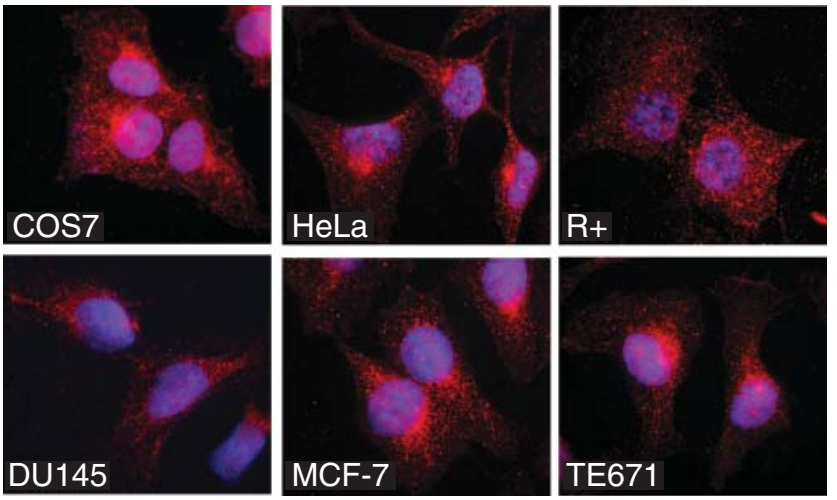
SUPPLEMENTARY FIGURES S1-S3

S1



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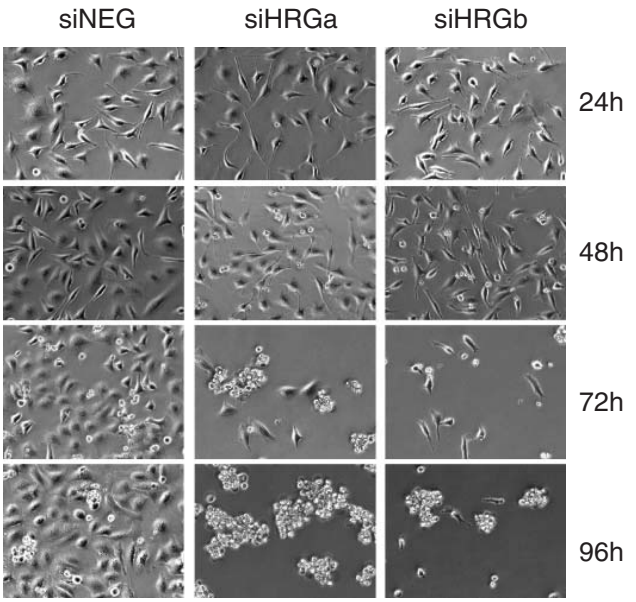

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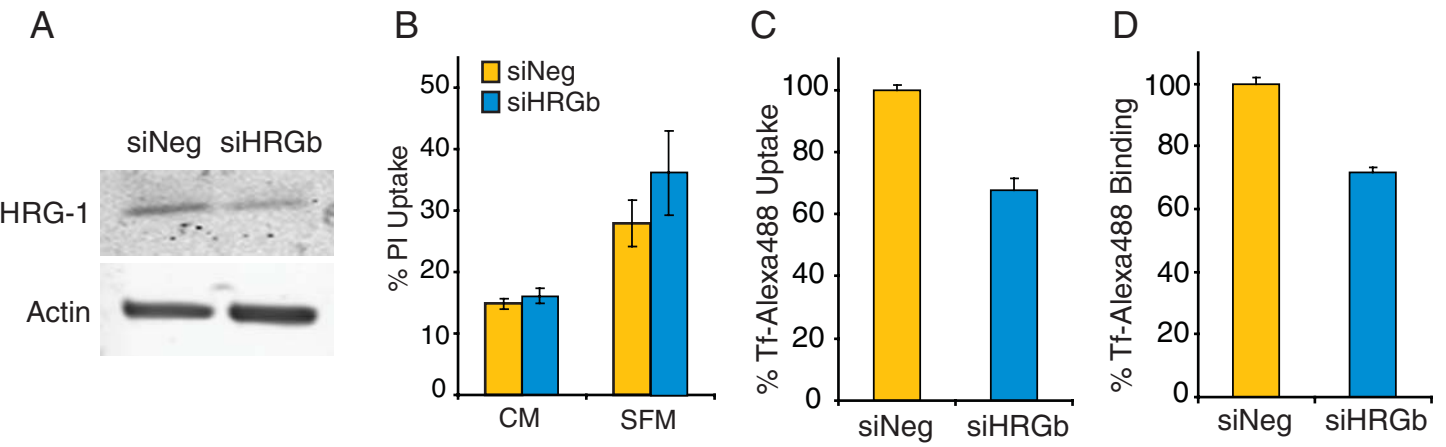
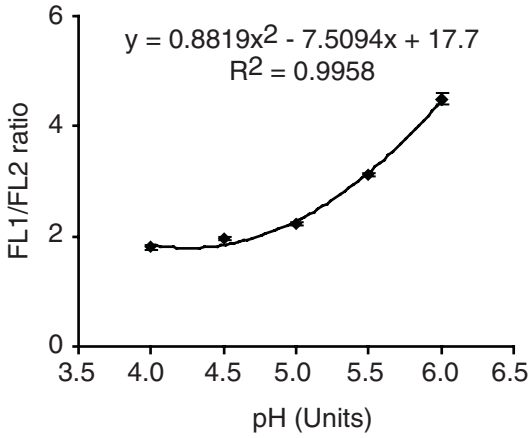


SUPPLEMENTARY FIGURES S4-S7

S4



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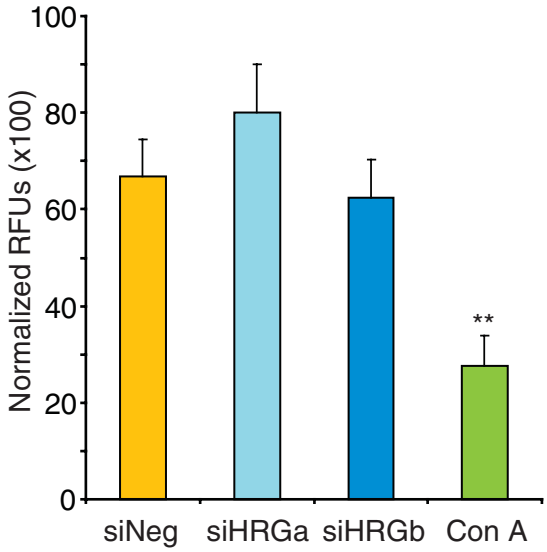

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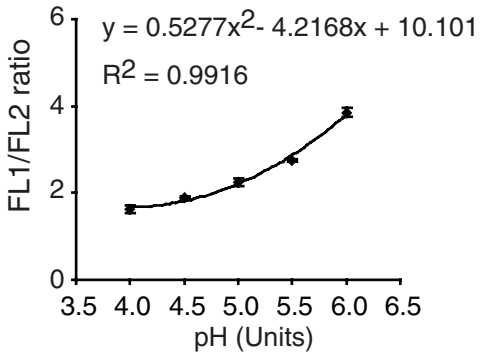

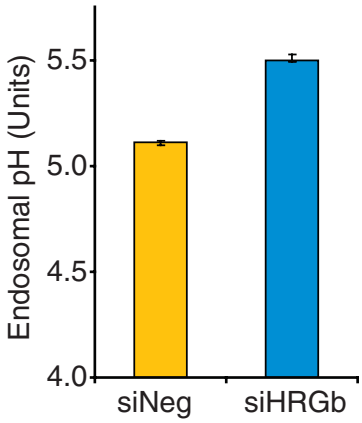

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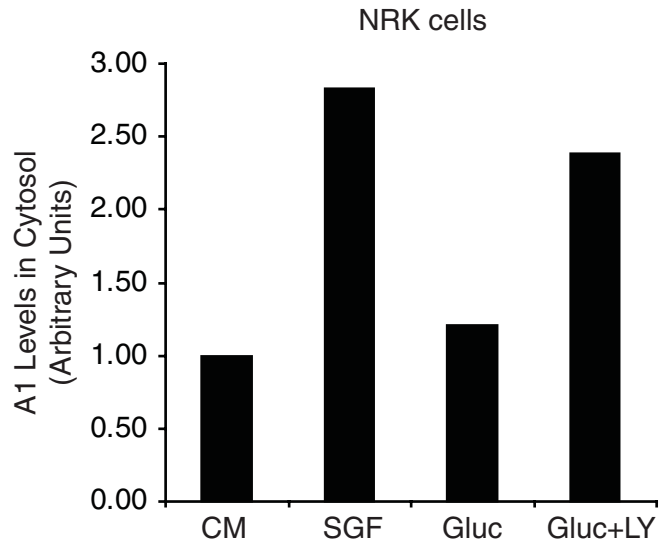

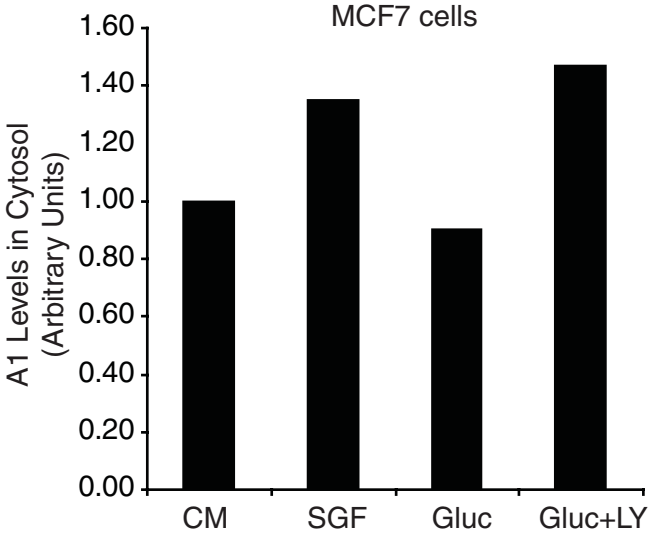

O'Callaghan et al Supplementary Figure Legends

Supplementary Figure S1

HRG-1 is expressed in cell lines of different origin. COS7, HeLa, R+, DU145, MCF7 and TE671 cells were fixed and stained for endogenous HRG-1, using Cy3-conjugated secondary antibody. Nuclei were visualised by Hoescht stain.

Supplementary Figure S2

HRG-1 suppression decreases cell viability. HeLa cells were transfected with 10nM *HRG-1*-specific siRNA or negative siRNA and the cultures were examined 24, 48, 72 and 96hrs later under 20X objective of a phase contrast microscope. A representative field for each sample is shown

Supplementary Figure S3

Effects of HRG-1 suppression in MCF-7 cells. MCF-7 cells were transfected with 10nM of either siNeg or siHRG-1b oligos to investigate the effects described for siHRG-1-transfected HeLa cells. (**A**) HRG-1 levels after siRNA transfection assessed by western blotting. (**B**) Cell viability 72 hours post-transfection assessed by Propidium Iodide uptake and analyzed by FACS (**C**) Transferrin-Alexa488 uptake. Cells were serum starved for 30 min and then incubated with 25 µg/µl Tf-Alexa488 for 15 min at 37 ºC. The amount of Tf-Alexa488 uptake was quantified by FACS and the percentage of Tf-Alexa Uptake plotted, relative to 100% set as the amount uptaken by control transfectants. Results are representative of three independent experiments with similar results. (**D**) Surface TfR levels were assessed in siRNA-transfected MCF-7 cells 24 hours post-transfection in triplicate wells. Cells were starved for 1 hour, transferred to ice and incubated with 25 µg/µl of Tf-Alexa488 diluted in cold PBS/0.1% BSA for 90 min. The cells were then washed extensively, harvested and the mean fluorescence quantified by FACS. The graph shows the percentage of surface bound Tf-Alexa488 considering siNeg levels as 100%. Results are representative of three independent experiments.

Supplementary Figure S4

pH Calibration curve HeLa cells. Endosomal pH value was estimated using FITC-dextran uptake, by measuring FITC mean fluorescence in the FL1 and FL2 channels

of the FACS, as described in Materials and Methods. The panel shows the calibration curve correlating pH values with FITC FL1/FL2 mean fluorescence ratio corresponding to the experiment shown in Fig 5D.

Supplementary Figure S5

Lysosomal pH estimation using LysoSensor Green. HeLa cells were transfected with siRNA oligos and 8 hours later replated in 96 well black plates. Next day (36 hours post-transfection) control cells were pretreated with 100nM Concanamycin A or Bafilomycin A for 1 hour, followed by incubation with complete medium containing 1 µM LysoSensor Grenn for 1 hour. Cells fluorescence was analyzed in a FlexiStation II fluorimeter (Molecular Devices). The graph shows the data as Relative Fluorescence Units (RFUs) normalized to cell numbers. ** $p<0.01$ using T-test.

Supplementary Figure S6

Endosomal pH in MCF-7 cells. MCF-7 cells with HRG-1 suppressed (Fig. S3) were assessed for endosomal pH using FITC-dextran uptake, by measuring FITC mean fluorescence in the FL1 and FL2 channels of the FACS, as described in Materials and Methods. The left panel shows the calibration curve correlating pH values with FITC FL1/FL2 mean fluorescence ratio. The right panel shows the estimated endosomal pH values for siRNA-transfected MCF-7 cells

Supplementary Figure S7

Validation of the quantification of cytosolic A1 levels as an assay to measure V-ATPase activity. Kidney NRK cells (left graph) were either held in complete media (CM), starved of serum and glucose for 16 hours (SGF), or stimulated with glucose after serum/glucose starvation in the absence or presence of 10 µM of the PI3K inhibitor LY294002 (Gluc+LY). The cells were lysed using a sucrose-based buffer and the cytosolic fractions purified as described in Materials and Methods. The amount of A1 present in the cytosol was quantified by western blot, using Actin as loading control and the A1/Actin ratio is shown in the graph. The increase in cytosolic

A1 levels after serum/glucose starvation correlates with the described disassembly of the V-ATPase in these conditions (16). Moreover, re-addition of glucose to the cells reduces cytosolic A1 levels back to CM levels and this is reverted by the presence of the PI3K inhibitor LY294002. Again, these changes in cytosolic A1 levels correlate with the previously described re-assembly of the V-ATPase after glucose stimulation, which is blocked by inhibition of the PI3K pathway (16). MCF-7 cells (right graph) were treated as described above, but the serum/glucose starvation lasted 4 hours. The cells were processed and the cytosolic A1 levels quantified as described above. The graph shows that MCF7 cells behave as NRK cells, but the changes in cytosolic A1 levels are not as prominent as in NRK. Nevertheless, the pattern of cytosolic A1 levels in MCF7 cells is the same as in NRK cells in all conditions analyzed.