A human genome-wide screen for regulators of clathrin-coated vesicle formation reveals an unexpected role for the V-ATPase

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Clathrin-mediated endocytosis is essential for a wide range of cellular functions. We used a multi-step siRNA-based screening strategy to identify regulators of the first step in clathrin-mediated endocytosis, formation of clathrin-coated vesicles (CCVs) at the plasma membrane. A primary genome-wide screen identified 334 hits that caused accumulation of CCV cargo on the cell surface. A secondary screen identified 92 hits that inhibited cargo uptake and/or altered the morphology of clathrin-coated structures. The hits include components of four functional complexes: coat proteins, V-ATPase subunits, spliceosome-associated proteins and acetyltransferase subunits. Electron microscopy revealed that V-ATPase depletion caused the cell to form aberrant non-constricted clathrin-coated structures at the plasma membrane. The V-ATPase-knockdown phenotype was rescued by addition of exogenous cholesterol, indicating that the knockdown blocks clathrin-mediated endocytosis by preventing cholesterol from recycling from endosomes back to the plasma membrane.

Clathrin-mediated endocytosis (CME) is the pathway used by cells to internalize a variety of proteins and lipids. It is essential for processes as diverse as nutrient uptake, regulation of signalling by hormones and growth factors, and recycling of synaptic vesicle membranes. Although much of the machinery responsible for CME has been identified and characterized, the mechanisms that regulate CME are much less well understood. Several recent studies have emphasized the complexity of these regulatory mechanisms1–4. For instance, an short interfering RNA (siRNA) library screen for genes involved in the endocytosis of transferrin and epidermal growth factor (EGF), both of which are taken up by CME, identified over 4,600 hits3. These genes most likely act at many different stages of the endocytic pathway, both upstream of CCV formation (for example, transcription and translation of transferrin and EGF receptors) and downstream (for example, trafficking between different types of endosomes and between endosomes and lysosomes).

Here, we set out to identify genes that specifically control the formation of CCVs at the plasma membrane (PM). We adopted a multi-step siRNA-based approach (Fig. 1), involving plate-reader-based assays to quantify the surface accumulation and internalization efficiency of model CCV cargo proteins, and a high-throughput microscopy-based assay to analyse the organization and morphology of clathrin-coated structures. Out of 92 top hits, we chose subunits of the V-ATPase for a more detailed analysis.

RESULTS

Genome-wide screening approach

Inhibition of CME leads to the accumulation of clathrin-dependent cargo proteins on the cell surface. Hence, for the primary screen, we designed an assay to identify siRNAs from a human genome-wide library that cause an increase in the surface levels of two model CCV cargo proteins: CD8-YXX8 and CD8-FXNPXY. These two constructs contain the extracellular/lumenal and transmembrane domains of a T-cell-specific protein, CD8, followed by very simple cytosolic tails containing the critical residues from either the YXX8 or the FXNPXY endocytic motif surrounded by alanines (Fig. 1). We have previously shown that both of these constructs are efficiently endocytosed in a clathrin-dependent manner5, and for the present study, they were stably transfected into HeLa cells and expressed under the control of the human cytomegalovirus promoter. For the purpose of our screen, CD8-YXXΦ and CD8-FXNPXY have two advantages over

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Received 20 July 2012; accepted 9 November 2012; published online 23 December 2012; DOI: 10.1038/ncb2652
endogenous CCV cargo proteins: their expression is not affected by receptor-specific signalling pathways, and their trafficking relies on a single clathrin-dependent motif.

**Surface accumulation of CCV cargo**

The siRNA library used for the primary screen targets 20,052 genes with SMARTpools of four siRNAs, arrayed in 267 96-well plates. Every plate also contained both positive controls (CLTC (clathrin heavy chain) and AP2M1 (AP-2 μ subunit) siRNAs) and negative controls (PLK1 siRNA, no siRNA and RISC-free siRNA; Supplementary Fig. S1). The siRNAs were reverse transfected into the CD8-YXXΦ and CD8-FXNPXY cell lines in duplicate (that is, 4 sets of plates in total). After 72 h, the cells were fixed and immunostained for surface CD8 (Alexa 488 mAb). To identify siRNAs causing non-clathrin-mediated changes in cell surface proteins, the cells were also stained for the major histocompatibility complex (MHC) class I subunit, β2m (Alexa 477). Hoechst stain was used as an indicator of cell number (Fig. 1a).

The fluorescence data for 52 spots across each well were collected in three channels using a plate reader (Fig. 2a). Figure 2b shows a plot of the Alexa 488 and Hoechst readings from a sample plate on which there was a strong hit, DNM2 (dynamin 2). Plots of all the raw data can be found at http://www.bioinformatics.cimr.cam.ac.uk/siRNA/Robinson_Screen. After excluding any non-viable knockdowns from further analysis, the ratio of Alexa 488 to Hoechst fluorescence intensity was used as a measure of CD8 accumulation on the PM. To control for plate-to-plate variability in staining and knockdown efficiency, the Alexa 488/Hoechst ratios from each plate were normalized to the positive (CLTC) and negative (no knockdown) controls (Supplementary Figs S2 and S3). There was a high correlation between the two technical repeats ($R^2 = 0.94$ for YXXΦ, 0.84 for FXNPXY) as well biological repeats ($R^2 = 0.65$ for YXXΦ, 0.89 for FXNPXY; Supplementary Fig. S4a,b).

We used strictly standardized mean difference (SSMD) to combine the duplicate measurements and rank SMARTpools according to both strength and reproducibility of phenotype (Fig. 2c and Supplementary Fig. S4c). An SSMD of greater than three was used as the threshold for hit selection. All of the hits for the CD8-YXXΦ cells had at least 2.85-fold more surface CD8 than the negative control, and all of the hits for the CD8-FXNPXY cells had at least 2.39-fold more surface CD8. For both cell lines, CLTC and DNM2 were among the top hits. AP2M1 was a strong hit only for the CD8-YXXΦ cells. DAB2, one of two main FXNPXY adaptors, was a weak hit for the...
CD8-FXNPXY cells but below the selection threshold, consistent with reports that both DAB2 and ARH have to be knocked down simultaneously to prevent internalization of FXNPXY-containing proteins. As expected, several SMARTpools caused an increase of both CD8-YXXΦ and CD8-FXNPXY ($R^2 = 0.47$). However, there was no correlation between the CD8 constructs and MHC-I ($R^2 = 0.01$; Fig. 2d). Supplementary Table S1 lists the 719 SMARTpools with SSMDs $>3$ for either or both of the motifs.

Selection of hits for secondary assays

To eliminate some of the potential false positives, we applied three exclusion criteria to the primary hit list (Fig. 2e). First, the siRNA targets were re-annotated according to version 25 of the RefSeq database, and where multiple or no target messenger RNAs were identified, a SMARTpool was excluded from the hit list. Second, we performed microarray analyses and excluded hits for which the target mRNA was represented on the microarray but not detected. Finally, we excluded hits where the increase of CD8 on the cell surface was accompanied by an increase in MHC-I, as these genes are unlikely to control CME. Out of the 334 hits remaining, the 241 top hits (Supplementary Table S1a) were selected for secondary screens, in which we used a set of different siRNA pools, chemically modified to reduce the off-target activity and designed with improved sequence selection algorithms (ONTARGETplus, OTP; refs 9,10).

Efficiency of CME

To confirm that knockdowns causing an increase in surface levels of CD8 specifically affect endocytosis, we determined the internalization efficiency (0 corresponds to inhibition in CLTC knockdown cells, and 1 to uptake efficiency in negative (no knockdown) controls; Fig. 3b). 28 SMARTpools against CLTC, AP2M1, DNM2 and DAB2 present in the library are colour-coded. (d) Correlation between the phenotypes for the different types of membrane protein: CD8-YXXΦ, CD8-FXNPXY and endogenous MHC-I. (e) Number and/or percentage of SMARTpools that fulfilled the different hit selection criteria for both YXXΦ- and FXNPXY-cargo (△) or for only one of the cargos (Y: YXXΦ, F: FXNPXY). SMARTpools with SSMD $>3$ were selected as primary-screen positives and analysed for sequence specificity according to RefSeq 25, mRNA expression (Supplementary Table S3) and phenotype specificity (increase on the cell surface specific for CCV-cargo alone, not accompanied by an increase of MHC-I), NA, not available.

Figure 2 Genome-wide primary screen for surface accumulation of CCV cargo. (a) Cells transfected with the siRNAs were fixed after 72 h and stained for surface CD8 (Alexa488), surface MHC class I (Alexa647) and DNA (Hoechst). Using a plate reader, 52 points were scanned in each of the three fluorescence channels, here shown on a sample plate for Alexa488. The fluorescence intensity of each point is colour-coded according to the scale presented (RFU, relative fluorescence units), and the corresponding plate map is presented in Supplementary Fig. S1. (b) Alexa488 (surface CD8-YXXΦ) and Hoechst readouts from one of the screening plates. Each point represents one well (one SMARTpool). Control wells are colour-coded according to the legend and grey points represent the library wells. Similar plots for each tested SMARTpool can be found at http://www.bioinformatics.cimr.cam.ac.uk/siRNA/Robinson_Screen. To analyse the data further, we calculated the ratio of Alexa488/Hoechst fluorescence intensity for each point and then normalized the ratios to the positive (CLTC) and negative (RISC-free, mock) controls (see Supplementary Fig. S4). The red line indicates the threshold for hit selection (SSMD $>3$) and the blue line the threshold for cell viability. SMARTpools against CLTC, AP2M1, DNM2 and DAB2 present in the library are colour-coded. (d) Correlation between the phenotypes for the different types of membrane protein: CD8-YXXΦ, CD8-FXNPXY and endogenous MHC-I. (e) Number and/or percentage of SMARTpools that fulfilled the different hit selection criteria for both YXXΦ- and FXNPXY-cargo (△) or for only one of the cargos (Y: YXXΦ, F: FXNPXY). SMARTpools with SSMD $>3$ were selected as primary-screen positives and analysed for sequence specificity according to RefSeq 25, mRNA expression (Supplementary Table S3) and phenotype specificity (increase on the cell surface specific for CCV-cargo alone, not accompanied by an increase of MHC-I), NA, not available.
SMART pools inhibited internalization of YXXΦ, 38 of FXNPXY, and 20 of these were shared between the two cargo proteins (Fig. 3b).

Morphology of clathrin-coated structures at the PM
To identify hits that affect the formation of endocytic CCVs, we designed an automated microscopy-based assay to characterize clathrin-coated structures (CCs) at the PM. We labelled the CCs with an antibody against CALM, an accessory protein that co-localizes with clathrin at the PM, but not on intracellular membranes (Figs 1c, 4). The prerequisite for quantitative high-throughput microscopy is obtaining good quality images, and in the case of PM CCs, the optimal focal plane is where cells adhere to the growth surface. Thus, as a reference, we used hydrophilic Cy3-labelled flow cytometry beads, which settle between the cells at the bottom of the dish. Whole-cell stain (WCS) was used to demark cell boundaries, and a SpotDetector BioApplication was used to identify CCs in each cell (Fig. 4a).

We screened the 241 selected siRNA pools by reverse transfecting cells in duplicate as before, then splitting the plates into four groups (Supplementary Table S2). Mitotic cells and cells from dense fields were excluded from the analysis (Fig. 4b, c). We focused on changes in total fluorescence intensity per spot and spot count per area (Fig. 4d–f) and selected 67 hits falling into three categories: increase in spot brightness (red), decrease in spot brightness (blue), and decrease in spot count without a change in brightness (yellow).

Functional networks within genes that affect CCS morphology and CME efficiency
The 92 hits combined from the two secondary assays (22 of which were shared hits) were analysed using the STRING database13. Four groups of functionally or genetically linked genes were identified (Fig. 5). CLTC, DNM2 and AP2M1 were correctly assigned to one network. The remaining three networks comprise: subunits of the vacuolar ATPase (V-ATPase; ATP6V0C, ATP6V0E, ATP6V1B2, ATP6V1D, ATP6V1F); genes involved in mRNA splicing (CDSCL, CDC40, SFRS3, SF3B3, SF3B4, SFPQ, SFRS3); and the histone acetyltransferase complex (RUVBL2, EP400, HTATIP (KAT5)). We decided to focus on V-ATPase for the present study.

Ultrastructure of CCs following depletion or inhibition of V-ATPase
Although V-ATPase plays an important role in endocytic trafficking by controlling the intraluminal pH of endosomes and lysosomes, a role in the formation of CCVs at the PM was unexpected. siRNAs against eight components of V-ATPase were present among the primary hits, and using the OTP pools, we verified that the knockdowns also inhibit internalization of an endogenous ligand, transferrin (Fig. 6a). To investigate the ultrastructure of the brighter CCs observed in V-ATPase-depleted cells (Fig. 6b), we turned to electron microscopy. Strikingly, knocking down V-ATPase caused the cells to accumulate large and irregular CCPs with average neck diameters of 220 nm (compared with 97 nm in control cells), whereas constricted pits were notably absent (Fig. 6c, d, e).
The inability of pits to constrict and the block in CME after V-ATPase depletion were surprising, as acute inhibition of the V-ATPase with bafilomycin A1 (BafA1) does not block transferrin internalization, but instead inhibits its recycling14 (Fig. 6f). However, when we incubated the cells with BafA1 for 24 h, transferrin internalization was completely abolished (Fig. 6f). This decrease in the efficiency of internalization
occurred progressively over the 24 h period, and resulted in a gradual accumulation of the transferrin receptor at the cell surface (Fig. 6g).

In a similar time-course experiment, we observed a corresponding increase in the brightness of CALM-labelled CCSs (Fig. 6h). Electron microscopy analysis revealed that at 17 h, BafA1-treated cells also accumulate wide, non-constricted CCSs (Fig. 6i–k). Together, these data suggest that siRNA-mediated depletion of different V-ATPase subunits, or inhibition of V-ATPase activity with BafA1, blocks CME by preventing CCPs from forming CCVs.

**V-ATPase inhibition and cytosolic acidification**

As V-ATPase is a proton pump and clathrin polymerization has been shown to be sensitive to pH (ref. 16), we suspected initially that V-ATPase depletion or BafA1 treatment might prevent CCV formation by decreasing the pH of the cytosol. Previous studies on unroofed cells have shown that cytosol acidification leads to an accumulation of arrested CCSs surrounded by membraneless microcages. Using a similar technique, we generated PM replicas from control and BafA1-treated cells to examine their three-dimensional architecture. The micrographs confirmed the abundance of enlarged CCSs and revealed an astounding variety in their morphologies (Fig. 7a,b).

Two other treatments that lead to a block in endocytosis coupled with microcage formation, hypertonic sucrose and potassium depletion, simultaneously block clathrin exchange. Therefore, we performed fluorescence recovery after photobleaching (FRAP) experiments on cells transfected with GFP-tagged clathrin light chain, cultured in the presence or absence of BafA1 (Fig. 7c,d). BafA1 treatment did not significantly affect the recovery kinetics, again arguing that clathrin is unlikely to be locked in microcages.

Finally, we measured the cytosolic and submembranous pH using soluble and PM-targeted SuperEcliptic (SE) pHluorin–mCherry fusion proteins (Fig. 7e). As the SEpHluorin fluorescence intensity decreases on protonation, the ratio of green to red signal can be used to estimate pH, as demonstrated by incubating the cells in buffers of different pH in the presence of an ionophore, nigericin (Fig. 7f,g). Treatment with BafA1 for 24 h resulted in only a modest pH decrease. As pH 6.5–6.8 has been consistently reported as the threshold at which CME is inhibited, we concluded that changes to cytosolic pH and microcage formation are unlikely to explain CME inhibition in the V-ATPase-depleted cells.

**CCV formation in cells lacking functional V-ATPase can be rescued with soluble cholesterol**

If the inhibition of CCV formation after V-ATPase inhibition cannot be attributed to a decrease in cytosolic pH, another possibility is that it could be a consequence of the alkalization of endosomes. It has been known for over 30 years that if endosomes cannot acidify, trafficking out of them is inhibited, and this is thought to be due to a general block in the formation of carriers. If an essential CCV membrane component were unable to recycle back to the PM, this would help to explain the BafA1 kinetics, because it would take time for this component to be depleted. A candidate for the missing component emerged from the observation that the morphology of the CCSs in V-ATPase-depleted cells is strikingly similar to the enlarged, shallow clathrin-coated invaginations that form after extraction of cholesterol from the PM with methyl-β-cyclodextrin. Similarly to the transferrin receptor, cholesterol recycles from endosomes to the PM (ref. 25), and consequently, in the presence of BafA1 it accumulates in endosomes and is depleted from the cell surface. We found that if we either knocked down V-ATPase or treated cells with BafA1 for 24 h, cholesterol (labelled with filipin) was lost from the PM and accumulated in an intracellular compartment, and we were able to quantify this change in three different cell lines using an automated microscopy-based assay (Fig. 8a,b). Double labelling indicates that the filamentous-positive compartment also contains trapped CCV cargo proteins (Supplementary Fig. S6). To investigate whether the loss of PM cholesterol could explain the block in endocytosis, we added exogenous cholesterol to the cells together with BafA1 or siRNAs targeting V-ATPase (Supplementary Fig. S7). We found that transferrin internalization was partially rescued (Fig. 8c,d), and there was also a rescue of CCS ultrastructure in PM replicas (Fig. 8e,f) and a corresponding decrease in CCS brightness (Fig. 8g).

**DISCUSSION**

To identify regulators of CCV formation, we carried out a multi-step siRNA-based screen. In the primary screen, hits were defined as siRNA pools that caused CCV cargo proteins with YXXΦ or FXNPXY motifs to accumulate on the PM. Using further selection criteria based on gene expression and siRNA pool specificity, we narrowed down the
**Figure 6** siRNA-mediated depletion of V-ATPase or prolonged inhibition with BafA1 leads to accumulation of large, non-constricted CCSs at the PM. (a) Efficiency of transferrin internalization after siRNA-mediated depletion of V-ATPase subunits. The cells were incubated with Alexa488-transferrin for 7 min, fixed and surface transferrin receptor was stained using a secondary Alexa647 antibody. Alexa488/Alexa647 ratios from three independent experiments normalized to the NTP control are plotted. (b) CALM immunofluorescence staining in ATP6V0C or ATP6V1B2-knockdown (kd) cells. (c,d) Electron microscopy analysis of control and V-ATPase-knockdown cells (combined ATP6V0C and ATP6V1B2 SMARTpools). (c) Representative micrographs of CCSs at the PM. The arrows indicate examples of CCPs connected to the PM within the section; the wedges indicate structures within 250 nm of the PM, likely to be PM-connected in a different section. (d) Size distribution of sectioned CCSs within 250 nm from the PM (marked with wedges in c). The lengths of the major and minor axis were measured. Blue lines indicate means of CCSs in control cells. (e) Size distribution of CCPs connected to the PM within the section (marked with arrows in c). The pit depth and neck width were measured. Grey lines indicate mean neck width. (f) Kinetics of transferrin (Tf) internalization in 24 h or 10 min BafA1-treated cells. The fraction of internalized [125I]transferrin is plotted for each time point. n = 3 independent experiments. (g) Efficiency of transferrin (Tf) internalization (left) and cell surface accumulation of transferrin receptor (TfR; right) after pre-treatment with BafA1 for 4, 10, 16, and 24 h. The assay was performed as described in a. n = 3 independent experiments. (h) Fold changes in the total spot intensity of CALM-labelled CCPs in a time-course experiment with BafA1. n = 3 independent experiments. (i) Electron microscopy analysis of the CCSs in cells treated with BafA1 for 24 h. (j) Size distribution of sectioned CCSs within 250 nm from the PM in BafA1-treated cells, analysed as described for d. (k) Size distribution of PM-connected CCPs in BafA1-treated cells, analysed as described for e. All error bars, ±s.e.m.
Figure 7 Inhibition of V-ATPase does not lock clathrin in microcages. (a,b) PM replicas from control and BafA1-treated cells. The cells were unroofed by sonication, fixed, critical-point dried and rotary shadowed. (c,d) Clathrin dynamics at the PM were monitored in cells treated with BafA1 or DMSO by FRAP analysis of clathrin-light-chain-GFP. (c) Images of the FRAP region (white rectangle) before and after photobleaching. (d) Corrected fluorescence recovery of the bleached region over time. Data from n = 3 independent experiments, with 45 and 34 cells analysed for DMSO and BafA1, respectively. Error bars, ± s.e.m. (e-g) pH measurements in intact cells. (e) Localization of the soluble and membrane-bound SEpHluorin-mCherry pH constructs (mCherry channel). (f) Cells transfected with the pH constructs were treated with BafA1 or DMSO for 24 h, collected in 20% FCS/PBS and analysed by flow cytometry. For reference, untreated cells were collected in K+/nigericin buffers of predetermined pH. The histograms represent the SEpHluorin/mCherry fluorescence intensity ratios. (g) Medians of SEpHluorin/mCherry ratios normalized to the median of untreated cells in each experiment. **P ≤ 0.001, *P ≤ 0.05.

list to 334 hits. Overall, the hit selection criteria applied are highly stringent and therefore may produce false negatives. However, this strategy allowed us to identify a small number of high-confidence hits, which we characterized in more detail.

To identify genes that control endocytosis at the step of CCV formation, we performed two secondary screens. First, we carried out an uptake assay, which allowed us to distinguish knockdowns that decrease the efficiency of CME from those that increase surface levels of CD8 for other reasons (for example, inefficient lysosomal degradation). Second, we carried out a microscopy-based assay, which allowed us to identify knockdowns that alter the morphology of CCs at the PM. As the intensity of CALM fluorescence peaks during vesicle scission28, an increase in spot brightness could correspond to an accumulation of deeply invaginated pits29 (for example, in...
**Figure 8** Soluble cholesterol rescues function and morphology of the PM CCPs in the V-ATPase-depleted cells. (a) Filipin staining in ATP6V0C-knockdown (CD8⁺) and NTP control (CD8⁻) cells. (b) Quantification of cholesterol depletion. Cholesterol was stained after a 24-h BafA1 treatment or 72 h after V-ATPase depletion. WCS was used to identify cell boundaries (green lines, top images). Top images are at two focal planes: at the cell base (offset −4 μm) to quantify fluorescence intensity at the periphery (PM pool, red mask within the region enclosed the by purple lines) and in the middle (offset −1 μm) to quantify intracellular fluorescence intensity (blue spots within the perinuclear region, yellow line). (See Supplementary Fig. S5.) Bottom left, the scatter plot compares intracellular and PM lipin staining intensity in control (blue) and BafA1-treated (red) wells (each dot represents one cell). Bottom right, the bar graph shows the levels of PM cholesterol for n = 3 independent experiments on BafA1-treated HeLa, RPE, and A431 cells, and after V-ATPase depletion (HeLa cells). BafA1 samples are normalized to DMSO, and V-ATPase knockdowns to NTP controls. Where indicated, the growth medium contained 20 μg/ml soluble cholesterol. (c) Distribution of Alexa488-labelled transferrin after a 7-min incubation at 37°C in control cells, BafA1-treated cells and BafA1-treated cells in the presence of 20 μg/ml soluble cholesterol. (d) Kinetics of ¹²⁵I-transferrin uptake in control cells, BafA1-treated cells and BafA1-treated cells in the presence of 20 μg/ml soluble cholesterol (Fig. 6e). The graph represents the fraction of internalized transferrin at different times. n = 3 independent experiments. (e,f) PM replicas of BafA1-treated cells, grown in the presence of 12.5 μg/ml soluble cholesterol for 24 h (Fig. 7a and Supplementary Fig. S8). (g) Total intensity of CALM-labelled spots (assay described in Fig. 4). BafA1-treated samples were normalized to DMSO, and V-ATPase knockdowns to NTP controls. Where indicated, the growth medium contained 20 μg/ml cholesterol. n = 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (h) Model of cholesterol distribution and function in the presence/absence of a functional V-ATPase. Cholesterol flip-flop between leaflets of the bilayer reduces membrane tension generated in high-curvature regions, such as CCP necks. Cholesterol is then recycled in vesicles from acidified endosomes back to the PM. After V-ATPase depletion or inhibition, endosomal pH increases, and cholesterol is retained. This causes PM cholesterol depletion and increases the energy needed to constrict CCP necks. All error bars, ±s.e.m.

From the final set of 92 hits, only CLTC, AP2M1 and DNM2 have been detected in CCVs by proteomics. This is, however, not surprising considering that individual knockdowns of accessory CCV components...
(for example, CALM (ref. 33) or epsins45) rarely lead to endocytic arrest. Thus, the siRNA-based approach seems to be biased towards proteins not physically incorporated into CCVs. Other proteins implicated in CCV formation, such as FCHo1/2 and Rab5A/B/C, were also not picked up as strong hits, possibly because they are encoded by functionally redundant genes (this functional redundancy also helps to explain why only one of the four AP-2 subunits was present in the list).

Although the purpose of this screen was to identify regulators of clathrin-mediated trafficking, the data we have generated can be mined to look for genes involved in other pathways as well. For instance, knockdowns that decrease surface expression of a particular protein may affect its secretion and/or recycling. In addition, for each knockdown, the MHC-I results are also available and will be an interesting data set to explore, especially because post-Golgi trafficking pathways of MHC-I are poorly characterized and 30% of MHC-I hits correspond to unknown proteins. All of our data can be accessed at http://www.bioinformatics.cimr.cam.ac.uk/siRNA/Robinson_Screen.

Out of our 92 hits, several are uncharacterized proteins, such as those encoded by HXL89 and LOC124220, which we have now validated with two or more distinct siRNAs, and which we intend to characterize more fully. Other hits have been at least partially characterized, and this enabled us to group them into three functional networks (plus the network containing coat components). One network consists of several components of the mammalian spliceosome. Interestingly, a number of CCV proteins exist in multiple splice variants, including the α and β subunits of AP-2 (ref. 35), clathrin light chains46 and dynamin 2 (refs 37,38). The spliceosome is also essential for cell-cycle progression39, and as endocytosis is arrested in mitotic cells40, further experiments will be required to resolve whether the endocytic block in spliceosome-depleted cells is due to incorrect splicing of coat proteins, or is a consequence of the mitotic arrest.

The second unexpected functional cluster contains subunits of the histone acetyltransferase complex. Histone acetylation plays an important role in the regulation of gene expression; however, three lines of evidence suggest that the block in CME may be due to changes in the acetylation of cytoplasmic proteins rather than histones. First, acetylation sites have been identified in three core components of CCVs: clathrin, AP-2 and dynamin 2 (ref. 41). Second, both RUVBL2 and KAT5 can translocate to the cytoplasm under certain conditions42,43. Third, in a hepatic cell line, WIF-B, a 30 min treatment with trichostatin A, a deacetylase inhibitor, was found to arrest deeply invaginated pits before fission45, and the short timescale argues against this effect being due to transcriptional regulation. Finding the key target(s) of the acetyltransferase complex will be critical for deciphering the role of acetylation in CME.

The third unexpected network, which we analysed in more detail, contains several V-ATPase subunits. We discovered that the block in CCV formation in V-ATPase-depleted cells can be explained by the retention of cholesterol in non-acidified endosomes, and its concomitant loss from the PM. At first glance, this phenotype is reminiscent of Niemann–Pick type C (NPC) disease, where mutations in pH-sensitive NPC1 or NPC2 lead to accumulation of cholesterol in endosomes and lysosomes46,47. However, in NPC cells, cholesterol is still exchanged with the PM (refs 48,49) and endocytosis still occurs50. In contrast, when endosomes are unable to acidify, trafficking out of them becomes inhibited. Although this phenomenon was first observed in the 1980s (ref. 21), it still awaits a molecular explanation, but endosome acidification is clearly important for the recycling of lipids as well as proteins.

Similarly, the precise function of cholesterol in CCV formation is not entirely clear. However, as transbilayer diffusion of cholesterol is fast (<1 s; refs 51,52) relative to the half-time of CCV formation (~100 s; ref. 28), it is possible that the spontaneous flip-flop of cholesterol could simply lower the membrane-bending energy. Thus, although the coat is still able to curve the membrane into a CCP in the absence of cholesterol, the energy required for the neck to constrict may be too great52 (Fig. 8h). In yeast, budding of CCVs from intracellular membranes requires a lipid flipase, Drs2p, consistent with the notion that the distribution of lipids between the two leaflets of the bilayer is important for membrane deformation53.

The role of V-ATPase in CME is particularly relevant in light of recent therapeutic strategies against cancer that target V-ATPase activity54. Interestingly, one such study demonstrated that BafA1-induced inhibition of cell growth can be rescued with cholesterol or iron55. The authors assumed that receptor-mediated endocytosis was still occurring normally in the BafA1-treated cells, but that iron and cholesterol were unable to be released in endosomes and lysosomes. However, the present study demonstrates that long-term BafA1 treatment inhibits CME, and this might account, at least in part, for the cytostatic effects of the V-ATPase inhibitors. V-ATPase activity has also been implicated in signalling by both Notch56,57 and Wnt (refs 58,59). Although again it was assumed that the molecules were still getting internalized, and that the phenotype was caused by pH-dependent events in the endosome, it is important to note that the Notch and Wnt pathways both require clathrin and dynamin60-63. Thus, the connection we have uncovered between endosome acidification, cholesterol trafficking and CCV formation may help to explain a number of observations on the role of V-ATPase in growth control and development.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

We would like to thank M. Boutros (DKFZ) for providing the siRNA reannotation data, S. Arden (CIMR) for mRNA isolation, J. Bauer (Centre for Microarray Resources, Department of Pathology, Cambridge) for analysis of the microarray data, S. Grinstein (University of Toronto) for the pH sensor plasmids and helpful suggestions, J. Skepper (Multi-Imaging Centre, Department of Anatomy, University of Cambridge) for help with the critical-point drying, all members of the Robinson laboratory, in particular G. Borner, for invaluable discussions, and P. Luzio and J. Kilmartin for reading the manuscript and helpful suggestions. This work was financially supported by the Wellcome Trust.

AUTHOR CONTRIBUTIONS

P.K. and M.S.R. designed the research; P.K. and C.S. carried out the genome-wide screen; P.K. and N.A.H. carried out the secondary screens; D.A.S. performed the thin-section analysis; D.A.S. and I.M.C. performed the unroofing; P.K. and N.A.H. performed the V-ATPase experiments; P.K., N.A.H., D.A.S., N.S. and J.W. analysed data; P.K. and N.S. designed the web page; P.K. and M.S.R. supervised the project; and P.K. and M.S.R. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/doifinder/10.1038/ncb2652

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METHODS

Antibodies, plasmids and other reagents. The following antibodies were used in the primary screen: mouse anti-CD8 (1:500, ATCC number: CRL-8014, LGC Promocochrom), rabbit anti-f-actin (A0072, Dako Cytomation, 1:500), Alexamarlabeled donkey anti-mouse IgG (A21202, 1:500) and Alexamarlabeled goat anti-rabbit IgG (A11024, 1:500, Invitrogen); in the CCS assay: goat anti-anti-CALM C-18 (sc-6433, Santa Cruz Biotechnology, 1:200), Alexa648-labeled donkey-anti-goat IgG (A11055, Invitrogen, 1:400); in the CD8 uptake assays: Alexa488-labeled anti-CD8 (MCA1226A488, Serotec, 1:50), anti-Alexa488 (A11094, Invitrogen, 1:47), anti-mouse IgG Alexa488 (A35711, Invitrogen, 1:400); in the transferrin uptake assays: anti-transferrin receptor (555534, BD Pharmingen, 1:200).

Bacterial transferrin was purchased from PerkinElmer-Cetus. Alexa488-labeled transferrin (Invitrogen) was made up according to the manufacturer’s instructions and used at 20 ng ml−1. Hoechst 33342 (Invitrogen) was used to add to the cells at a final concentration of 50 nM for 5 min before live-cell imaging.

BafA1 (Sigma-Aldrich) was dissolved in dimethylsulphoxide (DMSO) and used at a final concentration of 100 nM; a corresponding volume of DMSO (1:1,000, Sigma) was added as a control. Water-soluble cholesterol (40 mg g−1 material, balanced with MjCD, Sigma-Aldrich) was dissolved in H2O and added to the cells at the concentrations indicated in the text.

Filipin complex (F9765, Sigma) was dissolved in DMSO on the day and used at a concentration of 1 mg ml−1 (Sigma-Aldrich). The SepHLSuM–mCherry plasmids (cytosolic and PM-targeted) were a gift from S. Grinstein (University of Toronto, Canada). The CD63–EGFP C2 plasmid was a gift from P. Luzio (CIMR, UK). The GFP–clathrin light chain A plasmid was a gift from S. Boyle (University of Liverpool, UK). The pHLSuM–transferrin receptor plasmid was a gift from C. Merrifield (LEBS, France).

siRNAs. The human genome-wide siRNA library and other siRNAs were purchased from Dharmacon (Thermo-Fisher Scientific). The library contains 21,121 siGENOME SMARTpools (four siRNAs per gene) arrayed in 96-well plates; 20,052 SMARTpools were unique, 65 were duplicated within the library, and 2 were present in 3 different positions (these SMARTpools were used to estimate biological reproducibility). Two hundred and forty-one ONTARGETplus SMARTpools were used for secondary screening (Supplementary Table S2), siGENOME SMARTpools against CLTC (M-004000-01), AP2M1 (M-008170-00) and PLK1 (M-003290-01), chemically modified siRNA, impaired in uptake and processing by RISC (RISC-free, D-001220-01), and a non-targeting pool (NTP, D-001810-10) were used as controls.

Cell culture and DNA transfections. The HeLa-M cells9 were used throughout the study were grown in Dulbecco’s modified Eagle’s medium (D6465, DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin (Sigma-Aldrich). The HeLa-M cells expressing CD8-XX-Y or CD8-FXNPXX constructs were previously described10. Retinal pigmented epithelium (RPE) cells were grown in Nutrient Mixture F-12 Ham mixed 1:1 with DMEM containing 2 mM L-glutamine, and supplemented with 10% FBS, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin (Sigma-Aldrich). The A204 cells were grown in DMEM Glutamax-I (Gibco) with 10% FBS, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin (Sigma-Aldrich). All cells were grown at 37 °C with 5% CO2.

For DNA transfections, the cells were grown to 50% confluency and transfected for 24 h using a TransIT-HeLaMONSTER Transfection Kit (Mirus) with 2 µg plasmid DNA per well of a 6-well plate.

mRNA analysis. RNA was isolated from 106 CD8-XX-Y or CD8-FXNPXX cells collected (by trypsinization) using the Purescript RNA Purification Kit (Life Sciences Germany). Samples were processed and analysed at the Centre for Microarray Technologies (Department of Pathology, University of Cambridge). The analysis was performed using an Illumina HumanRef-8.v2 BeadChip array scanned on Illumina BeadStation 500 platform. The list of expressed genes was generated using low-stringency criteria to minimize the number of false positives. Variance-stabilizing transform and robust spline normalization algorithms11 from the lumi R package12 were used to process the signal intensity data. Using a F-value threshold of <0.01, 12,269 probes with a signal intensity above the background in at least one of the technical replicates of either of the cell line were selected (listed in Supplementary Table S3). MIAME-compatible data were submitted to GEO (accession: GSE39954).

siRNA reverse-transfection protocol. A 96-well plate with 10 µl of 1 µM pre-aliquoted siRNAs was used to generate 4 assay plates (Viewplates, Cat. No. 6005182, PerkinElmer; see Supplementary Fig. S1). First, 72 µl of Opti-MEM 1 (Invitrogen) was added to each well of the transfection plate. Oligofectamine (Invitrogen) was pre-incubated with Opti-MEM in a 1:8 ratio for 8 min, and 18 µl of the mix was added to each well. The transfection mix was incubated for 20 min at room temperature, and aliquoted into assay plates (20 µl per well). Next, 4,500 cells in 100 µl of DMEM (with penicillin/streptomycin and L-glutamine) were added to each well, and the plates were gently vortexed, and then incubated at 37 °C in 5% CO2 for 72 h. For knockdowns in 6-well plates, the volumes per well were scaled up 20x.

Genome-wide and secondary screens. Detailed protocols, methods for data analysis and hit selection strategy are provided in Supplementary Note.

Cholesterol redistribution assay. The cells were seeded in the 96-well plates 24 h before the analysis and fixed in 3.3% formaldehyde in PBS for 30 min at room temperature. The cells were then washed in PBS and stained with filipin (1:1,000 in PBS) and WCS green for 2 h. Cy5 beads (see CCS assay) in PBS were added before imaging. The images were acquired on the Arrayscan platform using beads as the focusing reference. Filipin was imaged at two focal plates: offset: −4 µm and −1 µm, and the WCS was imaged without an offset. The images were then analysed using a SpotDetector Bioapplication. PM cholesterol was quantified using the image with −4 µm offset where the fluorescence intensity was quantified within the peripheral circular region. The total fluorescence intensity of the spots per object within the ring region was used to estimate cholesterol loss from the PM. In the scatter plot in Fig. 8b, the amount of intracellular cholesterol was estimated using the average intensity of the spots identified within the circular region. For each condition, 1,000–9,000 cells were imaged in total in at least three independent experiments.

Identification of functional clusters. The STRING database was used to identify known and predicted protein–protein interactions between the hits (v8.3; ref. 13). Only interactions with scores above 0.7 are reported here.

Alexa488-labeled transferrin uptake assay. siRNA-transfected or BafA1-treated cells were incubated in serum-free DMEM for 45 min before a 7 min pulse with Alexa488-labeled transferrin at 37 °C. The cells were then washed and fixed (3.3% formaldehyde, 15 min) at 4 °C, surface transferrin receptor was labelled using an Alexa47 secondary antibody, and the DNA was stained with Hoechst. Fluorescence data were collected using the plate reader as described above. The Alexa488/Alexa47 and Alexa47/Hoechst ratios were used as a measure of endocytosis efficiency and transferrin receptor accumulation at the cell surface respectively. For the knockdown experiments, the ratios were normalized per experimental repeat by dividing by the mean value of the RISC-free control wells. For time-course experiments, triplicates within an experiment were averaged (Alexa488/Hoechst and Alexa47/Hoechst ratios calculated for each well), and for each time point, the values from the BafA1-treated wells were normalized to the mean of the wells treated with DMSO for the same length of time. The Alexa488/Alexa47 ratios were then calculated by dividing the normalized values.

125I-labeled transferrin uptake assay. The 125I-transferrin uptake assay has been previously described14. Briefly, control and treated cells were pre-incubated on ice with 125I-labeled transferrin in serum-free medium for 30 min at 4 °C, followed by incubations at 37 °C for various times. The medium was collected, surface-bound transferrin was stripped with a low-pH wash, and the cells were extracted with 1 M NaOH. Counts were quantified with a gamma counter. The graphs represent counts associated with intracellular transferrin as a fraction of the total.

Fluorescence microscopy. For FRAP experiments, the cells were transfected with clathrin light chain A–GFP, BafA1-treated 2 h post transfection, and imaged 24 h later in CO2-independent medium (Invitrogen) with a Zeiss LSM710 microscope equipped with a temperature-controlled incubator (37 °C). Fluorescence intensity data were collected in the bleach region (ROI1), a reference region within the same cell (ROI2) and a background region (ROI3). The measurements were performed at 2 s intervals for 180 s, with the beach following the fifth readout. Bleaching resulted in an average loss of 73 and 83% of fluorescence intensity for control and BafA1-treated samples respectively. The data for ROI1 were corrected for the background and post-bleach readout was subtracted from the measurements, and the results were divided by the average fluorescence intensity of the first five time points. Three independent experiments were performed; in each from 9 to 18 cells per condition were analysed.

For filipin staining, the cells were fixed for 30 min in 3.3% formaldehyde/PBS and incubated with filipin with 2 h. Alexa488 transferrin uptake for microscopy was performed for 7 min at 37 °C, and the cells were imaged at 37 °C for 15 min. Images were acquired using a Zeiss Axiovert 200M wide-field fluorescence microscope equipped with a Roper Scientific CoolSNAP of camera and a Plan-Apo ×63/1.4 oil-immersion lens using OpenLab software. The panels were assembled using Adobe Photoshop CS4.

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**Resin embedding of whole cells.** For thin-section electron microscopy, control, V-ATPase-depleted (using ATP6V0C and ATP6V1B2 SMARTpools) and BafA1-treated (16 h) cells were fixed in tissue culture dishes and prepared for electron microscopy as previously described. Ultrathin sections were cut, collected onto formvar/carbon-coated electron microscopy grids, and stained with uranyl acetate and lead citrate. The sections were observed in a Philips CM 100 transmission electron microscope (Philips Electron Optics) at 80 kV.

For quantitative analysis of the size of CCSs, micrographs were taken of cells sectioned perpendicular to the PM at a magnification of ×34,000 using a digital camera (Megaview III TEM; Soft Imaging System). For CCSs that were in continuity with the PM, the depth of the pit and the width of the neck were measured, whereas for cross-sectioned CCSs (within 250 nm of the PM), two axes were measured using iTEM software. For each condition, at least 93 CCSs were scored.

**PM replicas.** Control and BafA1-treated cells (24 h), with or without 12.5 μg ml⁻¹ cholesterol in the media, were grown on glass coverslips, washed in warm PBS, incubated for several seconds with polylysine in PBS and unroofed using sonication as described previously, except that sonication was performed in warm Hank’s solution (HBSS). The exposed PMs were immediately fixed in 2% gluteraldehyde in 25 mM HEPES, 125 mM K-acetate, 50 mM-Mg-acetate, at pH 7.3 and further processed by critical point drying and rotary shadowing, as described previously, and viewed in a Tecnai Spirit Biotwin 120 kV transmission electron microscope (FEI Company). The images were acquired with a Gatan Orius CCD (charge-coupled device) camera.

**pH measurements.** For pH measurements, cells were transfected with pH constructs 24 h before being assayed. The cells were collected in either 10% FCS/PBS or pH buffers (15 mM NaOH, 10 mM PIPES, 10 mM HEPES, 5 mM glucose, 1 mM Na₂HPO₄, 0.5 mM MgSO₄, 1 mM CaCl₂, 50 mM KCl and 90 mM potassium l-glutamate brought to the required pH using KCl or NaOH) and analysed on the BD LSRFortessa (BD Biosciences) Flow Cytometer using a 488 nm laser with 430/30 filters to excite SepHluorin and a 561 nm laser with 610/20 filters to excite mCherry. Moderate expressors gated in the mCherry channel were selected for analysis. For quantification, mCherry and SepHluorin were background-corrected, and the SepHluorin/mCherry ratio was calculated (using the derived parameter function in FlowJo Version 7.6). Then the median values of the ratio from BafA1-, DMSO- or pH-buffer-treated cells were normalized to untreated cells. A two-tailed, unpaired t-test was used to estimate statistical significance.

**Statistical methods.** Details of statistical analyses are provided in the subsections referring to the individual assays.

**Data access.** The microarray data were deposited in the GEO database (GSE39954). The MIARE-compatible screening data can be downloaded from: http://www.bioinformatics.cimr.cam.ac.uk/siRNA/Robinson_Screen.

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Figure S1. Summary of the reverse transfection protocol and plate layout in the primary screen. A 96-well plate with 10 μl of 1 μM pre-aliquoted siRNAs was used to generate four assay plates. 72 μl of Optimem was added into each well of the transfection plate. Oligofectamine was preincubated with Optimem in a 1:8 ratio for 8 min, and 18 μl of the mix was added to each well. The transfection mix was incubated for 20 min at RT, and aliquoted into assay plates (20 μl per well). Next, 100 μl of the cell suspension in DMEM (with P/S and L-glutamine), at a concentration of 45,000 cells/ml, was added to each well, and the plates were gently vortexed and incubated at 37°C, 5% CO₂ for 72 h. The final concentration of siRNA per well was 17 nM.
Supplementary Figure S2 (Kozik, Robinson)

| Total fluorescence of the 52 spots read in each well | CD8_readingSum | MHC_readingSum | DNA_readingSum |
| --- | --- | --- | --- |
| Scaling | CD8_scaled | MHC_scaled | DNA_scaled |
| Average fluorescence of the 3 Plk1 siRNA treated wells (empty due to cell death, background) | CD8_scaled_PlkM | MHC_scaled_PlkM | DNA_scaled_PlkM |
| Correction for background fluorescence | CD8_BGcorr | MHC_BGcorr | DNA_BGcorr |
| Phenotype: ratio of the reporter to Hoechst staining fluorescence | CD8_ratio | MHC_ratio | DNA_ratio |
| Average of the negative control wells (3 treated with RISC siRNA and 4 “mock” wells) | CD8_ratio_negCrtlM | MHC_ratio_negCrtlM | DNA_ratio_negCrtlM |
| Normalization to the negative controls | CD8_ratioNormNeg | MHC_ratioNormNeg | DNA_ratioNormNeg |
| Average CD8 fluorescence of the 3 wells treated with CLTC siRNA | CD8_ratioNormNeg_ClaM | MHC_ratioNormNeg_ClaM | DNA_ratioNormNeg_ClaM |

**Figure S2** Summary of the plate-wise data normalization steps (a) and graphs from a sample plate demonstrating data transformations (b-d). The sum of the fluorescence of the 52 data points per well was calculated, and the resulting numbers were scaled by dividing by 100,000 (CD8, MHC-I) or 1,000,000 (DNA) (see Figure 2b). The average of three wells treated with Plk1 siRNAs was calculated and subtracted from the corresponding channels to normalise for the background (b). The background-corrected values for the CD8 and MHC-I channels were divided by the Hoechst signal to normalise cell surface reporter fluorescence to cell number (CD8_ratio) (c). Next, the average of the signal in the negative control wells was calculated and subtracted from the CD8/Hoechst ratio, the average of the positive control values was calculated, and values obtained in the previous step were divided by the average of the wells with the SMARTpool targeting CLTC (d). Thus, a phenotypic score with a value close to 1 indicates a phenotype similar to clathrin, and a score with a value close to 0 indicates no change in the cell surface expression of CD8. These steps were repeated for each of the 1068 plates. In (b,c,d) a sample hit (DNM2) and a SMARTpool with no phenotype (DNASE1L1) are indicated on each of the plots.
Figure S3  Data normalization. (a) CD8/DNA fluorescence ratios of the control wells across the 267 screening plates with the CD8-YXXΦ (A_CD8_ratio) or CD8-FXNPXY (C_CD8_ratio) expressing cells before normalization. The wells are represented in the order in which the plates were scanned. Different types of technical variations are indicated: gradual drift of the controls during the screen (black arrows), and unusually low values in a plate batch, caused by either lower staining efficiency (decrease in both positive and negative controls, grey arrows) or transfection efficiency (decrease more obvious in positive controls, yellow arrow). The orange arrows indicate a new batch of cells. (b) Normalized CD8/Hoechst ratios for the two repeats of CD-YXXΦ (AB_CD8_ratioNorm) or CD8-FXNPXY (CD_CD8_ratioNorm).
Figure S4 Technical and biological reproducibility. (a) Correlation between the normalised CD8/Hoechst ratios (CD8_ratioNorm) from the duplicate plates. Data points from the wells with cell number above the viability threshold for CD8YXXΦ (A versus B) and CD8-FXNPXY (C versus D). (b, c) Correlation between the same SMARTpools on different positions in the library. Normalized CD8/Hoechst values for CD8-YXXΦ (AB_CD8_ratioNorm) and CD8-FXNPXY (CD_CD8_ratioNorm) are plotted in (b) and the corresponding SSMDs in (c). (d) The relationship between the normalized CD8/Hoechst ratio and SSMD. Examples of two different library plates with the CD8-YXXΦ expressing cells. After the CD8/Hoechst ratios were normalised to the negative and positive (CLTC) controls, the normalized ratios were used to calculate the SSMD. The blue line indicates a theoretical position for the points, if the duplicates were identical (SD = 0). The points with large error bars deviate from the line and fall down the rank. The red points indicate knockdowns with cell numbers below the viability threshold, which due to low fluorescence readings do not yield reproducible ratios. Hits with SSMDs > 3 (red line) are highlighted with a purple circle.
Filipin staining in BafA1-treated or V-ATPase kd cells. Sample filipin images acquired with the -4 μm offset from the Arrayscan-based assay to quantify cholesterol depletion from the PM (see Figure 8b). Scale bar: 20 μm.
Figure S6 Subcellular accumulation of cholesterol and transmembrane proteins in BafA1-treated cells. (a) Hela cells were transfected with transferrin receptor (TfR) conjugated to pHluorin, treated with BafA1 for 24 h, fixed for 30 min and stained with filipin for 30 min. On the merge filipin is false-coloured green and TfR-pHluorin red. Scale bar: 20 μm. (b) Cells expressing CD8-YXXΦ or CD8-FXNXPY were treated with BafA1 for 24 h, fixed in 3.3% PFA for 15 min, and permeabilised with 0.1% triton in PBS for 10 min. The cells were then stained for 1.5 h with anti-CD8-Alexa488 labelled antibody (MCA1226A488, Serotec, 1:50) and anti-TfR antibody (555534, BD Pharminogen, 1:200), which was pre-labelled using Alexa594 conjugated Fab fragments according to the manufacturer’s instructions (Zenon® labelling kit, Z25107, Invitrogen). The images were acquired using a CellObserver SD microscope equipped with an AxioCam MRm camera driven by Axiovision version 4.8 software and a Plan-Apo 63x/1.4 oil lens (all Carl Zeiss UK Ltd, Welwyn) and the panels assembled using Adobe Photoshop. Scale bar: 20 μm.
Figure S7 Endosomal/lysosomal pH in cells treated with BafA1 in the presence or absence of cholesterol. CD63-GFP expressing cells were treated with BafA1 in the presence or absence of 12.5 μg/ml soluble cholesterol. The cells were imaged in CO2-independent DMEM (Invitrogen) containing Lysotracker Red and either BafA1 or DMSO. Images were acquired as in Supplementary Figure S5. Scale bar: 20 μm.
**Figure S8** Overview of the plasma membrane in the “unroofed” cells. Low magnification images of cells treated with BafA1 for 24 h in the presence or absence of 12.5 µg/ml cholesterol.
Supplementary Table Legends

Table S1 Primary screen results for all SMARTpools classed as hits, where (a) list genes analysed in secondary screens and (b) lists genes that were not analysed further.

Table S2 Results from the secondary screens: CD8-YXXΦ/CD8-FXNPXY endocytosis and the CCS morphology assays.

Table S3 (a) List of genes expressed in the HeLa cells used in the screen. (b) Expression status for targets of each of the 20,052 SMARTpools.

Supplementary Note

Detailed screening protocols, methods for data analysis, and hit selection strategy.