

Supporting Information

Gap-Junction-Dependent Labeling of Nascent Proteins in Multicellular Networks

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Scheme S1. Chemical synthesis of Z-Aha and Z-Hpg

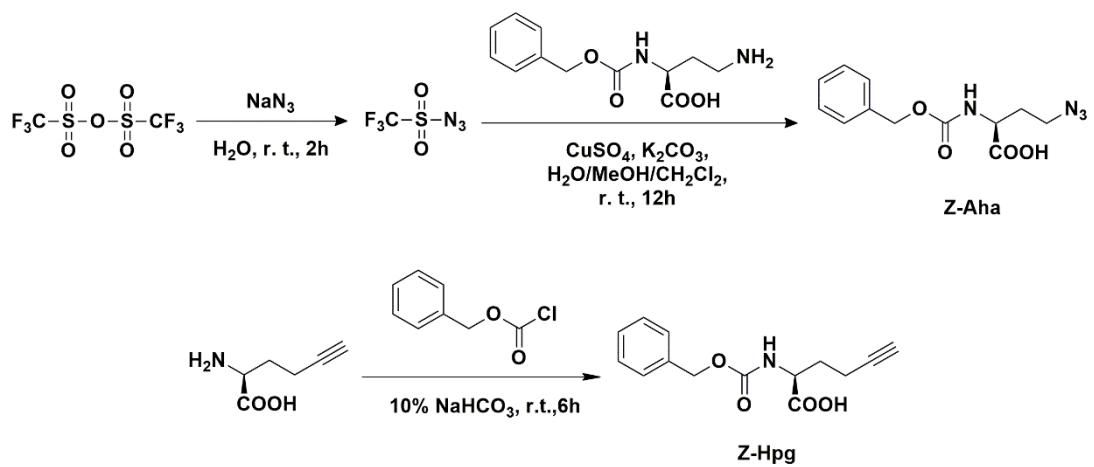


Table S1. Kinetic analysis of Z-cleaver-catalyzed conversion of Z-ncAAs to ncAAs.

| Substrate | Product | Conversion (%) | K_m (mM) | K_{cat} (s ⁻¹) | K_{cat}/K_m (mM ⁻¹ s ⁻¹) |
|-----------|---------|----------------|------------|------------------------------|---|
| Z-Aha | Aha | 69.0±3.8 | 2.51±0.37 | 5.36±0.53 | 2.16±0.33 |
| Z-Hpg | Hpg | 67.9±6.2 | 0.96±0.19 | 5.78±0.71 | 6.06±0.49 |

Values are shown as mean ± s.d. (n = 3 independent experiments).

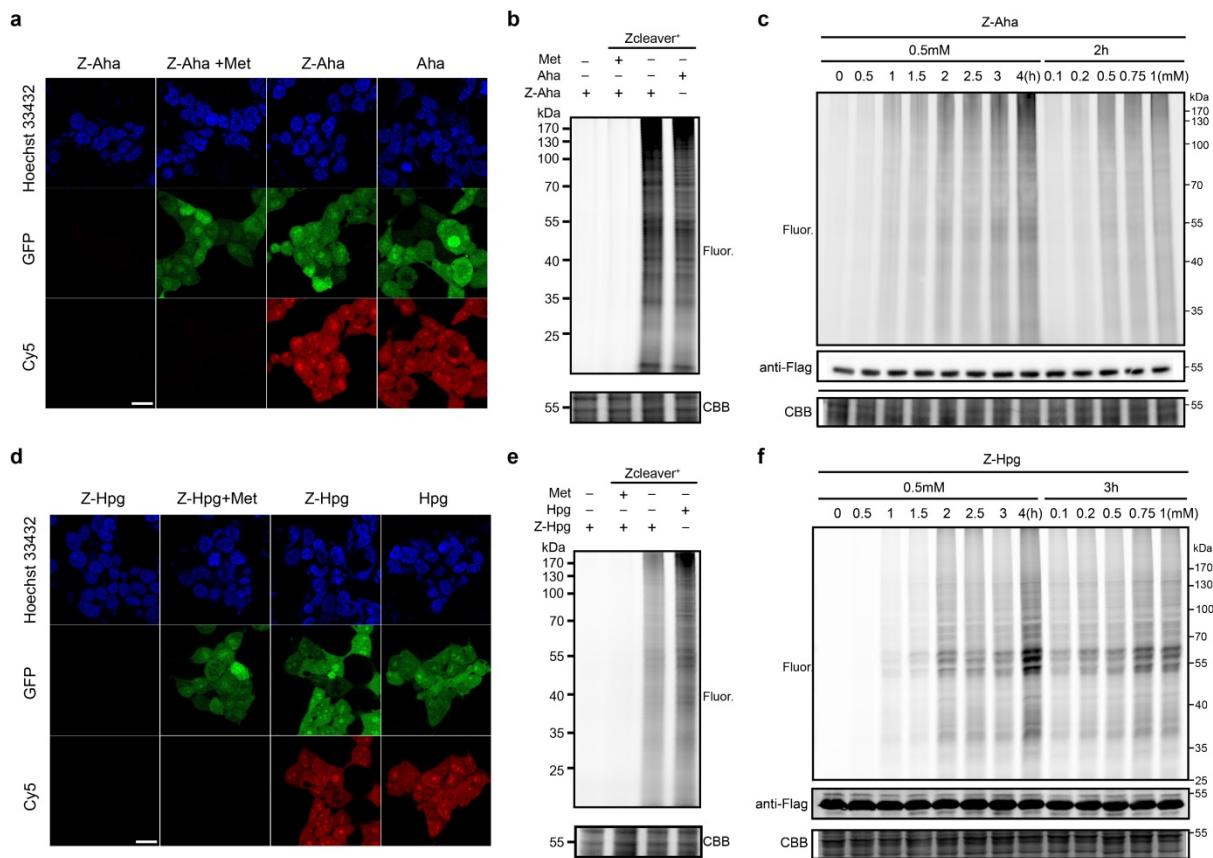


Figure S1. Conversion of Z-ncAAs to ncAAs in Zcleaver-expressing cells. (a) HEK293T cells were incubated with 0.5 mM Z-Aha, and HEK293T Zcleaver-T2A-GFP cells with 0.5 mM Met, 0.5 mM Z-Aha together with 0.5 mM Met, or 0.5 mM Aha for 4 h. The cells were then reacted with alkyne-Cy5 and stained with Hoechst 33342, followed by imaging with confocal fluorescence microscopy. (b) The cells treated with Aha or Z-Aha as in (a) were lysed, and the lysates were reacted with alkyne-Cy5 and analyzed by in-gel fluorescence scanning. (c) HEK293T Zcleaver cells were treated with 0.5 mM Z-Aha for varied durations of time or with Z-Aha at varied concentrations for 2 h, lysed, and reacted with alkyne-Cy5, and analyzed by in-gel fluorescence scanning. (d) HEK293T cells were incubated with 0.5 mM Z-Hpg, and HEK293T Zcleaver-T2A-GFP cells with 0.5 mM Met, 0.5 mM Z-Hpg together with 0.5 mM Met, or 0.5 mM Hpg for 4 h. The cells were then reacted with azide-Cy5 and stained with Hoechst 33342, followed by imaging with confocal fluorescence microscopy. (e) The cells treated with Hpg or Z-Hpg as in (d) were lysed, and the lysates were reacted with azide-AF488 and analyzed by in-gel fluorescence scanning. (f) HEK293T Zcleaver cells were treated with 0.5 mM Z-Hpg for varied durations of time or with Z-Hpg at varied concentrations for 3 h, lysed, and reacted with alkyne-Cy5, and analyzed by in-gel fluorescence scanning. In (a) and (d), scale bars, 20 μ m. In (b), (c), (e), and (f), Coomassie brilliant blue (CBB)-stained gels demonstrate equal loading. In (c) and (f), anti-Flag immunoblots represent Zcleaver expression.

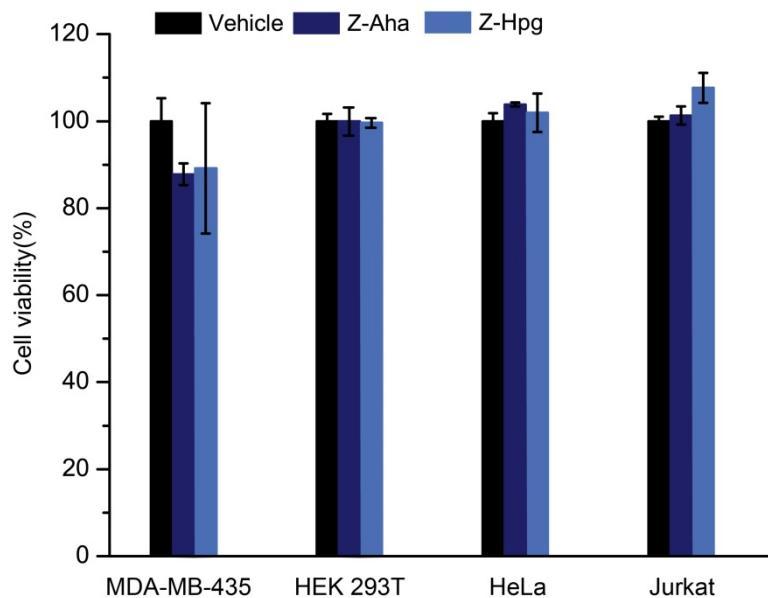


Figure S2. Cytotoxicity analysis of Z-ncAAs. MDA-MB-435, HEK293T, HeLa, and Jurkat cells were treated with 0.5 mM Z-Aha, 0.5 mM Z-Hpg, or vehicle for 4 h, followed by MTS assay. Error bars represent mean \pm s.d. (n = 3 independent experiments).

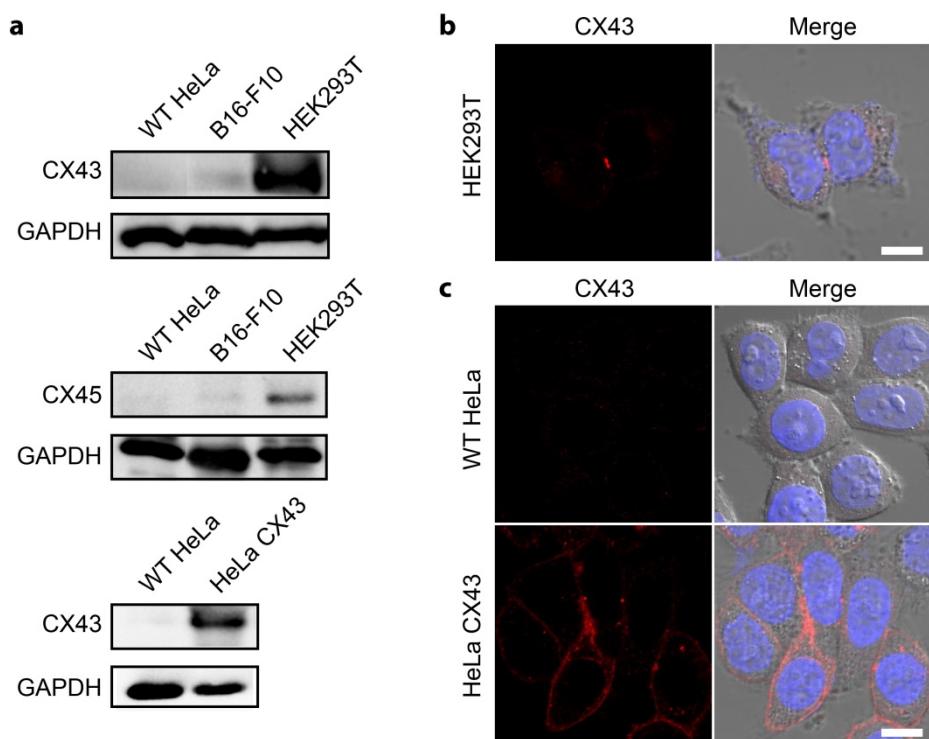


Figure S3. Expression level of connexins. (a) Immunoblotting analysis of CX43 and CX45 in WT HeLa, B16-F10, HEK293T, and HeLa CX43 cells. Immunoblotting of GAPDH demonstrates comparable loading. (b) Confocal fluorescence imaging of gap-junction-connected HEK293T cells. (c) Immunofluorescence imaging of CX43 expression in WT HeLa and HeLa CX43 cells. In (b) and (c) CX43 was stained with anti-CX43 antibody followed by a Alexa Fluor 555-conjugated secondary antibody (red signal) and Hoechst 33342 (nuclei; blue signal). Scale bar, 10 μ m.

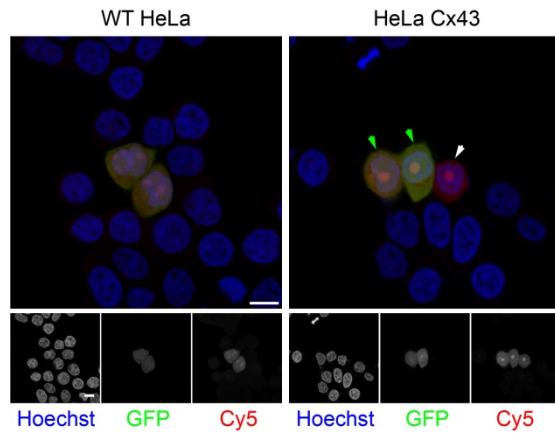


Figure S4. BORTAC-GJ labeling of gap-junction-connected cell networks composing of HeLa CX43 cells and Zcleaver-expressing HeLa CX43 cells. WT HeLa cells were cocultured with HeLa cells transfected with pZcleaver-IRES-GFP (left panel). HeLa CX43 cells were cocultured with HeLa CX43 cells transfected with pZcleaver-IRES-GFP (right panel). The cocultures were treated with 1 mM Z-Aha for 2 h, followed by reaction with alkyne-Cy5 and confocal fluorescence microscopy. Arrows highlight the HeLa CX43 cells (white arrows) connected with Zcleaver-expressing HeLa CX43 cells (green arrows), which were labeled by Z-Aha (Cy5). Scale bars, 10 μ m.

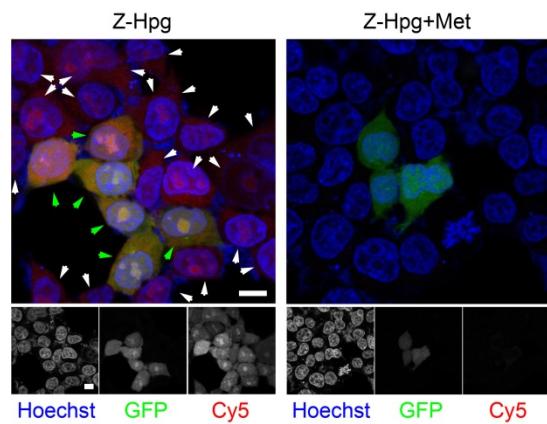


Figure S5. BORTAC-GJ labeling of the gap-junction-connected cell network by Z-Hpg. Co-culture of HEK293T Zcleaver-T2A-GFP and WT HEK293T cells were incubated with 0.5 mM Z-Hpg or 0.5 mM Z-Hpg in the present of 0.5 mM Met for 3 h, followed by reaction with azide-Cy5 and staining the nuclei with Hoechst 33342. Arrows highlight the network of WT HEK293T cells (white arrows) connected with HEK293T Zcleaver-T2A-GFP cells (green arrows), which were labeled by Z-Hpg (Cy5). Scale bars, 10 μ m.

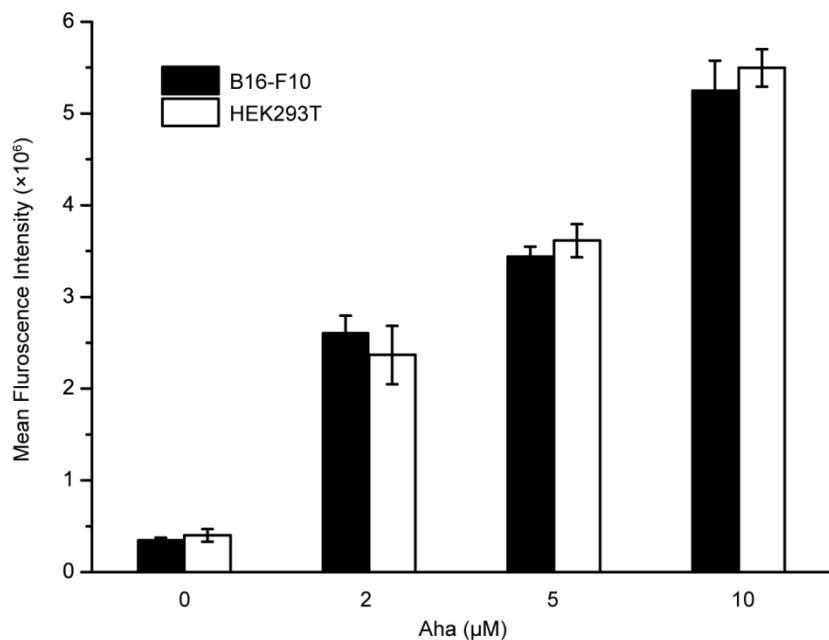


Figure S6. Evaluation of Aha metabolic incorporation efficiency in B16-F10 and HEK293T cells. HEK293T cells were labeled with the cell tracker CMFDA and cocultured with B16-F10 cells. The coculture was incubated with Aha at varied concentrations for 2 h, followed by reaction with alkyne-Cy5 and flow cytometry analysis. Error bars represent mean \pm s.d. ($n = 3$ independent experiments).

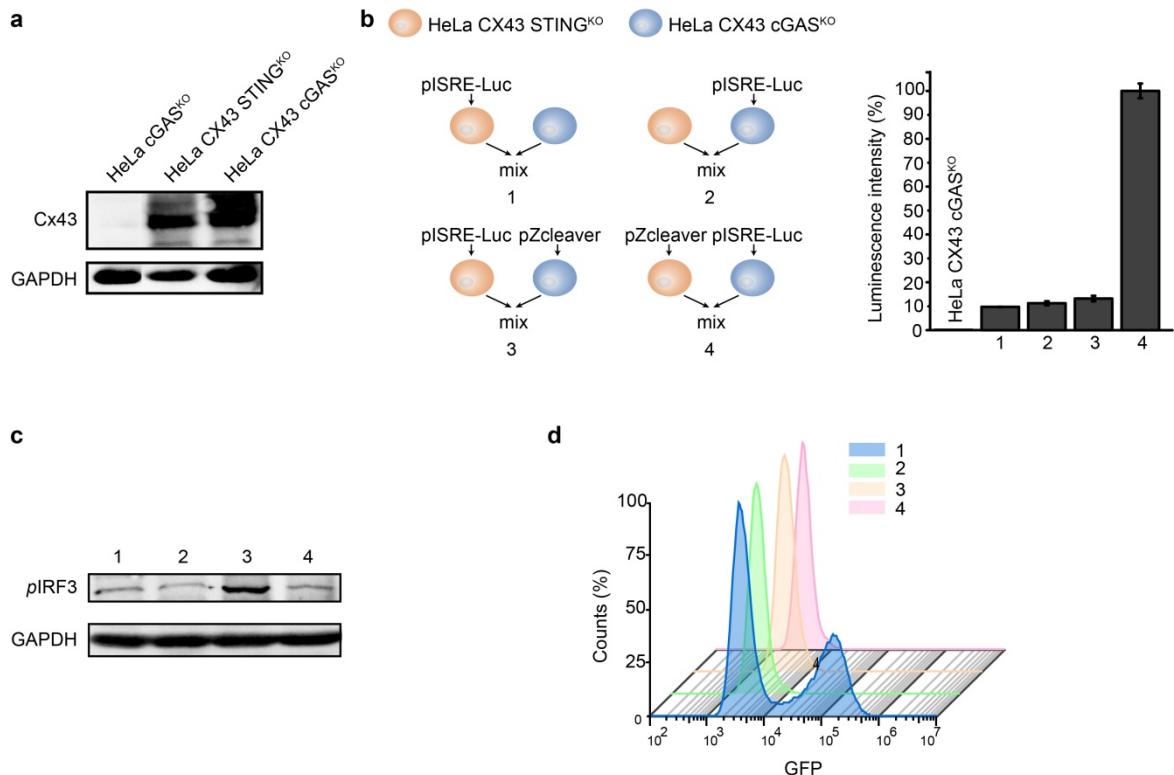


Figure S7. Bystander STING activation in HeLa CX43 cells. (a) Immunoblotting showing the CX43 expression in HeLa cGAS^{KO}, HeLa CX43 STING^{KO} and HeLa CX43 cGAS^{KO} cells. (b) HeLa CX43 STING^{KO} cells transfected with plasmid DNA encoding ISRE-luciferase (pISRE-Luc), vehicle, pISRE-luc, or Zcleaver expression plasmids (pZcleaver) were cocultured with HeLa CX43 cGAS^{KO} cells transfected with vehicle, pISRE-luc, pZcleaver, or pISRE-Luc, respectively. STING activation was assayed by luciferase reporter assay. Error bars represent mean \pm s.d. ($n = 3$ independent experiments). (c) HeLa CX43 STING^{KO} cells transfected with pZcleaver were mixed with HeLa CX43 cGAS^{KO} cells (Lane 3). HeLa CX43 cGAS^{KO} cells (Lane 1), HeLa CX43 STING^{KO} cells transfected with pZcleaver (Lane 2), and HeLa CX43 STING^{KO} cells transfected with pZcleaver mixed with HeLa CX43 cGAS^{KO} cells in the presence of the CBX inhibition (Lane 4) were shown as negative controls. Transactivation of STING was assayed by phosphorylation of IRF3 using immunoblotting. Immunoblotting of GAPDH demonstrates comparable loading. (d) HeLa CX43 cGAS^{KO} cells containing a stable ISRE-GFP plasmid (HeLa reporter cells) were cocultured with HeLa CX43 STING^{KO} cells transfected with pZcleaver (without fluorescent protein gene) (curve 1). HeLa reporter cells mixed with HeLa CX43 STING^{KO} cells (curve 2), HeLa CX43 STING^{KO} cells transfected with pZcleaver (curve 3) alone, and HeLa reporter cells alone (curve 4) were shown as negative controls. Transactivation of STING in reporter cells was assayed by the GFP expression using flow cytometry.

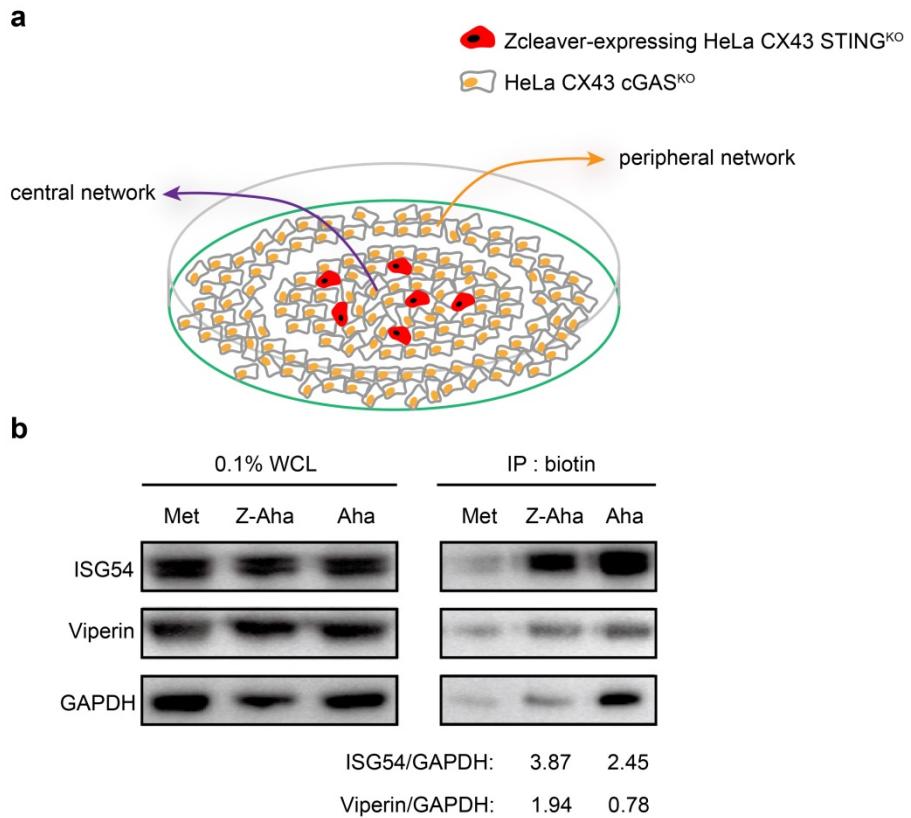


Figure S8. BORTAC-GJ labeling in heterogeneous cell networks. (a) Cells in culture dish were separated into two networks: the central network consisting of interconnected HeLa CX43 STING^{KO} cells transfected with the Zcleaver plasmid DNA and HeLa CX43 cGAS^{KO} cells at the 1:6 ratio; the peripheral network containing connected HeLa CX43 cGAS^{KO} cells. (b) The coculture was treated with 1 mM Met, 1.5 mM Z-Aha or 5 μ M Aha for 3 h, lysed, reacted with alkyne-biotin, followed by enrichment with streptavidin beads and immunoblotting analysis. Numbers below the gels show the enrichment ratio of ISG54 and Viperin using GAPDH as the internal control. WCL, whole cell lysate. Representative results are shown from three independent experiments.

Experimental Materials and Methods

Materials. Aha¹ and BTTAA² were synthesized with high purity as previously reported. Methionine and carbenoxolone disodium salt were purchased from Sigma-Aldrich. Hpg was obtained from Okeanos Tech. Co. Ltd. (Beijing, China). Cell Tracker CMFDA was purchased from Thermo Fisher Scientific. Alkyne-Cy5, azide-Cy5, azide-AF488 and alkyne-PEG4-biotin were purchased from Click Chemistry Tools. The following antibodies were used: mouse monoclonal anti-FLAG antibody (MBL), rabbit polyclonal anti-Connexin 43 (CX43) antibody (Abcam), rabbit polyclonal anti-Connexin 45 (CX45) antibody (Immunoway), rabbit polyclonal anti-phospho-IRF3 antibody (Immunoway), rabbit polyclonal anti-IFIT2 (ISG54) antibody (Immunoway), rabbit polyclonal anti-RSAD2 (Viperin) antibody (Immunoway), Alexa Fluor 555-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific), and streptavidin-conjugated agarose beads (Thermo Fisher Scientific).

Plasmids. The *Zcleaver* gene³ was ordered from Genewiz Inc. (Suzhou, China) and cloned into the pmCherry-N1, pIRES2-GFP and pcDNA3.1/*myc*-His(-)A vectors for expression in mammalian cells and the pQE-80L vector for expression in *E. coli*. The pISRE-Luc plasmid was purchased from Beyotime Company (Beijing, China) and the luciferase fragment was replaced by the *gfp* gene to generate the pISRE-GFP plasmid.

Cell culture. All cells were maintained at 37 °C and 5% CO₂. HEK293T, B16-F10, and HeLa cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. HeLa STING^{KO} and HeLa cGAS^{KO} cells, a kind gift from Prof. Zhengfan Jiang at Peking University, were cultured as WT HeLa cells. HeLa reporter cells were cultured in DMEM supplemented with 10% FBS, 600 µg/mL geneticin, 100 units/mL penicillin and 100 µg/mL streptomycin. Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate. MDA-MB-435 cells were cultured in Leibovitz's L-15 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Metabolic labeling. For Z-ncAAs or ncAAs metabolic labeling, cells were incubated in SFM medium (DMEM medium without methionine and with 1mg/mL bovine serum albumin) for 30 min. After depletion, the Z-ncAA or ncAA at the indicated concentration was added to the medium for an indicated duration of time.

Transfection and Lentiviral transduction. HEK293T cells stably expressing *Zcleaver* with a T2A linker (HEK293T *Zcleaver*-T2A-GFP and HEK293T *Zcleaver*-T2A-mCherry cells) and HeLa cells stably expressing CX43 (HeLa CX43, HeLa CX43 STING^{KO} and HeLa CX43 cGAS^{KO} cells) were generated by lentiviral transduction. HeLa reporter cell line was selected with gentamicin after transfected with the ISRE-GFP plasmid.

MTS assay. Cells were cultured a flat bottomed 96-well plate for 24 h and then treated with indicated compounds for 4 h, followed by adding the CellTiter 96[®] Aqueous One Solution Reagent (Promega) according to the manufacturer's instructions. After incubation for 2 h, the cell viability was determined by measuring 490 nm absorbance using a microplate reader.

Kinetic parameters of *Zcleaver*. *Zcleaver* (5 µg/mL) enzyme was incubated with Z-ncAAs at varied

concentrations at 37 °C for an indicated duration of time, followed by quenching the reactions with 1 M HCl. After dilution of the mixtures with Mill-Q water, 0.2 mM internal reference (such as Met) was added. Aliquots were taken and analyzed by LC-MS on an Waters ACQUITY UPLC I-Class SQD 2 system. The concentrations of ncAAs were determined from standard curves using the internal standard method. The conversion yields of Z-ncAAs by Zcleaver were measured using 30 µg/mL Zcleaver for a 30-min reaction.

In-gel fluorescence scanning. For mammalian cells, cell pellets were re-suspended in RIPA lysis buffer (1% Nonidet P 40, 1% sodium deoxycholate, 0.1% SDS, 50 mM triethanolamine pH 7.4, 150 mM NaCl) containing protease inhibitor cocktail (Complete ETDA-free, Roche, 1 tablet/50 mL). Cells were lysed by pipetting and agitation. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce) and normalized to 1 mg/ mL with lysis buffer. Samples were then reacted with 100 µM alkyne-Cy5, azide-Cy5 or azide-AF488, 500 µM CuSO₄, 1 mM BTTAA and 2.5 mM freshly prepared sodium ascorbate. The samples were vortexed on an IKA MS3 shaker for 2 h at r.t. The reacted samples were resolved on 10% or 12% SDS-PAGE gels and analyzed on a Typhoon FLA 9500 laser scanner (GE Healthcare).

Fluorescence Imaging. After metabolic labeling, cells were rinsed three times with PBS, fixed with 3.7% paraformaldehyde for 10 min, and rinsed three times with PBS. Cells were then permeabilized with 0.1% Triton-X100 in PBS for 15 min and rinsed for three times with PBS, followed by adding of PBS containing 40 µM alkyne-Cy5 or azide-Cy5, 2.5 mM sodium ascorbate, and BTTAA-CuSO₄ complex (200 µM CuSO₄, BTTAA:CuSO₄ in a 2:1 molar ratio) at r.t. After incubation for 20 min, the cells were washed four times with 1% Tween 20 in PBS, once with PBS, and stained with Hoechst 33342, followed by imaging on a ZEISS LSM 700 laser scanning confocal microscope.

Inhibition of gap junctions by carbenoxolone. HEK293T Zcleaver cells were cocultured with WT HEK293T overnight. Cocultured cells were changed into fresh SFM medium with 0.1 mM carbenoxolone for 30 min. The medium was then replaced by SFM containing 0.5 mM Z-Aha and 0.1mM carbenoxolone for 2 h.

Cell stimulation and transient transfection. Stimulation of HeLa CX43 STING^{KO} cells was performed by transient transfection with the pZcleaver-T2A-mCherry plasmid using X-tremeGENE (Roche) according to the manufacturer's instructions. After incubation for 48 h, DNA-stimulated and Zcleaver-expressing HeLa CX43 STING^{KO} cells were mixed with HeLa CX43 cGAS^{KO} cells for indicated durations of time, followed by imaging analysis or enrichment experiments.

Luciferase reporter assays. Fire-Lucy Assay Kit (Vigorous Biotechnology, Beijing, China) was used to assess the transactivation of the transiently expressed ISRE-Luc construct. Cells were lysed in Universal Lysis Buffer for 10 min at r.t. The cell lysates were incubated with the firefly luciferase substrate. Relative bioluminescence unit was measured by Synergy 4 Hybrid Microplate Reader with an integration time of 5 s .

Affinity enrichment of labeled proteins. The coculture of Zcleaver-expressing HeLa CX43 STING^{KO} and HeLa CX43 cGAS^{KO} cells in culture dish was incubated for 24 h, treated with Met, Z-Aha or Aha in SFM for indicated durations of time. Cells were washed with PBS and detached using 0.25% trypsin in EDTA. Cells were collected by centrifugation and lysed with RIPA buffer. After BCA determination and normalization, proteins were reacted with alkyne-biotin via CuAAC (100 µM alkyne-biotin, 2.5 mM sodium ascorbate, and BTTAA-CuSO₄ complex (500 µM CuSO₄,

BTTAA:CuSO₄ in a 2:1 molar ratio)) for 2 h at r.t. The proteins were precipitated by adding ice-cold methanol in a volume of 1:8 (sample : methanol) at -80°C overnight and the excess alkyne-biotin was removed. After washing twice with ice-cold methanol, the protein pellets were redissolved in 1.2 % SDS in PBS buffer. After diluting with 5-fold volume of PBS, streptavidin-agarose resins (Pierce) were added to the protein solution and incubated for 3 h at r.t. The resins were washed sequentially with 2% SDS in PBS, 8 M urea in PBS, 2.5 M sodium chloride in PBS, PBS for three times and water for three times.

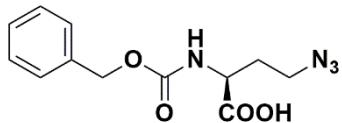
Construction of HeLa CX43 heterogeneous cell networks. Agarose hydrogels (1% w/v in Mill-Q water) were set into 10cm-diameter culture dishes and cut into ring-like slices with ~7 cm diameter and 1~2 mm width. HeLa CX43 STING^{KO} transfected with pZleaver-T2A-mCherry and HeLa CX43 cGAS^{KO} cells were added into the central space at an 1:6 ratio, and the same number of HeLa CX43 cGAS^{KO} cells were added in the peripheral space. After the cells adhered, the separating gels were removed.

Immunoblotting. Samples were resolved on 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore). After incubation for 1 h at r.t. in blocking buffer (5% non-fat milk in 1 × TBST (Tris buffered saline with 0.1% Tween-20, pH 8)), the membranes was incubated with anti-FLAG, anti-phospho-IRF3, anti-CX43, anti-CX45, anti-IFIT2, or anti-RSAD2, followed by the secondary antibody anti-rabbit-IgG-HRP or anti-mouse-IgG-HRP. For GAPDH detection, the membrane was incubated with anti-GAPDH-HRP.

Statistical analysis. Results are expressed as mean ± s.d. or mean ± s.e.m.

Chemical synthesis

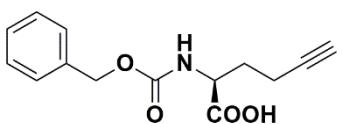
Synthesis of Z-Aha



Triflic azide was synthesized as previously described¹. To a solution of sodium azide (2.63 g, 40 mmol) in 7 mL water placed in an ice bath, 1.3 ml Tf₂O was dropwise added. The resulting mixture was stirred at r.t. for 2 h, followed by extracting triflic azide with methylene chloride.

1.02 g (4 mmol) Cbz-L-2,4-diaminobutyric acid, 0.84 g (6 mmol) K₂CO₃ and a catalytic amount (10 mg, 0.04 mmol) CuSO₄ were dissolved in 13 ml water and 26 ml methanol. Triflic azide extract was added dropwise into the mixture and reacted for 12 h at r.t.. After removing methanol and methylene chloride by rotary evaporation, the aqueous solution was extracted with ethyl acetate after the pH was adjusted to 3. The residue was purified by preparative HPLC chromatography to give the final product (0.40 g, 36% Yield). ¹H NMR (500 MHz, CD₃OD) δ 1.86-1.93 (m, 1H), 2.08-2.14 (m, 1H), 3.36-3.47 (dtd, 2H), 4.28 (dd, 1H), 5.04-5.13 (m, 2H), 7.23-7.41 (m, 5H). ¹³C NMR (125 MHz, CDCl₃) δ 31.36, 47.67, 51.67, 67.43, 128.21, 128.38, 128.62, 135.90, 156.14, 175.83. HRMS (ESI): Calcd for C₁₂H₁₃N₄O₄ [M-H]⁻: 277.10150, found 277.093131.

Synthesis of Z-Hpg

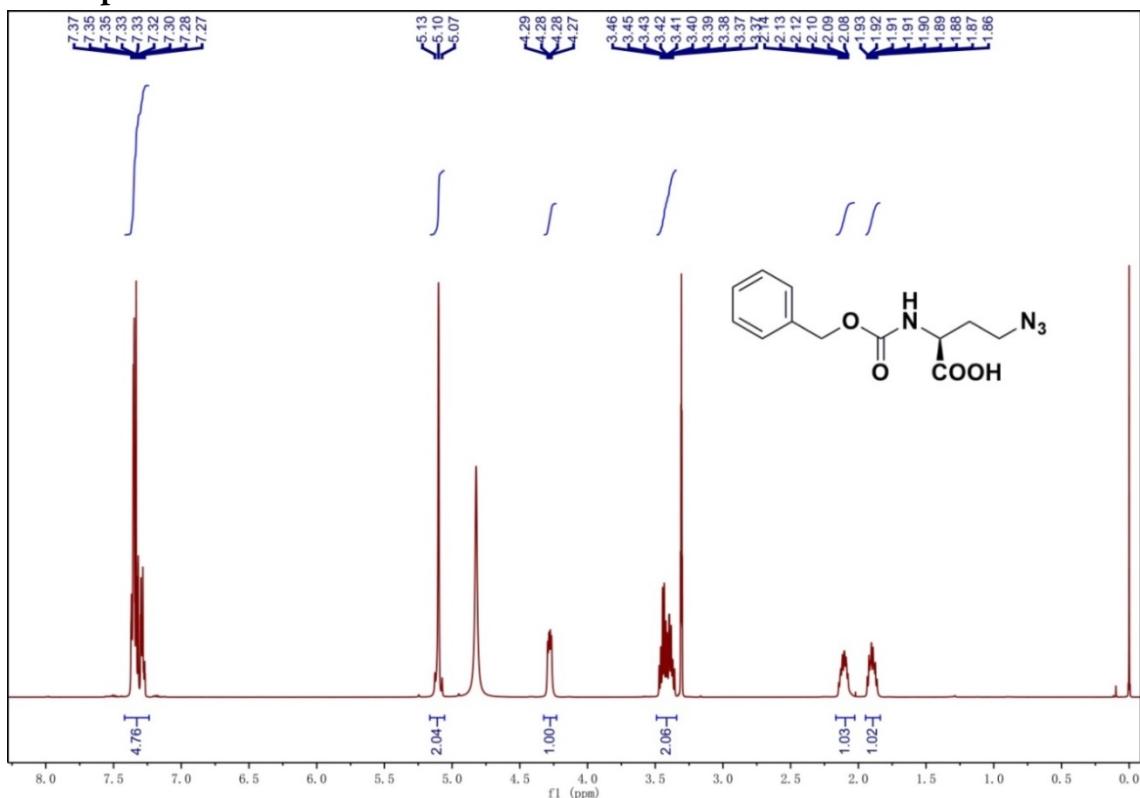


To a 10% NaHCO₃ aqueous solution, were added 0.40 g Hpg (3.1 mmol) and 0.70 g carbobenzoxy chloride (4.1 mmol). After reaction at r.t. for 6h, the mixture was extracted with ethyl acetate after the pH was adjusted to 6, washed by saturated NaCl solution, and purified by

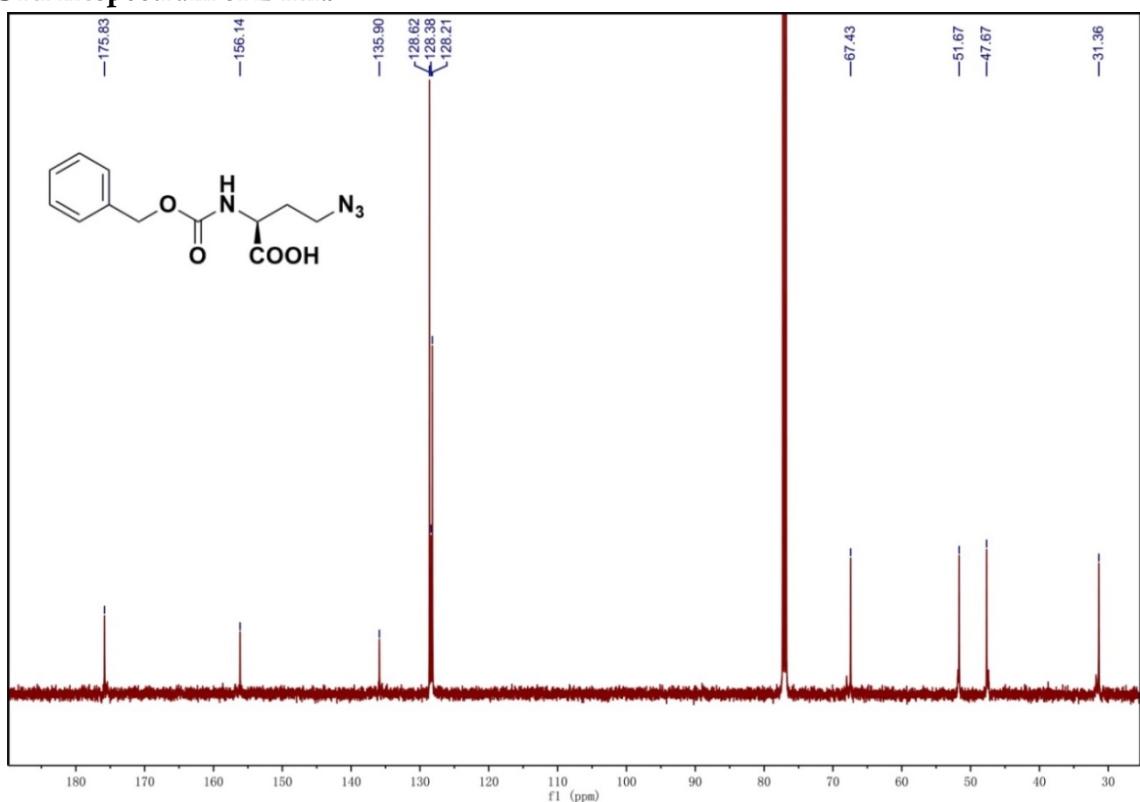
preparative HPLC chromatography to give the final product (0.21 g, 26% Yield). ^1H NMR (500 MHz, CD_3OD) δ 1.82-1.89 (m, 1H), 2.02-2.10 (m, 1H), 2.25 (t, 1H), 2.27-2.31 (m, 2H), 4.29 (dd, 1H), 5.09 (s, 2H), 7.26-7.37 (m, 5H). ^{13}C NMR (125 MHz, CD_3OD) δ 14.60, 30.38, 53.00, 66.26, 69.06, 82.08, 127.37, 127.58, 128.05, 136.80, 157.34, 174.11. HRMS (ESI): Calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_4$ [M-H] $^-$ 260.10011, found 260.09244.

Spectra of chemical compounds

^1H NMR spectrum of Z-Aha



^{13}C NMR spectrum of Z-Aha

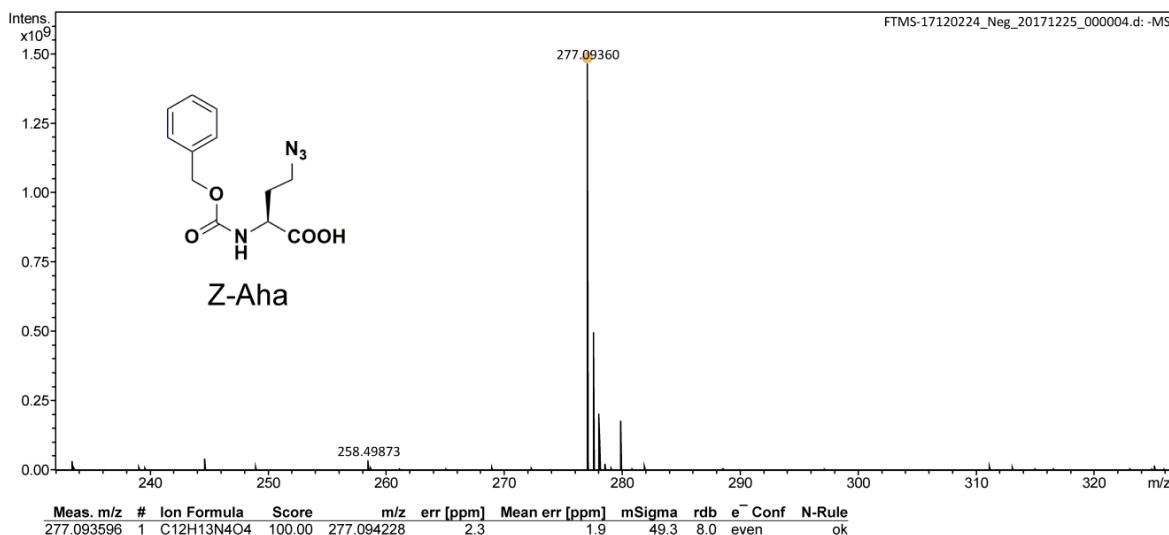


HRMS of Z-Aha

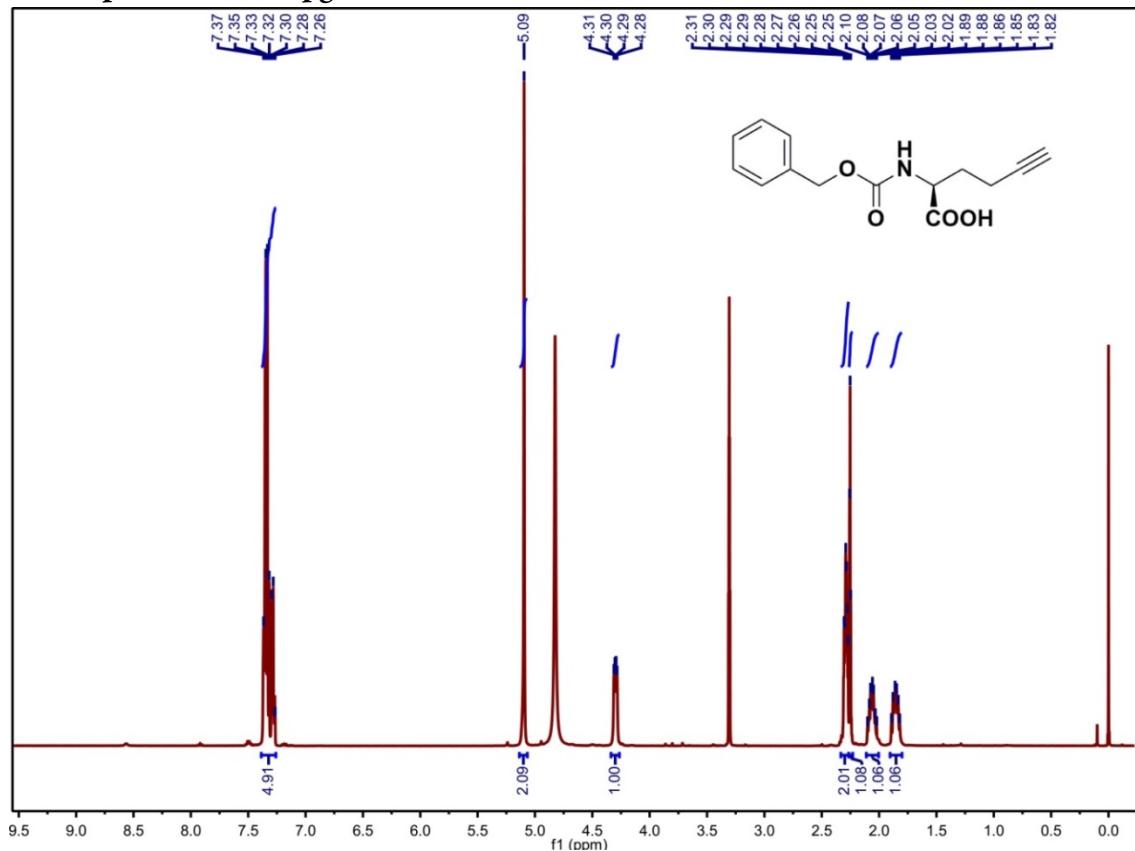
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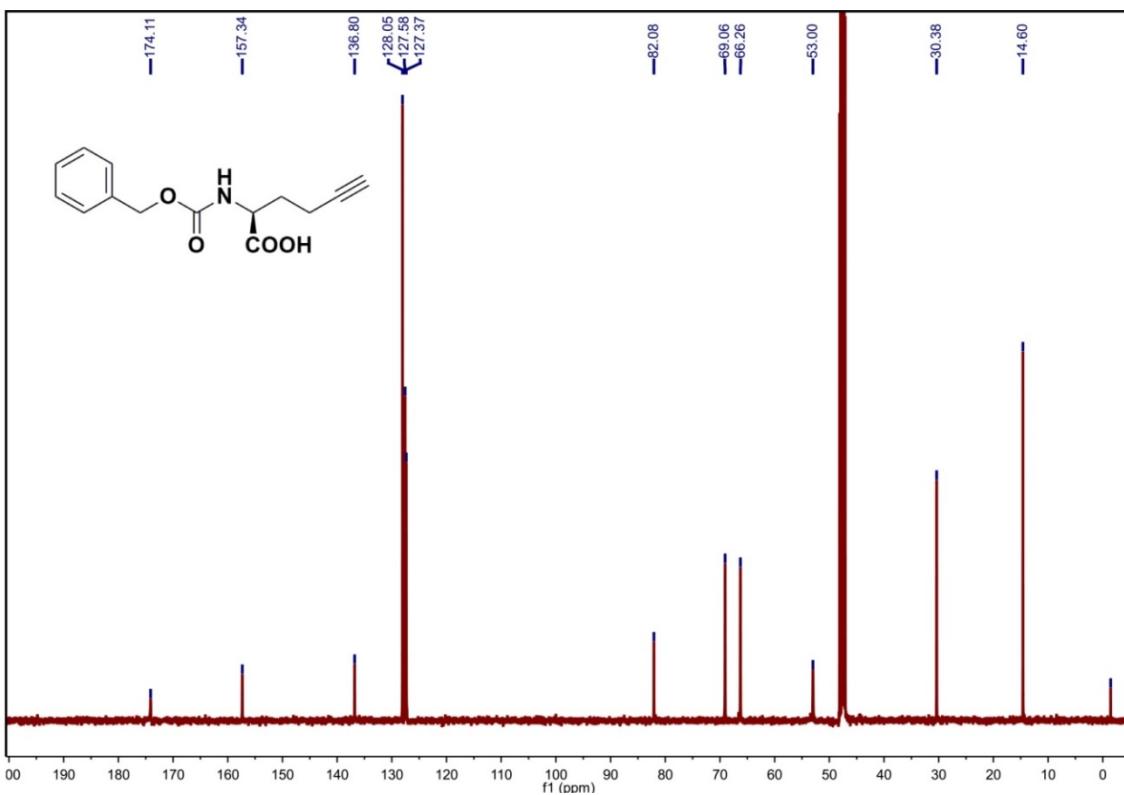
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¹H NMR spectrum of Z-Hpg



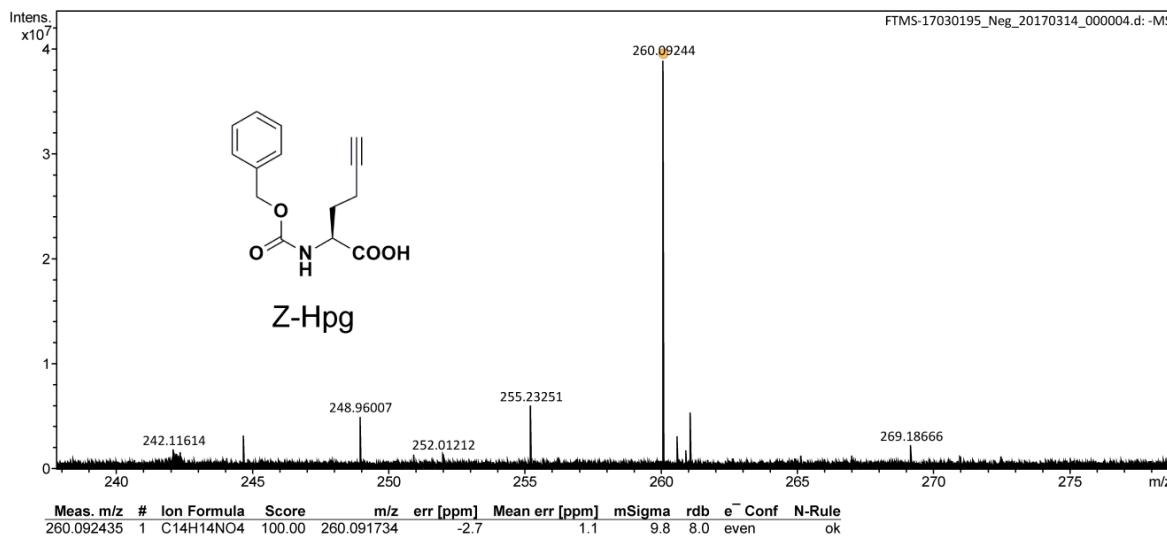
¹³C NMR spectrum of Z-Hpg



HRMS of Z-Hpg

Analysis Info

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| Sample | cbz-Hpg | Instrument | Bruker Solarix XR FTMS |
| Comment | | Operator | Peking University |



Supporting Reference

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- (3) Gauthier, N. P., Soufi, B., Walkowicz, W. E., Pedicord, V. A., Mavrakis, K. J., Macek, B., Gin, D. Y., Sander, C., and Miller, M. L. (2013) Cell-selective labeling using amino acid precursors for proteomic studies of multicellular environments. *Nat. Methods.* 10, 768-773.

List of abbreviations

| | |
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| STING | stimulator of interferon genes |
| cGAMP | cyclic GMP-AMP |
| ncAA | noncanonical amino acid |
| Z-ncAA | <i>N</i> -carbobenzyloxy-protected ncAA |
| WT HEK293T | wild-type HEK293T cells |
| HEK293T Zcleaver | HEK293T cells stably expressing Zcleaver |
| HEK293T Zcleaver-T2A-mCherry | HEK293T cells stably expressing Zcleaver-T2A-mCherry |
| HeLa CX43 | HeLa cells stably expressing CX43 |
| cGAS | cGAMP synthase |
| IFN | interferon |
| ISG | interferon-stimulated gene |
| HeLa cGAS ^{KO} | cGAS deficient HeLa cells |
| HeLa STING ^{KO} | STING deficient HeLa cells |
| HeLa CX43 cGAS ^{KO} | HeLa cGAS ^{KO} cells stably expressing CX43 |
| HeLa CX43 STING ^{KO} | HeLa STING ^{KO} cells stably expressing CX43 |
| HeLa reporter cells | HeLa CX43 cGAS ^{KO} cells containing a stable IFN-stimulated response element-GFP (ISRE-GFP) reporter plasmid |
| Zcleaver-expressing HeLa CX43 STING ^{KO} | HeLa CX43 STING ^{KO} cells transiently expressing Zcleaver |