Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Charge Designable and Tunable GFP as a Target pH-Responsive Carrier for Intracellular Functional Protein Delivery and Tracing

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Experimental Procedures

1. Reagents and Instruments

Reagents: Sodium phosphates (NaH_2PO_4 and Na_2HPO_4), Hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), ethylene diamine tetraacetic acid (EDTA), isopropyl-β-d-thiogalactoside (IPTG), phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from J&K Chemical (Guangzhou, China). Plasmid pUC19-*h19gfp* and pUC19-*h29gfp* were constructed by bio-seeker (Beijing, China). Plasmid pUC19-h39*gfp*, pUC19-*scgfp* and pUC19-*egfp* were constructed by Takara Co. (Dalian, China). Plasmid pUC57-*mifp*, pUC57-*rnase a* were constructed by Sangon Inc. (Shanghai, China). Other protein expression reagents including Tris and imidazole were purchased from Sangon (Shanghai, China). Cell culture medium (RPMI-1640), trypsin and fetal bovine serum (FBS) were purchased from Invitrogen (Gibco, USA). BCA protein determination reagent, 3-(4, 5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide (MTT), and penicillin-streptomycin were purchased from KeyGen (Nanjing, China). Phosphate buffer saline (PBS, pH 7.4) contained NaCl (136.7 mM), KCl (2.7 mM), Na_2HPO_4 (8.1 mM), and KH_2PO_4 (1.47 mM) was used as the bio-imaging buffer. 20× SSC buffer contained NaCl (3 M) and odium citrate (0.3 M), was used in situ fluorescent hybridization experiment. Three-dimensional (3D) cell culture hydrogel was purchased from Beaver Nanotechnologies (Suzhou, China). Hoechst 33258 staining solution and DNA (dT_{50} -biotin) were purchased from Sangon (Shanghai, China). Texas red-avidin was purchased from Thermo Fisher (USA). LysoTracker Red was purchased from Beyotime (Shanghai, China) Proteo-Juice protein transfection reagent was purchased from Merck (Novagen, German). Cancer cells were obtained from the cell bank of the central laboratory at Xiangya hospital (Changsha, China). All chemical reagents were of analytical grade and used without further purification, and all solutions were prepared with ultra-pure water (18.25 MΩ·cm) from the Millipore system.

Instruments: Cell viability assay was carried out on a SynergyTM Mx multi-mode microplate reader (BioTek, USA). Confocal fluorescence imaging of fluorescently stained cells was performed on a confocal laser scanning microscope (CLSM, Nikon A1 plus, Japan). All the fluorescence images were digitized and analyzed with Image J software. Flow cytometric analysis was performed on a Gallios flow cytometer (Beckman-Coulter, USA). Cell numbers were measured with a TC10 automated cell counter (Bio-Rad, USA). The sizes and images of 3D tumor spheroid were analyzed by cell imaging microporous plate detection system (BioTek cytation 5, USA) under a microscope of 4× objective lens. UV-Vis absorption value of GFPs were recorded on a Beckman Coulter DU-800

spectrophotometer (USA). Fluorescence spectra were measured on a QuantaMasterTM4 fluorescence spectrophotometer (PTI, Canada). pH value was measured by a pH meter (Mettler-Toledu, Sweden).

2. Construction, Expression and Purification of Charge Tunable GFPs

His_{19}GFP and His_{29}GFP were designed by mutating residues of the starting His_{39}GFP that were likely to tolerate substitution to positively charged amino acid (Lys or Arg) (Figure S1). The full-length gene, which is inserted in plasmid pUC19, was synthesized by bio-seeker (Beijing, China). The amino acid sequence of ScGFP, EGFP and His_{39}GFP were referred to the reported literature[1-4]. Genes encoding GFPs were inserted into pET-28a with restriction enzyme cutting sites of NdeI and EcoRI.

mIFP and RNase A were fused to C-terminal of His_{29}GFP, respectively. Sequence of mIFP and RNase A were referred to the reported literature[5,6]. Genes encoding mIFP and His_{29}GFP-mIFP were inserted into pET-28a with restriction enzyme cutting sites of NdeI/EcoRI and NdeI/XhoI, respectively. Genes encoding RNase A and His_{29}GFP-RNase A were inserted into pColdI with restriction enzyme cutting sites of EcoRI/HindIII and BamHI/HindIII, respectively.

The reconstructed plasmid was transformed into *E. coli* BL21 (DE3) by heat shock. Cells were grown in LB medium at 37 °C until OD600 (optical density at 600 nm) reached about 0.6, and then were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 15 °C for 24 h. Cells were harvested by centrifugation and re-suspended in binding buffer (10 mM Tris, 2 M NaCl, pH 7.4), and then lysed by sonication. Protein was purified by Ni-NTA agarose chromatography (ÄKTA, GE), and then the buffer was exchanged into desalination buffer (10 mM Tris, 100 mM NaCl, 50% glycerin, pH 7.4) by desalination chromatography (ÄKTA, GE). The purified GFPs and GFP-fusion proteins was quantified by absorbance at 488 nm with an extinction coefficient of 8.33×10^4 M^{-1} cm^{-1}, and then was stored at -80 °C. RNase A and mIFP were quantified by BCA protein determination reagent.

3. Analysis of the Theoretical Net Charge of GFPs

The charge of GFPs could be expressed by equation (Eq 1):

$$\text{theoretical charge} = \alpha \cdot m + \beta \cdot n - \varepsilon \cdot x - \zeta \cdot y + \theta \cdot z \quad \text{(Eq. 1)}$$

m, n, x, y and z denote the number of Lys, Arg, Asp, Glu, His respectively. α, β, θ denote the protonation degree of side chain in Lys, Arg and His (basic amino acid residue) respectively. While ε and ζ denote the deprotonation degree of side chain in Asp and Glu (acidic amino acid residue) respectively.

The relationship between pH and pK_a was demonstrate in the Henderson-Hasselbalch equation (Eq. 2).

$$\text{pH} = pKa + \log\left([\text{A}^-]/[\text{HA}]\right) \quad \text{(Eq. 2)}$$

$[A^-]$, $[HA]$ denote the deprotonated and the protonated amino acid residue, respectively. pK_a denotes the pK_a value of side chain (R) in amino acid. The protonation degree of basic amino acids was expressed by equation (Eq. 3):

$$\text{protonation degree} = [\text{HA}]/([\text{HA}] + [\text{A}^-]) \quad \text{(Eq. 3a)}$$

$$\text{protonation degree} = 1/\left[1 + 10^{(\text{pH} - pKa)}\right] \quad \text{(Eq. 3b)}$$

Substitute the reported pK_a value into equation (Eq. 3b), the calculated results showed that one Lys (R: -NH_2, pK_a = 10.54) or Arg residue (R: -CN_3H_4, pK_a = 12.48)[7] represent nearly one positive charge, due to nearly 100% protonation of Lys or Arg at pH 5.0-7.4.

The deprotonation degree of acidic amino acids was expressed by equation (Eq. 4):

$$\text{deprotonation degree} = [\text{A}^-]/([\text{HA}] + [\text{A}^-]) \quad \text{(Eq. 4a)}$$

$$\text{deprotonation degree} = 1/\left[1 + 10^{(pKa - \text{pH})}\right] \quad \text{(Eq. 4b)}$$

Substitute the reported pK_a value into equation (Eq. 4b), the calculated result showed that one Asp (R: -CH_2COOH, pK_a = 3.86) or Glu (R: -CH_2CH_2COOH, pK_a = 4.25)[8] residue represent approximately one negative charge for each amino acid, which was attributed to almost 100% deprotonation of Asp or Glu at pH 5.0-7.4.

By substituting equation (Eq. 3b) into (Eq. 1), the following expression is obtained:

$$\text{theoretical net charge} = \text{m} + \text{n} - \text{x} - \text{y} + \text{z}/\left[1 + 10^{(\text{pH} - pKa)}\right] \quad \text{(Eq. 5)}$$

pK_a donate the pK_a value (6.04) of imidazole side chain in His.[7]

4. Acid-Base Titration

The titration profile of acid-base titration was obtained using a pH meter. The proteins (5.39 μM) were dispersed in 150 mM NaCl solution, the solution was adjusted to pH 8.0 with NaOH (0.02 M). The diluted solution was titrated by stepwise addition of HCl (0.005 M) solution to obtain the titration profile.

5. Cell Culture

All the mammalian cells were cultured in RPMI-1640 culture medium at 37 °C with 5% CO_2. RPMI-1640 culture medium was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Amphotericin B). Cell numbers were measured with a TC10 automated cell counter (Bio-Rad, USA).

6. Live-Cell Imaging

Hela cells (2×10^4 cells/well) were seeded in a confocal dish (Cellvis, USA). After cultured for 24 h, cells were washed twice with PBS and incubated with protein (200 nM) in serum free medium (pH 6.5 or pH 7.4) for 40 min at 37 °C. After incubation, cells were washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein. Cell nucleus was labeled with Hoechst 33258 (1%, v/v) in PBS for 10 min and washed three times with PBS. Cell were imaged in prewarmed PBS solution at 37 °C and observed on confocal laser scanning microscope (CLSM, Nikon A1 plus). GFP and mIFP were visualized with a 488 and 650 nm excitation laser, respectively.

7. Flow Cytometry Assays

Hela cells (1.5×10^5 cells/well) were plated onto a 12-well plate. After 24 h incubation, cells were washed twice with PBS and incubated with protein (200 nM) in serum free medium (pH 6.5 or pH 7.4) for 40 min at 37 °C. Then cells were washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein, trypsinized, resuspended in 400 μL of PBS and placed on ice. Cells were analyzed by flow cytometry (Beckman-Coulter, USA) for GFP internalization (ex, 488 nm) or mIFP internalization (ex, 561 nm).

8. Endocytic Pathway Probes

Hela cells (2×10^4 cells/well) were seeded in a confocal dish (Cellvis, USA) and culture for 24 h. Afterwards, the cells were pre-incubated with several inhibitors specific for different endocytosis pathways[9,10] [chlorpromazine (CPZ, 10 μM) for clathrin-mediated endocytosis; nystatin (NYS, 27 μM) for cavelolin-mediated endocytosis; amiloride (AMI, 1 mM) and Wortmannin (WMN, 170 nM) for macropinocytosis] in full medium for 1 h at 37 °C, respectively. Following this incubation, cells were washed twice with PBS and incubated with His_{29}GFP (200 nM) in the presence of the inhibitors for another 40 min in serum free medium (pH 6.5), respectively. Cells were washed three times with 20 U/mL heparin in PBS. Cell nucleus was labeled with Hoechst 33258 (1%, v/v) in PBS for 10 min and washed. His_{29}GFP uptake was measured by CLSM. Semi-quantative results of cellular uptake were analyzed via Image J software.

9. Endosomal Trafficking of His_{29}GFP

Hela cells were treated for 40 min at 37 °C with His_{29}GFP or ScGFP (200 nM) diluted in serum free medium (pH 6.5). Following incubation, cells were washed three times with 20 U/mL heparin in PBS to remove extracellular protein. Then the cells were incubated for an additional 1, 2 and 4 h in full medium at 37 °C, respectively. Endosomes were labeled with LysoTracker Red (Beyotime, 75 nM) in full medium, referring to the standard procedure of specification. Cell nucleus was labeled with Hoechst 33258 (1%, v/v) in PBS for 10 min and washed. Then cells

were immediately observed using CLSM. The colocalization of GFP with endosomes was determined by calculation of the colocalization coefficient of the red and green channels using Image J software.

10. Fluorescent in Situ Hybridization to mRNA using an Oligonucleotide Probe

In situ hybridization method was referred to the reported literature[11]. Briefly, the oligonucleotide probe dT_{50}-biotin can hybridize to poly(A) tail of mRNA in cancer cells, and then a fluorescent labeled biomarker (texas red-avidin) can interact with dT_{50}-biotin by ligand-receptor interaction. The fluorescence of texas red can be used as RNA detection signal for image.

Hela cells (2×10^4 cells/well) were seeded in a slide. After cultured for 24 h, cells were washed twice with PBS and incubated with His_{29}GFP-RNase A or naked RNase A (200 nM) in serum free medium (pH 6.5 or pH 7.4). After incubation at 37 °C for 40 min, cells were washed three times with 20 U/mL heparin in PBS, incubated an additional 4 h in full medium at 37 °C. Fix cells with freshly made 2% paraformaldehyde in PBS at room temperature for 15 min, and wash cells in PBS (three times for 10 min each). Permeabilize cells with 0.5% Triton X-100 for 5 min on ice. Wash cells in PBS (three times for 10 min each). Rinse cells in 2 × SSC (two times for 10 min each). Hybridization buffer containing 2 μL of yeast tRNA (10 mg/ml), 2 μL of 20 × SSC, 4 μL of 50% dextran sulfate is added with 1 μL of oligo (~100 ng of oligo dT_{50} - biotin), 5 μL of deionized formamide, 2 μL of 10% BSA, and 4 μL of dH_2O. Final concentrations in the hybridization mixture are 5 ng/μL of probe, 25% formamide, 1 μg/μL yeast tRNA, 2 × SSC, and 10% dextran sulfate. The entire mixture is placed onto one cover ship. Invert cover ship onto a slide, seal with rubber cement, and incubate in a humid chamber at 42 °C overnight. Float the coverslips off the slide with 2 × SSC and wash in 2 × SSC (two times for 30 min each). Wash cells in 1 × SSC and 0.5 × SSC for 15 min each. Wash cells in 4 × SSC, 0.1% Triton X-100 at room temperature for 5 min. Incubate the cells in 4 × SSC / 1% BSA / 2 μg/mL texas red-avidin, for 30 min in a humid chamber at room temperature in the dark. Wash cells with 4 × SSC twice for 10 min each and then in 2 × SSC for 10 min. Samples were observed using CLSM.

11. Cytotoxicity Assay in Vitro

The cytotoxicity of His_{29}GFP-RNase A against cancer cells was determined by MTT assay. Briefly, HeLa cells (2×10^4 cells/well) were seeded on a 96-well plate and incubated for 24 h. Then full medium was replaced with serum free medium (pH 6.5 or pH 7.4) containing His_{29}GFP-RNase A, as well as His_{29}GFP, RNase A, and Proteo-Juice protein transfection reagent, respectively. Cells were washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein after 40 min post incubation at 37 °C. Then cells were received further incubation in 200 μL full medium for 24 h at 37 °C. Afterward, full medium was removed and MTT (5 mg/mL, 20 μL per well)

was added. Four hours later, the supernatant was replaced with 150 μL DMSO to dissolve the formazane. The optical density at 570 nm (OD_{570}) was determined via a microplate reader. Cell viability was calculated based on the formula: $\text{Cell viability} = [(OD_{sample} - OD_{buffer})/(OD_{cell} - OD_{buffer}] * 100\%$. OD_{sample} was OD_{570} value of cells with protein treatment, OD_{cell} was optical density of cells without protein treatment, OD_{buffer} was optical density of DMSO.

12. Three-dimensional (3D) Tumor Spheroid Model Analysis

HeLa cell tumoroids were prepared by cultivation in 3D cell culture hydrogel. The cultivation method was referred to the reported literature.[12] Firstly, cells were resuspended as single cell solution. For cell cultivation, cells were washed with PBS and resuspended in culture medium, which was supplemented with 20 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.5% BSA. Cells (1×10^4 cells /well) were seeded in a 12-well tissue culture plate coated with 1% low gelling temperature agarose, cultured in full medium with 5% CO_2 at 37 °C for 6 days. Then, cells were transformed to 3D hydrogel. Cell spheres were cultivated in 5% CO_2 at 37 °C and medium was updated once the culture medium nutrient was depleted. When the tumoroid size grow to 200-500 μm, tumor spheroids were incubated with proteins (200 nM) in serum free medium (pH 6.5 or pH 7.4) at 37 °C for 4 h. After incubation, tumor spheroids were washed three times with 20 U/mL heparin in PBS. The fluorescence of 3D tumor spheroids was analyzed by cell imaging microporous plate detection system (BioTek cytation 5, USA) under a microscope of 4 × objective lens. Fluorescence images were collected using the Z-scanning mode on the microscope. All images of Z-scanning were digitized and analyzed with the Image J software.

To analyze proliferation inhibition of 3D tumor spheroid model, 3D tumor spheroids (30 μm) were incubated with proteins (200 nM) in serum free medium (pH 6.5 or pH 7.4) at 37 °C for 4 h, washed by 20 U/mL heparin in PBS, and further incubated in full medium at 37 °C. The medium was updated once a day. The tumor size was measured every 2 days using the Z-scanning mode on the cell imaging microporous plate detection system (BioTek cytation 5, USA).

13. Protein Sequences

His_{29}GFP-RNase A

SKGEHLFHGHVPILVELKGDVNGHKFSVRGKGHGDATRGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCF
SRYPKHMKHHDFFKSAMPHGYVQERTISFKKDGHYKTRAEVKFEGHTLVNRIKLKGHDFKEHGNILGHK

LRYNFNSHHVYITADKHKNGIKAKFKIRHNVHDGSVQLADHYQQNTPIGRGPVLLPHNHYLSTRSHLSKD

PHEKRDHMVLLEFVTAAGIHHGHDEHYKEFGGGGSGGGGSGGGGSVDKETAAAKFERQHMDSSTSAAS

SSNYCNQMMKSRNLTKDRCRPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYSTMSITDCRETRS

SKYPNCAYKTTQANKHIIVACEGNPYVPVHFDASVDDDDDDDDDD

His_{29}GFP-mIFP

SKGEHLFHGHVPILVELKGDVNGHKFSVRGKGHGDATRGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCF

SRYPKHMKHHDFFKSAMPHGYVQERTISFKKDGHYKTRAEVKFEGHTLVNRIKLKGHDFKEHGNILGHK

LRYNFNSHHVYITADKHKNGIKAKFKIRHNVHDGSVQLADHYQQNTPIGRGPVLLPHNHYLSTRSHLSKD

PHEKRDHMVLLEFVTAAGIHHGHDEHYKGGSGGSGGSGGSSVPLTTSAFGHAFLANCEREQIHLAGSIQP

HGILLAVKEPDNVVIQASINAAEFLNTNSVVGRPLRDLGGDLPLQILPHLNGPLHLAPMTLRCTVGSPPRR

VDCTIHRPSNGGLIVELEPATKTTNIAPALDGAFHRITSSSSLMGLCDETATIIREITGYDRVMVVRFDEEG

NGEILSERRRADLEAFLGNRYPASTIPQIARRLYEHNRVRLLVDVNYTPVPLQPRISPLNGRDLDMSLSCLR

SMSPIHQKYMQDMGVGATLVCSLMVSGRLWGLIACHHYEPRFVPFHIRAAGEALAETCAIRIATLESFAQ

SQSKLE

14. Tables and Figures

Table S1. Charge calculation results of ScGFP and His_{39}GFP at a range of pH (pH 5.0-7.4).

	ScGFP	His_{39}GFP
pH 5.0	+45.2	+42.7
pH 6.0	+41.2	+27.4
pH 6.5	+38.6	+17.0
pH 7.4	+36.4	8.6

Table S2. Charge calculation results of green fluorescent proteins (GFPs) at pH 6.5 and pH 7.4.

	Number of His	Number of Arg/ Lys	Number of Asp/ Glu	Total charge	
				pH 6.5	pH 7.4
His_{29}GFP	29	37	20	+24.5	+18.2
His_{19}GFP	19	47	20	+31.9	+27.8
His_{39}GFP	39	27	20	+17.0	+8.6
ScGFP	10	56	20	+38.6	+36.4
EGFP	9	26	34	-5.7	-7.6

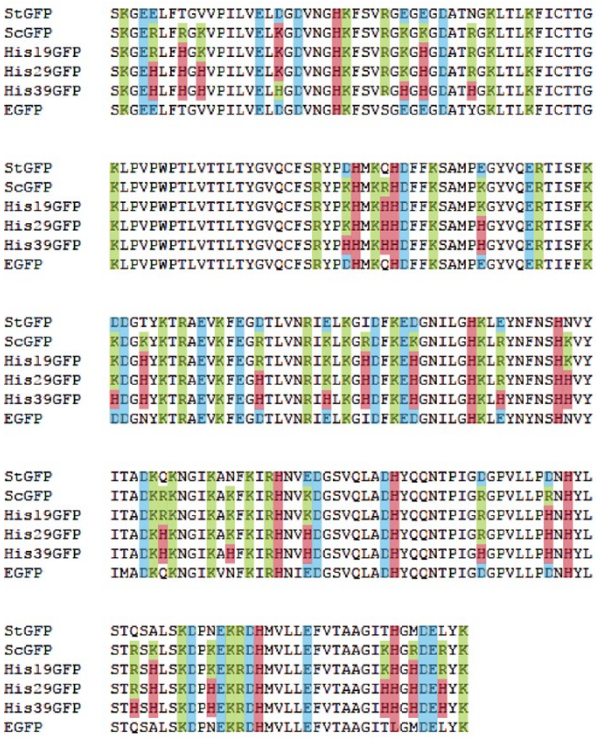

Fig. S1 Protein sequences of GFP variants, with His residues highlighted red, positively charged residues (Lys, Arg) highlighted green, and negatively charged residues (Asp, Glu) highlighted blue.

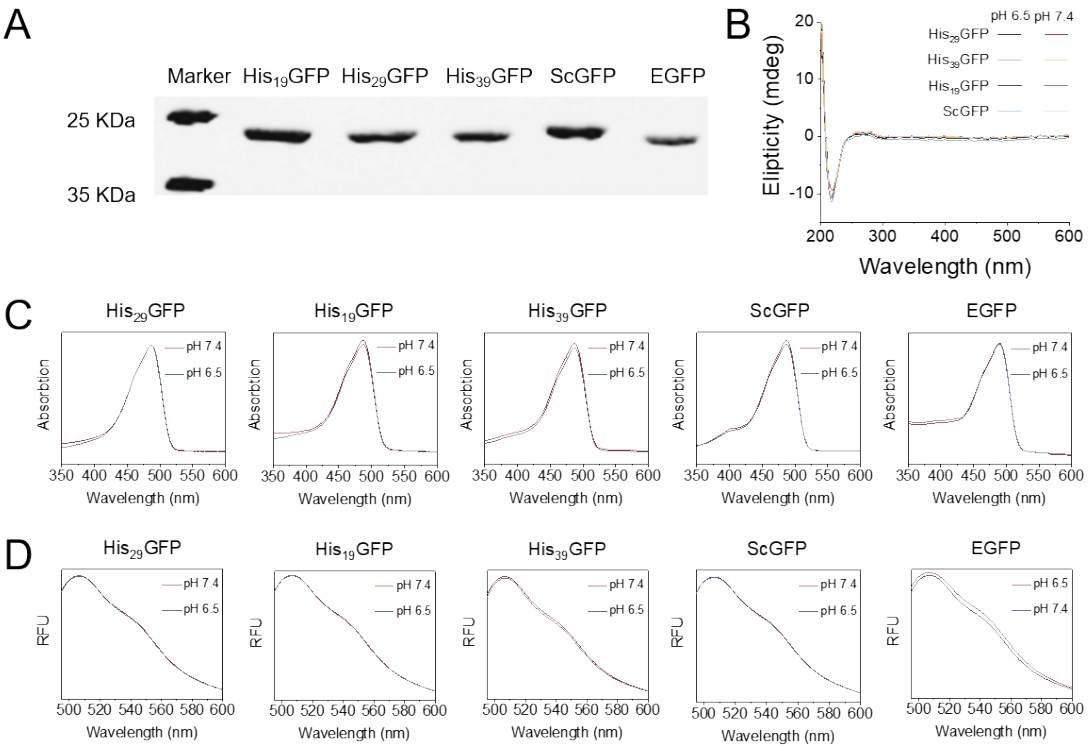

Fig. S2 Characterization of GFPs. (A) Analysis of GFPs by SDS-PAGE. (B) Circular dichroism spectra, (C) UV absorbtion spectra, and (D) emission spectra of GFPs at pH 6.5 and pH 7.4.

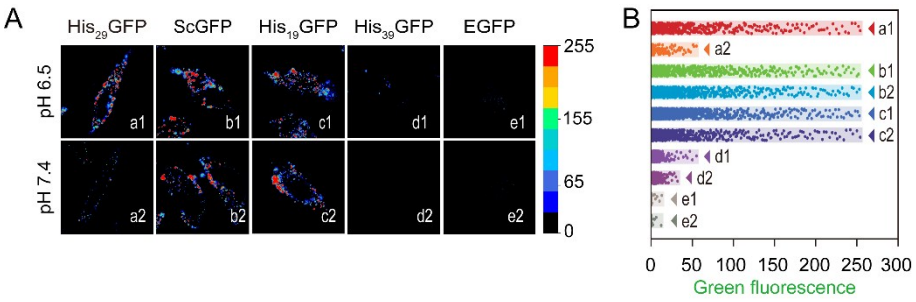

Fig. S3 (A) Corresponding color-coded fluorescence intensity of GFPs in Fig. 1C. (B) Fluorescence intensity distributions of each pixel in Fig. 1C. HeLa cells were incubated with different GFP (200 nM) in serum free medium at 37 °C for 40 min, washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein. Cells were imaged in prewarmed PBS solution at 37 °C and observed on confocal laser scanning microscope. GFP was visualized with a 488 excitation laser. Fluorescence images in Bitmap (BMP) format were analyzed by Python.

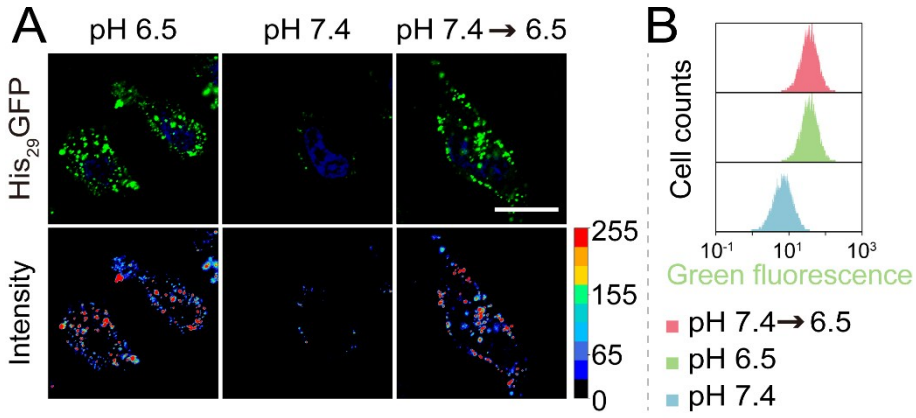

Fig. S4 (A) CLSM images of cellular uptake of $His_{29}GFP$ (200 nM). Top row: $His_{29}GFP$ fluorescence; bottom row: corresponding color-coded fluorescence intensity of $His_{29}GFP$. (B) Corresponding Flow cytometry results of cellular uptake of $His_{29}GFP$ in (A). Cells were treated with $His_{29}GFP$ (200 nM) in serum free medium at pH 6.5, pH 7.4, or pH modulated from 7.4 to 6.5 during incubation. Cells were incubated with $His_{29}GFP$ at each pH for 40 min at 37 °C. Scale bar is 20 μm.

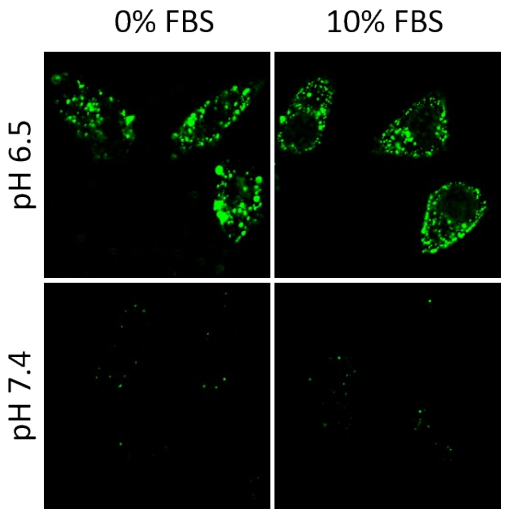

Fig. S5 The effect of serum on His_{29}GFP-RNase A internalization. HeLa cells in 1640 medium with 0% or 10% FBS were incubated with His_{29}GFP-RNase A (200 nM) for 40 min at 37° C. Cells were washed three times with 20 U/mL heparin in PBS to remove membrane-bound protein prior to image analysis.

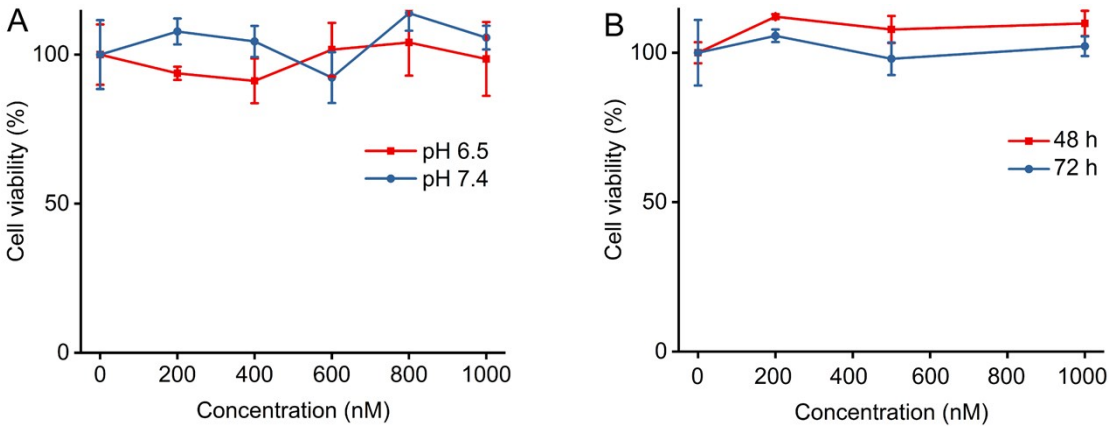

Fig. S6 Viability of Hela cells after exposure to His_{29}GFP. (A) Cells were incubated with His_{29}GFP in serum free medium (pH 7.4 or pH 6.5) for 40 min at 37 °C, washed three times with 20 U/mL heparin in PBS, and further cultured in full medium for 24 h at 37 °C before viability detection. (B) Cells were incubated with His_{29}GFP in full medium for 48 h or 72 h at 37 °C before viability detection. Cell viability was determined by MTT assay. Data points represent mean ± SD (n = 3).

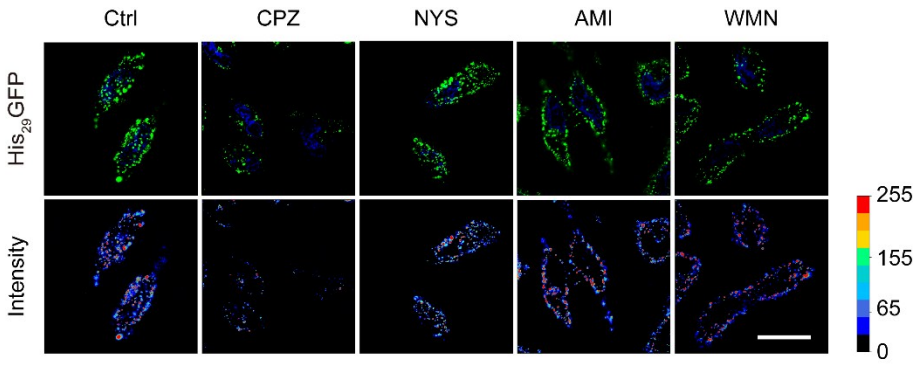

Fig. S7 Endocytic Pathway Probes. Hela cells were pretreated for 1 h with the indicated inhibitor prior to 40 min treatment with $His_{29}GFP$ (200 nM) in the continued presence of inhibitor, washed three times with 20 U/mL heparin in PBS. Cells were imaged in prewarmed PBS solution at 37 °C on confocal laser scanning microscope. Top row: GFP fluorescence; bottom row: corresponding color-coded fluorescence intensity of $His_{29}GFP$ analyzed by Python. Scale bar is 40 μm.

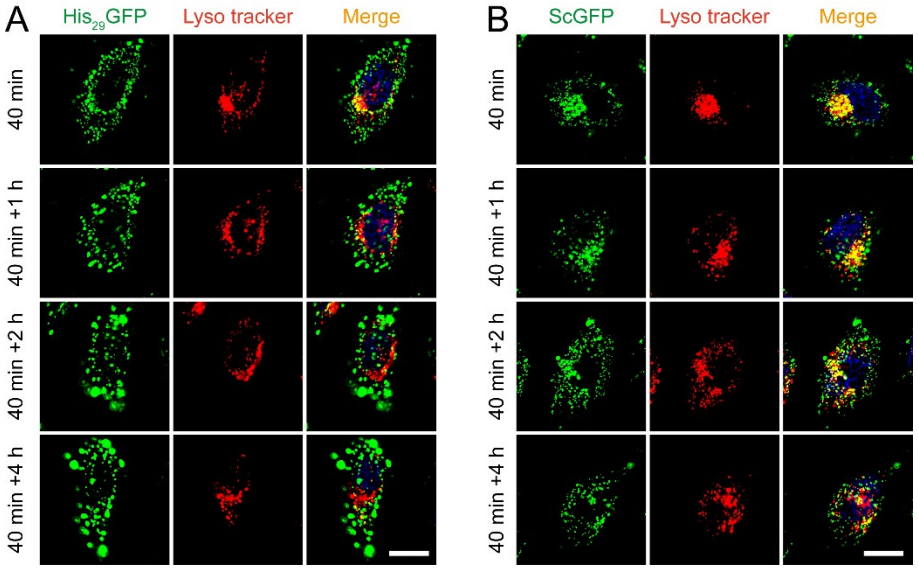

Fig. S8 Endosomal escape of $His_{29}GFP$ (A) and ScGFP (B). Cells were treated with $His_{29}GFP$ or ScGFP (200 nM) in serum free medium for 40 min at 37 °C, washed three times with 20 U/mL heparin in PBS, and subsequently incubated with full medium for an additional 1, 2, or 4 h at 37 °C. Late endosomes and lysosomes were stained by LysoTracker Red. Cell nucleus was labeled with Hoechst 33258. Cells were imaged on confocal laser scanning microscope. Red: LysoTracker Red; green: GFP; yellow: colocalization of red and green pixels; blue: Hoechst 33258. Scale bar is 20 μm.

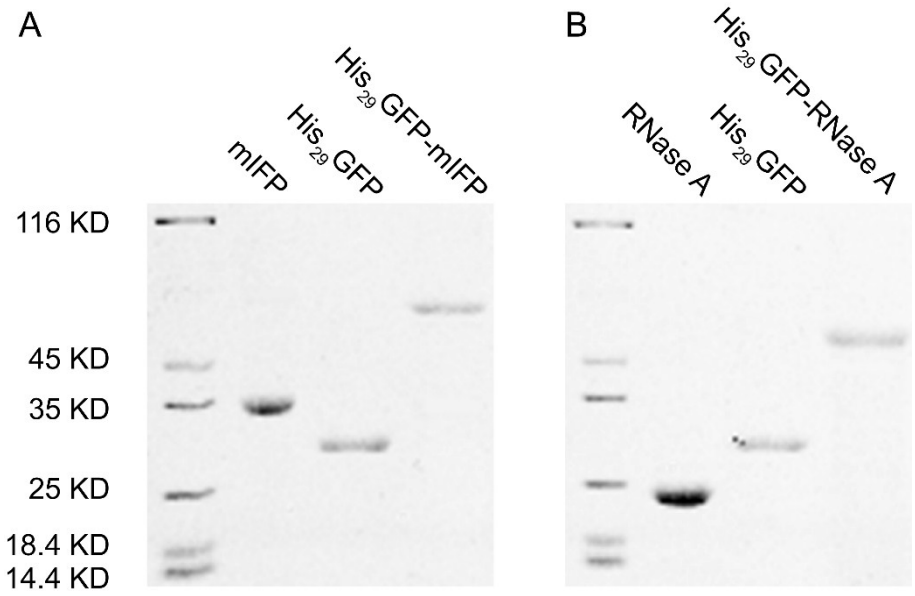

Fig. S9 12% SDS-PAGE of His_{29}GFP-mIFP (A) and His_{29}GFP-RNase A (B).

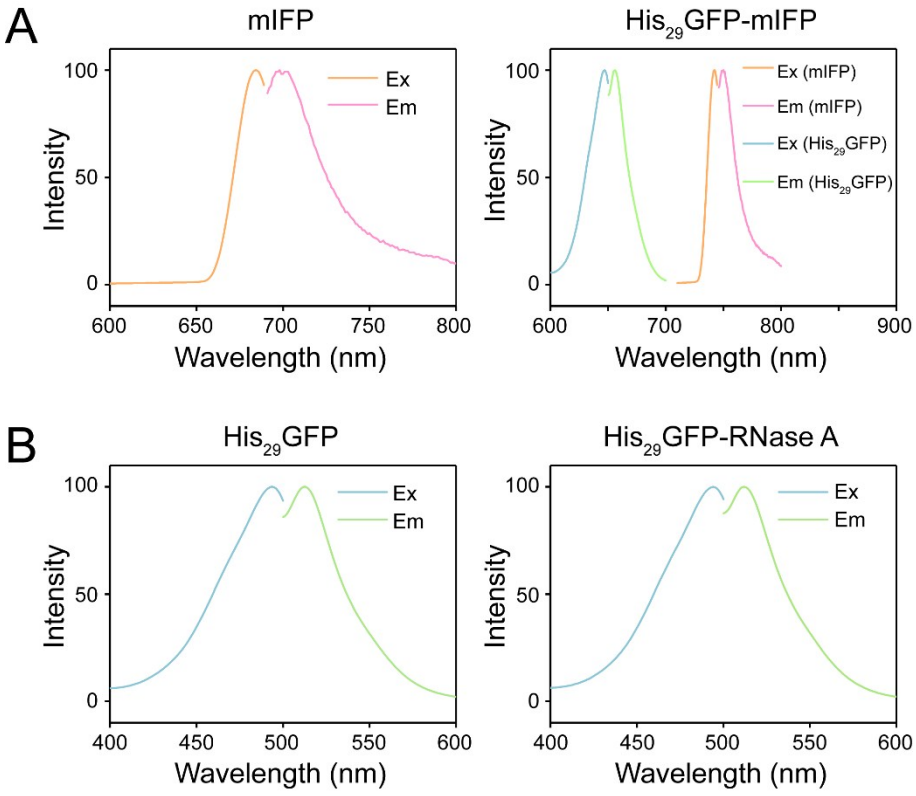

Fig. S10 Excitation and emission spectra of mIFP and His_{29}GFP-mIFP (A), His_{29}GFP and His_{29}GFP-RNase A (B).

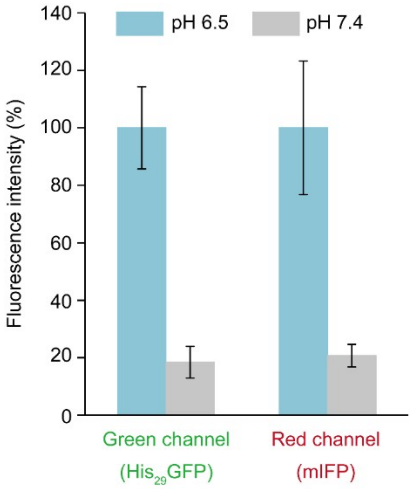

Fig. S11 Corresponding semi-quantitative results of His_{29}GFP-mIFP intensity in Figure 3B. HeLa cells incubated with His_{29}GFP-mIFP or mIFP (200 nM) in serum free medium (pH 6.5 or pH 7.4) for 40 min at 37 °C, washed three times with 20 U/mL heparin in PBS. Semi-quantitative results were obteined by analyzing fluorescence images with Image J software. Data points represent mean ± SD (n = 10).

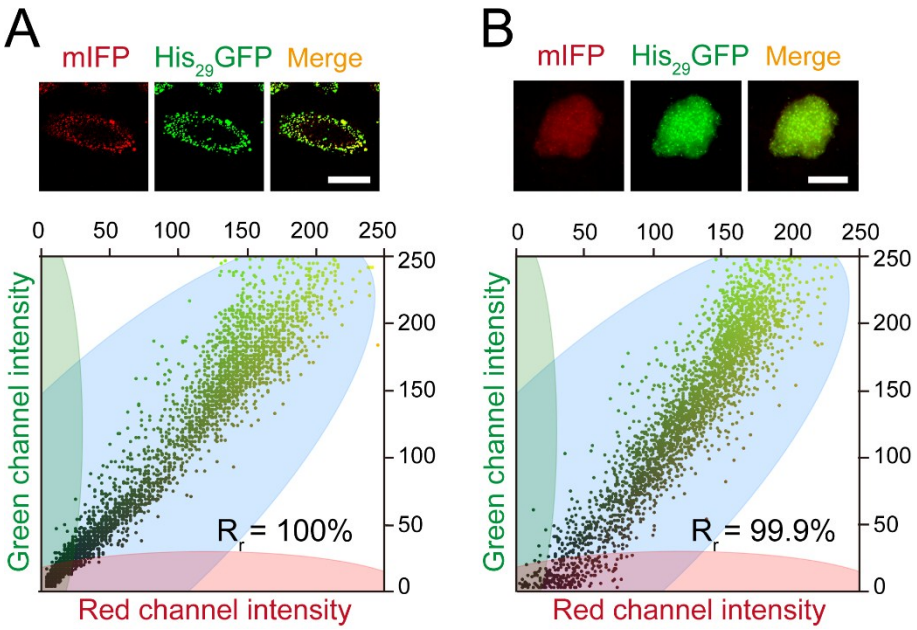

Fig. S12 (A) Fluorescence images (top) and fluorescence distribution of each pixel (bottom) of Hela cells treated with His_{29}GFP-mIFP (200 nM) in serum free medium (pH 6.5) for 40 min at 37 °C. Scale bar is 20 μm. (B) Fluorescence images (top) and fluorescence distribution of each pixel (bottom) of 3D tumor spheroid model treated with His_{29}GFP-mIFP (200 nM) in serum free medium (pH 6.5) for 4 h at 37 °C. Scale bar is 400 μm. Fluorescence distribution of each pixel was analyzed by Python.

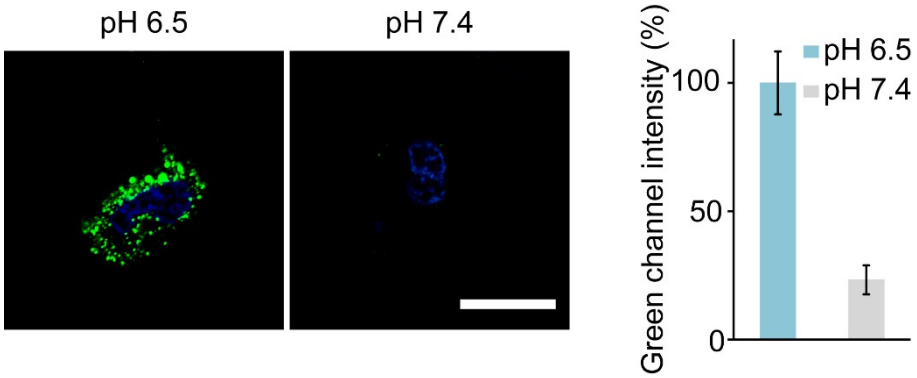

Fig. S13 CLSM images and corresponding semi-quantitative results of HeLa cells incubated with His_{29}GFP-RNase A. Hela cells were incubated with His_{29}GFP-RNase A (200 nM) in serum free medium (pH 6.5 or pH 7.4) for 40 min at 37 °C, washed three times with 20 U/mL heparin in PBS. Cells were imaged on confocal laser scanning microscope. Semi-quantitative results were obteined by analyzing fluorescence images with Image J software. The scale bar is 20 μm. Data points represent mean ± SD (n = 10).

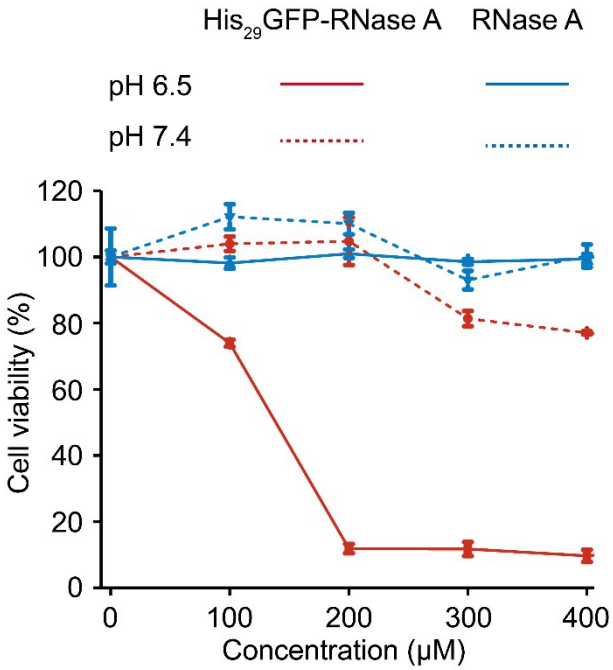

Fig. S14 Concentration-dependent cytotoxicity of His_{29}GFP-RNase A on Hela cells. Cells were treated with proteins in serum free medium (pH 6.5 or pH 7.4) for 40 min at 37 °C, washed three times with 20 U/mL heparin in PBS, and then further cultured for 24 h in full medium before detection. Cell viability was tested by MTT assay. Data points represent mean ± SD (n = 3).

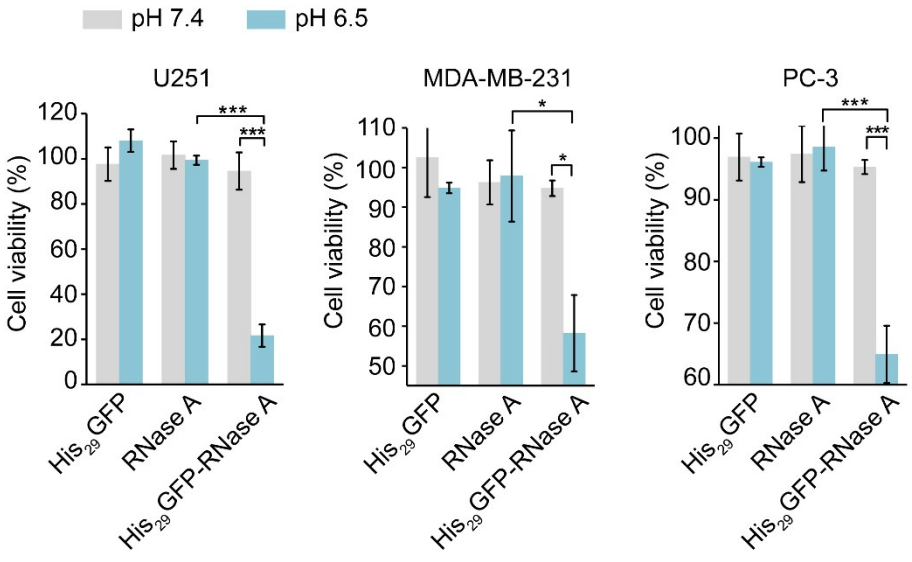

Fig. S15 Viability of U251, MDA-MB-231, PC-3 cells treated with His_{29}GFP-RNase A, His_{29}GFP and RNase A. Cells were incubated with each protein (200 nM) in serum free medium (pH 6.5 or pH 7.4) for 40 min at 37 °C, washed three times with 20 U/mL heparin in PBS, and then further cultured for 24 h in full medium before detection. Cell viability was tested by MTT assay. Data points represent mean ± SD (n = 3). *P < 0.05, ***p < 0.001.

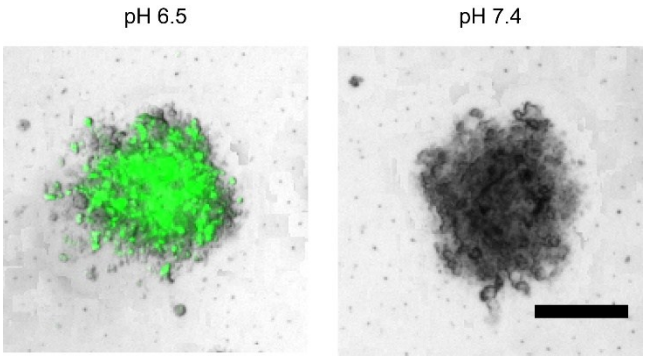

Fig. S16 Fluorescence images showing *in vitro* penetration of His_{29}GFP-RNase A in 3D tumor spheroid model of Hela cells. Cells were incubated with each His_{29}GFP-RNase A (200 nM) in serum free medium (pH 6.5 or pH 7.4) for 4 h at 37 °C, washed three times with 20 U/mL heparin in PBS. 3D tumor spheroids were imaged on cell imaging microporous plate detection system. Scale bar is 200 μm.

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