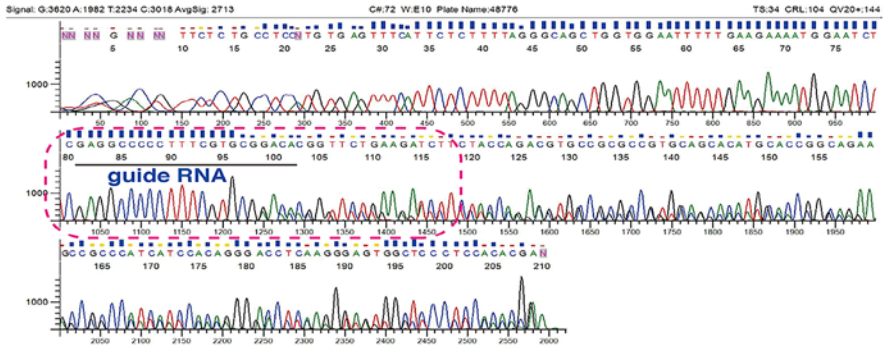
Figure S1. Targeted disruption of GAK using CRISPR/Cas9 genome editing. Genome sequences of the edited locus in selected colonies were confirmed by Sanger DNA sequencing. GAK, cyclin G-associated kinase; KO, knockout.

GAK-KO cells Cl. 1-1

Sequence primer (GAK-KO-F1): GCGTGAAACAGCCCTAGGTTCC



GAK exon 5: TCTCGAGGCCCCCTTTCGTGCG ACACGGTTCTGAAGATCT

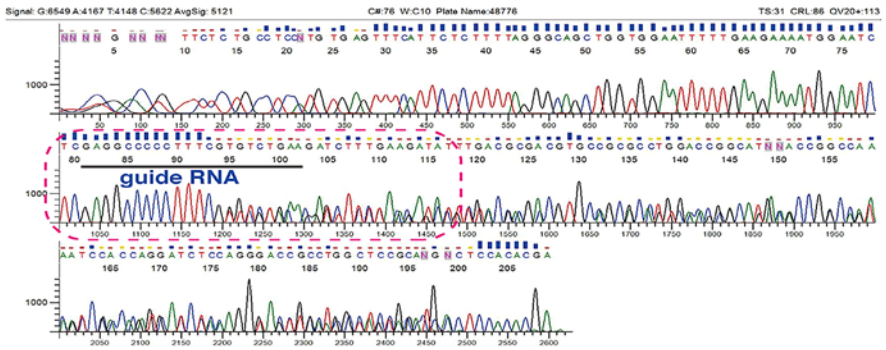
guide RNA

allele 1: TCTCGAGGCCCCCTTTCGTGCGG ACACGGTTCTGAAGATCT

allele 2: TCTCGAGGCCCCCTTTCGTGCGAAACACGGTTCTGAAGATCT

GAK-KO cells Cl. 1-2

Sequence primer (GAK-KO-F1): GCGTGAAACAGCCCTAGGTTCC



GAK exon 5: TCTCGAGGCCCCCTTTCGTGCG ACACGGTTCTGAAGATCT

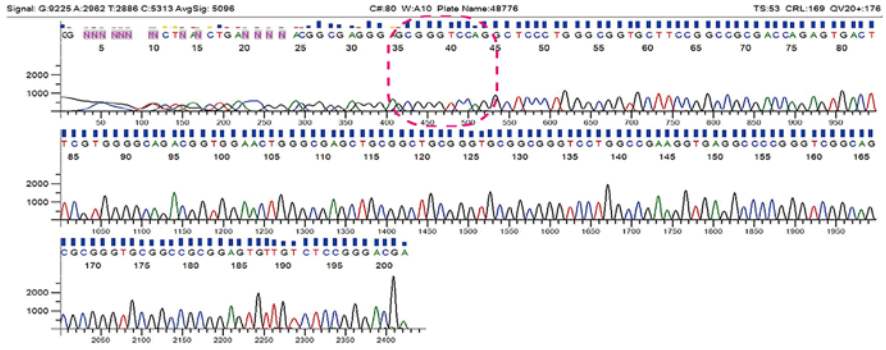
guide RNA

allele 1: TCTCGAGGCCCCCTTTCGTGCGTACACGGTTCTGAAGATCT

allele 2: TCTCGAGGCCCCCTTTCG---- -----GTTCTGAAGATCT

GAK-KO cells Cl. 2-1

Sequence primer (GAK-KO-F2): AGGCGGAAGATGGTGCACCTCC



GAK exon 1: GGCGAGGGAGCGGGAGCCCGAGCCCGACCACTCCGGCTGCCGCGGGGTGCGGCGCAGCCA

CCGCCATGTCGCTGCTGCAGTCGGCGCTCGACTTCTTGGCGGGTCCAGGCTCCCTGGGCG

guide RNA

alleles 1 & 2: GGCGAGGGAGCGGG----------

----------TCCAGGCTCCCTGGGCG

Figure S2. Continued.

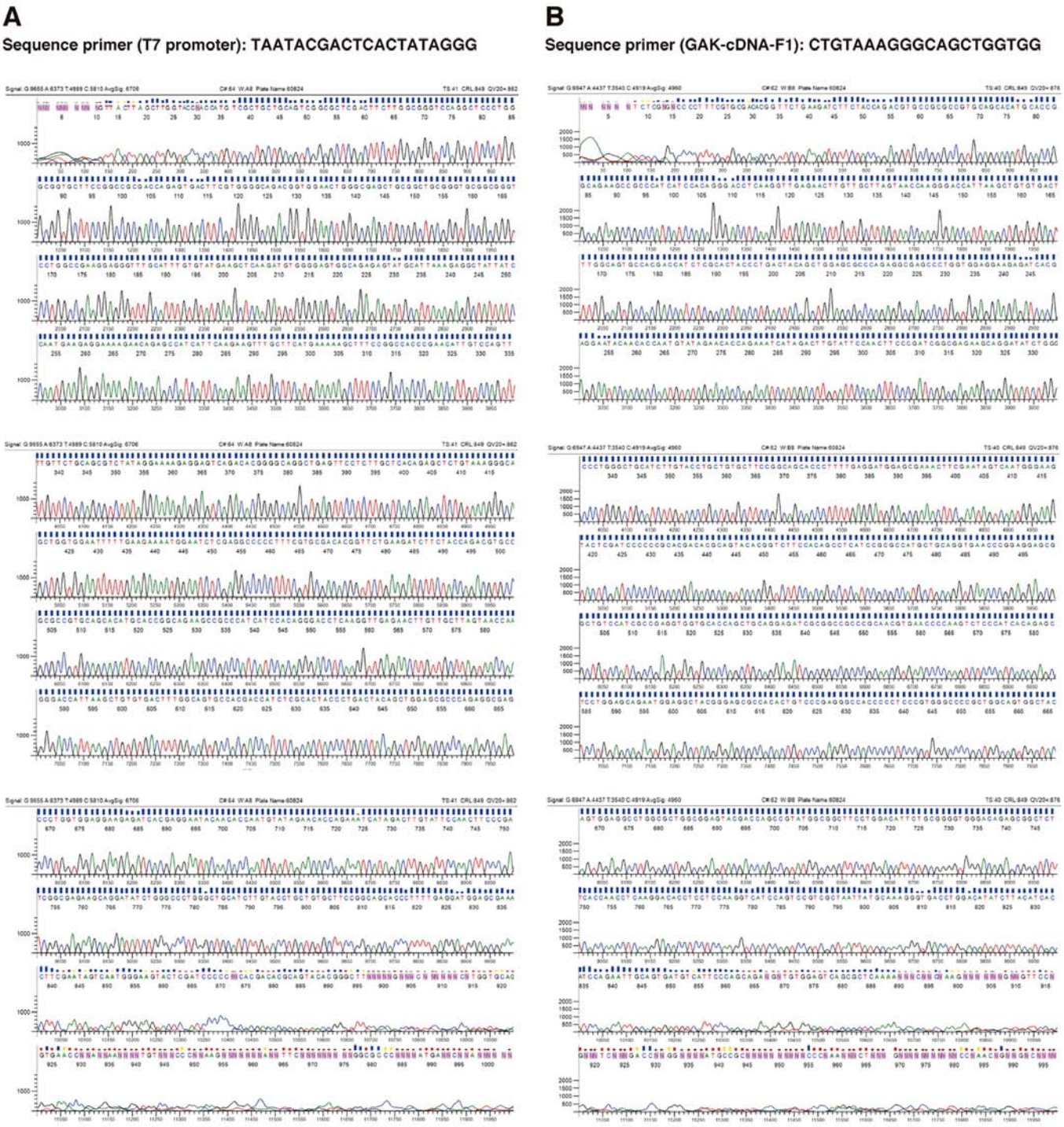
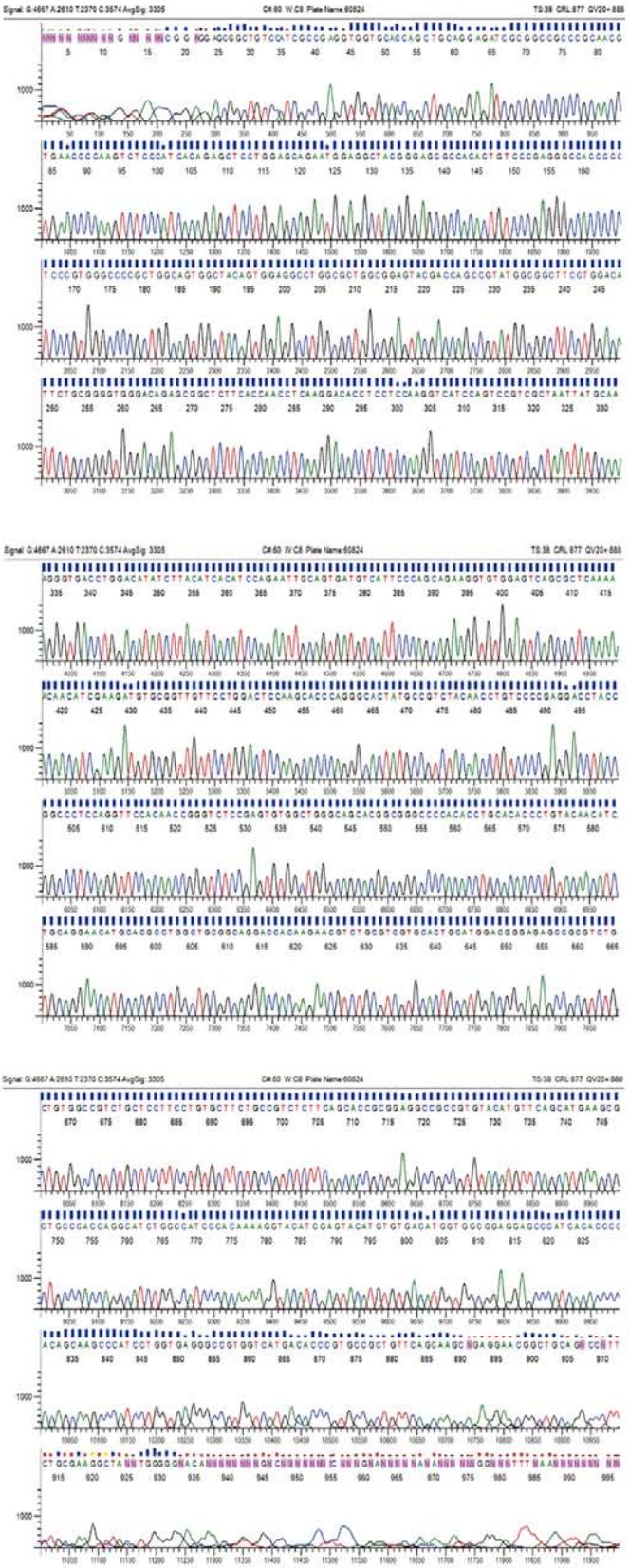


Figure S2. Continued.

C

Sequence primer (GAK-cDNA-F2): GTACACGGTCTTCCACAGCC



D

Sequence primer (GAK-cDNA-F3): CACACCCTGTACAACATCTG

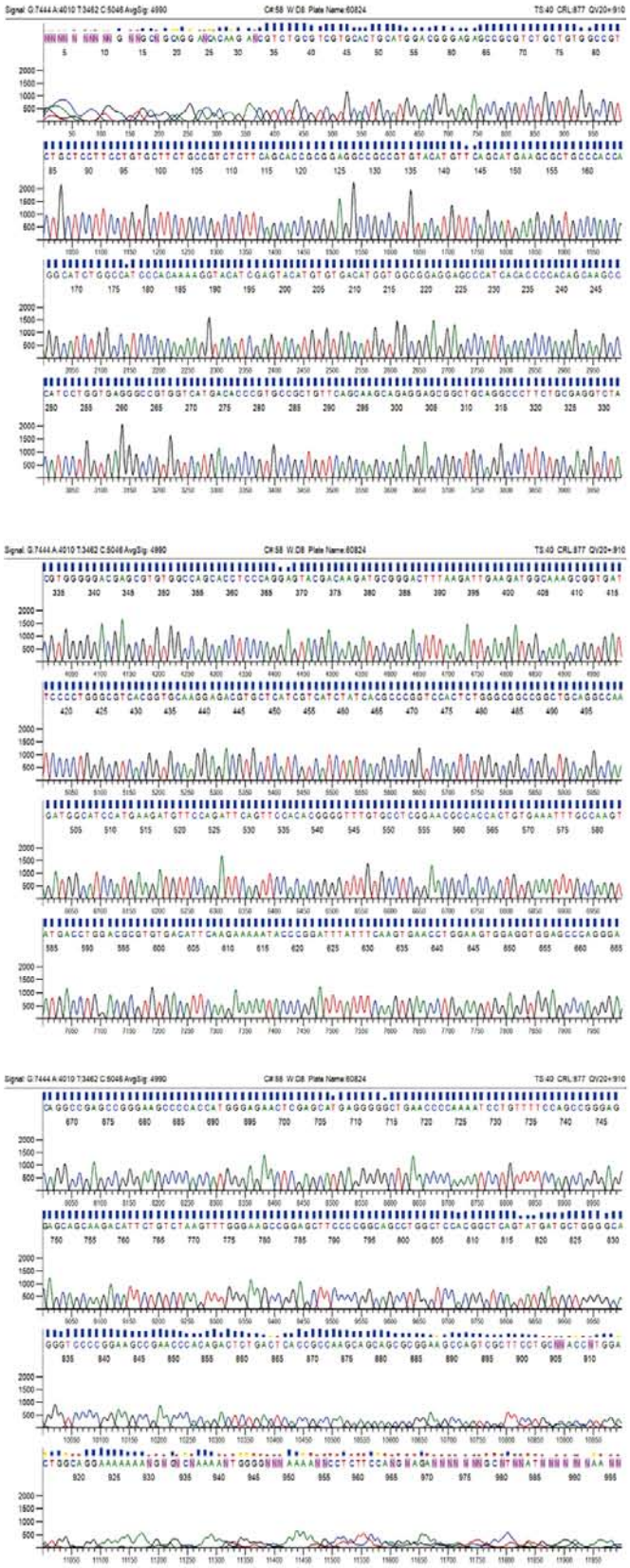
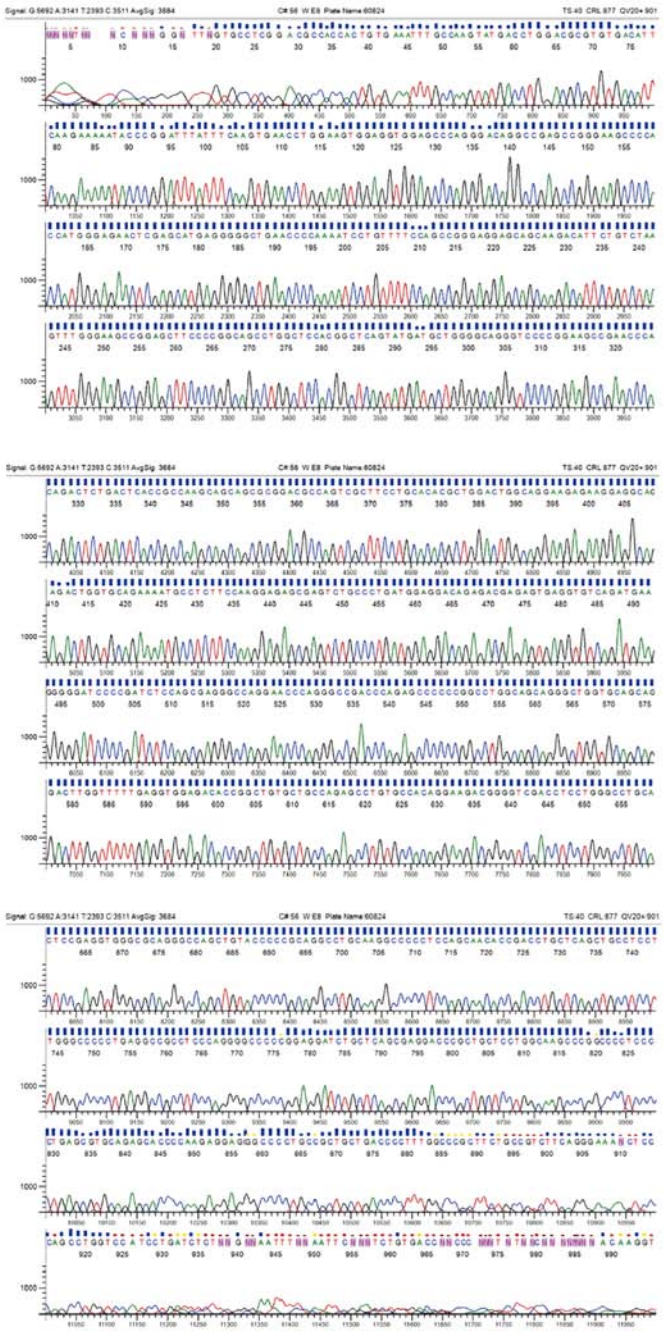


Figure S2. Continued.

E

Sequence primer (GAK-cDNA-F4): CCAAGATGGCATCCATGAAG



F

Sequence primer (GAK-cDNA-F5): CGAGAGTGAGGTGTCAGATG

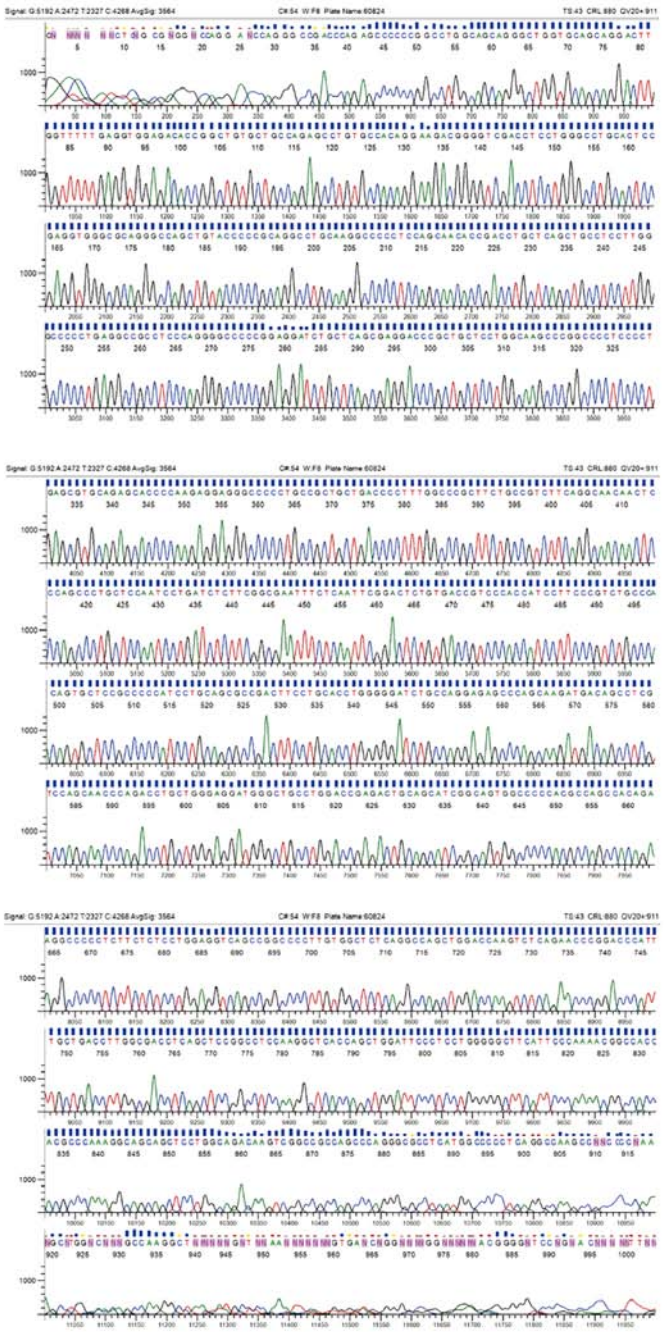
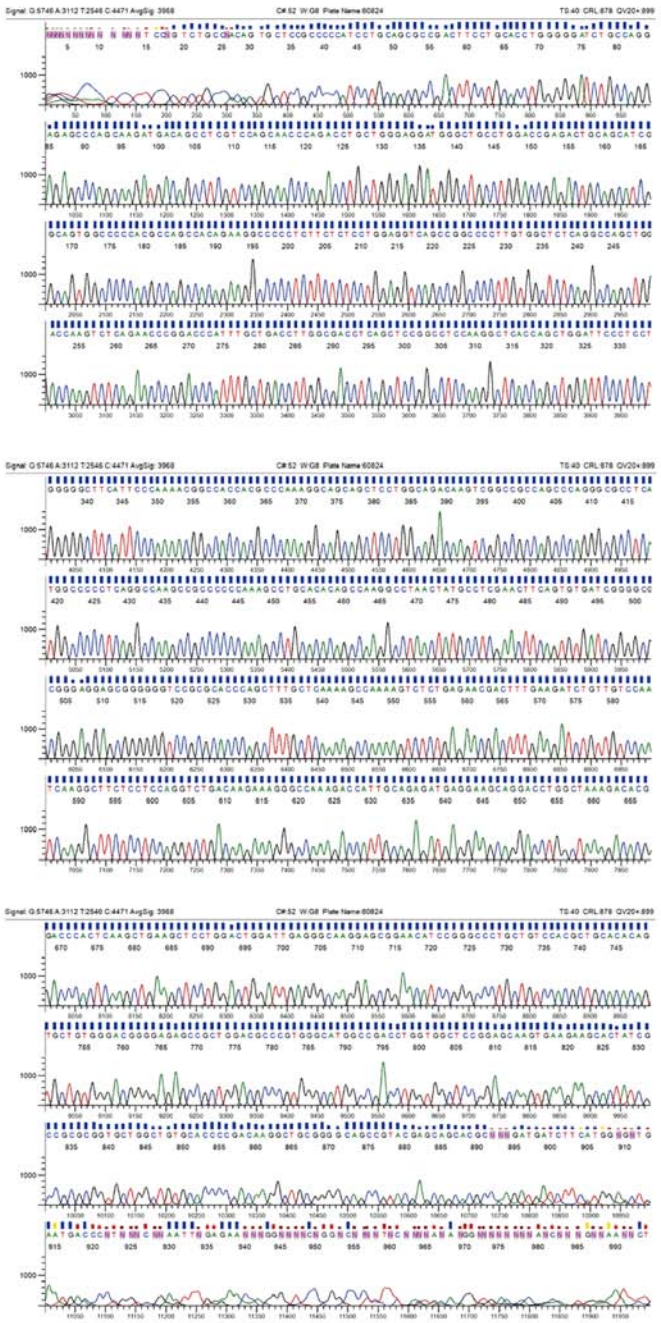


Figure S2. cDNA amplification of GAK by PCR. Cloned sequences were verified by Sanger sequencing using following primers: (A) T7 promoter, (B) GAK-cDNA-F1, (C) GAK-cDNA-F2, (D) GAK-cDNA-F3, (E) GAK-cDNA-F4, (F) GAK-cDNA-F5, (G) GAK-cDNA-F6, (H) GAK-cDNA-F7. GAK, cyclin G-associated kinase.

G

Sequence primer (GAK-cDNA-F6): GCGAATTTCTCAATTCGGAC



H

Sequence primer (GAK-cDNA-F7): CCCAGCTTTGCTCAAAAGCC

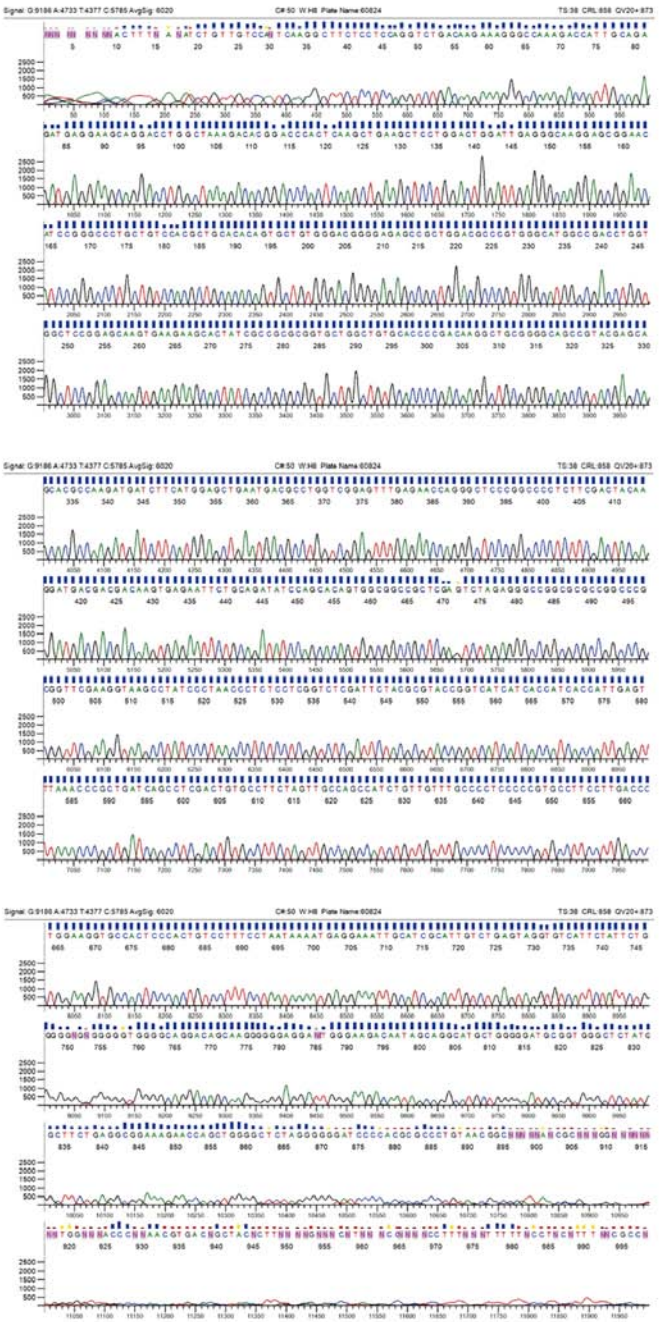


Figure S3. GAK disruption stagnates autophagic flux. (A) A549/GFP-LC3-mCherry-LC3ΔG and A549/GAK-KO/GFP-LC3-mCherry-LC3ΔG cells were cultured under basal-fed conditions, and the fluorescence intensities derived from GFP-LC3 and mCherry-LC3ΔG were monitored.Autophagic flux was determined as the relative intensity of GFP/mCherry ratios of the (B) microscopy images. Data were analyzed using an unpaired two-tailed Student's t-test. The box extends from the lower to the upper quartile, the middle line indicates the median, the X indicates the mean and the whiskers represent the minimum to maximum values, except for outliers, which are indicated by dots (A549/GFP-LC3-mCherry-LC3ΔG, n=8;A549/GAK-KO/GFP-LC3-mCherry-LC3ΔG, n=48). (C-F) Representative microscopy images for the data quantified in Fig. 1G-J. **P<0.01 vs. wild-type. GAK, cyclin G-associated kinase; KO, knockout; Baf, bafilomycin A1; Rap, rapamycin; HBSS, Hanks' balanced salt solution; Gln, glutamine.

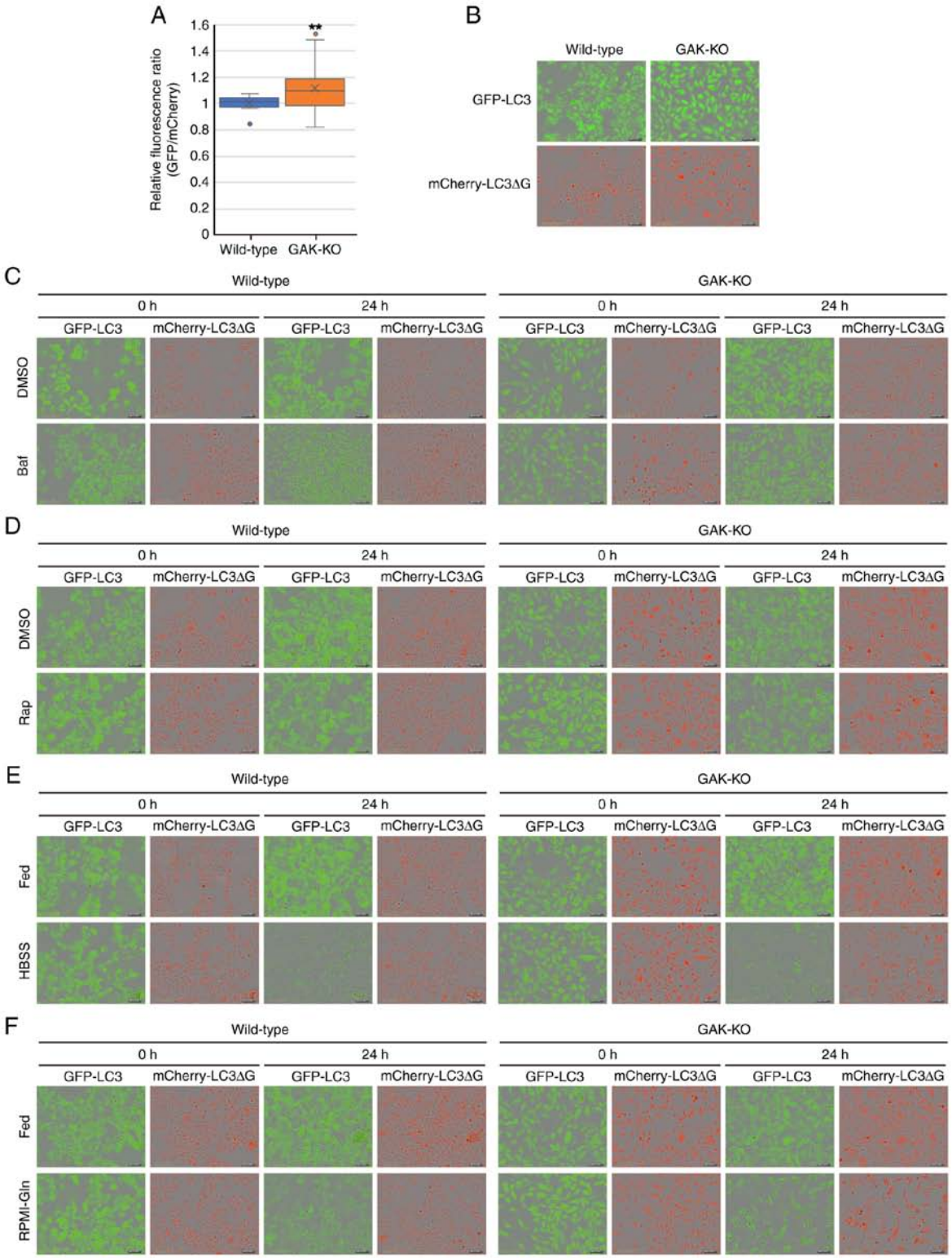


Figure S4. GAK disruption does not impair starvation-induced inhibition of mTOR signaling. Wild-type and GAK-KO cells were cultured under basal-fed, starvation (HBSS or RPMI-Gln for 2 h) or refed (starvation using HBSS or RPMI-Gln for 1 h, followed by fed conditions for 1 h) conditions. Phosphorylation levels of mTOR (Ser2448), S6K (Thr389), S6 (Ser235/236) and AMPK (Thr172) in wild-type and GAK-KO cells were (A) determined by immunoblotting and (B) semi-quantified. Data were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. Data are presented as the mean ± SD (n=3; except for p-S6K (Thr389)/total S6K, n=5). $^{*}P<0.05$ and $^{**}P<0.01$ vs. starved. GAK, cyclin G-associated kinase; KO, knockout; HBSS, Hanks' balanced salt solution; Gln, glutamine; S6, ribosomal protein S6; S6K, S6 kinase; p, phosphorylated.

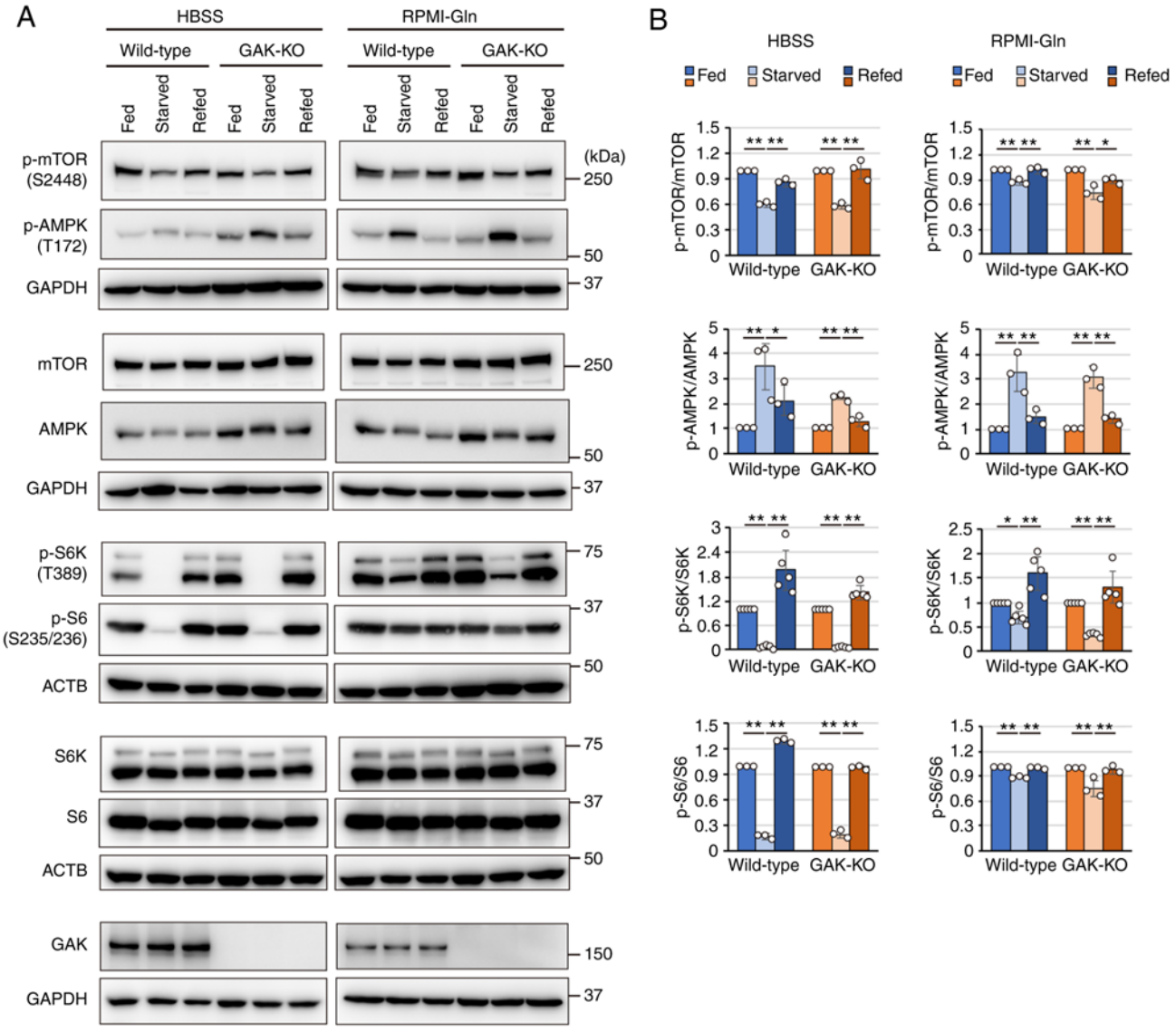


Figure S5. Impact of *GAK* disruption on the expression of lysosome-related genes. (A) Immunoblot analysis of LAMP1 and LAMP2 expression in wild-type and GAK-KO cells under basal-fed conditions. (B) Quantification of the relative protein expression of LAMP1 and LAMP2 in wild-type and GAK-KO cells. Data were analyzed using an unpaired two-tailed Student's t-test. Data are presented as the mean ± SD (n=3). **P<0.01 vs. wild-type. (C) Reverse transcription-quantitative PCR analysis of relative mRNA levels of *LAMP1* and *LAMP2* in wild-type and GAK-KO cells under basal-fed conditions. *GAPDH* was used as an internal control. Data were analyzed using an unpaired two-tailed Student's t-test. Data are presented as the mean ± SD (n=4). GAK, cyclin G-associated kinase; LAMP, lysosomal associated membrane protein; KO, knockout; ns, not significant.

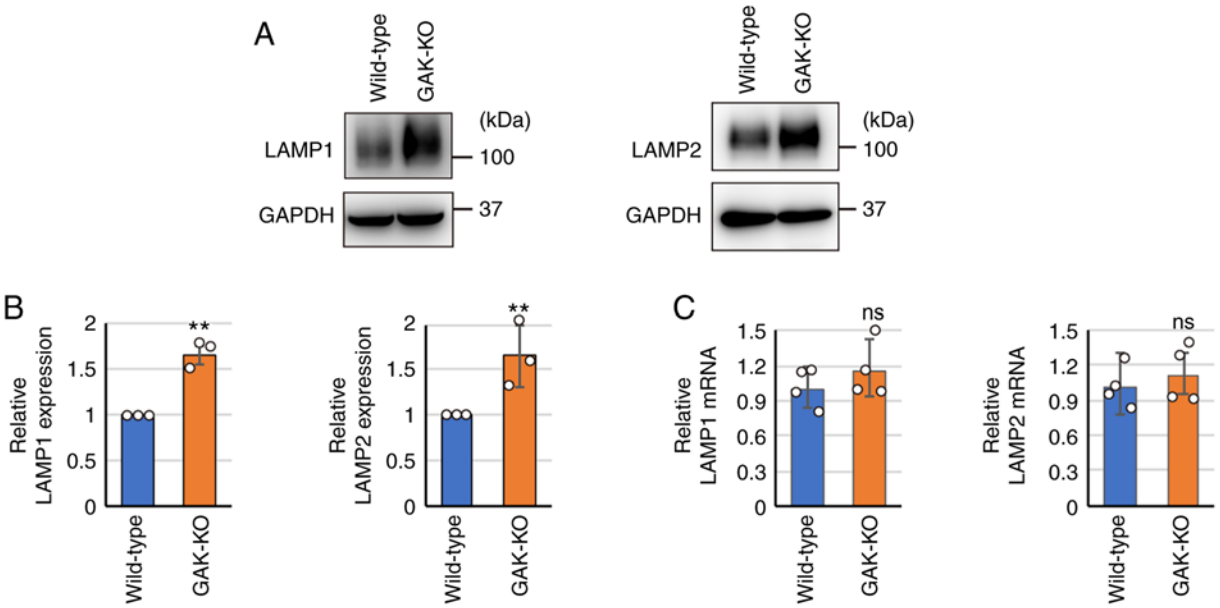


Figure S6. *GAK* disruption alters the intra-lysosomal conditions. (A) LysoSensor staining in wild-type, GAK-KO and GAK-rescued cells under basal-fed or starvation (RPMI-Gln for 8 h) conditions. Scale bar, 20 μm. (B) Relative mean fluorescence intensity of LysoSensor in wild-type, GAK-KO and GAK-rescued cells. Data were analyzed using one-way ANOVA followed by Dunnett's post hoc test. The box extends from the lower to the upper quartile, the middle line indicates the median, the X indicates the mean and the whiskers represent the minimum to maximum values, except for outliers, which are indicated by dots (wild-type/fed, n=33; GAK-KO/fed, n=21; GAK-rescued/fed, n=32; wild-type/starved, n=37; GAK-KO/starved, n=27; GAK-rescued/starved, n=34). **P<0.01 vs. wild-type. GAK, cyclin G-associated kinase; KO, knockout; Gln, glutamine; ns, not significant.

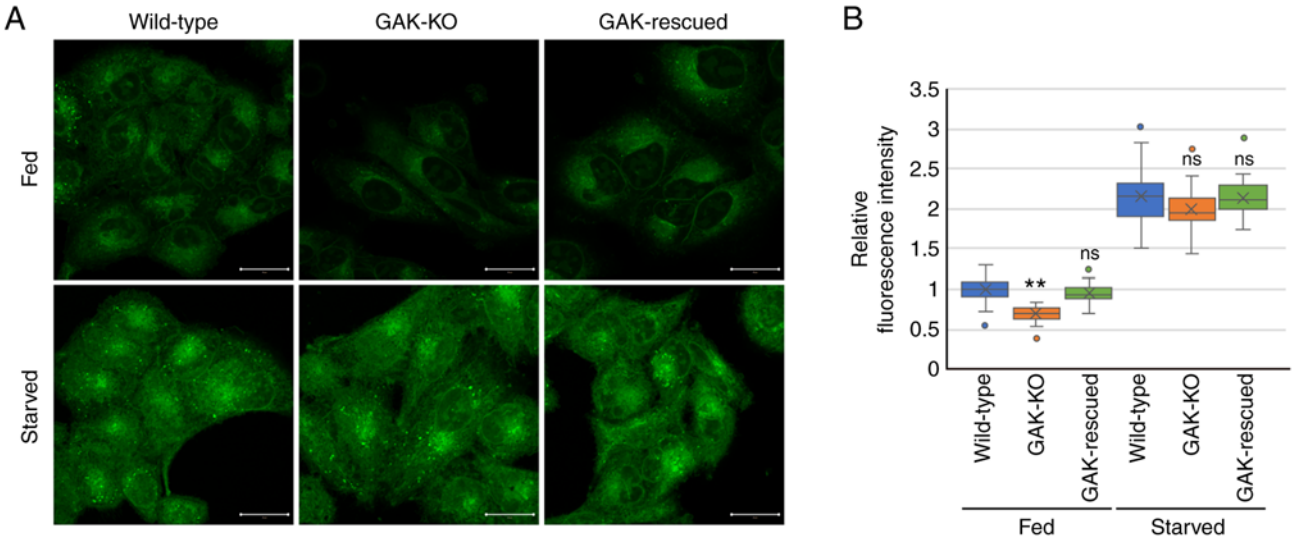


Figure S7. *GAK* disruption induces the accumulation of autophagosomes under starvation conditions. (A) Immunofluorescence analysis of LAMP2 (magenta) and LC3B (green) in wild-type and GAK-KO cells under basal-fed or starvation (RPMI-Gln for 4, 10 or 24 h) conditions. The dashed boxed regions are shown at a high magnification (x3) in theinset. Scale bar, 20 μm. (B) Number of enlarged LC3B-positive puncta was quantified in wild-type and GAK-KO cells using Imaris. Data were analyzed using one-way ANOVA followed by Dunnett's post hoc test. The box extends from the lower to the upper quartile, the middle line indicates the median, the X indicates the mean and the whiskers represent the minimum to maximum values, except for outliers, which are indicated by dots (wild-type/fed, n=37; wild-type/starved 4 h, n=24; wild-type/starved 10 h, n=29; wild-type/starved 24 h, n=46; GAK-KO/fed, n=16; GAK-KO/starved 4 h, n=20; GAK-KO/starved 10 h, n=18; GAK-KO/starved 24 h, n=26). *P<0.05 and **P<0.01 vs. fed. (C) Immunofluorescence analysis of LAMP2 (magenta) and LC3B (green) in wild-type, wild-type/sham-OE and wild-type/GAK-OE cells under basal-fed or starvation (RPMI-Gln for 10 or 24 h) conditions. The dashed boxed regions are shown at a high magnification (x3) in the inset. Scale bar, 20 μm. (D) GAK expression in wild-type, wild-type/sham-OE and wild-type/GAK-OE cells was confirmed by immunoblotting. (E) Number of enlarged LC3B-positive puncta in wild-type, wild-type/sham-OE and wild-type/GAK-OE was quantified using Imaris. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. The box extends from the lower to the upper quartile, the middle line indicates the median, the X indicates the mean and the whiskers represent the minimum to maximum values, except for outliers, which are indicated by dots (wild-type/fed, n=38; wild-type/sham-OE/fed, n=47; wild-type/GAK-OE/fed, n=50; wild-type/starved 4 h, n=51; wild-type/sham-OE/starved 4 h, n=56; wild-type/GAK-OE/starved 4 h, n=51; wild-type/starved 24 h, n=50; wild-type/sham-OE/starved 24 h, n=44; wild-type/GAK-OE/starved 24 h, n=40). **P<0.01 vs. wild-type/fed. GAK, cyclin G-associated kinase; LAMP, lysosomal associated membrane protein; KO, knockout; Gln, glutamine; OE, overexpression; ns, not significant.

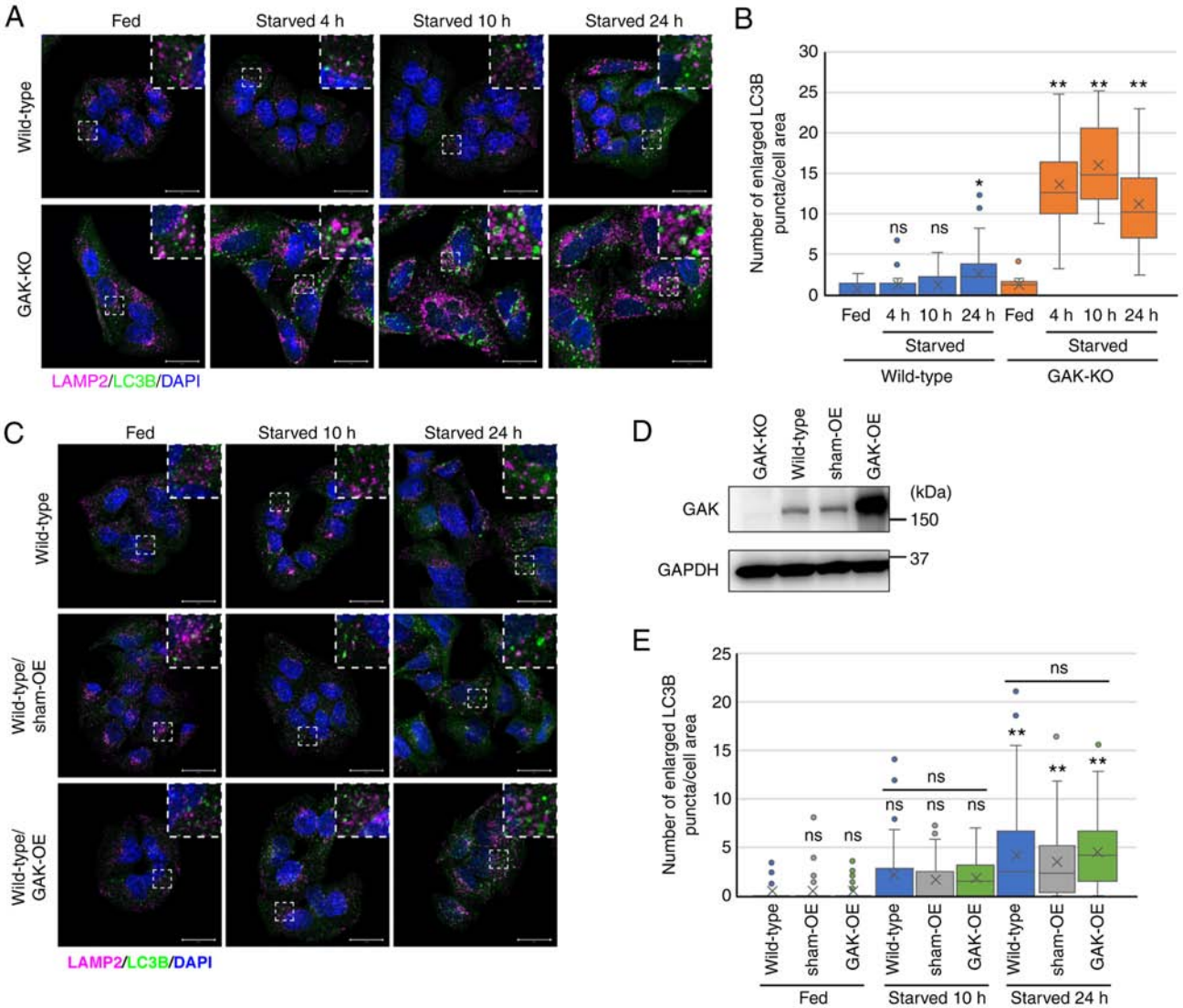


Figure S8. Chemical inhibition of GAK induces the accumulation of autophagosomes in various cell lines. (A) GAK expression in H596, PANC-1 and Hep-G2 cells was confirmed by immunoblotting. (B) Immunofluorescence analysis of LAMP2 (magenta) and LC3B (green) in H566, PANC-1 and Hep-G2 cells treated with 30 μM GAKi under basal-fed or starvation (RPMI-Gln for 8 h) conditions. The dashed boxed regions are shown at a high magnification (x3) in the inset. Scale bar, 20 μm. (C) Number of enlarged LC3B-positive puncta was quantified using Imaris. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. The box extends from the lower to the upper quartile, the middle line indicates the median, the X indicates the mean and the whiskers represent the minimum to maximum values, except for outliers, which are indicated by dots (H596/fed/DMSO, n=19; H596/fed/GAKi, n=17; H596/starved/DMSO, n=49; H596/starved/GAKi, n=48; PANC-1/fed/DMSO, n=21; PANC-1/fed/GAKi, n=19; PANC-1/starved/DMSO, n=25; PANC-1/starved/GAKi, n=46; Hep-G2/Fed/DMSO, n=36; Hep-G2/fed/GAKi, n=38; Hep-G2/starved/DMSO, n=73; Hep-G2/starved/GAKi, n=85). **P<0.01 vs. fed/DMSO; $^{\#\#}$P<0.01 vs. starved/DMSO. GAK, cyclin G-associated kinase; LAMP, lysosomal associated membrane protein; GAKi, GAK inhibitor; KO, knockout; Gln, glutamine; ns, not significant.

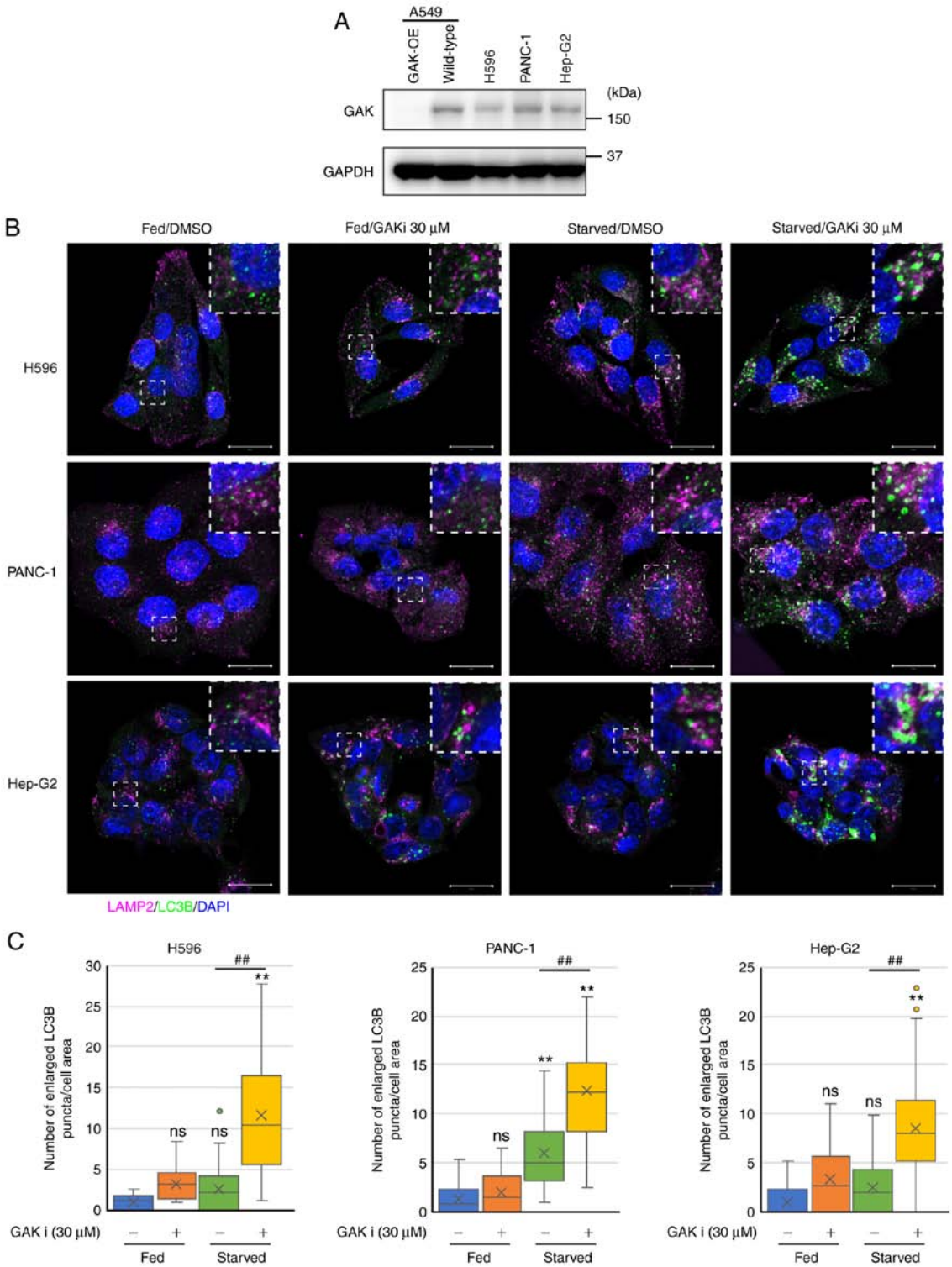


Figure S9. Chemical inhibition of GAK stagnates autophagic flux. Representative microscopy images for the quantified data presented in Fig. (A) 5D and (B) E. GAK, cyclin G-associated kinase; GAKi, GAK inhibitor; HBSS, Hanks' balanced salt solution; Gln, glutamine.

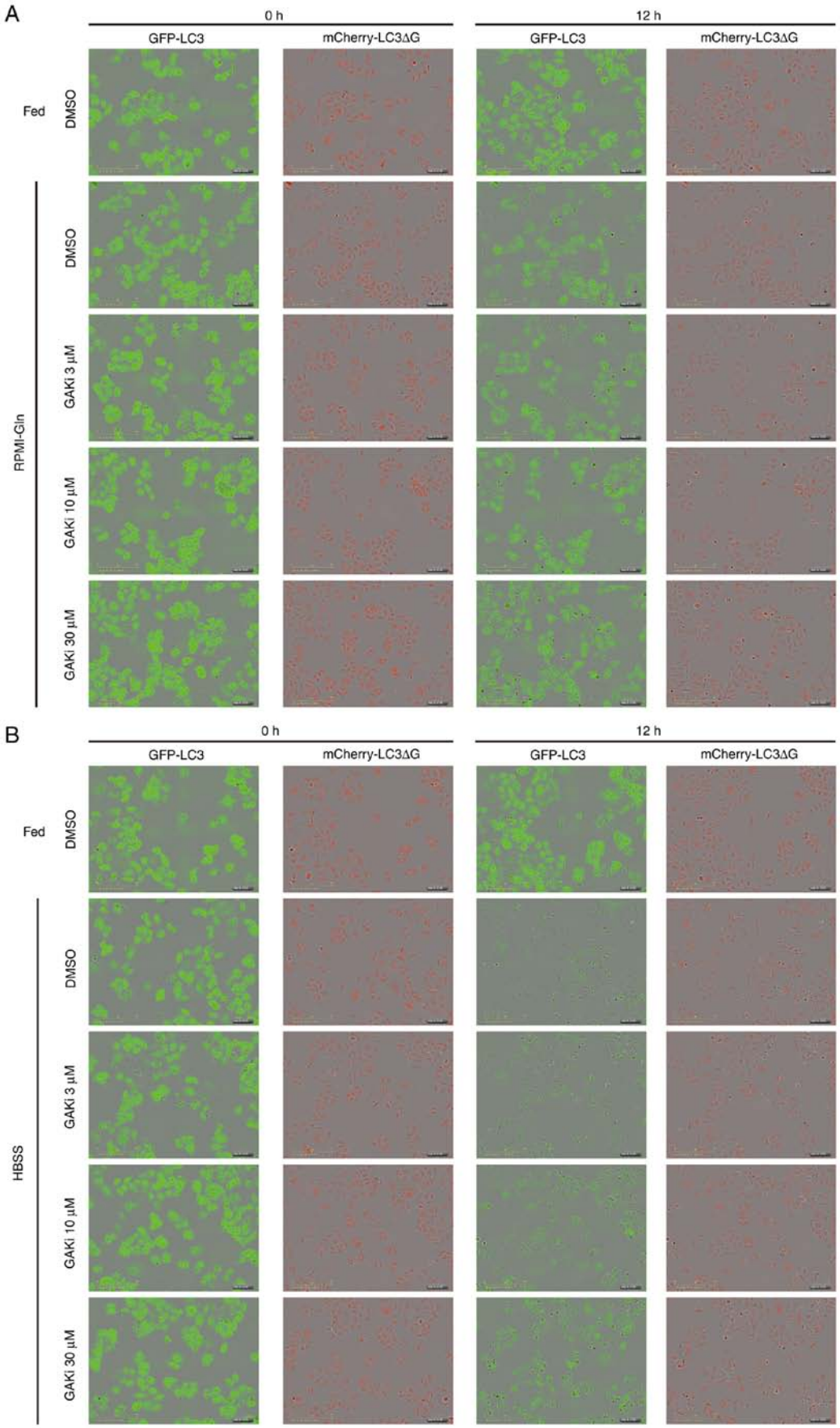


Figure S10. Rho-associated protein kinase inhibition eliminates the accumulation of autophagosomes and autolysosomes induced by GAK disruption. (A) Immunofluorescence analysis of LAMP2 (magenta) and LC3B (green) in GAK-KO cells (clones 1-2 and 2-1) under basal-fed, starvation(RPMI-Gln for 8 h) or starvation (RPMI-Gln for 8 h) with Y-27632 conditions. The dashed boxed regions are shown at a high magnification (x3) in the inset. Scale bar, 20 μm. (B) Number of enlarged LC3B-positive puncta was quantified using Imaris. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. The box extends from the lower to the upper quartile, the middle line indicates the median, the X indicates the mean and the whiskers represent the minimum to maximum values, except for outliers, which are indicated by dots (Cl.1-2/fed, n=17; Cl.1-2/starved, n=20; Cl.1-2/Y-27632/starved, n=14; Cl.2-1/fed, n=16; Cl.2-1/starved, n=16; Cl.2-1/Y-27632/starved, n=18). **P<0.01 vs. wild-type; $^{\#\#}$P<0.01 vs. GAK-KO. GAK, cyclin G-associated kinase; LAMP, lysosomal associated membrane protein; KO, knockout; Gln, glutamine; ns, not significant.

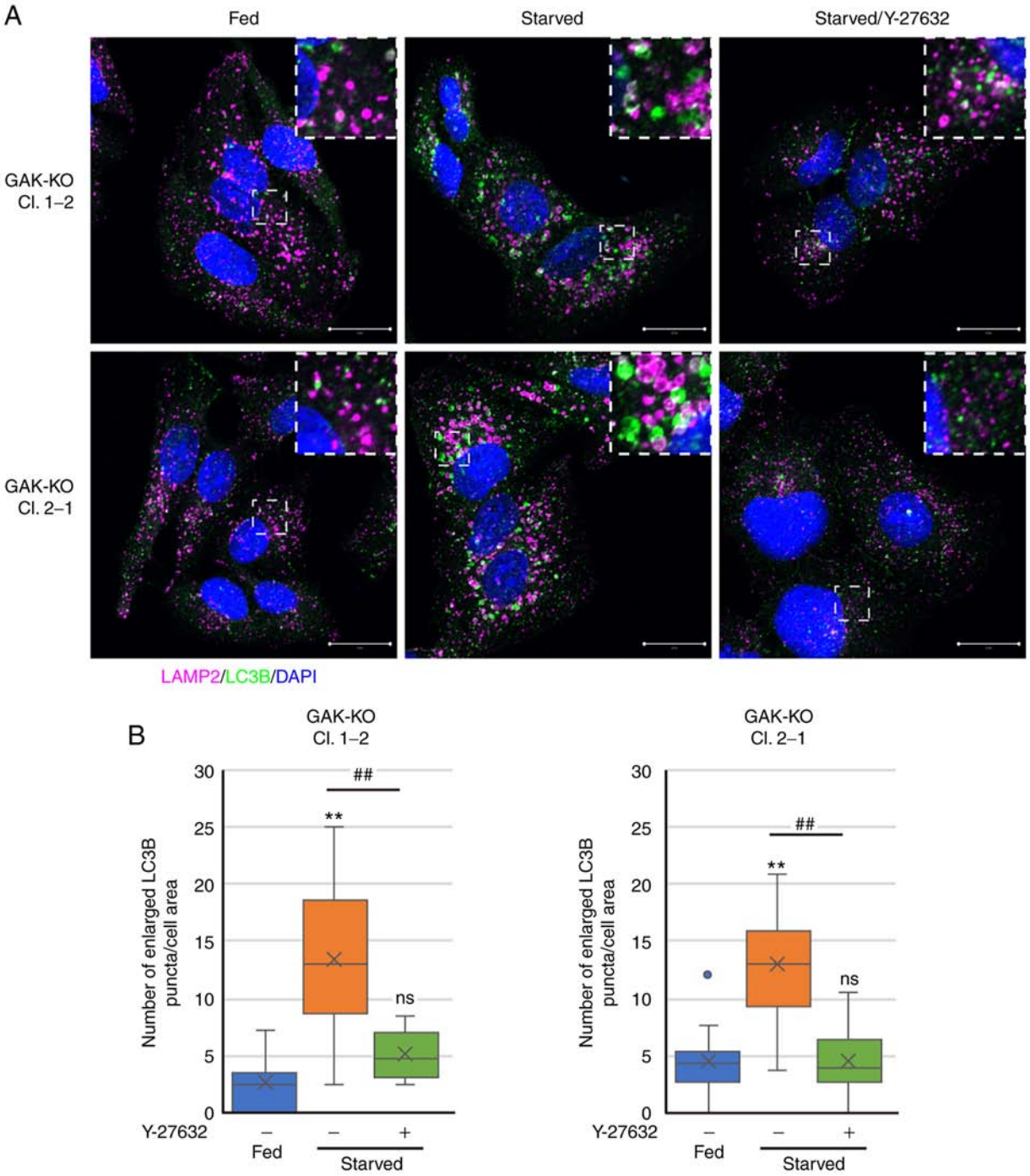


Figure S11. Rho-associated protein kinase inhibition does not impair autophagosome formation in GAK-KO cells. (A) Immunofluorescence analysis of LAMP2 (magenta) and LC3B (green) in wild-type cells and GAK-KO cells treated with the indicated concentrations of Y-27632 in the presence or absence of 10 nM Baf under starvation (RPMI-Gln for 8 h) conditions. Scale bar, 20 μm. (B) Immunofluorescence analysis of LAMP2 (magenta) and LC3B (green) in wild-type, GAK-KO, GAK-KO/shNT and GAK-KO/shROCK1 cells in the presence or absence of 10 nM Baf under basal-fed or starvation (RPMI-Gln for 8 h) conditions. Scale bar, 20 μm. GAK, cyclin G-associated kinase; KO, knockout; LAMP, lysosomal associated membrane protein; Gln, glutamine; sh, short hairpin RNA; NT, non-targeting; Baf, Bafilomycin A_1.

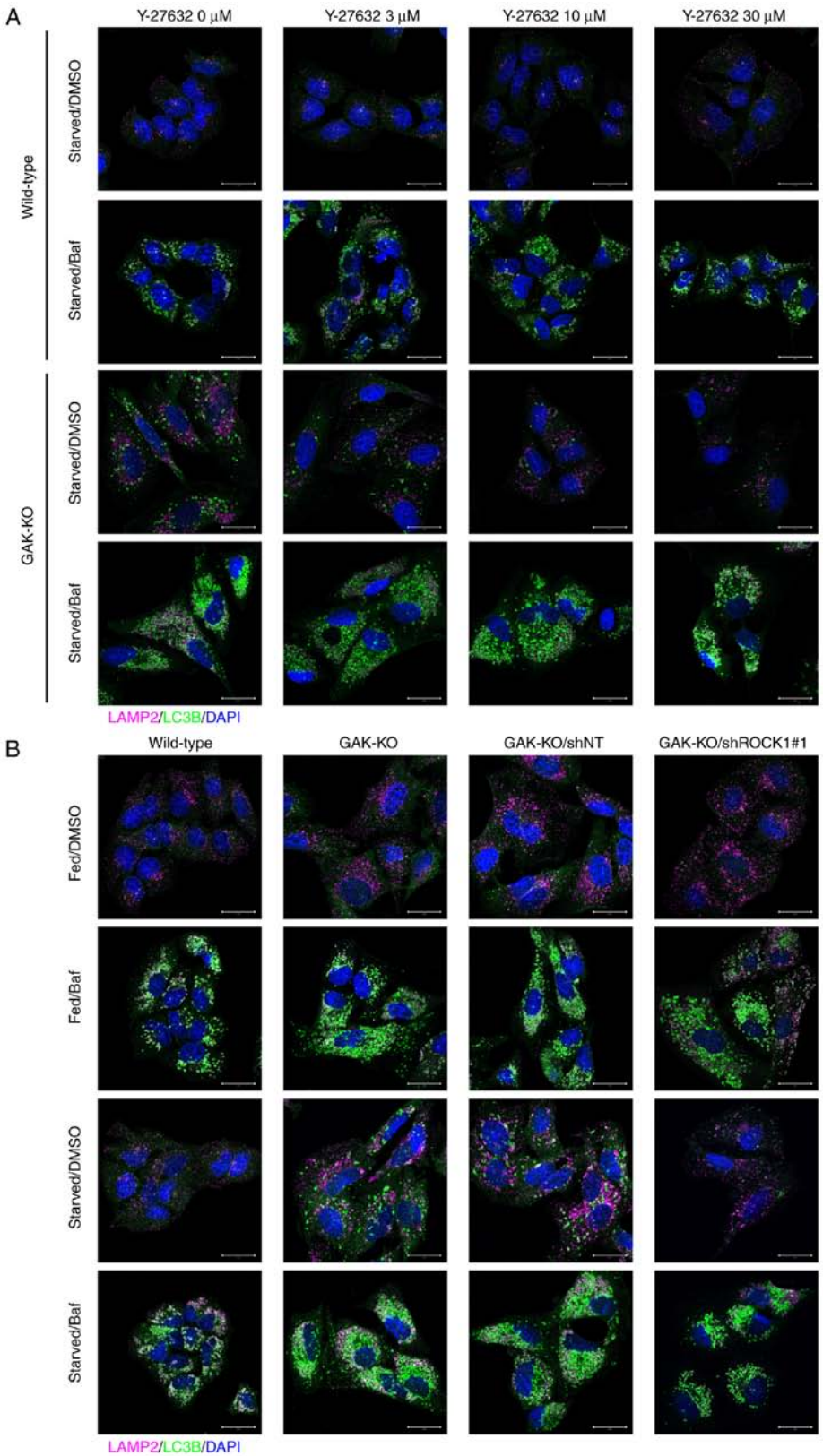


Figure S12. No interaction between GAK and ROCK1 in 293T cells. (A) Expression of GAK-FLAG and ROCK1-V5 in transfected 293T cells was confirmed by immunoblotting. (B) Immunoblot analysis of indicated proteins in total cell lysates or IPs of 293T cells expressing GAK-FLAG and ROCK1-V5. GAK, cyclin G-associated kinase; ROCK, Rho-associated protein kinase; IP, immunoprecipitate; IB, immunoblot.

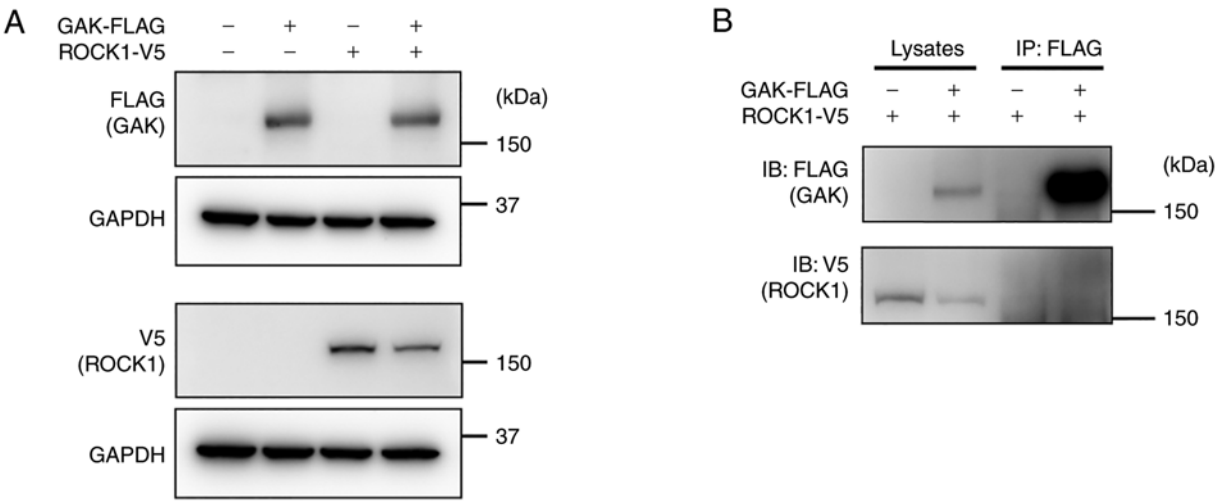


Figure S13. Schematic model for the role of GAK in the autophagy-lysosome system. GAK knockout caused impairment of autophagosome-lysosome fusion and autophagic lysosome reformation, accompanied by the accumulation of enlarged autophagosomes and autolysosomes during prolonged starvation. ROCK inhibition mitigated the effects induced by GAK knockout. These results indicated that GAK controlled lysosomal dynamics by regulating actomyosin during autophagy. GAK, cyclin G-associated kinase; ROCK, Rho-associated protein kinase; TFEB, transcription factor EB.

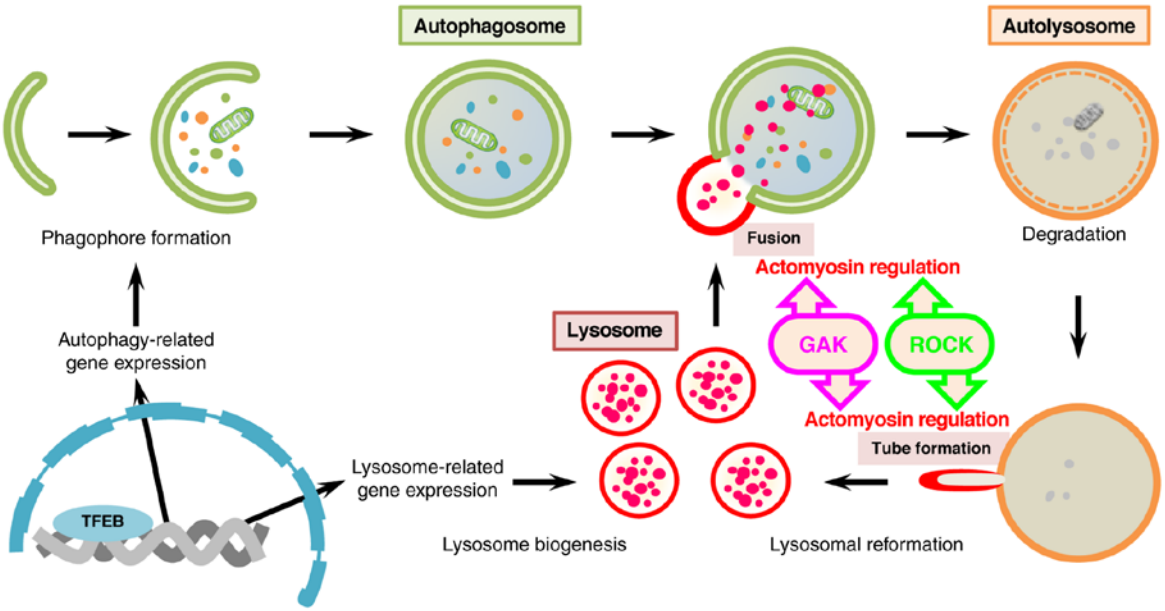


Table SI. Sequences of oligonucleotides and primers used in the present study.

A, Short hairpin RNA vectors

Oligonucleotide	Sequence (5'→3')
ROCK1 #1	F: CCGGCGGGTTGTTCAGATTGAGAAACTCGAGTTTCTCAATCTGAACAACCCGTTTTTTG
	R: AATTCAAAAAACGGGTTGTTCAGATTGAGAAACTCGAGTTTCTCAATCTGAACAACCCG
ROCK1 #2	F: CCGGGAGGTAAATGAACACAAAGTACTCGAGTACTTTGTGTTCATTTACCTCTTTTTTG
	R: AATTCAAAAAAGAGGTAAATGAACACAAAGTACTCGAGTACTTTGTGTTCATTTACCTC
Non-targeting	F: CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG
	R: AATTCAAAAACAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG

B, Quantitative PCR primers

Oligonucleotide	Sequence (5'→3')
LC3B	F: CGCACCTTCGAACAAAGAG
	R: CTTCTCACCCTTGTATCGTTCTATT
LAMP1	F: CAGATGTGTTAGTGGCACCCA
	R: TTGGAAAGGTACGCCTGGATG
LAMP2	F: GCACAGTGAGCACAAATGAGT
	R: CAGTGGTGTGTATGGTGGGT
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA

C, Primers used for sequencing

Oligonucleotide	Sequence (5'→3')
GAK-KO-F1	GCGTGAAACAGCCCTAGGTTCC
GAK-KO-F2	AGGCGGAAGATGGTGCACCTCC
T7 promoter	TAATACGACTCACTATAGGG
GAK-cDNA-F1	CTGTAAAGGGCAGCTGGTGG
GAK-cDNA-F2	GTACACGGTCTTCCACAGCC
GAK-cDNA-F3	CACACCCTGTACAACATCTG
GAK-cDNA-F4	CCAAGATGGCATCCATGAAG
GAK-cDNA-F5	CGAGAGTGAGGTGTCAGATG
GAK-cDNA-F6	GCGAATTTCTCAATTCGGAC
GAK-cDNA-F7	CCCAGCTTTGCTCAAAAGCC

ROCK, Rho-associated protein kinase; LAMP, lysosomal associated membrane protein; F, forward; R, reverse; GAK, cyclin G-associated kinase; KO, knockout.