table 1. The sequence of four designed specific ssDNA.

DNA	Sequence
S1	5’-ATTTATCACCCGCCATAGTAGACGTATCACC AGGCAGTTGAGACGAACATTCCTAAGTCTGAA-3’;
S2	5’-ACATGCGAGGGTCCAATACCGACGATTACA GCTTGCTACACGATTCAGACTTAGGAATGTTCG-3’;
S3	5’-ACTACTATGGCGGGTGATAAAACGTGTAGCA AGCTGTAATCGACGGGAAGAGCATGCCCATCC-3’;
S4	5’-ACGGTATTGGACCCTCGCATGACTCAACTGC CTGGTGATACGAGGATGGGCATGCTCTTCCCG-3’;

Table 2. The primers sequences of selected genes designed for qPCR.

mRNA	Primer pairs	
β-ACTIN	Forward	CCTAGACTTCGAGCAAGAGA
	Reverse	GGAAGGAAGGCTGGAAGA
Atg5	Forward	ACGATGACCTGTGTCGAACT
	Reverse	AAACCAAATCTCACTAACATCTTCT
Atg7	Forward	GAGAGTACATCCCCACCGTC
	Reverse	AGGGATCGTACACACCGACT
Atg12	Forward	GGCACCAGCTCTAGGCTTATAGTTG
	Reverse	GTTGTTCCACAGCATTTTCCATG
Atg13	Forward	AGCCCGGAGTCACTCTATGT
	Reverse	CCAAGTCGAGCCTGGACAAT
PIK3C3	Forward	CTTCAAGCATGGGGACGACT
	Reverse	ATTGGGTCCAGTCTCGCTTG
LC3	Forward	CGTCTTTGTGGGTTGGACCT
	Reverse	AGCGCCTGTTCATATGCCTC
Beclin 1	Forward	CGGCTCCTATTCCATCAAAA
	Reverse	CCACTTGAGATTCGTCAGCA
PI3K	Forward	CCCTGGCAAGTAACCATTCT
	Reverse	GTTACGTTCCAGAAATGCCA
AKT	Forward	CAGGTTCACCCAGTGACAAC
	Reverse	CTCCTTCACCAGGATCACCT
mTOR	Forward	AGAACCACATGCCACACAGT
	Reverse	CTTTGGCATTTGTGTCCATC

EXPERIMENTAL SECTION

Materials

Type II collagenase was obtained from Gibco. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution, Fetal bovine serum (FBS), 0.25% trypsin,

and phosphate-buffered saline (PBS) were obtained from Hyclone. Six pore plates were bought from Corning. FITC-labeled phalloidin was purchased from Sigma-Aldrich. DAPI and 4% paraformaldehyde were provided by Beyotime. Most of antibodies were bought from Abcam and some of them were purchased from CST. ssDNA was provided by Takara.

Cell cultures

The rat chondrocytes were isolated and collected from the knee articular cartilage of newborn 3-day old SD rats. In brief, cartilage tissues were cut into little pieces and first pretreated with 0.25% trypsin for 45 min in a sterile environment; then, they were washed with PBS 3 times to eliminate the trypsin, finally digested with 0.1% collagenase type II for 1-2 h in a 37 °C shaking bath. Fresh 10% FBS DMEM medium containing DMEM, 10% (v/v) FBS, and 1% (v/v) penicillin-streptomycin solution was used to neutralize enzyme activity in solution. the isolated chondrocytes were resuspended in fresh 10% FBS DMEM after the solution being centrifuged and discarding the supernatant, the chondrocytes in suspension since were cultured in the petri dish at incubator until passage II for usage as described previously [48-50].

Synthesis and characterization of TDNs

TDNs were prepared as reported previously[16-19]. In brief, four specifically designed and synthesized by TaKaRa Bio ssDNA (S1, S2, S3, S4) (Table 1) were combined in equimolar ratio in TM buffer (10 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0). The reaction solution was heated to 95 °C for 10 min, and then quickly cooled to 4 °C. Subsequently, the successfully self-assembled TDNs was characterized by 8% PAGE, determined by DLS in ddH_2O in 250 nM using a ZETAPals analyzer (Brookhaven Instruments, Holtsville, NY), and confirmed by AFM.

Transmission electron microscopy analysis

First of all, the chondrocytes treated with or without TDNs (250 nM) were fixed with 3% glutaraldehyde for 42 h at 4 °C. Secondly, the samples were decalcified with 20% EDTA. Subsequently, they were further fixed with 1% osmium tetroxide (OsO_4) for 2.5 h. The different concentration of ethanol (50, 70, 90, and 100%) were used to dehydrate the samples at 10-min intervals. After being incubating in the solution of alcohol and isoamyl acetate for 45 min, the samples kept on incubating isoamyl acetate for 45 min. After each step, use PBS to wash 3 times. The chondrocytes were embedded with gold-palladium. Finally, ultrathin sections were obtained using an ultramicrotome and examined by transmission electron microscope (Philips Electron Optics, Hillsboro, OR, USA).

Immunofluorescence

The chondrocytes in confocal dish of the two different groups, namely, treated with (experimental group) or without TDNs (control group), were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton™ X-100. The samples were blocked by 1% goat serum, and cultured with primary antibody solution (anti-ATG7, anti-ATG12, anti-Beclin, or anti-LC3 rabbit polyclonal antibody, 1:100 dilution). Next, the chondrocytes were incubated with secondary antibody (1:200 dilutions). since, the nuclei and cytoskeleton were stained with DAPI and phalloidin, respectively. After each step, wash with PBS 3 times. Finally, the samples were observed by confocal laser scanning microscopy as described previously [51-52].

Semi-quantitative PCR and quantitative PCR

RNA samples of chondrocytes treated with or without TDNs were collected, isolated, and purified via Trizol extraction method. After being melted in the RNase-free water, quantified by measuring absorbance at 260 nm by a spectrophotometer, all the RNA samples were reverse transcribed into cDNA using a synthesis kit. The expression of target mRNAs (Table 2) in each treatment group as normalized to the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated. Semi-quantitative PCR was accomplished with PCR kit (Mbi) and quantitative PCR (qPCR) was performed using SYBR® Green I PCR master mix and an ABI 7300 thermal cycler. The semi-quantitative PCR procedure consisted of a 30 s denaturization at 94°C, 30 s annealing cycle at 55–65°C, and 30 s elongation cycle, 28 to 35 amplification cycles at 72°C. Products were detected by 2% agarose gel electrophoresis in TAE buffer, and visualized by staining with Gel-Red. the data analysis was calculated by optical density with Image-Pro Plus 6.0. the procedure of qPCR was consisted of 3 min denaturation at 94 °C, 5 s annealing at 94 °C for 40 cycles, and 34 s elongation at 60 °C.

Western blotting analysis

The chondrocytes treated with or without TDNs were washed with ice-cold PBS 3 times to remove the residual medium. Lysis buffer was used to lyse and harvest total proteins. bicinchoninic acid assay (BCA) was applied to detect the concentration of the protein. Subsequently, the proteins were mixed with the SDS sample buffer at a ratio of 4:1(V/V), solubilized and boiled for 5min. Next, approximately 40 ug proteins were loaded onto 6%, 10%, and 12% (v/v) SDS polyacrylamide gels, separated, and then inserted into to a polyvinylidene fluoride membrane (PVDF). After incubation in blocking buffer, the membranes incubated with the following primary antibodies: anti-GAPDH, anti-ATG7, anti-ATG12, anti-LC3 and anti-Beclin. The blots were then incubated with secondary antibodies labeled anti-mouse or anti-rabbit and developed by chemiluminescence using ECL reagents.

Cell uptake assay

CY5-labeled ssDNA (CY5-ssDNA) and CY5-labeled TDNs (CY5-TDNs) were prepared for the fluorescence observation in cells. Chondrocytes were seeded on confocal culture at a density of 3 x 10^4 cells/ml and incubated for 12 h. They were then incubated with CY5-ssDNA (250 nM) and CY5-TDNs (250 nM) in fresh DMEM media for 6 h at 37℃, respectively. Next, cells were fixed 4% (w/v) paraformaldehyde. The cytoskeleton and nuclei were stained with FITC-labeled phalloidin and DAPI, respectively. After each step, wash with PBS 3 times. Cells uptake was imaged using a confocal laser scanning microscopy.

In vivo fluorescence imaging

Before imaging, mice were anesthetized with an intraperitoneal (IP) injection of 0.2 ml 10% chloral hydrate and were injected with 150 μl CY5-TDNs via the tail vein. At specified times (2 min, 10 min, 20 min, 30 min, 45 min, 60 min), the fluorescence images of the dorsal region of live mice were taken by a Maestro™ in vivo fluorescence imaging system by excitation at 645nm and emission at 680nm.

Statistical analysis.

Statistical analyses were performed using students t-test. There was markedly differential in statistics when the values of $p < 0.05$.

Abbreviations

TDNs	tetrahedral DNA nanostructures;
ATGs	autophagy-related genes
3D	three-dimensional
ssDNA	single-stranded DNA
LC3	microtubule-associated protein 1 light chain 3
PAGE	polyacrylamide gel electrophoresis;
TEM	transmission electron microscopy
PCR	polymerase chain reaction;
AFM	atomic force microscopy
FBS	fetal bovine serum;
DLS	dynamic light scattering;
OsO4	osmium tetroxide;
GAPDH	glyceraldehyde-3-phosphate dehydrogenase;
DMEM	Dulbecco’s Modified Eagle Media;
BCA	bicinchoninic acid assay;
PVDF	polyvinylidene fluoride membrane;

BSA	bovine serum albumin
PBS	phosphate-buffered saline;

Figure Legends

Fig S1. TDNs significantly enhanced the formation of autophagosomes in chondrocytes

Representative transmission electron microscope images showing ultrastructural changes in chondrocytes (red arrows indicate intracellular autophagosomes). The top row is the control group, while the row below is the TDN group.

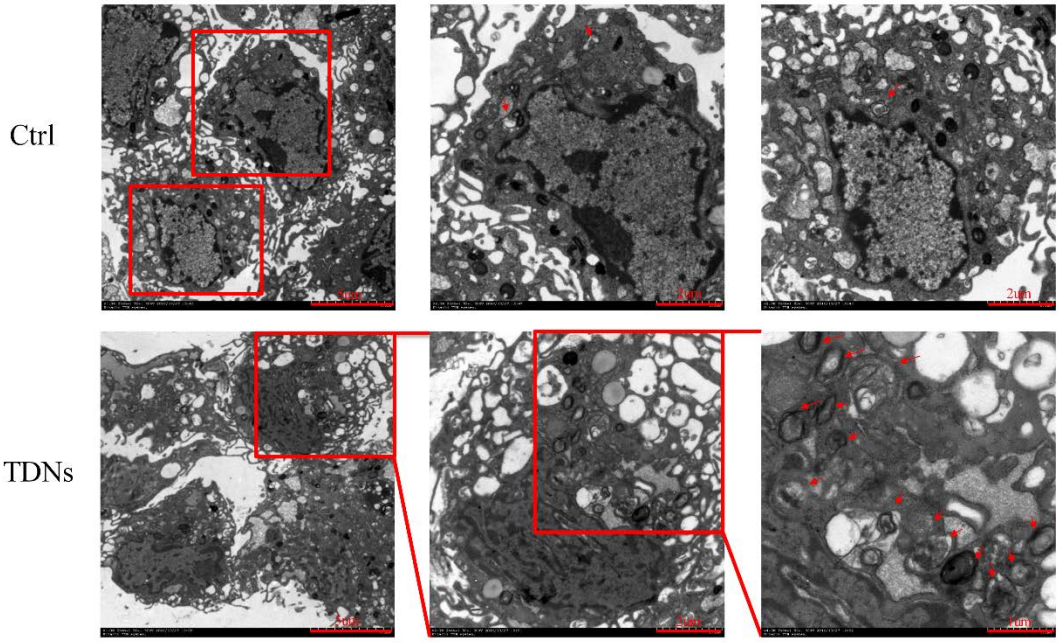


Fig S2. Effect of TDNs on autophagy-related genes and proteins expression in chondrocytes
(a) Comparisons of the expression levels of *Atg5*, *Atg7*, *Atg12*, *Atg13*, *Pik3c3*, Beclin, LC3 mRNA in chondrocytes between the control group and experimental group treated with TDNs by semi-quantitative PCR. (b) Quantification of the expression levels of autophagy-related genes after exposure to TDNs (250 nm) for 12 h. Data are presented as means ± SD (n = 5). Statistical analysis: *** $p < 0.001$. (c) Western blots showing the expression of essential proteins closely related to autophagy (ATG7, ATG12, Beclin, and LC3) after exposure on TDNs (250 nm) for 12 h. (d) Quantification of ATG7, ATG12, Beclin, and LC3 protein expression levels in chondrocytes treated with TDNs (250 nm) for 12 h. Data are presented as means ± SD (n = 5). Statistical analysis: *** $p < 0.001$. (e) Quantitative real-time PCR analysis of the expression of autophagy-related genes in cells treated with TDNs (250 nm) for 12 h. Data are presented as means ± SD (n = 5). Statistical analysis: * $p < 0.05$, ** $p < 0.01$.

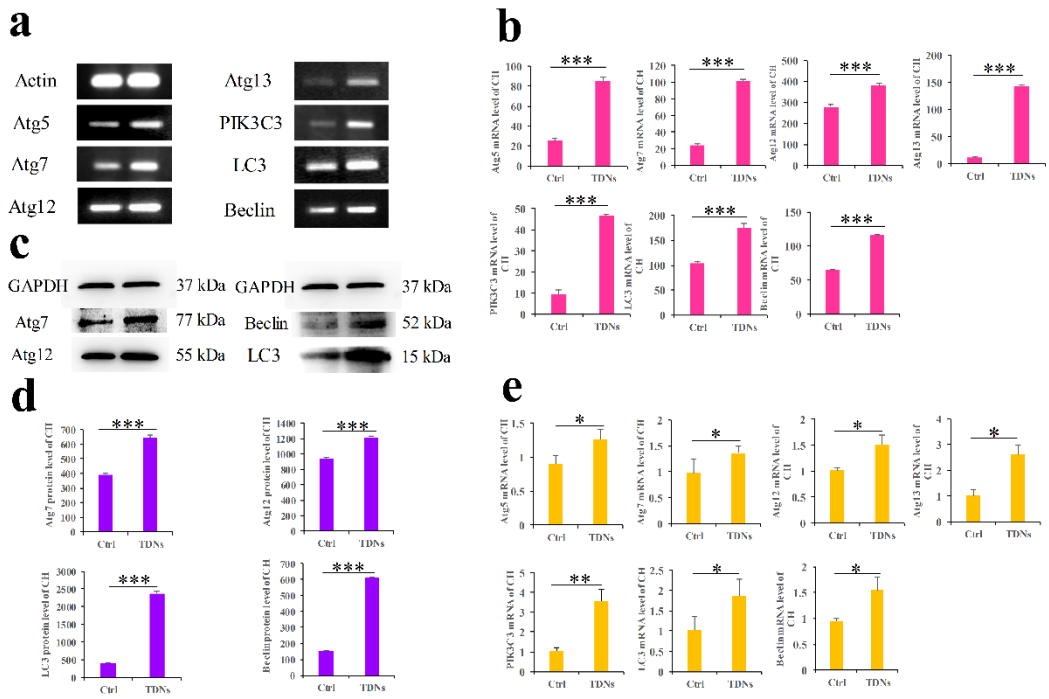


Fig S3. Immunofluorescence analysis of the expression levels of essential proteins

Confocal microscopic images of chondrocytes incubated without (control) or with TDNs (250 nM) for 12 h. Cells were stained to highlight cell nuclei (blue) and ATG7 (green) (a), ATG12 (green) (b) and Beclin (green) (c). Scale bars represent 25 μm.

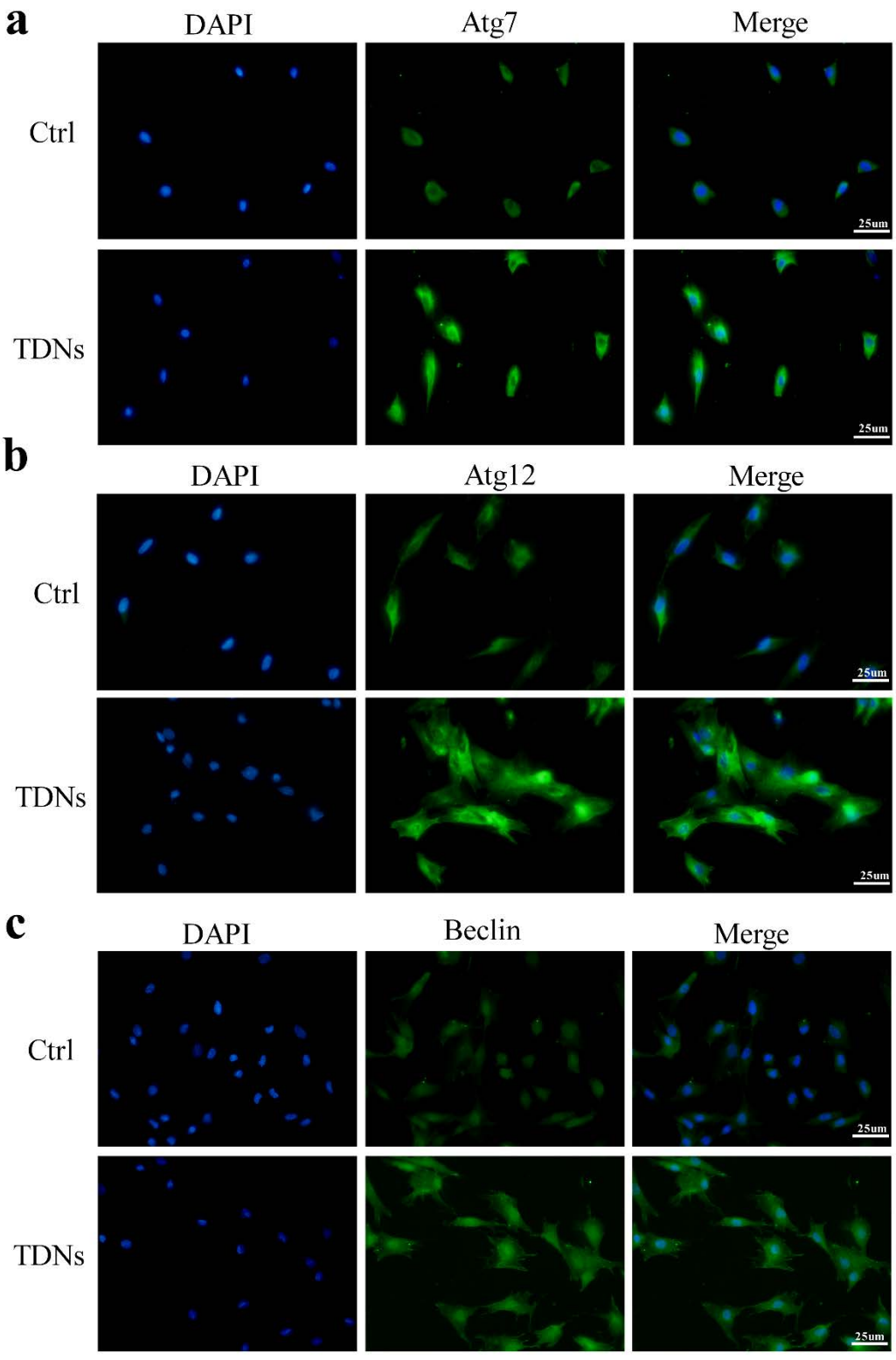


Fig S4. Expression levels of genes in the PI3K/Akt/mTOR signaling pathway were assessed by semi-quantitative PCR and qRT-PCR

(a) Semi-quantitative PCR of *Pi3k*, *Akt*, and *mTOR* mRNA after treatment with TDNs (250 nM) for 12 h. (b) Quantification of the expression levels of *Pi3k*, *Akt*, and *mTOR* mRNA after treatment with TDNs (250 nM) for 12 h. Data are presented as means ± SD (n = 3). Statistical analysis: ** $p<0.01$, *** $p < 0.001$. (c) Expression was also assessed by qRT-PCR after treatment with TDNs (250 nM) for 12 h. Data are presented as means ± SD (n = 3). Statistical analysis: * $p<0.05$, *** $p < 0.001$.

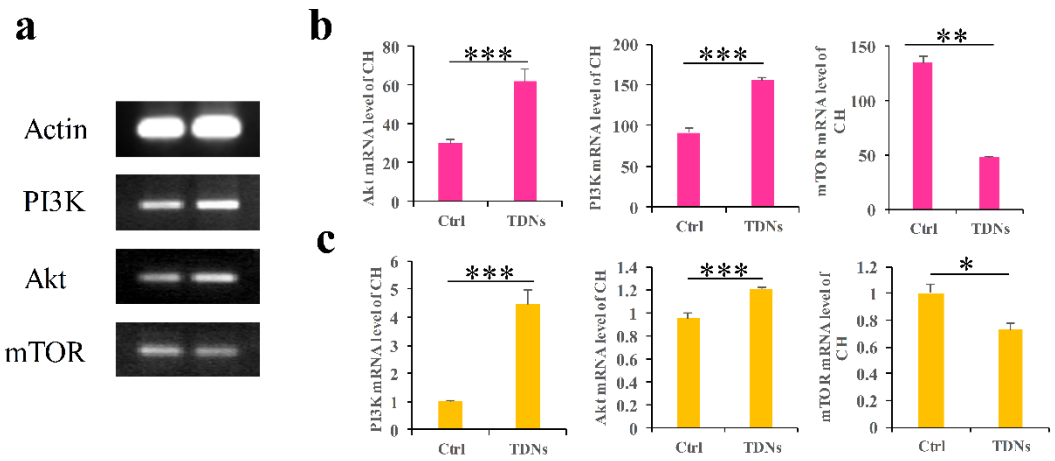
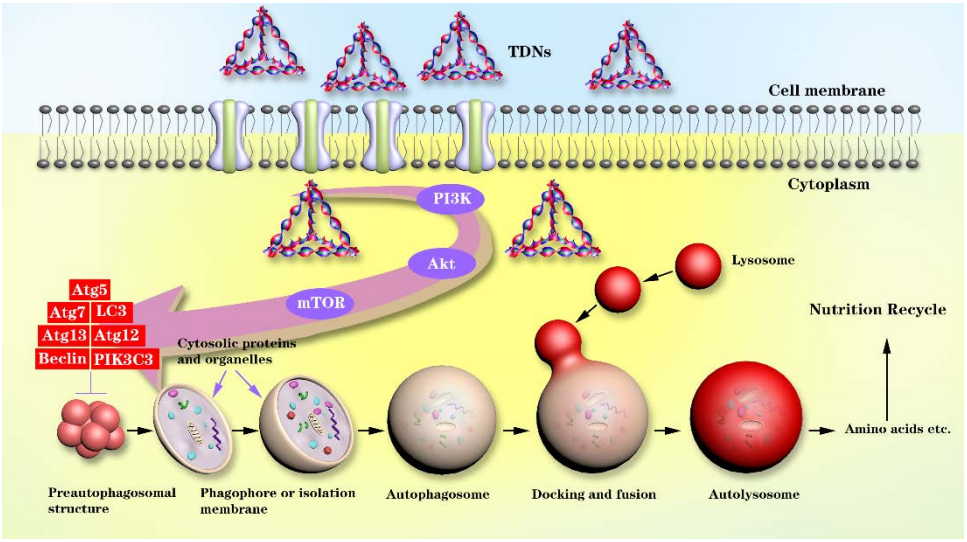


Fig S5. Schematic showing the proposed mechanism of how TDNs promote autophagy in chondrocytes.



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