Supporting Information for

Organelle-Specific Zinc Detection Using Zinpyr-Labeled Fusion Proteins in Live Cells

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Materials and Methods. *O*^{6}-(4-Aminomethyl-benzyl)guanine[1] and ZP1(6-CO_2H)[2] were prepared as previously described. *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, Applied Biosystems), anhydrous dimethylformamide (DMF, Aldrich), triethylamine (Aldrich), and deuterated methanol (Cambridge Isotope Labs) were purchased and used as received.

Reverse-phase HPLC purifications were carried out on an Agilent Technologies 1200 Series HPLC system. NMR spectra were acquired on a Varian 500 MHz spectrometer at ambient probe temperature (283 K) and referenced to the solvent. High-resolution mass spectra were collected by the staff at the MIT Department of Chemistry Instrumentation Facility.

Aqueous solutions were prepared using Millipore water. Molecular biology grade piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 99.999% KCl were purchased from Aldrich. Spectrophotometric measurements at pH 7.0 were conducted in 50 mM PIPES, 100 mM KCl aqueous buffer. In order to remove any adventitious metal ions, this buffer solution was treated with Chelex resin (Bio-Rad) according to the manufacturer's protocol. A 100 mM zinc(II) stock solution was prepared using 99.999% $ZnCl_2$ (Aldrich). Stock solutions of ZP1BG (1 mM) were prepared in DMSO, partitioned in 20-μL aliquots and stored in the dark at -20°C.

UV-vis spectra were recorded on a Varian Cary 50 Bio UV-visible spectrophotometer. Fluorescence spectra were recorded on a Quanta Master 4 L-format scanning spectrofluorimeter (Photon Technology International). The acquisition temperature was kept at 25 ± 1 °C by circulating water baths. Sample solutions were placed in quartz cuvettes (Starna) with 1-cm path lengths.

Synthesis of ZP1BG. ZP1(6-CO_2H) (10.0 mg, 0.012 mmol) and HATU (4.0 mg, 0.016 mmol) were combined in anhydrous DMF (1 mL) and stirred at room temperature for 30 min under a nitrogen atmosphere. *O*^{6}-(4-Aminomethyl-benzyl)guanine **1** (5.0 mg, 0.020 mmol) and triethylamine (0.01 mL) were added and the reaction mixture was stirred in the dark for 18 h under nitrogen. ZP1BG was isolated and purified from the reaction mixture by preparative reverse-phase HPLC employing a linear gradient of water/acetonitrile mixtures (85:15 to 72:28 over 20 min, 0.2% TFA) on a C18 column (Vydac 218TP1010, 10 × 250 mm) at a 4 mL/min flow rate. Lyophilization afforded ZP1BG as a bright orange solid (5.5 mg, 43%). ^{1}H NMR (500 MHz, CD_3OD): δ 4.47–4.56 (12H, m), 4.60 (2H, s), 5.61 (2H, s), 6.71 (2H, s), 7.41–7.52 (12H, m), 7.64 (1H, s), 7.92 (4H, t), 8.19 (1H, d), 8.29 (2H, m), 8.60 (4H, d). HR-MS (ESI): calcd. $[M + H]^+$, 1119.3219; found, 1119.3187.

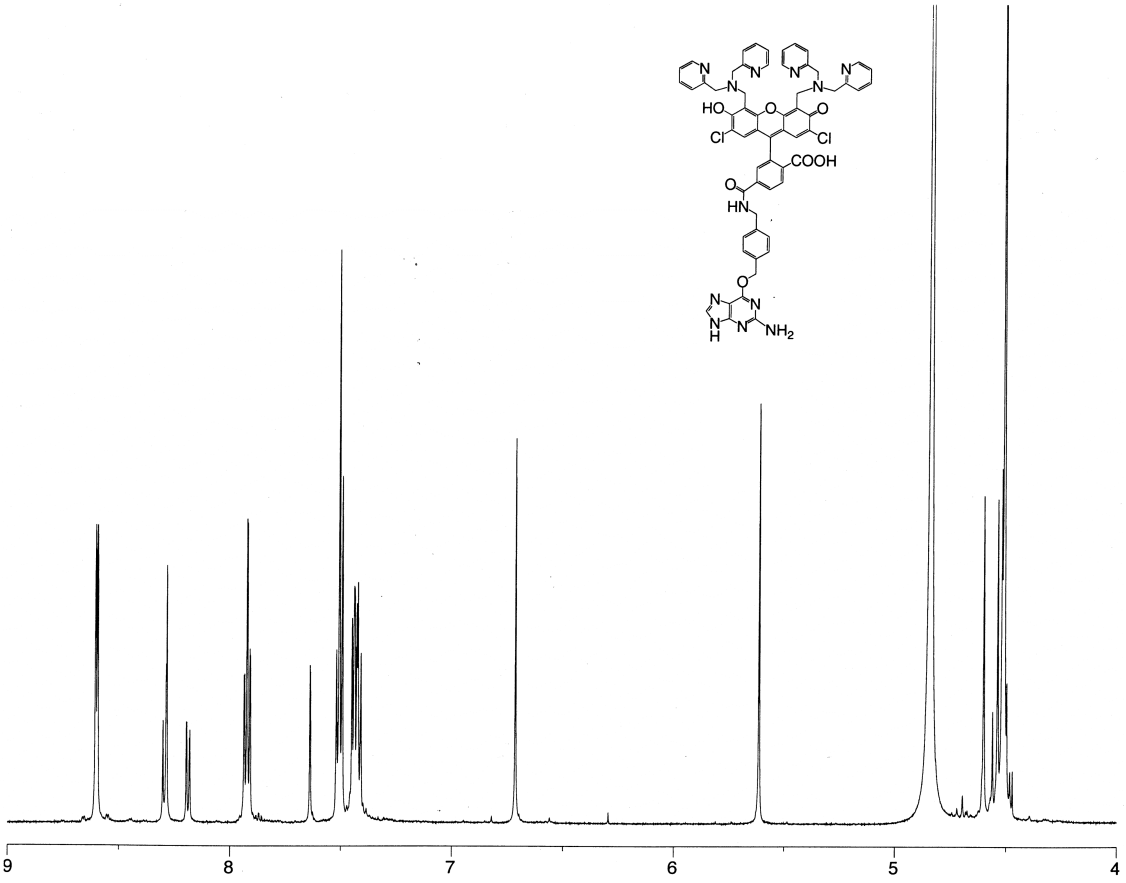

Figure S1. ^{1}H NMR spectrum (500 MHz, CD_3OD) of ZP1BG.

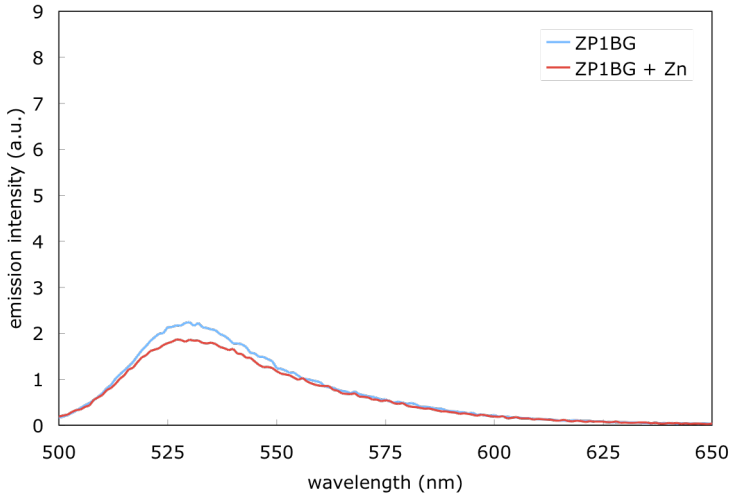

Figure S2. Fluorescence emission spectrum (λ_{ex} 490 nm) of ZP1BG (1 μM) in 50 mM PIPES buffer and 100 mM KCl at pH 7.0 before and after addition of 2 equiv. of $ZnCl_2$.

Expression and Purification of AGT-GST. AGT was expressed as a glutathione S-transferase (GST) fusion in *E. coli* cells using a pGEX2T-AGT construct as previously described.[1] The cell lysate was purified using a GE Healthcare ÄKTA™ Purifier equipped with a 1-mL GSTrap™ 4B column. Elution with 10 mM glutathione (in 20 mM Tris, 0.5 M KCl, pH 8.0) afforded the AGT-GST fusion. Protein-containing fractions were analyzed by SDS-PAGE, combined, and concentrated using a centrifugal filter (Amicon Ultra-15 3000 NMWL). Total protein concentration was determined using the Bradford assay. The protein solution was stored at -80°C.

In Vitro AGT Labeling and ESI Mass Spectrometric Analysis. AGT fusion protein samples (50 μM in elution buffer) were analyzed before and after incubation with ZP1BG (ca. 1 equiv). Electrospray mass data were collected by the staff of the Proteomics Core Facility at the MIT Koch Institute for Integrative Cancer Research. Samples were desalted and ESI mass spectra were acquired on a QSTAR Elite mass spectrometer and deconvoluted using the QSTAR BioTools software to generate molecular weight spectra (Figure S3).

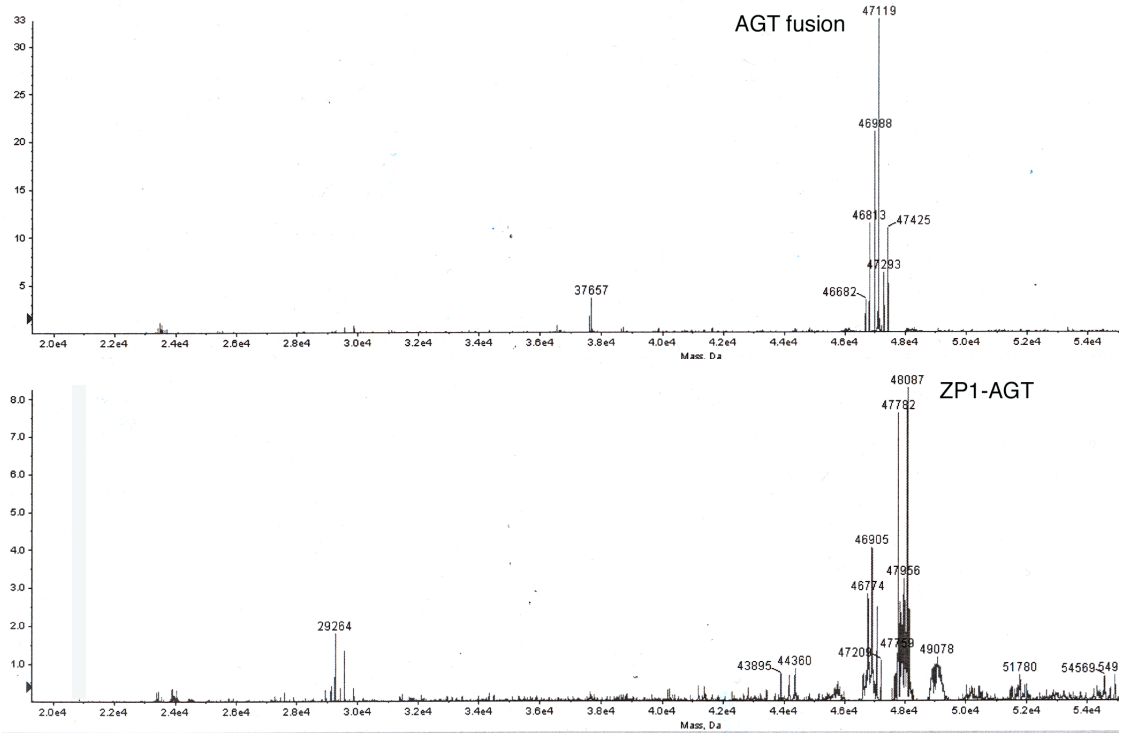

Figure S3. Molecular weight spectra of samples AGT (top panel) and ZP1-AGT (bottom panel) generated from the respective electrospray mass spectrometric data.

Table S1. Selection of peaks from the ESI mass spectra of the two protein samples showing a mass difference that is consistent with the calculated mass of the ZP1 label (968.3 Da).

MW(AGT sample)	MW(ZP1-AGT sample)	Mass difference
46813 Da	47782 Da	969 Da
46988 Da	47956 Da	968 Da
47119 Da	48087 Da	968 Da

Plasmid Construction. AGT51B cDNA was kindly provided by Prof. Kai Johnsson (Swiss Federal Institute of Technology, Lausanne, Switzerland).

For the preparation of pGolgi–AGT and pGolgi–mCherry, pCFP–Golgi (Clontech) was employed. It contains the Golgi-signaling sequence of β-1,4-galactosyltransferase[3] in front of CFP. The CFP coding sequence was removed by digestion of pCFP–Golgi with *BamHI* and *NotI*. AGT51B cDNA and mCherry[4] cDNA were PCR amplified using the forward and reverse primers listed below, which provided the *BamHI* and *NotI* restriction sites. The PCR products were purified, digested with *BamHI and NotI*, and ligated into the CFP site of *BamHI/NotI*-digested pCFP–Golgi.

For the preparation of pMito-AGT, oligonucleotides encoding the mitochondrial targets sequence of subunit VIII of cytochrome *c* oxidase,[5] which is identical to the sequence in pDsRed2–Mito (Clontech), were employed. The Mito1 and Mito2 sets of oligonucleotides listed below were annealed (Mito1-1 + Mito1-2: Mito2-1 + Mito2-2) and ligated (Mito1+Mito2 dsDNA) to provide the mito targeting insert with the appropriate sticky ends. The pGolgi–AGT construct was digested with *NheI* and *BamHI*, which removed the Golgi targeting sequence, and the mitochondrial sequence was ligated into the digested vector to afford pMito–AGT.

Primers and primer pairings:

5' AGT51B: CTGGATCCCATGGACAAGGATTGTGAAATGAAACGC
3' AGT51B: ATAGCGGCCGCTCAGTTTCGGCCAGCAGGCGGGG

5' mCherry: ATGGATCCAACCATGGTGAGCAAGGGCGAGGAGGAT
3' mCherry: ATGCGGCCGCTAACTTGTACAGCTCGTCCATGCC

Mito1-1: CTAGCATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGC
Mito 1-2: CCGGGCCGAGCCTGTCAAGCCCCGCAGCAGCAGCGGCGTCAGGACGGACATG

Mito 2-1: TCGGCCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGGGG
Mito 2-2: GATCCCCCAACGAATGGATCTTGGCGCGCGGCACTGGGAGCCG

Mammalian Cell Culture and Labeling Procedures. HeLa cells were cultured at 37 °C under a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone), glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Transient transfections were performed 24-30 h before imaging. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent according to the manufacturer's protocol.

For live cell imaging, cells were seeded in 35-mm poly-D-lysine coated glass-bottom culture dishes (MatTek Corporation). For AGT labeling, the growth medium was removed and replaced with the labeling solution containing 10 μM ZP1BG in complete DMEM. After a labeling incubation time of 1 h (37°C, 5% CO_2), the cells were rinsed with warm PBS (2 × 2 mL) and incubated for additional 30 min in serum-free DMEM. For multi-channel imaging and colocalization studies, staining of the nuclei or the mitochondria was accomplished in the latter incubation step by addition of Hoechst 33258 (Aldrich, final concentration: 4.5 μM) or Mitotracker Red (Invitrogen, final concentration: 0.2 μM), respectively. Cells were finally rinsed with warm PBS (1 × 2 mL) and warm serum-free DMEM (2 × 2 mL) immediately before imaging in serum-free DMEM (2 mL).

Zinc was added to the cultured cells as the pyrithione (2-pyridinethiol-1-oxide) salt using a 1:1 Zn(II)/pyrithione ratio. Stock solutions of $ZnCl_2$ (10 mM) and sodium pyrithione (20 mM) in DMSO were combined and diluted in PBS to a 1 mM concentration. The obtained solution was added to the cell culture dishes directly on the microscope stage to reach a 50 μM final concentration. Similarly, a stock solution of *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, 20 mM) in DMSO was diluted in PBS to a 2 mM concentration and then added to the culture dishes on the microscope stage to reach a 100 μM concentration.

Fluorescence Microscopy. The imaging experiments were performed using a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with an EM-CCD digital camera (Hamamatsu) and a MS200 XY Piezo Z stage (Applied Scientific Instruments). The light source was an X-Cite 120 metal-halide lamp (EXFO) and the fluorescence images were obtained using an oil-immersion objective at 63× magnification. The microscope stage was outfitted with an INC-2000 incubator, which maintained the samples at 37°C under a 5% CO_2 humidified atmosphere. Microscope operation and image processing were conducted using the Volocity software (Improvision). Exposure times for the fluorescence images ranged from 200 to 500 ms and were kept constant for each image series.

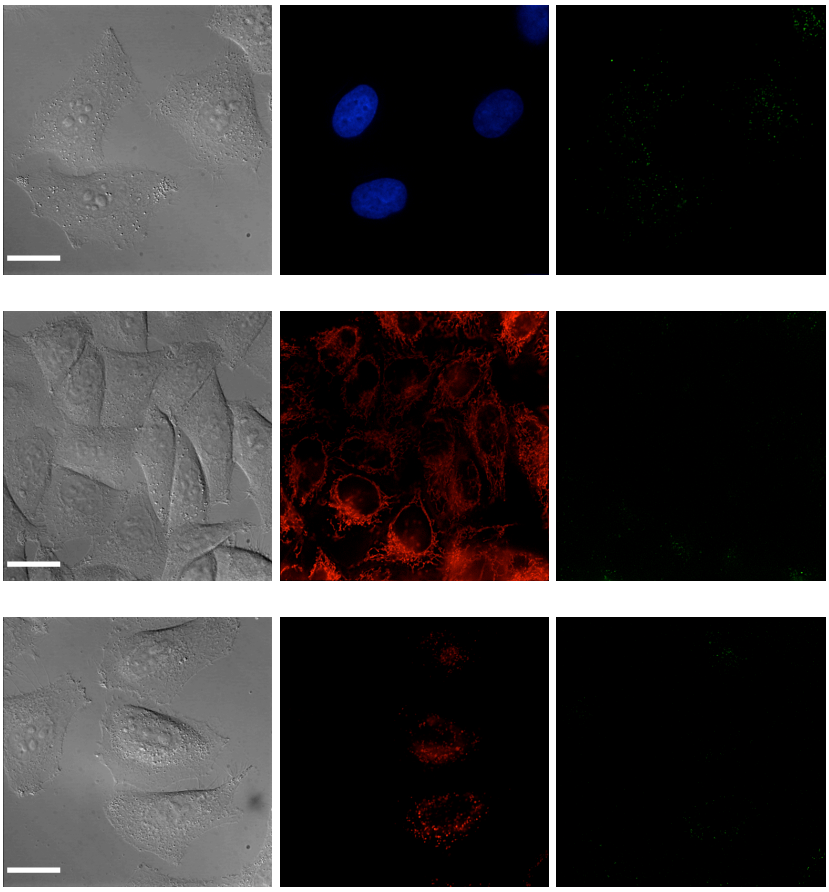

Figure S4. Distribution of ZP1BG (10 μM, 1 h) in HeLa cells that were not transfected with AGT-encoding plasmids. The nuclei were stained with Hoechst 33528 (4.5 μM, top panels), the mitochondria were stained with Mitotracker Red (0.2 μM, middle panels), and Golgi staining was performed by site-specific expression of mCherry (bottom panels).

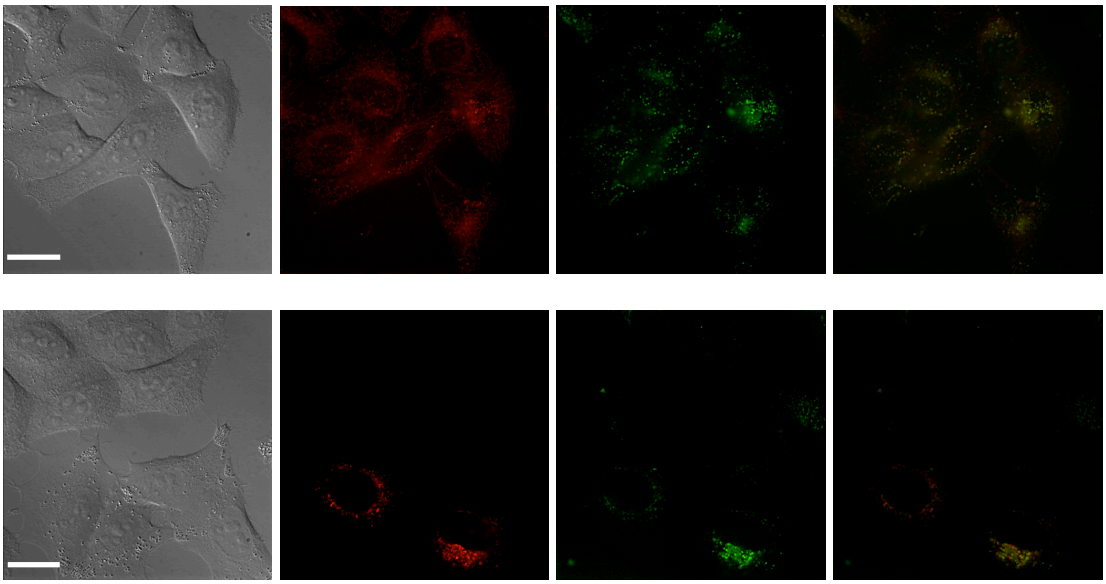

Figure S5. Full field of view for images shown in Figure 2 in the main text. HeLa cells transiently expressing AGT in the mitochondria (top panels) and in the Golgi apparatus (bottom panels) were labeled with ZP1BG (10 μM, 1 h). The organelles were marked in the red channel by staining with 0.2 μM Mitotracker Red (top panels) or by expression of the red fluorescent protein mCherry in the Golgi apparatus (bottom panels). From the left: (i) DIC image, (ii) emission from the organelle marker, (iii) emission from site-specifically localized probe, and (iv) overlay of the red and green fluorescence images. Scale bar: 25 μm.

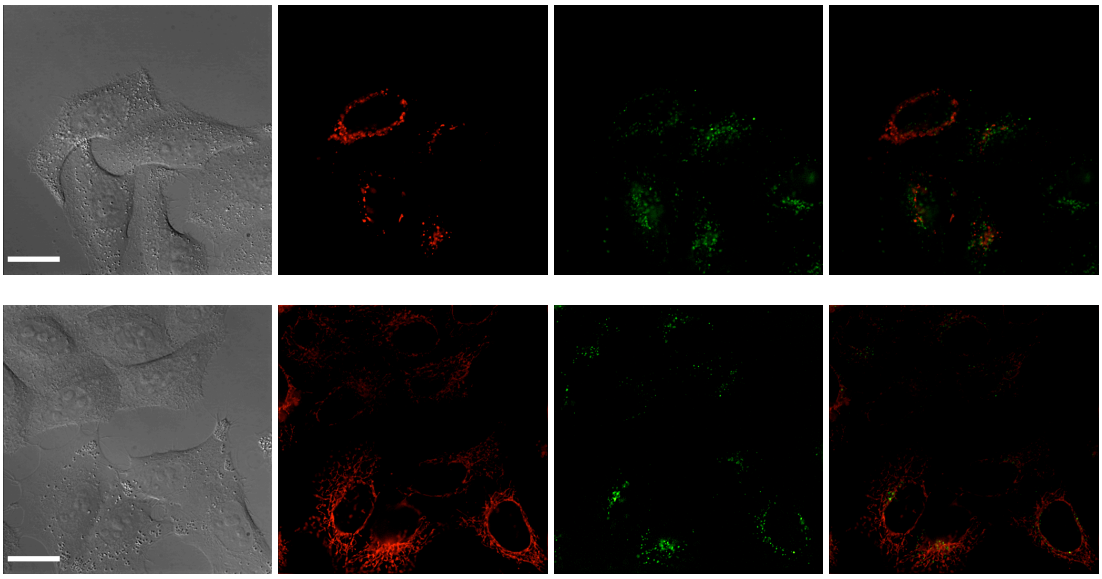

Figure S6. Cross-colocalization controls. Top panels: HeLa cells expressing AGT in the mitochondria and mCherry in the Golgi apparatus were labeled with ZP1BG (10 μM, 1 h). Bottom panels: HeLa cells expressing AGT in the Golgi apparatus were labeled with ZP1BG (10 μM, 1 h) and stained with Mitotracker Red (0.2 μM). From the left: (i) DIC image, (ii) emission from the organelle marker, (iii) emission from site-specifically localized probe, and (iv) overlay of the red and green fluorescence images. Scale bar: 25 μm.

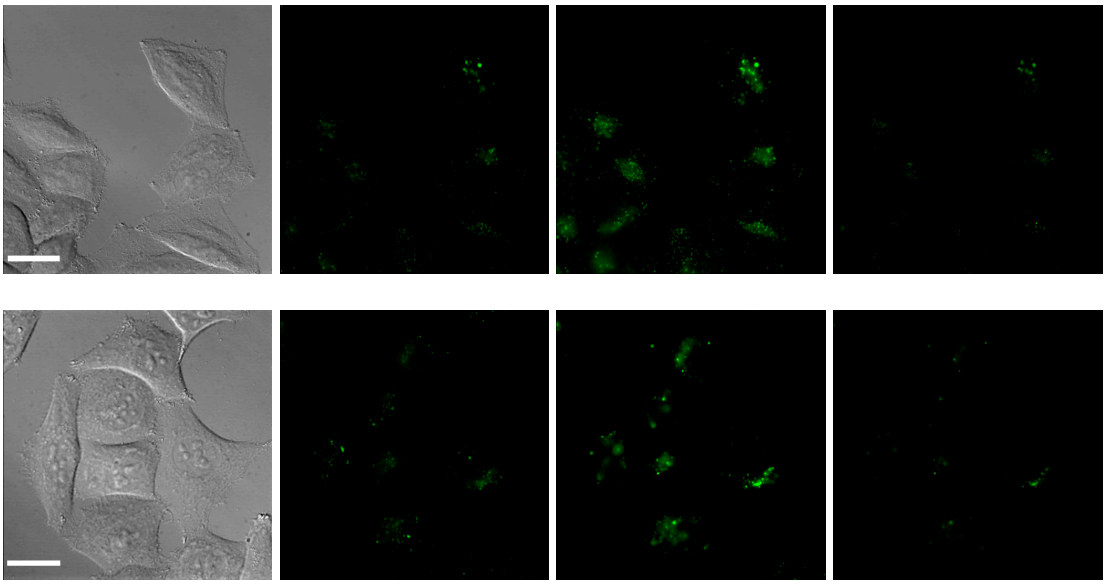

Figure S7. Full field of view for images shown in Figure 3 in the main text. Zinc-induced fluorescence response in HeLa cells expressing AGT in the mitochondria (top panels) and in the Golgi apparatus (bottom panels). From the left: (i) DIC image, (ii) labeling with ZP1BG (10 μM, 1 h), (iii) addition of Zn(II)/pyrithione (1:1, 50 μM, 5 min), and (iv) subsequent addition of TPEN (100 μM, 5 min). Scale bar: 25 μm.

References.

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