Supporting Information

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SI Materials and Methods

Reagents. Recombinant arginine deiminase (ADI), formulated with linear 20,000-molecular-weight polyethylene glycol molecules (ADI-PEG20), was generously provided by Polaris Pharmaceuticals, Inc.

Antibodies. Antibodies used in this study were as follows: mouse monoclonal anti-caspase 7 (10-1-62; BD Pharmingen), mouse monoclonal anti-caspase 8 (3-1-9; BD Pharmingen), mouse monoclonal anti-caspase 9 (2-22; BD Pharmingen), rabbit monoclonal E-cadherin (24E10; Cell Signaling Technology), rabbit polyclonal anti-Ku70 (Abcam), mouse monoclonal anti-lamin A/C (JoL3; Abcam), mouse monoclonal anti-LAMP1 (H4A3; Developmental Studies Hybridoma Bank), rabbit monoclonal NUP98 (C39A3; Cell Signaling Technology), rabbit polyclonal anti-gamma-H2A.X (phospho S139; Abcam), rabbit monoclonal anti-ATG5 (D5G3; Cell Signaling Technology), rabbit monoclonal anti-Histone H3 (D1H2; Cell Signaling Technology), and rabbit polyclonal anti-acetyl-Histone H2B (Cell Signaling Technology).

Cell Culture and Treatment. Prostate cancer cell lines CWR22Rv1, CWR22Rv1 GFP-LC3 (stably transfected), and PC3 were cultured in RMPI 1640 supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. Pancreas cancer cell lines Mia and L3.3 were culture in DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. All cells were maintained at 37 °C in a 5% (vol/vol) CO_2, 95% (vol/vol) air incubator. For autophagy induction, CWR22Rv1, CWR22Rv1 GFP-LC3, PC3, Mia, and L3.3 cells were seeded in six-well plates or glass-bottom dishes (P35GC-1.5; MatTek) and treated with ADI-PEG20 (0.3 μg/mL) for 24, 48, 72, 96, or 120 h the following day. For autophagy inhibition, CWR22Rv1 and CWR22Rv1 GFP-LC3 were treated with 3-methyladenine (3-MA, 1 mM; Sigma), ADI-PEG20 (0.3 μg/mL), or both for 24, 48, 72, 96, or 120 h the following day. All cell samples were collected at each time point and analyzed by immunofluorescence microscopy (see below). For antioxidant treatment, ADI-PEG20-treated CWR22Rv1 cells were incubated with or without N-acetyl cysteine (NAC, 10 μM, Sigma) for 96 h at 37 °C.

Arginine Deprivation. Arginine-depleted RPMI-1640 medium was prepared by adding back 0.05 g/L L-leucine (L8912; Sigma) and 0.04 g/L lysine (L5501; Sigma) into RMPI-1640 medium without L-arginine, L-leucine, lysine and Phenol Red (R1780; Sigma), and was supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. Arginine-depleted RPMI was sterilized by vacuum filtration system with a 47-mm membrane (Nalgene). Cells were initially cultured in regular RMPI-1640 until proper cell density was reached. Regular media was aspirated and cells were washed briefly with PBS and continued to incubate in arginine-depleted RPMI for 24, 48, 72, 96, or 120 h at 37 °C. Samples were collected at each time point and later analyzed by immunofluorescence microscopy (see below).

Microscope Image Acquisition and Image Analysis. For live-cell autophagy imaging, cells were seeded onto a 35-mm glass-bottom dish (1.5, poly-lysine coated; MatTek) or microfluidic plate (CellASIC) and visualized under a DeltaVision deconvolution microscope, which was also equipped with both environmental chamber and ONIX microfluidic perfusion system (CellASIC) to main constant temperature at 37 °C, humidity, and 5% (vol/vol) CO_2 concentration. For fixed-cell autophagy imaging, cells were seeded on coverslip (1.5 or 170-μm thickness) and fixed in 4% (vol/vol) paraformaldehyde/1× PBS (PFA) for 10 min at room temperature. After rinsing with washing buffer [Hepes buffered saline and 1% (vol/vol) BSA] three times, cells were permeabilized in permeabilization buffer [Hepes buffered saline, 1% (vol/vol) BSA, 2.5% (vol/vol) casein, and 0.05% (vol/vol) saponin (Sigma)] for 10 min at room temperature. Blocking buffer [Hepes buffered saline, 1% (vol/vol) BSA, and 2.5% (vol/vol) casein] was added for 1 h at room temperature. Primary antibodies used were described above, and appropriate secondary antibodies (Alexa Fluor 488, 555, 647; Invitrogen) were used after primary antibody incubation at 4 °C overnight. All coverslips were mounted over a microscope slide in Prolong Gold anti-fade reagent (Life Technologies).

For γH_2AX and 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunofluorescence microscopy and image analysis, cells were seeded on coverslip and fixed in PFA for 30 min at room temperature. After washing with PBS three times, blocking solution [0.1% (vol/vol) Triton X-100 and 5% (vol/vol) BSA in PBS] was added for 1 h at room temperature or 4 °C overnight. The slides are incubated with an anit-γH_2AX antibody (A300-081; Bethyl) or 8-OHdG (sc-66036; Santa Cruz) at 4 °C overnight and appropriate secondary antibodies (Alexa Fluor 568; Invitrogen) were used after primary antibody incubation. After washing with PBS, the coverslips were mounted over a microscope slide in Prolong anti-fade reagent that contained DAPI (P-36931; Life Technologies) and examined using a Stallion Digital imaging station (Carl Zeiss Microscopy).

Hypodiploid Cell (DNA Content <2n) Analyses Using Propidium Iodide. ADI-PEG20–treated cells, including the supernatant, were harvested and washed with PBS. Resuspended cells (in 300 μL PBS) were added with 700 μL of absolute cold ethanol dropwise with vortexing and fixed at −20 °C overnight. The cells were pelleted with centrifugation at 1,000 × *g* at 4 °C for 5 min. Supernatant was decanted carefully and washed twice in PBS at room temperature. Resuspended the cells (in 495 μL PBS) were added with 5 μL of RNase A (1 mg/mL) and incubated at 37 °C for 30 min to avoid the interference of RNA. Finally, 500 μL of PBS containing 10 μg/mL propidium iodide was added to make the finally concentration 5 μg/mL, and cells were stained for 15 min in darkness. The samples are subjected to flow-cytometric analysis for their DNA content histogram and the hypodiploid cell populations were scored (Gallios; Beckman Coulter).

Fluorescence-Based Methods to Measure CellROX Oxidation, MitoSOX Red Oxidation, Mitochondria Membrane Potential, and Autophagic Flux. Cells were stained with CellROX Deep Red (C10422, 5 μM; Molecular Probes) for 30 min before flow cytometry analyses (Gallios; Beckman Coulter). In a second fluorescent dye oxidation measurement, cells were incubated with MitoSOX Red (M36008, 5 mM; Life Technologies) for 45 min in serum-free DMEM. In this paper, elevation of CellROX Deep Red and MitoSOX Red fluorescence are used as the measure or indicator of increased nonmetabolic oxidation, which is consistent with oxidative stress. For mitochondrial membrane potential analyses, cells were incubated in DMEM with $DiOC_6$ (318426, 10 nM; Sigma) for 30 min before analyses. For autophagic flux analyses, CWR22Rv1/GFP-LC3 cells were harvested after ADI-PEG20 treatment for different time periods and immediately analyzed by flow cytometry. Data were collected from three independent

experiments and are presented in a histogram. The flow cytometry analyses were performed using FlowJo software (TreeStar).

Virus Production and Transduction. Target gene DNA in a lentiviral backbone (pLKO.puro or pSin) (21 μg), pΔ8.7 (14 μg), and pVSV-G (7 μg) were transfected using Lipofectamine 2000 (11668-019, Life Technologies) into HEK293T/FT cells that had been seeded in a T-175 flask and had reached 60% confluence on the day of transfection. On the second day after transfection, cells were treated with sodium butyrate (10 mM) to stimulate virus production. Media containing viruses were harvested on the fifth day and filtered through a 0.45-μm filter. For viral transduction, cells were treated with media containing viruses overnight in the presence of polybrene (8.3 μg/mL) and this was followed by selection with puromycin (1 μg/mL).

Whole-Cell Extracts and Immunoblotting. Cells were harvested and lysed on ice for 30 min in RIPA buffer (9806; Cell Signaling) containing complete protease inhibitor mixture (11836145001; Roche). The protein concentrations of whole-cell extracts were determined using a Bio-Rad Protein Assay Kit (500-0001; Bio-Rad). Approximately 40 μg of protein extract was mixed with an equal volume of 2× SDS loading buffer, boiled for 5 min, then separated by Tris-glycine SDS/PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk in PBST [PBS containing 0.05% (vol/vol) Tween 20] and incubated with primary antibodies at 4 °C overnight. The membranes were then washed with three times with PBST for 10 min, three times, and incubated with HRP-labeled secondary antibodies for 2 h at room temperature. Immunoblots were visualized using VersaDoc 5000 imaging system (Bio-Rad). All blots were performed with standard protocol.

Apoptosis and Mitotic Catastrophe Induction. CWR22Rv1 cells were cultured in RPMI supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. For UV-induced apoptosis, media was aspirated and cells were briefly washed with PBS. Cells were exposed to UV in UV Crosslinker box (Fisher Scientific) under optimal cross-link condition. Fresh RPMI-1640 was added, followed by 24 h incubation at 37 °C. For mitotic catastrophe induction, cells were treated with paclitaxel (100 nmol/L; Sigma) for 24 h at 37°C, and cell samples were collected the next day.

Oxygen Consumption Rate. Cellular mitochondrial function was measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer. The mitochondrial function was assayed by sequential injections of oligomycin (ATP synthase inhibitor), FCCP (carbonylcyanide *p*-trifluoromethoxy phenylhydrazone), mitochondrial oxidative phosphorylation protonophore and uncoupler, and rotenone (mitochondrial complex I inhibitor) to define basal oxygen consumption rate (OCR), ATP-linked OCR, proton leak, maximal respiratory capacity, reserve respiratory capacity, and nonmitochondrial oxygen consumption, all according to the manufacturer's instructions. Herein, basal OCR is used to represent the function of mitochondria. Briefly, 2×10^4 cells were seeded onto 24-well plates and incubated overnight before sequentially adding preoptimized concentrations of oligomycin, FCCP, and rotenone, in that order. After washing the cells with 1 mL seahorse buffer [DMEM without phenol red containing glucose (G7021, 4.5 g/L; Sigma), sodium pyruvate (11360070, 1 mM; Gibco), and glutamine (25030081, 4 mM; Gibco)], 600 μL seahorse buffer plus 60 μL each of oligomycin (75351, 50 μg/mL; Sigma), FCCP (C2920, 10 μM; Sigma), and rotenone (R8875, 10 μM; Sigma) was automatically injected. At the end of recording period, cells were collected and the individual cell numbers were determined using a trypan blue exclusion assay. OCR values were calculated after normalizing with the cell number and plotted as the mean ± SD.

8-OHdG ELISA. DNAs were isolated using DNeasy blood and tissue kit (69504; Qiagen) according to the supplier's protocols. Extracted DNAs were dissolved in water to 1 mg/mL and the DNA sample was converted to single-stranded DNA by incubating the sample at 95 °C for 5 min and rapidly chilling on ice. DNA samples were digested to nucleosides by incubating the denatured DNA with 10 units of nuclease P1 (N8630; Sigma) for 2 h at 37 °C in 20 mM sodium acetate (S2889; Sigma), pH 5.2, and followed with treatment of 10 units of alkaline phosphatase (M0290S; New England Biolabs) for 1 h at 37 °C in 100 mM Tris (T5941; Sigma), pH 7.5. The reaction mixture was centrifuged for 5 min at 6,000 × *g* and the supernatant was used for the 8-OHdG ELISA using Oxiselect oxidative DNA damage ELISA-kit (STA-320; Cell Biolabs) according to supplier's protocols.

SI Discussion

Somewhat similar formation of perinuclear autophagic vacuoles and uptake of leaked DNA have previously been described in envelopathies $Lmna^{H222P/H222P}$ mouse embryonic fibroblasts (1, 2). Consistent with the published literature (3, 4), our data illustrated an intimate relationship between the integrity of nuclear envelope, such as lamin, and DNA leakage. We postulated that that autophagosome membrane formation/fusion with nuclear membrane might play a role in DNA leakage. Alternatively, as the inner membrane first disintegrated/dissolved, followed by the outer membrane fused with autophagosome, damaged DNA exited from the nucleus at the weakened site and was captured by autophagosomes. The common theme of these phenomena is that they are all in response to genome instability, whether caused by nuclear envelope defects, micronuclei formation (1, 2), or reactive oxygen species from damaged mitochondria (this paper). Conceptually, it highlights the role of chromatophagy in the loss of genome integrity and order. Another nucleophagy-related phenomenon has been described in yeast, named piecemeal microautophagy of the nucleus (PMN) (5). The chromatophagy described here also differs from envelopathies- and micronuclei-associated nuclear-phagy: (*i*) a higher proportion of cells are affected, (*ii*) DNA is directly taken from nucleus instead of engulfing micronuclei—we never found any "free" micronuclei in the cytosol, and (*iii*) The DNA-containing autophagosomes are usually significantly larger and contain more DNA than micronuclei. Furthermore, rapamycin treatment induced PMN in *Saccharomyces cerevisiae* (6) but not chromatophagy in CWR22RV1 cells (this study). Finally, Fujiwara et al. have reported a novel type of lysosome-dependent autophagy, namely RNautophagy, targeting RNA (7) and DNautophagy targeting DNA (8). These authors demonstrated that RNA or DNA binds to the cytosolic tail of LAMP2C on the surface of the lysosomal membrane and further speculated that DNA could bind to the cytosolic tail of LAMP2C. This, however, could not entirely explain the recruitment of leaked DNA into autophagosomes. Additional studies are needed to distinguish the different types of chromatophagy.

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7. Fujiwara Y, et al. (2013) Discovery of a novel type of autophagy targeting RNA. *Autophagy* 9(3):403–409.

8. Fujiwara Y, et al. (2013) Direct uptake and degradation of DNA by lysosomes. *Autophagy* 9(8):1167–1171.

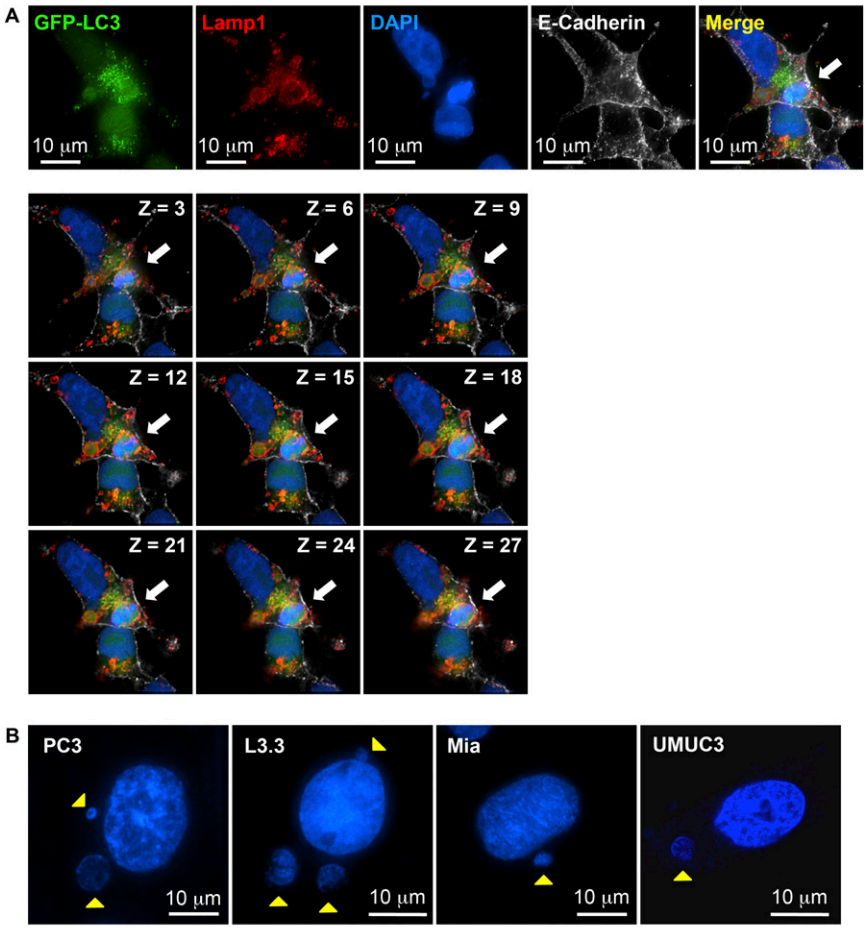


Fig. S1. Sustained arginine starvation induces DNA leakage. (*A*) E-cadherin staining in ADI-PEG20–treated cells (120 h) with DNA leakage phenotype reveals the complete cellular outline. White arrow indicates the leaked DNA localized within an intact cell (*Upper Right*). Additional Z-section images also show that both autophagosomes (GFP-LC3) and lysosomes (LysoTracker) colocalized with leaked DNA particle (white arrows) throughout all sections (*Lower*). (*B*) A similar phenotype could also be found in other prostate cancer cell line (PC3), pancreas cancer cell lines (Mia and L3.3), and a urinary bladder cancer cell line (UMUC3) with prolonged ADI-PEG20 treatment. Both Mia and UMUC3 cells are known to undergo caspase-dependent cell death with ADI-PEG20 treatment. Both cell lines were treated with 0.3 μg/mL ADI-PEG20 for 0, 24, 48, 72, and 96 h, and the DNA-leakage phenotype (yellow arrowhead) began to appear around 72 h.

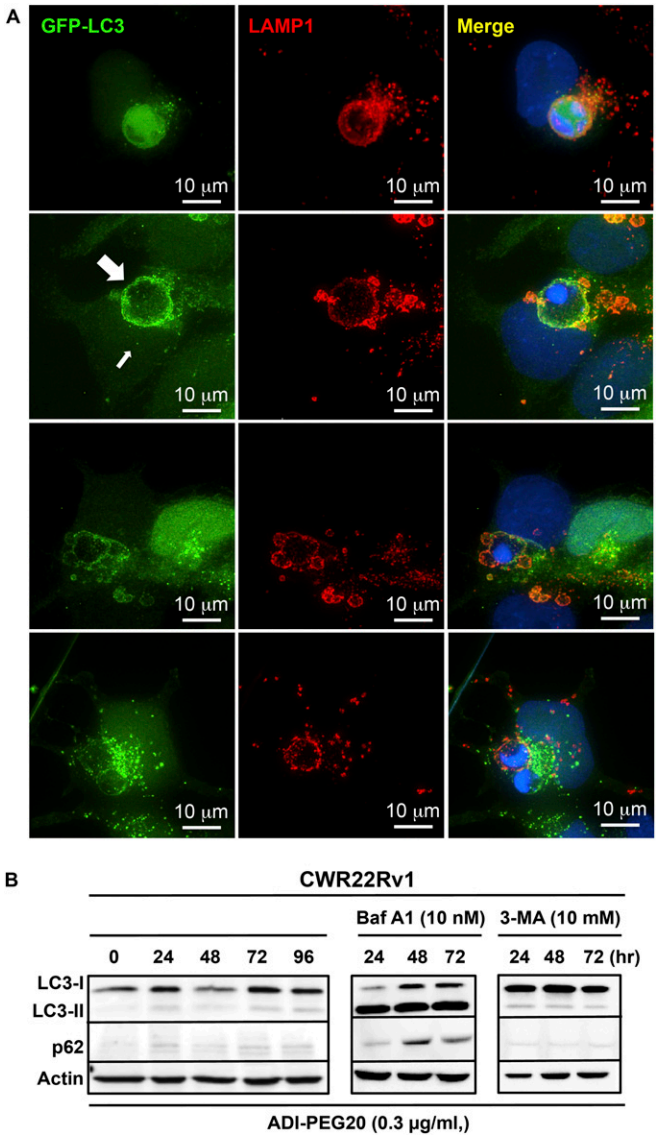


Fig. S2. Prolonged ADI-PEG20 treatment results in larger-than-normal autophagosome formation and colocalized with lysosome at 96 h posttreatment. (*A*) The marked size difference, compared with normal autophagosome (thin white arrow at small bright green dots compared with thick white arrow), is indicated by a thick white arrow. Merged images showing leaked DNA particles (DAPI) colocalized with giant-sized autophagosomes (GFP-LC3) and lysosomes (LAMP1). (*B*) CWR22Rv1 cells were treated with 0.3 μg/mL ADI-PEG20 for 0, 24, 48, 72, and 96 h. The abundance of LC3 and p62 were determined by Western blot analysis. As expected, there are no LC3 lipidation and p62 degradation but accumulation of LC3-I in CWR22Rv1 cells, suggesting the induction of an atypical autophagy by ADI-PEG20 treatment. CWR22Rv1 cells were treated with 0.3 μg/mL ADI-PEG20 for 0, 24, 48, and 72 h with or without Baf A1 or the addition of 3-MA for 6 h before cell harvesting. The abundance of LC3 and p62 was determined by Western blot analysis.

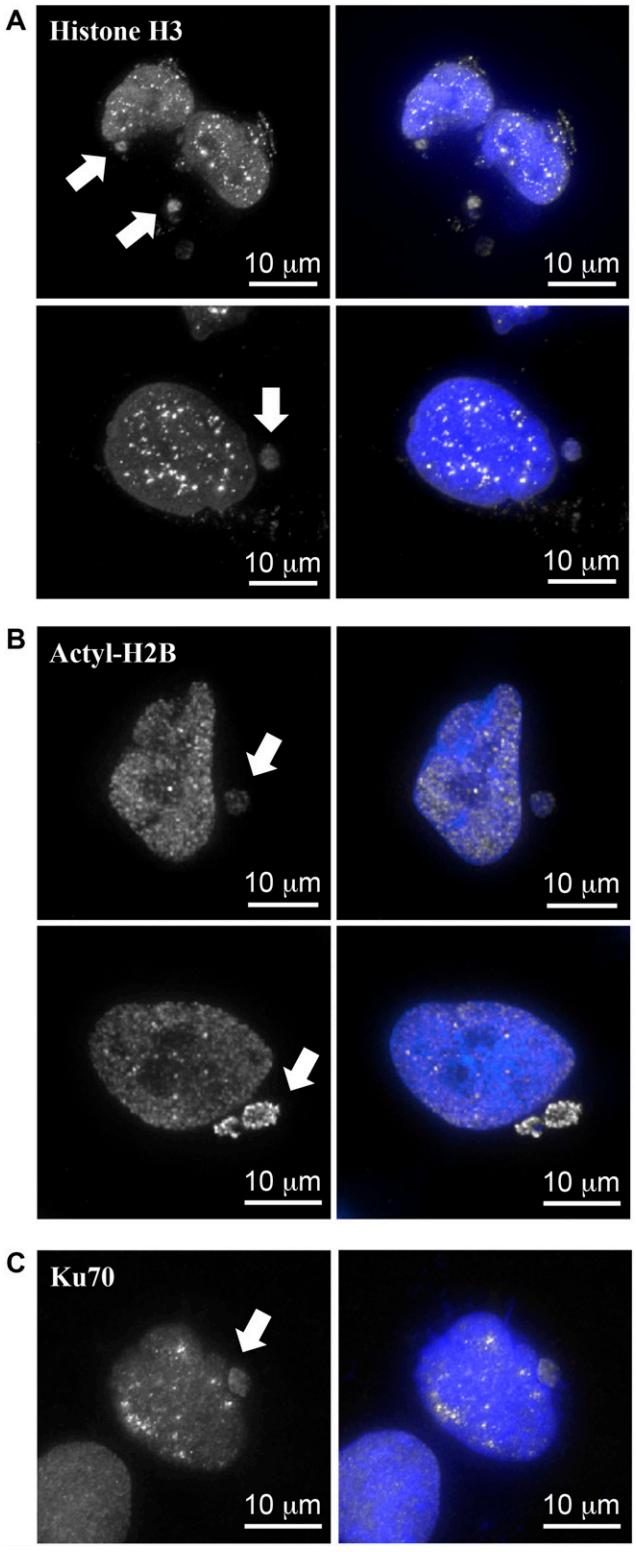


Fig. S3. Prolonged ADI-PEG20 treatment induces chromatophagy. CWR22Rv1 cells stained with DAPI (blue) and anti-histone H3 (*A*) or anti-acetyl-H2B (*B*) to show that histone H3 colocalized with leaked DNA outside of nucleus (white arrows). (*C*) Colocalization of double-strand-break binding protein Ku70 with leaked DNA (white arrows) could be found in ADI-PEG20–treated cells at later time points.

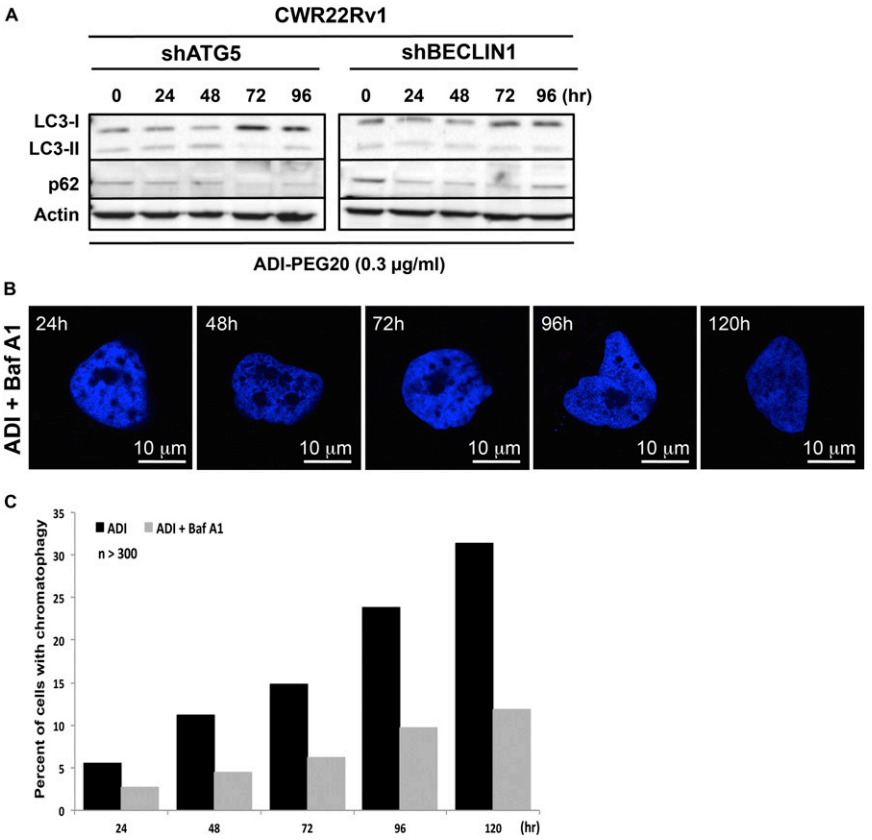
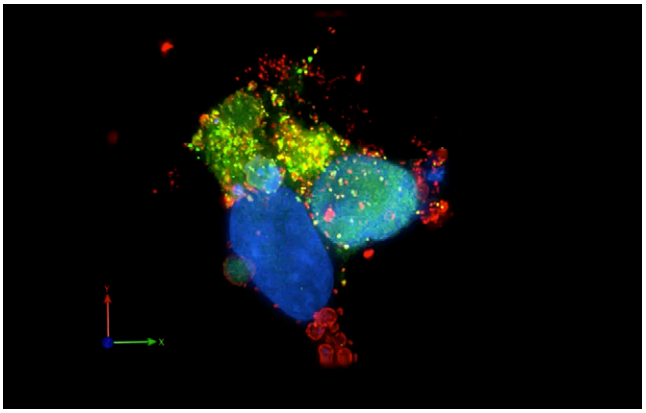


Fig. S4. Inhibition of autophagosome formation can reduce the occurrence of chromatophagy. (*A*) CWR22Rv1/shATG5 and shBECLIN1 cells were treated with ADI-PEG20 (0.3 μg/mL) for 0, 24, 48, 72, and 96 h. The abundance of LC3 and p62 was determined by Western blot analysis. There was a little LC3-I accumulation in shATG5 and shBECLIN1 cells, which might be due to the incomplete knockdown. (*B*) CWR22Rv1 cells were treated with ADI + Bafilomycin A1 (100 nM) for 24, 28, 72, 96, and 120 h and stained with DAPI to reveal the nucleus. Nuclear morphology was evaluated under fluorescence microscope with DAPI. (*C*) Percentage of cells with chromatophagy was counted under fluorescence microscope. Addition of Bafilomycin A1 with ADI treatment can reduce occurrence of chromatophagy.



Movie S1. CWR22Rv1 cells that have undergone ADI-PEG20 treatment for 96 h. Formation of a giant autophagosome (green) could be found fused with lysosome (red) and localized extremely close to the cell nucleus (blue). A three-dimensional deconvolution image showed that a portion of the giant autophagosome seemingly pushed into (or pulled out of) the nucleus, further suggesting that there could be an active "fusion" event between the membranes of the autophagosome and nucleus membrane.

Movie S1