Figures and figure supplements

The vacuolar-ATPase complex and assembly factors, TMEM199 and CCDC115, control HIF1α prolyl hydroxylation by regulating cellular iron levels

Anna L Miles et al
Figure 1. Depletion or inhibition of the V-ATPase stabilizes HIF1α in aerobic conditions. (A) Bubble plot depicting genes enriched in the forward genetic screen. Bubbles represent the genes enriched in the GFP\textsuperscript{HIGH} population compared to unmutagenised KBM7 cells expressing the HIF1α-
GFP<sup>ODD</sup> reporter. Proteins involved in V-ATPase assembly and function (green), canonical HIF1α regulation (purple), and the oxoglutarate dehydrogenase complex (blue) are highlighted, with the number of independent gene trap insertions indicated (brackets). (B) Pathway analysis of enriched genes in the KBM7 forward genetic screen. The top 114 genes enriched for multiple independent gene-trapping integrations in the GFP<sup>HIGH</sup> population compared to unmutagenised KBM7 cells expressing the HIF1α-GFP<sup>ODD</sup> reporter were analysed by gene ontology clustering for pathways significantly targeted in the screen. An individual gene enrichment p value < 0.1 was used as a threshold value for genes to be included in the pathway analysis. (C) Schematic of enriched gene trap insertion sites in the 5 V-ATPase subunits (ATP6V0D1, ATP6V1G1, ATP6AP1, ATP6V1A, ATP6V0A2) identified in the forward genetic screen. (Red = sense insertions, Blue = antisense insertions). (D, E) Validation of the V-ATPase subunits identified in the screen using CRISPR-Cas9 targeted gene editing in HIF1α-GFP<sup>ODD</sup> reporter (D) and wildtype (E) HeLa cells. Cells were simultaneously transduced with Cas9 and sgRNAs to ATP6V0D1, ATP6V1G1, ATP6AP1, ATP6V1A1, or ATP6V0A2. GFP levels were assessed by flow cytometry after 10 days (% GFP<sup>HIGH</sup> cells indicated) (D), and HIF1α levels measured by immunoblot (E). PHD2 and β2m were used as positive and negative controls respectively. (F, G) Chemical perturbation of V-ATPase function. Wildtype and HIF1α-GFP<sup>ODD</sup> HeLa cells were cultured in the presence of BafA (10 nM or 100 nM) for 24 hr and HIF1α levels measured by GFP fluorescence (F) or immunoblot (G).

DOI: 10.7554/eLife.22693.003
Figure 2. TMEM199 and CCDC115 are the human orthologues of the yeast Vma12p-Vma22p V-ATPase assembly complex. (A) Enriched gene trap insertion sites in TMEM199 identified in the forward genetic screen. (Red = sense insertions, Blue = antisense insertions). (B) Schematic for TMEM199

Figure 2 continued on next page
Figure 2 continued

(left) and Vma12p (right) membrane topology. TMEM199 and Vma12p demonstrate 23.89% sequence identity (Clustal Omega tool (EMBL-EBI)). (C, D) HIF1α-GFPODD reporter cells transduced with Cas9/TMEM199 sgRNA were sorted into GFPLOW (Lo) and GFPHIGH (Hi) populations by FACS (C), lysed, and immunoblotted for endogenous HIF1α and TMEM199 (D). PHD2 and β2m were used as positive and negative controls respectively, and β-actin served as a loading control. (E, F) TMEM199 reconstitution decreases HIF1α levels in TMEM199 deficient cells. TMEM199 KO clones were isolated following lentiviral transduction with sgRNA to TMEM199/Cas9 and serial dilution. Null clones were identified by immunoblot. A CRISPR resistant TMEM199 was overexpressed by lentiviral transduction in mixed populations of TMEM199 deficient cells (E) or clonal cells (F). HIF1α and TMEM199 levels were measured by immunoblot, and short and long exposures of TMEM199 levels are shown (E). (G) Co-immunoprecipitation coupled mass spectrometry. Wildtype HeLa cells and TMEM199 null cells were lysed in 1% NP-40 and immunoprecipitated for TMEM199 for 3 hr. Samples were validated by immunoblotting and submitted for mass spectrometry analysis. Proteins immunoprecipitated in wildtype HeLa compared to TMEM199 KO cells with a unique peptide count >2 are shown. (H) PyMOL structural alignment of CCDC115 (pink) and Vma22p (green) based on Phyre² predictions. (I) Immunoprecipitation of FLAG-CCDC115 with endogenous TMEM199 in wildtype (+) or TMEM199 deficient (-) HeLa cells. An unrelated FLAG tagged protein (FLAG-Ct) was used as a control. The lysate inputs and immunoprecipitated samples are shown. *non-specific band. (J, K) HIF1α-GFPODD reporter cells were depleted of CCDC115 by transduction with Cas9 and sgRNA. After 12 days, cells were sorted into GFPLow (Lo, grey box, left) and GFPHigh (Hi, grey box, right) populations by FACS (J), and immunoblotted for endogenous HIF1α (K). β-actin served as a loading control.

DOI: 10.7554/eLife.22693.004
Figure 3. TMEM199 and CCDC115 localise to the ER. (A) HeLa cells were homogenised and separated into membrane and cytosolic fractions by ultracentrifugation. The samples were analysed by immunoblotting for TMEM199. Calnexin was used as a loading control for membrane compartments. Figure 3 continued on next page.
whilst tubulin was used as a control for cytosolic fractions. (B, C) Representative immunocytochemical staining for endogenous TMEM199 (red) with the
ER marker KDEL, the golgi apparatus marker TGN46, early endosome marker EEA1, late endosome marker M6PR and lysosomal marker LAMP-1 (all in
green) (B). Scale bar represents 5 μm. Quantification of colocalisation for TMEM199 and the respective organelle markers using Pearson's Correlation
Coefficient (C) n ≥ 16 cells. (D, E) Confocal immunofluorescence microscopy of HeLa cells transduced with HA-CCDC115 (green) and endogenous
TMEM199 (red, top) or KDEL (red, bottom) (D). Scale bar represents 10 μm. Quantification of colocalisation for CCDC115 with TMEM199 or KDEL using
Pearson’s Correlation Coefficient (E) n ≥ 50 cells.
DOI: 10.7554/eLife.22693.005
Figure 4. TMEM199 and CCDC115 and are required for lysosomal degradation of EGFR and MHC Class I. (A) EGFR degradation assay for wildtype and BafA treated cells. HeLa cells were cultured in the presence or absence of 10 nM BafA for 24 hr. Cells were stimulated with EGF and lysed at the...
indicated times. Lysates were subjected to SDS-PAGE and immunoblotted for EGFR. β-actin was used as a loading control. (B, C) EGFR degradation assay for TMEM119 and CCDC115 deficient cells. HIF1α-GFP<sup>ODD</sup> cells were transduced with Cas9 and sgRNA to TMEM119 (B) or CCDC115 (C). After 14 days, cells were sorted into TMEM119 or CCDC115 sufficient (+/+ , GFP<sup>LOW</sup>), and TMEM119 or CCDC115 null (−/−, GFP<sup>HIGH</sup>) populations as described. Cells were then cultured for 24 hr before stimulation with EGF (100 ng/ml), harvested at indicated times and immunoblotted for EGFR. (D) MHC Class I degradation in HeLa cells expressing K3. HeLa-K3 cells were transduced with Cas9 and sgRNA to TMEM119, CCDC115, ATP6V1A1 or ATP6V0D1. After 14 days, cell surface MHC Class I levels were measured by flow cytometry (mAb W6/32). Wildtype HeLa cells were used as a control for total MHC Class I. Percentages of cells with MHC Class I at the cell surface are shown.

DOI: 10.7554/eLife.22693.006
Figure 5. TMEM199 and CCDC115 are required for acidification of endosomal compartments. (A, B) Live cell confocal microscopy of HIF1α-mCherryODD reporter cells transfected with the pH sensitive Tfnr-phl. HIF1α-mCherryODD reporter cells were transfected with Tfnr-phl and treated with 10nM BafA 24h (LM) and 10nM BafA 24h (HM).

Figure 5 continued on next page.
or without 10 nM BafA for 24 hr. Lower (LM) and Higher (HM) magnifications of representative BafA treated cells are shown (A). Quantification of intracellular Tfnr-phl/total Tfnr-phl fluorescence in BafA treated cells compared to no treatment (≥9 cells) (B). Additional control experiments are shown in Figure 5—figure supplement 1. (C, D) Live cell confocal microscopy of Tfnr-phl fluorescence in HIF1α-mCherryODD reporter cells depleted for TMEM199, CCDC115 or core V-ATPase subunits. TMEM199, CCDC115, ATP6V1A1 or ATP6V0D1 were depleted by sgRNA as described. After 10–12 days the cells were transfected with Tfnr-phl and live cell fluorescence measured after a further 24 to 48 hr (C). Quantification of intracellular Tfnr-phl/total Tfnr-phl fluorescence is shown (≥12 cells) (D). Representative wide field images are shown in Figure 5—figure supplement 1C. Scale bars represent 10 μm (A) or 5 μm (C). Values are mean±SEM.

DOI: 10.7554/eLife.22693.007
Figure 5—figure supplement 1. TMEM199 and CCDC115 are required for acidification of endosomal compartments. (A) pH clamping of HeLa cells expressing Tfnr-phl. HeLa cells were transfected with Tfnr-phl. After

Miles et al. eLife 2017;6:e22693. DOI: 10.7554/eLife.22693

Biochemistry | Cell Biology
Figure 5—figure supplement 1 continued

48 hr the cells were clamped at the indicated pH and fluorescence measured by live cell confocal microscopy. Differential interference contrast (DIC) microscopy confirmed the presence of intact cells at pH 5 and 6. (B) Tfnr-phl localisation in fixed cells. HIF1α-mCherry<sup>ODD</sup> reporter cells were transfected with Tfnr-phl as described. After 24 hr the cells were plated on cover slips and treated with or without 10 nM BafA for a further 24 hr. Cells were fixed (4% paraformaldehyde (PFA)) prior to confocal microscopy. (C) Representative wide field images of Tfnr-phl fluorescence in HIF1α-mCherry<sup>ODD</sup> reporter cells depleted for TMEM199, CCDC115 or core V-ATPase subunits. Scale bars represent 20 μm (A), 5 μm (B), and 10 μm (C).

DOI: 10.7554/eLife.22693.008
Figure 6. Disrupting the V-ATPase activates HIF1 and HIF2. (A) Immunoblot of HIF1α levels in response to the proteasome inhibitor MG132, the V-ATPase inhibitor BafA, the lysosomotropic agent Chloroquine, and the oxidative metabolism inhibitor NH₄Cl. (B) Immunoblot of HIF1α levels in HeLa cells with or without 10nM BafA. (C) Immunofluorescence images of HIF1α and TMEM199 expression in HeLa cells. (D) Flow cytometry analysis of HIFα-GFP expression in cells transfected with different sgRNAs. (E) Western blot analysis of HIF1α, CA9, and β-actin levels in HIFα-GFP reporter cells treated with different sgRNAs. (F) Immunoblot of HIF2α, HO-1, and β-actin levels in HIFα-GFP reporter cells treated with different V-ATPase inhibitors. Figure 6 continued on next page.
in response to BafA treatment at 0.5, 1, 2, 4, 8 and 24 hr. (C) Confocal immunofluorescence microscopy of WT (top) and BafA (bottom) treated HeLa cells stained for endogenous HIF1α. Cells were treated in the presence or absence of 10 nM BafA for 24 hr before immunofluorescence staining with HIF1α (white). Cells were mounted using DAPI (blue) and visualised by confocal microscopy. Scale bar represents 10 μm. (D) Immunocytochemical staining to examine HIF1α stabilisation in TMEM199 depleted HIF1α-GFPODD reporter cells. HIF1α-GFPODD reporter cells were depleted of TMEM199 using CRISPR-Cas9 genetics and stained for HIF1α (white), TMEM199 (red) and DAPI (blue). Scale bar represents 20 μm. (E–G) Levels of HIF1α or HIF2α and their target genes in cells depleted of V-ATPase subunits. HIF1α-GFPODD reporter cells were transduced with sgRNA to the indicated V-ATPase subunits as described. After 14 days, cell surface CA9 was measured by flow cytometry (E). Levels of HIF1α, HIF2α and their targets CA9 and HO-1 were measured by immunoblot (F, G). DOI: 10.7554/eLife.22693.009
Figure 7. V-ATPase depletion or inhibition stabilises HIF1α in a non-prolyl hydroxylated form. (A) HIF1α stabilisation in ATG16 null HeLa cells. HeLa cells and ATG16 null cells were treated with increasing concentrations of BafA (10 nM and 100 nM) before immunoblotting for HIF1α. (B) HIF1α levels Figure 7 continued on next page
following depletion of HSC70 and LAMP2A in aerobic conditions. HSC70 and LAMP2A depleted cells were generated using CRISPR-Cas9 gene editing with three individual sgRNAs (g1, g2, g3). HIF1α, LAMP2A and HSC70 levels were visualised by immunoblot. Untreated (Ct) and BafA treated HeLa cells were used as controls. (C) HIF1α levels following siRNA-mediated depletion of HSC70. HeLa cells were transfected with siRNA to HSC70 or an siRNA control (Ct), and HIF1α or HSC70 levels measured by immunoblot after 96 hr. Cells were treated with or without 10 nM BafA for 24 hr prior to lysis. (D) LAMP2A deficient HeLa cells were treated with or without 10 nM BafA for 24 hr. Three different sgRNAs were used (g1, g2, g3). (E, F) Immunoblot of total HIF1α and the prolyl hydroxylated form in response to MG132, DMOG, BafA and Chloroquine (E). Quantification of immunoblots represented using ImageJ analysis (F) (n = 3). (G, H) In vitro prolyl hydroxylation of the HIF1αODD protein following incubation with lysates from WT, BafA and DMOG treated HeLa cells. The levels of hydroxylated HIF1α were measured using a prolyl hydroxy-HIF1α specific antibody (G). Quantification of the in vitro hydroxylation assay using ImageJ analysis (H) (n = 3). Values are mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

DOI: 10.7554/eLife.22693.010
Figure 8. Disrupting V-ATPase activity decreases intracellular iron levels. (A, B) V-ATPase inhibition leads to intracellular iron depletion. (A) HeLa cells were treated with BafA (10 nM or 100 nM) or 100 µM DFO for 24 hr. HIF1α, IRP2, NCOA4 and ferritin (ferritin heavy chain 1, FTH1) levels were measured.

Miles et al. eLife 2017;6:e22693. DOI: 10.7554/eLife.22693
by immunoblot. (B) HIF1α-GFP<sup>ODD</sup> reporter cells transduced with Cas9 and sgRNA targeting V-ATPase components (TMEM199, CCDC115, ATP6V0D1 and ATP6V1A1) were sorted into GFP<sup>LOW</sup> (Lo) and GFP<sup>HIGH</sup> (Hi) populations as described. The lysates were immunoblotted for HIF1α, IRP2, or NCOA4. β-actin served as a loading control. (C) Iron chelation prevents HIF1α hydroxylation. In vitro prolyl hydroxylation of the HIF1α<sup>ODD</sup> protein following incubation with lysates from WT or DFO treated lysates (100 μM for 24 hr) as previously described. DMOG served as a control for PHD inhibition. (D–F) In vitro hydroxylation of PHD activity in DFO or BafA treated lysates supplemented with ferrous iron. Lysates from control, DFO (D) or BafA (E) treated HeLa cells were extracted as previously described, incubated with the HIF1α<sup>ODD</sup> protein, and supplemented with increasing concentrations of iron chloride (FeCl<sub>2</sub>, Fe(II)). Prolyl-hydroxylated HIF1α<sup>ODD</sup> levels were visualised by immunoblot and quantified by densitometry for the BafA treated lysate (F) (n = 3). Values are mean±SEM. *p<0.05, **p<0.01 Fe(II) compared to no treatment in BafA treated cells. NS=not significant. DOI: 10.7554/eLife.22693.011
Figure 9. Iron supplementation restores HIF1 activity to basal levels following V-ATPase inhibition in cell lines and primary cells. (A–D) Iron reconstitution in BafA or DFO treated HeLa cells. (A, C) HIF1α-GFPODD reporter cells were treated with BafA (10 nM or 100 nM), or 100 μM DFO for 24 hours. (E–G) HEK293T and Fibroblasts cells were treated with BafA (10 nM or 100 nM) or 100 μM DFO for 24 hours. (H) HeLa cells treated with BafA (10 nM or 100 nM) or 100 μM DFO for 24 hours. (I) HEK293T cells treated with EGF for 0, 45, 180, 360 minutes. (J) CA9 mRNA, VEGF mRNA, GLUT1 mRNA expression in WT, BafA, and BafA+Fe(III). (K–M) ATP6V1A1, CCDC115, TMEM199 sgRNA were used to knock down protein expression in HeLa cells. **Figure 9 continued on next page**
Figure 9 continued

hr with 50 μM iron citrate (Fe(III)) (red), 200 μM Fe(III) (blue) or no iron (green), and GFP levels analysed by flow cytometry. (B, D) Wildtype HeLa cells were treated with or without BafA (10 nM or 100 nM) or DFO (100 μM) and Fe(III) (50–200 μM) as described, and endogenous HIF1α levels were measured by immunoblot. (E–G) HEK293T cells (E), human dermal fibroblasts (F) and RCC10 VHL null and VHL reconstituted cells (G) were treated with BafA (10 nM) with or without the addition of 50 μM iron citrate (Fe(III)). HIF1α levels were visualised by immunoblot. β-actin served as a loading control. (H) HeLa cells were treated with 20 μM MG132 for 2 hr or 10 nM BafA for 24 hr with or without the addition of 50 μM iron citrate. (I) EGFR degradation assay for BafA treated cells following iron treatment. HeLa cells were cultured with 10 nM BafA for 24 hr, with or without 50 μM iron citrate (Fe(III)), and stimulated with EGF as previously described. EGFR, NCOA4, and ferritin (FTH1) levels were visualised by immunoblot. β-actin was used as a loading control. (J) RT-qPCR analysis of HIF1α and its target genes in response to BafA and iron citrate treatment (n ≥ 2). (K–M) Populations of mixed CRISPR KO cells for ATP6V1A1 (K), TMEM199 (L) and CCDC115 (M) were treated with 50 μM iron citrate for 24 hr and HIF1α levels measured by immunoblot. Values are mean±SEM. *p<0.05, **p<0.01. NS = not significant.

DOI: 10.7554/eLife.22693.012
Figure 10. Disrupting transferrin uptake leads to iron-dependent HIF1 activation. (A) The effect of iron treatment on HIF1α stabilisation in the presence or absence of BafA. HeLa cells were treated with 50 μM iron citrate (red) or 10 nM BafA (blue) for the indicated times and HIF1α levels measured by IB: HIF1α and IB: β-actin. (B) Control, PHD2, NCOA4 sgRNA3. (C) NCOA4 sgRNA: Ct, PHD2 1, 2, 3. (D) Control, IRP2 sgRNA3. (E) IRP2 sgRNA: Ct, PHD2 1, 2, 3. (F) Control, TFR sgRNA1. (G) 50 μM Fe(III) TFR sgRNA1. (H) Ct, BalA, PHD2 sgRNA, TFR sgRNA1, sgRNA2. Fe(III) - + - + - + +

Miles et al. eLife 2017;6:e22693. DOI: 10.7554/eLife.22693

Research article
Biochemistry | Cell Biology
Figure 10 continued

immunoblot. β-actin served as a loading control. (B–E) The effect of NCOA4 or IRP2 depletion on HIF1α levels and intracellular iron levels. HIF1α-GFPODD reporter HeLa cells were transduced with Cas9 and sgRNAs to NCOA4 (B, C) or IRP2 (D, E) and HIF1α levels measured by GFP accumulation (B, D) top) and immunoblot after 8 (IRP2) or 12 days (NCOA4) (C, E). Cell surface transferrin levels were measured by flow cytometry (B, D) bottom). IRP2 and ferritin (FTH1) levels were visualised by immunoblot (C, E). An sgRNA to PHD2 was used as a control. (F) HIF1 activation following depletion of the transferrin receptor. HIF1α-GFPODD reporter HeLa cells were transduced with Cas9 and sgRNAs to the transferrin receptor. HIF1 activation was measured by GFP accumulation and cell surface expression of CA9 (top). Depletion of the transferrin receptor was measured by flow cytometry (bottom) (G, H) Iron reconstitution restores HIF1α turnover in transferrin receptor deficient cells. HIF1α-GFPODD reporter HeLa cells were transduced with Cas9 and sgRNAs to the transferrin receptor as described, and 50 µM ferrous citrate added to media for 24 hr. HIF1α levels were measured by flow cytometry for GFP fluorescence (G) or immunoblot (H). IRP2, NCOA4 and FTH1 were visualised to measure intracellular iron. β-actin served as a loading control. Additional experimental examples with alternative sgRNAs to NCOA4, IRP2 or the transferrin receptor are shown in Figure 10—figure supplement 1. TFR=transferrin receptor.

DOI: 10.7554/eLife.22693.013
Figure 10—figure supplement 1. Disrupting transferrin uptake leads to iron-dependent HIF1 activation. (A) Flow cytometry of mixed populations of NCOA4 sgRNA-targeted HIF1α-GFPODD reporter HeLa cells 8 days to 12 days post transduction. Three different sgRNA were used. (B) HIF1α-GFPODD reporter.

Miles et al. eLife 2017;6:e22693. DOI: 10.7554/eLife.22693

Research article

Biochemistry | Cell Biology
Figure 10—figure supplement 1 continued

reporter and transferrin receptor levels using sgRNA2 to the transferrin receptor. (C) HIF1α-GFP<sup>ODD</sup> reporter and transferrin receptor levels using two different sgRNA to IRP2. (D) HIF1α levels following depletion of IRP2 at different time points post transduction. Mixed populations of IRP2 sgRNA-targeted HIF1α-GFP<sup>ODD</sup> reporter HeLa cells 10 or 12 days post transduction using three different sgRNA. (E) HIF1α-GFP<sup>ODD</sup> reporter levels in mixed populations of transferrin deficient cells (using sgRNA2 to the transferrin receptor) with or without iron citrate treatment. TFR=transferrin receptor.

DOI: 10.7554/eLife.22693.014