Supplementary information to the article

Novel inducer of innate immunity HO53 stimulates autophagy in human airway epithelial cells

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Supplementary Figure S1

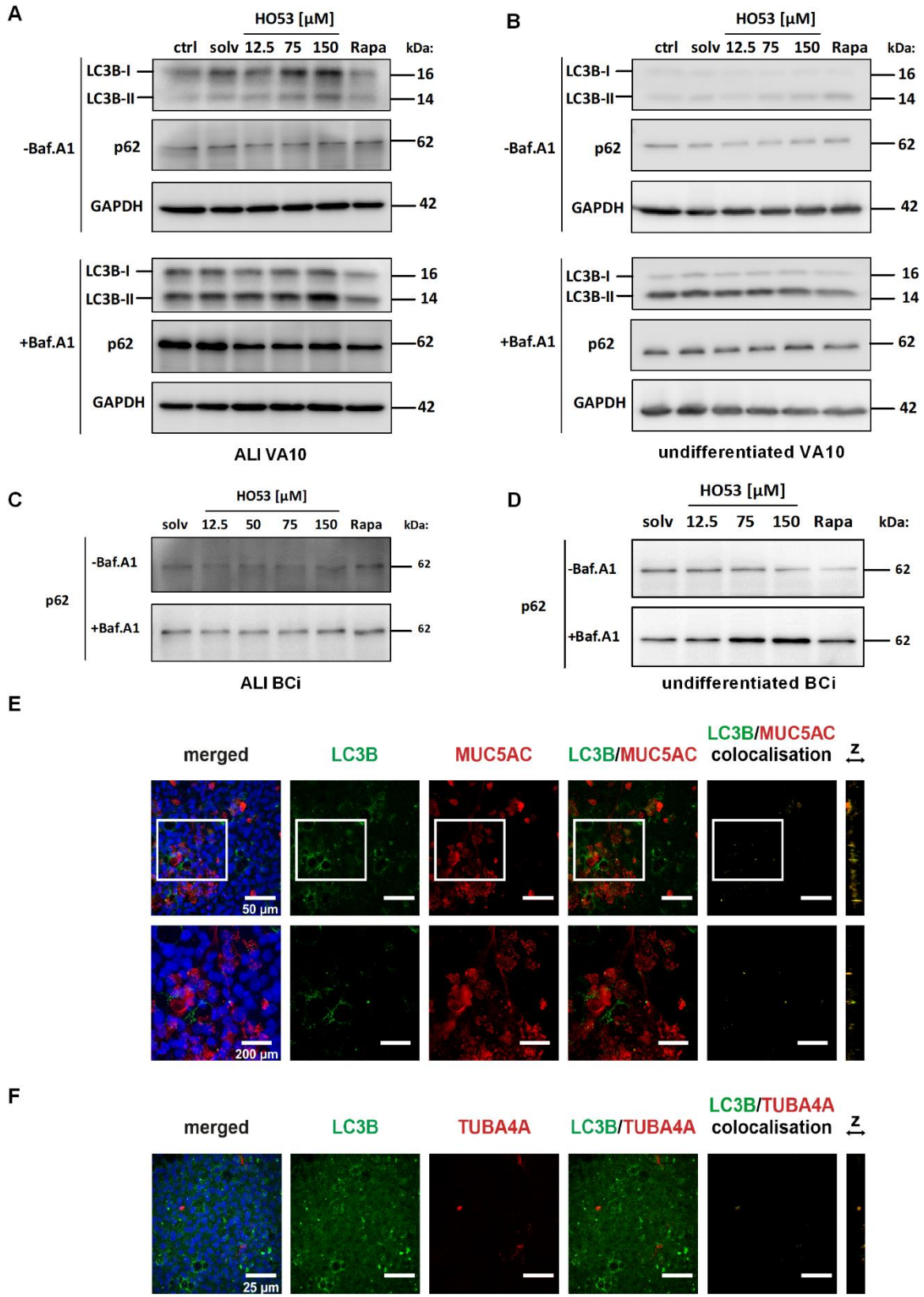


Fig. S1. Effect of HO53 treatment on induction of autophagy in VA10 cells and different cell populations of differentiated BCi cells. BCi and VA10 cells were stimulated for 24 h with different doses of HO53, 250 nM rapamycin (Rapa) used as a positive control and DMSO (final concentration of 0.3%) used as a solvent control (solv) in combination with (+Baf.A1) or without (-Baf.A1) Bafilomycin A1 (100 nM). Treatment of differentiated VA10 and BCi cells was performed by addition of the compound to the lower chamber of the trans-well insert. The level of both autophagy markers LC3B processing and p62 in **A)** air-liquid interphase cultured VA10 and **B)** undifferentiated VA10, and p62 marker in **C)** air-liquid interphase cultured BCi and **D)** undifferentiated BCi was evaluated by Western blotting. Representative pictures of n=3 independent experiments where GAPDH was used as a loading control. Full-length blots are presented in Supplementary Figure S7. Co-localization of LC3B puncta clusters (green) with **E)** mucous cells stained with antibodies against mucin 5AC (MUC5AC, mucous cells marker; red) and **F)** ciliated cells stained with antibodies against acetylated (K40) α-tubulin (TUBA4A, ciliated cells marker; red) with extracted signal from co-localized LC3B/MUC5AC and LC3B/TUBA4A (yellow) from merged channels. Differentiated bronchial epithelial BCi cell layers were treated with HO53 (75 µM) and Bafilomycin A1 (100 nM) for 24 h. Representative pictures of one experiment with scale bar 25 µm, 50 µm and 200 µm for magnified area in white frame.

Supplementary Figure S2

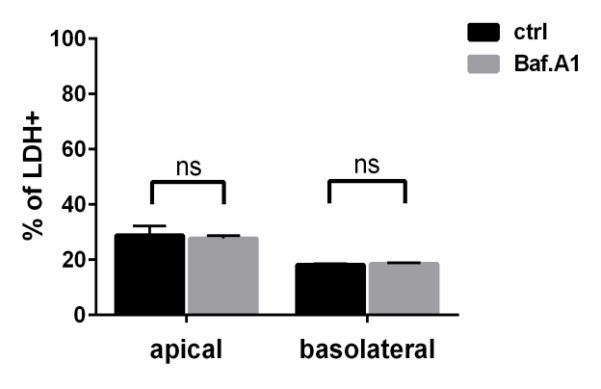
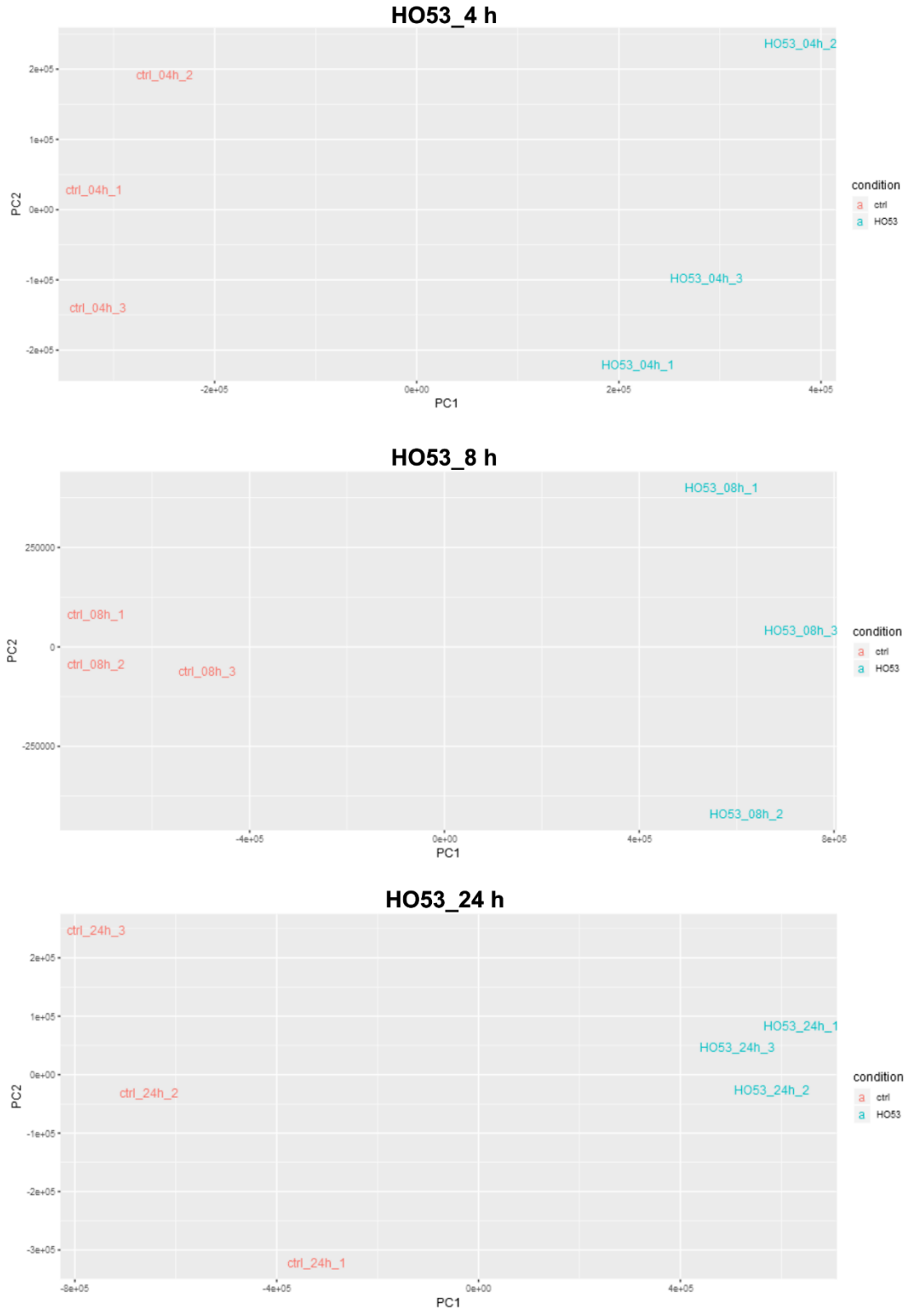


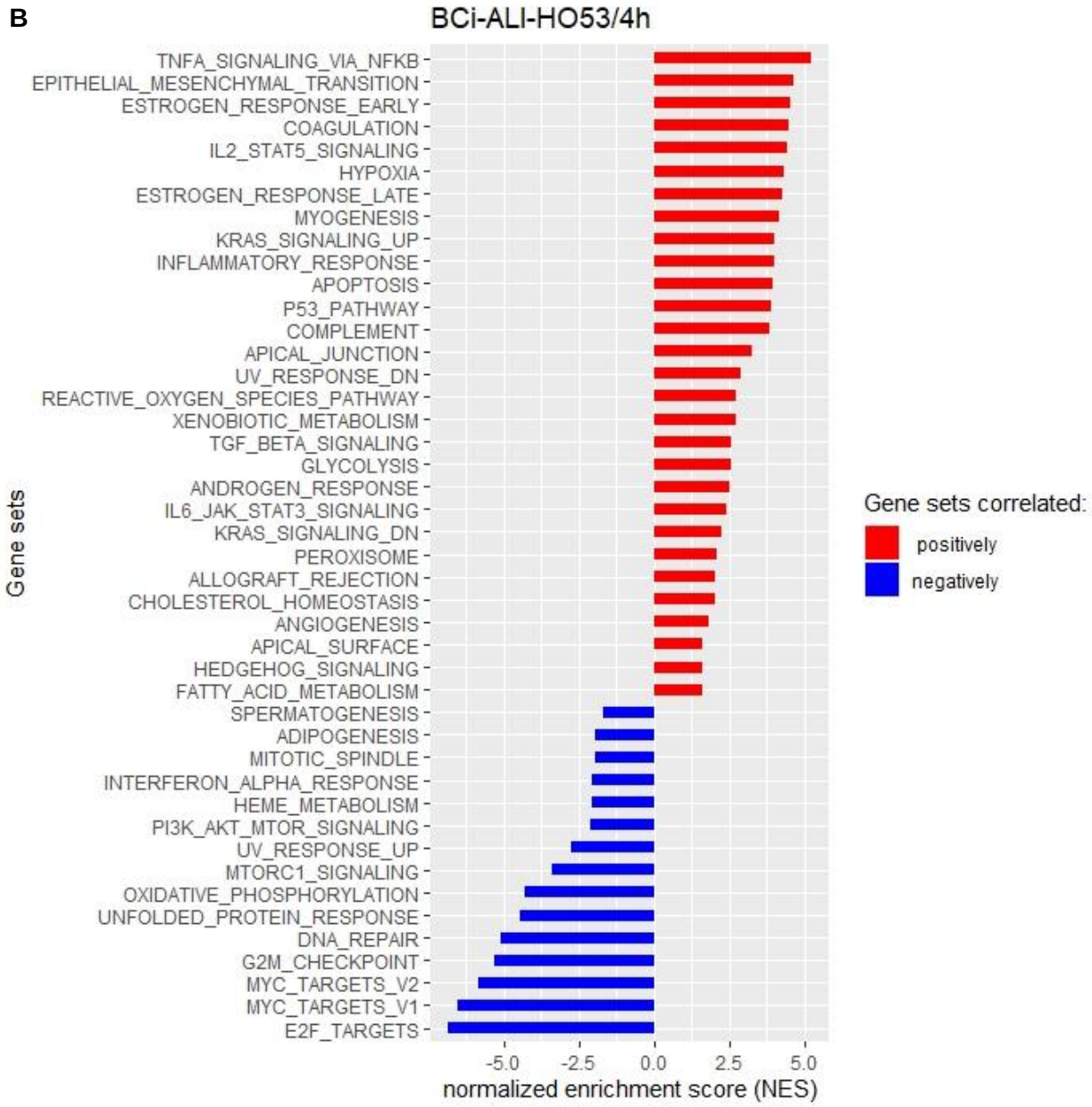
Fig. S2. Cytotoxic effect of Bafilomycin A1 on bronchial epithelial BCi cells differentiated in air-liquid interphase (ALI) culture. Bafilomycin A1 at 100 nM was added to the lower chamber of the trans-well insert (basolateral side) for 24 h. Cytotoxicity of Bafilomycin A1 was measured as LDH release to the culture medium on both, apical and basolateral side of the trans-well insert and presented as a percentage of LDH release in positive control (100%). Data present average ± SD from n=3 independent experiments analyzed by two-way ANOVA with Sidak post-hoc test, where ns – non significant.

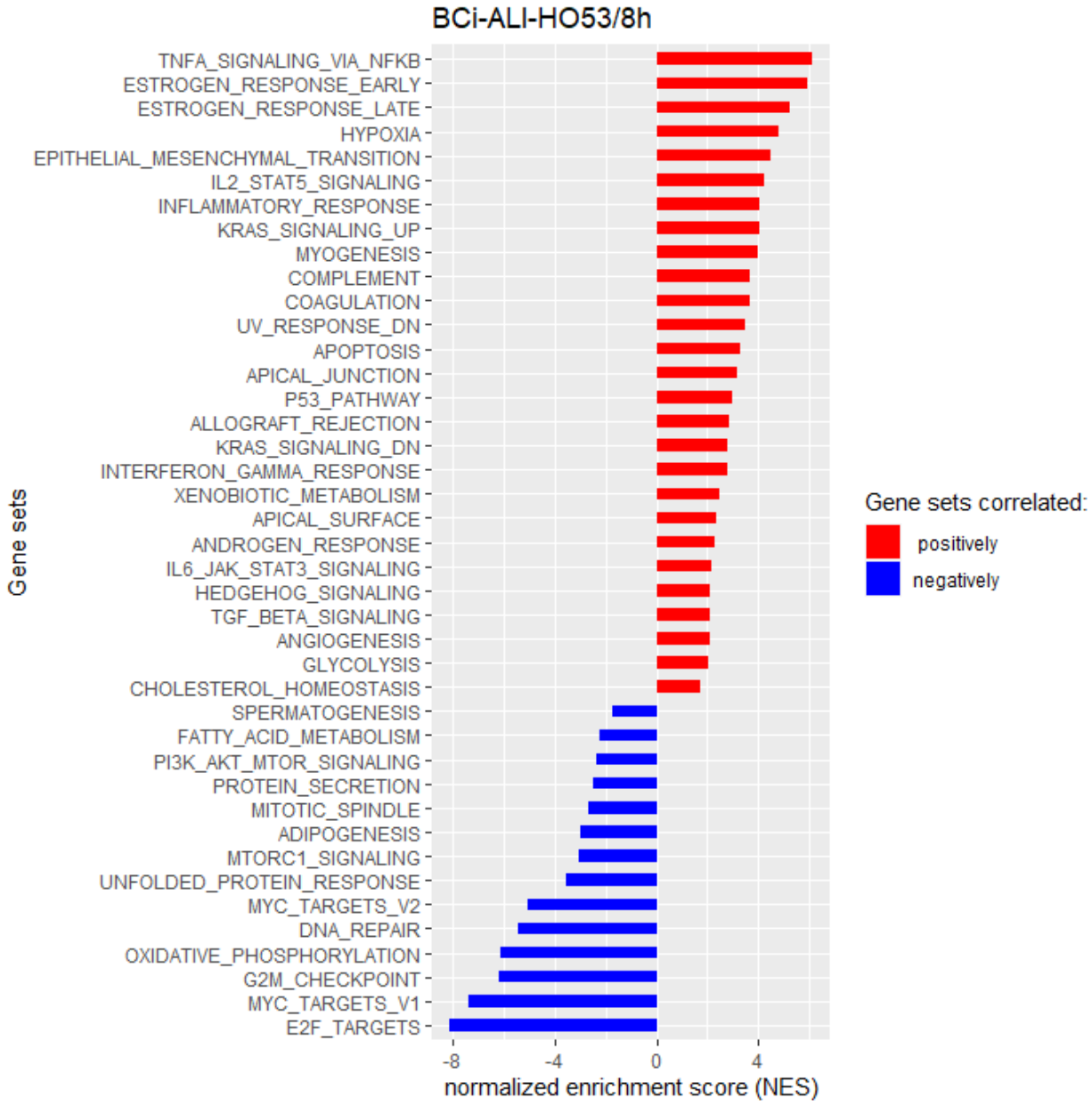
Supplementary Figure S3

A



B


BCi-ALI-HO53/4h
TNFA_SIGNALING_VIA_NFKB
EPITHELIAL_MESENCHYMAL_TRANSITION
ESTROGEN_RESPONSE_EARLY
COAGULATION
IL2_STAT5_SIGNALING
HYPOXIA
ESTROGEN_RESPONSE_LATE
MYOGENESIS
KRAS_SIGNALING_UP
INFLAMMATORY_RESPONSE
APOPTOSIS
P53_PATHWAY
COMPLEMENT
APICAL_JUNCTION
UV_RESPONSE_DN
REACTIVE_OXYGEN_SPECIES_PATHWAY
XENOBIOTIC_METABOLISM
TGF_BETA_SIGNALING
GLYCOLYSIS
ANDROGEN_RESPONSE
IL6_JAK_STAT3_SIGNALING
KRAS_SIGNALING_DN
PEROXISOME
ALLOGRAFT_REJECTION
CHOLESTEROL_HOMEOSTASIS
ANGIOGENESIS
APICAL_SURFACE
HEDGEHOG_SIGNALING
FATTY_ACID_METABOLISM
SPERMATOGENESIS
ADIPOGENESIS
MITOTIC_SPINDLE
INTERFERON_ALPHA_RESPONSE
HEME_METABOLISM
PI3K_AKT_MTOR_SIGNALING
UV_RESPONSE_UP
MTORC1_SIGNALING
OXIDATIVE_PHOSPHORYLATION
UNFOLDED_PROTEIN_RESPONSE
DNA_REPAIR
G2M_CHECKPOINT
MYC_TARGETS_V2
MYC_TARGETS_V1
E2F_TARGETS
Gene sets
-5.0
-2.5
0.0
2.5
5.0
normalized enrichment score (NES)
Gene sets correlated:
positively
negatively


BCi-ALI-HO53/8h
TNFA_SIGNALING_VIA_NFKB
ESTROGEN_RESPONSE_EARLY
ESTROGEN_RESPONSE_LATE
HYPOXIA
EPITHELIAL_MESENCHYMAL_TRANSITION
IL2_STAT5_SIGNALING
INFLAMMATORY_RESPONSE
KRAS_SIGNALING_UP
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COMPLEMENT
COAGULATION
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APOPTOSIS
APICAL_JUNCTION
P53_PATHWAY
ALLOGRAFT_REJECTION
KRAS_SIGNALING_DN
INTERFERON_GAMMA_RESPONSE
XENOBIOTIC_METABOLISM
APICAL_SURFACE
ANDROGEN_RESPONSE
IL6_JAK_STAT3_SIGNALING
HEDGEHOG_SIGNALING
TGF_BETA_SIGNALING
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GLYCOLYSIS
CHOLESTEROL_HOMEOSTASIS
SPERMATOGENESIS
FATTY_ACID_METABOLISM
PI3K_AKT_MTOR_SIGNALING
PROTEIN_SECRETION
MITOTIC_SPINDLE
ADIPOGENESIS
MTORC1_SIGNALING
UNFOLDED_PROTEIN_RESPONSE
MYC_TARGETS_V2
DNA_REPAIR
OXIDATIVE_PHOSPHORYLATION
G2M_CHECKPOINT
MYC_TARGETS_V1
E2F_TARGETS
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0
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normalized enrichment score (NES)
Gene sets correlated:
positively
negatively

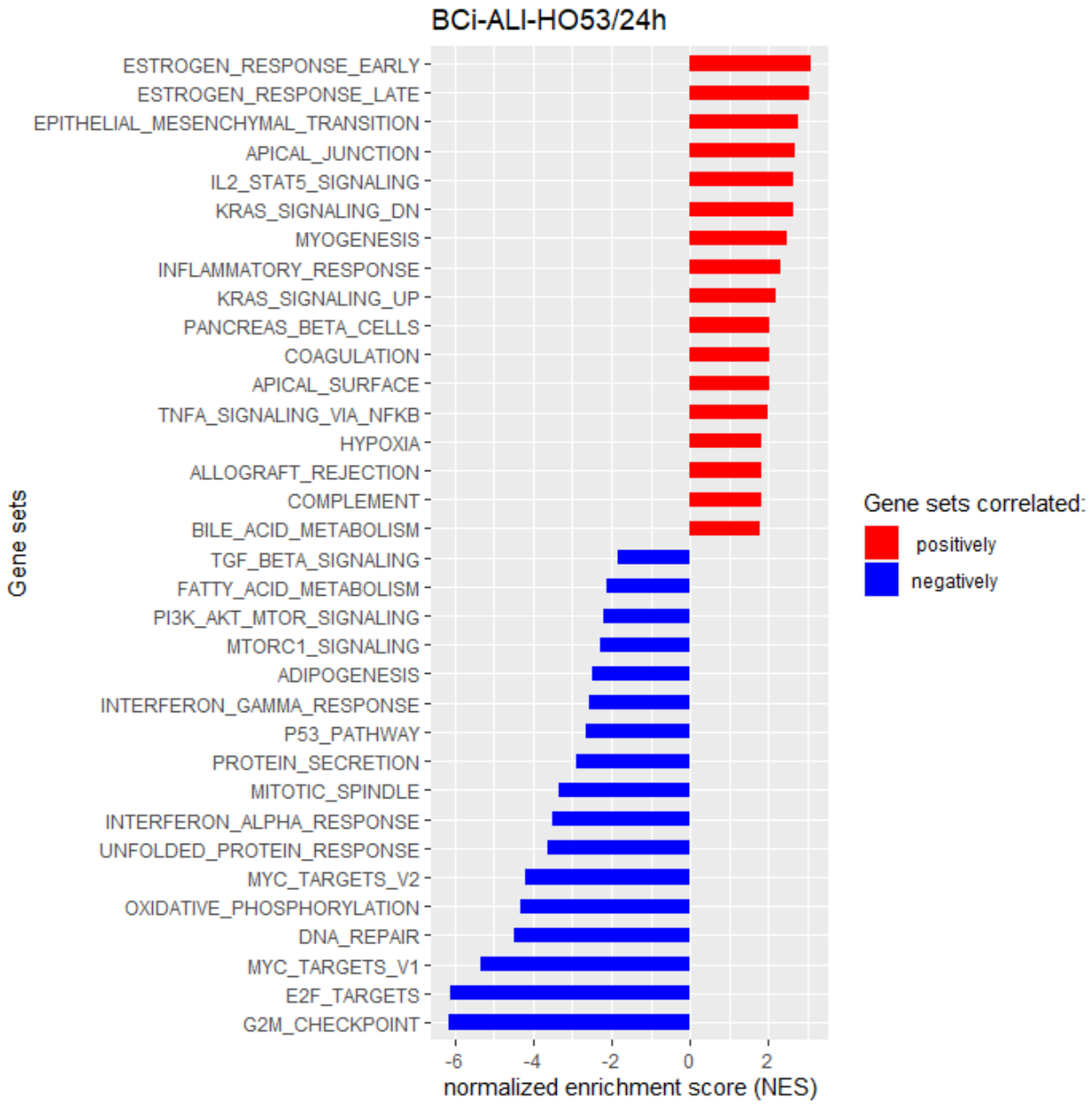


Fig. S3. Transcriptome analysis of HO53 treated differentiated BCi cells. A) Principal component analysis (PCA) of Kallisto/sleuth output data for triplicates from control and HO53 treated groups. PCA plots present clustering of triplicates from the control group in comparison to triplicates from HO53 treated groups for each time point 4 h (HO53/ctrl-4h-1/2/3), 8 h (HO53/ctrl-8h-1/2/3) and 24 h (HO53/ctrl-24h-1/2/3) treatment. Estimated counts were used as a unit of principal component analysis. **B)** Gene set enrichments analysis (GSEA) of the expression data from differentiated in air-liquid interphase culture BCi cells after 4 h, 8 h and 24 h of treatment

with HO53. All affected gene sets by HO53 for each time point were presented as positively correlated gene sets (red, NES > 0) and negatively correlated gene sets (blue, NES < 0) with FDR ≤ 0.05. NES – normalized enrichment score; FDR/ q-value – false discovery rate.

Supplementary Figure S4

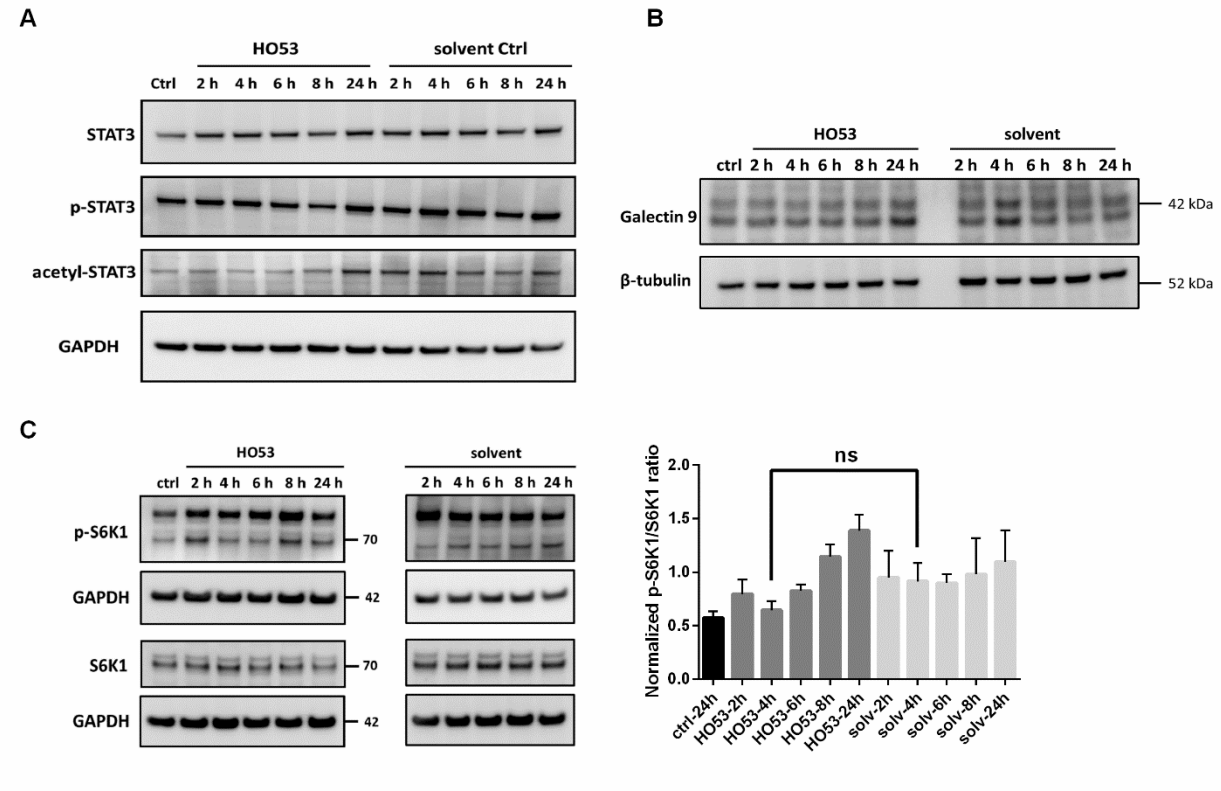


Fig. S4. Induction of autophagy by HO53 in differentiated BCi cells is not mediated by STAT3 and galectin 9. A) Changes in STAT3 phosphorylation (Tyr705) and STAT3 acetylation (Lys685) analysed by Western blot after 2, 4, 6, 8 and 24 h of treatment with HO53 (75 µM) and solvent control (solvent Ctrl; 0.3% final concentration of DMSO). GAPDH served as a loading control and the experiment was performed n=1. **B)** Expression level of galectin 9 at different time points of HO53 and solvent control treatment analyzed by Western blotting. β-tubulin was used as a loading control. The representative Western blot of n=3 independent experiments. **C)** Activation of mTOR signaling pathway analyzed by Western blotting of phosphorylated S6K1 (Thr389) at different time points of HO53 treatment. Quantification of Western blots was performed in two steps: first, by measurements of bands intensity and normalization to GAPDH (loading control) and then, by calculation of normalized ratio for phospho-S6K1 (Thr389) to total S6K1. The

representative Western blot of n=4 independent experiments. Quantitative comparison was done on the samples run in one experiment on separate gels/blots processed in parallel. Statistical analysis was performed by one-way ANOVA with Sidak post-hoc test, where ns – non-significant versus corresponding solvent control. Full-length blots are presented in Supplementary Figure S7.

Supplementary Figure S5

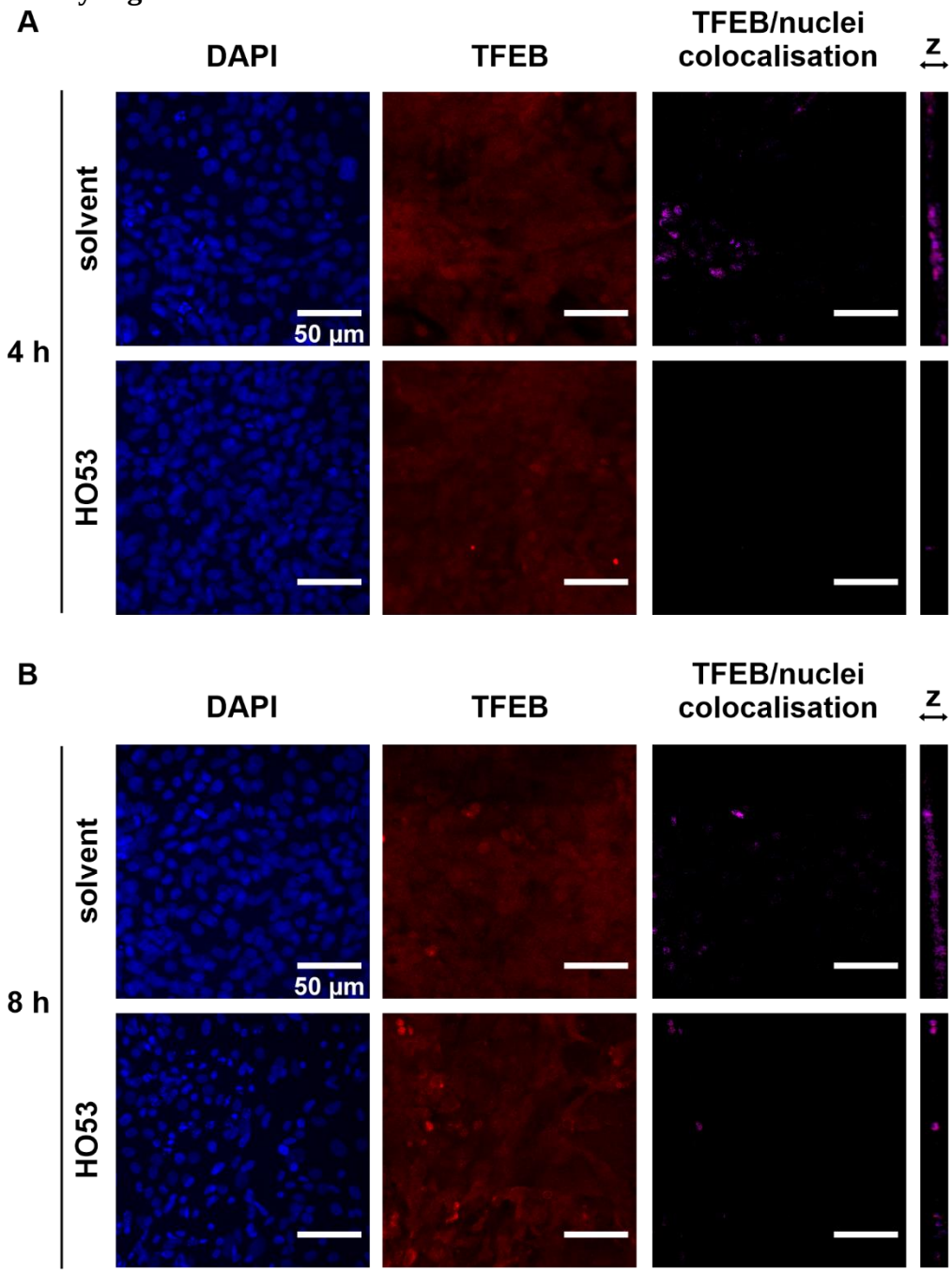


Fig. S5. Nuclear translocation of TFEB in differentiated BCi cells upon HO53 treatment at different time points. TFEB nuclear translocation after **A)** 4 h and **B)** 8 h of treatment with 75 µM HO53 analyzed by confocal imaging of nuclei (blue, DAPI), TFEB (red) and extracted signal from co-localized TFEB/nuclei (pink) from merged channels with scale bar 50 µm. Representative images from at least 3 different areas of ALI filter from n=3 independent experiments.

Supplementary Figure S6

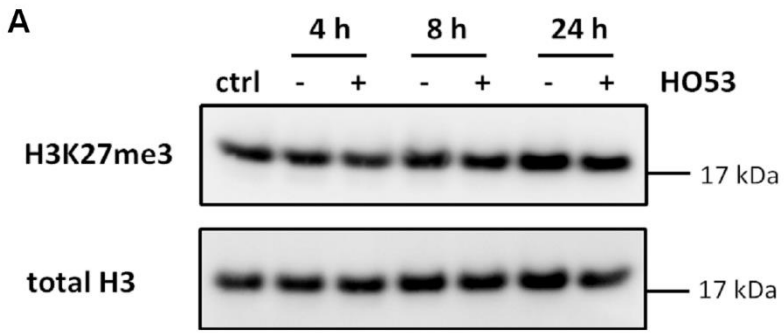


Fig. S6. Treatment with HO53 does not affect H3K27 trimethylation level. The representative Western blot of H3K27 trimethylation and total H3 level (loading control) analyzed by Western blotting in untreated (ctrl) and treated with solvent (-) and 75 µM HO53 (+) ALI BCi cells at indicated time points. DMSO at final concentration of 0.3% was used as a solvent control. The representative Western blot of n=3 independent experiments. Full-length blots are presented in Supplementary Figure S7.

Supplementary Figure S7

Figure 1A

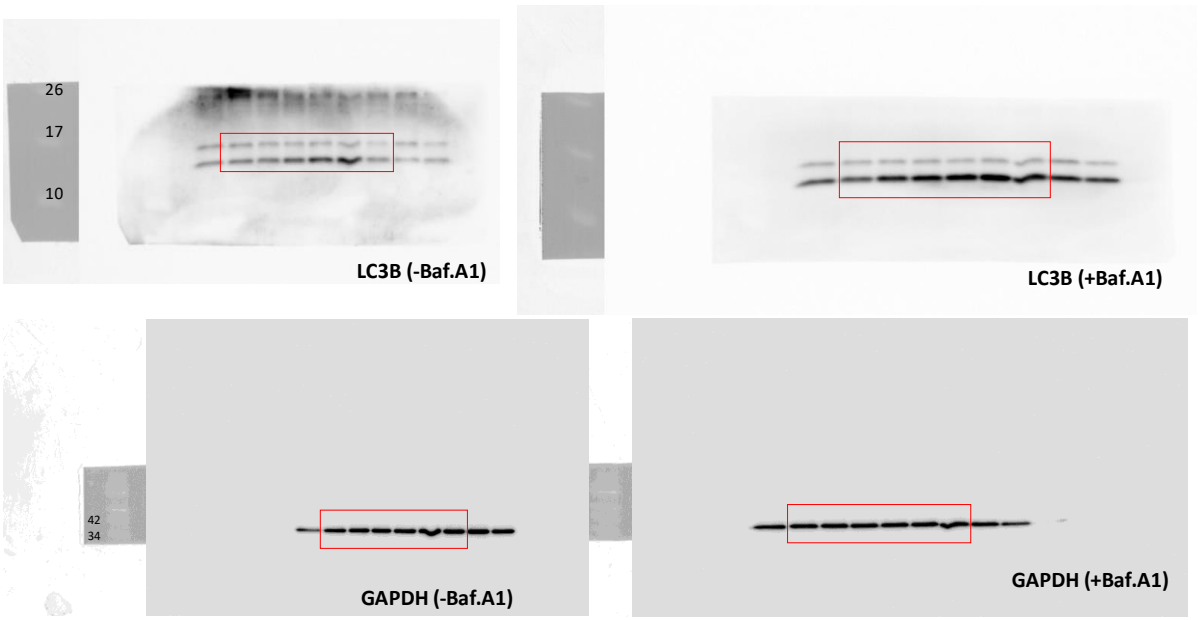


Figure 1B

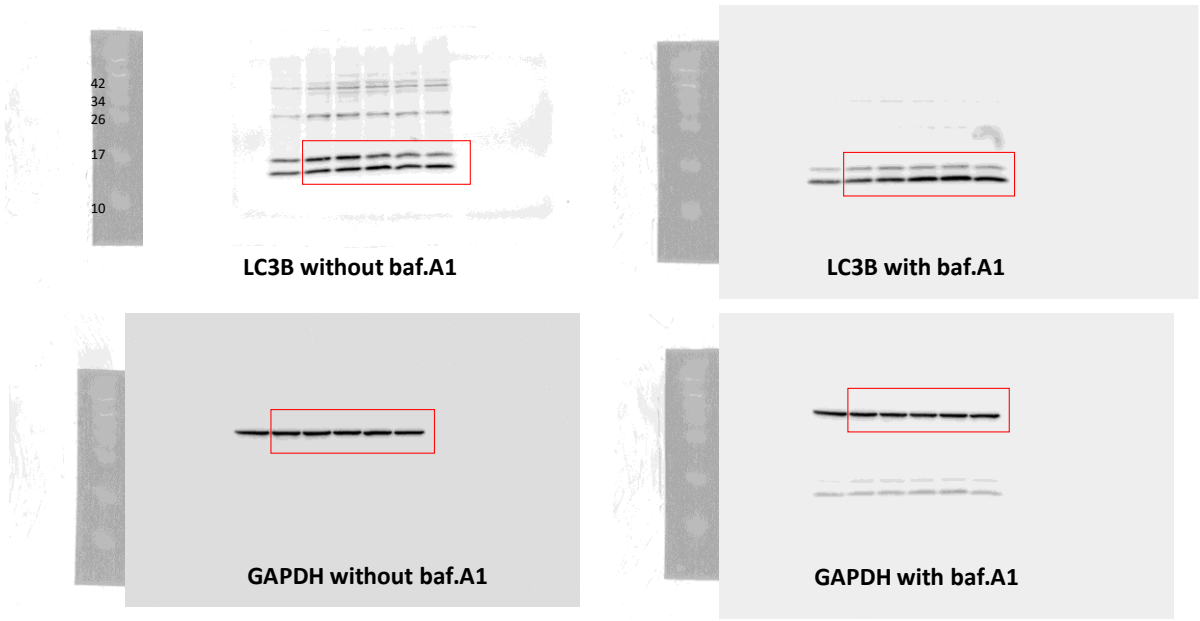


Figure S1A

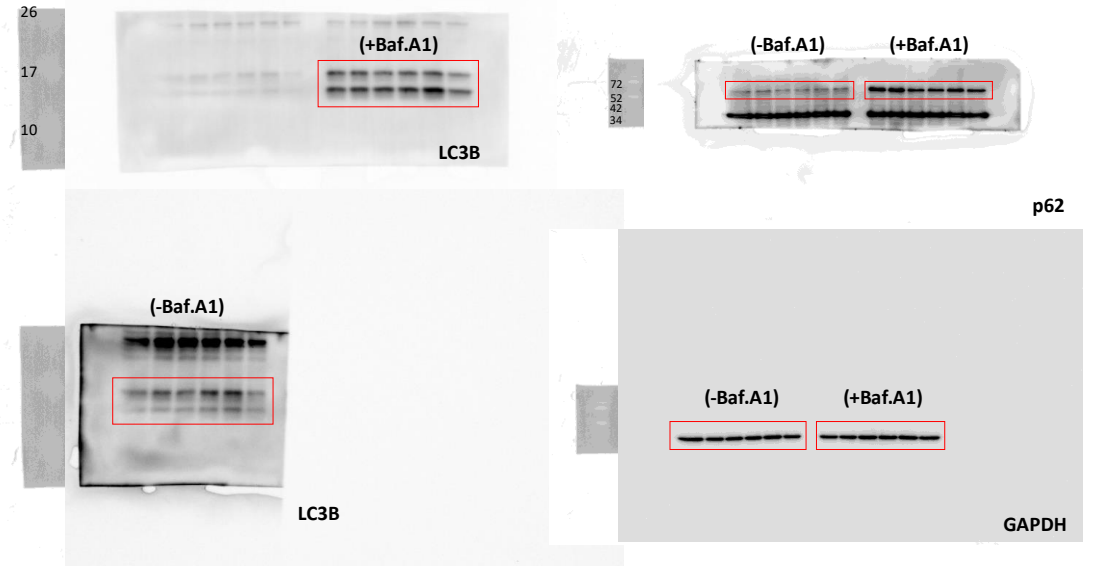


Figure S1B

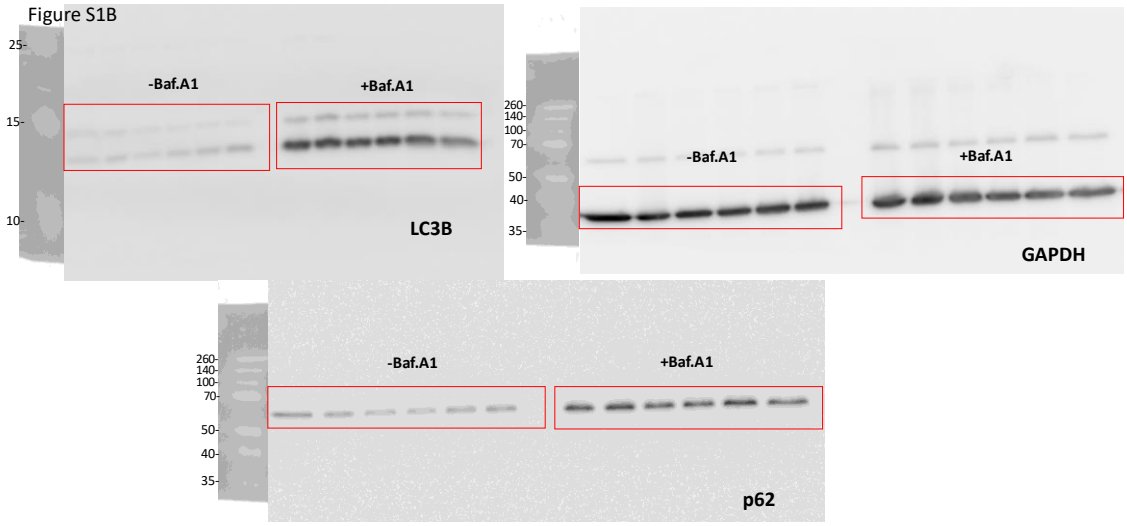


Figure S1C

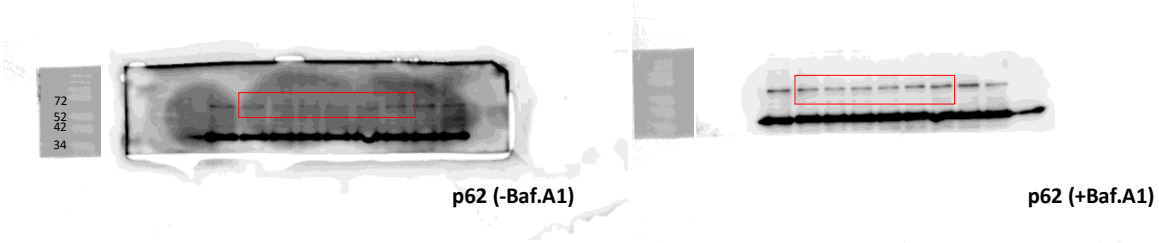


Figure S1D

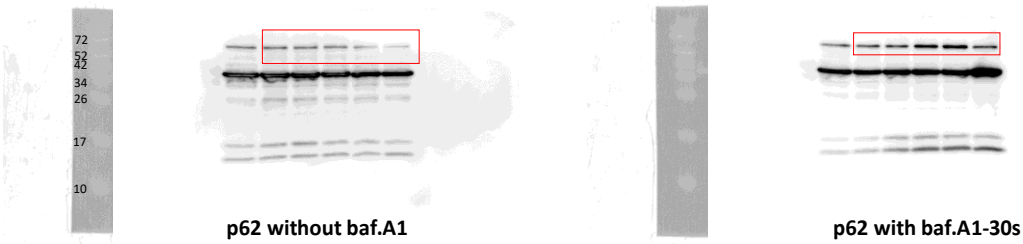


Figure 3D

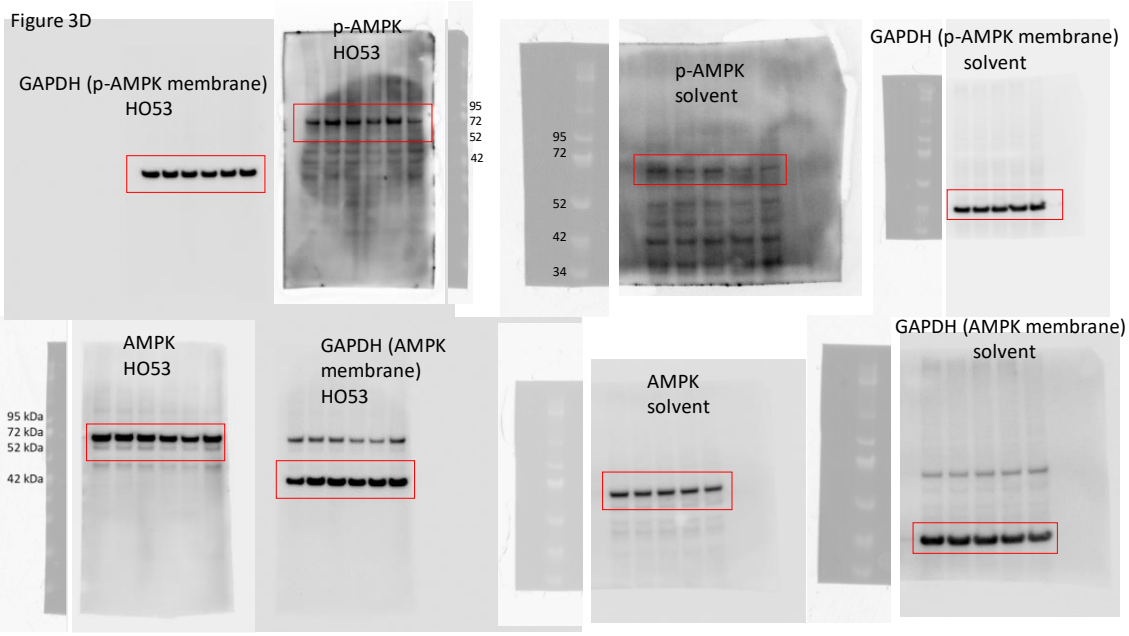


Figure 3E

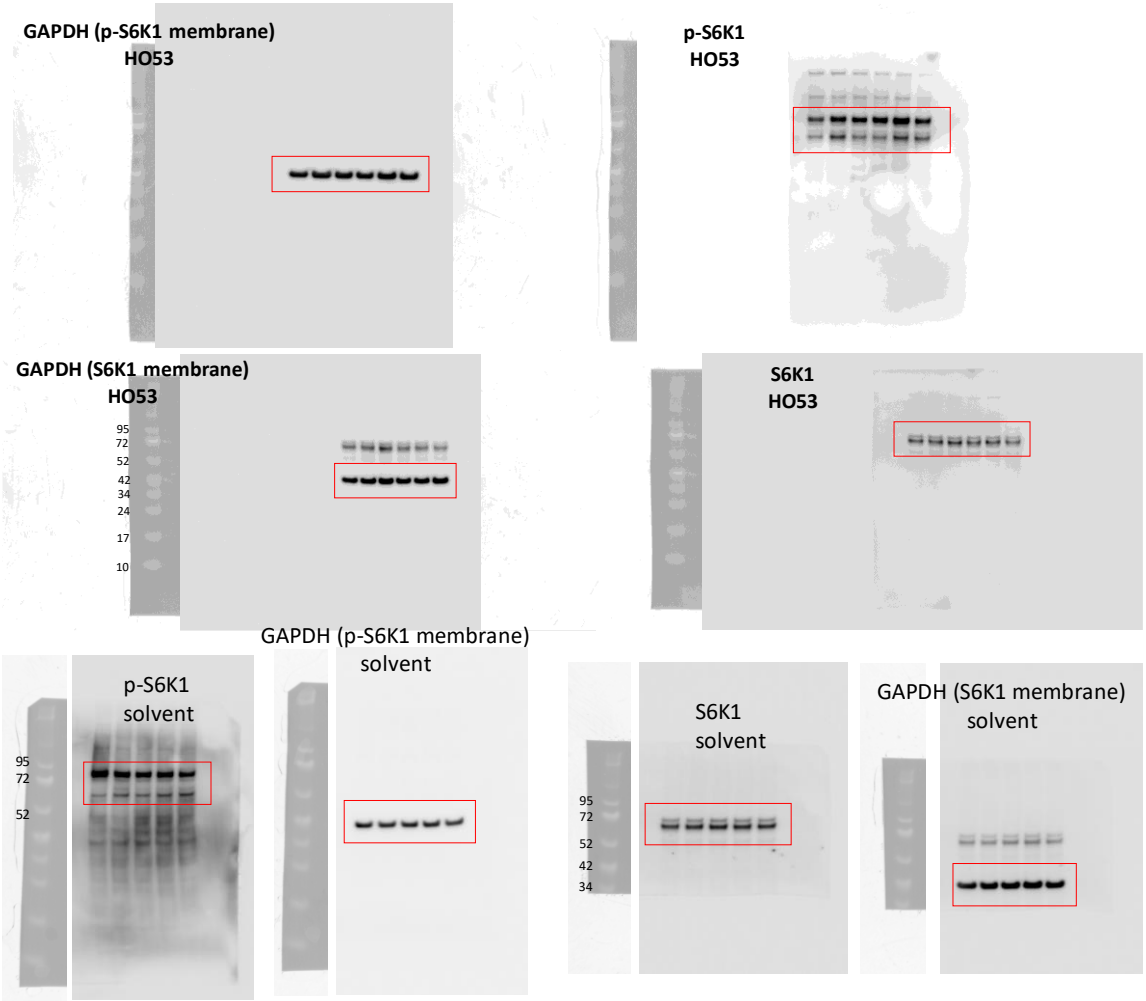
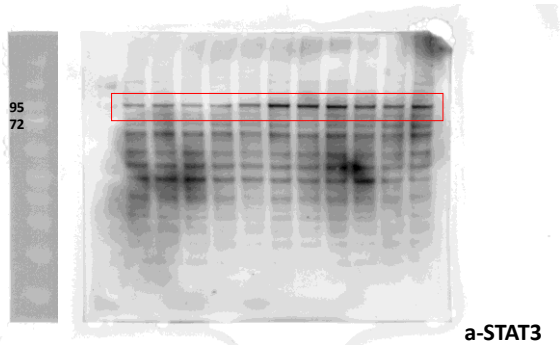


Figure S3A



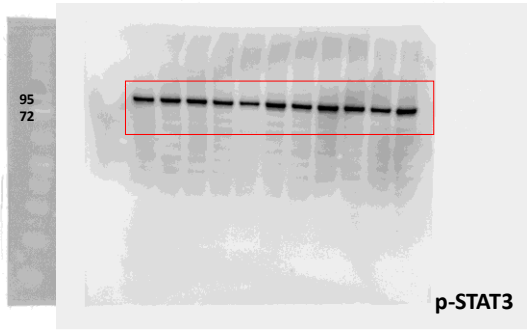
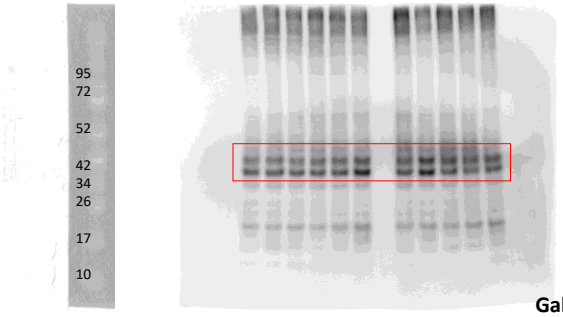


Figure S3B



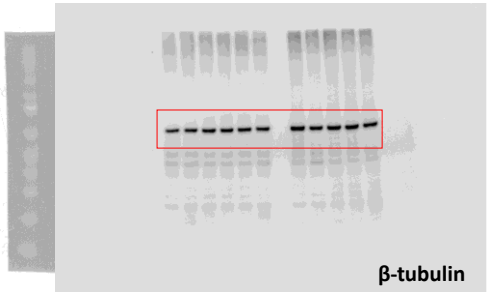


Figure 5F

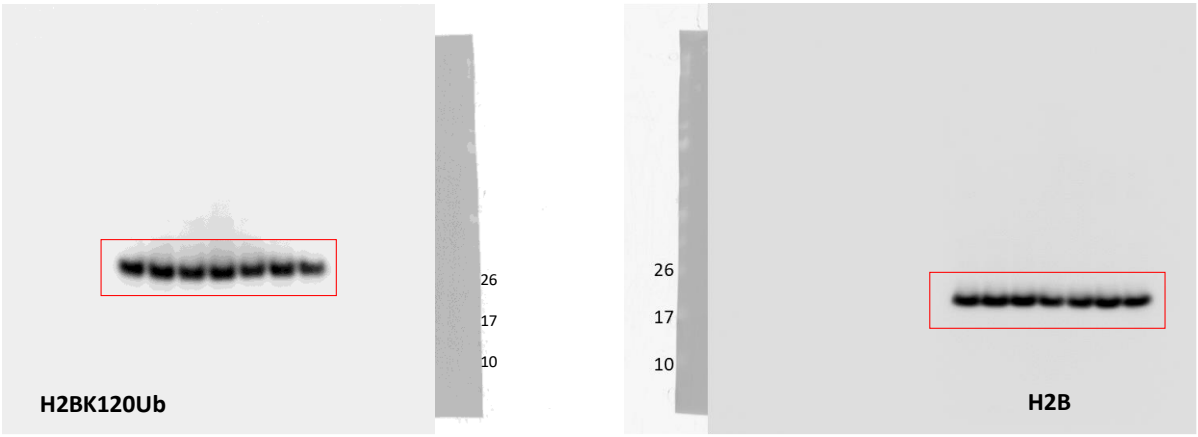


Figure S5

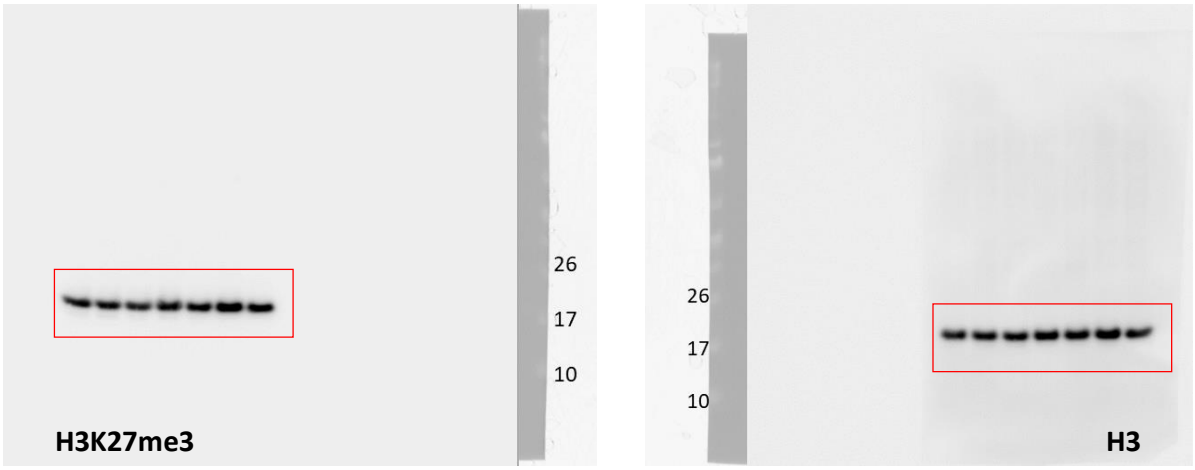


Fig. S7. Display of full-length Western blot pictures.

Supplementary Table S1. List of primary antibodies used in this study.

Antibodies	Source	Identifier
Rabbit acetyl-Stat3 (Lys685)	Cell Signalling Technology	#2523
Mouse AMPKα (F6)	Cell Signalling Technology	#2793
Rabbit GAPDH (14C10)	Cell Signalling Technology	#2118
Rabbit LC3B	Cell Signalling Technology	#2775
Rabbit SQSTM1/p62 (D1Q5S)	Cell Signalling Technology	#39749
Rabbit phospho-AMPKα (Thr172) (D4D6D)	Cell Signalling Technology	#50081

Mouse phospho-p70 S6 kinase (Thr389) (1A5)	Cell Signalling Technology	#9206
Rabbit phospho-Stat3 (Tyr705) (D3A7)	Cell Signalling Technology	#9145
Rabbit p70 S6 kinase (49D7)	Cell Signalling Technology	#2708
Mouse Stat3 (124H6)	Cell Signalling Technology	#9139
Rabbit TFEB	Cell Signalling Technology	#4240
Rabbit Galectin-9	Cell Signalling Technology	#54330
Rabbit β-Tubulin	Cell Signalling Technology	#2128
Rabbit Tri-Methyl-Histone H3 (Lys27)	Cell Signalling Technology	#9733
Mouse Ubiquityl-Histone H2B (Lys120)	Cell Signalling Technology	#5546
Mouse Histone H3	Cell Signalling Technology	#3638
Rabbit Histone H2B	Cell Signalling Technology	#8135
Mouse Occludin (OC-3F10)	Invitrogen	#33-1500
Mouse Mucin 5AC	Abcam	ab3649
Mouse alpha Tubulin (acetyl K40)	Abcam	ab24610

Supplementary Table S2. List of primers used in this study.

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
LGALS9B/C	CGTCCCCTTTTCTGGGACTA	CGTGTTGCACACCACATACC
PRKAA2	GCTATGAAGCAGCTGGATTTTGA	GCTGAGGTGTTGAGGAACCA
MAP1LC3A	TGAACTGAGCTGCCTCTACC	GAGGGACAACCCTAACACG
MAP1LC3C	GAGGAAGTTGCTGGAATCCG	GTAAAAGGCTTCCGTGGCTC
ATG4B	GGACATCAACGAGGCCTAC	CAACGTAGCCGATGAAGTAGT
ATG16L1	GCACCAAGAGGAACTGACTGA	AAAGCTTAGTGCGCAGGTCT
ATG16L2	CTTCGGGACCGTACGCAA	GACCAGTGATGGGACTTGGT
USP44	CCTGATGGAAACTGGGCGA	CATGTTTGCACGTATCCATTGC
EHMT2	CTCCGACGTGTGGTTTGC	GACACAGGGAATGGGCAC
EZH2	TGCTTCCTACATCGTAAGTGC	TGGGGTCTTTATCCGCTCAG
TSC2	TTGATGACACCCCCGAGAAG	CGGACCACATGTTCAGACAC
EEF2	GACATCACCAAGGGTGTGCAG	GCGGTCAGCACACTGGCATA