Supplementary Information

MEK/ERK-mediated oncogenic signals promote secretion of extracellular vesicles by controlling lysosome function

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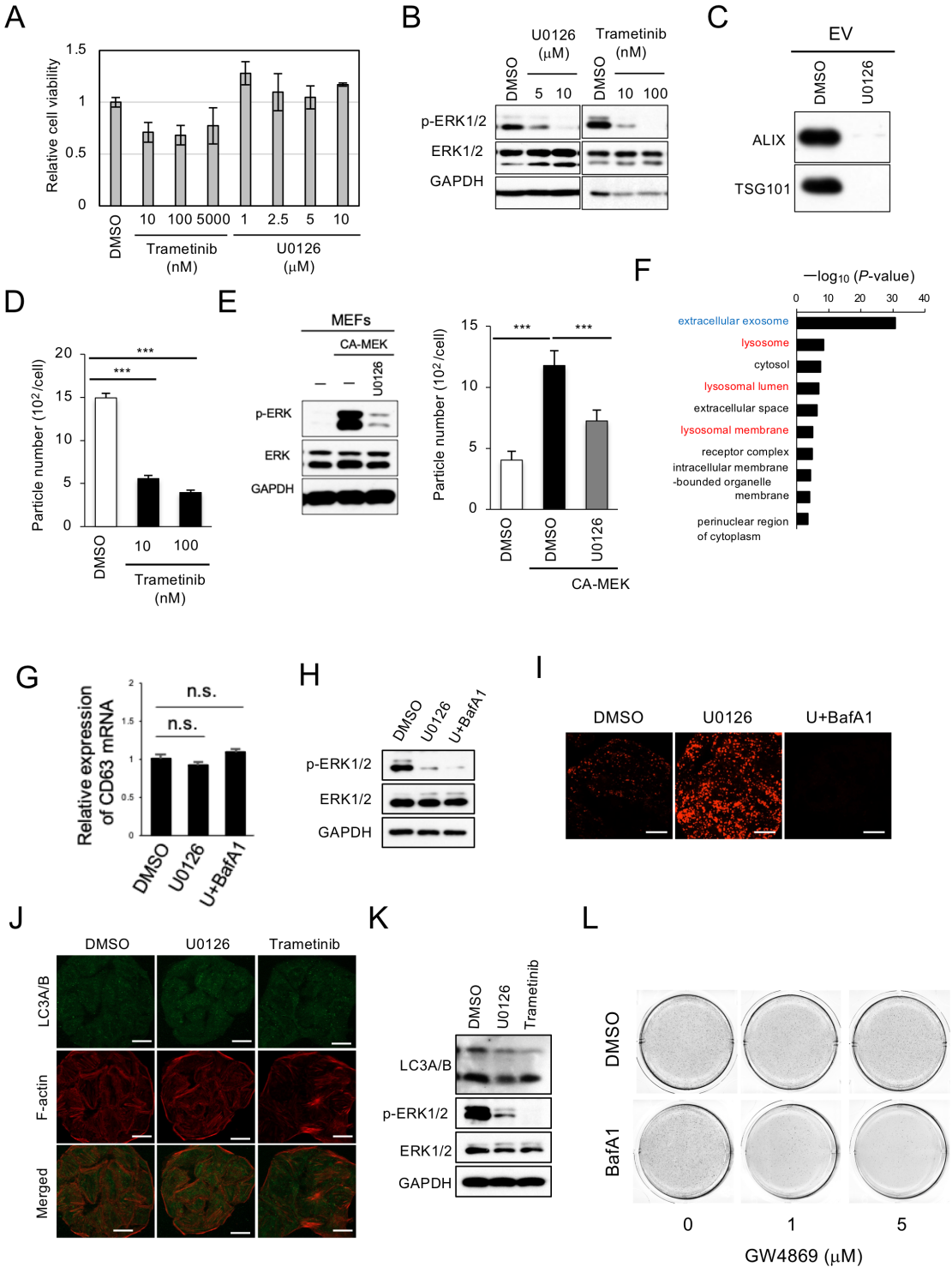

Fig. S1. MEK/ERK activation increases EV secretion. (***A***) Cell viability of HT29/CD63Nluc cells at different concentrations of trametinib or U0126. (***B***) Total cell lysates from HT29/CD63Nluc cells treated with U0126 or trametinib for 24 h at the indicated concentrations were immunoblotted with the indicated antibodies. (***C***) EV lysates from HT29/CD63Nluc cells treated with or without U0126

(10 μM) were immunoblotted with the indicated exosome markers. (***D***) HT29 cells treated with trametinib at the indicated concentrations were examined by NTA for quantification of isolated EV particles. (***E***) MEFs expressing constitutively active MEK (CA-MEK) were treated with or without U0126 (10 μM) for 24 h, and whole-cell lysates were immunoblotted with the indicated antibodies (left panels). NTA for the quantitative measurement of isolated EVs from each MEF line (right panel). (***F***) Gene Ontology analysis of >2-fold ($log_2FC = 1.0$) upregulated genes in U0126-treated as compared with DMSO-treated HT29 cells (GSE18232). The analysis was performed using the DAVID bioinformatics tool. (***G***) Expression of *CD63* in HT29 cells treated with DMSO, 10 μM U0126, and 50 nM BafA1, respectively, for 24 h. (***H***) Total cell lysates from HT29 cells treated with DMSO or 10 μM U0126 and 50 nM BafA1 for 24 h were immunoblotted with the indicated antibodies. (***I***) Lysotracker Red staining of the HT29 cells indicated in (H). (***J***) LC3 immunostaining of HT29 cells treated with DMSO, U0126 (10 μM), or trametinib (100 nM) for 24 h. (***K***) Total cell lysates from cells indicated in (J) were immunoblotted with the indicated antibodies. (***L***) HT29 cells treated with DMSO or 1 nM BafA1 and/or GW4869 (GW; 1 or 5 μM) were used for the soft-agar colony formation assay. Representative dishes are shown. Scale bar = 10 μm. Data represent the mean ± standard deviation of three independent measurements. Statistical analysis was performed using one-way ANOVA test. *$P < 0.05$, *** $P < 0.001$; *n.s.*, not significant.

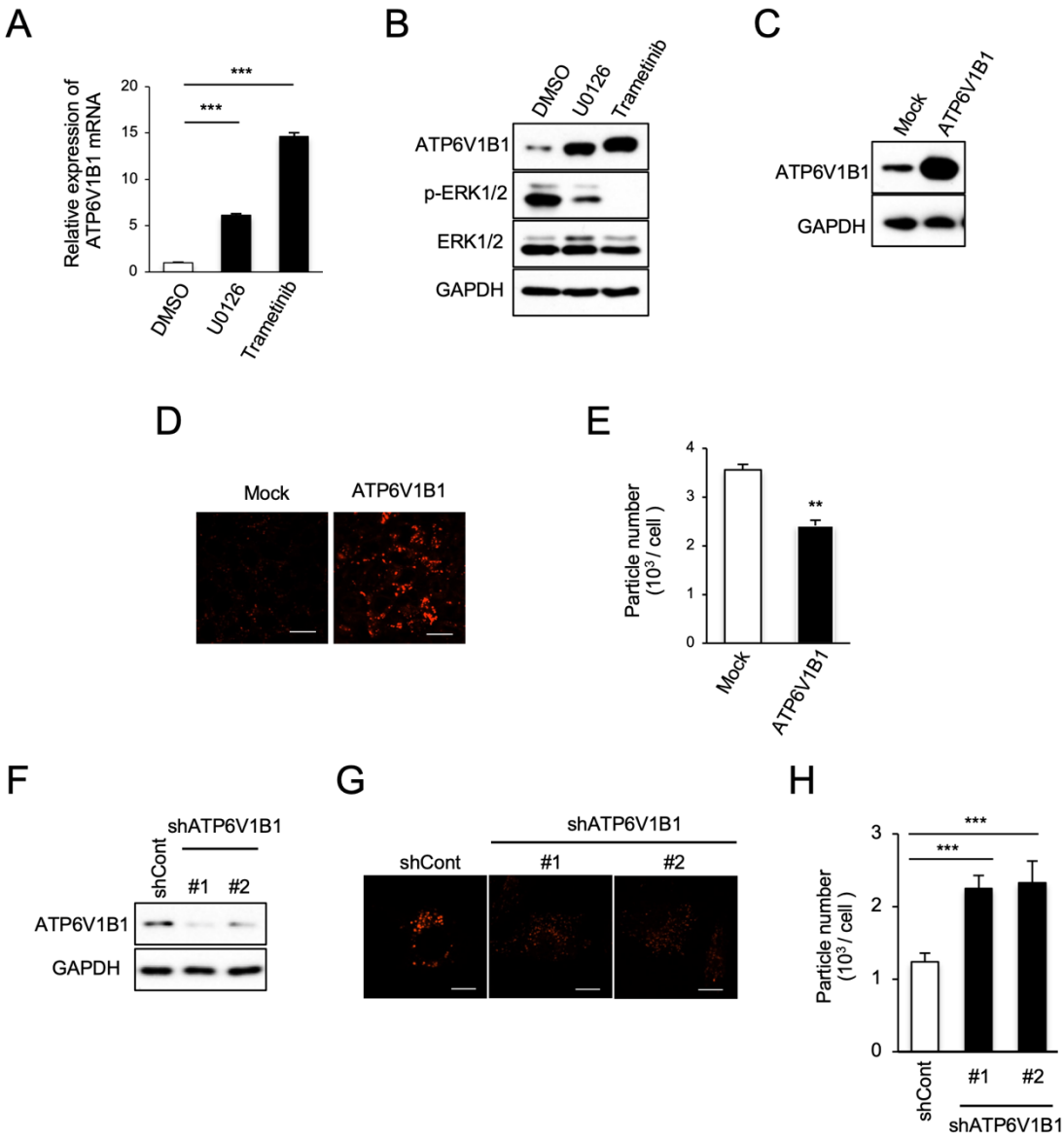

Fig. S2. MEK/ERK suppression promotes lysosomal acidification. (***A***) Expression analysis of *ATP6V1B1* in HT29 cells treated with DMSO, U0126 (10 μM), or trametinib (100 nM) for 24 h. (***B***) Total cell lysates from HT29 cells treated with DMSO, 10 μM U0126, or 100 nM trametinib for 24 h were immunoblotted with the indicated antibodies. (***C***) HT29 cells expressing mock or *ATP6V1B1* were immunoblotted with the indicated antibodies. (***D***) HT29 cells used in (C) were subjected to staining by Lysotracker Red DND-99. (***E***) NTA for the quantitative measurement of particles isolated from cells indicated in (C). (***F***) Total cell lysates from MEFs transfected with *ATP6V1B1* shRNA were immunoblotted with anti-ATP6V1B1. (***G***) The cells indicated in (F) were subjected to Lysotracker Red staining. (***H***) NTA for the quantitative measurement of isolated particles from the cells indicated in (F). Scale bar = 10 μm (D, G). Data represent the mean ±

standard deviation of three independent measurements. Statistical analysis was performed using one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$.

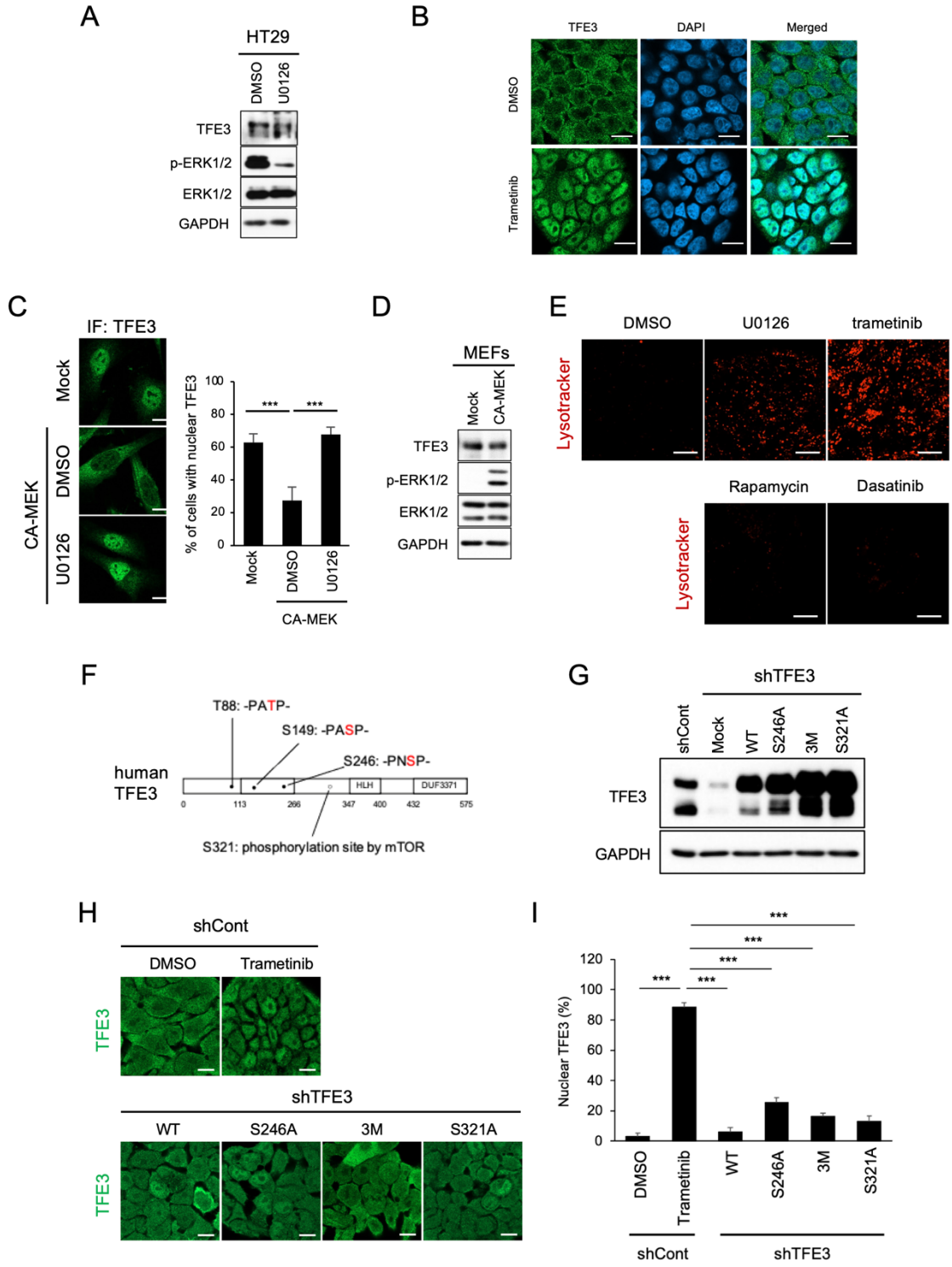

Fig S3. Nuclear localization of TFE3 by MEK/ERK inactivation. (***A***) Total cell lysates from HT29 cells treated with DMSO or U0126 (10 μM) for 24 h were immunoblotted with the indicated

antibodies. (***B***) TFE3 immunostaining and nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) of HT29 cells treated with DMSO or trametinib (100 nM) for 24 h. (***C***) TFE3 immunostaining of parental and MEK-transformed (CA-MEK) MEFs treated with DMSO and U0126 (10 μM), respectively, for 24 h (left panels). Quantification of the ratio (%) of cells with nuclear localization of TFE3 (n > 150 cells, pooled from three independent experiments). (***D***) Cell lysates from MEFs expressing mock or CA-MEK were immunoblotted with the indicated antibodies. (***E***) Lysotracker Red staining of HT29 cells treated with DMSO, U0126 (10 μM), trametinib (100 nM), rapamycin (200 nM), or dasatinib (50 nM) for 24 h. (***F***) Sequences of ERK substrate motif in human TFE3. (***G***) The indicated shRNA-resistant TFE3 mutants were expressed in HT29 cells transfected with *TFE3* shRNA. 3M: T88A/S149A/S246A. Total cell lysates were subjected to immunoblotting with the indicated antibodies. (***H***) TFE3 immunostaining of the HT29 cells indicated in (*G*). (***I***) Quantification of the ratio (%) of cells with nuclear localization of TFE3 (n > 50 cells, pooled from three independent experiments). Scale bar = 10 μm (B, C, E, H). Data represent the mean ± standard deviation of three independent measurements. Statistical analysis was performed using one-way ANOVA test. ***$P < 0.001$.

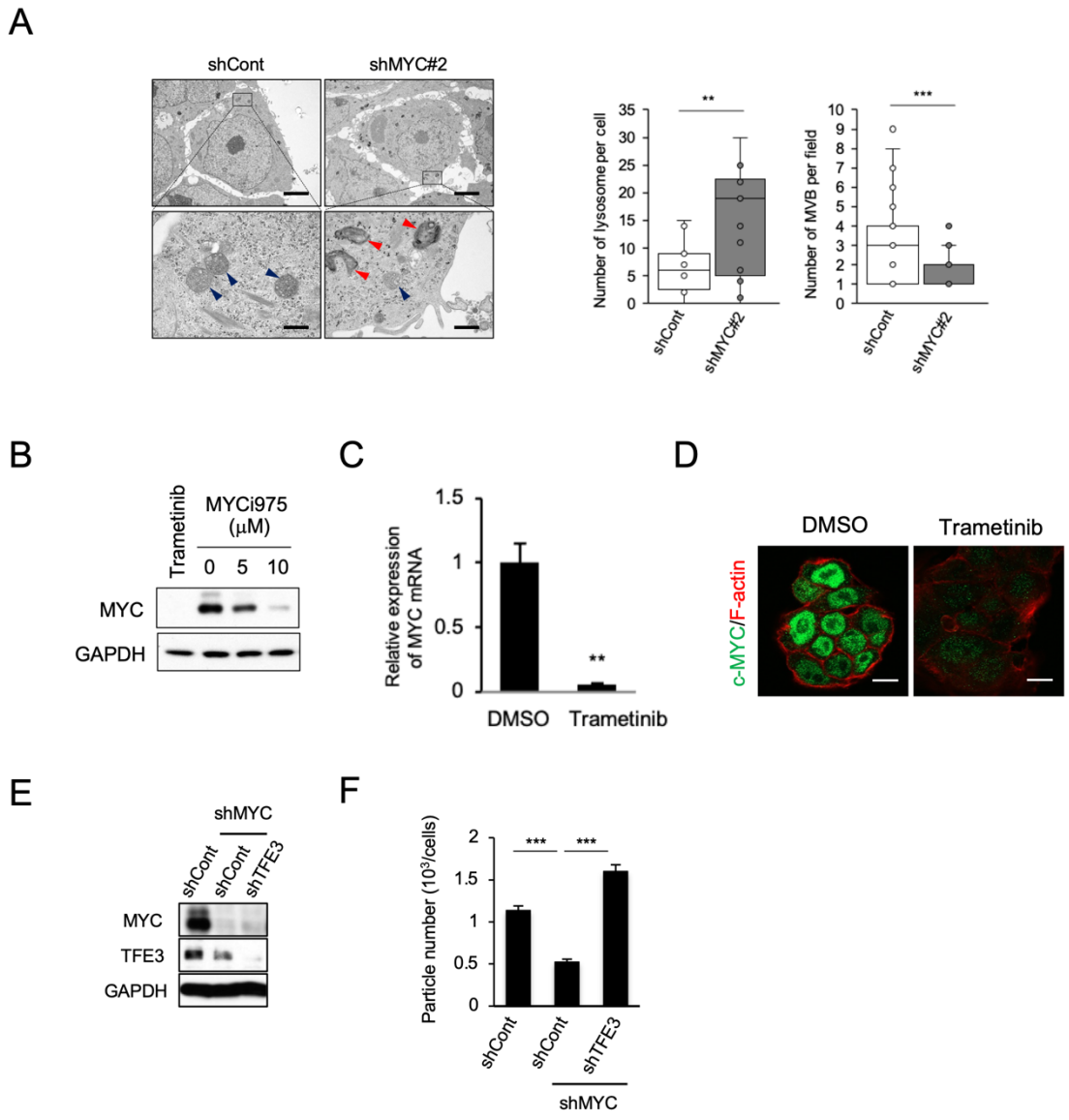

Fig. S4. MYC downregulation activates lysosome function. (***A***) Electron microscopy images of MVBs (blue arrowheads) and lysosomes (red arrowheads) in HT29 cells expressing control (shCont) or *MYC* (shMYC) shRNAs. Scale bar = 5 μm (upper panels). Boxed areas with dotted line are enlarged in the lower panels (scale bar = 500 nm). Quantification of the number of lysosomes per cell (n = 13) and MVBs in >20 fields per condition. Boxes represent the interquartile range, and the line inside the box represents the median value. (***B***) Cell lysates from HT29 cells treated with different concentrations of trametinib (100 nM) or MYC inhibitor (MYCi975) for 24 h were immunoblotted with the indicated antibodies. (***C***) Real-time qRT-PCR analysis of *MYC* in HT29 cells treated with or without trametinib (100 nM) for 24 h. (***D***) Intracellular localization of c-MYC (green) in HT29 cells treated with or without trametinib (100 nM) for 24 h were analyzed by immunostaining. Actin filaments were visualized by Alexa 594-phalloidin staining (red). Scale bar

= 10 μm. (***E***) Total cell lysates from HT29 cells transfected with shRNA were immunoblotted with the indicated antibodies. (***F***) NTA for the quantitative measurement of isolated particles from the cells indicated in (E). Data represent the mean ± standard deviation of three independent measurements. Statistical analysis was performed using one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$.

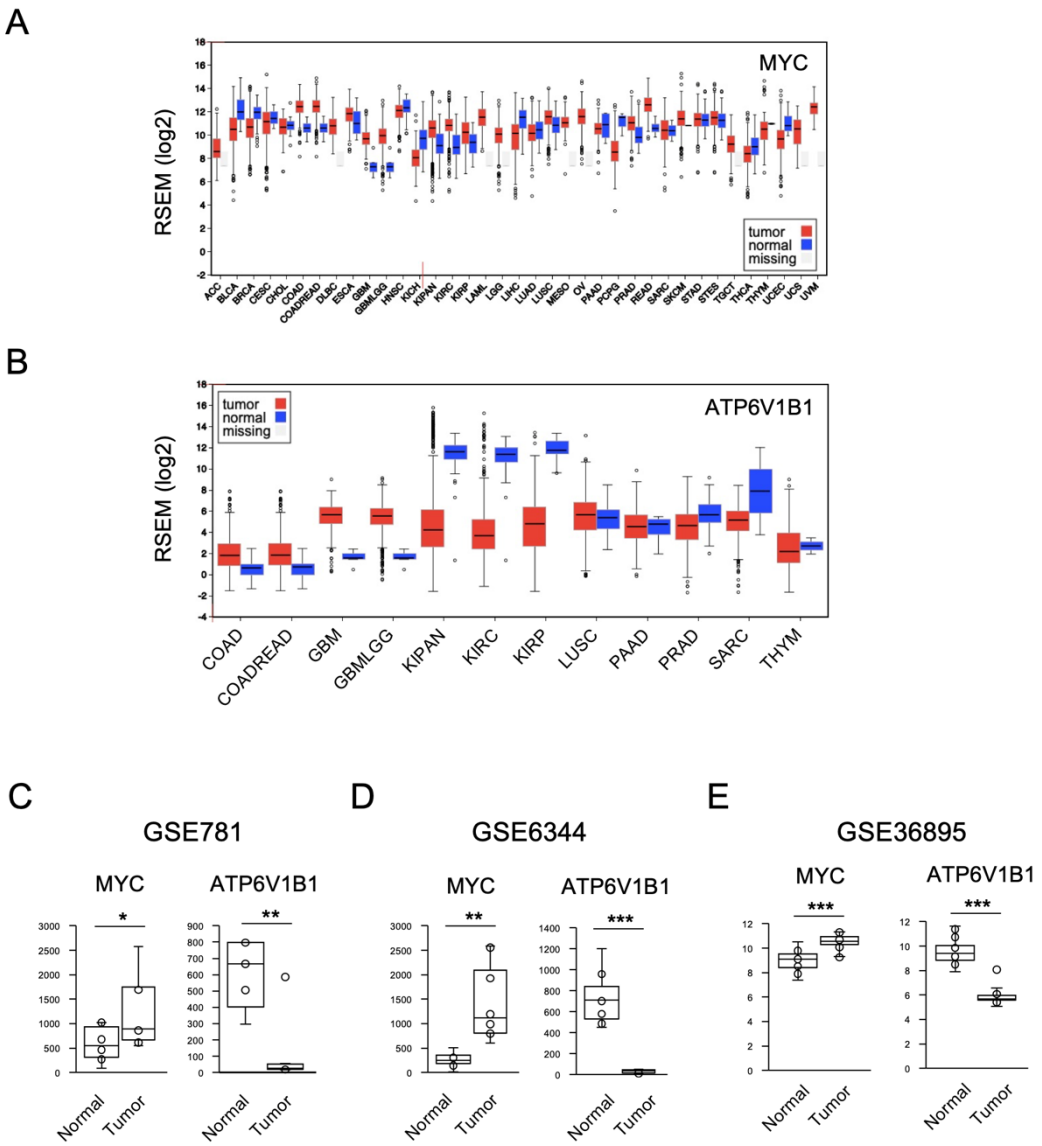

Fig. S5. Expression of *MYC* and *ATP6V1B1* in cancer tissues. *MYC* (***A***) and *ATP6V1B1* (***B***) levels in several types of tumor and adjacent normal tissues. The expression of each gene in primary tumor (red) and normal tissue (blue) was analyzed (TCGA dataset). ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; COADREAD, colorectal adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; GBMLGG, LGG glioma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIPAN, pan-kidney cohort (KICH+KIRC+KIRP); KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute

myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; STES, stomach and esophageal carcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCSU, uterine carcinosarcoma; UVMU, uveal melanoma. *MYC* and *ATP6V1B1* expression in renal cell carcinoma tissue as compared with that in normal kidney tissues according to re-analysis of (***C***) GSE781, (***D***) GSE6344, and (***E***) GSE36895. Boxes represent the interquartile range, and the line inside the box represents the median value. Statistical analysis was performed using one-way ANOVA test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

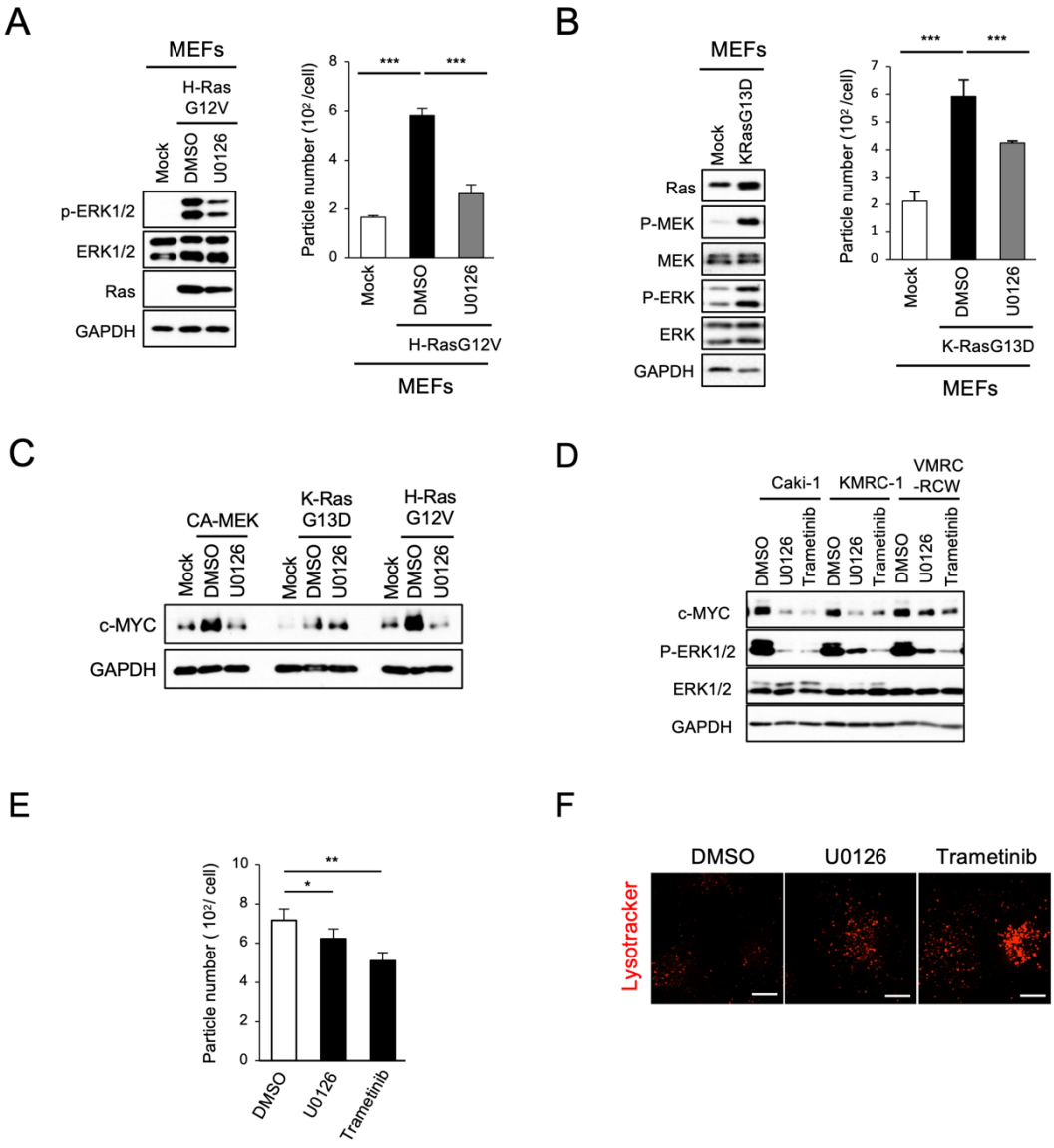

Fig. S6. MEK inactivation induces downregulation of *MYC* and EV secretion. MEFs expressing gain-of-function mutants of (***A***) H-Ras (G12V) and (***B***) K-Ras (G13D) were treated with or without U0126 (10 μM) for 24 h, and whole-cell lysates were immunoblotted with the indicated antibodies (left panels). NTA for the quantitative measurement of EVs isolated from each MEF line (right panel). (***C***) MEFs expressing constitutively active MEK (CA-MEK) or gain-of-function mutants of H-Ras (G12V) and K-Ras (G13D) were treated with or without U0126 (10 μM) for 24 h, and whole-cell lysates were immunoblotted with the indicated antibodies. (***D***) RCC cells (Caki-1, KMRC-1, and VMRC-RCW) were treated with DMSO, U0126 (10 μM), or trametinib (100 nM) for 24 h and subjected to immunoblotting with the indicated antibodies. (***E***) Caki-1 cells indicated in (D) were subjected to exosome preparation, followed by NTA for the quantification of isolated particles. (***F***) Lysotracker Red staining of the Caki-1 cells indicated in (D). Scale bar = 10

μm. Data represent the mean ± standard deviation. Statistical analysis was performed using one-way ANOVA test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

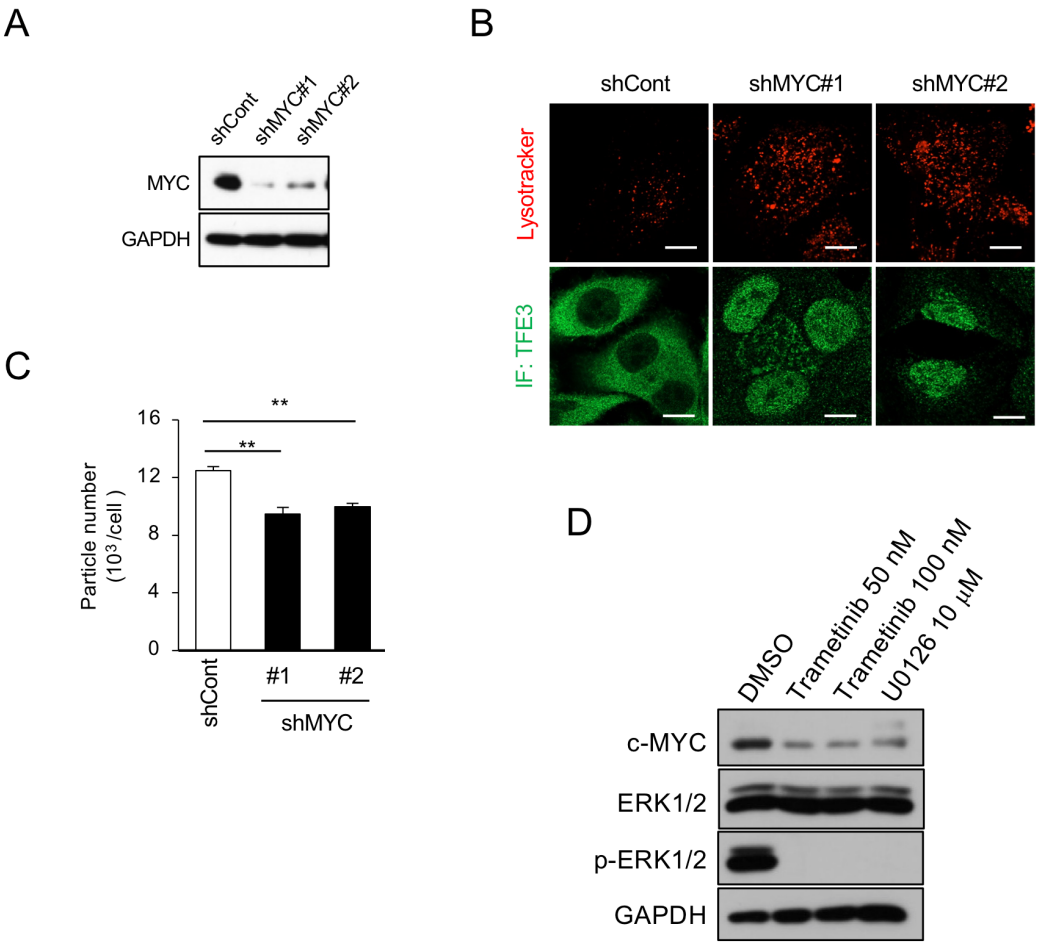

Fig. S7. Disruption of *MYC* expression suppresses EV secretion by activating lysosome function in prostate cancer cells. (***A***) Cell lysates from PC3 prostate cancer cells expressing control (shCont) or *MYC* (shMYC) shRNAs were immunoblotted with the indicated antibodies. (***B***) Cells indicated in (A) were stained with Lysotracker Red or for anti-TFE3 (green). Scale bar = 10 μm. (***C***) NTA of the isolated particles was performed in the cells indicated in (A). (***D***) MDA-MB-231 cells were treated with DMSO, U0126 (10 μM), or trametinib (50 or 100 nM) for 24 h and subjected to immunoblotting with the indicated antibodies. Data represent the mean ± standard deviation of three independent measurements. Statistical analysis was performed using one-way ANOVA test. $^{**}P < 0.01$.

Table S1. Primers used in this study

Target gene		Sequence
CD63	F	TAAGGAGGGCTGTGTGGAGAA
CD63	R	AGGCAGCAGGCAAAGACAA
MYC	F	CCTCGGATTCTCTGCTCTCCT
MYC	R	TTCCTCATCTTCTTGTTCCTCCTC
Lysosomal enzyme（Human）		
GAA	F	AGATTGTAAGGTTTGCCCTCCTC
GAA	R	CATACCCCACACCTCCACCT
CTSS	F	GAAGAGAGCCCACTAATTCAAGGA
CTSS	R	AGGAGCACACCAAGAGCACA
ARSA	F	GAATGAGGAAACTGAGGTGCAGA
ARSA	R	TCAAATCCCAGCCCAACC
TPP1	F	TGAAATGCTGTGAGCTTGACTTG
TPP1	R	AGATGAGATGCGGAGGGAGA
FUCA1	F	ACGACAAAGCATCACGAAGG
FUCA1	R	AGAGCTGTTCCCAATTCACCA
MAN2B2	F	AGGCTAATGGCAGGAAATGGT
MAN2B2	R	GCTGGCTTGATTGAGTGTGG
CTSD	F	CCACACACACCCACACACTC
CTSD	R	GGGGAAAACCACAGAACAAAAC
ASAH1	F	GGCCCCAGCCTACTTTATCC
ASAH1	R	ACCACATACCATCTACCCTGCTT
V-ATPase（Human）		
ATP6V1B1	F	AGGACTGTGTGCAGCGTGA
ATP6V1B1	R	TCTGGGAGGGTGAAGTGGA
Internal control（Human）		
GAPDH	F	GCAAGAGCACAAGAGGAAGAGAG
GAPDH	R	GAGGGGAGATTCAGTGTGGTG
18S rRNA	F	CAACACGGGAAACCTCACC
18S rRNA	R	TGTGTGGACTTGGGAGAGGA

GAA: alpha glucosidase, CTSS: cathepsin S, ARSA: arylsulfatase A, TPP1: ripeptidyl peptidase 1,
FUCA1: alpha-L-fucosidase 1, MAN2B2: mannosidase alpha class 2B member 2,
CTSD: cathepsin D, ASAH1: N-acylsphingosine amidohydrolase 1,
ATP6V1B1: ATPase H+ transporting V1 subunit B1, GAPDH: glyceraldehyde-3-phosphate dehydrogenase