Supporting Table 1. Characteristics of HCC patients

Variables	n
Gender	
Male	**100**
Female	**47**
Age (yr)	
Range	**26-86**
Median	**59**
< 60	**80**
≧ 60	**67**
Viral status	
HBV [a]	**77**
HCV [b]	**67**
HBV and HCV	**3**
Cirrhosis	
Non-cirrhosis	**82**
Cirrhosis	**65**
Recurrence	
Non-recurrence	**61**
Recurrence	**86**
AFP [c]	
< 100	**86**
≧ 100	**61**
Tumor size	
< 4 cm	**62**
≧ 4 cm	**85**

[a] HBV: hepatitis B virus

[b] HCV: hepatitis C virus

[c] AFP: alpha-fetoprotein.

Supporting Table 2. Correlation of cyclin D1, BECN1 and SQSTM1 expression with clinicopathological parameters of paired specimens of HCC patients

	Gender	Age	Tumor size	HBV vs. HCV	Cirrhosis	Recurrence
Cyclin D1 [a]	0.773	0.408	0.032*	0.156	0.565	0.300
BECN1 [a]	0.001*	0.577	0.574	0.705	0.709	0.174
SQSTM1 [a]	0.271	0.430	0.792	0.437	0.606	0.051
Cyclin D1 & BECN1 [b]	0.718	0.734	0.041*	0.399	1.000	0.294
Cyclin D1 & SQSTM1 [b]	1.000	0.480	0.004*	0.724	0.725	0.205
Cyclin D1 & BECN1 & SQSTM1 [b]	0.850	0.721	0.011*	1.000	0.475	0.273

1. All the numbers are *P* values. The significance is set at $P < .05$ (*).

2. The definitions of gender, age, tumor size, viral status, cirrhosis and recurrence are the same as in Supplementary Table 1.

a: Single factor analysis by two-tailed Student's t-test. Results were obtained by IHC staining for cyclin D1, BECN1, and SQSTM1 expression level in the tissue array.

b: Fisher's exact test was used for multiple factor analysis, by which high cyclin D1, low BECN1, and high SQSTM1 expression together was defined as the high-risk group, as shown in Figure 6D. The relationship between high-risk and low-risk groups was compared by this method.

Supporting Materials and Methods

Cell lines and reagents

Hepatoma cell lines (Hep 3B and Huh7), MEF-*Atg5(+/+)*, MEF-*Atg5(-/-)*, MEF-*Sqstm1(+/+)*, and MEF-*Sqstm1*(-/-) were maintained in Dulbecco's modified Eagle's medium (GIBCO, 12100-061) with 10% fetal bovine serum (Biological Industries, 04-001-1A) and incubated at 37 ºC in a 5% CO_2 incubator (1). Amiodarone (Sigma, A8423), rapamycin (Sigma, R0395), chloroquine (Sigma, C6628), and MG132 (Sigma, C2211) were purchased from Sigma.

MTT assay

Cells (4×10^3/well) in the 96-well plates received different treatments for 24 h and 48 h. MTT solution (Sigma, M2128) (0.05 mg/ml in DMEM medium) was added to each well at 37°C for 3 h. The medium was removed and 100 μl of DMSO (Sigma, D4540) was added. Cell proliferation was determined by measuring the cell lysate at the optical density of 540 nm wavelength using a 96-well multiscanner autoreader (Thermo Scientific, Santa Clara, USA).

Flow cytometry analysis for cell cycle

Cells (2×10^5/well) in the 6-well plates received different treatments for 24 h and 48 h. Cells were fixed with 70% ethanol and stored at -20℃ overnight. Cells stained with the cell cycle buffer (contain PI, 0.04 mg/ml, 0.1% Triton-X 100 and Rnase)

followed by flow cytometry analysis.

Flow cytometry analysis for cell viability

Cells (2×10^5/well) were stained with the propidium iodide (PI, 0.04 mg/ml) (Sigma, P4170) followed by flow cytometry analysis.

Autophagosome purification

Hep 3B cells (1×10^6/well) were treated with amiodarone for 24 h followed by CQ treatment for another 24 h. Cells in 10% sucrose (Sigma, S0389) were mixed with 0.5 ml of the buffer (1 M Hepes/0.1 M EDTA) and homogenized using a Dounce homogenizer. This homogenate was diluted with homogenization buffer (HB; 0.25 M sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.3) containing 0.5 mM glycyl-l-phenylalanine 2-naphthylamide (GPN) and 1% DMSO. After incubation for 7 min at 37 °C, the tubes of GPN-treated homogenate were centrifuged at 4000 *rpm* for 2 min to collect the supernatant. A Nycodenz gradient was prepared by diluting isotonic (36% w/v) Nycodenz (Axis-Shield, 1002424) with HB to obtain a top layer of 9.5% Nycodenz and a bottom layer of 22.5% Nycodenz. The Nycodenz gradient was centrifuged at 28000 *rpm* in a SW28 rotor (Beckman) overnight and the gradients were divided into three fractions. The interface band was diluted with HB and layered on top of a discontinuous gradient of 33% Percoll (GE Healthcare, 17-0891-01) in HB on top of 22.5% Nycodenz in HB, and centrifuged for 1 h at 20000 *rpm* in the SW28 rotor.

Autophagosomes formed a band at the lower interface, from which it was recovered for analysis. Percoll was removed from the fractions of the autophagosomes by mixing with isotonic 60% (w/v) iodixanol in water, which was centrifuged for 30 min in the SW40 rotor at 20000 *rpm*. The autophagosome band was collected from the interface band (2).

Immunofluorescent staining

Cells (1×10^5/well) were seeded on a slide. Cells under different conditions were fixed in 4% formaldehyde for 20 min. The slide was incubated for 30 min in 0.1% Triton X-100 in PBS. Anti-LC3 (Medical and Biological Laboratories, PM036), anti-SQSTM1 (Medical and Biological Laboratories, PM045), or anti-cyclin D1 (Abcam, ab16663) antibodies were added on the slide and kept overnight at 4°C. The primary antibody was labeled using the specific secondary antibody with Alexa Fluor 405 (Invitrogen, A31553), Alexa Fluor 488 (Invitrogen, A11008), or Alexa Fluor 568 (Invitrogen, A11004). The fluorescent change of the cells was investigated under a confocal microscope (Olympus, FV-1000, Tokyo, Japan).

Immunoprecipitation

Cells (1×10^6/well) were harvested in lysis buffer and 1 mg of cellular protein was incubated with specific antibodies at 4 ºC overnight. Protein G agarose bead (50 µl) (GE Healthcare, 17-0618-01) was mixed with the immuno-complexes and collected

after centrifugation by adding SDS-PAGE sample buffer and boiling for 10 min. Western blotting was conducted followed by reaction with anti-LC3, anti-cyclin D1, or anti-SQSTM1 antibodies.

BrdU incorporation assay

Cells (1×10^5/well) were seeded in the six-well tray and treated with different conditions. The cells were grown in 0.04 mg/ml bromodeoxyuridine (BrdU) (Sigma, B5002) containing medium for 30 min. For cell cycle progression analysis, the cells were synchronized by serum free starvation for 24 h and then followed by BrdU containing medium with different treatment for 0, 8 and 16 h (3). Cells were fixed in acidic ethanol at -20 ºC for 10 min, and then incubated in 2N HCl for 10 min. Anti-BrdU polyclonal antibody (GE Healthcare, RPN202) at a dilution of 1:400 was added to the well at 4 ˚C overnight. The primary antibody was detected by FITC-conjugated goat anti-Mouse IgG and the nucleus was defined by propidium iodide staining under a fluorescent microscope (Olympus) (4).

Immunohistochemical staining

Slides of paraffin sections treated with anti-cyclin D1 (Abcam, ab134175), anti-SQSTM1, anti-BECN1 (Abcam, ab62472), or anti-ATG5 antibody were processed as described elsewhere (4). Briefly, the slides were labeled with biotin-linked secondary antibody followed by streptavidin (Dako Cytomation, K3461)

treatment for 30 min at room temperature. The slides were treated with AEC solution for 10 min at room temperature and then counterstained with 10% hematoxylin (Muto Pure Chemicals Co, Ltd, 3000-2) and mounted by glycerol gelatin (Sigma, GG1).

Plasmid Construction

The human p-cyclinD1-HA-WT and p-cyclinD1-HA-$K_{33-238}R$ plasmids were gift of Dr. E. Dmitrovsky (Dartmouth-Hitchcock Medical Center, USA). Wild-type cyclin D1 was mutagenized to generate T286A mutant by site-directed mutagenesis using the Quik Change II Site-Directed Mutagenesis Kit (Agilent Technologies, 200523) and verified by sequencing.

Detection of cyclin D1 messenger RNA expression by real-time PCR

Total RNA of cells was extracted using a single-step method with TRIzol reagent (Invitrogen). For reverse transcription-polymerase chain reaction (RT-PCR), the cDNA template was synthesized using 1μg of total RNA with an oligo-dT primer and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, USA). The sequences of PCR primers were as follows:

Cyclin D1 (Human) Forward: 5'-TCAGCCCTGGATTGTTC-3'

Cyclin D1 (Human) Reverse: 5'-AACTTGGGGGCCTACAGCAC-3'

β-actin (Human) Forward: 5'-GGCGGCACCACCATGTACCT-3'

β-actin (Human) Reverse: 5'-AGGGGCCGGACTCGTCATACT-3'

Cyclin D1 (Mouse) Forward: 5'-GAGCCATCCAAACTGAGGA-3'

Cyclin D1 (Mouse) Reverse: 5'-CTCTCCACCTTCTGCCAAA-3'

β-actin (Mouse) Forward: 5'-CTGGAACGGTGAAGGCGA-3'

β-actin (Mouse) Reverse: 5'-GACCATCCTCCTCTTAGGAGTG-3'

Colony formation

Hep 3B cells (1×10^6/well) were transiently transfected with HA-vector or mutant cyclin D1 (p-cyclin D1-HA-$K_{33\text{-}238}R$) [each of the 17 lysines (K) between amino acid 33 to 238 was replaced by arginine (R)] plasmid DNA. Cells were seeded in six-well trays and soft agar analysis was conducted following the protocol described elsewhere (5).

Small hairpin RNA (shRNA) lentiviral infection system

Hep 3B cells (1×10^6/well) were infected with sh-GFP (control), sh-Atg5 or sh-Atg7 lentivirus and incubated at 37 ºC in a 5% CO_2 overnight. Cells were selected by puromycin (2 μg/ml) for 2 weeks. Lentiviral shRNA sequences:

GFP: ACAACAGCCACAACGTCTATA

ATG5-1: CCTTTCATTCAGAAGCTGTTT

ATG5-2: CCTGAACAGAATCATCCTTAA

ATG7-1: GCCTGCTGAGGAGCTCTCCAT

ATG7-2: CCCAGCTATTGGAACACTGTA

Xenograft mouse model

Female, 6-week-old NOD/SCID mice were obtained from the Laboratory Animal Center of National Cheng Kung University. The animals were maintained in a pathogen-free facility under isothermal conditions with regular photoperiods. The experimental protocol adhered to the regulation of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the university. Six-week-old female NOD/SCID mice were injected subcutaneously with Hep 3B cells, Hep 3B cells expressing HA-vector or HA-cyclin D1-K_{33-238}R gene. After tumor cell injection for 3 days, followed by intraperitoneal injection of amiodarone (30 mg/kg) every 3 days for 20 days. Mice were then sacrificed and the tumor weight was measured.

Orthotopic rat model of liver tumor formation

Sprague Dawley (SD) rats were purchased from the Laboratory Animal Center of NCKU. The experimental protocol complied with Taiwan's Animal Protection Act and was approved by the Laboratory Animal Care and Use Committee of NCKU. Four-week-old male SD rats were prepared as previously described (2). Rat hepatoma N1-S1 cells were orthotopically inoculated into the rat livers, followed by intraperitoneal injection of amiodarone (30 mg/kg) or DDW. Rats were sacrificed to collect the livers one week after orthotopical injection.

Hepatitis B virus X protein (HBx) transgenic mice

HBx transgenic mice (C57B/6 background) were generated as previously described.(6) Six- and 16-month-old *HBx* transgenic mice were sacrificed and analyzed.

Statistical analysis

All data are shown as the mean ± SEM. Differences between the experimental and control groups were analyzed by two-tailed Student's *t*-test. Fisher's exact test was used to compare the relationship between BECN1 and cyclin D1, or SQSTM1 and cyclin D1 with clinicopathological parameters. The survival analysis of the HCC patients was conducted by Kaplan-Meier plot, and the Log Rank Test was performed using GraphPad Prism software.

Supporting Figure legends

Supporting Figure 1. High cyclin D1 expression correlates with poor overall survival of HCC patients

(A) The protein expression levels of cyclin D1, SQSTM1, and BECN1 in 20 HCC patients were determined by immunoblotting. N: Non-tumor; T: tumor. (B) The overall survival rate of HCC patients within 4 years after surgery as determined by Kaplan-Meier analysis. *P* values were obtained by long rank test. A total of 147 paired HCC patients were divided into two groups. High cyclin D1 expression group (n=60) showed cyclin D1 expression in the tumor tissue vs. cyclin D1 expression in the adjacent non-tumor tissue > 1-fold and low-cyclin D1 expression group (n=87).

Supporting Figure 2. Cyclin D1 expression correlates with autophagy progression

(A) The Hep 3B cells were infected with lentivirus harboring sh-GFP (control), sh-Atg5, or sh-Atg7 sequence and then treated with amiodarone (5 μM) for 48 h. Cyclin D1 mRNA expression was detected by real-time PCR analysis. (B) MEF-*Atg5(+/+)* and MEF-*Atg5(-/-)* cells treated with amiodarone for 48 h. Cyclin D1 mRNA expression was detected by real-time PCR analysis. (C) Hep 3B and Huh 7 cells were treated with various concentrations of rapamycin and amiodarone for 24 h and 48 h. Cell number was measured by MTT assay. (D) Hep 3B cells (5×10^5/well)

treated with rapamycin or amiodarone for 48 h. Cyclin D1 mRNA expression was detected by real-time PCR analysis.

Supporting Figure 3. Detection of ubiquitinated cyclin D1 in the presence of amiodarone

(A) Hep 3B cells (1×10^6/well) were treated with or without amiodarone (5 μM) for 24 h and total protein extraction was immunoprecipitated by anti-cyclin D1 followed by immunoblotting to evaluate the level of ubiquitin on cyclin D1 by anti-poly-ubiquitin antibody. (B) Hep 3B cells (1×10^6/well) were transfected with wild-type (p-cyclin D1-HA-WT) or mutant cyclin D1 plasmid (p-cyclin D1-HA-$K_{33\text{-}238}$R) and selected by G418 (600 μg/ml) for 2 weeks. Total protein was extracted from total lysates and the expression of HA tag was detected by anti-HA antibody. (C and D) Hep 3B cells were transfected with wild-type (p-cyclinD1-HA-WT) or mutant type cyclin D1 (p-cyclinD1-HA-$K_{33\text{-}238}$R) plasmid and selected by G418 (600 μg/ml) for 2 weeks. Both cell lines were treated with or without amiodarone (5 μM) for 24 h. Total protein extraction was immunoprecipitated by the anti-HA antibody followed by immunoblotting to evaluate the level of ubiquitin by the anti-poly-ubiquitin antibody in (C). Cyclin D1 expression level was detected by immunoblotting after amiodarone treatment for various times in (D).

Supporting Figure 4. Cyclin D1 phosphorylation (Thr-286) is essential for its selective recruitment and binding with LC3

(A) Hep 3B cells treated with amiodarone for various times. The protein expression of GSK-3β was determined by immunoblotting. (B) Hep 3B cells were treated with amiodarone (5 μM) for 24 h followed by CQ (50 μM) treatment for another 24 h. The proteins in the PNS and AP were analyzed by immunoblotting for the expression levels of p-cyclin D1 (Thr-286), LC3, Hsp 60 and calreticulin. (C) Immunogold-labeled p-cyclin D1 (Thr-286) (12 nm gold bead) in the purified double-membrane autophagosomes were detected under TEM. Scale bar= 100 nm. (D and E) Hep 3B cells were transfected with wild-type (p-cyclinD1-HA-WT) or mutated phosphorylation type of cyclin D1 (p-cyclinD1-HA-T286A) plasmid and selected by G418 (600 μg/ml) for 2 weeks. Cells were treated with or without amiodarone (5 μM) for 24 h and total protein extraction was immunoprecipitated by anti-HA in (D) and by anti-LC3 in (E), followed by immunoblotting to evaluate the protein expression level.

Supporting Figure 5. SQSTM1 is a receptor of the ubiquitinated-cyclin D1 to interact with LC3

Hep 3B cells (1×10^6/well) were treated with or without amiodarone (5 μM) for 24 h

and total protein extraction was immunoprecipitated by the anti-cyclin D1 antibody to evaluate the interaction of NBR1 with cyclin D1 in (A), optineurin with cyclin D1 in (B) and NDP52 with cyclin D1 in (C). (D) MEF-*Sqstm1(+/+)* and MEF-*Sqstm1(-/-)* cells were treated with amiodarone (15 μM) for 24 h and the protein extraction was immunoprecipitated with the anti-LC3 antibody followed by immunoblotting to evaluate the expression of cyclin D1 and LC3. (E) MEF-*Sqstm1(+/+)* and MEF-*Sqstm1(-/-)* cells were synchronized by serum-free starvation for 24 h and then DNA synthesis was measured by BrdU incorporation after amiodarone treatment for 0, 8, and 16 h. Scale bar=20 μm. Quantification is shown in the diagram. Data represent means ± SEM (n=5).

Supporting Figure 6. Both autophagic activity and autophagosomes were increased by amiodarone in the liver tumor of an orthotopic rat model

Rat N1-S1 hepatoma cells were orthotopically inoculated into the rat livers, followed by intraperitoneal injection of DDW or amiodarone (30 mg/kg). Rats were sacrificed to collect the livers one week after orthotopical injection (n=5). (A) Protein was extracted from the tumors of DDW and amiodarone treatment groups, and the expression of LC3 and β-actin was detected by Western blotting. (B) Tumor sections of amiodarone treatment were further examined under TEM. Scale bar=2 μm; White box is further

enlarged. Scale bar=500 nm. The arrows point to the double-membrane autophagosomes.

Supporting Figure 7. Liver tumor was suppressed by induction of autophagic activity in a xenograft mouse model

(A) The Hep 3B cells ($5x10^6$) were inoculated s.c. into NOD/SCID mice (n=5). After tumor cell injection for 3 days, mice were injected i.p. with amiodarone (30 mg/kg) every 3 days for 20 days. Mice were sacrificed and the tumor weight was measured at 20 days. Scale bar=1 cm. (B) The same cells as (A) were inoculated into NOD/SCID mice (n=4). After tumor cell injection for 3 days, mice were injected i.p. with rapamycin (3 mg/kg) every 3 days for 21 days. Mice were sacrificed and the tumor weight was measured at 21 days. Scale bar=1 cm. (C) The sections from tumors were derived from Hep 3B cells with or without autophagy inducers (amiodarone or rapamycin) treatment. Cyclin D1 was labeled with red fluorescent tag and LC3 protein was labeled with green fluorescent tag in the sections of tumor tissues. Scale bar=10 μm. Data represent means ± SEM (n=5). (D) and (E). The sections that were the same as (C) were labeled with anti-cyclin D1, anti-SQSTM1, and anti-Ki67 antibodies to detect protein expression in the tissue by IHC. Scale bar=20 μm. Cyclin D1, SQSTM1, and Ki67 levels were determined by ROI followed by HistoQuest analysis and shown

as bar graphs. Data represent means ± SEM (n=5).

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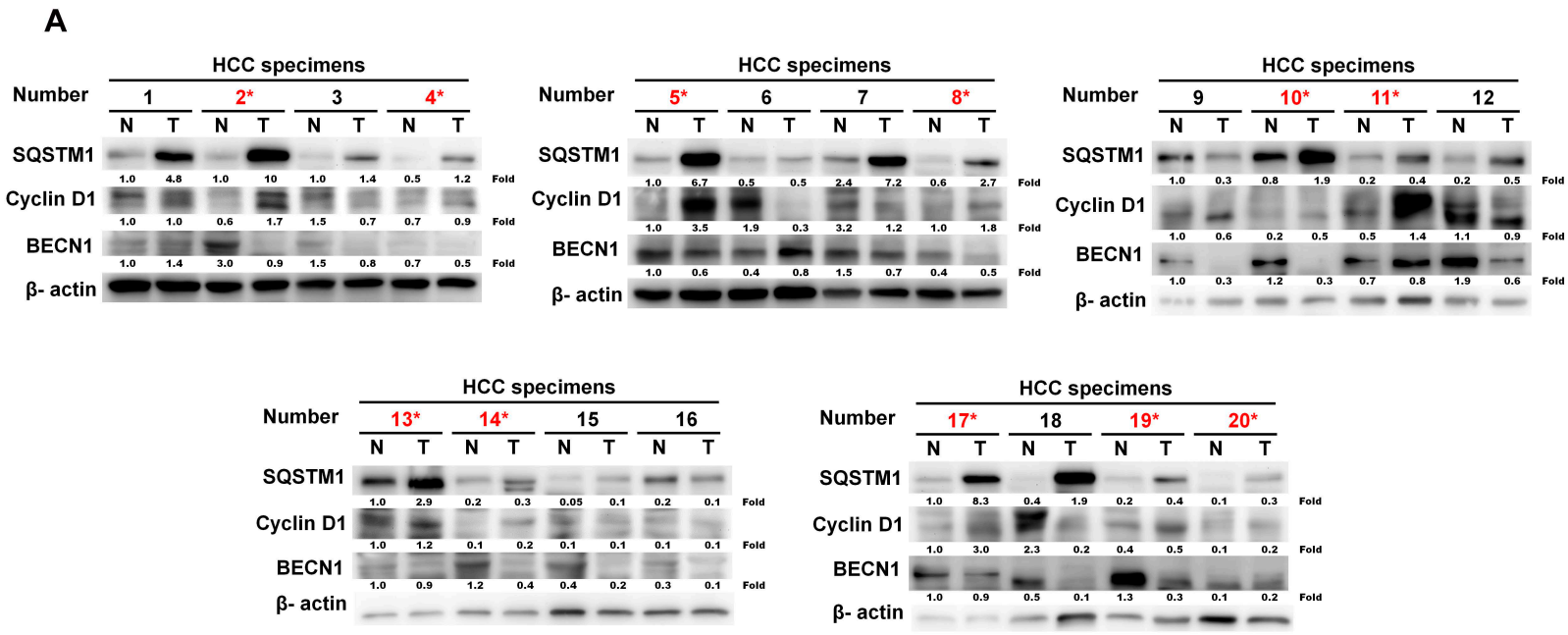
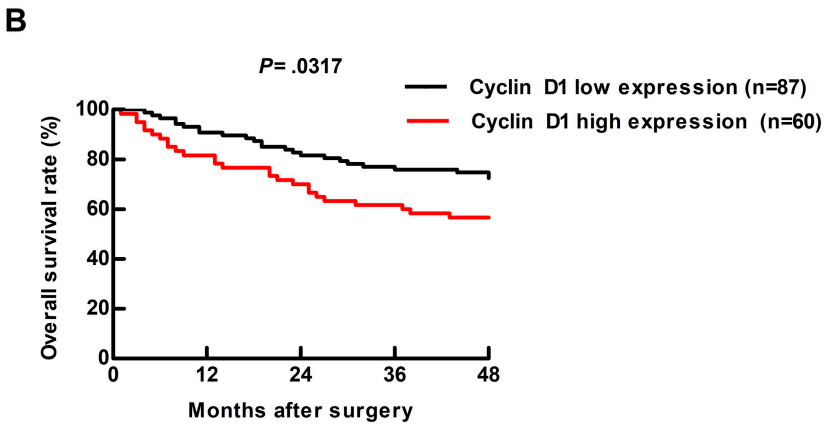
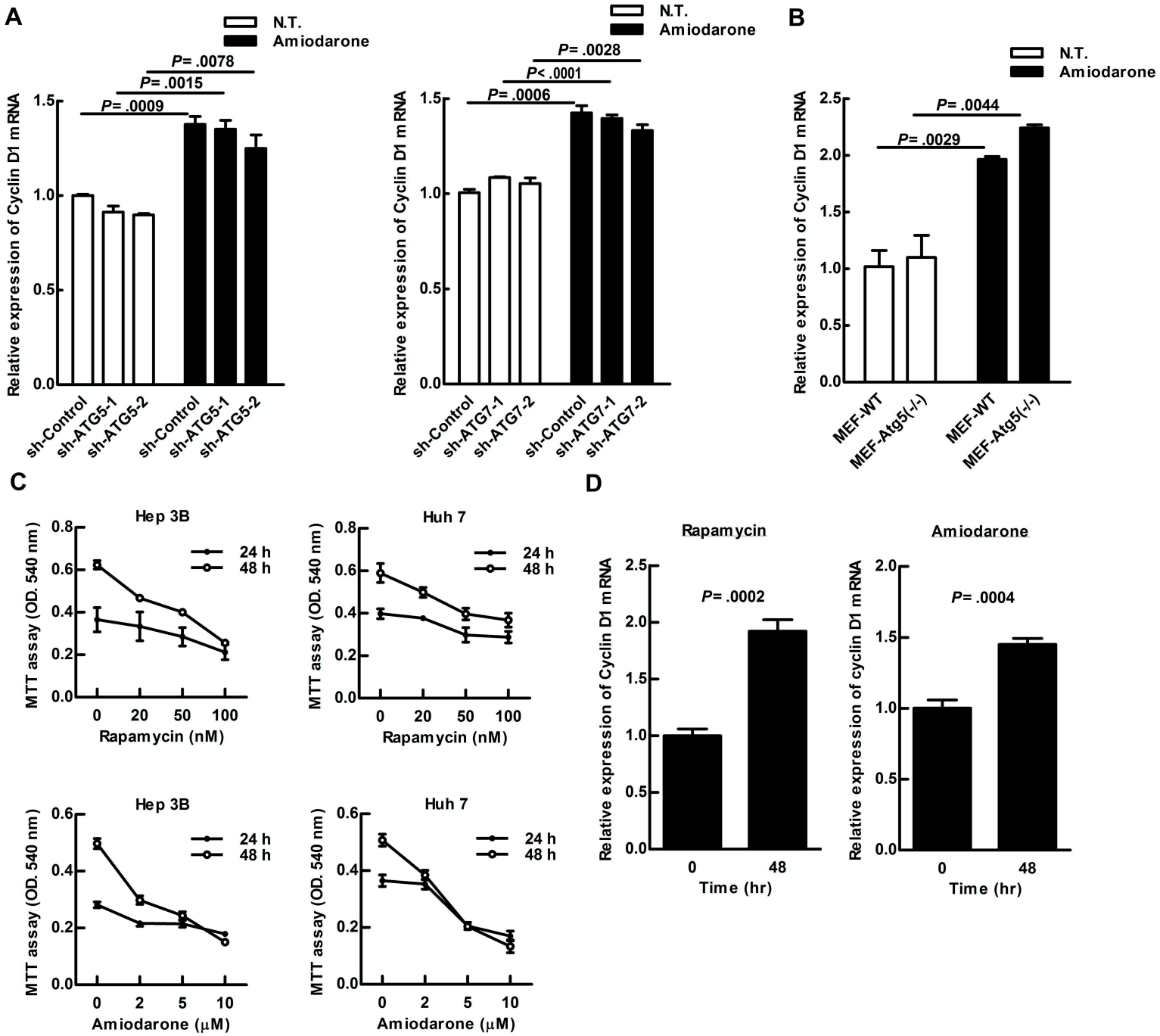
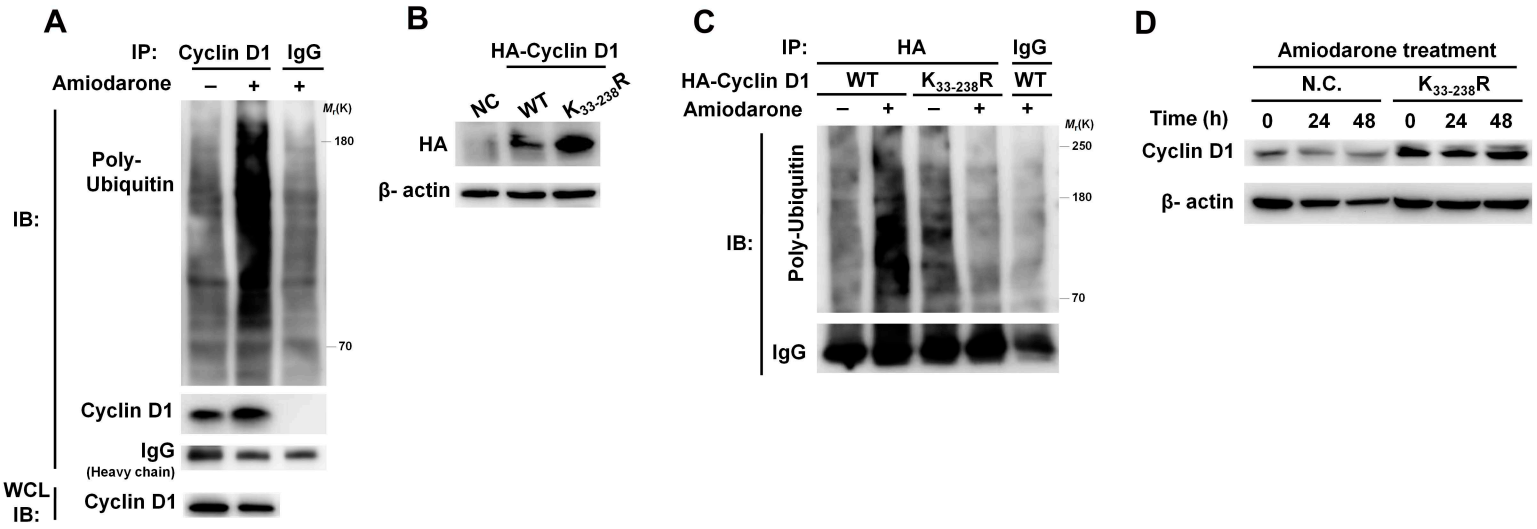




Supporting Figure 1



Supporting Figure 2



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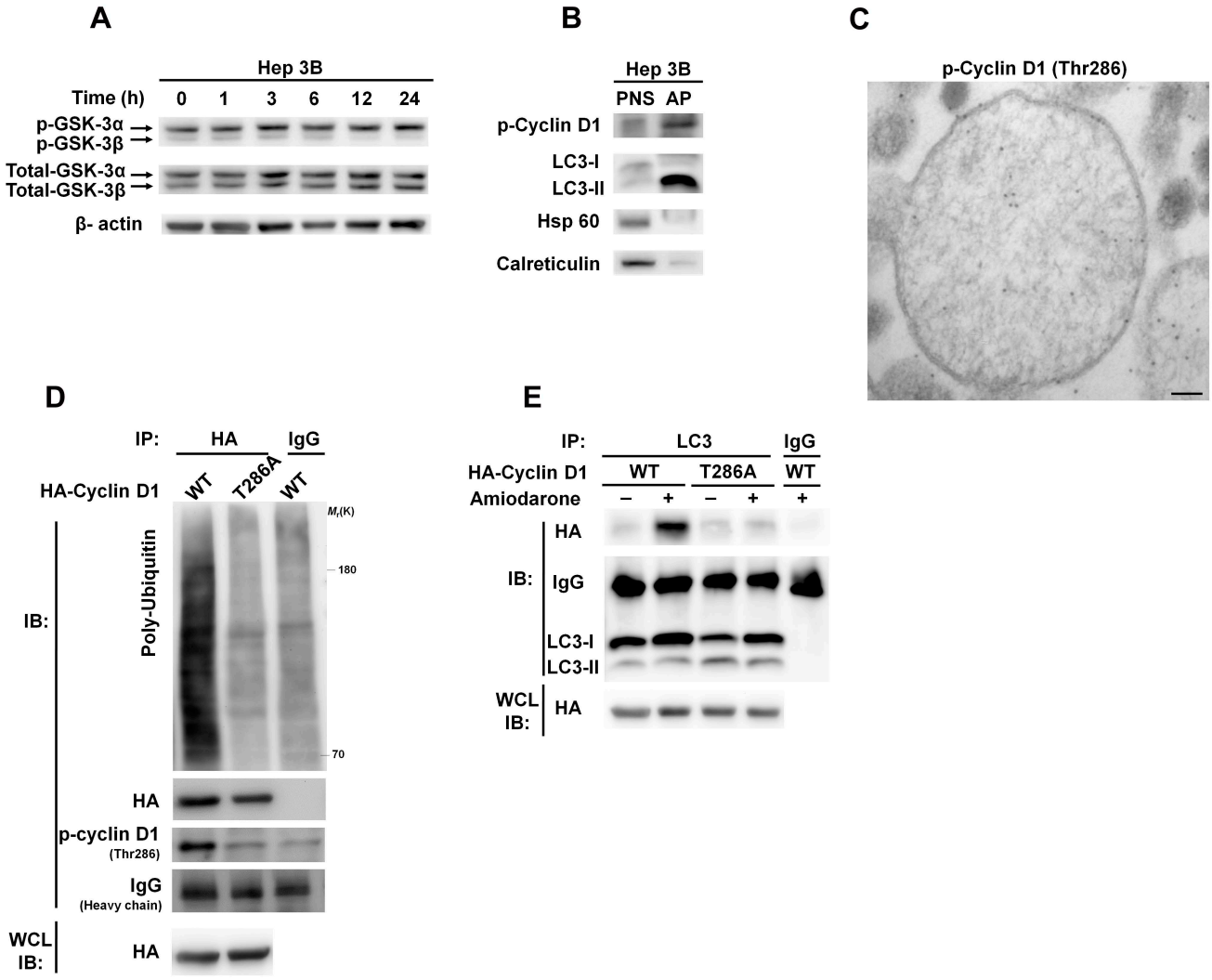
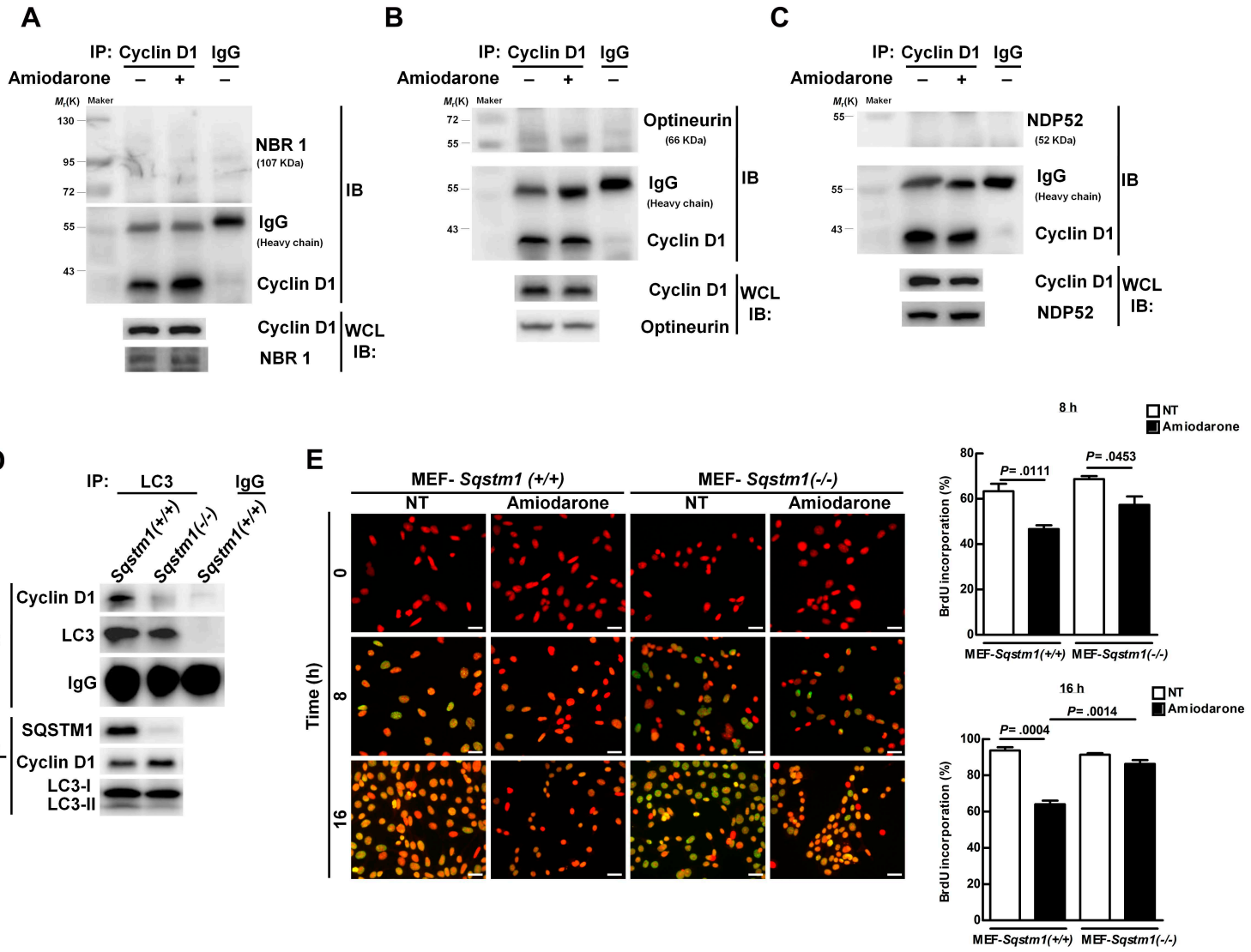
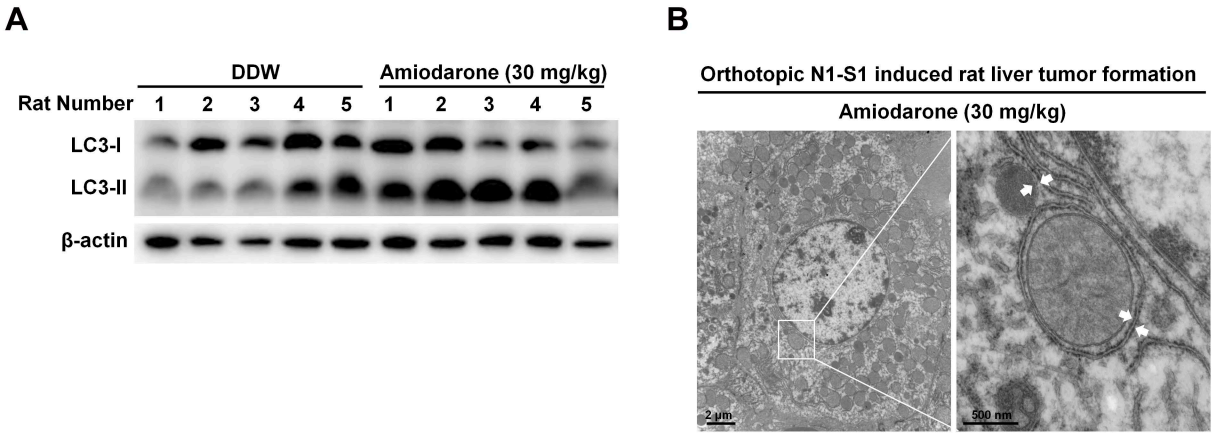


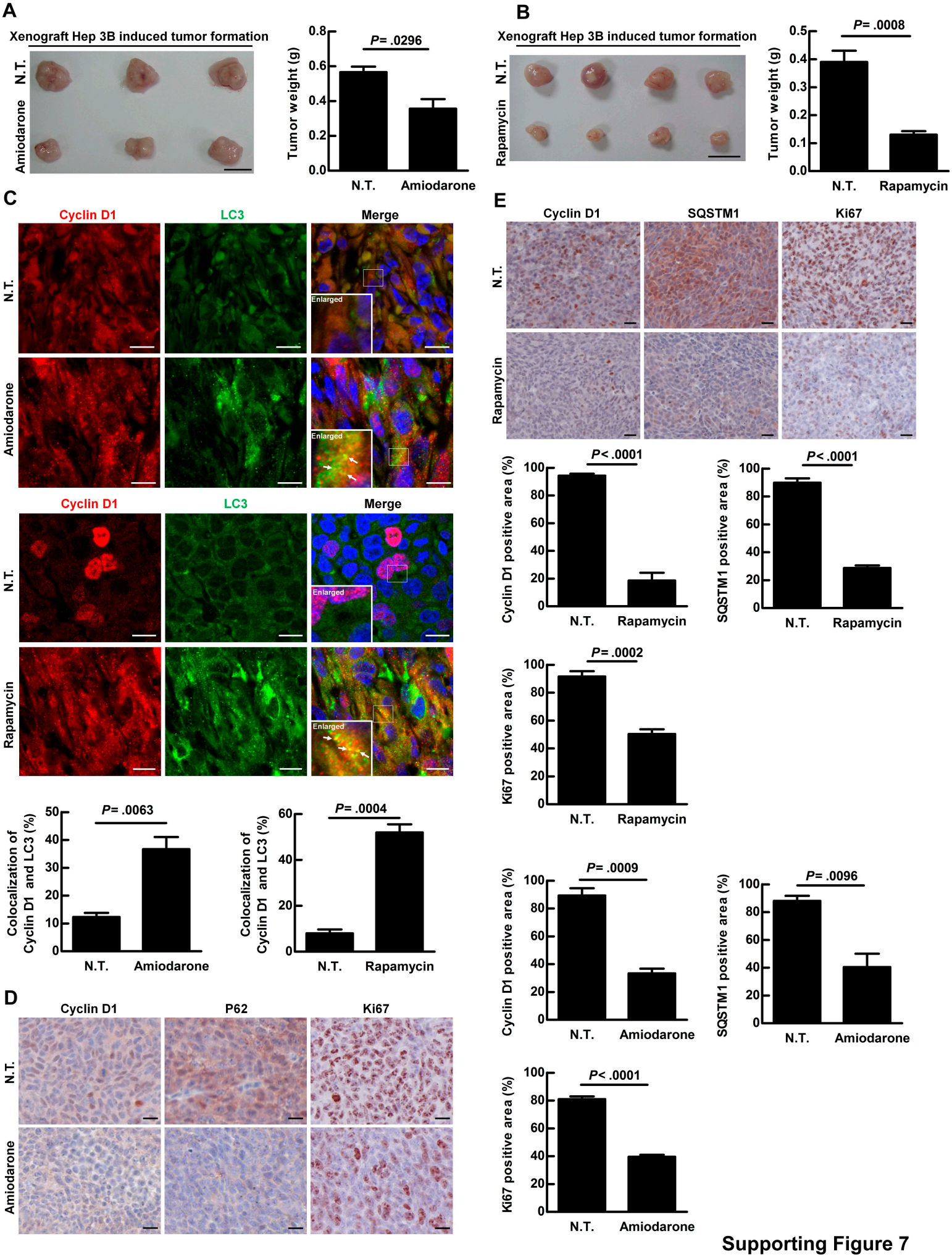
Supporting Figure 4



Supporting Figure 5



Supporting Figure 6



Supporting Figure 7