STAT3 associates with vacuolar H\(^+\)\text{-ATPase} and regulates cytosolic and lysosomal pH

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Dysregulated intracellular pH is emerging as a hallmark of cancer. In spite of their acidic environment and increased acid production, cancer cells maintain alkaline intracellular pH that promotes cancer progression by inhibiting apoptosis and increasing glycolysis, cell growth, migration, and invasion. Here we identify signal transducer and activator of transcription-3 (STAT3) as a key factor in the preservation of alkaline cytosol. STAT3 associates with the vacuolar H\(^+\)\text{-ATPase} in a coiled-coil domain-dependent manner and increases its activity in living cells and in vitro. Accordingly, STAT3 depletion disrupts intracellular proton equilibrium by decreasing cytosolic pH and increasing lysosomal pH, respectively. This dysregulation can be reverted by reconstitution with wild-type STAT3 or STAT3 mutants unable to activate target genes (Tyr705Ph and DNA-binding mutant) or to regulate mitochondrial respiration (Ser727Ala). Upon cytosolic acidification, STAT3 is transcriptionally inactivated and further recruited to lysosomal membranes to reestablish intracellular proton equilibrium. These data reveal STAT3 as a regulator of intracellular pH and, vice versa, intracellular pH as a regulator of STAT3 localization and activity.

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INTRODUCTION

Tumorigenesis proceeds via an evolutionary process, in which a succession of genetic changes provide the transforming cells with a set of acquired capabilities that enable tumor growth and dissemination.\(^1\) These traits include sustained proliferative signaling, metastatic capacity, activation of angiogenesis, replicative immortality, reprogrammed energy metabolism, as well as escape from cell death, growth suppressors, and immune destruction. Besides these well-established hallmarks of cancer, the pH gradient reversal, i.e., acidification of extracellular pH (pH\(_e\)) from ~7.4 in normal cells to 6.5–7.0 in cancer cells, while maintaining alkaline cytosolic pH (pH\(_c\)) of normal cells (~7.2) or further alkalinizing it to values as high as 7.6 in cancer cells, is emerging as a universal hallmark of cancer observed in malignant tumors regardless of the pathology, genetics, and origin.\(^2\) The reversal of the pH gradient is an early event in tumorigenesis and its maintenance reinforces metabolic adaptation, tumor cell survival, invasion, immune evasion, and drug resistance. For instance, glycolytic flux essential for metabolic reprogramming is stimulated by alkaline cytosol,\(^3\) whereas the activation of apoptosis-inducing caspases depends on mild acidification of the cytosol.\(^4\) In parallel, the acidification of the extracellular space promotes tumor immune escape and effective proteolytic degradation of extracellular matrix by invading tumor cells.\(^5,6\) Thus, in line with genome instability, pH gradient reversal could be considered as an underlying cellular requirement for acquiring and maintaining several other cancer traits during tumorigenesis. Yet, our knowledge of its formation and maintenance is rather rudimentary. Hitherto, plasma membrane-localized ion transporters, including Na\(^+\)/H\(^+\) exchanger 1 (NHE1), proton-linked monocarboxylate transporters and vacuolar H\(^+\)\text{-ATPase} (V-ATPase), as well as carbonic anhydrases, have been identified as proteins contributing to the cancer-associated increase in net acid extrusion.\(^4\) In addition to the acid removal via the plasma membrane, V-ATPase pumps protons from the cytosol into intracellular vesicles of the endo-lysosomal compartment, especially late endosomes and lysosomes, which serve as major intracellular proton stores.\(^8\) For simplicity, we hereafter refer to all organelles detected by fluorescent dextran loading or staining for V-ATPase subunits or lysosome-associated membrane proteins LAMP1 or LAMP2 as lysosomes. Compared with normal cells, most invasive cancer cells have an enlarged and highly acidic lysosomal compartment, more peripherally localized lysosomes, and an increase in lysosomal exocytosis.\(^11,13\) Thus, the lysosomal V-ATPase may contribute to the establishment and maintenance of the reversed pH gradient of cancer cells by removing cytosolic protons to the lysosomal lumen, from where they can be effectively discarded to the extracellular space via lysosomal exocytosis.

V-ATPase is a large multi-subunit complex composed of 14 different proteins that are organized into a water soluble, ATP-hydrolyzing V\(_1\) domain, and a membrane-embedded V\(_0\) proton channel, which function together by coupling the energy of ATP hydrolysis to the transport of protons across the lipid bilayer.\(^8,10\)
The V-ATPase-mediated acidification of lysosomal lumen is essential not only for the cargo degradation but also for the cellular metabolism in general, e.g., through the regulation of several key signaling pathways, including mechanistic target of rapamycin complex 1 and Notch pathways. Furthermore, V-ATPase activity has an important role in cancer cells by enhancing their metastatic potential, chemotherapy resistance, and survival in the acidic tumor environment. V-ATPase activity has an important role in cancer cells by promoting malignant transformation and cancer progression by phosphorylated STAT3 is localized to mitochondria, where it may independently and in a long time, it has been assumed that STAT3-mediated promotion of tumor growth depends entirely on its well-described transcriptional activities. STAT3 has, however, been demonstrated to control cell metabolism and migration in a transcription-independent manner. A small pool of acetylated and Ser727-phosphorylated STAT3 is detached from its transcriptional activation domain. Its transcriptional activity depends on the phosphorylation of Tyr705 in the transcriptional activation domain. Phosphorylated STAT3 can form homodimers or heterodimers with other STAT proteins via reciprocal SH2-phosphotyrosine interactions and translocate into the nucleus to activate gene transcription. In normal cells, the transcriptional activation occurs rapidly in response to cytokine signaling and is transient, whereas oncogenes with tyrosine kinase activity, e.g., c-Src and activated members of epidermal growth factor receptor family, keep STAT3 constitutively active in many cancers. For a long time, it has been assumed that STAT3-mediated promotion of tumor growth depends entirely on its well-described transcriptional activities. STAT3 has, however, been demonstrated to control cell metabolism and migration in a transcription-independent manner. A small pool of acetylated and Ser727-phosphorylated STAT3 is localized to mitochondria, where it may promote malignant transformation and cancer progression by supporting the optimal function of mitochondrial electron transport chain and inhibiting the production of reactive oxygen species. Furthermore, association of cytoplasmic STAT3 with a mictotubule-destabilizing protein stathmin potentiates microtubule polymerization and cell movement. Thus, transcriptional and cytoplasmic activities of STAT3 may function in concert to promote tumorigenesis.

Inspired by our finding of lysosome-associated STAT3, we investigated its role in intracellular pH regulation. Here we demonstrate that STAT3 associates with the lysosomal V-ATPase complex, stimulates its ATPase activity, and contributes to the maintenance of the alkaline cytosol and acidic lysosomal lumen.

RESULTS

STAT3 localizes to the lysosomal membrane

Prompted by the punctate lysosome-like pattern of RFP-STAT3 in live A549 non-small cell lung cancer cells, in which the N2 terminus of the endogenous STAT3 gene is tagged with a red fluorescent protein (RFP) using transcription activator-like effector nuclease-mediated knock-in, we investigated the putative lysosomal localization and function of STAT3. For this purpose, we took advantage of the above-mentioned A549-RFP-STAT3 cells and confirmed that in addition to a diffuse cytoplasmic localization, RFP-tagged endogenous STAT3 formed numerous cytoplasmic puncta in untreated cells (Fig. 1a). Labeling the organelles of A549-RFP-STAT3 cells with appropriate fluorescent markers revealed that the majority of the RFP-STAT3 puncta colocalized with lysosomes labeled with cascade blue dextran or blue fluorescent protein (BFP) fused to either LAMP1 or LAMP2, whereas no significant colocalization was observed between RFP-STAT3 puncta and markers of mitochondria, early endosomes, or Golgi apparatus (Fig. 1a). The marker for endoplasmic reticulum stained large parts of the cytoplasm and therefore the resolution of confocal microscopy did not allow a proper analysis of its colocalization with RFP-STAT3 (Fig. 1a). To test whether endogenous STAT3 localized to lysosomes in other cell types, we generated SKOV3 ovarian carcinoma cells with enhanced green fluorescent protein (EGFP) fused to the N2 terminus of the endogenous STAT3 gene using CRISPR-Cas9 gene editing. Akin to A549 cells, EGFP-tagged endogenous STAT3 formed cytoplasmic puncta that colocalized with lysosomes in live SKOV3 cells (Supplementary information, Fig. S1a). Notably, the punctate pattern and lysosomal localization of STAT3 in A549-RFP-STAT3 and SKOV3-EGFP-STAT3 cells was largely abolished by treatments commonly used to permeabilize fixed cells for immunocytochemistry, i.e., methanol, saponin and triton (Supplementary information, Fig. S1b and data not shown), which may explain why the abundant lysosomal localization of STAT3 has not been discovered earlier. Accordingly, co-staining of the endogenous, non-tagged STAT3 and ATP6V01D, a lysosomally localized subunit of V-ATPase, in fixed and saponin-permeabilized HeLa cervix cancer cells showed less colocalization of STAT3 and lysosomes than observed by live-cell imaging (Supplementary information, Fig. S1c). Confirming its association with HeLa cell lysosomes, ~5% of total cellular STAT3 protein associated with lysosomes immunopurified with antibodies against the cytosolic tail of LAMP1 or captured with a magnet after iron-dextran loading of the lysosomal compartment (Fig. 1b, c).

Although the nuclear and mitochondrial localizations of STAT3 depend on the phosphorylation of Tyr705 and Ser727, respectively, the phosphorylation status of these sites in lysosomal STAT3 did not show clear preference to either modification (Fig. 1b, c). To find out on which side of the lysosomal membrane STAT3 resided, we exposed intact lysosomes to proteinase K in the presence or the absence of a digitonin detergent. Unlike luminal cathepsin D protein, which was effectively digested by proteinase K only in the presence of detergent, STAT3 was completely digested in the absence of detergent (Fig. 1d). These data suggest that STAT3 resides on the cytosolic surface of the lysosomal membrane and should therefore be able to be mobilized by an appropriate stimulus. Correspondingly, treatment of A549-RFP-STAT3 cells with interleukin 6, a potent inducer of nuclear localization and transcriptional activity of STAT3, triggered a rapid decline in cytoplasmic STAT3-positive puncta and a simultaneous accumulation of STAT3 in nuclei (Fig. 1e). Taken together, these data suggest that in the absence of activating stimuli, a fraction of cellular STAT3 is loosely attached to the cytosolic side of lysosomal limiting membrane.

STAT3 interacts with V-ATPase on the lysosomal membrane via its coiled-coil domain

To understand the putative function of lysosomal STAT3, we searched for STAT3-interacting proteins in lysosomal extracts of HeLa cells transiently transfected with Flag-tagged STAT3 (STAT3-Flag). Nanoscale-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) analysis of proteins co-immunoprecipitating with STAT3-Flag identified eight subunits of the V-ATPase complex as potential STAT3-binding partners (Fig. 2a). As described above, V-ATPase is a large multi-subunit complex composed of a cytosolic ATP-hydrolyzing V0 domain and a membrane-embedded V1 proton channel. Of the eight V-ATPase subunits identified as putative STAT3-associating proteins, one was a Vc subunit (ATP6V0D1) and seven were V0 subunits, and ATP6V1A was the strongest candidate based on the amount of protein co-immunoprecipitating with STAT3-Flag, the number of peptides identified, and repeated identification in...
independent experiments (Fig. 2a). Similar association of endogenous STAT3 with the V-ATPase complex was demonstrated by its ability to co-precipitate with endogenous ATP6V1A as analyzed by immunoblotting and nano-LC-MS/MS (Fig. 2b; Supplementary information, Fig. S2a and b), and to co-precipitate with the V-ATPase complex purified with hemagglutinin-tagged ATP6V1A (Fig. 2c). Super resolution-structured illumination microscopy (SR-SIM) confirmed the close proximity of endogenous STAT3 and ATP6V0D1 in intact HeLa cells (Fig. 2d, e). Likewise, proximity ligation assay (PLA), which detects protein colocalization with single-molecule resolution, gave strong, punctate PLA signals for STAT3 and ATP6V0A1 in HeLa cells, as well as in non-transformed...
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Fig. 1 STAT3 localizes to the lysosomal membrane. a Representative images of A549-RFP-STAT3 cells labeled with the indicated organelle markers (blue). Values, mean percentage of RFP-STAT3 puncta colocalizing with the indicated organelle marker ± SD of three independent experiments with ≥ 10 cells/sample analyzed in each. Colocalization analysis was not applicable (NA) in KDEL-BFP-labeled cells due to the diffuse staining. The areas marked with white squares are magnified in upper right corners. Images of live cells were taken with 60× magnification using Zeiss LSM700 confocal microscope. See Supplementary information, Fig. S1 for colocalization of STAT3 and lysosomes in other cells. b Representative immunoblots of STAT3 and the indicated organelle markers in total cell lysates at the indicated time points (FT) and immunoprecipitates (eluate) from HeLa cells. α = 3. c Representative immunoblots of the indicated proteins in total cell lysates or the indicated fractions of HeLa cells (left) and quantification of P-Y705-STAT3 and P-S727-STAT3 levels relative to total STAT3. d Representative immunoblots (top) and quantification (bottom) of the indicated proteins in lysates of HeLa cell lysosomes purified by iron-dextran method and left untreated or treated with 10 µg/ml proteinase K and 100 µg/ml digitonin for 10 min at 25 °C when indicated. CTS, cathepsin D. e Representative images (left) and quantification of cytosolic RFP-STAT3 puncta (right) in A549-RFP-STAT3 cells left untreated or treated with 100 µg/mL IL6 for 30 min and stained with Hoechst 33342. SD of three independent experiments, with ≥ 10 cells/sample. 

pancreatic duct epithelial cells (H6C7) and mammary fibroblasts (HMFD3), but not in their STAT3-depleted counterparts (Fig. 2f, g). Notably, PLA for STAT3 and other abundant lysosomal membrane proteins LAMP1 and CD63 gave practically no signal (Fig. 2h).

To characterize the association between STAT3 and the V-ATPase complex, we performed mutagenesis of STAT3 and investigated the ability of the Flag-tagged STAT3 mutants to associate with the V-ATPase complex by PLA employing antibodies against Flag and ATP6V1A in HeLa cells. The first 138 amino acids of the NH2-terminus of STAT3 failed to colocalize with ATP6V1A, whereas NH2-terminal STAT3 fragments of 321 or 688 amino acids showed roughly similar colocalization to the wild-type protein (Fig. 3a, b; Supplementary information, Fig. S3a). Thus, the coiled-coil domain residing between the amino acids 138 and 321 appeared to be essential for the interaction. To corroborate this hypothesis, we deleted a highly conserved sequence between residues 239 and 280 of the STAT3 coiled-coil domain (Supplementary information, Fig. S3b). Correspondingly, immunoblot analyses of antibodies against Flag and ATP6V1A in HeLa cells. The ability of Y705F and DNA-binding defective mutants of STAT3 to associate with the V-ATPase as analyzed by PLA (Fig. 3b), we investigated its ability to rescue the lysosomal pH phenotype of HeLa-STAT3-KO-11 cells after transient transfection. Contrary to the wild-type STAT3, which significantly reduced the lysosomal FITC/TMR ratio following a transient transfection, STAT3-CCM failed to do so (Supplementary information, Fig. S4b). To validate the role of STAT3 in the acidification of the lysosomal compartment, we measured the volume of the acidic compartment of HeLa cells loaded with Lysotracker Green by flow cytometry. In line with increased lysosomal pH observed by FITC/TMR ratio measurements in HeLa-STAT3-KO-11 clones, the STAT3-depleted cells had a significantly lower capacity to accumulate Lysotracker Green than the control cells, and this phenotype was effectively rescued by the reconstitution with the wild-type STAT3 or its Y705F, S727A, and DNA-binding defective mutants (Fig. 4c; Supplementary information, Fig. S4c and d). Further supporting the role of STAT3 in the acidification of lysosomes, HeLa-STAT3-KO cells had reduced maturation of cathepsin B (CTSB) and longer half-life of lysosomal dextran conjugated to pH-insensitive AlexaFluor 488 (Fig. 4d, e).

STAT3 stimulates V-ATPase-mediated hydrolysis of ATP. The ability of Y705F and DNA-binding defective mutants of STAT3 to effectively rescue the lysosomal pH phenotype in STAT3-KO cells strongly suggested that STAT3 acidifies lysosomes independently of its transcriptional activity. To substantiate this hypothesis, we sequenced the transcriptomes of HeLa-C4 and HeLa-STAT3-KO cells and compared the expression levels of mRNAs for lysosomal proteins (Supplementary information, Fig. S5a and b). STAT3 depletion did not reduce the expression of mRNAs for major V-ATPase subunits (Supplementary information, Fig. S5a). On the contrary, several of them and their corresponding proteins (e.g., ATPV1A, ATPV1B2, and ATP6V0D1) were upregulated in STAT3-KO cells (Fig. 5a and Supplementary information, Fig. S5a). Out of all the genes whose protein products according to Gene Ontology (www.geneontology.org/page/go-enrichment-analysis) or Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/pathway.html) datasets localize to lysosomes, only 13 and 10 genes had over 1.5-fold reduced and increased expression, respectively, in HeLa-STAT3-KO cells as compared with control cells (Fig. 5b and Supplementary information, Fig. S5b). To our knowledge, none of the altered genes, except for the upregulated V-ATPase subunits, have been reported to regulate lysosomal pH. These data suggest that STAT3 stimulates lysosomal acidification by a posttranscriptional mechanism. Thus, we tested whether

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STAT3 regulated the assembly of V₁ and V₀ domains of the V-ATPase complex by investigating their proximity by PLA employing antibodies against ATPV1A and ATP6V0D1. The PLA was validated by the efficient reduction in the number of PLA puncta upon small interfering RNA (siRNA)-mediated depletion of either ATPV1A or ATP6V0D1 (Fig. 5c, d). HeLa-STAT3-KO-11 cells had as many PLA puncta as the control cells, indicating that STAT3 does not control the assembly of the V-ATPase complex (Fig. 5d). In line with this, the significant reduction in the colocalization of STAT3 with ATP6V1A and ATP6V0D1 following the siRNA-mediated depletion of ATP6V0D1 and ATP6V1A, respectively, suggested that STAT3 associates with the assembled V-ATPase complex.
Cytosolic pH regulates STAT3 localization and STAT3 localization regulates cytosolic pH

Inspired by the ability of STAT3 to enhance the V-ATPase-mediated transfer of protons from the cytosol to lysosomes, we tested whether its subcellular localization could be regulated by changes in intracellular pH. To disrupt the intracellular pH equilibrium, we starved cells for amino acids to enhance the V-ATPase activity, or exposed them to five short (15–60 min) treatments that acidify the cytosol (Supplementary information, Fig. S6a), i.e., (i) V-ATPase inhibitor bafilomycin A1, (ii) lysosomal membrane destabilizer leucyl-leucine methyl ester (LLOMe), (iii) nicrosamide, a protonophore that carries protons across membranes, (iv) ethylisopropyl amiloride (EIPA), an inhibitor of plasma membrane NHE1, in the absence of NaHCO3/CO2 buffer system or (v) in the presence of propionic acid (propionate). All tested treatments significantly enhanced the association of RFP-STAT3 with lysosomes in A549-RFP-STAT3 cells as demonstrated by increased RFP-STAT3 puncta formation and significantly enhanced lysosomal fluorescence intensity of RFP-STAT3 (Fig. 6a).

In HeLa cells, all tested treatments triggered a significant increase in lysosomal STAT3 as demonstrated by significantly increased STAT3/LAMP1 ratios in immunoblots of lysosomal lysates (Fig. 6b). These increases were likely due to the translocation of STAT3 from other cellular compartments, as none of the short treatments induced detectable changes in the expression levels of STAT3 or LAMP1 (Fig. 6b, c). In addition, all cytosol-acidifying treatments effectively reduced the phosphorylation of Tyr705-STAT3 in HeLa and A549 cells (Fig. 6c, d; Supplementary information, Fig. S6b). In agreement with the dependence of the transcriptional activity of STAT3 on P-Tyr705, cytosolic acidification reduced the levels of proteins encoded by known STAT3 target genes, CCND1 and BIRC5, as analyzed by immunoblotting (Fig. 6d), CCND1 mRNA as analyzed by quantitative PCR (qPCR) (Fig. 6e), and mRNA levels of CCND1, MYC, and 179 other predicted STAT3 target genes as analyzed by whole transcriptome sequencing (Supplementary information, Fig. S6c). Thus, acidification of the cytosol triggers a rapid dephosphorylation of P-Tyr705-STAT3 accompanied by translocation of STAT3 to the lysosomes, where it can promote the neutralization of the acidic cytosol by enhancing the proton pumping activity of the V-ATPase complex.

Most cancers depend on aerobic glycolysis to generate ATP, and several oncogenes enhance this process by upregulating the expression of glucose transporters and glycolytic enzymes. This results in increased cytosolic lactate production. Thus, we tested whether oncogene-mediated activation of glycolysis and subsequent acid production are associated with enhanced translocation of STAT3 to the lysosomes. For this purpose, we used MCF7 cells with inducible expression of N-terminally truncated active form of ErbB2 oncogene, p95NErbB2, which enhances glycolysis by upregulating lactate dehydrogenase expression.

Immunopurified lysosomes from MCF7-p95NErbB2 cells had over ten times higher STAT3/LAMP1 ratio than control MCF7 cells, whereas the level of total STAT3 was similar (Fig. 6f and Supplementary information, Fig. S6d). Increased lysosomal localization of STAT3 in MCF7-p95NErbB2 cells was confirmed by PLA for STAT3 and LAMP1 or CD63 in HeLa-C-4 clone. Nuclei were counterstained with DAPI. Cell images in f–h were taken with 60× magnification using Zeiss LSM700 confocal microscope. The optimal slice thickness (~350 nm) was defined by the Zeiss zen software. The images of H6C7 cells in g are integrated stacks. Scale bar, 10 µm. Error bars, SD of three independent experiments with ≥ 10 cells analyzed/sample. P-values were calculated by two-tailed, homoscedastic Student’s t-test (f) or one-way ANOVA combined with Dunnett’s multiple comparisons test (h)
Fig. 3  STAT3 interacts with V-ATPase via its coiled-coil domain.  

**a** Domain structure of STAT3 with mutations used in **b** indicated below. SH2, Src homology 2 domain; TAD, transactivation domain. **b** Quantification of PLA (anti-Flag and anti-ATP6V1A) puncta in HeLa cells expressing the indicated Flag-tagged STAT3 constructs (top). Error bars, SD of three independent experiments with ≥10 cells analyzed/sample. P-values were calculated by one-way ANOVA combined with Dunnett’s multiple comparisons test. Rough estimates of the relative expression levels of Flag-tagged STAT3 constructs are shown below the histogram as percentages of the expression of the wild-type STAT3. See Supplementary information, Fig. S3b for representative immunoblots. Bottom, representative images of PLAs taken with 60× magnification using Zeiss LSM700 confocal microscope. The optimal slice thickness (~350 nm) was defined by the Zeiss zen software. Lysosomes were visualized with cascade blue dextran. Scale bar, 10 µm. **c** Representative immunoblots of the indicated proteins in total cell lysates or the indicated fractions of HeLa-STAT3-KO cells reconstituted with STAT3-Y705F (20 µg protein/lane). n = 3
when not in complex with the other subunits (K.M., unpublished data), we were not able to verify the direct association between STAT3 and ATP6V1A in vitro. The significant reduction of the PLA signal from ATPV0D1 and STAT3 upon depletion of the ATP6V1A subunit supported, however, the idea that STAT3 associated with V-ATPase via the ATP-hydrolyzing V1 complex. This was further substantiated by the ability of STAT3 to enhance the ATPase activity of the V-ATPase complex in vitro. The characterization of the molecular details of this association will require extensive structural analyses of the entire membrane-embedded V-ATPase complex, which are out of the scope of this study.

The V-ATPase controls lysosomal luminal pH in a tightly regulated manner that promptly responds to extracellular and intracellular cues. This requires constant and precise control of the speed of...
STAT3 regulates intracellular pH homeostasis via lysosomal pH regulation

MATERIALS AND METHODS
Reagents and resources
Sources of reagents and resources used are listed in Supplementary information, Table S1.

Cell culture
Human A549 non-small cell lung carcinoma (male), HeLa cervix carcinoma, and SKOV3 ovarian carcinoma were obtained from American Type Culture Collection (ATCC). The cells were authenticated by ATCC and used within 6 months after thawing. Human pancreatic duct epithelial cells (H6C7) were purchased from Kerastat. Human immortalized mammary fibroblasts (HMFM) were kindly provided by Michael J. O’Hare (Ludwig Institute for Cancer Research, London, UK). Lentivirus was generated by the Institute for Cancer Research, London, UK.

Reagents and resources
Sources of reagents and resources used are listed in Supplementary information, Table S1.
Human MCF7 mammary carcinoma cells stably transfected with inducible pTRE-p95ErbB2 or corresponding pTRE vector were established and cultured as described previously. The expression of p95ErbB2 was induced by washing off the tetracycline (1 µg/ml) three passages before the experiment. H6C7 cells were cultured in serum free media (SFM). Other cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (complete medium), and for H6C7 cells also with 2 mM glutamine. Cells were maintained at 37°C and 5% CO2. All cell lines were found negative for mycoplasma using Venor GeM Classic PCR kit.
To perform site-directed mutagenesis of STAT3, pCDNA3.1-DYK-STAT3 was PCR amplified using KOD Xtreme™ Hot Start DNA Polymerase and primers listed in Supplementary information, Table S2. Larger deletions (ΔDB and ΔSH2) were performed as described previously, using primers listed in Supplementary information, Table S2. Amplified DNA digested with DpnI was amplified in Escherichia coli DH5α. The obtained sequence-verified STAT3 regulates cellular pH equilibrium

Table S2. Larger deletions (ΔDB and ΔSH2) were performed as described previously, using primers listed in Supplementary information, Table S2. Amplified DNA digested with DpnI was amplified in Escherichia coli DH5α. The obtained sequence-verified
plasmids and EF-STAT3-Y705F-UbcGFP were used as templates for the construction of STAT3 expression plasmids described in Supplementary information, Table S3.

Transfections
If not otherwise stated, transfections were performed using TurboFectin 8.0 or Lipofectamine 3000 transfection agents according to the manufacturer’s instructions.

siRNAs were transfected with Lipofectamine RNAmax at 20 nM according to the manufacturer’s protocol and cells were analyzed 72 h later.

To create stable cell lines, lentiviral and packaging plasmids were transfected into subconfluent Lenti-X™ 293T cells. After 48 h, virus particles were collected with PEG-it™ Virus Precipitation Solution according to the manufacturer’s instructions. HeLa cells growing on six-well plates (2 x 10^6 cells/well) were treated with 5 µl of the obtained virus suspension supplemented with 8 µl protamine sulfate and salt-injected by centrifugation at 2,400 x g for 90 min. After 48 h, cells were transferred to 10 cm petri dish and the medium was supplemented with 200 µl µg/mL hygromycin B. After ~3 weeks of selection, stable clones were picked and analyzed by immunoblotting to identify successfully transfected clones.

For transposon-mediated transfection, expression plasmids and supertransposase vector (1:1) were co-transfected into HeLa cells growing on six-well plates (2 x 10^6 cells/well). After 48 h, cells were transferred into a 10 cm petri dish and the medium was supplemented with 1 µg/mL puromycin. After ~2 weeks of selection, stable clones were picked and validated by immunoblotting.

CRISPR/Cas9-mediated gene editing
STAT3-knockout cells were generated using CRISPR/Cas9-mediated gene editing as described previously. Single-guide RNA targeting STAT3 was created by cloning appropriate primers into pxprX01 and transfected into HeLa, H6C7, or HMF3 cells. After 2 weeks of selection in medium containing 1 µg/mL puromycin, clones were picked up and validated by immunoblotting and sequencing.

The EGFP-STAT3 tagging CRISPR gRNA was generated by cloning the guide sequences Cr-Uni and Cr-STAT3 into the pMA-SpCas9-g1 and pMA-SpCas9-g2 gRNA expression vectors, respectively. The pAAV-EGFP-MMEJ vector was generated by amplifying the EGFP coding sequences from pFlC3 (kindly provided by Dr. Yoshimori) with specific primers listed in Supplementary information, Table S3. The PCR product was digested by Ncol and ligated into a pAAV-MCS vector. All plasmids were further validated by Sanger sequencing.

To generate the SKOV3-EGFP-STAT3 cells, SKOV3 cells growing on six-well plates (2.5 x 10^5 cells/well) were transfected with 400 ng pAAV-EGFP-MMEJ, 100 ng pMA-STAT3-gRNA, 100 ng pMA-MMEJ-gRNA, 300 ng pSpCas9(BB)-2A-Puro (PX459) and 100 ng pNeDaKo-Neo employing XtremeGENETM 9 DNA transfection reagent according to the manufacturer’s instructions. Cells were passaged to a 10 cm petri dish 24 h later and after an additional 24 h, the medium was supplemented with 0.7 µg/mL G418 for 10 days. G418-resistant cells were further expanded and enriched for EGFP-positive cells by fluorescence-activated cell sorting (FACS core facility, Aarhus University).

Labeling of lysosomes and other organelles
Organelles were visualized 2-3 days after transfection with the indicated plasmids encoding appropriate organelle markers fused to BFP, i.e., pLAMP1-BFP or pLAMP2-BFP for lysosomes, pTagBFP2-ManNl-N10 for Golgi, pTagBFP2-Rab5a-7 for early endosomes, mITO-BFP for mitochondria, and BFP-KDEL for endoplasmic reticulum. Alternatively, lysosomes were visualized by loading the cells with 0.4 µg/mL cascade blue dextran (10KDa) or 0.25 mg/mL AlexaFluor 594-dextran (10KDa) for 1 h in 37°C cell incubator, followed by washing with Dulbecco’s phosphate-buffered saline (DPBS) and 5 h incubation in 37°C cell incubator. The samples were then analyzed either live or after 20 min fixation in 4% paraformaldehyde (PFA) with Zeiss LSM700 confocal laser scanning microscope with 60× objective.

Subcellular fractionation and lysosome purification
Subconfluent cells grown in 15 cm petri dishes in 20 mL complete medium were treated for 24 h with 2 mM iron-dextran solution (53 mg/mL in deionized water; prepared essentially as described previously), washed in DPBS, and ruptured on ice in SCA buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose) with freshly added 1× protease inhibitor cocktail using a Dounce glass homogenizer (BioVision, 1998-1) until ~90% of cells were permeable for trypan blue. Ruptured cells were centrifuged at 1,000 x g for 10 min at 4°C. Nuclear pellets were lysed in TBS lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% CHAPS, 1% octyl-β-glucoside) with 1× protease inhibitor cocktail and the supernatant containing cytosol and organelles was loaded to LS MACS Separation Columns on octoMACSTM Separator magnet stand. First, 1 mL of eluate was collected, columns were washed twice with 0.5 mL SCA buffer, and lysosomes were eluted with 1 mL SCA buffer after removing the columns from the magnet. Both eluates were centrifuged at 20,000 x g for 20 min, supernatant from the first eluate (cytosol) was collected and pellets (organelles without lysosomes and lysosomes) were lysed in TBS lysis buffer with 1× protease inhibitor cocktail.

Alternatively, lysosomes were immunopurified with anti-LAMP1 antibodies. Subconfluent cells growing on petri dishes were scraped in ice-cold DMEM, washed three times with cold
Fig. 7 Lysosomal STAT3 protects cells against cytosolic acidification. a Cytosolic pH in the indicated HeLa clones determined by image analysis of cells loaded with pHrodo™ Green AM. Standard curve is displayed on the right. b, c Cell death of the indicated HeLa cell clones (b) and HeLa-STAT3-KO-11 cells transiently transfected with vector or STAT3-Y705F (c) left untreated, starved in EBSS for 6 h, or treated with 25 µM EIPA + 50 mM propionate for 24 h was determined by propidium iodine uptake. Error bars, SD of ≥ 3 independent experiments. P-values were calculated by one-way ANOVA combined with Dunnett’s multiple comparisons test (a, b) or by two-tailed, homoscedastic Student’s t-test (c).

phosphate-buffered saline (PBS), and resuspended in SCA-low EDTA buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, pH 7.5) with freshly added protease and phosphatase inhibitor cocktail. Cell suspension (6 x 10⁷ cells in 800 µL) was homogenized in gentleMACS using program “tissue: h_mito_tissue_01” and centrifuged at 750 x g for 5 min at 4 °C. The supernatant (light membrane fraction) was collected and rotated with 0.225 µg primary antibody at 4 °C for 15 min before adding 10 µL MicroBeads coupled to goat anti-rabbit antibody for 1 h. The suspension (3 x 150 µL) followed by 500 µL SCA-low EDTA buffer was applied to MS Columns equilibrated with SCA-low EDTA buffer supplemented with 25 U/ml benzonase in the magnetic field of the octoMACSTM Separator. After removing the column from the magnetic stand, lysosomes were eluted with 3 x 100 µL SCA-low EDTA buffer.

Immunodetection

Immunoblotting. Cells were lysed in Laemmli sample buffer (125 mM Tris, pH 6.7, 20% glycerol, 140 mM SDS) supplemented with complete protease inhibitor cocktail. After addition of 0.05 M dithiothreitol and bromphenol blue, boiling and separation by 4%–20% gradient SDS-polyacrylamide gel electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes using Bio-Rad Trans-Blot Turbo system. Membranes were blocked with PBS containing 5% milk and 0.1% Tween-20, and stained with the indicated primary antibodies and appropriate peroxidase-conjugated secondary antibodies listed in Supplementary information, Table S1. CTB antibody was kindly provided by Dr. Ekkehard Weber (Martin Luther University Halle-Wittenberg, Halle, Germany). The signal was detected with Clarity Western ECL Substrate and Luminescent Image Reader, and quantified by densitometry with Image Studio Lite software.

Immunocytochemistry. Cells grown on glass coverslips were fixed with 4% PFA or ice-cold methanol, quenched with 50 mM ammonium chloride in DPBS, permeabilized and blocked in 5% goat serum, 1% bovine serum albumin (BSA) in DPBS supplemented with 0.3% Triton X-100 or 0.1% saponin, and stained with the indicated primary and secondary antibodies listed above. Coverslips were mounted with Prolong Gold Antifade mounting medium with DAPI. Images were acquired using a Zeiss LSM700 microscope with Plan-Apochomat 63×/1.40 Oil DIC M27 objective and Zen 2010 software (all equipment and software from Carl Zeiss, Jena, Germany). Pinholes were set so that the section thickness was equal for all channels and ≤ 1 AU. Cell contours (n > 20) were defined manually and green and red thresholds were set up in single channel mode and retained for all samples in an experiment. SR-SIM was performed using a 63×, 1.4 numerical aperture, oil-immersion objective lens, and an sCMOS PCO.edge camera mounted on an Elyra PS.1 microscope. Samples were illuminated with 488 and 561 nm lasers passed through diffraction gratings of 34 and 42 µm, respectively. Processing was performed with Zen software black edition 2012 and the channels were aligned in x, y, z according to a matrix calculated with an image of 100 nm beads recorded in the same conditions as the sample. Three-dimensional analyses were done by Fiji software and remodeling was done by Zen 2012 black software.

Immunoprecipitation. Cells washed with PBS and scraped off in SCA buffer supplemented with 1× protease inhibitor cocktail, were ruptured using a Dounce glass homogenizer until ~90% of cells were permeable for trypan blue. The supernatant obtained after 10 min centrifugation at 1,000 x g at 4 °C was further centrifuged at 20,000 x g for 20 min at 4 °C and the pellet (organelles) was lysed in TBS lysis buffer supplemented with 1× protease and phosphatase inhibitor cocktails on ice. The obtained lysate (150 µg protein) was precleared by 1 h incubation with 10 µL control IgG conjugated with magnetic beads and the supernatant aspirated on magnetic stand was rotated for 2 h with 20 µL specific antibodies (rabbit anti-HA or mouse anti-FLAG) or appropriate control IgG conjugated to magnetic beads at 4 °C. Magnetic purifed beads were washed three times with TBS lysis buffer, once with TBS with 300 mM NaCl at 4 °C, and proteins were eluted in 25 µL Laemmli lysis buffer for 5 min at 95 °C before immunoblot analysis.

Proximity ligation assay. PLA assay was performed with Duolink In Situ Red Starter Kit according to the manufacturer’s instructions. Briefly, cells grown on coverslips were fixed with 4% PFA for 20
min, permeabilized in buffer 1 (PBS with 1% BSA and 0.3% Triton X-100) for 10 min and blocked in Duolink blocking buffer. Samples were then incubated for 18 h at 4°C with the indicated primary antibodies diluted in buffer 1 supplemented with 5% goat serum, washed 3×5 min in buffer 1, and incubated with Duolink secondary antibodies for 1 h at 37°C. After washing 2×5 min in Duolink washing buffer A, samples were incubated for 30 min at 37°C with 1:40 dilution of Duolink ligase in Duolink ligation buffer and washed 2×2 min in Duolink washing buffer A. Finally, samples were incubated for 100 min at 37°C with 1:80 dilution of Duolink polymerase in Duolink amplification buffer, washed for 2×10 min in 1× Duolink washing buffer B and for 1 min in 0.01x washing buffer B, and mounted with ProLong™ Gold Antifade Mountant. Images were taken by LSM700 with 60× magnification and analyzed by open source Image J software.

In vivo crosslinking
In vivo crosslinking was performed by incubating cells in 3 mL fresh culture media containing 2.5 mM dithiobis(succinimidyl propionate) (DSP) for 10 min at 37°C and 5% CO₂. DSP was quenched by adding 330 µL of 1 M Tris-HCl (pH 8.0) and incubating for another 10 min. After two washes with cold PBS, cells were lysed in 500 µL TBS lysis buffer supplemented with 1× protease and phosphatase inhibitor cocktails on ice for 20 min.

Protein identification by mass spectrometry
Anti-FLAG immunoprecipitation from lysates of lysosomal extracts from HeLa cells transfected with pBCMV-STAT3-Flag-puro or empty vector 3 days earlier, and anti-STAT3 and mlgG2a immunoprecipitation from total lysates of HeLa cells after in vivo crosslinking were performed according to the protocol described above. Interacting proteins were identified and quantified by nano-LC-MS/MS, essentially as previously described. Briefly, each gel lane was cut into 1×1 mm pieces and cysteine residues were blocked by reduction and alkylation using tris(2-carboxyethyl) phosphine and iodoacetamide, respectively. In-gel digestion was performed using trypsin and resulting peptides were extracted from gel pieces using acetonitrile and trifluoroacetic acid, and finally purified on PepClean C-18 Spin columns. LC-MS/MS was performed on an EASY nanoLC coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer. Peptide samples were separated on a PepMap C-18 reverse phase column (25 cm length, 75 µm inner diameter, and 2 µm particle size) and eluted by a 90 min linear gradient of acetonitrile (4%-40%) containing 0.1% formic acid. The MS was operated in a data-dependent mode, automatically switching between MS and MS2 acquisition, with mass resolution of 70,000 and 17,500, respectively. Up to ten most intense ions were fragmented per every full MS scan by higher-energy collisional dissociation. Dynamic exclusion of 10 s was applied and ions with single charge or unassigned charge states were excluded from fragmentation. MaxQuant software version 1.5.2.8 was applied for protein identification and label-free quantification by means of peptide peak areas. The MS raw files were searched against a database consisting of 20,197 Homo sapiens protein sequences downloaded from UniProt. Carbamidomethylation of cysteines was set as fixed modification, whereas methionine oxidation and protein N-terminal acetylation were set as dynamic modifications. The false discovery rate (FDR) was assessed by searching against a reverse decoy database and FDR thresholds of protein and peptide identification were both set to 0.01.

pH measurements
Lysosomal pH was estimated based on the fluorescence intensity ratio of pH-sensitive FITC and pH-insensitive TMR essentially as described previously. In brief, cells were loaded with 2.5 mg/mL 70 kDa dextran coupled to FITC and TMR in complete medium for 18 h, washed with DPBS, and incubated for additional 5 h with complete medium without dextran. Images of dextran-loaded cells were acquired by LSM700 confocal laser scanning microscope (Zeiss) and the FITC/TMR ratio was calculated by open source Image J software. Standard curves used to estimate lysosomal pH were created by similar analysis of cells incubated with a series of Hapes buffers (145 mM KCl, 10 mM glucose, 1 mM MgCl₂, 10 µM nigericin, 20 mM Hepes) with pH ranging from 4.5 to 7.0. After transient transfections, pH changes were expressed as arbitrary units based on FITC/TMR ratio, the lower ratio indicating higher pH.

The relative volume of the acidic compartment of trypsinized cells stained with 5 min with 75 nM Lysotracker Green DND-26 and washed in PBS was estimated by flow cytometry analysis using BD FACSVersa™ instrument. Data were collected with BD FACSuite v1.0.6 and analyzed by FlowJo V10 software.

To estimate the cytosolic pH, cells washed with Live Cell Imaging Solution were incubated for 30 min in 37°C in the same solution containing 1:1,000 dilution of pHrodo™ Green AM Intracellular pH Indicator and 1:100 dilution of PowerLoad™ concentrate, washed with Live Cell Imaging Solution, and analyzed by LSM700 confocal laser scanning microscope. Standard curves used to estimate cytosolic pH were created by similar analysis of cells incubated with a series of pH calibration buffers (pH 4.5, 5.5, 6.5, and 7.5) supplemented with 10 µM valinomycin and 10 µM nigericin (Intracellular pH Calibration Kit).

STAT3 recombinant protein generation
To produce recombinant N-terminally truncated and C-terminally superfolder-GFP-tagged STAT3 (∆N-STAT3-sfGFP), the sequence coding for amino acid residues 127–770 of STAT3 was PCR amplified with primers listed in Supplementary information, Table S2 and ligated into the cloning site of the pETM11SU-M03sfGFP vector. The obtained pETM11SU-M03ANSTAT3sfGFP plasmid was used to transform competent E. coli BL21 Star™(DE3) cells. After 18 h shaking at 16°C, cells were lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole, 0.5 mM diithiothreitol (DTT)) supplemented with 1× complete EDTA-free protease inhibitor cocktail, 1× BugBuster Protein Extraction Reagent, and 50 units/mL Benzonase Nuclease, and centrifuged at 21,000×g for 45 min at 4°C. The supernatant was filtered (0.2 µm) and passed through Ni-NTA affinity resin (Qiagen, 30210), which was washed in lysis buffer before elution of N-terminally hexahistidine- and SUMO3 domain-tagged recombinant ∆N-STAT3sfGFP protein in Tris buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl) supplemented with 300 mM imidazole. The protein was dialyzed against Tris buffer overnight at 4°C in the presence of 200:1 (mol/mol) of in house-purified SenP2 protease to cleave off the N-terminal tags. Finally, the protein was loaded on the Superdex 200 size-exclusion chromatography column (GE Healthcare), and the fractions containing the ∆N-STAT3sfGFP were pooled and stored at −80°C. sfGFP control protein was prepared in a similar manner using pETM11SU-M03sfGFP vector for transformation.

V-ATPase assay
To purify V-ATPase, two 15 cm petri dishes with subconfluent HeLa cells were transfected with pcDNA3.1HA-ATP6V1A plasmid (5 µg/plate). Three days later, cells were scraped off in SCA buffer supplemented with 1× protease inhibitor cocktail and ruptured using a Dounce glass homogenizer until ~90% of cells were permeable for trypan blue. Ruptured cells were centrifuged at 1,000×g for 10 min and the obtained supernatant was further centrifuged at 20,000×g for 20 min. The pellet was lysed in 450 µL TBS lysis buffer supplemented with 1× protease inhibitor cocktail, incubated on ice for 30 min, and centrifuged at 20,000×g for 1 min. After determining the protein concentration, the supernatant containing 150 µg protein was precleared by rotation with 12 µL magnetic bead-conjugated rabbit IgG for 1 h at 4°C. The
precleared supernatant aspirated on magnetic stand was then rotated with 20 µL magnetic bead-conjugated rabbit mAb against HA for 2 h at 4 °C and the supernatant was aspirated on magnetic stand. The beads were washed 3× 5 min in 500 µL of TBS lysis buffer and suspended in 50 µL of V-ATPase reaction buffer (40 mM HEPES, pH 7.5, 1 mM MgCl2, 100 mM KCl, 0.25 mM Sucrose, 5 µg/mL Oligomycin, 1 mM DTT) supplemented with 1× protease inhibitor cocktail.

To measure the V-ATPase activity, 15 µL anti-HA beads (V-ATPase) were mixed with 4.5 µg ΔN-STAT3sfGFP or 4.5 µg sfGFP (negative control) and the volume was adjusted to 150 µL with V-ATPase reaction buffer. The reaction was started by adding 1 µM ATP (final concentration) and incubating the samples for 30 min at 30 °C. The samples and free phosphate standard dilutions were then transferred to 96-well plates (50 µL/well) and the reactions were stopped by adding 100 µL BIOMOL Green Reagent. Plates were incubated at 25 °C for 20–30 min before determining OD620nm in a Varioskan Flash Multimode Reader. The amount of ATP hydrolyzed was calculated using the standard curve created.

RNA sequencing

The mRNA and non-coding RNAs were enriched by removing rRNA with RNaseH. Target RNAs were fragmented into short fragments in the fragmentation buffer and cDNAs were synthesized using the RNA fragments as templates for N6 random primer, followed by end reparation and ligation to adapters. The quantity and quality of the cDNA libraries were assessed using an Agilent 2100 BioAnalyzer. Finally, the libraries were sequenced on the BGISEQ-500 with 50 single-end read.

Sequencing reads that contained adapters, had low quality, or aligned to rRNA were filtered off before mapping. Clean reads were aligned to the hg19 UCSC RefSeq (RNA sequences, GRCh37) using bowtie2. Fragments per kilobase of transcript per million (FPKM) were aligned to rRNA were filtered off before mapping. Clean reads mapping reads values were obtained by transforming the fragments in the fragmentation buffer and cDNAs were synthesized with EC Plan-Apochromat 63×/1.40 Oil DIC M27 objective. Fluorescence intensities were determined by open source ImageJ software.

Dextran degradation assay

Subconfluent cells were loaded with 0.4 mg/mL AlexaFluor 488-dextran (10 kDa) for 20 min and washed twice with PBS. The samples for the analysis of AlexaFluor 488-dextran uptake were then fixed with 4% PFA for 20 min, whereas the parallel samples for the analysis of AlexaFluor 488-dextran degradation were incubated in complete medium for additional 4 h before fixation. Images were acquired using a Zeiss LSM700 microscope with EC Plan-Apochromat 63×/1.40 Oil DIC M27 objective. Fluorescence intensities were determined by open source ImageJ software.

Data resources

The MS proteomics data have been deposited to the ProteomeXchange Consortium (www.ebi.ac.uk/pride) via the PRIDE partner repository with the dataset identifier PXD006788. GEO accession number for RNA-Seq data is GSE108495. Other datasets generated during the current study will be available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

B.L. designed and performed the great majority of experiments, analyzed the data, and wrote the first draft of the manuscript. J.P. performed and analyzed the MS experiments. Y.L. and L.L. generated SKO3V-EF-GFP-STAT3 cells. A.C. performed the qPCR analyses and assisted in RNA extractions, cell line characterization, and SR-SIM. J.H. and F.X. performed RNA-Seq and bioinformatics analyses, which were supervised by X.L. K.K.B.C. performed antibody-based lysosome purification and K.M. prepared recombinant proteins and assisted in the optimization of other protocols. M.J. designed the overall study, supervised the experiments, assisted in the data analyses, and wrote the final draft of the manuscript. All authors contributed to the final text and approved it.

ADDITIONAL INFORMATION

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