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Supplemental Information

Histone Acetylation Regulates Intracellular pH

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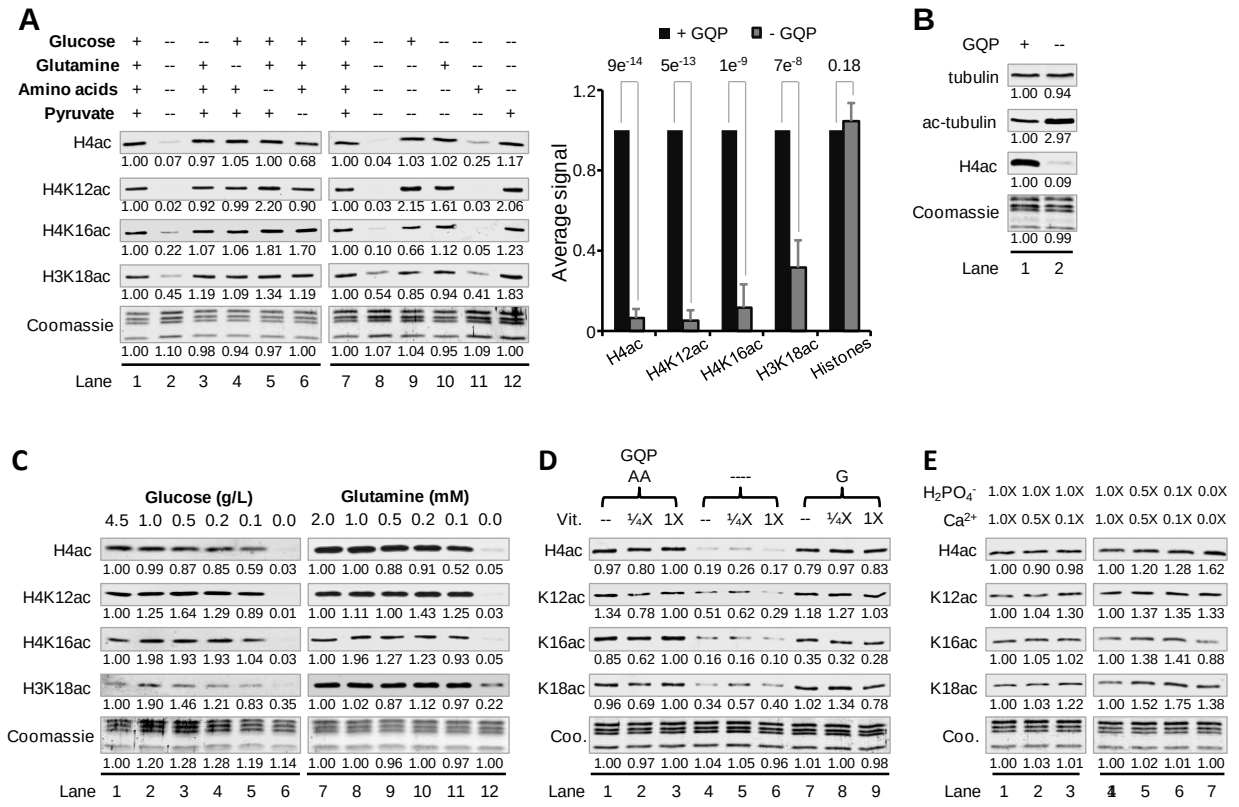

Figure S1, Related to Figure 1. Minimal Levels of Glucose, Glutamine or Pyruvate Maintain Global Levels of Histone Acetylation Independently of Vitamins and Salts. (**A**) Western blots (WBs) of histone acetylation in 231 cells cultured for 16 hrs in DMEM salts and vitamins with the indicated ac-CoA sources at concentrations found in standard DMEM. The bar graph shows average values ± standard deviation from ten independent experiments and p values derived from the Student's t-test. (**B**) WBs of tubulin and histone H4 acetylation from 231 cells

under conditions of glucose (G), glutamine (Q) and pyruvate (P) deprivation. (**C**) WBs of histone acetylation from 231 cells cultured at the indicated concentrations of G or Q in DMEM salts and vitamins. (**D**) WBs showing the effects of varying vitamins concentration on histone acetylation with the indicated ac-CoA source. (**E**) WBs showing the effects of varying concentrations of Ca^{2+} and phosphate on histone acetylation in 231 cells. Lane 1 in each panel – except for panel D – is the reference condition, the values of which are set to 1. In panel D, the reference condition is lane 3.

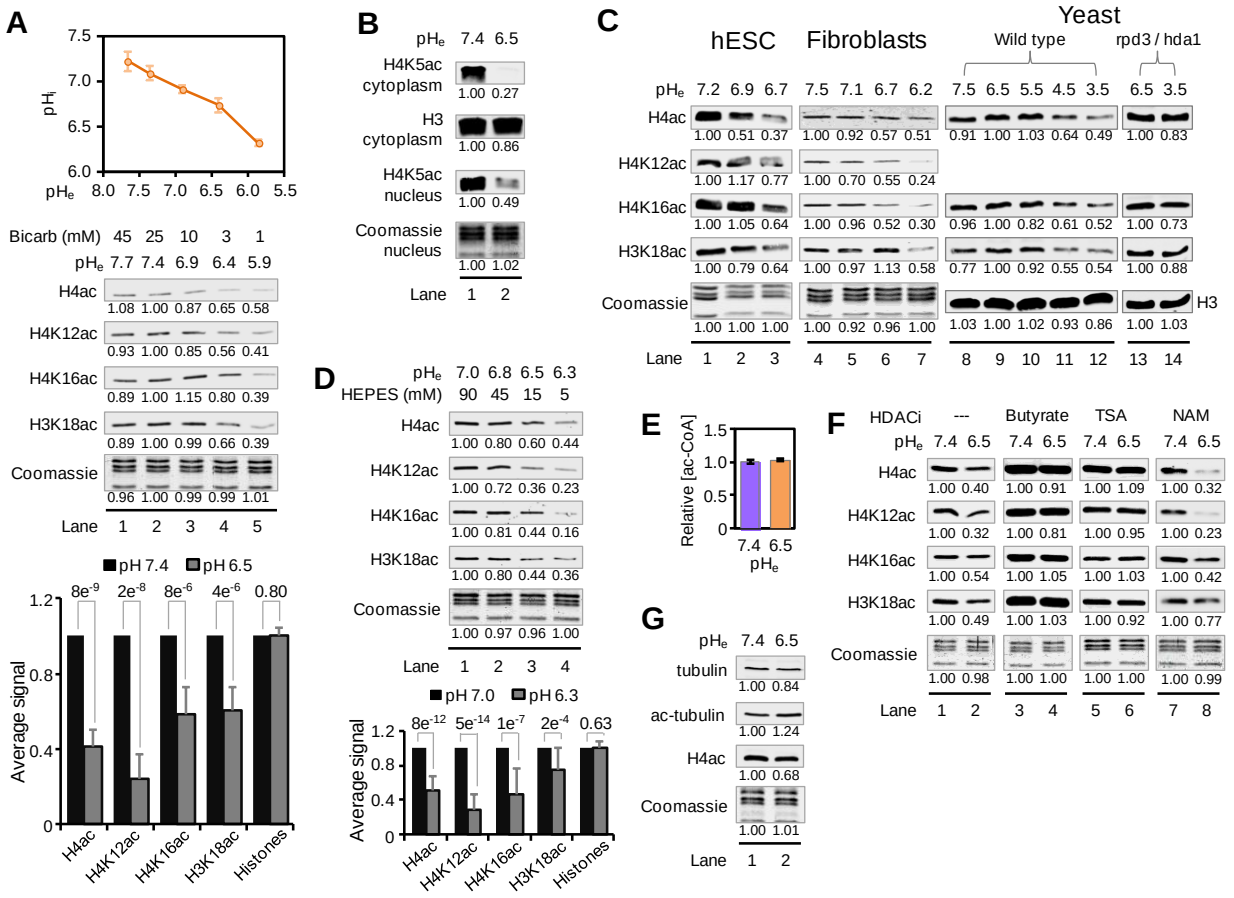

Figure S2, Related to Figure 2. Global Levels of Histone Acetylation Change in Response to pH Alterations. (**A**) Measurements of pH_i (mean ± standard deviation) as a function of pH_e and effects on histone acetylation in 231 cells cultured at the indicated pH_e for 16 hrs in complete DMEM. The reference condition is set to 25 mM bicarbonate (lane 2), as this approximates the

normal physiological concentration. The bar graph shows average values ± standard deviation from ten independent experiments and p values derived from the Student's t-test. (**B**) Western blot (WB) analysis of H4K5ac in 231 cells in the indicated histone fractions and pH. (**C**) WBs of acetylation in H1 human embryonic stem cells cultured for 3 hrs in m-TESR medium at the indicated pH_e, or IMR90 normal primary lung fibroblasts treated for 16 hrs in complete DMEM at the indicated pH_e or budding yeast (YDS2) grown for 4 hrs in YPD at the indicated pH. Histone H3 was used as loading control for the yeast experiments. Note that pH has little effect on histone acetylation in the strain with Rpd3 and Hda1 deleted. (**D**) WBs of histone acetylation in 231 cells cultured in media buffered with HEPES. The bar graph shows average values ± standard deviation from twenty one independent experiments and p values derived from the Student's t-test. (**E**) Ac-CoA measurements (mean ± standard deviation) of 231 cells treated for 4 hrs at the indicated pH_e. (**F**) WBs of histone acetylation in 231 cells that were treated with or without 5 mM sodium butyrate, 500 nM TSA or 2 mM nicotinamide (NAM) for 6 hrs in complete DMEM at pH_e 7.4 followed by incubation at the indicated pH_e for 4 hrs. (**G**) WBs of tubulin and histone H4 acetylation in 231 cells treated for 16 hrs at the indicated pH values.

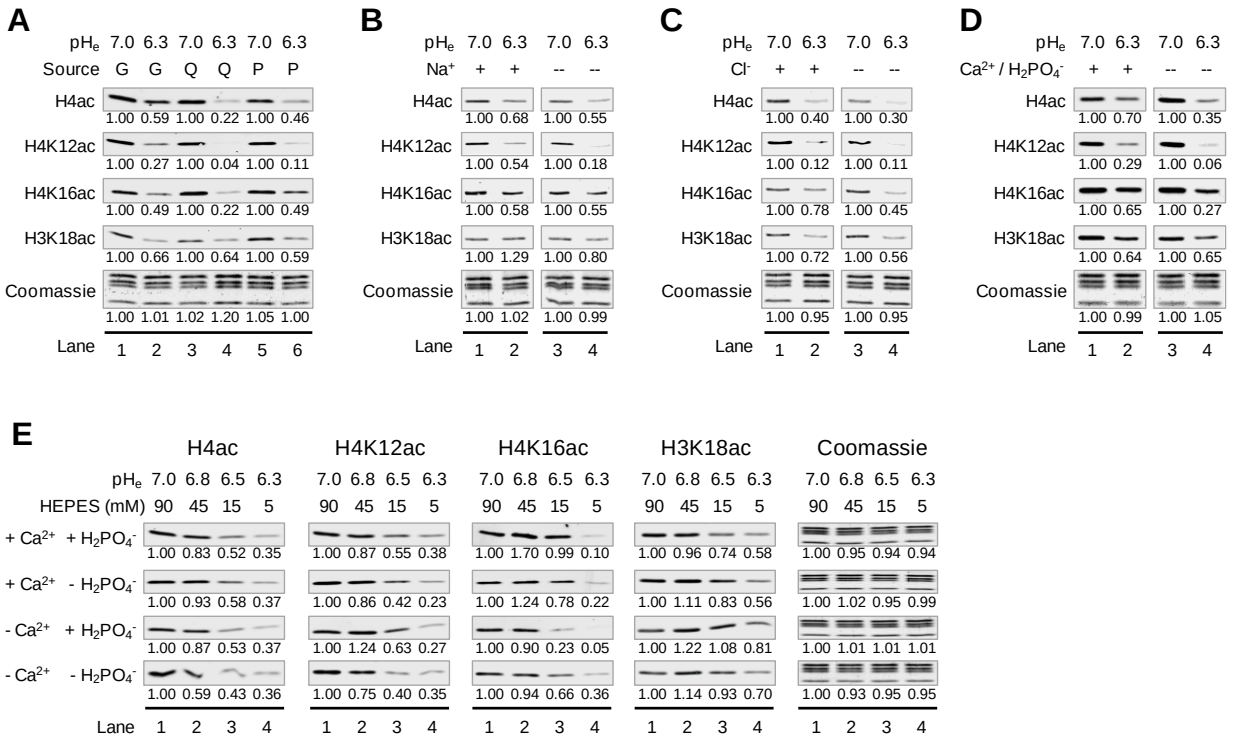

Figure S3, Related to Figure 3. Changes in Histone Acetylation Levels in Response to pH Do Not Require Specific Carbon Sources or Salts. Western blots of histone acetylation in 231 cells cultured for 16 hrs in DMEM salts at pH_e 7.0 or 6.3 (**A**) including the indicated carbon source or lacking (**B**) Na^+, (**C**) Cl^-, and (**D-E**) Ca^{2+} and phosphate. In panel **D**, cells were starved of Ca^{2+} and phosphate for 3 days prior to pH_e treatment, while in panel **E**, Ca^{2+} and phosphate were removed from the culture medium at the time of pH_e treatment.

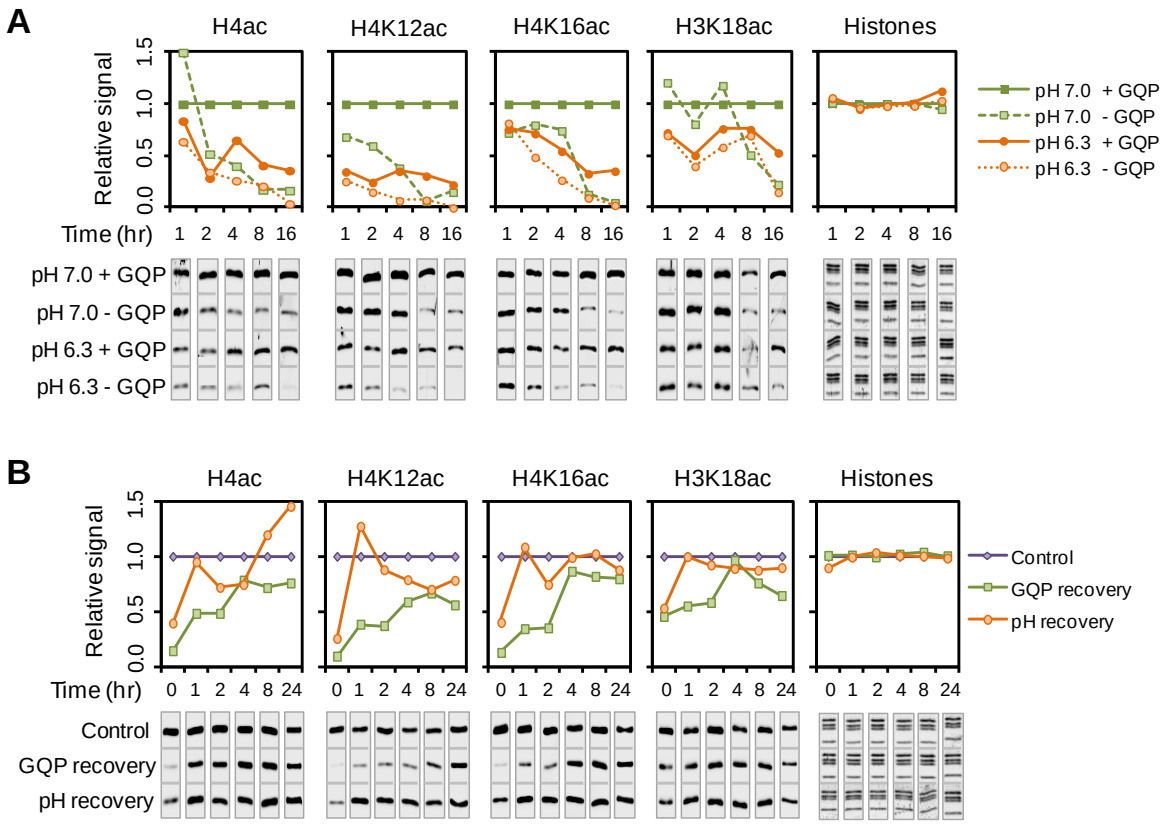

Figure S4, Related to Figure 5. Nutrient Availability and pH Alter Acetylation Dynamics Differently in 231 Cells. Western blots of histone acetylation in 231 cells cultured in DMEM salts (**A**) for the indicated amount of time and pH_e in the presence (+) and absence (-) of GQP and (**B**) at pH_e 7.4 with GQP (control), pH_e 7.4 without GQP (for GQP recovery) or pH_e 6.5 with GQP (for pH recovery) for 16 hrs followed by treatment for the indicated amount of time in medium at pH_e 7.4 with GQP. Membrane images were cropped in order to place them below the graph at the corresponding time points.

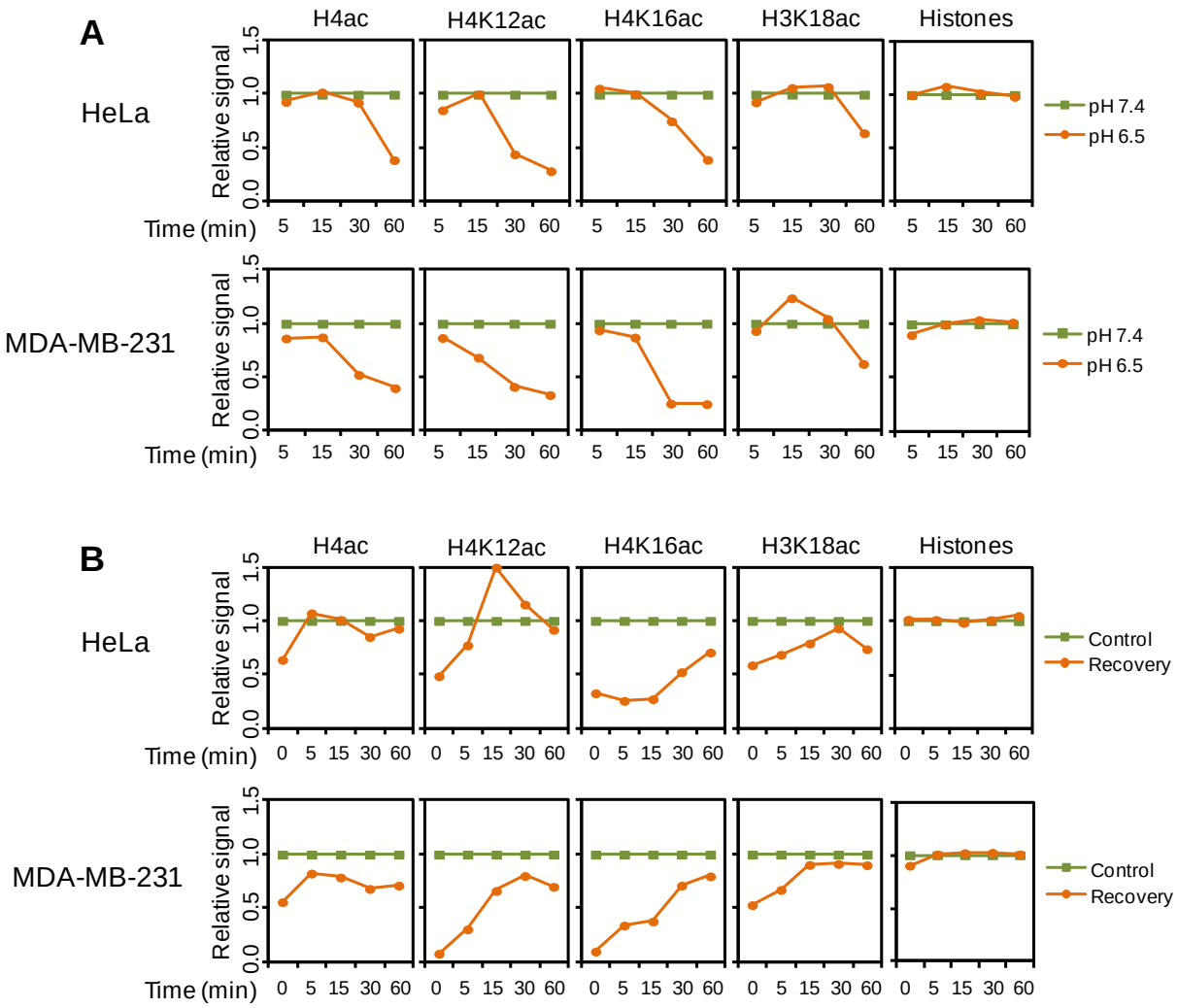

Figure S5, Related to Figure 6. Loss and Recovery of Acetylation by pH Treatment is Rapid. Western blots of histone acetylation from HeLa or 231 cells cultured in DMEM (**A**) for the indicated time and pH, or (**B**) for 16 hrs in DMEM at pH 7.4 (control) or 6.5 (recovery) followed by treatment at pH 7.4 for the indicated amount of time. Note that the recovery of acetylation begins within 5 min.

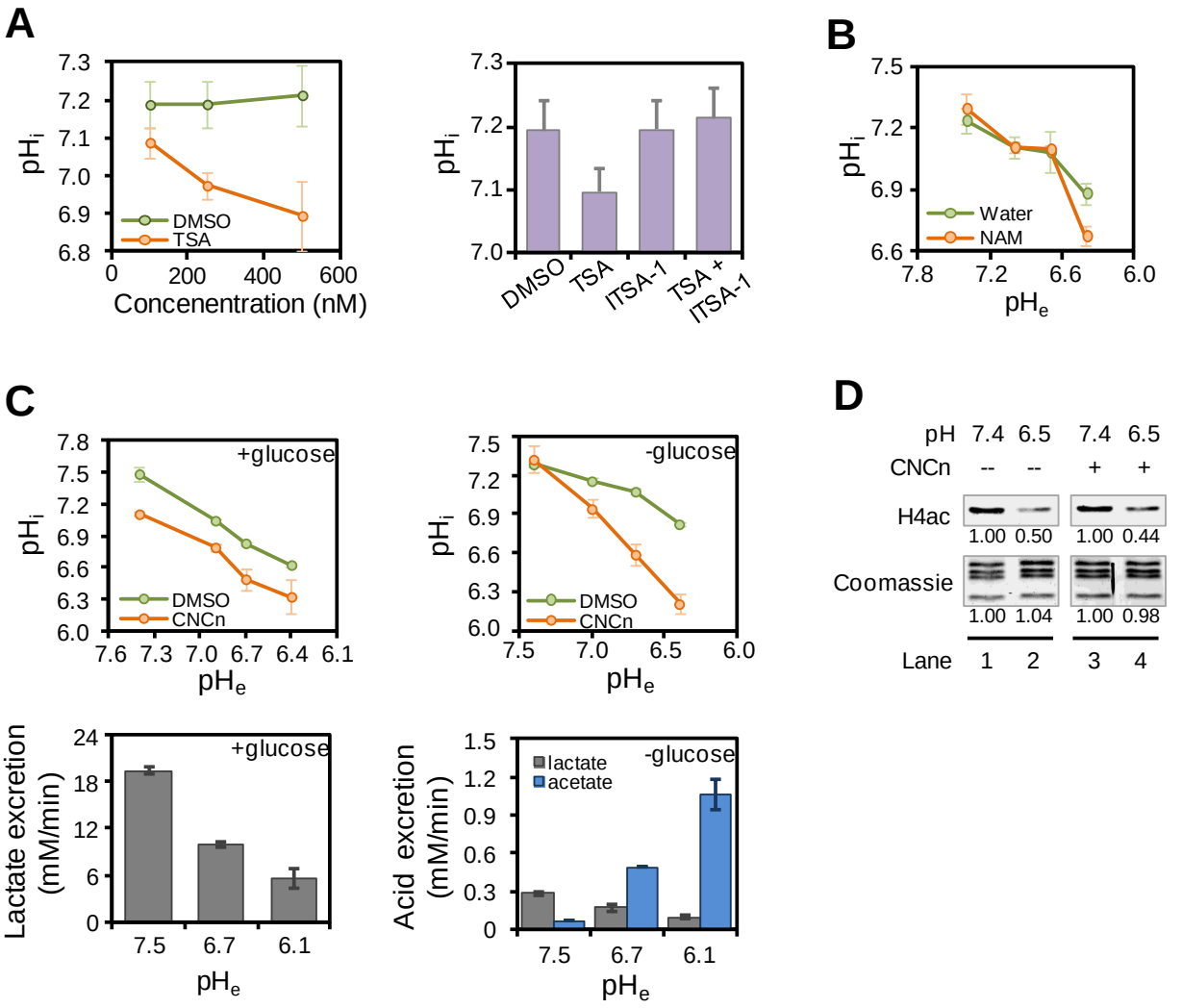

Figure S6, Related to Figure 7. Global Histone Acetylation and Deacetylation is Linked to pH_i. pH_i of 231 cells treated in standard DMEM overnight with (**A**) the indicated concentration of TSA (left panel); or 250 nM TSA, 50 µM ITSA-1 or both (right panel); or (**B**) 2 nM nicotinamide (NAM). (**C**) pH_i of 231 cells in which MCT function was inhibited by treatment for 1 hr with 10 mM CNCn in DMEM with (top left panel) and without (top right panel) glucose along with rates of acetate and lactate excretion (bottom panels) under identical conditions. Note that in absence of glucose, acetate excretion at high pH is reduced to minimal levels. In contrast, rate of acetate excretion at low pH is not dependent on the presence of glucose. (**D**) Western blots of histone acetylation from 231 cells treated for 4 hrs in DMEM at pH 7.4 with 10 mM CNCn followed by treatment with 10 mM CNCn at the indicated pH_e for an additional 2 hrs. Data in **A-C** is presented as mean ± standard deviation.

Table S1, Related to Figure 4. Gene Ontology Analysis of Gene Expression Changes in HeLa Cells in Response to pH.

Gene Ontology Terms	Benjamini p value
Up-regulated >2-fold at low pH	
Histone core	3.21E-07
Basic-leucine zipper (bZIP) transcription factor	5.78E-05
MAPK signaling pathway	1.63E-04
Regulation of transcription from RNA polymerase II promoter	2.77E-04
Positive regulation of nitrogen compound metabolic process	3.72E-04
Positive regulation of cellular biosynthetic process	8.12E-04
Response to endogenous stimulus	7.19E-03
Down-regulated >2-fold at low pH	
Plasma membrane	2.92E-06
Glycoprotein	1.75E-05
Extracellular matrix	8.22E-04

Supplemental Experimental Procedures

Cell culture

HeLa, MDA-MB-231 and IMR90 cells were generally cultured in DMEM without glucose, glutamine, phenol red, sodium pyruvate and sodium bicarbonate (Sigma #D5030) to which was added 1 g/L glucose, 2 mM glutamine, 1 mM sodium pyruvate 26 mM sodium bicarbonate and 1X antibiotic/antimycotic (Gibco #15240). Medium pH was adjusted to 7.2 by the addition of HCl before being filter sterilized. Dialyzed fetal bovine serum (FBS) (Gibco#26400) was re-dialyzed against 150 mM NaCl using 10,000 molecular weight cutoff dialysis tubing (SpectrumLabs#08670252) to remove trace nutrients (Wice et al., 1981). Dialysis was performed at 4^{o}C for 2 hrs for a total of 3 times in 6 L dialysate each time. Redialyzed serum was added to media at a concentration of 5%. The use of dialyzed serum significantly improved the reproducibility of data.

Media preparation

Custom media were prepared where appropriate as indicated in the main text. DMEM consists of salts, vitamins, amino acids, glucose, glutamine, pyruvate and bicarbonate. For experiments in DMEM salts, we followed the recipe from Sigma (#E7510) with the exception that glucose and phenol red were omitted. Experiments in which sodium was omitted were done using the same salt recipe but substituting an equimolar mixture of lithium chloride and potassium chloride for sodium chloride, HEPES for sodium bicarbonate, and potassium phosphate for sodium phosphate. Experiments in which chloride was omitted were done by substituting sodium nitrate for sodium chloride, potassium nitrate for potassium chloride, and calcium nitrate for calcium chloride. Experiments in which calcium and phosphate were omitted were done by simply excluding them from the salt formulation since they contribute very little to the overall tonicity of the medium. For experiments in DMEM salts and vitamins, we added vitamins purchased from Sigma (#M6895) to our DMEM salts solution. For experiments in complete DMEM we used Sigma powder DMEM (#D5030) which was prepared as described above. The pH of the media was varied by altering the buffering component of the media.

Adding different amounts of bicarbonate resulted in the pH as shown in figures 2A and S2A when incubated at 37^{o}C and 5% CO_2. Media pH was also adjusted by adding varying amounts of HEPES and then adjusting the pH to 7.2 by the addition of acid or base. This resulted in the pH as shown in figures 2D and S2D when incubated at 37^{o}C and 5% CO_2. All pH_e measurements were taken by bubbling 5% CO_2 into the media for 30 minutes (min) prior to taking measurements using a standard pH meter. Media pH did not change more than 0.2 units during any of the experiments performed. For H1 hESCs, the pH of media was varied by adding adequate acid while bubbling 5% CO_2. The pH of yeast growth media (YPD) was adjusted by addition of acid or base.

Western blotting

Western blots were performed on acid-extracted histones and on whole cell lysates and results were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). This system utilizes fluorescently-labeled secondary antibodies which allow for fluorescent intensity values to be recorded upon excitation with a fluorescent scanner. These values are reported as relative values below images of the fluorescently scanned membranes which were converted to grayscale. Westerns using the histone antibodies were performed with 15% polyacrylamide gels while those done on the whole cell lysates were run on 4-12% Bis-Tris gradient gels (Invitrogen #NPO321BOX). In both cases, samples were prepared to sufficient volume to load all gels for the experiment which were run in parallel. Transfer was done using the iBlot system with Immobilon-FL membrane (Millipore #IPFL00010) replacing the PVDF membrane provided (Invitrogen #IB4010-01). Primary antibodies used were H4ac (Millipore #07-352), H4K16ac (Active Motif #39167), H3K18ac (Active Motif #39587), H4K5ac (Millipore #07-327), H3K4me2 (Abcam #32356), K3K9me2 (Millipore #07-441), H3K27me3 (Millipore #07-449), H3K36me2 (Millipore #07-274), H3 (Abcam #1791), tubulin (BD Biosciences #556321), acetyl-tubulin (Sigma #T6793), MCT1 (Sigma #HPA003324); H4K12ac was kindly provided by Michael Grunstein.

Acid extracted histones were prepared from a 10-cm plate of cells at roughly 70% density. All reagents used were chilled to 4^{o}C and spins were done using a chilled microfuge

maintained at 4°C. Briefly, nuclei were collected following hypotonic lysis of cells and then acid-extracted with H_2SO_4. The proteins in the extraction were collected by TCA precipitation and dissolved in 100 µl of 95°C H_2O. Protein concentration was estimated with the BCA assay (Thermo Scientific #23225) using the manufacturer's protocol. The histone solution was then diluted in an equal volume of 2X SDS protein buffer (SPB) and boiled for 5 min. Histone protein concentration was then normalized between samples by using the Odyssey system to quantify the four histone bands on SimplyBlue (Invitrogen #LC6060) stained gels loaded with 1 µg of protein (as determined by BCA). The loading amount was then adjusted to ensure even loading of histones. This method of loading normalization consistently resulted in an even loading of histone proteins much better than using various antibodies recognizing total histone H3 or H4. Even loading of histone proteins was a critical factor in accurately assessing levels of modification.

Whole cell lysates were prepared from a 10-cm plate by washing the cells with PBS and then adding 750 µl of 1X SPB. Samples were then boiled and concentration was approximated between samples by comparison of SimplyBlue stained gels using the Odyssey system. Histone bands as well as several other prominent bands were quantified and used to adjust the loading amounts for subsequent westerns. Preparation of yeast histones by TCA-precipitation was performed as described previously (Peng et al., 2008) and loading between samples was normalized using results from immunoblots against total H3.

^{3}H-Acetate excretion assay

Culture medium pH is typically established by the concentration of bicarbonate in the medium and the level of CO_2 in the atmosphere. Cells in culture are usually grown in medium containing 26 mM bicarb in a 37°C incubator at 5% CO_2 which results in a medium pH of around 7.4. In this particular assay, cells were taken in and out of a 37°C incubator frequently during the course of the experiment. This did not allow for a constant concentration of CO_2; therefore, the experiment was performed at 37°C at atmospheric CO_2 conditions. As such, in order to obtain media of varying pH, we added the amount of bicarbonate that would have resulted in varying pH at 5% CO2 as shown in figures 2A and S2A. We then adjusted the pH of

the solution by the addition of acid or base to equal the pH that would have been attained at 5% CO2 with the given amount of bicarbonate. Medium at pH 7.4 contained 25 mM bicarbonate, pH 6.8 had 7 mM bicarbonate, pH 6.4 had 3 mM bicarbonate and pH 5.9 had 1 mM bicarbonate.

To start the experiment, $2x10^5$ cells were plated per well of a 6-well plate in DMEM (pH 7.4) with 10% FBS. 48hrs later cells were labeled for 1 hour (hr) in 3 ml DMEM (pH 7.4) with 66 µCi/ml of ^{3}H-acetate (Moravek #634). Following the label, a 30 minute chase was performed in 3 ml DMEM (pH 7.4). Chase medium was then removed for counting purposes and 1 ml of DMEM (pH 7.4) was incubated for 10 min to assess the rate of label extrusion prior to changes in media pH. (This rate is indicated in graphs as time point 0.) Beginning at time point 0, cells were incubated in 1 ml media of varying pH for different time intervals. At each time point, 800 µl of media from each well was collected for counting purposes, the residual media was discarded and 1 ml fresh media was added. The appearance of label was monitored at each time point for each medium pH by a liquid scintillation counter. Counts were normalized between experiments using time point 0 to correct for differences between labeling efficiency.

For experiments with α-cyano-4-hydroxycinnamate (CNCn; Sigma #595667), the reagent was dissolved in DMSO as a 500 mM stock and added to media at a final concentration of 10 mM (Wahl et al., 2002). Control media were prepared by diluting DMSO into medium at a ratio of 1:50. The pH of CNCn and DMSO control media was adjusted after CNCn or DMSO addition and was then used during the chase and all subsequent steps. Experiments with TSA were performed by diluting a 5 mM TSA in DMSO solution (Sigma #T1952) to a final concentration of 500 nM. Control media were prepared by diluting DMSO into medium at a ratio of 1:10,000. Cells were incubated in TSA (or DMSO control) media for 24 hrs prior to labeling. All subsequent steps were done using TSA or DMSO control media.

Intracellular pH measurement

Adherent cells were grown on 35-mm poly lysine coated glass bottom culture dishes (MatTek, Ashland, MA) to 40% confluency in DMEM plus 10% FBS and 1% Anti-Anti (Gibco #15240). All pH_i measurement solutions were bubbled with 5% CO_2 and contained the

appropriate amount of bicarbonate to obtain the desired pH according to the Henderson-Hasselbach equation. Cells were washed with Earle's balanced salt solution (EBSS) and then loaded with 5 nmol/ml of the pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5-(and-6)carboxyfluorescein, acetoxymethylester (BCECF-AM; Molecular Probes #B1170) in EBSS for 25 min in the 37°C incubator with 5% CO_2. pH_i was measured immediately after loading by obtaining measures of fluorescent intensity at an emission wavelength of 535 nm, with alternating excitation wavelengths of 495 and 440. Images were obtained with an Axiovert 200M Zeiss florescent microscope equipped with a Chroma BCECF filter set (Chroma #71001a) and a high-resolution video camera (Axio CAM MRm, Zeiss, Germany). Ratio between 495/440 channels was calculated for a field of cells by AxioVision 4.8 and Slidebook 4.2 after subtracting background in each channel. During the experiment, cells were superfused with 37°C EBSS at with a chamber insert diamond medium bath (Warner Instruments, Hamden, CT) at a rate of 1 ml/min while pH was monitored continuously.

pH_i of non-adherent T-cells was measured using a four-laser digital BD LSRII flow cytometer as described previously with some modifications (Chow and Hedley, 2001). Cells were resuspended in loading solution (EBSS with 26 mM bicarbonate and 20 mM HEPES with the pH adjusted to 7.4 by the addition of sodium hydroxide) and loaded with 5 nmol/ml of 5-(and-6)-Carboxy SNARF®-1, Acetoxymethyl Ester, Acetate (SNARF-1; Molecular Probes #C1272) for 30 min at 37^{o}C and 5% CO_2. After loading, cells were resuspended in loading solution or high potassium calibration solutions at various pH containing 10 μM nigericin. After 20 min of incubation at atmospheric conditions cells were loaded in the flow cytometer and fluorescent intensities were collected at emission wavelengths of 580 nm and 640 nm for an excitation wavelength of 488 nm using linear amplification. Fluorescent ratios of 640/580 nm were used for determination of pH_i. Data were analyzed using Flow Jo software.

Ratios from both types of experiments were converted to pH by in situ calibration for each set of experiments using nigericin and high potassium isotonic solution as described previously (Nehrke, 2006).

Supplementary References

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