Supplementary Material for

Akt-Mediated Regulation of Autophagy and Tumorigenesis Through Beclin 1 Phosphorylation

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SUPPLEMENTARY ONLINE MATERIAL

MATERIALS AND METHODS

Plasmids

The following Addgene plasmids were used: Addgene plasmid 15294 (pBabe-Puro-Myr-Flag-AKT1) (*1*), Addgene plasmid 1764 (pBABE-puro) (*2*), Addgene plasmid 1765 (pBABE-hygro), Addgene plasmid 8449 (pUMVC), Addgene plasmid 8454 (pCMV-VSV-G) (*3*), Addgene plasmid 10879 (pLKO.1 TRC control) (*4*), Addgene plasmid 12260 (psPAX2), Addgene plasmid 12259 (pMD2.G), Addgene plasmid 9008 (pcDNA3 Myr HA Akt1), Addgene plasmid 9031 (pcDNA T7 Akt1 K179M T308A S473A) (*5*), Addgene plasmid 15266 (pBabe-Neo-Myr-Flag-AKT1) (*1*), Addgene plasmid 1767 (pBABE-neo) (*2*), Addgene plasmid 18064 (keratin 18 (pcDNA3)), Addgene plasmid 18059 (keratin 18 R89C), and Addgene plasmid 18061 (keratin 18 S33A) (*6*). Vimentin-GFP and vimentin (S39A)-GFP were a gift from D. Lev (*7*). Site-directed mutagenesis of Flag-Beclin 1 was performed using QuikChange XL (Stratagene); wild-type (WT) and mutant constructs were sequenced to confirm that no secondary mutations were introduced. Flag-Beclin 1 constructs (WT, S234A, S295A, and S234A/S295A (AA)) were cloned into pBABE-puro and pBABE-hygro backbones to generate retroviral transfer vectors. Lentiviral-compatible shRNAs against Beclin 1 (RMM4431-101259746, sense: ACAGGAGCTGGAAGATGTGGAA; RHS4430-98520944, sense: AGCCAATAAGATGGGTCTGAAA) and vimentin (RHS3979-9596530, sense:

CGCCATCAACACCGAGTTCAA; RHS4430-99138515, sense: CGCGCTTCCTGGAGCAGCAGAA) were ordered from Open Biosystems.

Antibodies and Additional Reagents

The following antibodies were used for western blot analyses or immunoprecipitation: 14-3-3 (pan) (Santa Cruz, sc-629), 4E-BP1 (Cell Signaling, 9644), p4E-BP1 Thr37/46 (Cell Signaling, 3929), actin (Santa Cruz, sc-47778), Akt (Cell Signaling, 9272), pAkt Ser473 (Cell Signaling, 4058), Atg14 (MBL, PD026), goat anti-Beclin 1 (Santa Cruz, sc-10086), mouse anti-Beclin 1 (Santa Cruz, sc-48341), rabbit anti-Beclin 1 (Santa Cruz, sc-11427), cytokeratin 18 (DC-10) (Santa Cruz, sc-6259), Flag and Flag-agarose (Sigma, F1804), GFP (Invitrogen, A11122), Ki-67 (Abcam, ab16667), LC3 (Novus, NB100-2220), Myc (Santa Cruz, sc-40), p62 (ProGen, 03-GPP62-C), mouse anti-vimentin (Santa Cruz, sc-6260), and rabbit anti-vimentin (Cell Signaling, D21H3). Phospho-Beclin 1 S^{234} and S^{295} antibodies were generated by PhosphoSolutions. Briefly, synthetic peptides corresponding to phosphorylated and dephosphorylated S^{234} and S^{295} of Beclin 1 were used to immunize four rabbits (two per phosphopeptide). Sera were first purified over a phosphopeptide affinity column and then over a dephosphopeptide affinity column. Additional reagents used in the study include: Lipofectamine 2000 (Invitrogen), active recombinant GST-Akt1 (R&D Systems, 1775-KS), Torin1 (gift from D. Sabatini) (*8*), Earle's Buffered Salt Solution (EBSS) (Invitrogen), TUNEL staining kit (Apoptag® peroxidase *In Situ* Apoptosis Detection Kit; Chemicon International), Immunohistochemistry (ABC Elite Kit, Vector Laboratories), puromycin (Sigma), geneticin® (G418) (Gibco), hygromycin (Sigma).

Cell Culture

HEK293T, HeLa, HeLa/GFP-LC3 (*9*), U87-MG and U87-MG + PTEN (gift from P. Mischel and R. DeBerardinis), and Rat2 (ATCC) cells were maintained in DMEM supplemented with penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 10% FBS (Gibco). MDA-MB-231 (ATCC) cells were maintained in RPMI supplemented with penicillin/streptomycin and 10% FBS. MCF10-DCIS (gift from G. Pearson) cells were maintained in 1:1 DMEM:F12 supplemented with penicillin/streptomycin (Gibco), 5% horse serum (Invitrogen), hEGF at 20ng/mL (Lonza), hydrocortisone at 0.5 μg/ml (Sigma), Cholera toxin at 100 ng/ml (Sigma), insulin at 10 μg/ml (Gibco), and 10% FBS (Gibco). WM793B and 451Lu cells (Coriell) were maintained in 4:1 keratinocyte-SFM:Leibovitz L-15 (Invitrogen) supplemented with penicillin/streptomycin (Gibco), insulin at 5 μg/ml (Gibco), and 5% heat-inactivated FBS (Gibco). All cells were maintained in growth-enhanced treated tissue culture flasks (TPP, 90076). For starvation experiments, cells were washed 2X with D-PBS and then incubated for 150 min in either EBSS (starvation medium) or medium containing 1:1 OPTI-MEM:DMEM, 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco), and 1 mM sodium pyruvate (Gibco) (normal medium). For the detection of endogenous phosphorylated Beclin 1 S^{295} in human cancer lines, cells were grown to confluence on two 150-mm dishes and medium was refreshed 120 min prior to harvesting the cells.

For siRNA experiments in HeLa cells, $1.5x10^5$ cells were plated in 60-mm dishes for 24 h. Cells were then transfected with a non-targeting siRNA #3 (Thermo Scientific), or siRNAs targeting human YHAWB (14-3-3β) (Thermo Scientific, L-008766-00-0005) or

YHAWE (14-3-3ε) (Thermo Scientific, MQ-017302-02-0002), at 20 nM using Lipofectamine 2000. Cells were incubated for 48 h, washed, and then transiently transfected with Flag-Beclin 1 and vimentin-GFP. For other transient transfection experiments in HeLa cells, cells were transfected 24 h prior to lysis and co-immunoprecipitation experiments. DN-Akt1 was transfected at a ratio of 3:1 compared to myr-Akt1 to achieve similar levels of protein expression of exogenous Akt1. For GFP-LC3 immunoflourescence assays in HeLa cells, $5x10^4$ HeLa/GFP-LC3 cells were plated directly onto the Lipofectamine transfection mix into 4-well chamber slides (Lab-Tek, 154526). Thirty-six h later, cells were washed and treated with normal or starvation medium for 2 h prior to fixation with 4% PFA. GFP-LC3 puncta were analyzed as previously described (*10*). For GFP-LC3 immunofluorescence assays in Rat2 cells, 7.5 x 10^4 Rat2 cells were plated into chamber slides 24 h prior to transfection with the GFP-LC3 plasmid. Twenty-four h later, cells were fixed and analyzed. In experiments involving vimentin shRNA knockdown, cells were grown in normal medium or subjected to EBSS starvation for 2 h prior to fixation.

Retroviral and Lentiviral Gene Delivery

VSV-G pseudotyped MuLV viruses were generated by transient transfection of HEK293T cells with pUMVC and VSV-G as previously described (*2*). Briefly, HEK293T cells were triple transfected using Lipofectamine 2000 with the transfer vector (e.g. pBABE-puro), pUMVC packaging plasmid, and VSV-G envelope plasmid in a 10:9:1 ratio. The medium was refreshed 12 h later. Forty-eight h after initial transfection, the supernatant with viral particles was filtered through a 0.5 μm filter. The filtered virus

was mixed with ~30% fresh medium and polybrene was added to 6 μm/ml. This mixture was placed on target cells and placed 2X again at 12 h intervals. Retrovirally-transduced cells were selected in 2 μg/ml puromycin for two days, 100 μg/ml hygromycin for 2 days, or 600 μg/ml G418 for 4 days.

VSV-G pseudotyped lentiviruses were generated by transient transfection of HEK293T cells with pUMVC and VSV-G as previously described (*2*). Briefly, HEK293T cells were triple transfected using Lipofectamine 2000 with the transfer vector (e.g. pLKO puro), psPAX2 packaging plasmid, and pMD2.G envelope plasmid in a 4:3:1 ratio. Lentivirally-transduced cells were selected in 2 μg/ml puromycin for 2 days.

Immunprecipitations and Western Blot Analyses

HeLa (100-mm dish) and HEK293T (60-mm dish) cells were harvested 24-28 h after transfection for co-immunoprecipitation assays. HEK293T cells were rinsed off the plate with ice-cold PBS, washed, and then lysed in IP lysis buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1x phosphatase inhibitor mix (Pierce), and 1x protease inhibitor mix (Roche)). HeLa cells were rinsed with ice-cold PBS and then scraped into IP lysis buffer. Cell lysates were rotated at 4°C for at least 30 min, and then the soluble fraction was isolated by centrifugation at 14,000 rpm for 10 min at 4°C. A fraction of the clarified lysate was used for western blot analysis. Primary antibodies or agarose-conjugated antibodies (Flag- and Myc-) were added to the lysates and rotated for at least 4 h at 4°C, and then 20-60 μl of a Protein G sepharose slurry (50% v/v) (Santa Cruz) was added for at least 1 h. Immunoprecipitates were washed 3X with cold lysis buffer. Whole cell lysates and immunoprecipitated proteins were boiled in 30-40 μl

sample buffer, separated by SDS-PAGE on precast 4-15% gels (BioRad), transferred, and blotted with the antibodies described.

For analysis of endogenous protein interactions, 150-mm dishes of HeLa cells and Rat2 cells were used to generate whole cell lysates. For solubilization of keratin and vimentin, cells were lysed in Empigen lysis buffer (20 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 2.8% Empigen BB (Sigma), 1x Halt Phosphatase Inhibitor Cocktail (Pierce), and 1x Protease Inhibitor Cocktail (Roche)). Empigen lysates were rotated at 4°C for at least 30 min, and then the soluble fraction isolated by centrifugation at 14,000 rpm for 10 min at 4°C. Lysates were the diluted to 1.4% Empigen with lysis buffer without detergent prior to addition of the primary antibody. For analysis of the effects of starvation on co-immunoprecipitation, cells were incubated in starvation or normal medium for 2 h immediately prior to harvesting. For proteins requiring immunoprecipitation and detection by the same antibody (keratin 18, GFP), an IP-Western kit (Genscript) was employed according to the manufacturer's instructions. For detection of endogenous p-Beclin S^{295}, the primary antibody (Phosphosolutions) was washed for 4 x 6 min in PBS-Tween0.1% and incubated with mouse/human-adsorbed donkey anti-rabbit IgG-HRP (Santa Cruz, sc-2305) at 1:4000 for 1 h prior to developing. For experiments involving immunoprecipitation and western blot detection of Beclin 1, a mouse anti-Beclin 1 monoclonal Ab (described above) was used for immunoprecipitation and a rabbit anti-Beclin 1 polyclonal Ab (described above) was used for western blot analysis.

***In vitro* Kinase Assay**

Flag-Beclin 1 or Flag-Beclin 1 AA was transfected into HEK293T cells (100 mm dish). Twenty-four h later, cells were lysed with IP lysis buffer *without* phosphatase inhibitor and immunoprecipated with anti-Flag-agarose as described above. The agarose-bound Flag-Beclin 1 was washed in IP buffer 3X and then treated with 20U of Lambda Phosphatase (NEB) in phosphatase buffer for 30 min at 30°C. The dephosphorylated slurry was washed 3X with 1% Triton X-100, 0.1% SDS, 20mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 1x Halt Phosphatase Inhibitor Cocktail (Pierce), and 1x Protease Inhibitor Cocktail (Roche), and then washed 3X with Akt kinase assay buffer (Cell Signaling). The slurry was incubated with 1 μg GST-Akt1, 0.2 mM ATP, and kinase assay buffer (Cell Signaling) in a total volume of 50 μl for 30 min at 30°C. The reactions were stopped by the addition of 50 μl 2x sample buffer (BioRad), boiled, separated by SDS-PAGE, transferred and blotted with phosphospecific antibodies as described above. For Akt inhibitor treatments, the slurry was first incubated with MK-2206 (1 μM) (Selleckcehm), Akt inhibitor X (1 μM) (Calbiochem), or DMSO control for 15 min on ice prior to the addition GST-Akt1.

Lipid Kinase Assay

Cells were lysed in IP lysis buffer. Immunoprecipitates were obtained using a mouse anti-Beclin 1 (Santa Cruz, sc-48341) antibody. The IP mixture was washed 2X with lysis buffer, 2X with TNE (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and phosphatase inhibitor mix), and then resuspended in 50 μL TNE. Beads were incubated in 80 μl kinase buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 20 μg phosphoinositide (Avanti polar lipids), 45 μM ATP, 10 μCi ^{32}P-ATP) at

22 °C for 20 min. The reaction was stopped by adding 20 μl 8M HCl and the lipids were extracted with 160 μl $CHCl_3$:MeOH (1:1; v/v) followed by centrifugation at 6000 rpm for 5 min. Phosphorylated lipids (20 μl) were spotted on a thin-layer chromatography (TLC) plate and separated with chloroform/methanol/water/ammonium at a ratio of 60:47:11.3:2.

Soft Agar Assay for Colony Formation

Assays were performed in quadruplicate in 6-well plates. Base agar consisted of 0.5% agar (BD Biosciences), 1x DMEM, 1 μg/ml puromycin, and 500 μg/ml G418 and 10% FBS. Top agar consisted of 0.3% agar, 1x DMEM, 10% FBS with 2.5 x 10^4 Rat2 cells per well. Cells were incubated in 5% CO_2 at 37^0C for 21 days. To quantify the colonies, three randomly selected images of each replicate were captured as grayscale images on an inverted dissecting scope (Zeiss SteREO Discovery.V12). The size and number of colonies were determined using the "Analyze Particles" algorithm in ImageJ. The images, colony number, and colony size data are representative of 2 independent retroviral transduction experiments.

Tumor Xenograft Studies

To measure tumor formation by Akt1- and Beclin 1-retrovirally transduced Rat2 fibroblasts, 6 week-old NOD SCID® mice (JAX Stock Number 001303, Jackson Laboratories) were shaved on the right flank one day prior to injection and injected subcutaneously with 1×10^7 tumor cells in 200 μl of PBS. For all Rat2 cell lines expressing myr-Akt1, 9-10 mice per cell line were injected and for cell lines lacking myr-Akt1, 5 mice per cell line were injected. Tumor growth was monitored by daily

measurements of tumor length (L) and width (W) and tumor volume was estimated using the formula (tumor volume = ½(L × W^2)). After 21 days, mice were sacrificed and the tumors were dissected and weighed. Tumors were fixed in 3% paraformaldehyde, embedded in paraffin, sectioned, and stained by H & E. TUNEL staining was performed according to the manufacturer's instructions, using Sigma *FAST*™ 3, 3′-diaminobenzidine (DAB) tablets as the peroxidase substrate. Immunohistochemical staining of paraffin-embedded tumor tissues was performed using Ki-67 (1:100) and p62 (1:1000) primary antibodies and the ABC Elite immunoperoxidase kit according to the manufacturer's instructions. All animal procedures were performed in accordance with institutional guidelines and with approval from the Institutional Animal Care and Use Committee.

Immunoflourescence and Imaging

For colocalization immunoflourescence assays, $7.5x10^4$ Rat2 fibroblasts were plated into 4-well chamber slides (Lab-Tek, 154526) for at least 24 h. Slides were fixed with methanol at -20°C for 5 min and processed for immunoflourescence as previously described (*11*). For primary antibody incubation, slides were incubated with rabbit anti-Beclin 1 (Santa Cruz, sc-11427) and mouse anti-vimentin (Santa Cruz, sc-6260) or mouse anti-Beclin 1 (Santa Cruz, sc-48341) and rabbit anti-vimentin (Cell Signaling, D21H3) overnight at 4°C. Slides were embedded with Prolong Gold with DAPI (Invitrogen). Z-stacks were acquired with a Zeiss AxioImager M2 microscope equipped with a Photometrics CoolSnap HQ2 camera and a Zeiss PLAN APO 40X/1.3 NA objective using the same acquistion times for each sample. Z-stacks were deconvolved with AutoDeBlur (Bitplane) using a blind deconvolution algorithm (30 iterations, medium

noise) and Imaris version 7.4.0 (Bitplane) was then used to examine the deconvolved images. Background signals from samples stained with secondary antibody alone and acquired with identical exposure times for each antibody pair were used to determine thresholds for positive signal. Representative images were chosen and exported from the Slide module in Imaris after examining >50 cells in triplicate for each condition using identical settings for each set of control versus experimental conditions.

Statistical Analysis

A t-test was used for direct comparison of means between two different groups. For data involving multiple comparisons, ANOVA analysis was used with Tukey–Kramer adjustment for multiple comparisons. For comparisons of the magnitude of changes in response to a treatment between two different cell types, a 2-way ANOVA test was used. Linear mixed effect models were used to compare the different changes (slopes of linear curves, Fig. 2D) among groups. The model compares the differences between groups, while accounting for the correlation within the same subject.

SUPPLEMENTARY TABLES

Supplementary Table 1

This table describes the histopathological characteristics of xenografts formed by Akt1-transformed Rat2 fibroblasts.

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Supplementary Table 1. Histopathological Characteristics of Xenografts formed by Akt1-Transformed Rat2 Fibroblasts

Xenograft Genotype	Sample	Pattern of growth	Infiltrative edges	Nuclear pleomorphism present	Mean No. Mitotic Figures per High Power Field (400x)
myr-Akt1 + Vector	1	Fascicular and storiform	+	-	6.3
myr-Akt1 + Vector	2	Fascicular and storiform	+	+	6.3
myr-Akt1 + Vector	3	Fascicular and storiform	+	+	6.3
myr-Akt1 + Vector	4	Fascicular and storiform	+	-	10.7
myr-Akt1 + Vector	5	Fascicular and storiform	+	-	6.7
myr-Akt1 + Vector	6	Fascicular and storiform	+	-	8.3
myr-Akt1 + Vector	7	Fascicular and storiform	+	+	13.7
myr-Akt1 + Vector	8	Fascicular and storiform	+	-	10.0
myr-Akt1 + Vector	9	Fascicular and storiform	+	-	8.0
myr-Akt1 + Beclin 1	10	Fascicular and storiform	+	+	8.0
myr-Akt1 + Beclin 1	11	Fascicular and storiform	+	+	7.7
myr-Akt1 + Beclin 1	12	Fascicular and storiform	+	-	7.3
myr-Akt1 + Beclin 1	13	Fascicular and storiform	+	+	9.7
myr-Akt1 + Beclin 1	14	Fascicular and storiform	+	+	8.0
myr-Akt1 + Beclin 1	15	Fascicular and storiform	+	-	9.0
myr-Akt1 + Beclin 1	16	Fascicular and storiform	+	+	9.7
myr-Akt1 + Beclin 1	17	Fascicular and storiform	-	+	10.3
myr-Akt1 + Beclin 1	18	Fascicular and storiform	+	-	5.7
myr-Akt1 + Beclin 1 AA	19	Disorganized	-	-	3.3
myr-Akt1 + Beclin 1 AA	20	Disorganized	-	-	4.3
myr-Akt1 + Beclin 1 AA	21	Disorganized	-	-	3.7
myr-Akt1 + Beclin 1 AA	22	Disorganized	-	-	3.0
myr-Akt1 + Beclin 1 AA	23	Focal loose fascicles	-	+	3.3
myr-Akt1 + Beclin 1 AA	24	Disorganized	-	-	1.7
myr-Akt1 + Beclin 1 AA	25	Focal loose fascicles	-	-	5.7
myr-Akt1 + Beclin 1 AA	26	Disorganized	-	-	3.3
myr-Akt1 + Beclin 1 AA	27	Focal loose fascicles	+	-	5.3
myr-Akt1 + Beclin 1 AA	28	Disorganized	-	-	1.7

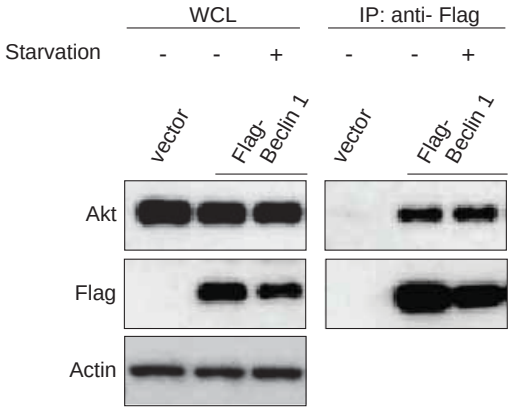

Fig. S1. Active Akt interacts with Beclin 1 in a nutrient-independent manner. Co-immunoprecipitation of Akt with anti-Flag antibody in HeLa cells co-transfected with HA-myr-Akt1 and a control vector or Flag-Beclin 1 during growth in normal medium or in EBSS for 2 h. WCL, whole cell lysates.

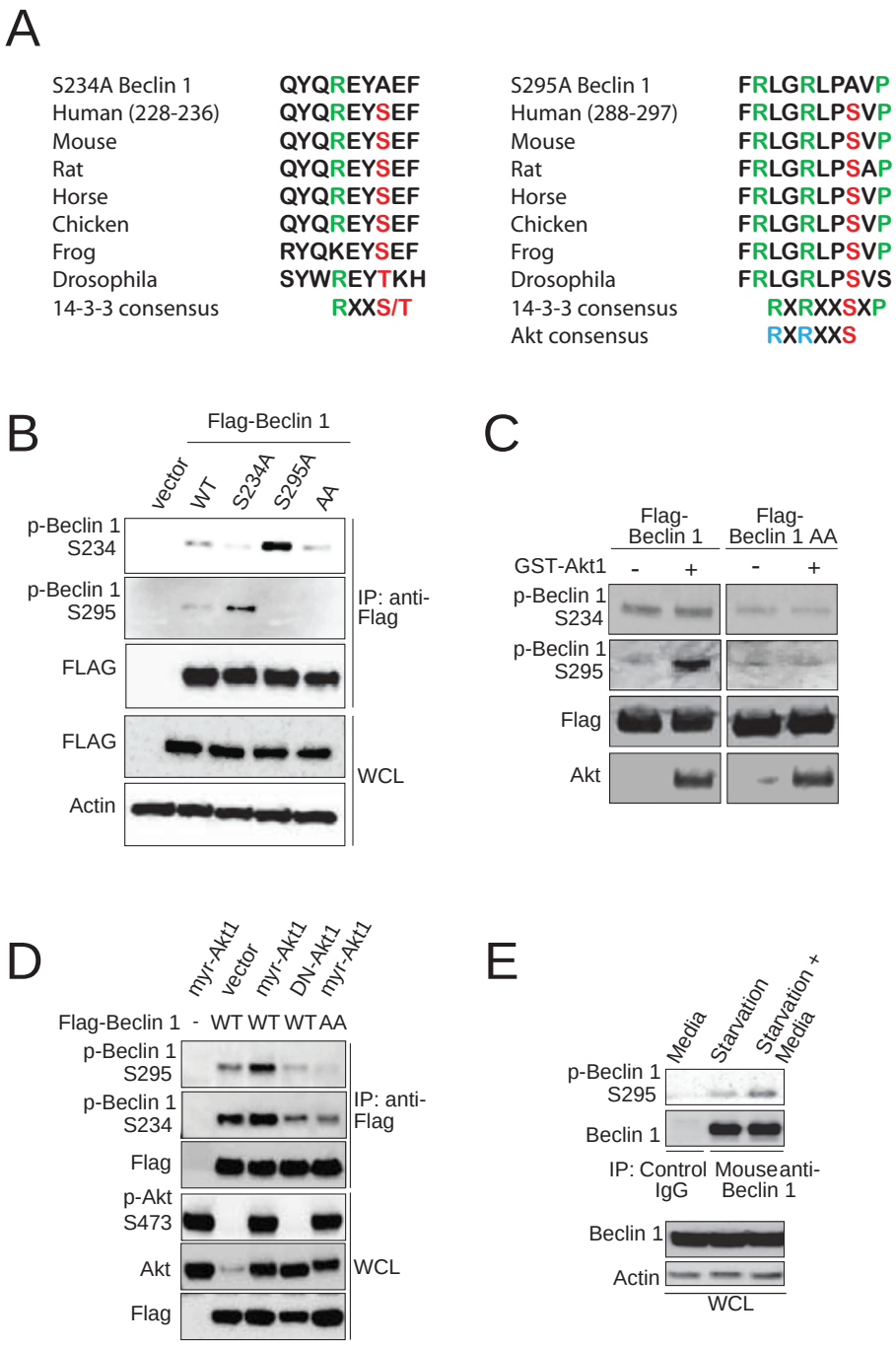

Fig. S2. Characterization of phosphorylation sites in Beclin 1. **(A)** Alignment of the two conserved phosphorylation sites in Beclin 1 (S^{234} and S^{295}). Both sites are predicted to be 14-3-3 binding motifs. Ser^{295} is also predicted to be a possible Akt substrate. **(B)** Western blot analysis of wild-type Beclin 1, Beclin 1 S234A, Beclin 1 S295A, and Beclin 1 S234A/S295A (AA) constructs using phosphospecific antibodies generated against phosphorylated S^{234} and phosphorylated S^{295}. HeLa cells were transfected with control vector or the indicated Flag-Beclin 1 constructs. **(C)** *In vitro* kinase assay to detect Akt phosphorylation of Beclin 1. Lysates from HEK293T cells transfected with Flag-Beclin 1 or Flag-Beclin 1 AA (S234A/S295A) were immunoprecipitated with anti-Flag, and the immunoprecipitates were used as substrates for purified, active GST-Akt1. Beclin 1 phosphorylation was detected using the indicated phosphospecific antibodies. **(D)** Effects of Akt on phosphorylation of Beclin 1 S^{295} and Beclin 1 S^{234} in HeLa cells transfected with myr-Akt1 or DN-Akt1 and Flag epitope-tagged wild-type Beclin 1 or Beclin 1 AA constructs as indicated. **(E)** Comparison of Beclin 1 S^{295} phosphorylation during starvation conditions or following 15 min of exposure to normal medium after starvation. WCL, whole cell lysates.

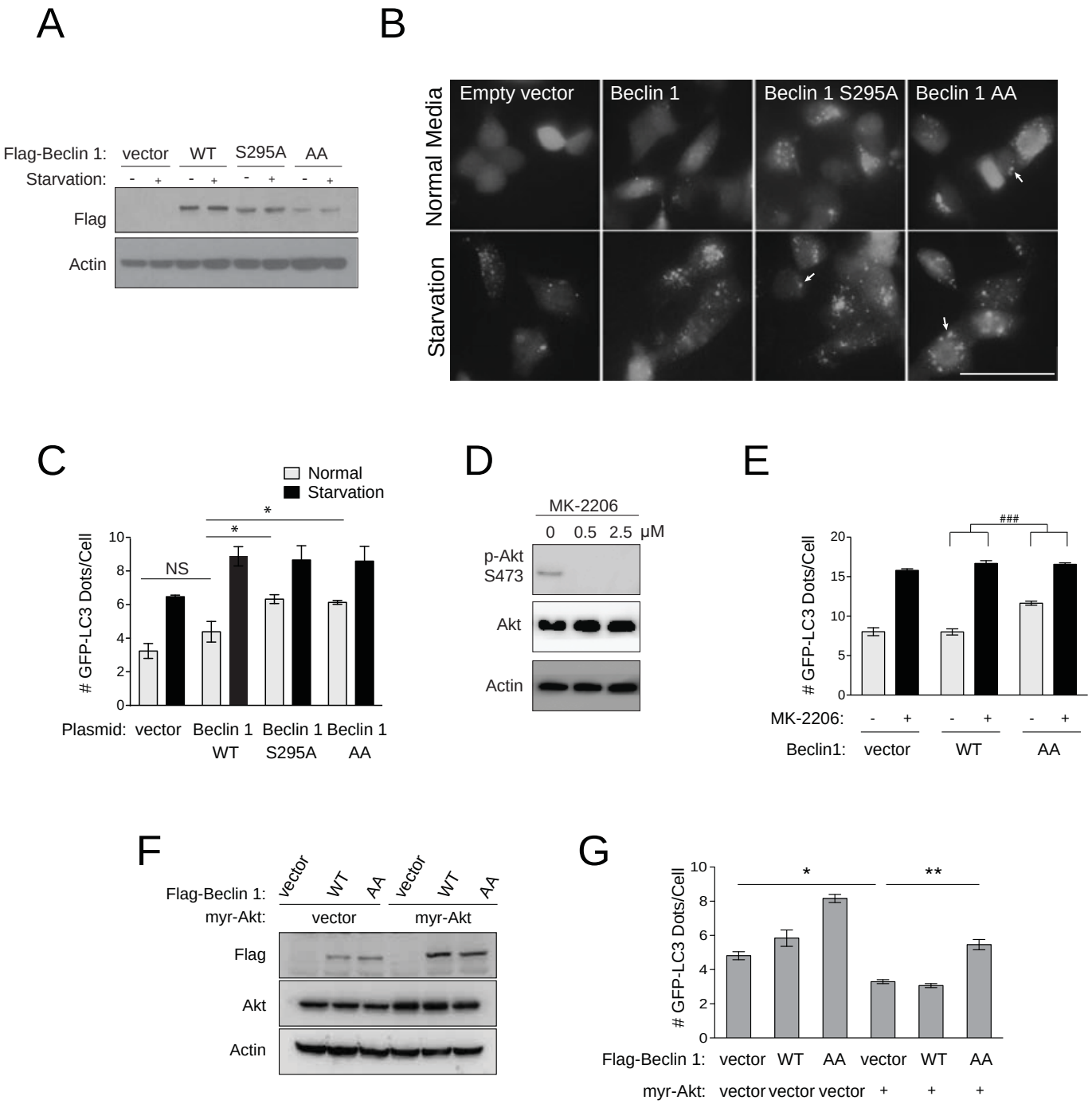

Fig. S3. Effects of Beclin 1 phosphorylation site mutants and Akt on autophagy in MCF7 cells. **(A)** Western blot detection of Flag-Beclin 1 expression in MCF7 cells transfected with indicated Beclin 1 constructs during growth in normal medium and in starvation conditions. **(B-C)** Representative images **(B)** and quantitation **(C)** of GFP-LC3 dots in MCF7 cells transfected with the indicated Beclin 1 expression plasmids in normal conditions or starvation conditions (EBSS for 2 h). Arrows in **(B)** denote representative autophagosomes. **(D)** Western blot analysis of Akt activation, as measured by phosphorylation of Akt S^{473}, in MCF7 cells treated with indicated amount of the Akt inhibitor MK-2206 for 1 h. **(E)** Quantitation of the number of GFP-LC3 dots per cell in MCF7 cells transfected with indicated plasmids with or without treatment with an Akt inhibitor (MK-2206, 2.5 μm for 2 h). **(F)** Western blot analyses to detect levels of Flag-Beclin 1 and Akt expression in MCF7 cells transfected with indicated plasmids. **(G)** Quantitation of the number of GFP-LC3 puncta in MCF7 cells co-transfected with indicated Beclin 1 and myr-Akt1 expression plasmids and grown in normal medium. For (C. E, and G), bars represent mean ± SEM of triplicate samples with >50 cells analyzed per sample. Similar results observed in 3 independent experiments. NS, not significant, **P*<0.05; ***P*<0.01, Tukey test. $^{###}$P<0.001; 2-way ANOVA. Scale bar, 50 μm.

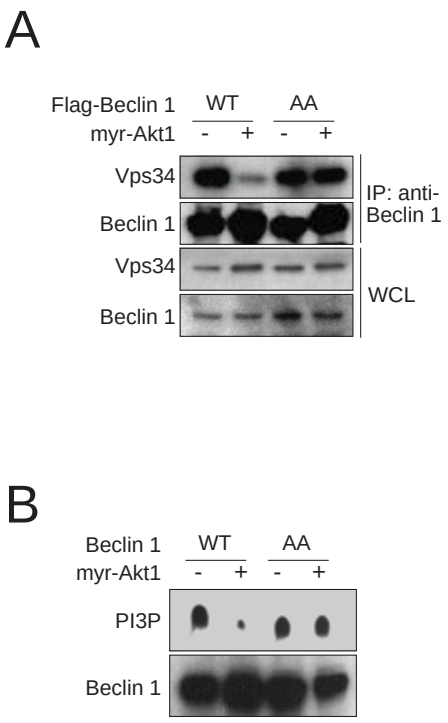

Fig. S4. Effects of active Akt on Beclin 1/Vps34 interactions and Beclin 1-associated Vps34 lipid kinase activity. **(A)** Immunoprecipitation of endogenous Vps34 with anti-Beclin 1 in Rat2 cells stably transduced with indicated Flag-Beclin 1 and Akt constructs. **(B)** Vps34 lipid kinase activity in Rat2 cells transduced with indicated retroviral vectors and immunoprecipitated with anti-Beclin 1. Upper gel, PI3P from lipid kinase assay; lower gel, protein immunoblot detection of Beclin 1 in immunoprecipitates.

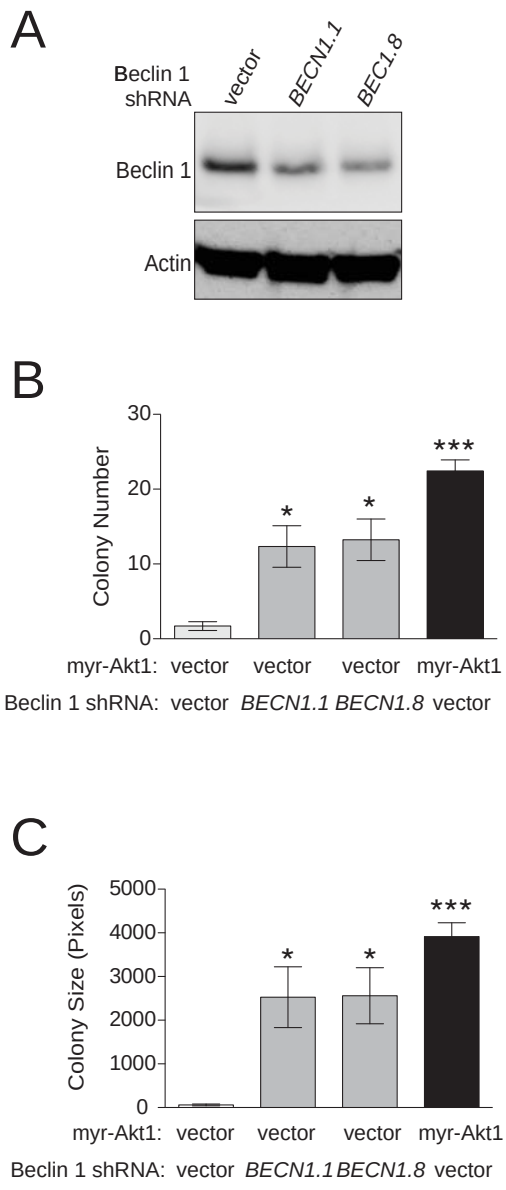

Fig. S5. Effects of Beclin 1 knockdown in Rat2 fibroblasts on colony formation in soft agar. **(A)** Western blot detection of Beclin 1 in Rat2 fibroblasts stably transduced with lentivirus vectors expressing indicated Beclin 1 shRNAs. **(B)** Quantitation of numbers of soft agar colonies formed by Rat2 fibroblasts transduced with indicated vectors (Beclin 1 shRNA or myr-Akt1 expression). **(C)** Quantitation of the size of soft agar colonies formed by Rat2 fibroblasts transduced with indicated vectors. For **B**-**C**, bars represent mean ± SEM of triplicate samples with ≥12 random images analyzed per sample by ImageJ. **P*<0.05, ***P*<0.01,*** *P*<0.001; t-test for comparisons with the first bar in graph.

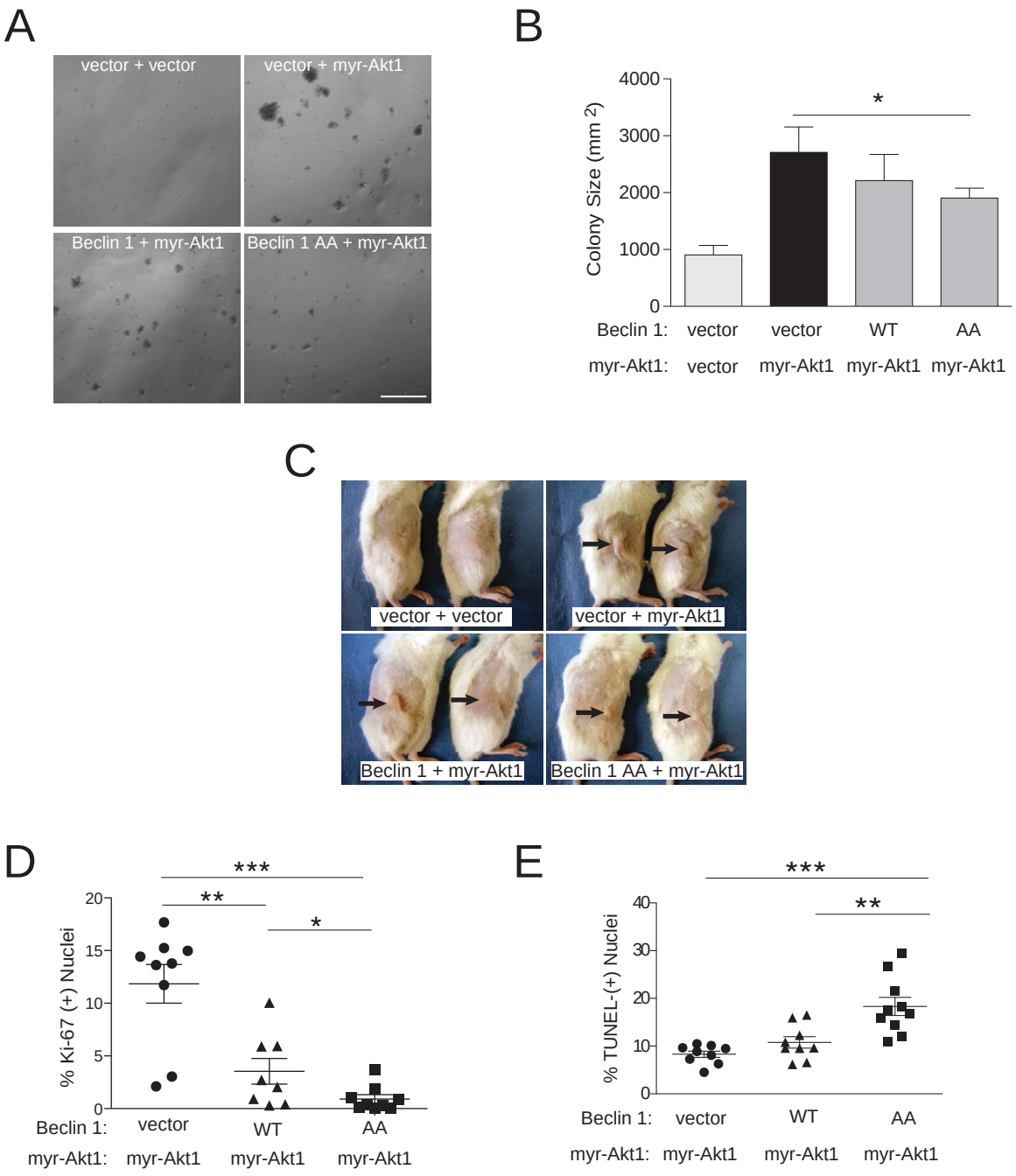

Fig. S6. Effects of Beclin 1 overexpression on Akt-mediated Rat2 fibroblast transformation. **(A)** Images of soft agar colonies formed by Rat2 fibroblasts tranduced with indicated myr-Akt1 and Beclin 1 vectors. Colonies were imaged 21 days after plating. Scale bar, 500 μm. **(B)** Quantitation of the size of soft agar colonies formed by Rat2 fibroblasts transduced with indicated myr-Akt1 and Beclin 1 vector. Bars represent mean ± SEM of triplicate samples of ≥12 random images analyzed by ImageJ. **P*<0.05; t-test. **(C)** Representative photomicrographs of tumors formed in NOD SCID® mice 21 days after subcutaneous injection prior to necropsy and tumor dissection. **(D-E)** Quantitation of percentage of cells in indicated tumor genotype with nuclear Ki-67 (D) or TUNEL (E) staining. Results represent mean ± SEM for all tumors in each genotype (9-10 per group). **P*<0.05,***P*<0.01,****P*<0.001, Tukey test.

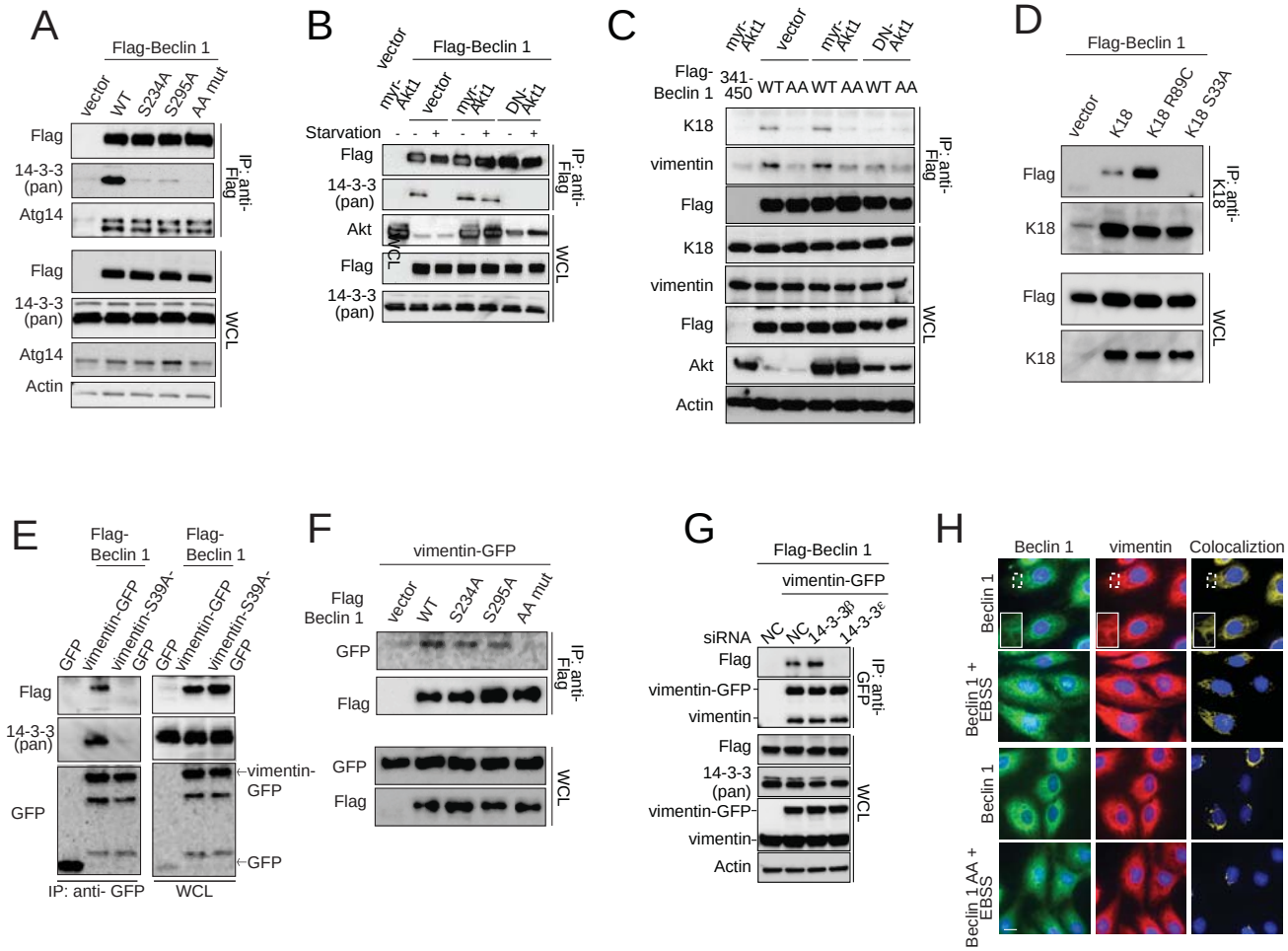

Fig. S7. Interactions between Beclin 1, 14-3-3 proteins, and intermediate filament proteins. **(A)** Co-immunoprecipitation of 14-3-3 proteins with Flag-Beclin 1 in HeLa cells transfected with indicated wild-type or mutant Beclin 1 plasmids. **(B)** Immunoprecipitation of 14-3-3 proteins with Flag-Beclin 1 in HeLa cells transfected with indicated Flag-Beclin 1 and Akt1 expression plasmid with or without starvation for 2 h. **(C)** Immunoprecipitation of keratin 18 (K18) or vimentin with Flag-Beclin 1 in HeLa cells transfected with indicated Flag-Beclin 1 and Akt constructs. Lane labeled 341-450 represent a construct expressing Flag epitope-tagged Beclin 1 amino acids 341-350. **(D)** Co-immunoprecipitation of Flag-Beclin 1 with wild-type or mutant forms of keratin 18 (K18) in HEK293T cells transfected with Flag-Beclin 1 and indicated K18 constructs. The K18 R89C mutation is known to increase 14-3-3 binding and the K18 S33A mutation is known to decrease 14-3-3 binding (6). **(E)** Co-immunoprecipitation of Flag-Beclin 1 and 14-3-3 proteins with vimentin-GFP in HeLa cells cotransfected with Flag-Beclin 1 and indicated vimentin constructs. Vimentin S39 has previously been shown to be an Akt substrate (*7*). Arrows indicate GFP and vimentin-GFP bands. **(F)** Co-immunoprecipitation of vimentin-GFP with Flag-Beclin 1 in HeLa cells co-transfected with vimentin-GFP and indicated Flag-Beclin 1 constructs. **(G)** Effects of siRNAs that target 14-3-3ß or 14-3-3ε on Flag-Beclin 1 immunoprecipitation with vimentin-GFP in HeLa cells transfected with indicated plasmids. **(H)** Localization of Flag-Beclin 1 with endogenous vimentin in Rat2 cells transduced with the indicated vector with or without treatment starvation (EBSS) for 2 h. For primary antibody incubation, slides were incubated with rabbit anti-Beclin 1 (Santa Cruz, sc-11427) and mouse anti-vimentin (Santa Cruz, sc-6260) overnight at 4°C.

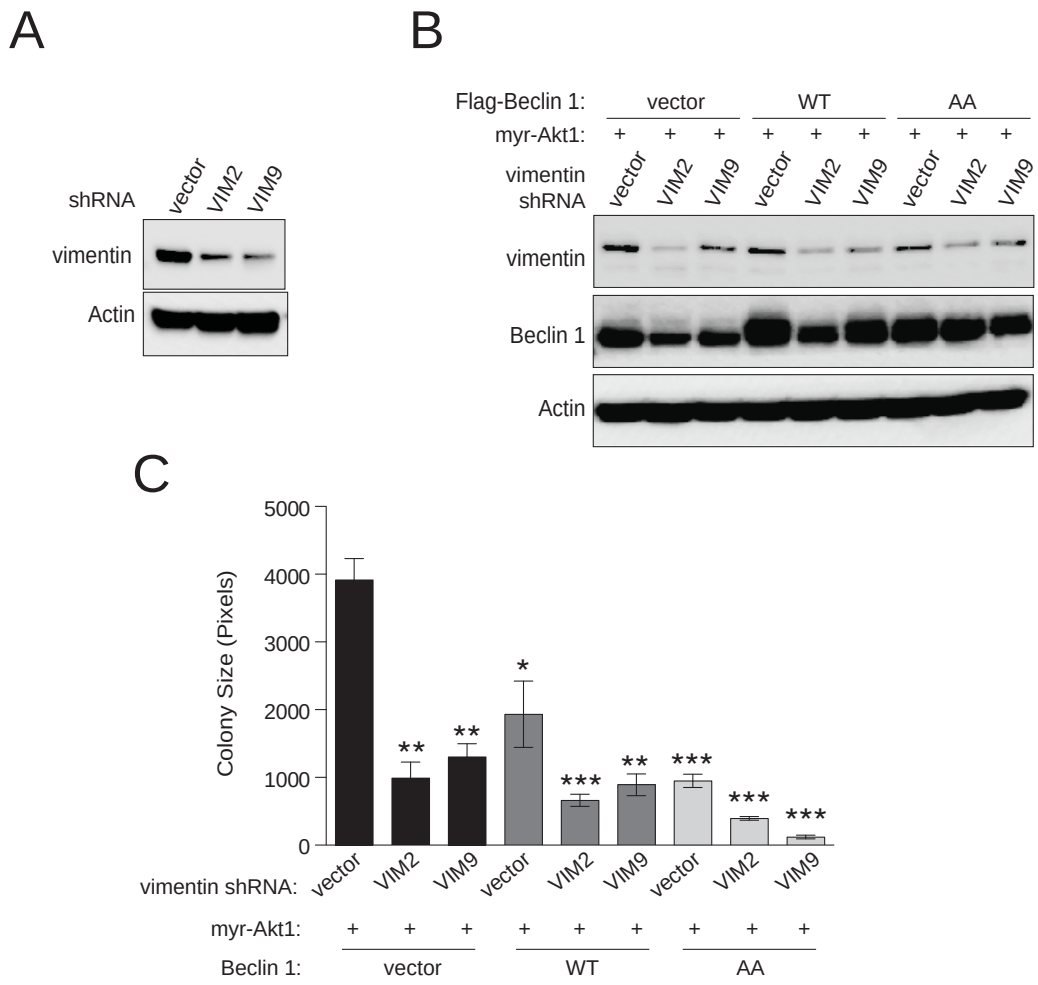

Fig. S8. Effects of vimentin knockdown in Rat2 fibroblasts on colony formation in soft agar. **(A)** Western blot detection of vimentin in Rat2 fibroblasts transduced with lentivirus vectors expressing indicated vimentin shRNAs and then transiently transfected with GFP-LC3. **(B)** Western blot detection of vimentin in Rat2 fibroblasts stably transduced with lentivirus vectors expressing indicated vimentin shRNAs. **(C)** Quantitation of the size of soft agar colonies formed by Rat2 fibroblasts transduced with indicated vectors. Bars represent mean ± SEM of triplicate samples with ≥10 random images analyzed per sample by ImageJ. **P*<0.05, ****P*<0.001; t-test for comparisons with the first bar in graph. VIM, vimentin.