

ATNC: Versatile Nanobody Chimeras for Autophagic Degradation of Intracellular Unligandable and Undruggable Proteins

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SUPPLEMENTARY INFORMATION

Methods and Experimental Details

Mammalian cell culture

HeLa (Cat# CL-0101) and OVCAR3 (Cat# CL-0178) cells were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, P.R. China), short tandem repeat (STR) identified and proven to be HIV-1, HBV, HCV, mycoplasma, and other microorganisms free before culturing. Other reagents such as full DMEM (Dulbecco's modified Eagle's medium) and PBS (phosphate buffered saline) were also confirmed to be mycoplasma free before usage. HeLa cell culture was maintained at 37°C under 5 % CO₂ in high glucose (4.5 g·L⁻¹) DMEM (Cat# SH30243.01, HyClone) containing 4 mM L-glutamine and sodium pyruvate and supplemented with additional 10 % fetal bovine serum (FBS, Cat# SV30087.03, HyClone), 1 % non-essential amino acid (NEAA, 100×), and 1 % penicillin-streptomycin (100×). OVCAR3 cell culture was maintained at 37 °C under 5 % CO₂ in RPMI-1640 (Cat# PM150110, Pricella) medium supplemented with additional 20 % FBS (Cat# SV30087.03, HyClone), 0.01 mg·ml⁻¹ insulin (Cat# MX0904, MKBio), 1 % non-essential amino acid (NEAA, 100×), and 1 % penicillin-streptomycin (100×). Trypsin-EDTA (Cat# SH30042.01, HyClone) and PBS (Cat# SH30256.01, HyClone) were used in subculturing. HeLa cells were subcultivated in a ratio of 1:5~10 while OVCAR3 cells were subcultivated in a ratio of 1:3~4.

Plasmid construction

Plasmid vectors, such as pTXB1, EGFP-C1 and EGFP-N1 were obtained from commercial vendors. These parental vectors may be further engineered, such as introducing a His₆-tag, or replacing EGFP by other fluorescent proteins to give modified versions of the parental vector for cloning. Subcloning and Gibson cloning-like methods were employed to construct the desired plasmids. For subcloning, fragments of interest were directly cut from the parent plasmid using appropriate restriction enzymes, or amplified by PCR from plasmids containing the desired genes using hyPerFUision high-fidelity polymerase (Cat# 1032, APEX BIO), gel purified, digested with restriction enzymes and purified again. The gene fragments were ligated into appropriate vectors using T4 DNA ligase. Multiple fragments were assembled by stepwise subcloning or one-step multi-fragment Gibson cloning. Most genes were obtained via custom gene synthesis from Comate Bioscience Co., Ltd. (Changchun, P.R. China). Alternatively, genes were obtained from Miaoling Plasmid Sharing Platform. Mito-sequence: ESGDASGSGSGSRAQASNSKLIKSAEDEKAKEEPPGNHRIVILAMLAIQVFLGALIKIIQLRKNN. List of primers for cloning plasmids that involve PCR amplifications, and more details about how the plasmids were constructed were provided as Supplementary Data.

Transfection

Transient transfection was typically performed in an 8-well (Cat# 155409) Lab-Tek® imaging chamber from Thermo Scientific using Lipo8000™ transfection reagent from Beyotime Biotechnology (Cat# C0533). Typically, 0.25 µg DNA was dissolved in 12.5 µl

gibco opti-MEM (Cat# 31985-062, Life technologies) and then 0.4 μl Lipo8000™ transfection reagent was added and mixed via gentle pipetting. Then this mixture was added into an imaging chamber well seeded with 2.5×10^4 cells that were already adhesively attached on the bottom in 250 μl full DMEM. The cells were maintained under 5% CO_2 at 37 °C for around 2 h. Then the medium was replaced by warm full DMEM and the cells were further incubated under 5% CO_2 at 37 °C for over 20 h. For co-transfection of more than one plasmid, the quantity of DNA used in this protocol implies the total amount of plasmids.

Confocal microscopy

Live cells were imaged in phenol red free Dulbecco's Modified Eagle Medium (Cat# 21063-29, Life Technologies) supplemented with additional 10 % FBS, 1 % sodium pyruvate, 1 % NEAA and 1 % penicillin-streptomycin at 37 °C under 5 % CO_2 . Microscopy was performed using Zeiss LSM 880 inverted confocal laser scanning microscope equipped with an Airyscan super resolution module. Zeiss Plan- APOCHROMAT 60 \times /1.4 oil DIC objective was primarily used for imaging; for larger field of views, Zeiss Plan-APOCHROMAT 40 \times /0.95 DICIII objective was used, such as in EdU assays. Confocal images were typically acquired in 12-bit depth at 512 \times 512 resolution. 405 nm laser diode was used to excite blue-colored fluorophore such as mTagBFP2, 488 nm argon laser was used to excite green-colored fluorophores such as EGFP, and HeNe laser 543 nm or HeNe laser 594 nm was used to excite red-colored fluorophores such as mCherry. In most cases, the basic imaging setup parameters were configured applying the "Smart-Setup" function with typical parameters set as follows: scan speed 8, pixel dwell time 1.54 μs , number of averaging 4, line mode in one-direction scanning, and pinhole 89.9 μm . All confocal microscopic images aside from EdU experiments were performed 24 h after transfection.

Preparation of the cyclic cell-penetrating peptide Cys-cR10*

The cyclic decaarginine Cys-cR10* peptide features a cyclic rR ring (r = D-Arg, R = L-Arg) plus a (Gly)₅ linker with an N-terminal cysteine and a C-terminal -CONH₂ group. The peptide was synthesized via standard solid phase peptide synthesis from Rink amide resin. After the synthesis of liner R10* fragment, intramolecular cyclization was performed to bridge the Lys side chain (-NH₂ group) and Glu side chain (-COOH group). Afterwards, Cys-(Gly)₅ tail was sequentially added to the cyclic-R10* moiety followed by TFA deprotection and HPLC purification. Cys-cR10* peptide was obtained in a high purity of over 95% and confirmed using mass spectrometry. C₈₄H₁₆₀N₅₀O₁₉S, exact mass: 2205.28, M.W.: 2206.56, found m/z 736.4 [M+3H]³⁺, 552.6 [M+4H]⁴⁺, 442.3 [M+5H]⁵⁺.

Protein expression and purification

General protocol

pTXB1 vector was used to express intein-tag fused nanobody chimeras for subsequent expressed protein ligation (EPL). These plasmids for protein expression were first transformed into *E. coli* Rosetta 2a cells and the transformants were selected on ampicillin (100 mg·L⁻¹) agar plates depending on the antibiotic resistance of the plasmids. A single colony was used to inoculate 50-100 ml of LB medium containing 100 mg·L⁻¹ ampicillin and shaken at 240 rpm for 8-10 hours or overnight at 37 °C. 30-50 ml of the preculture was used to further inoculate ~1.8 L fresh LB medium containing 100 mg·L⁻¹ ampicillin and

additional chloramphenicol ($33 \text{ mg}\cdot\text{L}^{-1}$). The absorbance at 600 nm (OD₆₀₀) of the inoculated culture should be controlled between 0.05 to 0.1 in this inoculation step. Then the culture was shaken at 180 rpm at 37°C for a few hours (typically 2-3 h) until OD 600 reached 0.5-0.6. Then 0.5 ml isopropyl β -D-thiogalactoside (IPTG) stock solution (1M) was added (final \sim 0.27 mM) to induce protein expression at 16 °C overnight. Sometimes protein expression time and temperature needed to be optimized in order to achieve an optimal expression for some particular proteins.

Later, cells were harvested by centrifugation at $13881\times g$, at 4 °C for 15 min and washed once with PBS ($4149\times g$, 10 min). The bacterial pellet was resuspended in lysis buffer (pH 8.0, PBS supplemented with additional 0.5 M NaCl, 3 % glycerol, w/o 3 mM β -mercaptoethanol (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF)). For relatively smaller volumes of bacterial cell suspensions (< 40 ml), bacterial cells were typically lysed via ultra-sonification at 80 W for 30 min or 60 W for 45 min (1 s sonification followed by 3 s interval) on ice. For batch processing or larger volumes of cell suspensions, cells were typically lysed using ultra-high-pressure homogenizer cooled by a bench chiller for 2-3 cycles under 800-900 bar at 4 °C. The lysate was cleared by high-speed centrifugation ($74766\times g$, 45 min, 4 °C) and the supernatant was loaded onto a gravity Ni-NTA column (2-5 ml resin). The Ni-NTA column was washed and then the His-tag fused protein was eluted using step-gradient of imidazole (50, 100, ..., until 500 mM) solutions. Alternatively, GE ÄKTA Pure machine equipped with a HisTrap FF column was used to purify His-tagged protein via gradient elution (0 \rightarrow 500 mM imidazole) by combining buffer A (pH 8.0 PBS, 0.5 M NaCl, 3 % glycerol, w/o 3 mM BME) and buffer B (pH 8.0 PBS, 0.5 M imidazole, 0.5 M NaCl, 3 % glycerol, 3 mM BEM). Ionic exchange or size exclusion chromatography may be further applied if additional purifications are necessary. The obtained proteins were typically concentrated, buffer exchanged in buffer A, aliquoted, snap frozen in liquid nitrogen, and stored under -80 °C.

General EPL protocol for the preparation of nanobody-LC3B-Cys intermediate

Nanobody-LC3B-intein precursors were expressed as a fusion chimera with a C-terminal Mxe GyrA intein-His₆ tag by cloning the respective gene into pTXB1 vector. Afterwards, this fusion chimera was expressed, purified and buffer exchanged in Buffer A (pH 8.0 PBS, 0.5 M NaCl, 3 % glycerol). Typically, the Mxe GyrA intein fusion protein was reconstituted to around $20 \text{ mg}\cdot\text{ml}^{-1}$ before cysteine cleavage. For 1 volume of nanobody-LC3B-intein-His₆ solution, 1/2 volume of pH 8.0 sodium 2-mercaptoethanesulfonate (MENSNa) stock solution (2 M) was added as the intein cleavage reagent and 1/2 volume of pH 8.0 4-mercaptophenylacetic acid (MPAA) stock solution (1.1 M) was added as the expressed protein ligation catalyst. Afterwards, 66 mM L-cysteine solution in PBS buffer was added to the reaction solution at a final concentration of 0.8 mM. The reaction mixture was incubated at 4 °C for 3 days. The ligated product was then purified via step-gradient (0 \rightarrow 500 mM imidazole) reverse gravity Ni-IMAC chromatography using high affinity Ni-charged Resin FF (Cat# L00666-25, GenScript). In this process, cleaved intein-His₆ fragment will bind onto the resin and pure nanobody-LC3B-Cys intermediate will be eluted out.

Stepwise protocol for the preparation of nanobody-LC3B-SS-cR10* ATNC degrader

- Nanobody-LC3B-Cys chimera was buffer exchanged (1-2×) into Buffer A (pH 8.0 PBS, 0.5 M NaCl, 3 % glycerol), degassed via brief sonification, add 2 equiv. TCEP (20 mM stock), flushed with Ar, incubate for 45 min and degassed via sonification again.
- Add 8 equiv. DTNP (100 mM in 0.5 M Na₂HPO₄) for 45-60 min to activate Cys and the reaction solution will soon turn yellowish; buffer exchange (2-3×) into pH 9.0 Disulfidization Buffer (50mM HEPES, 0.5M NaCl) to remove the excess of DTNP.
- Degas via brief sonification, portion-wise add 3 equiv. of 15 mM stock Cys-cR10* in DMSO, incubate on ice for a few hours or overnight; buffer exchange (1-2×) into pH 7.2 PBS and the nanobody-LC3B-SS-cR10* degrader product in PBS was obtained, then aliquoted, snap frozen in liquid nitrogen and stored under -80 °C before use.

Western blot (WB) analysis

HeLa or OVCAR3 cell line was plated in 24-well plates at a density of 6×10^4 cells per well or 8-well imaging chamber at a density of 2.5×10^4 cells per well, and then left to grow overnight to allow cell adhesion. For the first form of ATNC degradation using expressed intrabodies, cells were subjected to transfection with nanobody-LIR/LC3B chimera (e.g. GBP-mCherry-LC3B) and w/o co-transfection of other necessary plasmids. Unless otherwise specified, 48 hours later cells were washed (2×PBS), collected using 2× SDS-PAGE loading buffer (120 μl/well for 24-well plates, 40 μl/well for 8-well chamber), and then boiled at 90 °C for 5 minutes before subsequent gel electrophoresis and WB analysis. For the second form of CIP-operated ANTC degradation, cells were transfected with necessary plasmids and 24 h later, (+)-ABA dimerizer at final 20 μM was added to the cell culture, incubated for 1.5 h, washed (2×PBS), and then cell samples were collected 46.5 h later for WB analysis (for Fig. 4d-g); alternatively, 24 h after transfection, (+)-ABA at specified final concentrations was added to the cell culture and incubated for 48 h, and then the cell samples were collected and subjected to WB analysis (for Fig. 4h-i). For the third form of ATNC degradation, cell-permeable nanobody-LC3B-Cys-SS-cR10* chimeras at specified final concentrations were added to cell culture and incubated for 24 hours; subsequently, cell lysate samples were collected and subjected to WB analysis. For study of the effect of rapamycin (Rap) or chloroquine (CQ) on degradation, Rap or CQ was added 2 h after transfection, and microscopic imaging was performed 22 h post-drug addition while WB analysis was performed 46 h post-drug addition.

For WB analysis, cell lysate samples were first subjected to 12% or 15% SDS-PAGE gel electrophoresis (180V, 45 min), transferred to nitrocellulose (NC) membrane (PALL, Cat. #: 66485) on ice applying constant 400 mA current for 30 min in rapid WB transferring buffer (Genefist, Cat. #: GF1816). The NC membrane was blocked using 5 % skim milk (Biosharp, Cat. #: BS102) in 1× TBST at room temperature for 1.5 h. Then, the blocked NC membrane was labeled by primary antibodies diluted in 5 % skim milk at 4 °C overnight. These primary antibodies include: GFP-tag rabbit mAb antibody (Zenbio, Cat. #: R24437) diluted at a ratio of 1:1000, mCherry-Tag antibody (BIOS, Cat. #: bs-41161R) diluted at a ratio of 1:2000, RhoA rabbit pAb antibody (Zenbio, Cat. #: 346086) diluted at a ratio of 1:1000, TOMM20 rabbit mAb antibody (Zenbio, Cat. #: R25952) diluted at a ratio of 1:1000, HE4 rabbit pAb antibody (Zenbio, Cat. #: 381649), diluted at a ratio of 1:400, β-actin rabbit mAb antibody (ABclonal, Cat. #: AC026) diluted at a ratio of 1:50000, and GAPDH

polyclonal antibody (Bioworld, Cat. #: AP0063) diluted at a ratio of 1:5000. The next day, NC membrane was washed by TBST (3×5min), incubated with secondary antibody using HRP-conjugated goat anti-rabbit IgG antibody (Zenbio, Cat: #: 511203) diluted in 5 % skim milk at a ratio of 1: 5000 at room temperature for 1h, and washed by TBST (3×10min) before imaging. For WB signal detection, the membrane was developed with a mixture of high-sensitivity luminescent liquid (Biosharp, Cat. #: BL523B). Densitometry of developed bands was measured and analyzed using LI-COR Odyssey Fc imaging system

EdU cell proliferation assay

The EdU cell proliferation assay was performed using an EdU cell proliferation detection kit (Cat# C10310-1, RiboBio). Briefly, 1×10^4 OVCAR3 cells harvested at exponential phase were seeded in a 96-well imaging plate and allowed to grow overnight. Drug solutions at specified final concentrations in full DMEM was used to treat the cells and incubate at 37 °C under 5% CO₂ for 24 h. Then, the drug solution was replaced by EdU solution at a final concentration of 50 μM. It was incubated for 2 hours at 37°C under 5% CO₂ as recommended for general cancer cell lines. Then, each well was washed by PBS (2×5 min) to remove the excess of EdU, added with 100 μl of fixative solution (4% PMA in PBS) and incubated for 30 min at room temperature. Then 100 μl of 2 mg·ml⁻¹ glycine solution was added to each well and shaken at RT for 5 min to quench the fixative. Glycine solution was removed and each well was washed by 200 μl PBS and shaken at RT for 5 min. PBS was removed and each well was added with 200 μl cell permeabilization solution (0.5% TritonX-100 in PBS) and shaken at RT for 10 min. The fixed cells were further washed by PBS (1×5 min) before labeling.

Before fluorescent labeling by click reaction, 1× Apollo labeling solution that contains the red color Apollo567 dye (Cat# C10310-1, RiboBio), catalyst and other necessary reagents were freshly prepared according to the manufacture's guidance. For example, 1 ml of 1× Apollo labeling solution could be prepared by sequentially adding 938 μl DI-H₂O, 50 μl Apollo reaction buffer (reagent B), 10 μl Apollo catalyst solution (Cu²⁺, buffer C), 3 μl Apollo 567 dye (reagent D) and ~9 mg Apollo additive (sodium ascorbate, reagent E). 200 μl freshly prepared 1× Apollo labeling solution was added into each well, shielding from light, and shaken at RT for 30 min to complete the click labeling. Labeling solution was removed, and the cells in each well were washed by permeabilization solution (0.5% TritonX-100 in PBS) again (3×10min). Permeabilization solution was removed and the cells were wash by PBS (1×5 min). Finally fresh PBS was added and the labeled cells were ready for confocal microscopy imaging. Hoechst could be used to label the nucleus if necessary.

Image analysis

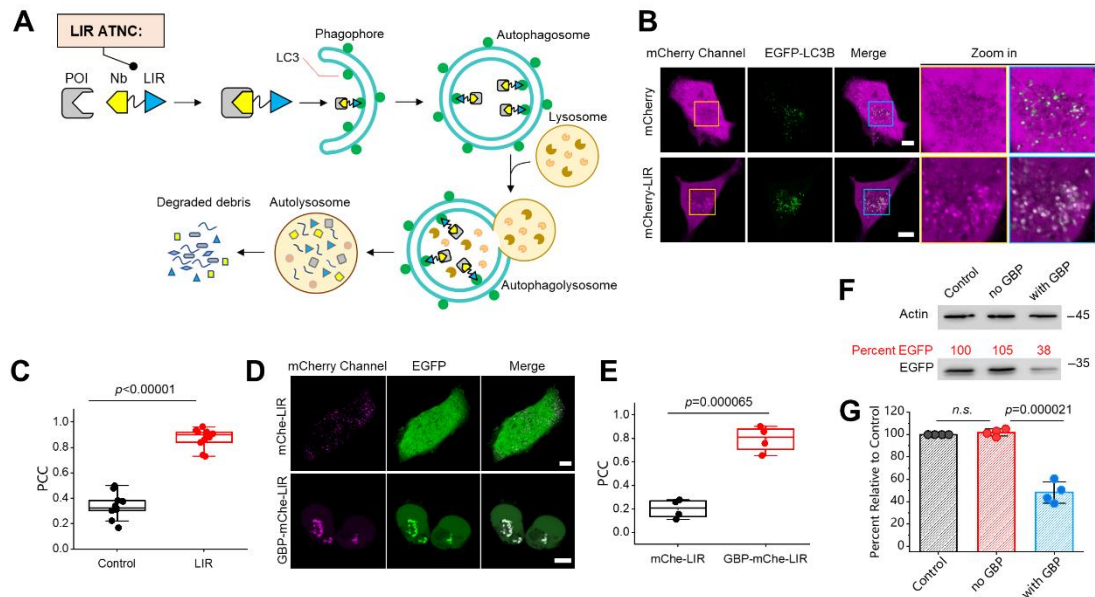
Microscopic images were analyzed and processed with ImageJ/Fiji and prepared for presentation using Microsoft Office PowerPoint. Image manipulations were restricted to adjustment of brightness level (i.e. linear stretch), background subtraction, cropping, rotating, scaling, and false color-coding using Look-Up Tables (LUT). Pearson's correlation coefficient (PCC) was employed for colocalization analysis using the "Manders_Coefficients.class" plugin for ImageJ/Fiji. Usually, there is only a single cell within an imaging field; otherwise, a single cell was first selected using the polygon selections tool and then remove extra cells (Edit/Clear outside) prior to PCC analysis. The

images were converted to 8-bit depth (Image/Type/8-bit) prior to analysis, and typically no less than ten cells were analyzed for each PCC analysis.

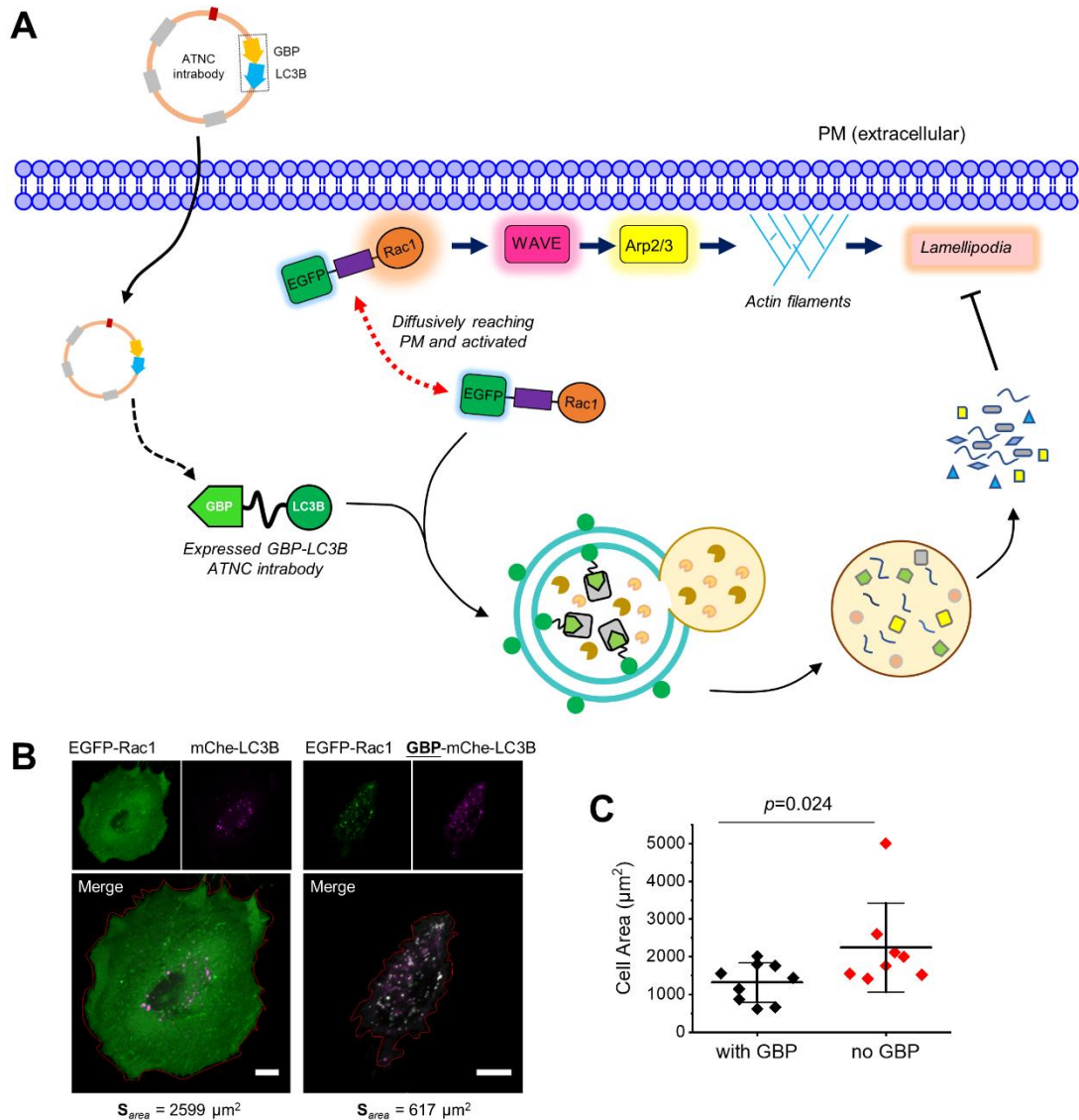
Statistics and reproducibility.

All microscopic imaging experiments were representative of at least three independent repeats if not otherwise stated. EdU experiments were performed twice with similar results and images from only one imaging section was used for analysis to keep fluorescence intensity readout consistent. Representative SDS-PAGE images were from at least three independent repeats with similar results. Origin and Microsoft Excel were used for plotting, data fitting, graphing and statistical analysis. All box plots show mean (square), median (bisecting line), bounds of box (75th to 25th percentiles), outlier range with 1.5 coefficient (whiskers), and minimum and maximum data points (lower/ upper whiskers). Student's *t*-tests were used to compare two experimental conditions. Unless otherwise specified, one-sided unpaired *t*-tests were performed, as for example cells with or without drug/inducer treatment. When necessary, stars were used to denote *P*-values for indicated statistical tests (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$). Exact *P*-values were indicated for critical experiments.

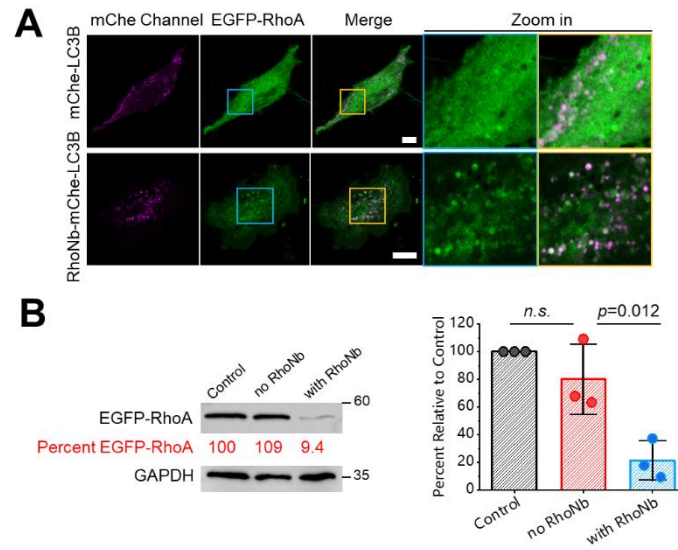
Supplementary Figures



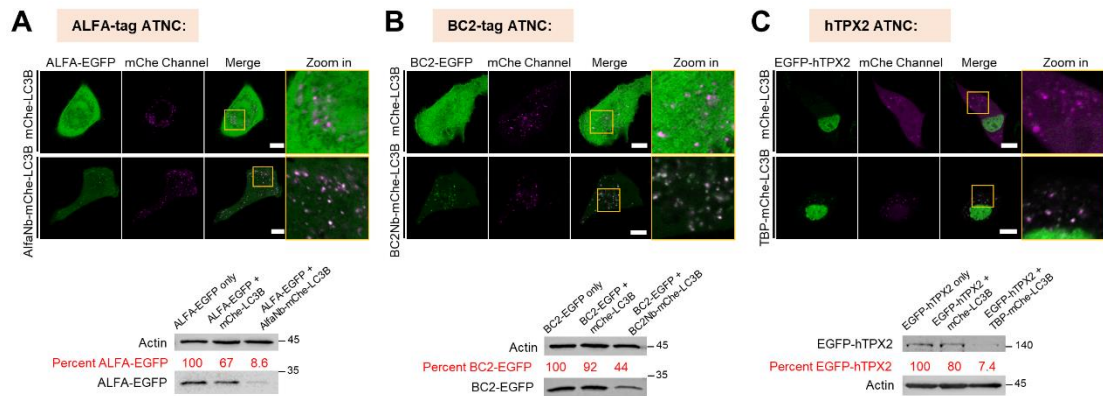
Supplementary Figure 1. Design and evaluation of LIR-based ATNC: (A) Schematic overview of working mechanism of LIR-based ATNC (*i.e.* nanobody-LIR chimera) that recruits protein of interest to autophagic degradation pathway. (B) Representative confocal microscopic images of live HeLa cells coexpressing EGFP-LC3B and mCherry-LIR or mCherry (*i.e.* control) revealed that mCherry-LIR showed clear colocalization with EGFP-LC3B labeled autophagic puncta. (C) Pearson's correlation coefficient (PCC) analysis of the colocalization between EGFP and mCherry channels ($n=11$ cells for both). (D) Representative confocal microscopic images of live HeLa cells coexpressing EGFP and GBP-mCherry-LIR or mCherry-LIR showed that EGFP was degraded only in the presence of GBP-mCherry-LIR along with the formation of autophagic puncta. (E) PCC colocalization analysis between EGFP and mCherry channels ($n=4$ cells). (F) Representative WB result (48 h) of the HeLa cell sample for control (EGFP only), no GBP group (EGFP + mCherry-LIR), and GBP group (EGFP + GBP-mCherry-LIR). (G) Statistical quantification of the WB degradation degree ($n=4$ experiments).



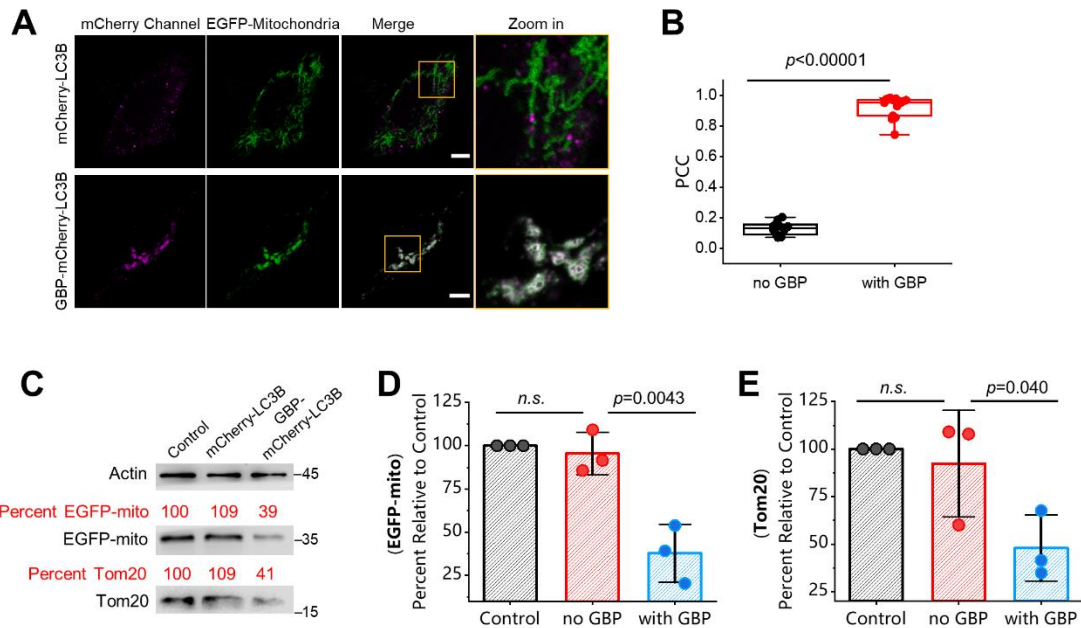
Supplementary Figure 2. Intracellularly expressed GBP-LC3B ATNC intrabody is a general and easy-to-implement platform for study cellular functions by down-regulating EGFP-fused proteins using only a plasmid as the key reagent: (A) Study the effect of down-regulation of Rac1, a small GTPase involved in actin cytoskeleton function, on the formation of lamellipodia in live HeLa cells. Intracellularly expressed GBP-LC3B ATNC intrabody degrades EGFP-Rac1 and subsequently off-regulates the formation of lamellipodia. (B) Representative confocal micrographs reveal that expressed GBP-mChe-LC3B ATNC degrades overexpressed EGFP-Rac1 and changes HeLa cells from a highly extended state (robust lamellipodia pattern, area $S = 2599 \mu m^2$) to a rather shrink state (lamellipodia inhibited, area $S = 617 \mu m^2$). Scale bar: 10 μm . (C) Statistical comparison of the cell areas in (B) for cells coexpressing EGFP-Rac1 and GBP-mChe-LC3B (with GBP group; $n=9$ cells) or mChe-LC3B (no GBP group; $n=8$ cells). Abbreviations: WAVE: WASp-family verprolin-homologous protein; Arp2/3: actin-related proteins-2/3; mChe: mCherry.



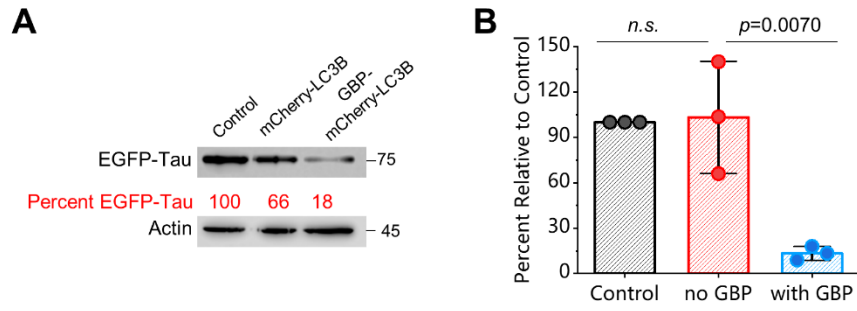
Supplementary Figure 3. RhoNb-mCherry-LC3B ATNC degrades overexpressed EGFP-RhoA in live HeLa cells: (A) Confocal microscopic images of live HeLa cells coexpression of EGFP-RhoA and mCherry-LC3B or RhoNb-mCherry-LC3B (24 h). (B) Representative WB result (48 h) and statistical comparison of the degradation result of the control sample (EGFP-RhoA only), no RhoNb group (EGFP-RhoA + mCherry-LC3B) and with RhoNb group (EGFP-RhoA + RhoNb-mCherry-LC3B) (n=3 experiments).



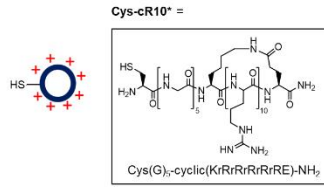
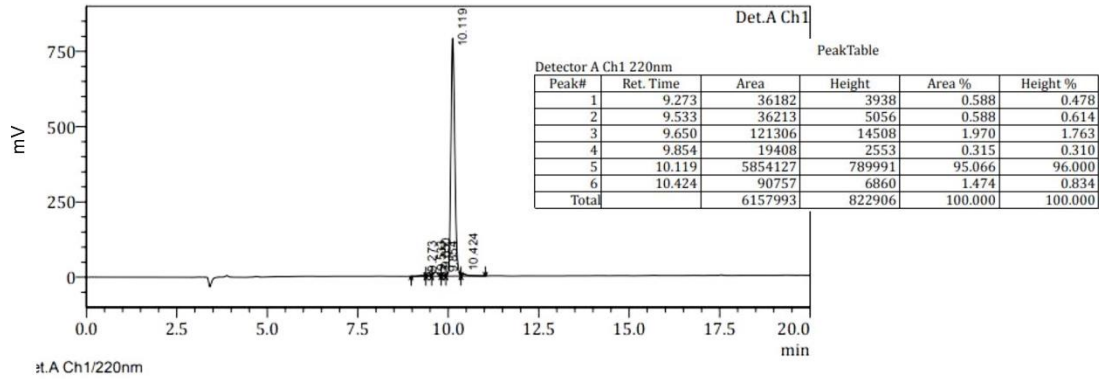
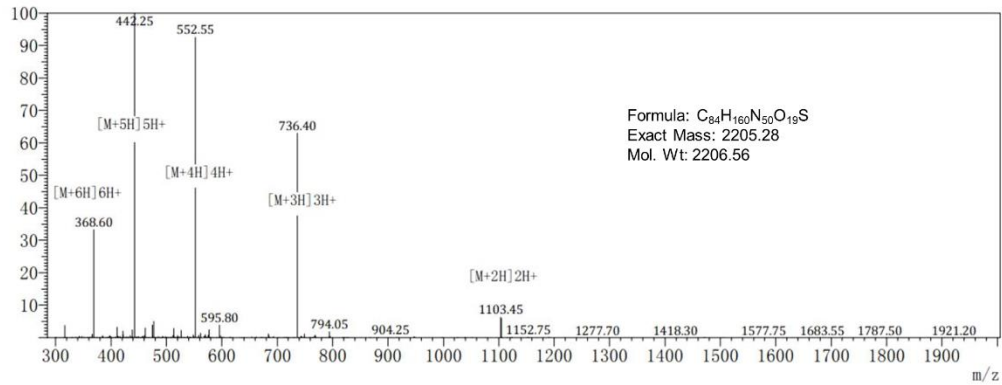
Supplementary Figure 4. Further demonstration of the modularity of ANTC for degradation of alternative cellular targets: (A) ALFA-tag ATNC that features an ALFA-tag nanobody (AlfaNb) effectively degrades ALFA-EGFP in live HeLa cells. (B) BC2-tag ATNC featuring a BC2-tag nanobody (BC2Nb) effectively degrades BC2-EGFP in live HeLa cells. (C) hTPX2 ATNC that features a hTPX2 nanobody (TPX2 binding protein, TBP) effectively degrades the nucleus localization EGFP-hTPX2 protein in live HeLa cells. Confocal micrographs (24 h) of live HeLa cells coexpressing EGFP-fused protein and mCherry-LC3B or Nb-mCherry-LC3B showed the entrapment of EGFP-fused protein into autophagic puncta; WB analysis (48 h) confirmed degradation of these EGFP-fusions. GFP antibody was used as the primary antibody in these blots. Scale bar: 10 μ m. Abbreviations: mCherry: mCherry.



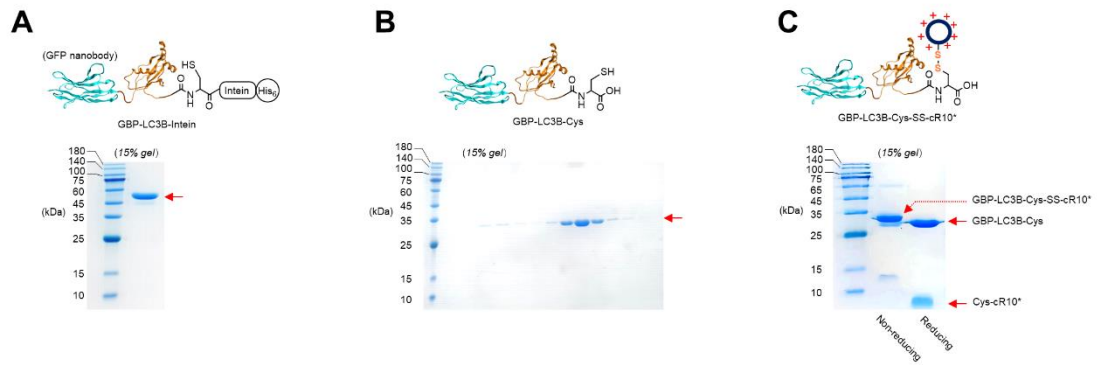
Supplementary Figure 5. Degradation of the organelle mitochondria via ATNC: (A) Mitochondria in live HeLa cells were labeled by expressing EGFP-mito (mito: mitochondria targeting sequence; see Methods for its peptide sequence information), and the cells were expressed with GBP-mCherry-LC3B (experiment) or mCherry-LC3B (control). Representative confocal microscopic images revealed that mitochondria were encapsulated in autophagic puncta only in the presence of GBP-mCherry-LC3B chimeric degrader but not in the control cells. (B) Pearson's correlation coefficient (PCC) analysis of the colocalization between EGFP and mCherry channel (control: expressing only EGFP-mito; $n=12$ cells for no GBP group; $n=13$ cells for with GBP group). (C) WB result (48 h) of the degradation of EGFP-mito revealed a clear degradation of EGFP-mito and a key mitochondria membrane marker Tom20 protein. (D-E) Statistical quantification of degradation of EGFP-mito and Tom20 ($n=3$ experiments); means. One-sided Student's t -test was used in this figure, $n.s.$: non-significant; see Methods for description of box plots; bar graphs denote mean \pm standard deviation (SD) in this figure.



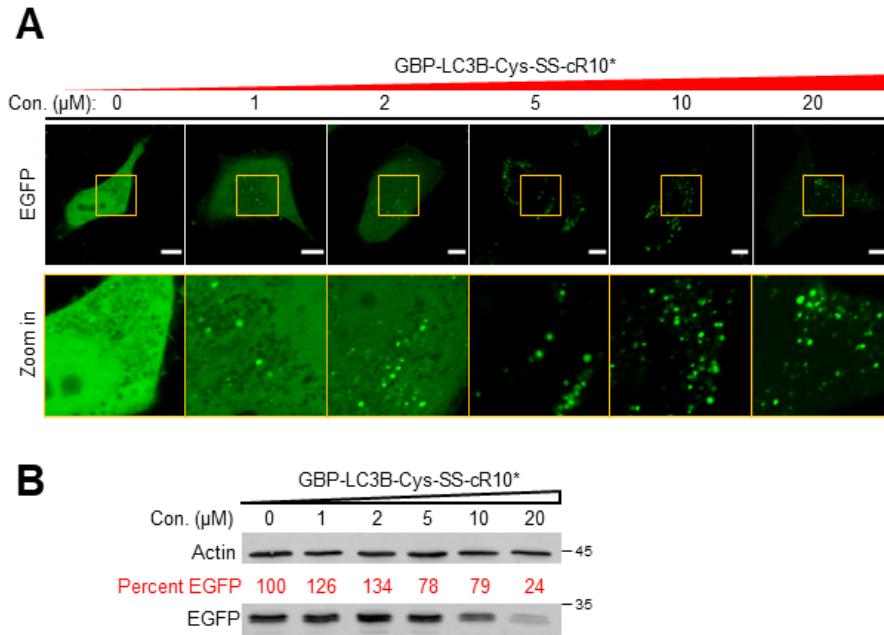
Supplementary Figure 6. Degradation of the prone-to-aggregate protein Tau using ATNC: (A) WB result revealed a degradation of EGFP-Tau protein (Tau: the P301L aggregating mutant form of Tau; control: cells expressing only EGFP-Tau). (B) Statistical quantification of degradation of EGFP-Tau (n=3 experiments); one-sided Student's *t*-test was used, *n.s.*: non-significant; bar graphs denote mean \pm SD.

A**B HPLC:****C MS:**

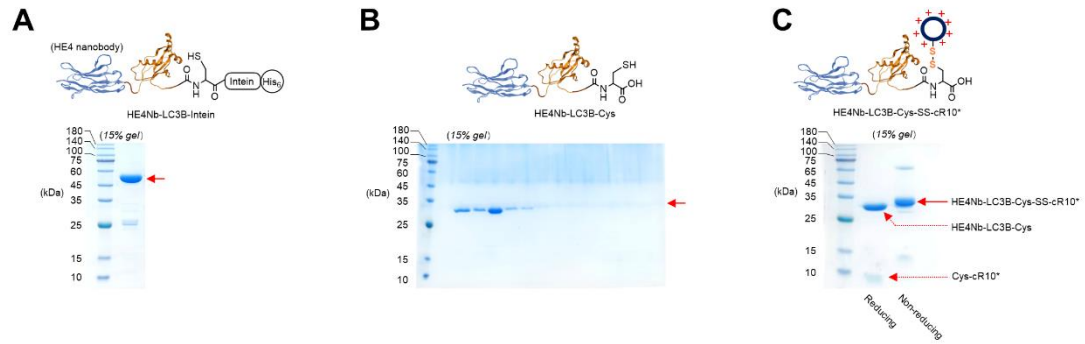
Supplementary Figure 7. Chemical structure and HPLC-MS characterization of Cys-cR10*: (A) The symbol for Cys-cR10* and the peptide sequence and chemical structure of Cys-cR10*. (B) HPLC analysis of Cys-cR10* revealing a purity of 96.0 %. (C) ESI-MS spectrometry analysis of Cys-cR10*.



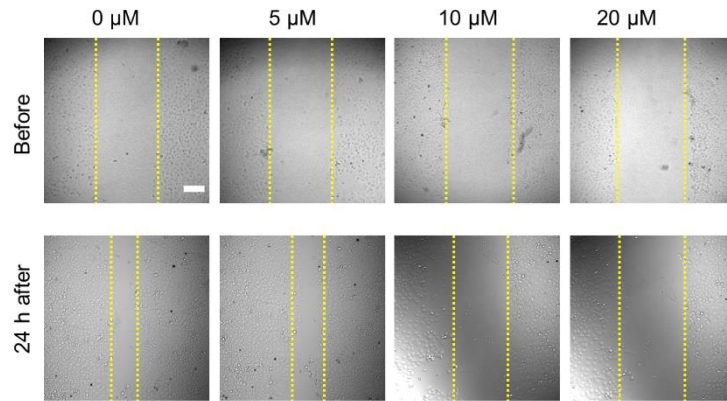
Supplementary Figure 8. SDS-PAGE characterization of the adduct, intermediates and the product for the preparation of GBP-LC3B-Cys-SS-cR10* (GLR): Representative SDS-PAGE gel images of the adduct GBP-LC3B-intein (A), intermediate GBP-LC3B-Cys (B) and the product GBP-LC3B-Cys-SS-cR10* (C); GBP: green fluorescent protein binding protein, i.e. GFP nanobody.



Supplementary Figure 9. Characterizations of GBP-LC3B-Cys-SS-cR10* (GLR)-based degradation of EGFP via confocal microscopic and WB analysis: (A) Representative confocal micrographs of live HeLa cells expressing EGFP treated with gradient concentrations of GBP-LC3B-Cys-SS-cR10* (GLR) for 24 h. (B) WB analysis results of the cell lysate sample 24 h after drug treatment.

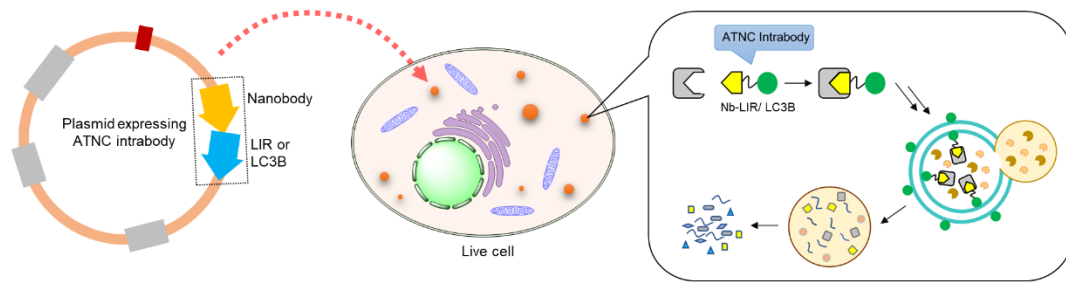


Supplementary Figure 10. SDS-PAGE characterization of the adduct, intermediates and the product for the preparation of HE4Nb-LC3B-Cys-SS-cR10* (GLR): Representative SDS-PAGE gel images of the adduct HE4Nb-LC3B-intein (A), intermediate HE4Nb-LC3B-Cys (B) and the product HE4Nb-LC3B-Cys-SS-cR10* (C); HE4Nb: HE4 nanobody.

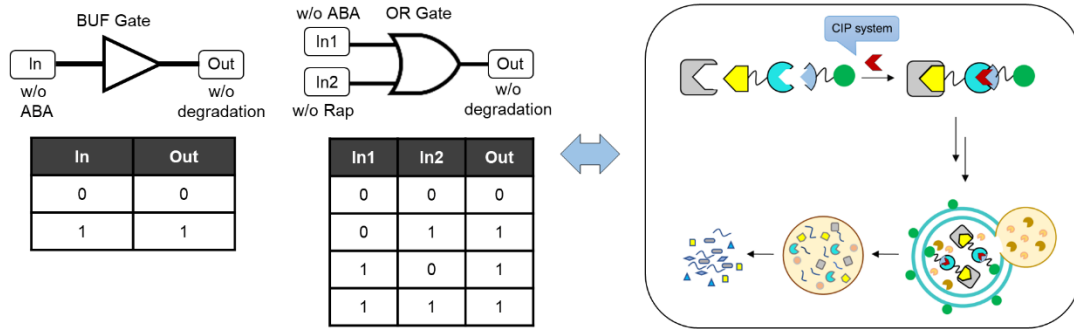


Supplementary Figure 11. Scratch-wounding cell migration assay for evaluation of cell migration after treatment of gradient concentrations of the HLR drug: Live OVCAR3 cells were scratched before drug treatment and imaged 24 hour after drug treatment. The approximate boundary of the scratched region was shown in yellow dashed line. 10× objective was used for imaging; scale bar: 200 μm .

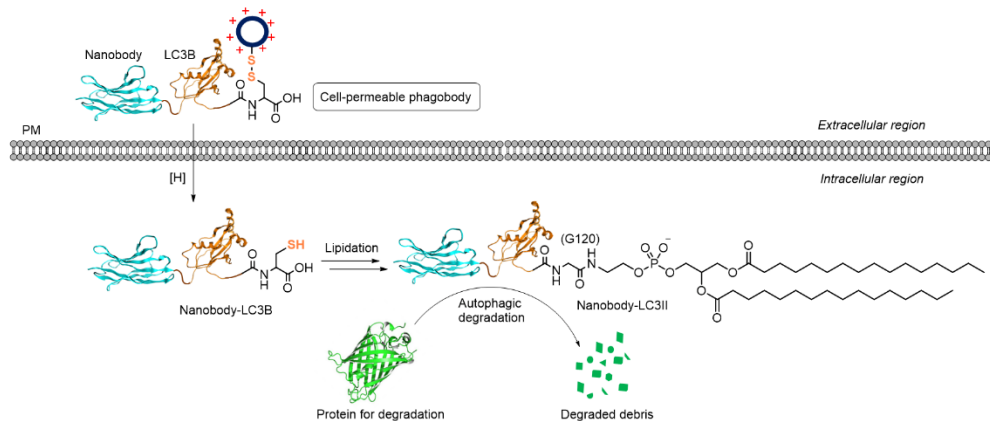
Form 1: ATNC intrabodies allows ease of implementation



Form 2: CIP-operated logic gated ATNC



Form 3: Cell-permeable ATNC drugs



Supplementary Figure 12. Three ways for implementation of ATNC methodology. ATNC can be versatilely implemented in three forms: The first form is so-called ATNC intrabodies that simply expresses an ATNC chimeras inside live cells using no more than a plasmid; this approach is very easy to apply and wins in terms of simplicity. The second form is chemically induced proximity (CIP)-controlled ATNC that allows logic-gated and also dose-dependent control of the degradation degree by ATNC. The third is in the form of cell-permeable phagobodies that hold therapeutic potential; unligandable and undruggable targets of therapeutic relevance can be degraded via drug-treatment.