Supporting Information

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SI Materials and Methods

Cell Culture and Reagents. HeLa cells were grown in DMEM containing 10% (vol/vol) FBS and penicillin/streptomycin at 37°C and 5% CO_2. Transfections were carried out according to manufacturer's instructions by using either GeneJuice (Novagen) or Lipofectamine 2000 (Invitrogen). The CD8-chimera constructs (1) were a kind gift from Scottie Robinson (University of Cambridge, Cambridge, United Kingdom).

The amino acid sequences of the latter parts of CD8 proteins were as follows:

CD8-WT KRLKRRRVCKCPRPVVKSGDKPSLSARYV*
CD8-CIMPR KRLKYKKERREMVMSRLTNCCRRSANVSY
KYSKVNKEEEADENETEWLMEEIQPPAPR
PGKEGQENGHVAAKSVRAADTLSALHGDE
QDSEDEVLTLPEVKVRPPGRAPGAEGGPP
LRPLPRKAPPPLRADDRVGLVRGEPARRG
RPRAAATPISTFHDDSDEDLLHV*
CD8-8xA KRLKRRRIPAAAAAAAAV*
CD8-YAAL KRLKRRRIPAAYAALAAV*
CD8-EAAALL KRLKRRRIPAEAAALLAV*
CD8-FANPAY KRLKRRRIPAFANPAYAV*

H2B-GFP, H2B-mCherry, and GFP-LCa were available from previous work (2). Mouse monoclonal anti-clathrin heavy chain antibody was purified from hybridoma CRL-2228 (ATCC). All reagents were from Sigma, unless stated otherwise.

Flow Cytometry. Analysis of surface and internalized CD8 was performed as described (1). Briefly, HeLa cells expressing CD8 constructs were synchronized in S phase with 2 mM thymidine for 18 h, at the G_2/M checkpoint with 9 μM RO-3306 for 18 h (3) and in M-phase either by 30-min release from RO-3306 treatment or with 40 ng/mL nocodazole for 16 h. Thymidine and RO-3306 arrested cells were trypsinized, whereas cells in mitosis (nocodazole-arrested or those released for 30 min from RO-3306 block) were gently isolated by shake off. We estimate that interphase-synchronized cell populations and (after shake off) mitotic populations were >99% pure. Cells were then centrifuged at 4,000 × *g* for 5 min and resuspended in growth medium containing mouse anti-human CD8 Alexa488-conjugated antibody (1:50, MCA1226A488; Serotec) at 37 °C for 40 min to allow internalization. Subsequently, cells were washed in ice-cold 1% (wt/vol) BSA/PBS before incubation with rabbit anti-Alexa488 (1:67, A11094; Invitrogen) and anti-mouse IgG Alexa633-conjugated secondary antibodies (1:200; Invitrogen), at 4 °C for 30 min. Cells were then washed and fixed in 3.7% (wt/vol) paraformaldehyde before analysis by flow cytometry (FACSCalibur; Becton Dickinson). For quantification of internalized anti-CD8 Alexa488, cells expressing equivalent amounts of surface CD8 construct were gated, as described (1). The gate was a rectangle that spanned the *x* axis and excluded the nontransfected cells and those with very high amounts of CD8 on the surface (20–200 a.u. in Fig. 1*C*). As a control representing endocytic block, interphase cells were incubated with hypertonic sucrose (0.45 M) during the uptake assay (4). For each condition, 1×10^4 cells were measured and after gating, $>1 \times 10^3$ events were analyzed. This number is far in excess of the tens of cells analyzed by light microscopy in previous studies (5).

For analysis of transferrin uptake, synchronized cells were trypsinized or isolated by shake off and starved for 30 min in serum-free media. Cells were then incubated with 50 μg/mL Alexa488-conjugated transferrin for 5 min at 4 °C, then moved to 37 °C and incubated for 10 min. Cells were pelleted, washed in ice cold 1% (wt/vol) BSA/PBS, acid-washed twice, and fixed before flow cytometric analysis. For surface bound and internalized transferrin analysis cells were incubated with Alexa488-labeled transferrin as described above. After an acid wash, cells were resuspended in ice cold 1% (wt/vol) BSA/PBS containing 50 μg/mL Alexa647-conjugated transferrin (Invitrogen) and incubated for 30 min on ice. Cells were then washed, fixed, and analyzed by flow cytometry. For each sample, 1×10^4 events from each sample were acquired, and geometric means from four independent experiments were used for comparison and statistical analysis.

Analysis of internalized and surface transferrin receptor (TfR) was performed by using a similar method to CD8 labeling. HeLa cells were synchronized with thymidine, RO-3306 (10 μM), or nocodazole for 18 h. After synchronization, cells were harvested appropriately (cells synchronized in M phase by using RO-3306 were released for 60 min from the drug treatment), centrifuged, and resuspended in growth medium containing mouse anti-human TfR-FITC conjugated antibody (1:5, SAB4700518; Sigma-Aldrich). Samples were preincubated on ice for 20 min to allow antibody binding to the surface receptors before transfer to 37 °C for 20 min for the internalization to occur. Control (4 °C) samples were not transferred to 37 °C but kept on ice. As a control representing endocytic block, thymidine-treated cells were incubated with hypertonic sucrose (0.5 M) during the 37 °C uptake assay. After incubation with the antibody, cells were washed in ice-cold 1% BSA/PBS, before incubation with rabbit anti-FITC (1:50, 71–1900; Invitrogen) and anti-mouse IgG Alexa633-conjugated antibodies at 4 °C for 30 min. Cells were then washed, fixed, and analyzed by flow cytometry. For each sample, 1×10^4 events were acquired and geometric means with no gating from three independent experiments were used for comparison and statistical analysis.

Immunofluorescence and Live-Cell Labeling. HeLa cells on glass coverslips were fixed with 3% paraformaldehyde and 4% sucrose in PBS (wt/vol). For steady-state localization of CD8 constructs, cells were permeabilized with 0.5% Triton X-100 in PBS (vol/vol) for 10 min. For staining of surface transferrin receptor, no permeabilization was carried out. Cells were blocked [3% (wt/vol) BSA and 5% (vol/vol) goat serum in PBS] before addition of primary antibodies, followed by washing and secondary antibody incubation (where necessary). Finally, cells were washed and mounted in Mowiol containing 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI).

For immunofluorescence analysis of the CD8 uptake assay, cells were prepared and stained as above except cells were grown on coverslips with no synchronization and anti-mouse IgG Alexa568-conjugated secondary antibody was used.

For live-cell imaging of CD8 uptake, cells were cotransfected with H2B-mCherry and the CD8 constructs. Cells were synchronized by using RO-3306 as above, released for 25 min, then transferred to CO_2-independent media and a 37 °C, humidified microscope chamber (Okolab S.r.l). Cells in prometaphase or metaphase were identified by H2B-mCherry. Alexa488-conjugated anti-CD8 antibody was then added at 1:100 dilution in CO_2-independent media, and images were acquired every 30 s for 30 min.

For transferrin uptake experiments, the cells were incubated for 45 min at 37 °C in serum-free DMEM. Cells were first incubated on ice for 5 min followed by addition of 50 μg/mL Alexa488-conjugated transferrin (Invitrogen) in serum-free me-

dia. After 30 min on ice, cells were transferred to a 37°C/5% CO_2 incubator for 7 min (adequate time to allow substantial uptake but sufficiently short so that cells are unlikely to have progressed significantly through the cell cycle during the course of the uptake). Cells were then acid-washed (0.1 M glycine and 150 mM NaCl, pH 3) to remove surface-bound transferrin before fixation (3% paraformaldehyde and 4% sucrose in PBS) for 15 min. Subsequently, cells were processed for immunofluorescence as above and stained with rabbit anti-transferrin receptor (CBL47; Chemicon) followed by anti-rabbit Alexa568 secondary antibody (1:500; Invitrogen).

For FM4-64 uptake experiments, HeLa cells transiently expressing H2B-GFP were incubated with FM4-64 (15 μM; Invitrogen) for 10 min. Excess dye was washed from the plasma membrane by using normal extracellular solution (136 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 10 mM Hepes, 2 mM $CaCl_2$, and 10 mM glucose at pH 7.4) for 10 min.

Microscopy. Microscopy was performed on a Leica confocal microscope SP2 with a 63× (1.4 N.A.) oil-immersion objective. Alexa488 and Alexa568 were excited by using Ar/Kr 488 nm or He/Ne 543 nm laser lines, respectively. DAPI was excited by using a multiphoton laser. Excitation and collection of emission were performed separately and sequentially. *Z*-series were acquired by using the software's optimized z-depth between each slice (0.122 μm). For live-cell experiments, either a Nikon Ti fluorescence microscope and DS-Qi 1Mc camera (CD8 experiments) or an Olympus IX81 fluorescence microscope and Orca-ER camera (FM dye experiments) was used with a 60× oil-immersion objective.

Data Analysis. Flow cytometry data were analyzed, plots were generated in CellQuest (Becton Dickinson), and histograms were made in WinMDI. Quantification of transferrin uptake and surface transferrin receptor from confocal *Z*-series was performed in Image J by thresholding the stack, drawing around cell outlines, and measuring each section. The background-corrected mean intensities for all slices were summed to give the total intensity for each cell. Alternative methods of quantification produced the same result. Image J was used to create the maximum intensity projections shown in supporting information. Data were plotted in Igor Pro-6.2 (Wavemetrics). Figures were assembled in Adobe Photoshop. For statistical testing, one-way ANOVA with Tukey's or Dunnett's post hoc test was used to compare multiple groups. Where only two groups were compared, Student's *t* test was used in Instat (GraphPad).

1. Kozik P, Francis RW, Seaman MN, Robinson MS (2010) A screen for endocytic motifs. *Traffic* 11:843–855.
2. Royle SJ, Bright NA, Lagnado L (2005) Clathrin is required for the function of the mitotic spindle. *Nature* 434:1152–1157.
3. Vassilev LT, et al. (2006) Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc Natl Acad Sci USA* 103:10660–10665.
4. Heuser JE, Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* 108:389–400.
5. Boucrot E, Kirchhausen T (2007) Endosomal recycling controls plasma membrane area during mitosis. *Proc Natl Acad Sci USA* 104:7939–7944.

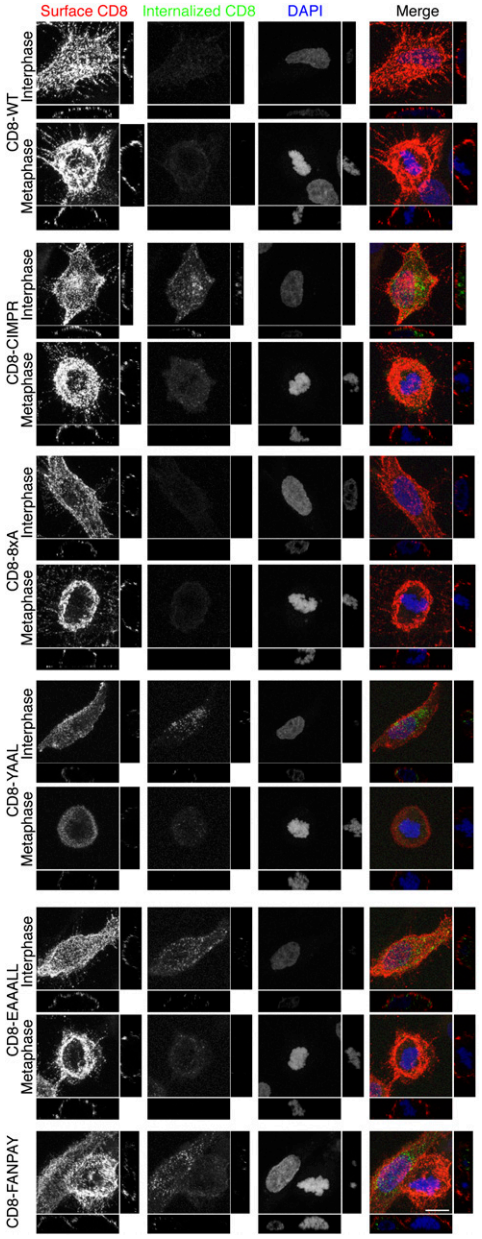

Fig. S1. Mitotic inhibition of endocytosis: CD8 trafficking in interphase or early mitotic cells. 3D confocal reconstructions of representative cells expressing each CD8 construct. A maximum intensity projection of a confocal *Z*-series is shown en face (*x-y*) or as *y-z* and *x-z* orthogonal sections for each channel. (Scale bar: 10 μm.)

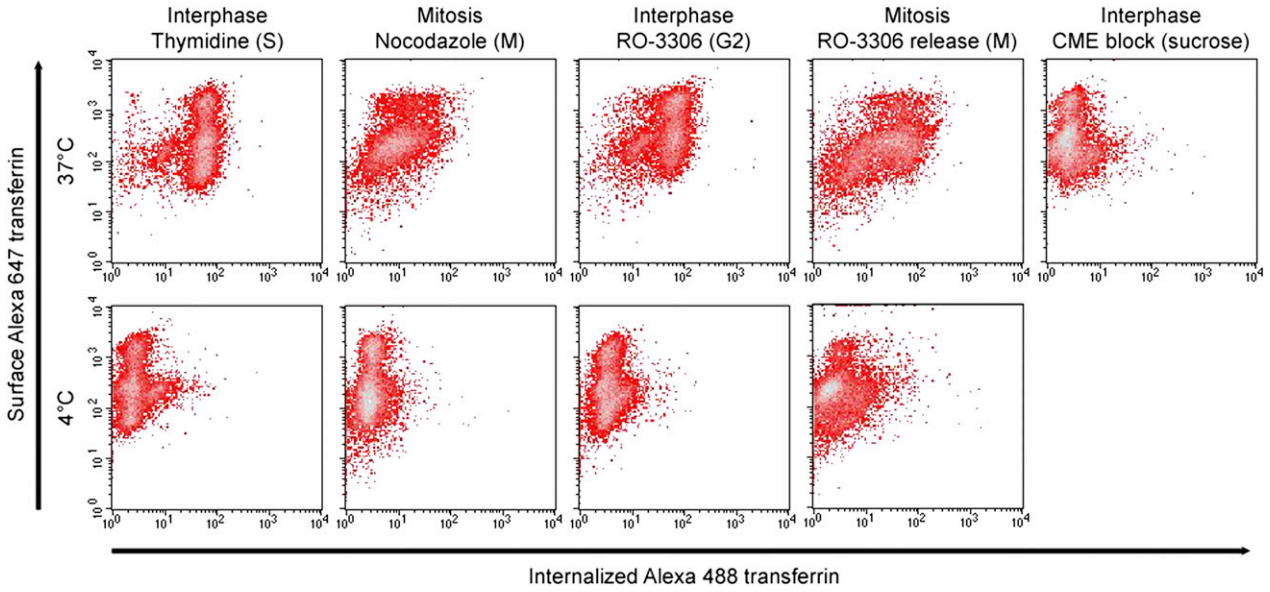

Fig. S2. Transferrin uptake is inhibited during early mitosis despite abundant surface TfR. Quantification of transferrin uptake and surface transferrin by flow cytometry. Results from a single experiment are shown. Plots of internalized versus surface transferrin are shown. Note that there is (*i*) no decrease in the amount of surface transferrin in any condition and (*ii*) the leftward shift of the plots from mitotic cells. Plots are pseudocolored to show the density of points.

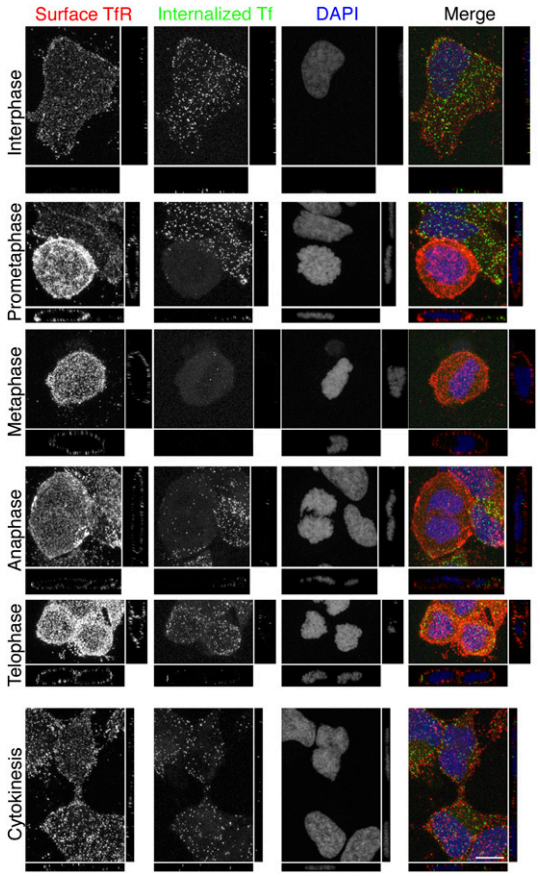

Fig. S3. Transferrin uptake is inhibited during early mitosis despite abundant surface TfR. 3D confocal reconstructions of the cells that are shown in Fig. 4. A maximum intensity projection of a confocal *Z*-series is shown en face (*x-y*) or as *y-z* and *x-z* orthogonal sections for each channel. (Scale bar: 10 μm.)