

A novel live cell imaging assay reveals regulation of endosome maturation

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1 **Abstract (186 words)**

2
3 Cell-cell communication is an essential process in life, with endosomes acting as key
4 organelles for regulating uptake and secretion of signaling molecules. Endocytosed material
5 is accepted by the sorting endosome where it either is sorted for recycling or remains in the
6 endosome as it matures to be degraded in the lysosome. Investigation of the endosome
7 maturation process has been hampered by the small size and rapid movement of endosomes
8 in most cellular systems. Here, we report an easy versatile live-cell imaging assay to monitor
9 endosome maturation kinetics, which can be applied to a variety of mammalian cell types.
10 Acute ionophore treatment led to enlarged early endosomal compartments that matured
11 into late endosomes and fused with lysosomes to form endolysosomes. Rab5-to-Rab7
12 conversion and PI(3)P formation and turn over were recapitulated with this assay and could
13 be observed with a standard widefield microscope. We used this approach to show that Snx1-
14 and Rab11-dependent endosomal recycling occurred throughout endosome maturation and
15 was uncoupled from Rab conversion. In contrast, efficient endosomal acidification was
16 dependent on Rab conversion. The assay provides a powerful tool to further unravel various
17 aspects of endosome maturation.

18 **INTRODUCTION**

19

20 Endosomes are central organelles in orchestrating cell interactions with the extracellular
21 environment, whether by regulating the composition of signalling molecules at the plasma
22 membrane or by facilitating uptake and digestion of certain nutrients or degrading toxic or
23 no longer needed material. Their wide range of functions is accomplished through a
24 sequential and highly regulated process known as endosome maturation (Huotari and
25 Helenius, 2011; Podinovskaia and Spang, 2018; Spang, 2016). Early endosomes accept
26 incoming cargo from the endocytic vesicles and undergo extensive sorting to package
27 selected cargo into recycling vesicles for the return to the cell surface or to the Golgi, whereas
28 membrane cargo destined for removal is internalised into intraluminal vesicles (ILVs) for its
29 subsequent degradation in endolysosomes. As these sorting endosomes mature into late
30 endosomes, now mainly containing cargo destined for degradation, they no longer accept
31 cargo from the cell surface and acquire properties necessary for their interaction with
32 lysosomes. Upon fusion with lysosomes, late endosomes form endolysosomes, whose highly
33 acidic and hydrolytic milieu facilitates degradation of the remaining cargo and regeneration
34 of the lysosome (Guerra and Bucci, 2016). Throughout this maturation process, the Golgi
35 apparatus supports the endosomal activities by supplying components essential for the
36 progression of endosome maturation, such as proton pump subunits, lysosomal hydrolases,
37 and factors necessary for selective recruitment of GTPases to the endosome (McDermott and
38 Kim, 2015; Nagano et al., 2019).

39 As endosomes complete their sorting tasks and mature, they undergo extensive
40 changes to their properties to aid their divergent functions. The selective recruitment of
41 GTPases, Rab5 and Rab7 to early and late endosomes, respectively, ensures specificity of
42 interaction with other organelles, such as endocytic vesicles and other early endosomes for
43 Rab5-positive endosomes, and lysosomes for Rab7-positive endosomes (Balderhaar and
44 Ungermann, 2013; Solinger and Spang, 2013). The process of displacement of Rab5 at early
45 endosomes by Rab7 at late endosomes is defined as Rab conversion (Poteryaev et al., 2010;
46 Rink et al., 2005). Additionally, early endosomes contain the signalling lipid PI(3)P, which is
47 further phosphorylated to PI(3,5)P₂ in late endosomes (Hsu et al., 2015). These lipids serve as
48 organelle identity molecules, facilitating recruitment of components, such as sorting and
49 tethering factors, necessary for endosomal function (Schink et al., 2013). Endosomal

50 acidification is another essential change that must take place for endosomes to mature, with
51 pH ~6.5, 5.5 and 4.5 characterising early endosomes, late endosomes and lysosomes,
52 respectively (Casey et al., 2010). These changes in GTPase recruitment, PIP composition and
53 acidification status, among others, must be tightly coordinated to ensure unidirectional and
54 aligned adjustments to endosome identity and purpose for the endocytic system to operate
55 properly. However, coordination of such processes during endosome maturation is poorly
56 understood.

57 A major setback in understanding the kinetics of endosome maturation is lack of
58 experimental systems, which would allow us to monitor endosomes at individual endosome
59 level over prolonged periods of time. The small size of the endosomes and their rapid
60 movement within the cell makes it impractical to track maturing endosomes as they rapidly,
61 within seconds or minutes, move out of field of focus or get lost among other vesicles in the
62 dense perinuclear space. Phagosomes have provided a unique way of studying certain aspects
63 of endosome maturation, allowing for uniform size and synchronisation (Naufer et al., 2018;
64 Podinovskaia et al., 2013). However, these are a specialised subset of endosomes that are
65 involved in minimal amount of sorting and proceed rapidly through endosome maturation,
66 and therefore are not suitable for defining kinetics of classic endosome maturation. Given the
67 present lack of suitable approaches to study endosome maturation, enlarging endosomes
68 might offer a solution to observing individual endosomes over time.

69 We found that acute nigericin treatment followed by washout led to the formation of
70 enlarged Rab5 positive endosomes that undergo Rab5-to-Rab7 conversion with anticipated
71 kinetics in different cell lines. Other hallmarks of endosome maturation such as PI(3)P, SNX1
72 and Rab11 recruitment and acidification likewise occurred. Finally, matured late endosomes
73 fused with lysosomes, resulting in functional endolysosomes. Our minimally invasive assay
74 provides an inexpensive and robust way to evaluate relative kinetics of key mediators of
75 endosome maturation at individual endosome level. This assay does not require any
76 specialized equipment, and maturation is detected with the ease of a conventional widefield
77 microscope. We used this assay to investigate whether Rab conversion influences recycling
78 to the plasma membrane and endosomal acidification. We found that recycling to the plasma
79 membrane did not strongly correlate with Rab conversion. Fusing GalT to ratiometric
80 pHlemon (Burgstaller et al., 2019) (GalT-pHlemon) allowed us to follow the degradation
81 pathway to the lysosome and to demonstrate that acidification is slowed down when Rab

82 conversion is blocked, suggesting that Rab conversion is required for efficient acidification
83 during endosome maturation.

84

85

86 RESULTS

87

88 Short nigericin treatment induces enlarged endosomes that undergo Rab conversion

89 Endosomes are highly dynamic and motile organelles and, given their small size and
90 frequently indistinct and changing shape, are highly uncooperative to monitoring over
91 extended periods of time by microscopy. The ability to follow dynamic events, such as Rab
92 conversion, at individual endosome level is pivotal for unravelling the mechanisms of
93 endosome maturation. Therefore, we sought a minimally invasive way to enlarge endosomes
94 to make them more distinct and traceable over time. We discovered that a 20-min nigericin
95 treatment of HeLa cells, stably expressing mApple-Rab5 and GFP-Rab7, followed by washout
96 led to the formation of enlarged Rab5- and Rab7-positive endosomes ([Fig 1A](#)). Nigericin is an
97 ionophore known to reversibly permeabilise membranes to protons and K⁺ ions. Indeed, the
98 short nigericin treatment disrupted intracellular pH gradient, which re-established within 30
99 min of washout as visualised by Lysotracker accumulation in treated cells ([Fig 1B](#)). The
100 presence of the enlarged Rab5-positive endosomes was often transient but could also last for
101 longer times, whereas enlarged Rab7-positive endosomes persisted until complete recovery
102 of Rab5 and Rab7 morphology by 20 h ([Fig 1A](#)). We hypothesised that the enlarged Rab5-
103 positive early endosomes mature to Rab7-positive late endosomes. Therefore, we followed
104 individual endosomes at 1 min intervals, starting from enlarged spherical compartments
105 devoid of either Rab5 or Rab7, and we could indeed observe transient recruitment of Rab5
106 and its subsequent displacement by Rab7 ([Fig 1C, video Fig 1C supplement 1](#)), consistent with
107 previous descriptions of Rab conversion events (Del Conte-Zerial et al., 2008; Poteryaev et al.,
108 2010; Skjeldal et al., 2021). Hence, acute nigericin treatment leads to enlarged compartments
109 that are capable of recruiting Rab5 and undergoing Rab conversion.

110 We frequently found that Rab5 recruitment was initiated after a Rab5-positive
111 endocytic vesicle or endosome was touching or fusing with an enlarged compartment ([Fig 1C](#);
112 [Fig 1, figure supplement 1A](#)), suggesting either kiss-and-run or fusion with a Rab5-positive
113 structure drove Rab5 recruitment. Therefore, endocytic events from the plasma membrane
114 are also required to form enlarged Rab5 positive early endosomes. We also observed

Figure 1

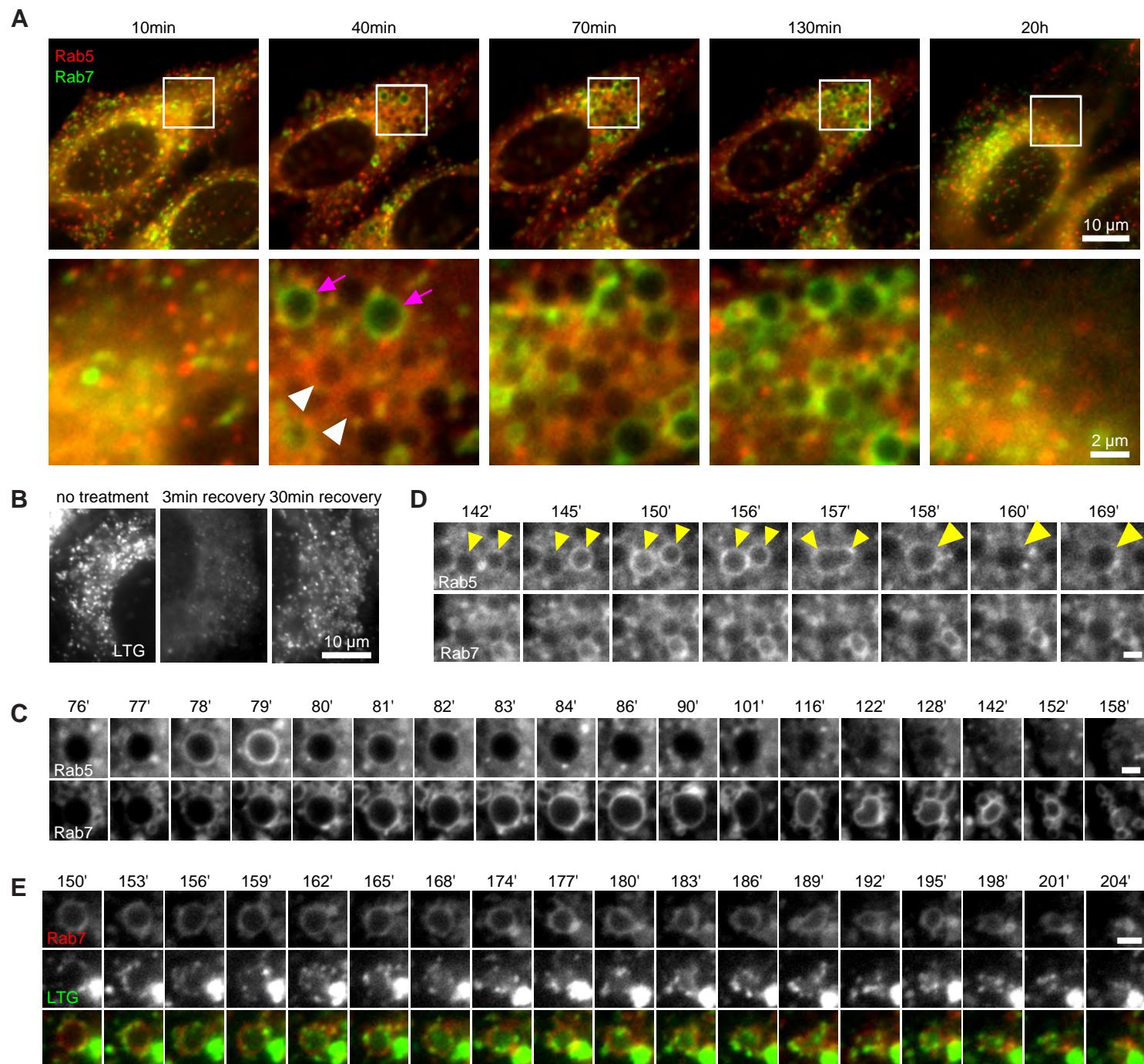


Figure 1. Rab conversion and completion of endolysosomal stages of endosome maturation can be observed in enlarged endosomes, induced with short nigericin treatment.

Nigericin was added to HeLa cells at 10 μ M for 20 min and washed away, and cells were imaged by time-lapse microscopy. Recovery times are specified relative to removal of nigericin.

(A,C,D) Cells stably expressing mApple-Rab5 and GFP-Rab7.

(A) Images to show enlarged Rab5- (white arrows) and Rab7- (magenta arrows) positive compartments and return to normal morphology by 20 h.

(B) Lysotracker Green (LTG) was added to cells during and following nigericin treatment. Images show rapid re-accumulation of Lysotracker in treated cells.

(C) The enlarged endosome was selected to show Rab5 recruitment, Rab conversion and endolysosomal stages of endosome maturation.

(D) An example of homotypic fusion of two Rab5-positive endosome and subsequent Rab conversion.

(E) Cells stably expressing mApple-Rab7 were imaged in the presence of Lysotracker Green. An enlarged Rab7-positive endosome was selected to show accumulation of Lysotracker concomitant with the loss of spherical shape and a reduction in size of the maturing endolysosome. (C-E) Scale bar = 2 μ m.

115 homotypic Rab5 endosomal fusions, a hallmark of early endosomes, indicating that the
116 enlarged Rab5-positive structures behaved as bona fide early endosomes ([Fig 1D, video Fig](#)
117 [1D supplement 1](#)). Following Rab conversion, the spherical Rab7-positive endosomes
118 persisted over a range of several minutes to several hours and remained Lysotracker-
119 negative, with lysosomes seen as Lysotracker-positive puncta circling around the endosomes.
120 Once the endosome acidified sufficiently to accumulate Lysotracker, it lost its spherical shape
121 and got torn apart until no longer detectable ([Fig 1C and E, video Fig 1E supplement 1](#)),
122 consistent with endolysosomal stages of endosome maturation. Fusion of the enlarged
123 endosomes with Dextran-AF488-loaded lysosomes was apparent through accumulation of
124 Dextran-AF488 in the enlarged endosomes ([Fig 1, figure supplement 1B](#)). Thus, acute nigericin
125 treatment could induce the formation of large early endosomes, which could be observed to
126 mature into late endosomes, and subsequently fuse with lysosomes and undergo
127 endolysosome-to-lysosome maturation. This acute treatment may provide the basis of a
128 powerful assay that could be employed to follow individual maturing endosomes.

129 How common is this phenomenon of the enlarged endosome induction by acute
130 pharmaceutical treatment? First, we checked whether acute treatment with another
131 ionophore, monensin, or the weak base NH₄Cl, which perturbs the pH gradient, would have
132 a similar effect. Indeed, we observed transient Rab5 recruitment and the more extended
133 Rab7 recruitment at the enlarged endosomes of NH₄Cl pre-treated cells, and likewise, gradual
134 Rab7 recruitment following acute monensin treatment ([Fig 1, figure supplement 2](#)).
135 Therefore, interfering with ion homeostasis and membrane potential appear to solicit a
136 similar stress response as nigericin, resulting in formation of enlarged endosomes. Second,
137 we investigated whether this effect was cell line specific or more generally applicable. We
138 tested the epithelial cell line HEK293, the fibroblast-like cell line COS1 and the neuronal line
139 Neuro2. In all three cell lines we could observe enlarged endosomes that were either Rab5 or
140 Rab7 positive ([Fig 1, figure supplement 3](#)). Therefore, enlarged endosome induction is not
141 restricted to nigericin treatment of HeLa cells but rather is applicable to a wide range of
142 experimental systems.

143

144 **TGN membranes transition into endosomes after acute nigericin treatment**

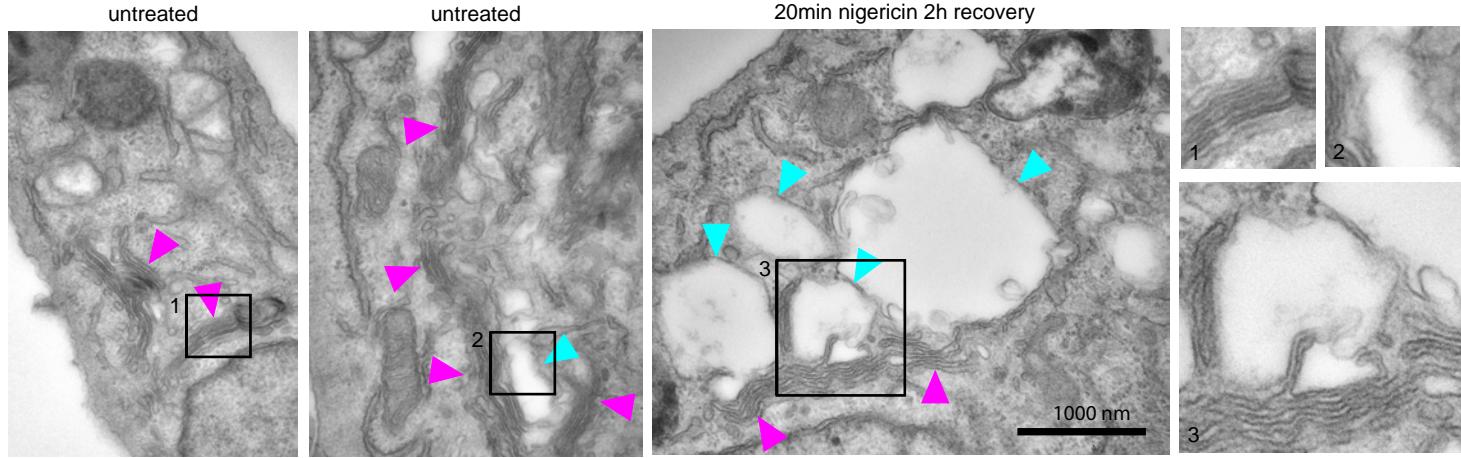
145 Short nigericin treatment led to enlarged endosomes, however, they did not start out as
146 Rab5-positive entities ([Fig 1C](#)). Therefore, we investigated the origin of the membranes for

147 these compartments. Electron microscopy images revealed that the enlarged compartments
148 originate at the trans face of the Golgi (Fig 2A), in line with previous reports of ionophore
149 treatment leading to the swelling of the trans-Golgi leaflet (Ledger et al., 1980; Morre et al.,
150 1983). We ruled out contribution from the autophagy pathway by staining mApple-Rab5
151 expressing cells with LC3b antibody and showing no detectable autophagy induction or LC3b
152 presence at the enlarged endosomes at 60 min post nigericin treatment (Fig.2, figure
153 supplement 1A). To determine whether the swollen TGN membranes would enter the
154 endosomal pathway, we performed immuno-electron microscopy with HeLa cells stably
155 expressing trans-Golgi marker GaIT-GFP after acute nigericin treatment. The micrographs
156 demonstrate the presence of GaIT in the enlarged trans-Golgi network (TGN) compartments
157 and in ILVs of multivesicular bodies at later time points (Fig 2B). Therefore, the membranes
158 that acquire Rab5 and convert to Rab7 positive endosomes are probably derived from the
159 TGN. This swelling of the TGN is likely a transient response to the acute stress because after
160 48 h the Golgi had recovered from the treatment (Fig 2C). Consistent with this notion, we
161 occasionally observed swollen Golgi leaflets also in untreated cells signifying a process that
162 occurs naturally in the cell, which we are uniquely amplifying with acute perturbation (Fig 2A).
163 Indeed, the cells continued to grow and divide (Fig.2, figure supplement 1B), and after an
164 initial slow start, the nigericin-treated cells recover their doubling rate within 24 h (Fig.2,
165 figure supplement 1C). In line with previous reports (Merion and Sly, 1983), our findings
166 indicate that short nigericin treatment induces reversible changes and has minimal impact on
167 cell health.

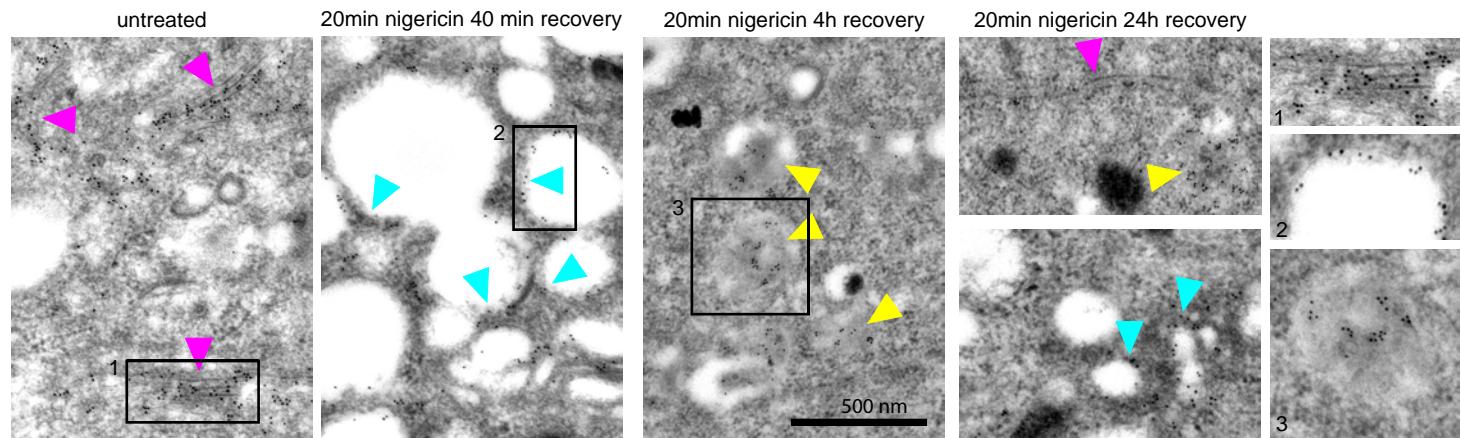
168 To corroborate our results, we monitored HeLa cells stably expressing GaIT-GFP by
169 fluorescence microscopy following acute nigericin treatment. Golgi vesiculation was observed
170 within 15 min of nigericin washout (Fig 3A, video Fig 3A supplement 1). We observed similar
171 results when we used monensin as ionophore (Fig.2, figure supplement 1D). A large fraction
172 of these vesicles would adopt early endosomal identity because individual GaIT-positive
173 structures acquired Rab5 over time (Fig 3A), as also observed by immuno-electron microscopy
174 (Fig 3B). Moreover, similar to the transiently transfected GaIT-GFP, endogenous GaIT
175 persisted in the endosomes (Fig 3C), consistent with the observations of its subsequent
176 internalisation into ILVs (Fig 2B and 10D), and Golgi morphology was fully recovered within
177 48h (Fig.2, figure supplement 1E). The contribution of cargo from the endocytic pathway to
178 the enlarged compartments was evidenced by the addition of Dextran-AF488 for 1 h to the

Figure 2

A



B



C

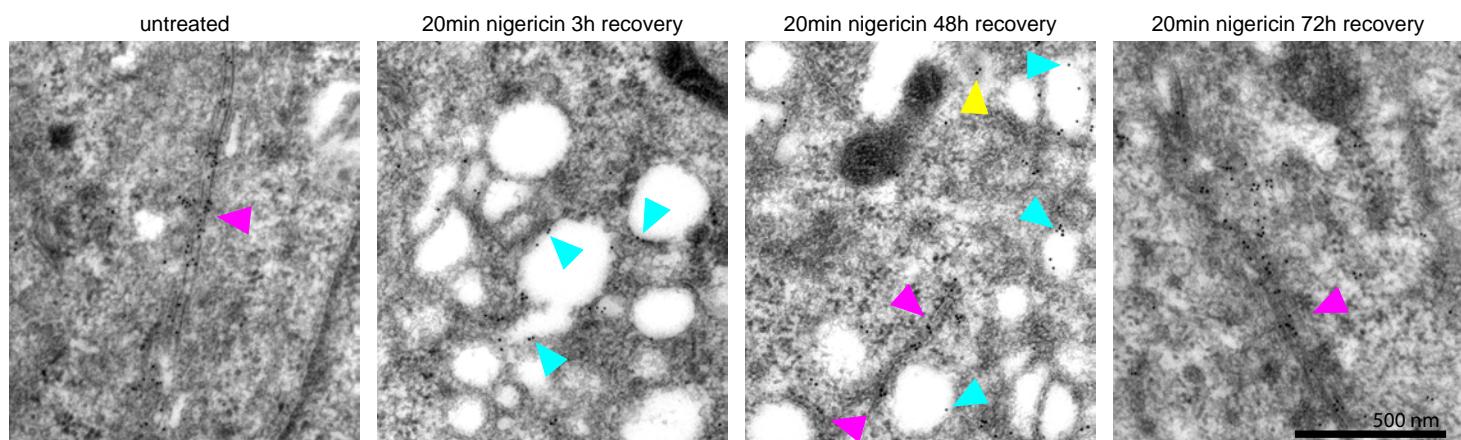


Figure 2. Nigericin-induced enlarged compartments originate at the Golgi and contain trans-Golgi marker GALT, later found in ILVs, with most enlarged compartments resolved by 48 h.

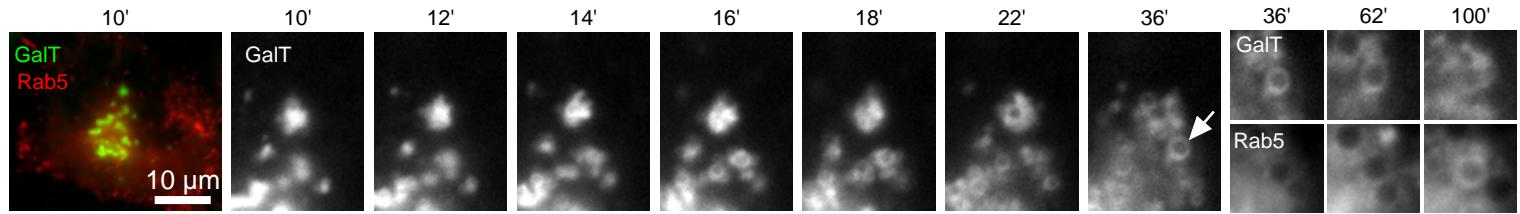
Nigericin was added to HeLa cells for 20 min and washed away, and cells were processed for electron microscopy (A-C), imaged by time-lapse microscopy (D) or harvested for counting (E) at specified times after the wash.

(A) Cells stained with osmium tetroxide and potassium hexacyanoferrate reveal large spherical compartments (cyan arrows) originating at the trans-face of the Golgi (magenta arrows) in nigericin-treated cells and, occasionally, in untreated cells.

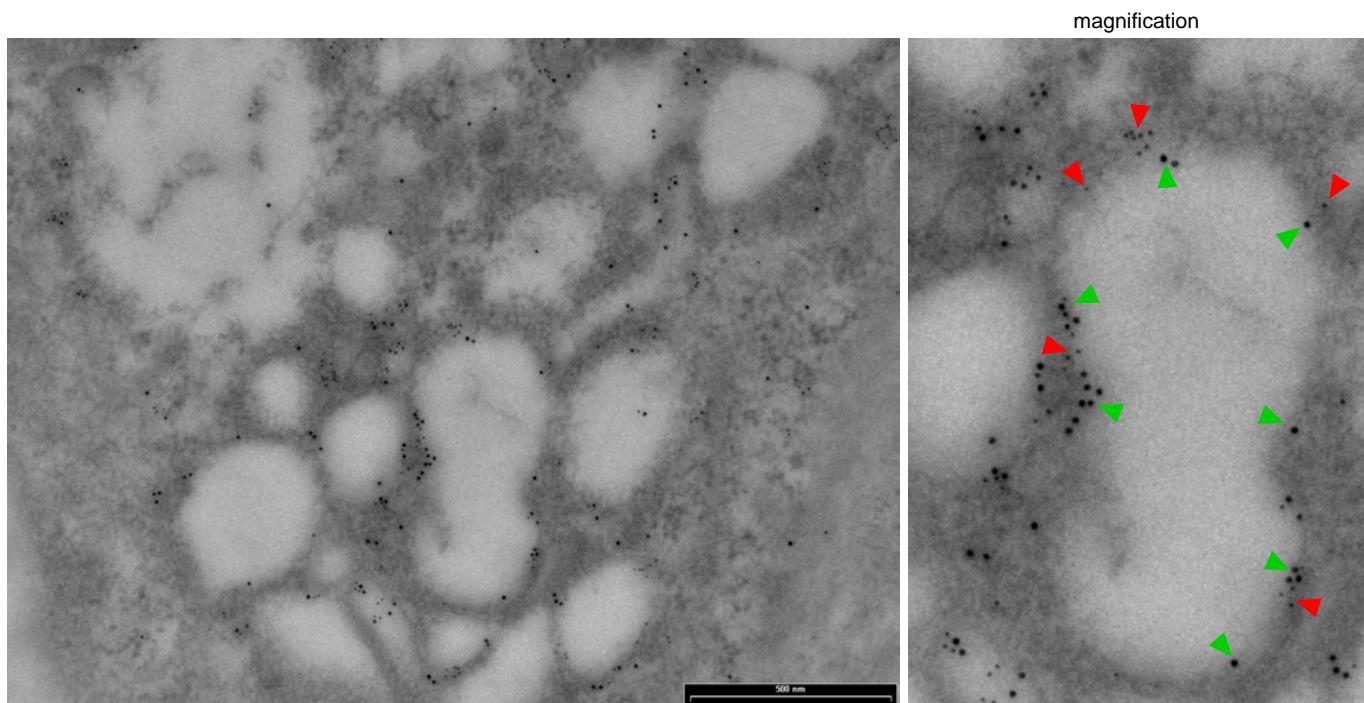
(B,C) Cells stably expressing GALT-GFP were stained with anti-GFP and 12 nM immuno-Gold to reveal GALT-GFP at the Golgi (magenta arrows), the limiting membrane of the enlarged compartments (cyan arrows) as well as in ILVs of the enlarged MVBs at later time points (yellow arrows).

Figure 3

A



B



C

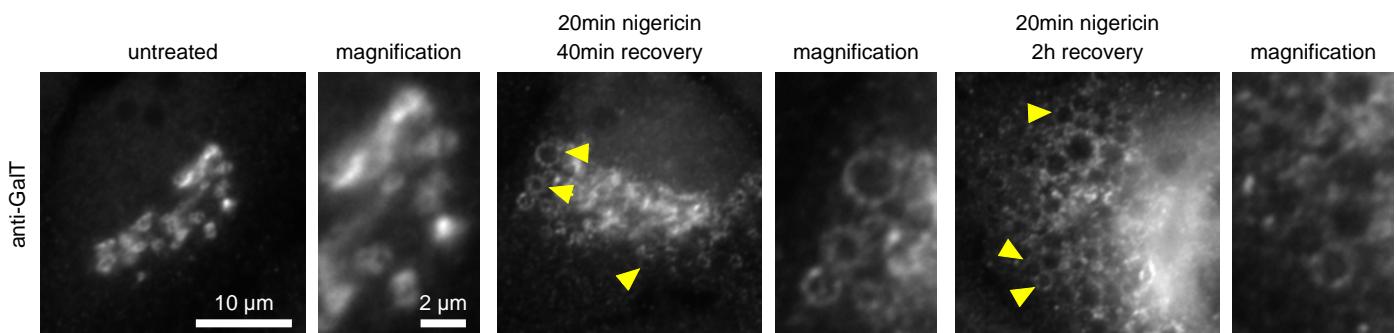


Figure 3. Short nigericin treatment leads to trans-Golgi vesiculation and subsequent acquisition of Rab5.

Nigericin was added to HeLa cells for 20 min and washed away, and cells were imaged by time-lapse microscopy (A), processed for electron microscopy (B) or for immunofluorescence (C) at specified times after the wash.

(A,B) HeLa cells stably expressing GalT-GFP and transiently transfected with mApple-Rab5.

(A) Representative kinetic of Golgi vesiculation post nigericin treatment as visualised with GalT-GFP. The selected vesiculated compartment (arrow), initially negative for Rab5 subsequently becomes positive for both markers.

(B) Immuno-EM image of cells at 2 h post recovery, with 12 nm Gold labelled GFP (green arrows) and 5 nm Gold labelled mApple (red arrows) present at the enlarged compartments. Scale bar = 500 nm.

(C) Images of cells stained with anti-GalT to reveal endogenous GalT presence at the enlarged compartments.

179 cell medium of nigericin-treated cells and its detection in the Rab5-positive enlarged
180 endosomes after washing the dextran away ([Fig.2, figure supplement 1F](#)). Taken together,
181 our findings suggest that acute nigericin treatment leads to enlarged Golgi-derived
182 compartments that are able to acquire early endosomal identity and mature into late
183 endosomes. Thereby, acute nigericin treatment provides us with a means to generate
184 functionally competent enlarged endosomes that can be monitored at individual endosome
185 level by widefield microscopy over extended periods of time to define the kinetics of a wide
186 range of mediators of endosome maturation.

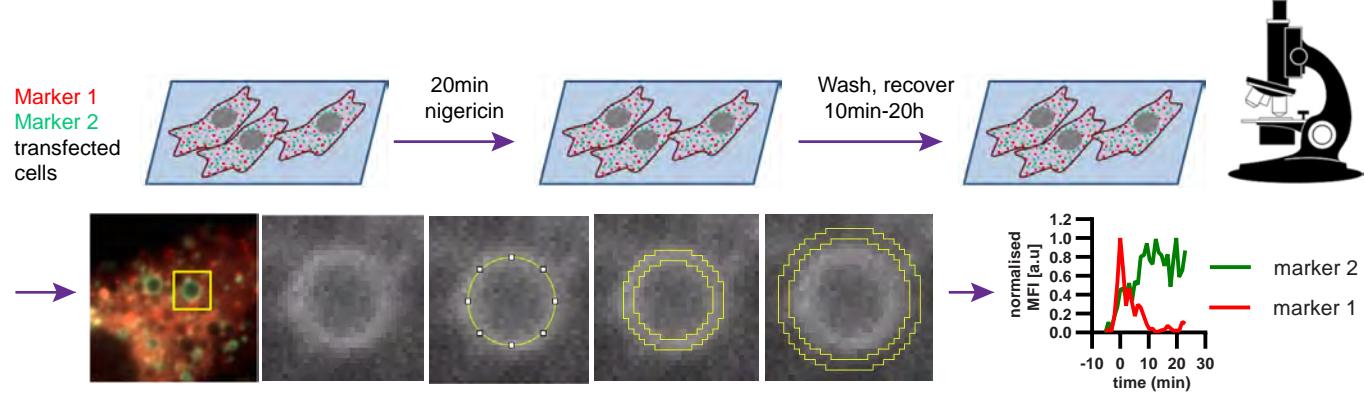
187

188 **Rab conversion occurs with anticipated kinetics on enlarged endosomes**

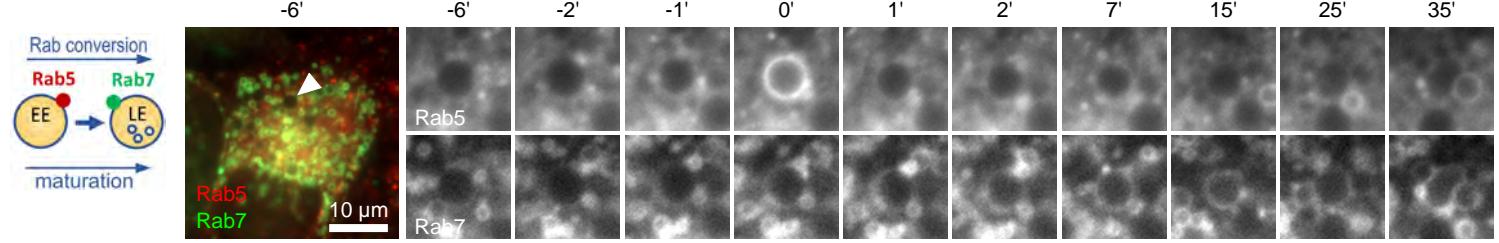
189 Having established a novel assay to study endosome maturation, we used it first to revisit the
190 kinetics of Rab conversion. The formation of enlarged early endosomes was asynchronous
191 and therefore we imaged over several hours without significant loss of fluorescence signal.
192 We captured many events of Rab conversion for further analysis and signal quantification.
193 Endosomes that were initially negative for Rab5 and acquired Rab5 during the time course
194 were chosen for analysis. For quantification purposes, we measured the mean fluorescence
195 intensity of the rim of the enlarged endosome at all time points when the endosome was
196 detectable ([Fig 4A](#)). Following transient Rab5 recruitment, all selected endosomes underwent
197 Rab conversion. Initially Rab5 was recruited uniformly to the rim of the endosome, but could
198 segregate also into distinct domains, before becoming completely displaced by Rab7 ([Fig 4B,](#)
199 [Fig. 4, figure supplement 1A](#)). Consistent with previous findings, Rab5 levels dropped when
200 Rab7 reached about 50% of its maximal level ([Fig. 4B-D](#)) (Del Conte-Zerial et al., 2008;
201 Poteryaev et al., 2010; van der Schaar et al., 2008). Moreover, Rab conversion was completed
202 within 4 min after its initiation, which is similar to previously reported observations (Del
203 Conte-Zerial et al., 2008; Poteryaev et al., 2010; Rink et al., 2005). Once Rab5 was fully
204 removed, Rab7 plateaued off showing stable presence at the late endosome ([Fig 4D; Fig. 4,](#)
205 [figure supplement 1B](#)). Occasionally, Rab5 produced multiple peaks, with Rab7 plateauing off
206 after the latest Rab5 peak ([Fig. 4, figure supplement 1C and D](#)). Such Rab5 behaviour may
207 indicate the reversible nature of endosome maturation and existence of checkpoints to
208 ensure alignment of parallel processes. Our results closely agree with Rab conversion kinetics
209 in other systems and further refine Rab conversion kinetics in human cells. Empowered by
210 this strict sequential kinetics of Rab5 and Rab7 in maturing endosomes, we next explored the

Figure 4

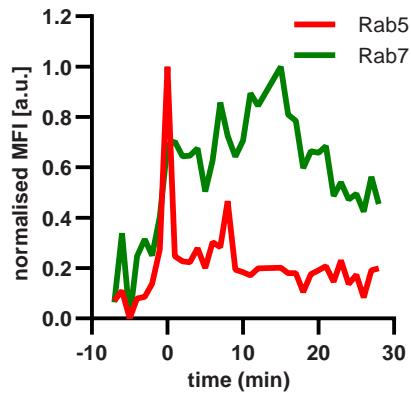
A



B



C



D

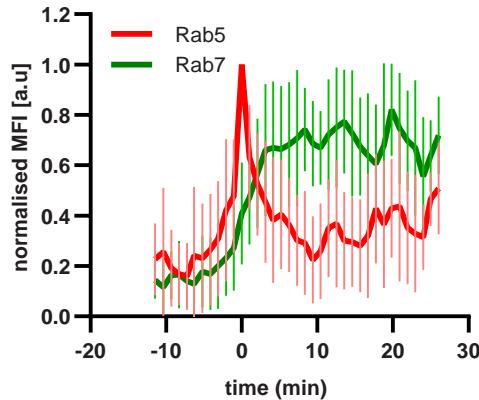


Figure 4. Enlarged endosomes recruit Rab5 and undergo Rab conversion with anticipated kinetics

(A) Scheme to show experimental flow, starting with transfection of cells of choice with selected markers, followed by short nigericin treatment, and time-lapse microscopy during the recovery phase, with subsequent quantification of mean fluorescence intensity (MFI) of the chosen markers at the rim of the enlarged endosomes, and the resulting kinetic plots of background-subtracted MFI normalised for maximum and minimum values over the entire time course of the endosome. Since endosome maturation is asynchronous, relative time is calculated by using Rab5 peak as a reference for Rab conversion and set to t=0. The plot shown in the scheme represents the kinetic of the images in Figure 1C (marker 1 as Rab5 and marker 2 as Rab7).

(B,C,D) HeLa cells, stably expressing mApple-Rab5 and GFP-Rab7 were treated for 20 min with nigericin, washed and imaged over a 3 h period.

(B) Time-lapse images of a representative endosome to show transient Rab5 recruitment and its subsequent displacement by Rab7.

(C) Corresponding graph of MFI of Rab5 and Rab7 at the rim of the endosome in (B) during and around the time of Rab conversion. Numerical data for all analyzed endosomes is available in Figure 4D - Source Data 1.

(D) Averaged Rab5 and Rab7 kinetics of 27 endosomes. Error bars represent standard deviation. Representative graph of three independent experiments.

Figure 5

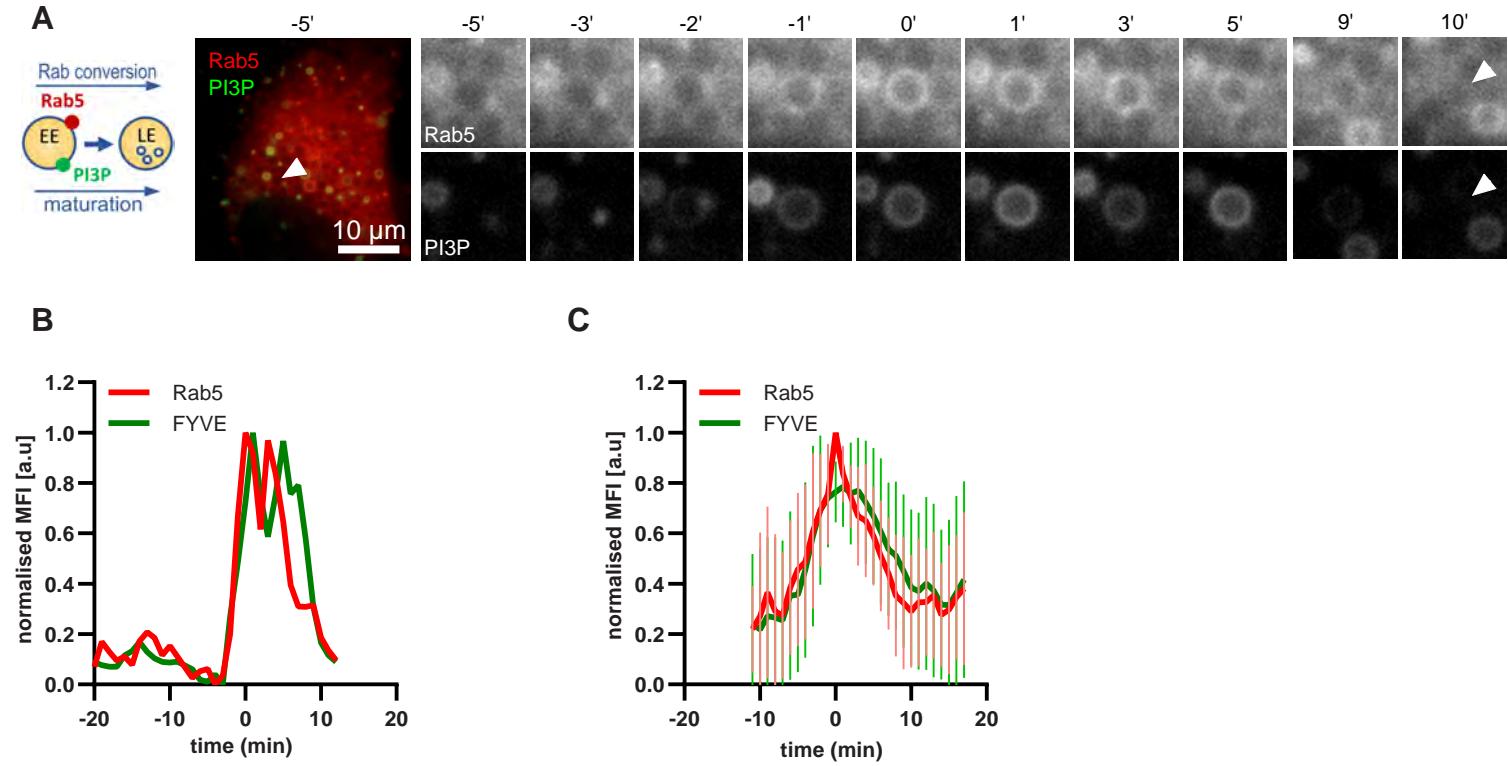


Figure 5. PI(3)P is recruited to endosomes concomitantly with Rab5.

HeLa cells, stably expressing mApple-Rab5 and transiently transfected with the PI(3)P marker, GFP-FYVE, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A) Time-lapse images of a representative endosome to show transient Rab5 recruitment accompanied by PI(3)P.

(B) Corresponding graph of normalised mean fluorescence intensity of Rab5 and FYVE at the rim of the endosome in (A) over the time the endosome was detectable.

(C) Averaged Rab5 and PI(3)P kinetics of 19 endosomes. Error bars represent standard deviation. Representative graph of three independent experiments. Numerical data for all analyzed endosomes is available in Figure 5C - Source Data 1.

211 kinetics of other mediators of endosome maturation relative to either Rab5 or Rab7
212 recruitment, using the maximum peak of Rab5 or the 50% of the maximal fluorescence
213 intensity of Rab7 as reference point for Rab conversion.

214

215 **PI(3)P levels peak concomitantly with Rab5 levels**

216 Driving early endosome identity, Rab5 recruits the PI(3)P kinase VPS34 and forms a positive
217 feedback loop with PI(3)P (Zerial and McBride, 2001). Coincidence detection of PI(3)P levels
218 and the Rab5GEF Rabex5 by the Rab7 GEF Mon1/CCZ1 was proposed to drive Rab conversion
219 and endosome maturation (Poteryaev et al., 2010) and subsequently trigger the formation of
220 PI(3,5)P₂ (Compton et al., 2016; Dove et al., 2009). We analyzed cells expressing mApple-
221 Rab5 and the PI(3)P marker GFP-FYVE. As expected, Rab5 and PI(3)P appeared concomitantly
222 on enlarged early endosomes ([Fig 5, video Fig 5A supplement 1](#)). However, after Rab5 peaked,
223 we observed a slight delay in the disappearance of GFP-FYVE ([Fig. 5C, Fig. 5, figure supplement](#)
224 [1](#)), suggesting that the onset of PI(3)P to PI(3,5)P₂ conversion occurs with some delay.
225 Nevertheless, our data are consistent with a tight temporal and spatial regulation of PI(3)P
226 levels on endosomes during maturation.

227

228 **Snx1 recruitment is initiated with Rab5 recruitment and can persist during Rab7 stages**

229 A major property of endosomes is their ability to undergo extensive sorting, to recycle
230 components back to the cell surface and to the Golgi and to internalise membrane cargo
231 destined for ILV-mediated degradation. Our electron microscopy data provides evidence that
232 the nigericin-induced enlarged endosomes are capable of ILV formation and internalisation
233 of the GalT marker ([Fig 2B](#)). Additionally, the presence of Snx1-GFP in transient punctate
234 microdomains or tubular protrusions at the enlarged endosomes suggests active sorting from
235 the endosome to the plasma membrane and the Golgi ([Fig 6A](#)). To determine whether the
236 Snx1-mediated sorting is coordinated with Rab conversion, we analyzed cells co-expressing
237 Snx1-GFP and mApple-Rab5 ([Fig 6A](#)), and recorded the presence of Snx1 at the enlarged
238 endosomes as they acquired and removed Rab5. Snx1 assembly on endosomes occurred
239 concomitantly with Rab5 recruitment ([Fig 6 A-C; video Fig 6A supplement 1; Fig. 6, figure](#)
240 [supplement 1 A and B](#)), pointing to a potential coordination. Snx1 assembly at the endosomes
241 was highly dynamic, forming one or multiple domains at a time ([Fig. 6, figure supplement 1](#)
242 [C](#)). We observed weak correlation of Snx1 and Rab5 localization in discrete domains on early

Figure 6

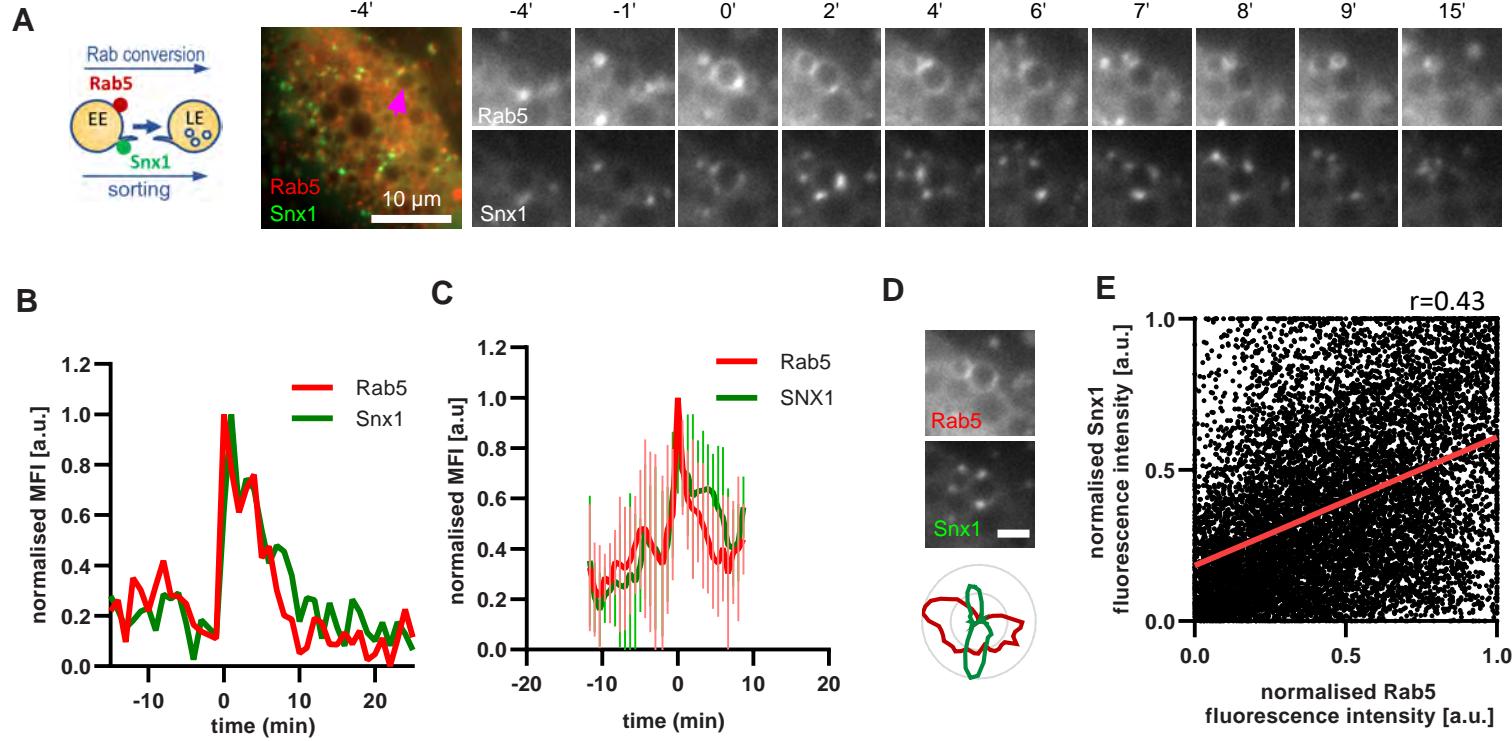


Figure 6. Snx1 subdomain formation at the endosomes initiates with Rab5 recruitment and peaks during Rab conversion stages.

HeLa cells, stably expressing mApple-Rab5 and transiently transfected with Snx1-GFP, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A) Time-lapse images of a representative endosome to show Snx1 subdomain formation relative to Rab5 recruitment.

(B) Corresponding graph of normalised mean fluorescence intensity (MFI) of Rab5 and Snx1 at the rim of the endosome in (A) over the time the endosome was detectable.

(C) Averaged Rab5 and Snx1 kinetics of 12 endosomes. Error bars represent standard deviation. Representative graph of three independent experiments. Numerical data for all analyzed endosomes is available in Figure 6C - Source Data 1.

(D) Images of Rab5 and Snx1 at an enlarged endosome and a corresponding line profile of normalised fluorescence intensity along the rim to show co-existence as well as independence of subdomains of the two markers. Scale bar = 2 μm.

(E) Correlation plot of normalised fluorescence intensity of Rab5 and Snx1 as measured in (D) for 14 endosomes for a total of 118 time points, and a corresponding regression line. Pearson's correlation $r=0.43$. Pooled data from two independent experiments. Numerical data for all analyzed endosomes is available in Figure 6E - Source Data 1.

Figure 7

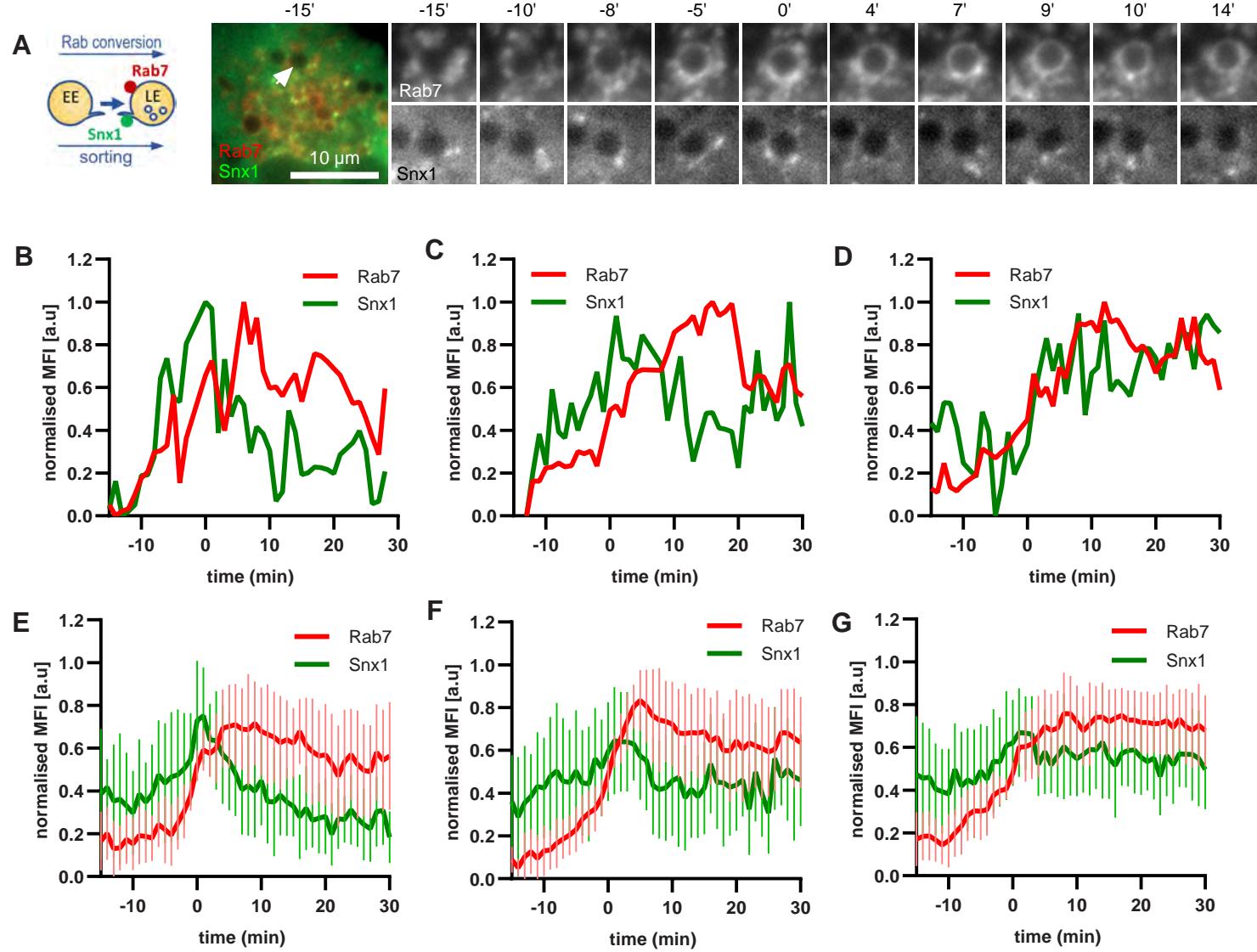


Figure 7. Snx1 subdomain formation at the endosomes initiates with Rab5 recruitment, peaks during Rab conversion stages and continues in late endosomes.

HeLa cells, stably expressing mApple-Rab7 and transiently transfected with Snx1-GFP, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A) Time-lapse images of a representative endosome to show Snx1 subdomain formation relative to and Rab7 recruitment.

(B) Corresponding graph of MFI of Rab7 and Snx1 at the rim of the endosome in (A) over the time the endosome was detectable, to show Snx1 peaking during Rab conversion.

(C,D) Additional graphs of MFI of Rab7 and Snx1 at the rim of endosomes to show the second Snx1 peak (C) or continuing Snx1 presence (D).

(E,F,G) Averaged Rab7 and Snx1 kinetics binned into the three patterns of Snx1 recruitment as observed in (B,C,D), representing 19, 21 and 20 endosomes for the single peak, double peak and continuing presence of Snx1, respectively. Error bars represent standard deviation. Three independent experiments were performed, and data pooled. Numerical data for all analyzed endosomes is available in Figure 7 - Source Data 1.

243 endosomes ([Fig 6D and E](#)). Moreover, Snx1 levels either declined during Rab conversion or
244 persisted for a while. Our data indicate that Snx1 recruitment on early endosomes occurs
245 simultaneously with Rab5, but that Snx1 microdomains could either co-exist with or exist
246 independently of Rab5, suggesting that Rab5 may promote Snx1 recruitment but is not
247 essential for its maintenance or dynamics at endosomes.

248 To corroborate the apparent lack of strict coordination between Rab5 removal and
249 Snx1 persistence at the endosomes, we co-expressed Snx1-GFP and mApple-Rab7 ([Fig 7A](#),
250 [video Fig 7A supplement 1](#)). Consistent with Snx1 presence during the Rab5 phase, Snx1
251 recruitment peaked during early stages of Rab7 recruitment, when the endosome is expected
252 to be Rab5-positive ([Fig 7B-D](#)). In about one third of all Rab7-positive endosomes analysed,
253 Snx1 recruitment was transient and was no longer present after Rab7 peaked or levelled off
254 to indicate completed Rab conversion ([Fig 7B and E](#)). In another third of analysed endosomes,
255 Snx1 initially displayed the same kinetics, but was recruited back again to the late Rab7-
256 positive endosome ([Fig 7C and F](#)), suggesting that Rab5 may be dispensable for Snx1
257 recruitment to late endosomes. In the remaining subset of endosomes, Snx1 peaked and
258 persisted throughout endosome maturation ([Fig 7D and G](#)). Analysis of Rab7 and Snx1
259 domains at the endosome again revealed weak correlation of Rab7 and Snx1 domains ([Fig. 6](#),
260 [figure supplement 1D and E](#)). Taken together, our data suggest that sorting into recycling
261 pathways is most likely initiated very early on endosomes but can also persist on late
262 endosomes, indicating a continuous process independent of Rab conversion.

263

264 **Interaction of early and late endosomes with Rab11 proceeds independently of Rab5 or**
265 **Rab7**

266 To further support our hypothesis that sorting can occur continuously from early to late
267 endosomes, we examined the behaviour of a key component of the recycling pathway to the
268 plasma membrane, Rab11. If our hypothesis were correct, we would expect Rab11 to show
269 similar behaviour as Snx1. Therefore, we investigated the dynamics of GFP-Rab11 in relation
270 to Rab5 or Rab7 positive endosomes. Rab11 docks on the tubular part of maturing/sorting
271 endosomes and promotes the recycling of cargo to the plasma membrane (Solinger et al.,
272 2020; van Weering et al., 2012). Surprisingly, Rab11 positive vesicles contacted early
273 endosomes even before strong Rab5 recruitment ([Fig 8A-C](#), [video Fig 8A supplement 1](#)).
274 Moreover, Rab11 interaction with endosomes appeared to be independent of Rab5, Rab7, or

Figure 8

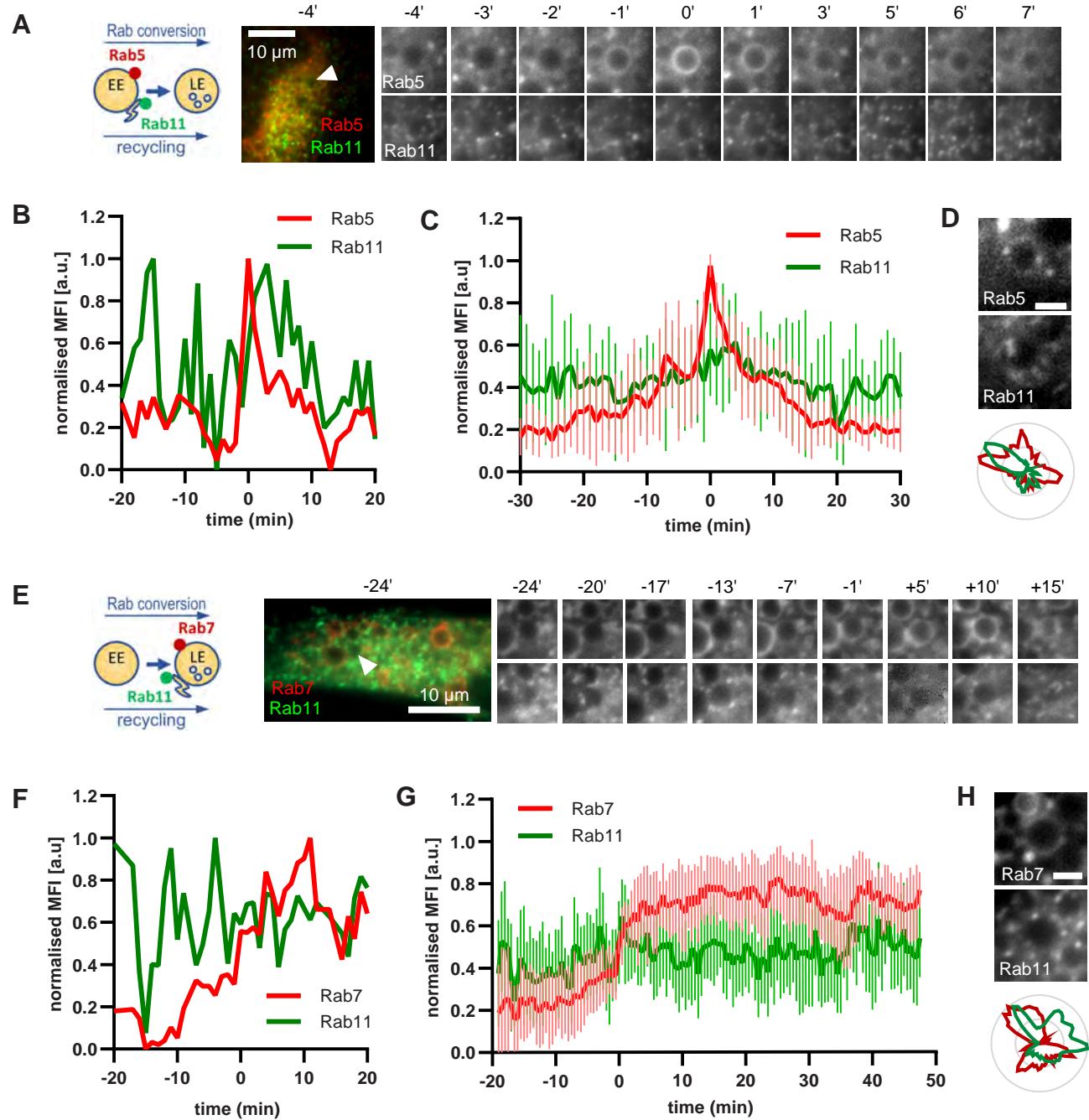


Figure 8. Rab11 interacts with the maturing endosome independently of Rab5 or Rab7.

HeLa cells, stably expressing mApple-Rab5 (A-D) or mApple-Rab7 (E-H) and transiently transfected with GFP-Rab11, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A,E) Time-lapse images of a representative endosome to show continuous Rab11 interaction with the maturing endosome relative to Rab5 (A) or Rab7 (E) recruitment.

(B,F) Corresponding graphs of normalised mean fluorescence intensity of Rab5 (B) or Rab7 (F) and Rab11 at the rim of the endosome in (A) or (E), respectively, over the time the endosome was detectable.

(C,G) Averaged Rab5 (C) or Rab7 (G) and Snx1 kinetics of 16 and 15 endosomes, respectively. Error bars represent standard deviation. Representative graphs each of three independent experiments. Numerical data for all analyzed endosomes is available in Figure 8C - Source Data 1 and Figure 8G - Source Data 1.

(D,H) Images of Rab5 (D) or Rab7 (H) and Rab11 at an enlarged endosome and corresponding line profiles of normalised fluorescence intensity along the rim to show co-existence as well as independence of subdomains of Rab11 and the two markers. Scale bar = 2 μm. Numerical data for analyzed endosomes is available in Figure 8D and S8 - Source Data 1 and Figure 8H and S8 - Source data 1.

275 Rab conversion as it continued throughout endosome maturation (Fig. 8, video Fig 8E
276 supplement 1; Fig. 8, figure supplement 1). Thus, our data indicate that, similar to sorting,
277 recycling to the plasma membrane appears to be largely decoupled from Rab conversion.
278

279 **GalT-pHlemon is a reliable reporter for pH measurements along the endocytic pathway**

280 Both, Rab conversion and acidification are essential for endosome maturation but how the
281 two are coordinated is poorly understood. To follow endosomal acidification at individual
282 endosome level, we have tested several available endosomal pH sensors, such as mApple-
283 Lamp1-pHluorin and NHE6-pHluorin2 (Ma et al., 2017), however, we found that these sensors
284 were predominantly retained in the ER following transient transfection in our system. We
285 exchanged the pHluorin tag on Lamp1 and NHE6 with the recently developed pH-responsive
286 ratiometric probe, pHlemon (Burgstaller et al., 2019), but this did not improve the export
287 from the ER. Therefore, we replaced the GFP of the GalT-GFP construct with pHlemon. Since
288 GalT was present at the Golgi-derived enlarged compartments, and later in Rab5-positive
289 endosomes and ILVs (Fig 2B, 3A-B), we hypothesised that the sensor anchored to GalT will
290 illuminate the entire endosome maturation pathway, from early endosomes to
291 endolysosomes. The pHlemon probe consists of yellow and mTurquoise2 fluorescent proteins
292 in tandem, with YFP reducing and mTurquoise2 slightly increasing fluorescence upon
293 acidification in a reversible manner (Burgstaller et al., 2019). Untreated cells expressing the
294 GalT-pHlemon sensor displayed a characteristic Golgi-ribbon appearance in both YFP and CFP
295 channels as well as punctate appearance of CFP signal alone, indicative of highly acidified
296 lysosomes or endolysosomes (Fig 9A). We could reliably detect YFP/CFP ratios over the pH
297 4.0-7.5 range (Fig 9B, Fig. 9, figure supplement 1), allowing for accurate pH measurements of
298 the entire endolysosomal pathway. Our sensor designated pH 6.2 for the Golgi-ribbon
299 structures and pH 4.0-5.7 for the lysosomes and endolysosomes, as detected by the CFP
300 puncta, in untreated cells (Fig 9C). Most importantly, our sensor located to the nigericin-
301 induced enlarged endosomes and indicated a pH range between 5.5 and 6.6 at 50 min
302 washout, reflective of the different stages of maturation (Fig 9D, video Fig 9D supplement 1).
303 Therefore, GalT-pHlemon is a useful tool to read-out pH in the endosomal system.
304

305 **Endosomal acidification is most pronounced during Rab conversion**

Figure 9

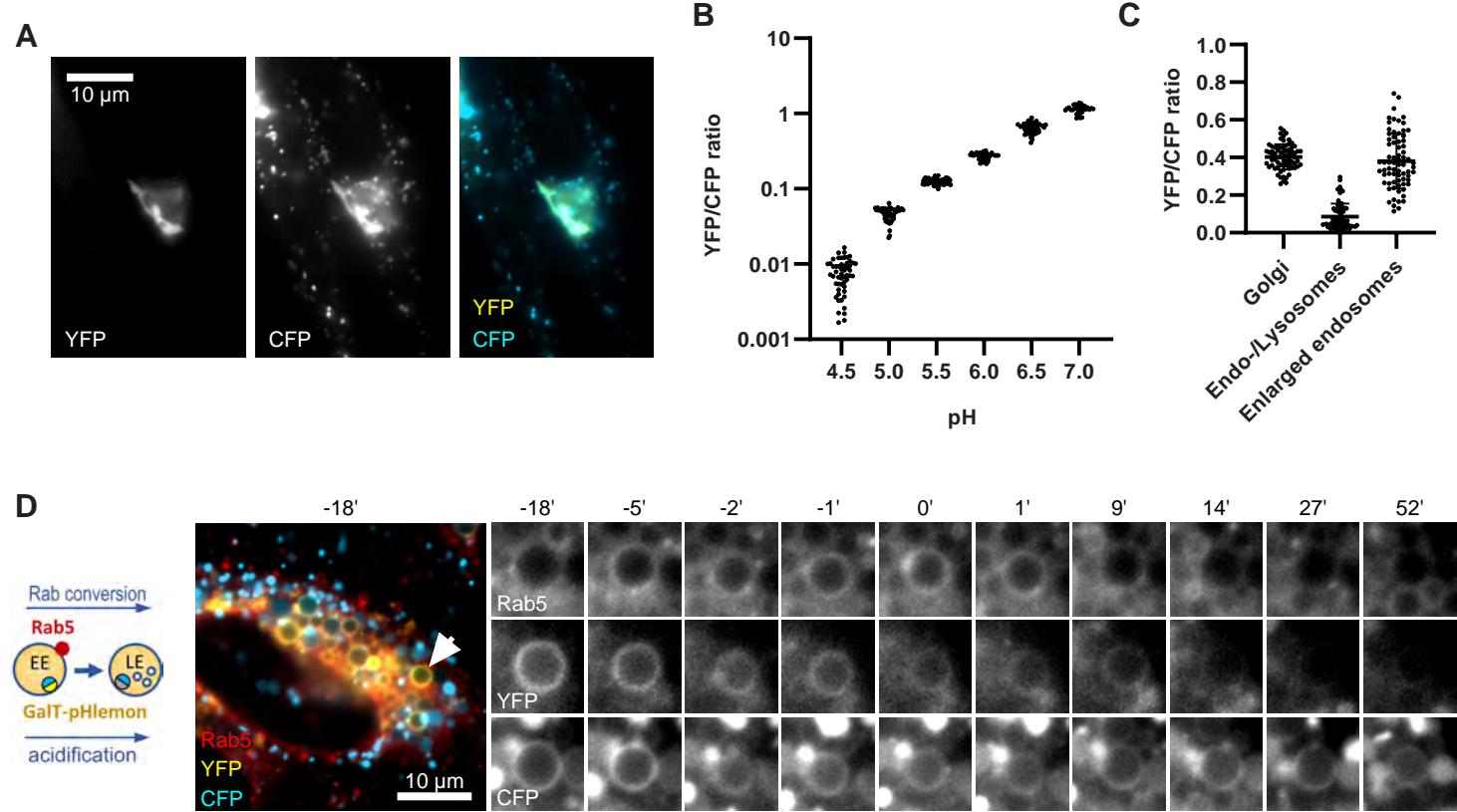


Figure 9. GalT-pHlemon sensor detects endosomal acidification.

(A-C) HeLa cells were transiently transfected with the ratiometric pH sensor, GalT-pHlemon.

(A) Images of cells to show Golgi-ribbon distribution of GalT-pHlemon in both YFP and CFP channels as well as cytosolic CFP-filled puncta in CFP channel only, representing highly acidified organelles.

(B) Graph to show robust response of GalT-pHlemon sensor to pH 4.5-7.0 range as displayed by YFP/CFP ratio measurements in cells incubated with calibration buffers of specified pH values. Numerical data for all analysed Golgi ROIs is available in Figure 9B - Source Data 1.

(C) YFP/CFP measurements of GalT-pHlemon in the Golgi ribbon, endo-/lysosomes (cytosolic CFP puncta), as well as in the enlarged endosomes post 20 min nigericin treatment and 100 min recovery. Numerical data for all analysed organelle ROIs is available in Figure 9C - Source Data 1.

(D) HeLa cells, stably expressing mApple-Rab5 and transiently transfected with GalT-pHlemon, were treated for 20 min with nigericin, washed and imaged over 3 h. Images show GalT-pHlemon sensor localising to the enlarged transiently Rab5-positive endosome and changing YFP and CFP intensity consistent with endosomal acidification.

Figure 10

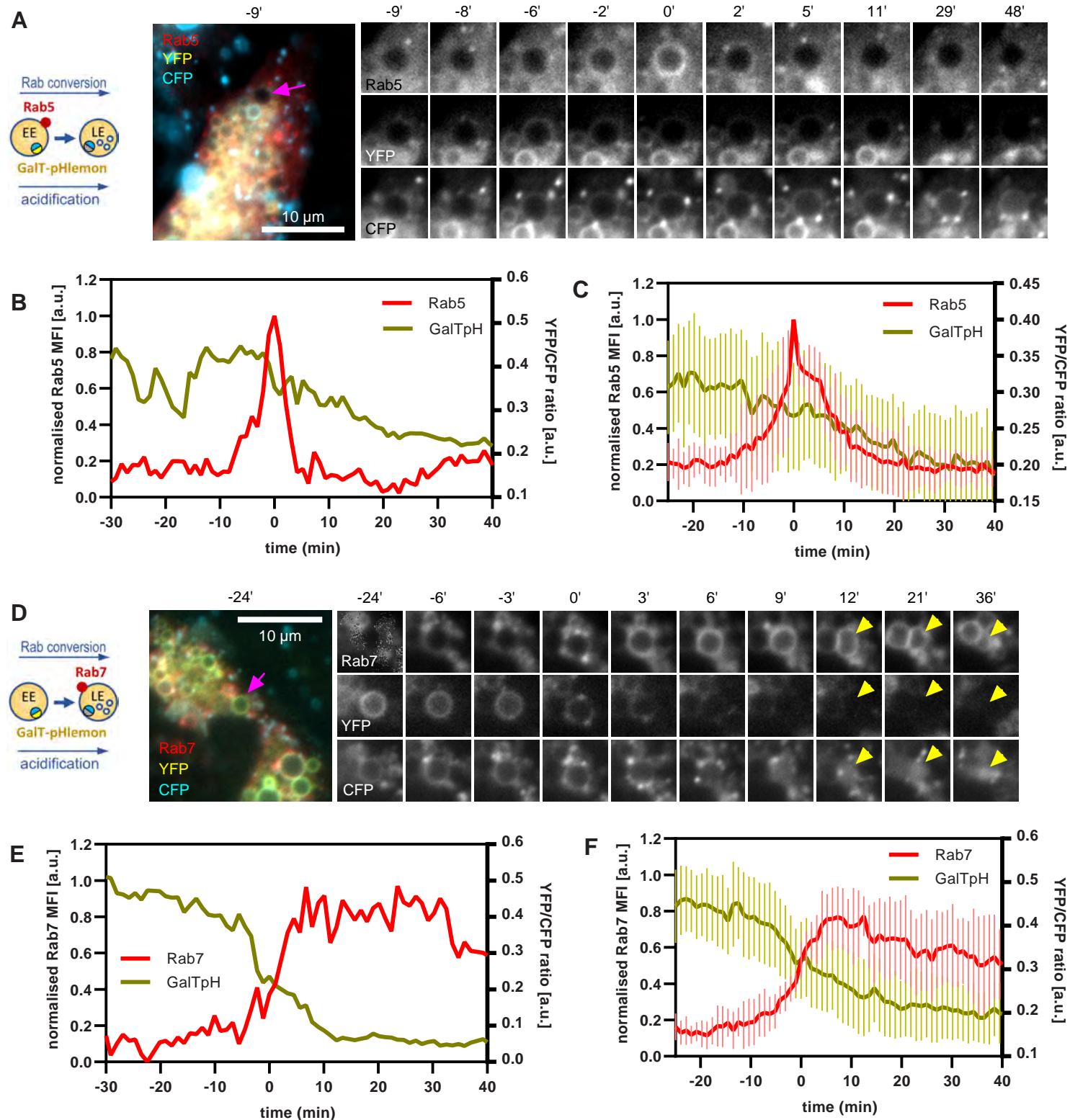


Figure 10. GaT-pHlemon sensor detects endosomal acidification, which correlates with Rab conversion.

HeLa cells, stably expressing mApple-Rab5 (A-C) or mApple-Rab7 (D-F) and transiently transfected with GaT-pHlemon, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A,D) Time-lapse images of a representative endosome to show association of Rab conversion with acidification, as detected by the decrease in the YFP signal and relatively constant CFP at the rim.

(B,E) Corresponding graphs of normalised mean fluorescence intensity of Rab5 (B) or Rab7 (E) and YFP/CFP ratio of the endosomal GaT-pHlemon signal at the rim of the endosome in (A) or (D), respectively, during and around the time of Rab conversion.

(C,F) Averaged Rab5 (C) or Rab7 (F) and GaT-pH kinetics of 15 and 18 endosomes, respectively. Error bars represent standard deviation. Pooled data from two independent experiments. Numerical data for all analysed endosomes is available in Figure 10C - Source Data 1 and Figure 10F - Source data 1.

306 Equipped with a sensor locating to endosomes and responding to endosomal pH changes, we
307 investigated the kinetics of endosomal acidification relative to Rab5 and Rab7 recruitment,
308 using cells stably expressing mApple-Rab5 or mApple-Rab7 and transiently transfected with
309 GaltT-pHlemon. The nigericin-induced enlarged endosomes showed a dramatic decrease in
310 YFP signal, which always coincided with Rab5-positive stages of endosome maturation ([Fig 10A, video Fig 10A supplement 1](#)) and with early phases of Rab7 recruitment ([Fig 10D, video Fig 10D supplement 1](#)). Ratiometric quantifications of intraluminal YFP and CFP signals
311 revealed relatively stable YFP/CFP ratio prior to Rab5 recruitment, followed by a sharp
312 decrease during Rab5 recruitment and Rab conversion, and stabilisation of a new, lower
313 YFP/CFP ratio in Rab7-positive endosomes ([Fig 10B, C, E and F](#)). Conversion of YFP/CFP ratios
314 to pH values indicates an average pH of 6.6 in early endosomes prior to Rab5 recruitment and
315 a final pH of 5.7 in Rab7 endosomes. Our data indicate that the biggest pH drop occurs
316 concomitantly with Rab5-to-Rab7 exchange, pointing to a regulation of acidification during
317 endosome maturation.
318

319

320

321 **Impaired Rab conversion is associated with slower endosomal acidification**

322 If endosomal acidification is dependent on the progression of endosome maturation, then
323 blocking endosome maturation by impairing Rab conversion should undermine acidification.
324 To block Rab conversion, we knocked out the Ccz1, a subunit of the Rab7GEF, which has been
325 shown to promote Rab conversion ([Fig. 11, figure supplement 1A](#)) (Nordmann et al., 2010;
326 Poteryaev et al., 2010; van den Boomen et al., 2020). Ccz1 depletion abolished Rab7
327 recruitment to, and Rab5 removal from, the nigericin-induced enlarged endosomes ([Fig 11A, videos Fig 11A supplement 1-3](#)). Ccz1-deficient Rab5-positive endosomes could engage in
328 homotypic fusion and interact with Rab5-positive enlarged compartments but did not mature
329 to classical endolysosomes [Figure 11, figure supplement 1B, Fig 1C](#)). These perturbations
330 could be efficiently rescued by expression of wild-type Ccz1 in Ccz1 knock-out cell lines ([Fig. 11A, videos Fig 11A supplement 1-3](#)). To ensure that in rescue experiments we selected for
331 analysis only the cells expressing Ccz1, and not untransfected cells, we appended a far-red
332 fluorophore mNeptune2 via the T2A peptide linker to Ccz1, resulting in expression of the two
333 separate proteins in the transfected cells ([Fig. 11, figure supplement 2A](#)). The mNeptune2 was
334 tagged with NLS, targeting it to the nucleus, to minimise interference with the mApple signal
335 at the endosomes ([Fig. 11, figure supplement 2B](#)). Hence, we have generated Ccz1 knock-out
336

Figure 11

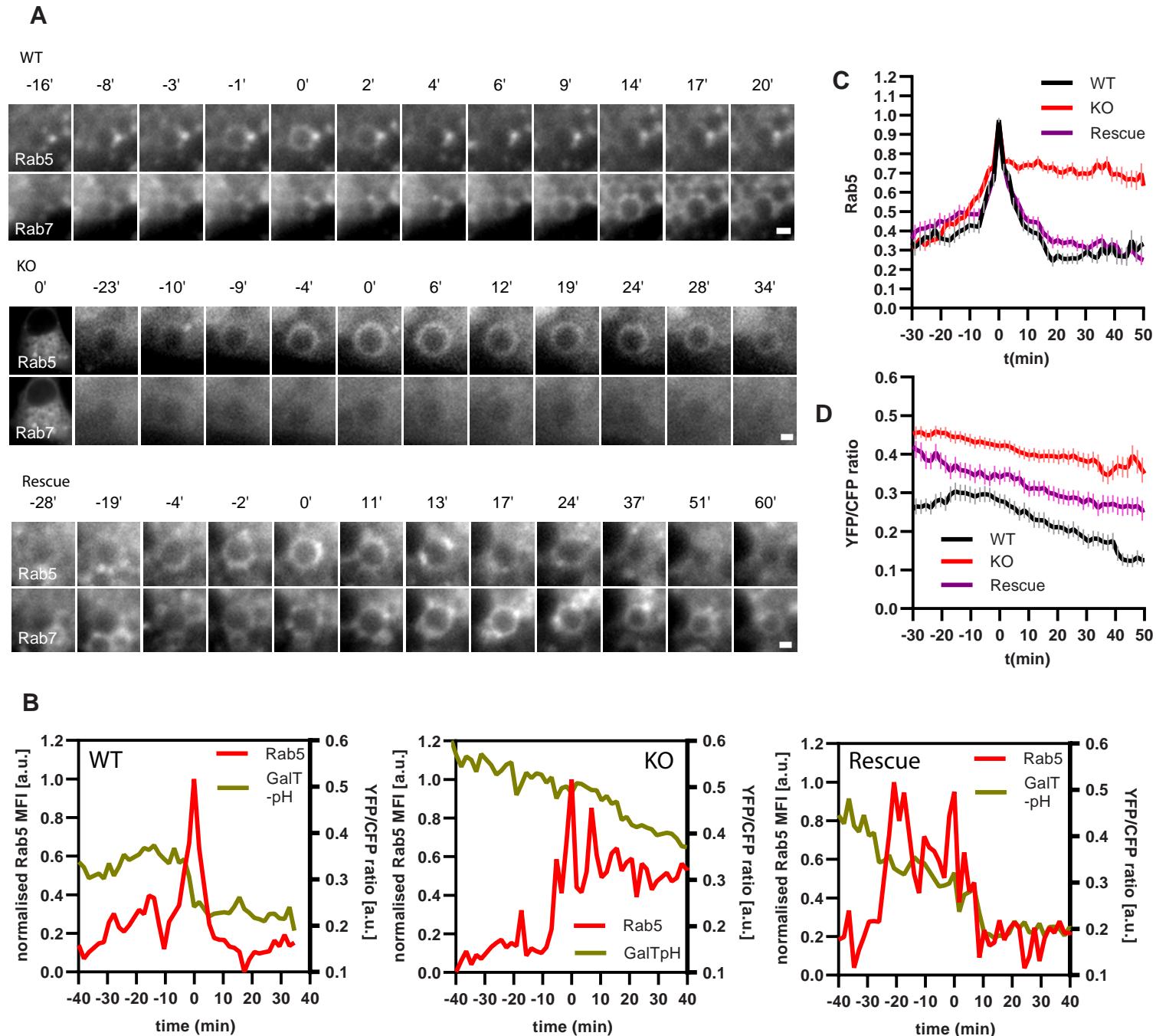


Figure 11. Ccz1 KO disrupts Rab conversion and delays endosomal acidification.

HeLa cell lines with wild-type (WT) Ccz1 and knocked-out Ccz1 (KO) were transiently transfected with mApple-Rab5 and either GFP-Rab7 (A) or GalT-pHlemon (B-D). Ccz1 expression plasmid was co-transfected for 72 h for rescue experiments. Nigericin was added to cells for 20 min and washed away, and cells were imaged by time-lapse microscopy, as described in Figure 4A.

- (A) Time-lapse images of representative endosomes to show absence of Rab7 recruitment and the associated lack of displacement of Rab5 in KO cells, compared to the expected Rab conversion in WT and rescue cells. Scale bar = 1 μ m.
- (B) Graphs of normalised mean fluorescence intensity of Rab5 and YFP/CFP ratio of the endosomal GalT-pHlemon signal in representative endosomes during and around the time of Rab conversion / Rab5 peak, in WT, KO and rescue cells.
- (C,D) Averaged kinetics of Rab5 recruitment (C) and corresponding GalT-pHlemon YFP/CFP ratios (D) for WT, KO and rescue cells, in 54, 54 and 56 endosomes, respectively. Error bars represent SEM. Pooled data from three independent experiments using different Ccz1 clones. Numerical data for all analysed endosomes is available in Figure 11 - Source Data 1.

Figure 12

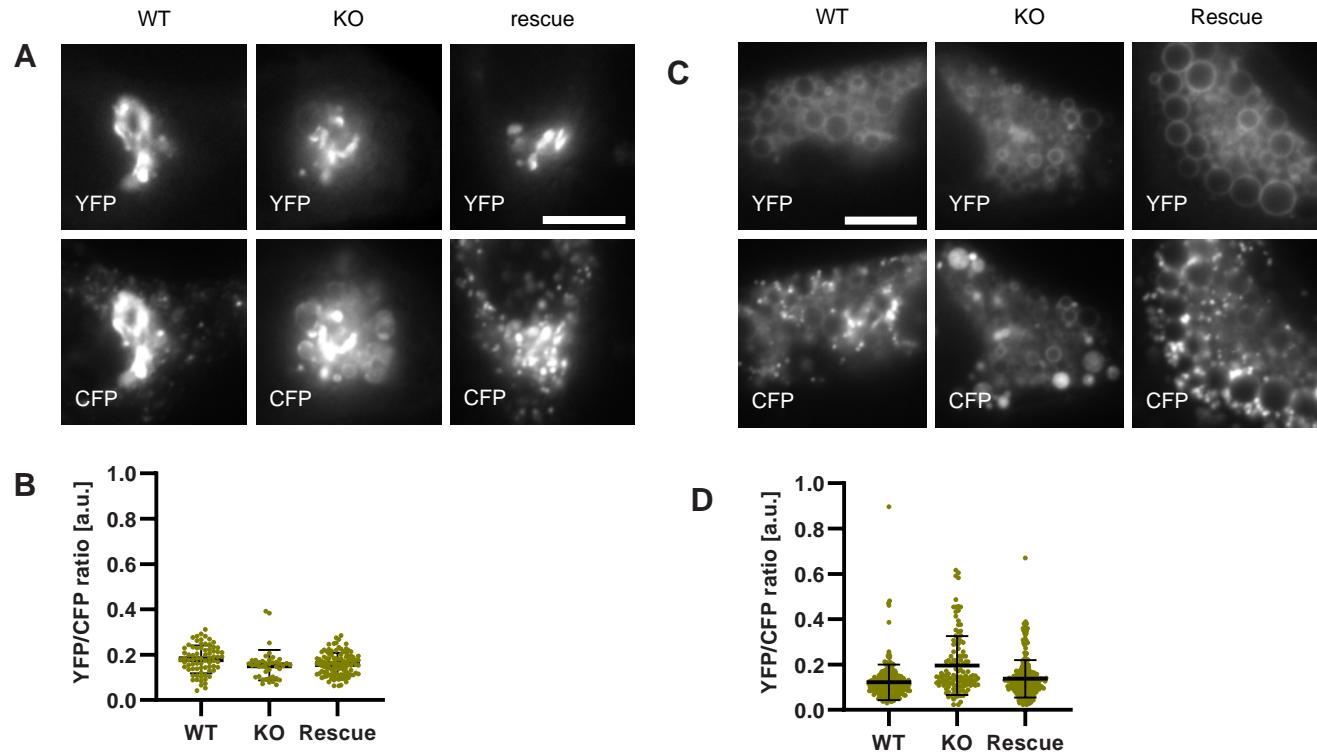


Figure 12. Lysosomes of Ccz1 knockout cells can acidify to the same extent as wild-type cells, with some delay.

HeLa cell lines with wild-type (WT) Ccz1 and knocked-out Ccz1 (KO) were transiently transfected with Galt-pHlemon. Ccz1 expression plasmid was co-transfected for 72 h for rescue experiments. WT, KO and rescue cells were left untreated (A,B) or treated with 20 min nigericin followed by 50 min recovery (C,D).

(A) Images of cells showing Golgi and highly acidified organelles as visualised with Galt-pHlemon. Acidified organelles appear as puncta in WT and rescue cells and as larger round CFP-filled compartments in KO cells. Scale bar = 10 μ m.

(C) Images of cells pre-treated with nigericin have dispersed trans-Golgi and re-acidified organelles as in (E). Scale bar = 10 μ m.

(B,D) Corresponding measurements of YFP/CFP ratio of the Galt-pHlemon sensor in the acidified organelles. Numerical data for all analysed endosomes is available in Figure 12B - Source Data 1 and Figure 12D - Source Data 1.

338 cell lines that showed impaired Rab conversion and could be efficiently rescued with the Ccz1
339 rescue construct.

340 To test our hypothesis that impaired Rab conversion compromises endosomal
341 acidification, we expressed mApple-Rab5 and GalT-pHlemon in Ccz1-KO and control cells, and
342 monitored the YFP/CFP ratio kinetics of the pH sensor on Rab5-positive endosomes ([Fig 11B](#),
343 [Fig. 11, figure supplement 1B](#)). While in the control and Ccz1 rescue cells, acidification
344 occurred with similar kinetics as observed above ([Fig 10B and C](#)), in Ccz1 KO cells the
345 acidification was strongly delayed ([Fig 11B-D](#)). Nevertheless, acidification occurred eventually
346 after a long delay in Ccz1 KO cells. In line with this conclusion, Ccz1 KO cells have grossly
347 enlarged CFP-filled puncta and compartments, reflective of their enlarged lysosomes and
348 acidified hybrid compartments as also observed with Lysotracker staining of Rab5-positive
349 compartments ([Fig 12A](#), [Fig 12, figure supplement 1](#)). The CFP-positive compartments in
350 untreated cells showed no differences in pH between Ccz1-replete and Ccz1-deficient cells,
351 ranging from pH 5.7 to pH 4.0, indicative of endolysosomes and lysosomes, respectively ([Fig](#)
352 [12B](#)). Furthermore, following disruption of pH by nigericin treatment and washout, wild-type
353 cells restored their lysosomal pH, while Ccz1 KO cells displayed a wide range of pH in
354 pHlemon-filled compartments, ranging from pH 6.4 to pH 4.0 ([Fig 12C and D](#)). Taken together,
355 our data suggest that Rab conversion is driving efficient endosomal acidification.

356

357

358 **DISCUSSION**

359

360 Despite the pivotal importance of endosomal transport for cell survival and communication,
361 tissue organization and development in all eukaryotic cells, endosomes largely escape largely
362 precise temporal and spatial observations because of their small size and mobility. Several
363 approaches using enlarged endosomes with low motility have successfully been used in the
364 past, however, these were restricted to a particular cell type and organism or a limited subset
365 of endosomes (Poteryaev et al., 2010; Skjeldal et al., 2021; van Weering et al., 2012). We
366 report here an easy, reliable and inexpensive method to enlarge endosomes in a variety of
367 different cell types and follow them over their entire lifetime - from initial formation and
368 subsequent maturation to endolysosome formation and lysosome maturation. Importantly,
369 our acute nigericin treatment takes advantage of the natural, physiological cellular stress

370 response and does not cause any autophagy induction, lysosomal damage, cell death or
371 growth retardation. Moreover, this assay does not require any special equipment; a standard
372 fluorescence microscope equipped with commonly used filters and a camera are sufficient.
373 We validated our assay by comparing our measured parameters of endosome maturation
374 with published data. Most importantly, Rab5-to-Rab7 conversion progressed with previously
375 published kinetics (Poteryaev et al., 2010; Rink et al., 2005; Skjeldal et al., 2021; van der
376 Schaar et al., 2008). In addition, PI(3)P was produced on Rab5 endosomes at about the same
377 rate, in which Rab5 levels increased, consistent with the positive feedback loop between Rab5
378 and the PI(3)P kinase Vps34 (Zerial and McBride, 2001). While the PI(3)P levels coincided with
379 the Rab5 peak, we observed a short delay in decrease of the FYVE-GFP. This finding is
380 consistent with a previous report, which described *C. elegans* Rab7GEF recruitment to PI(3)P-
381 rich endosomes (Poteryaev et al., 2010) to drive Rab conversion. Additionally, Vps34 removal
382 and subsequent PI(3)P turnover is dependent on low luminal pH as characteristic of late
383 endosomes (Naufer et al., 2018; Podinovskaia et al., 2013). It is conceivable that the slight
384 delay in reducing PI(3)P levels might provide directionality during Rab conversion. Thus, our
385 assay faithfully recapitulates endosome maturation as described in other model systems.

386 We used the established assay to determine whether there is a strict coordination
387 between recycling to the plasma membrane and Rab conversion. There is evidence in the
388 literature that recycling to the plasma membrane only happens from Rab5 compartments or
389 from Rab5 and Rab7 compartments (van Weering et al., 2012). Our data indicate that
390 recycling to the plasma membrane can happen throughout the lifetime of an endosome. We
391 observed that SNX1 was recruited simultaneously with Rab5 probably mediated through its
392 PX domain (Peter et al., 2004), consistent with the reported coordination between the two
393 proteins (van Weering et al., 2012). The PX domain recognises PI(3)P, which is recruited
394 concomitantly with Rab5 to maturing endosomes. However, even though in about one third
395 of the endosomes, there seemed to be temporal coordination between SNX1 and Rab5
396 removal from the endosome, sorting persisted in the remaining Rab7-positive endosomes. In
397 addition, we did not observe any spatial coordination on the endosomal membrane as the
398 SNX1 and Rab5 or Rab7 domains appeared to move independently. Moreover, Rab11
399 contacted maturing endosomes irrespective of whether they were Rab5 or Rab7 positive.
400 Therefore, our data suggest that the onset of recycling is coordinated with the arrival of Rab5,
401 at least for Snx1, but the process itself is independent of Rab conversion, as previously

402 suggested (Rojas et al., 2008). Consistently, we have shown previously that when Rab
403 conversion is blocked, Rab11 localization and Rab11-dependent recycling are not affected in
404 *C. elegans* oocytes (Poteryaev et al., 2010; Poteryaev et al., 2007).

405 Although acidification of endosomes is required for endosome maturation
406 (Podinovskaia and Spang, 2018), how this process is regulated remains poorly understood.
407 Since Rab conversion is a major driver of endosome maturation, we asked whether Rab
408 conversion regulates endosomal acidification. Unfortunately, all pH sensor probes we tried
409 turned out not to be useful because they were mostly stuck in the ER. While in neurons, in
410 which the probes have mainly been applied, this might be less of an issue, in our system this
411 has prevented any meaningful analysis. We, hence, developed a new probe based on the
412 ratiometric pHlemon and GALT, which localises to Golgi but enters also the endolysosomal
413 pathway. With this new probe, we showed that Rab conversion is required for efficient
414 acidification. Over extended times, acidification of endosomes still occurred in absence of Rab
415 conversion and we speculate that this acidification can help drive fusion with lysosomes.
416 Moreover, this effect may explain why a *sand-1* mutant in *C.elegans*, knockdown of Mon1a
417 and b, or a Ccz1 KO in mammalian cells is not lethal (Poteryaev et al., 2010; Poteryaev et al.,
418 2007). How Rab conversion promotes a drop in pH, we can only speculate at this point. It is
419 possible that the activity of the V-ATPase is upregulated during Rab conversion, driven by
420 Rab7 effectors such as RILP (Bucci et al., 2000; De Luca and Bucci, 2014; De Luca et al., 2014),
421 and is accompanied by the arrest in interactions with less acidified endocytic vesicles. It is also
422 conceivable that there is a simple regulatory loop such as phosphorylation and
423 dephosphorylation of V-ATPase subunits, however, the potential kinase or phosphatase
424 regulators remain to be identified. Additionally, proton channels such as Nhe6 have been
425 reported to finetune endosomal pH, and their role in endosome acidification kinetics remains
426 to be explored. Finally, multiple factors are known to interact with the V-ATPase; their role in
427 controlling the acidification during Rab conversion remains to be established.

428 In conclusion, we have developed and validated a straightforward live-cell imaging
429 assay and used it to define kinetics and regulation of mediators of endosomal maturation.
430 This assay will be invaluable for addressing outstanding questions relating to the regulation
431 and potential coordination of processes during endosome maturation. Given its applicability
432 to different cell types, it may also be useful in establishing cell-type specific differences in the
433 regulation of endosomal transport. The assay has been designed to make it accessible and

434 applicable in most cell biology laboratories and proved to be a powerful tool to further our
435 understanding of endosome biology.

436

437

438

439 MATERIALS AND METHODS

440

441 Cell culture

442 HeLa CCL2 and HeLa Kyoto- α cells were grown at 37°C and 5% CO₂ in high-glucose Dulbecco's
443 modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum
444 (FCS, Biowest), 2 mM L-Glutamine, 1 mM Sodium Pyruvate, and 1x Penicillin and Streptomycin
445 (all from Sigma).

446 For transient cell transfections, cells were plated into 6-well plates to reach 70%
447 confluence the following day and transfected with 0.5 μ g plasmid DNA complexed with Helix-
448 IN transfection reagent (OZ Biosciences). Cells were used in imaging assays at 48 h post
449 transfection. For the Ccz1 rescue experiments, given the larger size of the plasmid, 2 μ g
450 plasmid DNA was used, and cells were transfected for 72h.

451

452 For cell growth assays, cells were plated into 12-well plates at 10,000 cells per well. The
453 following day, a sample was taken for counting (0 h time point), and the remaining cells were
454 incubated in complete medium with or without nigericin for 20 min, washed, incubated for
455 specified time periods and collected for counting. Both conditions were reaching 90%
456 confluence by 72 h. Three wells were measured per condition. Doubling time was calculating
457 using the formula

$$458 (t_1 - t_2) * \frac{\log 2}{\log(\frac{c_2}{c_1})}$$

459 where c1 and c2 are cell counts for two consecutive time points t1 and t2.

460

461 DNA constructs

462 mApple-Rab5a, mApple-Rab7a, GalT-mCherry, mNeptune2-C1 and mApple-Lamp1-pHluorin
463 plasmids were a gift from Michael Davidson (Addgene # 55944, 54945, 55052, 54836, 54918).
464 GFP-Rab11a was a gift from Richard Pagano (Addgene # 12674) (Choudhury et al., 2002).

465 EGFP-2xFYVE was a gift from Harald Stenmark (Addgene # 140047) (Gillooly et al., 2000).
466 Lamp1-GFP was a gift from Esteban Dell'Angelica (Addgene #34831) (Falcon-Perez et al.,
467 2005). pSpCas9(BB)-2A-GFP (pX458) and pSpCas9(BB)-2A-puro (pX459) were a gift from Feng
468 Zhang (Addgene #48138 and #48139) (Ran et al., 2013). Snx1-turboGFP and Ccz1-myc were
469 from Origene (#RG201844 and RC222195). GFP-Rab7a was generated by substituting mApple
470 in the Rab7a plasmid with GFP from GFP-Rab11a using NheI and Xhol restriction sites.

471 For stable GalT-GFP cell line generation, a sequence encoding GalT-EGFP (B4GALT1
472 ORF minus the catalytic moiety) was generously provided by Jennifer Lippincott-Schwartz
473 (Howard Hughes Medical Institute, Ashburn, VA). GalT-EGFP was amplified using primers with
474 restriction site overhangs for NotI and PstI, and subcloned into the Retro-X Q vector pQCXIP
475 (Takara Bio). The GalT-EGFP plasmid for transient transfections was generated from GalT-
476 mCherry plasmid and the EGFP insert from LAMP1-EGFP, using NEBuilder HiFi Assembly
477 cloning kit (New England Biolabs, NEB) with the primers designed by the NEBuilder Assembly
478 Tool ([table 1](#)) following manufacturer's instructions.

479 For Ccz1 rescue experiments, the myc tag was removed from the Ccz1-myc plasmid
480 using NEB site-directed mutagenesis (SDM) kit (NEB) following manufacturer's instructions
481 and primers selected using NEBaseChanger tool ([table 1](#)). Nuclear localisation sequence (NLS)
482 was cloned at the N terminal of mNeptune2 using NEB SDM kit with the mNeptune2-C1
483 plasmid following manufacturer's instructions and primers listed in table S1. To generate NLS-
484 mNeptune2-T2A-ccz1 and NLS-mNeptune2-T2A-ccz1-myc, primers were designed with
485 NEBuilder Assembly Tool (NEB) as listed in [table 1](#) to generate PCR products from NLS-
486 mNeptune2, pSpCas9(BB)-2A-GFP and ccz1 plasmids. Purified PCR products for NLS-
487 mNeptune2 and T2A peptide were ligated together using forward primer for mNeptune2 and
488 reverse primer for T2A in a PCR reaction with 5 cycles at 50°C and 25 cycles at 63.7°C (i.e. the
489 annealing temperature of the two primers). Purified NLS-mNeptune2-T2A and ccz1 backbone
490 PCR products were assembled together using NEBuilder HiFi Assembly cloning kit (NEB).

491 For the pH sensor constructs, we used pHlemon, which consists of mTurquoise2 and
492 EYFP in tandem with a 21bp linker in between (Burgstaller et al 2019). Separate constructs
493 for mTurquoise2+half-the-linker and half-the-linker+EYFP were synthesized by Twist
494 Bioscience ([table 1](#)). The two sequences were cloned separately into pCR Blunt II-Topo vectors
495 (Invitrogen). For the GalT-pHlemon plasmid, primers were designed with NEBuilder Assembly
496 Tool ([table 1](#)) to generate PCR products for the mTurquoise2 and EYFP as well as the GalT

497 backbone without the tag from GalT-mCherry plasmid. Purified PCR products were assembled
 498 together using NEBuilder HiFi Assembly cloning kit.

499

500 **table 1. Oligonucleotide sequences used to generate specified constructs**

Oligonucleotide description/ purpose	Oligonucleotide sequence(s)	
Generate ccz1 plasmid without myc tag using site-directed mutagenesis		
Remove myc from ccz1-myc plasmid	taaacggccggccgcgtt	atccaagaagaagatgttgtgaactgcgttgac
Generate mNeptune2 plasmid with NLS inserted at N-terminal using site-directed mutagenesis		
Append NLS to mNeptune2	gcggaaggctgtctaaaggcgaaagagc	ttttttttggcatggggcaccggtag
Generate NLS-mNeptune2-5-T2A-ccz1 plasmid using NEBuilder HiFi Assembly		
Amplify NLS-mNeptune2	tgcgcatttcgttacagctcgccatg	gatctggccgcgcgtccatgcacaaagaagcgg
Amplify T2A from px458	ccggccgcgcgtcagccattggccaggattctcctc	gctgtacaaggaaattcggcagtggagag
Amplify ccz1 plasmid	ggcgatcgccgcggcagatc	atggctgcagcggcggcc
Generate Topo plasmids with mTurquoise2 and EYFP sequences		
mTurquoise2 +half-the-linker sequence	atggtgagcaaggcgaggagctgttacccgggtggccatcctgtcgagctggacggcactaaacggccaca agttcagcgtgtccggcgaggcgatggcgatccacctacggcaagctgaccctgaagttcatctgcaccacggcaag ctggccgtgccctggcccacccctgtgaccacccctgtctggcgatcgtgtccgcgttgcaccatccatggccatcatgg agcacgacttcaagtccgcattgcgcaggctacgtccaggaggccaccatcttcaggacgcggcaactaca agaccgcgcgaggtaagttcgagggcgacccctgtgaccgcacccatgtcgactgacttgcaggatcaggag cgcaacatcctgggcacaagctggaggataactacttcaggacgcacgtctatatcccgccgacaaggcaga ggcatcaaggccaacttcaagatccgcacaacatcgaggacggccgtgcagctccgcaccactaccaggaga ccccatcgccgacggccctgtctccgcacaaccactaccctgacgcaccctgtcaagctgagcaa gagaagcgcgatcatggctgtggagttcgatccgcgggatcactctggcatggacgcagctgtacaagG GTGGAGGCG	
half-the-linker +EYFP sequence	GTAGCGAATTCatggtgagcaaggcgaggagctgttacccgggtggccatcctgtcgagctggacggc gtaaacggccacaagttcagcgtgtccggcgaggcgatggcgatccacctacggcaagctgaccctgaagttcatctg caccacggcaagctgcctgtccctggcccacccctgtgaccacccctgtgcaccatccatggccgtc gaccatcgaggacttcaagtccgcattgcgcaggctacgtccaggaggccaccatcttcaggac gacggcaactacaaggacccgcgcgaggtaagttcgagggcgacccctgtgaccgcacgt acttcaaggaggacggcaacatcctgggcacaagctggaggataactacaacaggccacaacgt caaggcacaacgtctatcatggcc caaggcacaacggcatcaaggtaacttcaagatccgcacaacatcgaggacggcagcgtgc ctaccaggagaacccccatcgccgacggccctgtgtccgcacaaccactaccctg gacgcacttccatggccgttgcaccgcgggatcactctggcatggac gagctgtacaagta	
Generate GalT-pHlemon plasmid using NEBuilder HiFi Assembly (substitute mCherry with pHlemon)		
Amplify GalT plasmid minus mCherry	agcggccgcgactcttagat	ggggcgaccgggtggatc
Amplify mTurquoise2	aggatccaccggcgccaccatggtagcgaggcaaggcgat	aattcgctaccgcctccacccttgcac
Amplify EYFP	ggtagggcggtcgaaattcatggtag	gatcttagatcgccgcgttacttgtacagctcg
Generate GalT-EGFP plasmid using NEBuilder HiFi Assembly (substitute mCherry with EGFP)		

Amplify GalT plasmid minus mCherry	agcggccgcgactctagat	ggtggcgaccgggtggatc
Amplify EGFP from Lamp1-EGFP	aggatccaccggtcgccaccatggtagagcaaggcgag	gatctagagtcgcggccgtttacttgtacagctcgccatg

501

502

503 **Fluorescent cell line generation**

504 To generate GalT-EGFP stable cell lines, Phoenix Amphi packaging cells (from the Nolan lab,
505 Stanford University) were grown in complete medium supplemented with 1 mM sodium
506 pyruvate and transfected with pQCXIP-GalT-EGFP using FuGENE HD (Promega) to produce
507 viral particles. The viral supernatant was harvested after 48-72 h, passed through a 0.45 µm
508 filter, supplemented with 15 µg/ml polybrene, and added to target HeLa-α cells. The next day,
509 complete medium with 1.5 µg/ml puromycin was added. Following selection, cells were
510 subjected to cell sorting on a FACSaria III (BD Biosciences) to obtain a cell pool with
511 homogenous expression levels. HeLa-α-GalT-EGFP were maintained in complete medium
512 supplemented with 1.5 µg/ml puromycin at 37°C in 5% CO₂.

513 Stable expression of mApple-Rab5 in HeLa cells was achieved by transfection of HeLa
514 CCL2 cells with the mApple-Rab5a plasmid and three rounds of bulk-sorting by FACS at 15, 33
515 and 61 days post transfection. The cell line stably expressing mApple-Rab7a were generated
516 as previously described (Wu et al., 2020). Briefly, HeLa CCL2 cells were transfected with the
517 mApple-Rab7a plasmid, FACS-sorted for mApple-positive cells at 20 days and into 96-well
518 plates 15 days later for clonal expansion. To generate the cell line with stable expression of
519 both, mApple-Rab5 and GFP-Rab7, stably-expressing mApple-Rab5 cells were transfected
520 with GFP-Rab7 and FACS-sorted at 7 days post transfection for a bulk population of mApple-
521 positive and GFP-positive cells and, a further 11 days later, into 96-well plates for clonal
522 expansion. The doubly-positive colonies were bulk-sorted once again 50 days later to remove
523 cells that were no longer expressing either marker.

524

525 **CRISPR-Cas9 knock-out of Ccz1**

526 Ccz1 has a homolog, Ccz1b, which differs by 4 nucleotides and is identical in amino acid
527 sequence. Guide RNAs (gRNA) were designed to target both genes simultaneously. The
528 CRISPR strategy was described previously (Ran et al., 2013; Solinger et al., 2020). Briefly,
529 based on gRNAs targeting the first and last exons of Ccz1, two double-stranded

530 oligonucleotide sequences were cloned one each into the two plasmids, Px458 (GFP) and
531 Px459 (Puro) and transfected into HeLa CCL2 cells. Plasmids without the inserts were used as
532 controls. After 24 h of transfection, cells underwent 24 h selection with puromycin, followed
533 by single-cell FACS sorting of GFP-positive cells for clonal expansion. Clones showing >90%
534 reduction in Ccz1 expression were used for evaluation of endosome maturation. Three
535 different ccz1-deficient (KO) clones and three different control clones (WT) were used in
536 experiments to obtain the three biological replicates.

537

538 **Live cell imaging**

539 Cells were plated into 8-well imaging chambers (Miltenyi) the day before imaging to reach
540 50-70% confluence on the day of imaging. Just prior to imaging, cell medium was replaced
541 with pre-warmed imaging buffer (5 mM Dextrose, 1 mM CaCl₂, 2.7 mM KCl, 0.5 mM MgCl₂ in
542 PBS supplemented with 10% FCS and Penicillin and Streptomycin). Where specified, cells
543 were treated with 10 µM Nigericin (AdipoGen, 10 mM stock in Ethanol) or 5 µM Monensin
544 (Sigma, 50 mM stock in Ethanol) prior or during imaging. Where specified, 100 nM Lysotracker
545 Green (Molecular Probes, 1mM stock in DMSO) or 10nM Lysotracker Red in imaging buffer
546 was added 20 min prior to imaging.

547 Cells were imaged at 37 °C using an inverted Axio Observer microscope (Zeiss) with a
548 Plan Apochromat N 63×/1.40 oil DIC M27 objective and a Photometrics Prime 95B camera.
549 Filters with standard specifications for CFP, GFP, YFP, dsRed and APC were used to image
550 mTurquoise2, GFP, YFP, mApple and mNeptune2, respectively. To minimise overexpression
551 artifacts, cells were selected for imaging that expressed minimal amount of each fluorescent
552 marker that was sufficient to produce good quality signal. For time-lapse experiments, to
553 monitor endosome maturation kinetics, cells were treated with nigericin for 20 min, washed
554 4 times in imaging buffer and imaging chamber mounted onto an automated microscope
555 stage. Several fields of view (FOV) were selected for imaging per condition. The microscope
556 was programmed to image all FOVs sequentially and repeat the imaging at 1-2 min intervals.
557 The obtained images corresponded to the recovery time between 40 min and 190 min post
558 nigericin treatment. For experiments comparing Ccz1 WT to KO clones, with and without
559 rescue, all four conditions were imaged in a single time-lapse experiment. All experiments
560 were performed three times on different days.

561

562 **Single endosome analysis and quantification**

563 Using the time-lapse images, endosomes were selected for analysis with the following
564 criteria. For mApple-Rab5 expressing cells, endosomes initially devoid of Rab5 and later
565 acquiring and subsequently losing Rab5 were identified. For mApple-Rab7 expressing cells,
566 endosomes initially devoid of Rab7 and subsequently acquiring Rab7 and stabilising its
567 expression were identified. This ensured that the entire Rab conversion event was captured
568 in the kinetic.

569 To quantify the recruitment of markers to the endosome, an oval selection tool in Fiji
570 was used to draw a circular region-of-interest (ROI) closely following the rim of the endosome
571 in the channel for the most visible marker or by predicting the location of the rim if cases
572 where the enlarged endosome was negative for both markers and appeared as a dark circle
573 ([Fig 4A](#)). For less circular endosomes and endolysosomes, the ROI was adjusted using the
574 elliptical or a free-hand selection tool. ROIs were adjusted for every time point where the rim
575 of the endosome could be unambiguously identified. Mean fluorescence intensity (MFI) of a
576 two-pixel wide rim at the ROI was recorded in all channels. A larger two-pixel wide rim three
577 pixels away from the endosome was generated with a macro based on the original ROI, and
578 MFI was calculated as a measure of background for each time point ([Fig 4A](#)). We found that
579 adjusting the MFI at the rim of endosome for this background MFI minimised noise and
580 produced data reflective of visual assessment of marker presence at the endosome. For
581 intraluminal pH measurements, the circular ROI at the rim of the endosome was
582 reduced by 1 pixel and the total MFI of the reduced ROI was calculated in both YFP and CFP
583 channels. A ROI in a field where no cells were present was measured to obtain background
584 values. This approach was found to produce pH measurements as accurate as the modified
585 rim measurements, in which select pixels were removed to exclude interference from the
586 highly acidified vesicles interacting with the enlarged endosome. For the subdomain
587 measurements, two-pixel thick segmented lines with spline fit were drawn around the full
588 perimeter of the endosome starting at the top, and histogram measurements were obtained
589 of fluorescence intensity along the length of the line.

590 Endosomal recruitment marker measurements were background-subtracted and
591 adjusted for the minimum and maximum values of the entire measured kinetic, to represent
592 a range from 0 to 1. The pH measurements were kept as background-adjusted YFP/CFP
593 ratios. For averaging, kinetics were aligned for Rab5 peak or for Rab7 at 50% of its final

594 maximum value, representing the point of Rab conversion ([Fig 4C-D](#)). At least 10-20
595 endosomes from at least 3-10 cells were used in analysis and each experiment was repeated
596 at least 3 times. Means, standard deviations, SEM, Pearson's correlation and linear regression
597 were calculated in GraphPad Prism.

598

599 **pH calibrations**

600 GalT-pHlemon images provided us with pH-responsive YFP/CFP ratio measurements. To
601 convert YFP/CFP ratios to pH values, GalT-pHlemon expressing cells were incubated in buffers
602 of known pH containing 5 µM Nigericin, 50 µM Monensin and 100 nM Concanamycin A
603 (Sigma, 100 µM stock in DMSO) to disrupt intracellular proton gradients. The buffers
604 consisted of 138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Glucose, 10 mM
605 HEPES (for pH 5.5-9.0) or 10 mM MES (for pH 4.0-5.0), and pH was adjusted with NaOH or
606 HCl. Images were taken at 15 min after adding the buffers to the cells. Golgi ribbon structures
607 were selected with a segmented line tool in Fiji, and MFI was calculated in both channels and
608 subtracted for background measured in a field devoid of cells. The equation for the line of
609 best fit was generated in GraphPad Prism based on log(dose) response curve with variable
610 slope, where log(dose) is pH and response is YFP/CFP values (sigmoidal four-parameter dose-
611 response curve; [Fig S9](#)).

612

613 **Electron microscopy**

614 HeLa CCL2 cells were grown in 10 cm dishes, treated for 20 min with nigericin and left to
615 recover. At specified times, cells were fixed in DMEM containing 2.5% glutaraldehyde and 3%
616 formaldehyde for 2 h at room temperature. Cells were washed with PBS and cell membranes
617 stained with 2% osmium tetroxide and 1% potassium hexacyanoferrate in H₂O for 1 h at 4°C.
618 Following a wash with water, cells were further stained for proteins and nucleic acids with 2%
619 uranyl acetate in H₂O overnight at 4°C. Samples were subsequently dehydrated in
620 acetone/H₂O in stepwise increases in acetone concentration of 20%, 50%, 70%, 90% and 3x
621 100%. The samples were infiltrated with 50% epon embedding medium in acetone for 1 h at
622 room temperature, and subsequently with 100% epon resin overnight. Next day, fresh epon
623 resin was added and samples were polymerised for 24 h at 60°C. Sections of 60–70 nm were
624 collected on carbon-coated Formvar-Ni-grids and were viewed with a Phillips CM100 electron
625 microscope

626 To prepare cells for immunolabeling, HeLa cells stably expressing Galt-GFP were
627 prepared as previously described (Beuret et al., 2017). Sections were stained sequentially
628 with rabbit anti-GFP (1:100; Abcam 6556) and goat anti-rabbit coupled to 10 nm gold particles
629 (BB International). For dual labelling, HeLa cells stably expressing mApple-Rab5 were
630 transiently transfected with GalT-EGFP, prepared for immunolabelling as above, and stained
631 sequentially for GalT-GFP and mApple-Rab5. The sections were blocked with PBST
632 (PBS+0,05% Tween20) supplemented with 2% BSA for 20 min, incubated overnight at 4°C with
633 anti-GFP (1:100, Abcam), washed five times for 5 min with PBS and incubated for 2 h at room
634 temperature (RT) with donkey anti-rabbit coupled with 12 nm Gold (Jackson Immuno
635 Research). The wash step was repeated and the single-stained sections were subsequently
636 fixed for 2 min with 1% glutaraldehyde in PBS, washed with PBS and blocked for 10 min with
637 the blocking solution. Following a 2 h incubation at RT with anti-mCherry (1:100, St John's
638 Laboratory STJ140001), the sections were washed with PBS, blocked with PBS supplemented
639 with 2% fish gelatin (Sigma) for 10 min and incubated with mouse anti-goat (1:100, Jackson
640 Immuno Research) for 90 min. The wash and the 2% fish gelatin block steps were repeated
641 and the sections were incubated with goat anti-mouse coupled with 5 nm Gold (1:100,
642 BBInternational) for 2 h. The double-stained sections were washed five times for 5 min with
643 PBS and three times for 2 min with H₂O, and subsequently stained for 10 min with 2% uracyl
644 acetate and 2 min Reynold's solution.

645

646 **Immunostaining**

647 HeLa cells were plated onto coverslips 24 h prior to 20 min nigericin treatment and recovery.
648 At specified times post nigericin treatment, cells were fixed in 2% paraformaldehyde,
649 permeabilised with 0.1% Triton X-100, blocked in PBS containing 2% BSA and 5% goat serum,
650 and stained with anti-Galt (1:50, Sigma HPA010807) followed by AF594-conjugated goat anti-
651 rabbit and DAPI. Coverslips were mounted onto glass slides with Fluoromount G (Southern
652 Biotech) and sealed with nail polish. Following z-stack image acquisition in DAPI and dsRed
653 channels, images were deconvolved using Huygens deconvolution online software tool.

654

655 **Supplementary methods**

656 **Cell culture**

657 HEK293 and Neuro2A cells were grown at 37°C and 5% CO₂ in high-glucose Dulbecco's
658 modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum
659 (FCS, Biowest), 2 mM L-Glutamine, 1 mM Sodium Pyruvate, and 1x Penicillin and Streptomycin
660 (all from Sigma). Cos-1 cells were grown in low-glucose DMEM supplemented as above. For
661 live cell microscopy, imaging chambers were pre-coated with poly-L-lysine to enhance
662 attachment of these three cell types.

663

664 **DNA constructs**

665 mTagBFP2-Rab7 was generated by replacing Rab5 in mTagBFP2-Rab5a (Addgene #55322, gift
666 of Michael Davidson) with Rab7 from mApple-Rab7 (Addgene #54945) using BamHI and Xhol
667 restriction sites.

668

669 **Live cell imaging**

670 For endolysosomal labelling, 0.5 mg/mL Dextran-AF488 (10,000 MW, Molecular Probes, 10
671 mg/mL stock in water) was pulsed into cells for 17 h and chased for 4 h into lysosomes. For
672 endosomal dextran labelling, 0.5 mg/mL Dextran-AF488 was pulsed into cells for 65 min post
673 nigericin treatment and subsequently washed away for imaging within the following 15 min.

674

675 **qRT-PCR**

676 RNA was extracted and purified from cells using RNeasy kit or using the Trizol-Chloroform
677 extraction and isopropanol precipitation, following manufacturer's instructions. cDNA was
678 reverse-transcribed using GoScript reverse transcriptase primed with a mix of Oligo(dT)s and
679 random hexamers (Promega). qRT-PCR was performed using GoTaq qPCR master mix
680 (Promega) and primers specific for spanning the intron junction between exons 3 and 4 of
681 ccz1 (ACATTTAGCCCCATCAAAACCTGC, CCGAACAAACCATGACCATCC). Ccz1 expression was
682 normalized for β-actin expression.

683

684 **Western blotting and antibodies**

685 HeLa cells transiently transfected with NLS-mNeptune2-5-T2A-ccz1-myc were lysed in lysis
686 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH8.0, protease inhibitors) and denatured in
687 Laemmli buffer at 65°C for 5 min. Samples were resolved by SDS-PAGE and transferred onto
688 nitrocellulose membrane. Samples were blocked in milk, stained with anti-myc (1:2,000,

689 Sigma 9E10) and HRP-conjugated anti-mouse, and revealed with WesternBright ECL HRP
690 substrate (Advansta).

691

692 Immunofluorescence

693 HeLa cells stably expressing mApple-Rab5 were plated onto coverslips 24 h prior to
694 treatment. Cells were left untreated, treated for 16 h with bafilomycin (100 nM, Enzo Life
695 Sciences, 100 µM stock in DMSO), or treated with nigericin for 20min followed by a 60 min
696 washout. Following treatment, cells were fixed for 10 min with methanol at -20°C, blocked in
697 PBS containing 2% BSA and 5% goat serum, and stained with anti-LC3b (1:400, Cell Signaling
698 Technology #3868) followed by AF488-conjugated goat anti-rabbit. Coverslips were mounted
699 onto glass slides with Fluoromount G (Southern Biotech) and sealed with nail polish. Following
700 z-stack image acquisition, images were deconvolved using Huygens deconvolution online
701 software tool.

702

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708

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710

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820 **Supplementary Figures:**

821 Figure 1, Figure Supplement 1. Enlarged compartments interact with early endosomes and
822 acquire intraluminal content from the endolysosomal pathway.

823 Figure 1, Figure Supplement 2. Enlarged endosomes that undergo Rab conversion can be
824 induced by different treatments.

825 Figure 1, Figure Supplement 3. Enlarged endosomes that undergo Rab conversion can be
826 induced in different cell types.

827 Figure 2, Figure Supplement 1. Cell growth is unaffected by nigericin treatment, which
828 induces reversible Golgi vesiculation.

829 Figure 4, Figure Supplement 1. Enlarged endosomes occasionally display multiple Rab5
830 peaks and form Rab5 subdomains.

831 Figure 5, Figure Supplement 1. PI(3)P is recruited to endosomes concomitantly with Rab5.

832 Figure 6, Figure Supplement 1. Dynamic Snx1 recruitment suggest active sorting at the
833 enlarged endosome.

834 Figure 8, Figure Supplement 1. Rab11 interacts with the maturing endosome independently
835 of Rab5 or Rab7.

836 Figure 9, Figure Supplement 1. GalT-pHlemon sensor responds to pH changes in a sigmoidal
837 dose-response manner.

838 Figure 11, Figure Supplement 1. Validation and characterisation of Ccz1 knockout cell lines.

839 Figure 11, Figure Supplement 2. Validation and characterisation of Ccz1 the rescue
840 construct.

841 Figure 12, Figure Supplement 1. Characterisation of Ccz1 knockout cell lines.

842

843 **Source data files**

844 Figure 4D - Source Data 1. Quantification of Rab5 and Rab7 recruitment at endosomes.

845 Figure 5C - Source Data 1. Quantification of Rab5 and PI(3)P recruitment at endosomes.

846 Figure 6C - Source Data 1. Quantification of Rab5 and Snx1 recruitment at endosomes.

847 Figure 6E - Source Data 1. Quantification of Rab5 and Snx1 subdomains at endosomes.

- 848 Figure 7 - Source Data 1. Quantification of Rab7 and Snx1 recruitment at endosomes.
- 849 Figure 8C - Source Data 1. Quantification of Rab5 and Rab11 recruitment at endosomes.
- 850 Figure 8D and S8 - Source Data 1. Quantification of Rab5 and Rab11 subdomains at
851 endosomes.
- 852 Figure 8G - Source Data 1. Quantification of Rab7 and Rab11 recruitment at endosomes.
- 853 Figure 8H and S8 - Source data 1. Quantification of Rab7 and Rab11 subdomains at
854 endosomes.
- 855 Figure 9B - Source Data 1. Quantification of GalT-pHlemon signal in cells in calibration
856 buffers of known pH.
- 857 Figure 9C - Source Data 1. Quantification of GalT-pHlemon signal in Golgi ribbon, endo-
858 /lysosomal puncta, and enlarged endosomes.
- 859 Figure 10C - Source Data 1. Quantification of Rab5 recruitment and GalT-pHlemon signal at
860 endosomes.
- 861 Figure 10F - Source data 1. Quantification of Rab7 recruitment and GalT-pHlemon signal at
862 endosomes.
- 863 Figure 11 - Source Data 1. Quantification of Rab5 recruitment and GalT-pHlemon signal at
864 endosomes in Ccz1 WT, KO and rescue cells.
- 865 Figure 12B - Source Data 1. Quantification of GalT-pHlemon signal in endo-/lysosomal
866 puncta in Ccz1 WT, KO and rescue cells.
- 867 Figure 12D - Source Data 1. Quantification of GalT-pHlemon signal in endo-/lysosomal
868 puncta in Ccz1 WT, KO and rescue cells post nigericin treatment.
- 869 Figure S4C - Source Data 1. Cell growth numbers and quantification post nigericin
870 treatment.
- 871 Figure S7C - Source Data 1. Quantification of Rab5 and Snx1 subdomains at endosomes.
- 872 Figure S7E - Source Data 1. Quantification of Rab7 and Snx1 subdomains at endosomes.
- 873 Figure S9 - Source Data 1. Sigmoidal dose-response curve to define relationship between
874 GalT-pHlemon signal and pH.
- 875 Figure S10A - Source Data 1. Raw RT-PCR data for Ccz1 expression levels in Ccz1 WT vs KO
876 cells.
- 877 Figure S11A - Source Data 1. Original Western Blot images for NLS-mNeptune-T2A-myc
878 expression.
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Figure 1, Figure supplement 1

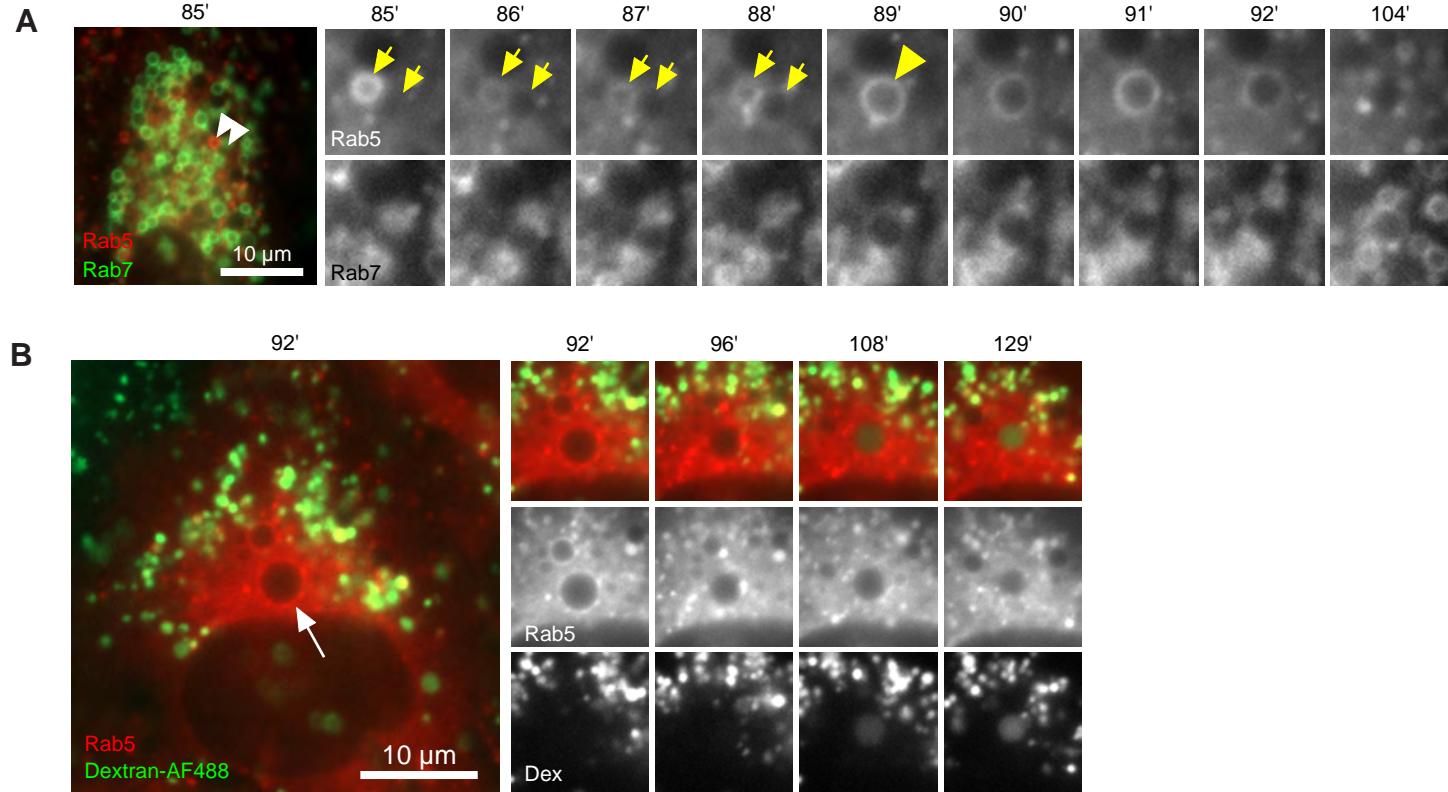


Figure 1, Figure supplement 1 . Enlarged compartments interact with early endosomes and acquire intraluminal content from the endolysosomal pathway.

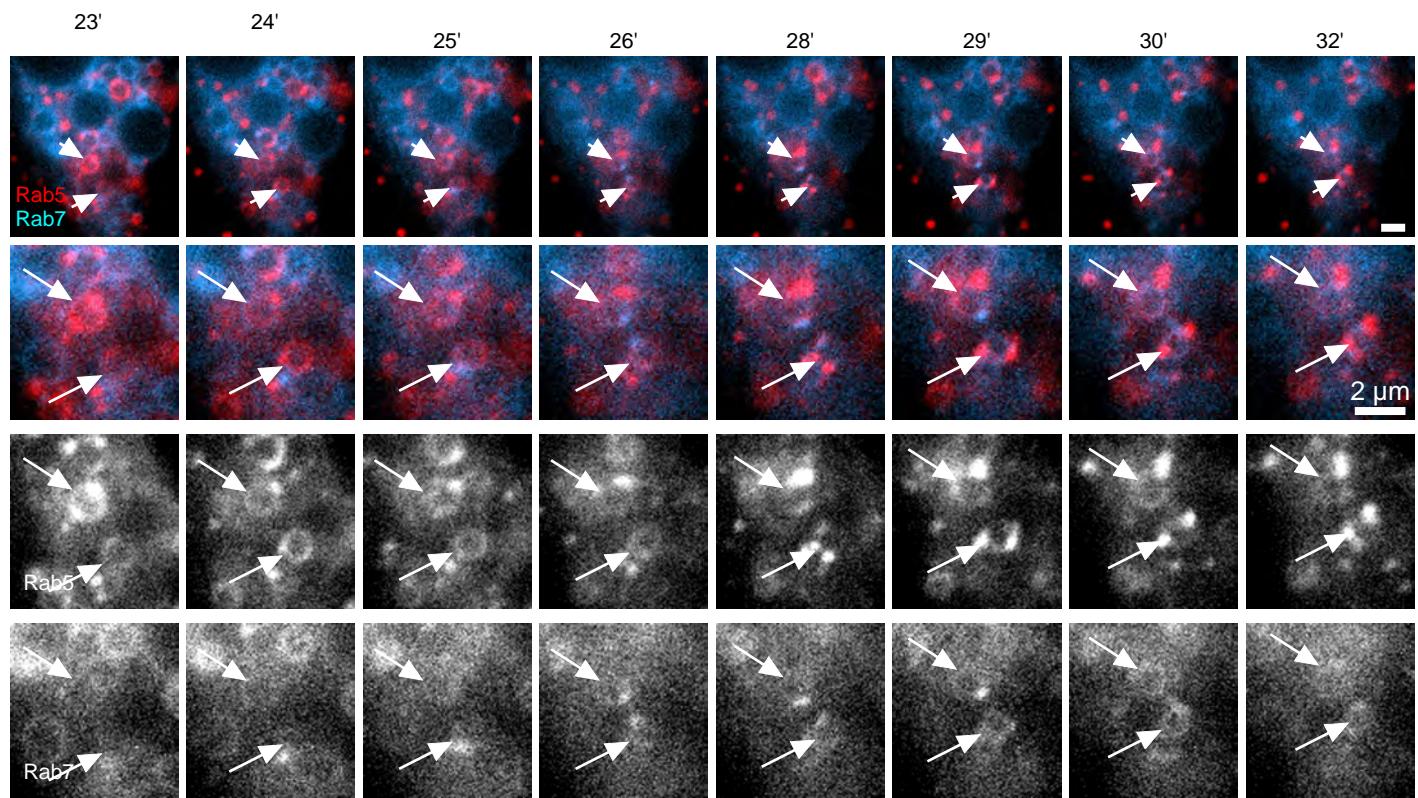
Nigericin was added to HeLa cells, stably expressing mApple-Rab5 and GFP-Rab7 (A) or mApple-Rab5 alone (B), for 20 min and washed away, and cells were imaged by time-lapse microscopy. Recovery times are specified relative to removal of nigericin.

(A) Example to show an enlarged compartment devoid of Rab5 fusing with Rab5-positive endosome to subsequently undergo Rab conversion.

(B) Example to show an enlarged Rab5-positive endosome later acquiring Dextran-AF488, which had been pulse-chased into the endolysosomal pathway (4h pulse, 1.5 h chase) prior to nigericin treatment. Recovery times are specified relative to removal of nigericin.

Figure 1, Figure Supplement 2

A



B

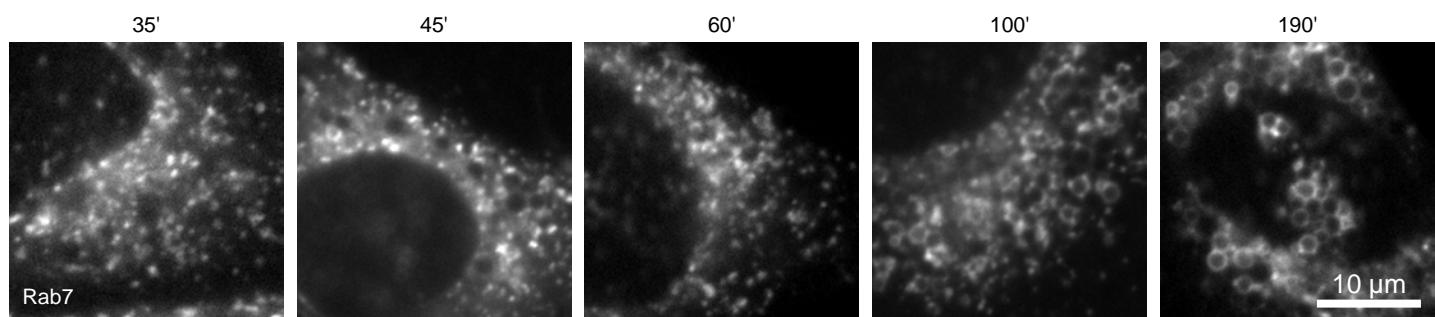


Figure 1, Figure Supplement 2. Enlarged endosomes that undergo Rab conversion can be induced by different treatments.

(A) HeLa cells stably expressing mApple-Rab5 and transiently transfected with BFP-Rab7 were treated for 4 h with ammonium chloride, washed and allowed to recover for 10 min prior to imaging. Arrows point to two Rab5-positive endosomes, which subsequently acquire Rab7.

(B) HeLa cells stably expressing mApple-Rab7 were treated for 20 min with monensin, washed and imaged at specified times after the wash. Representative images of cells show accumulation of enlarged Rab7-positive endosomes at later time points.

Figure 1, Figure Supplement 3

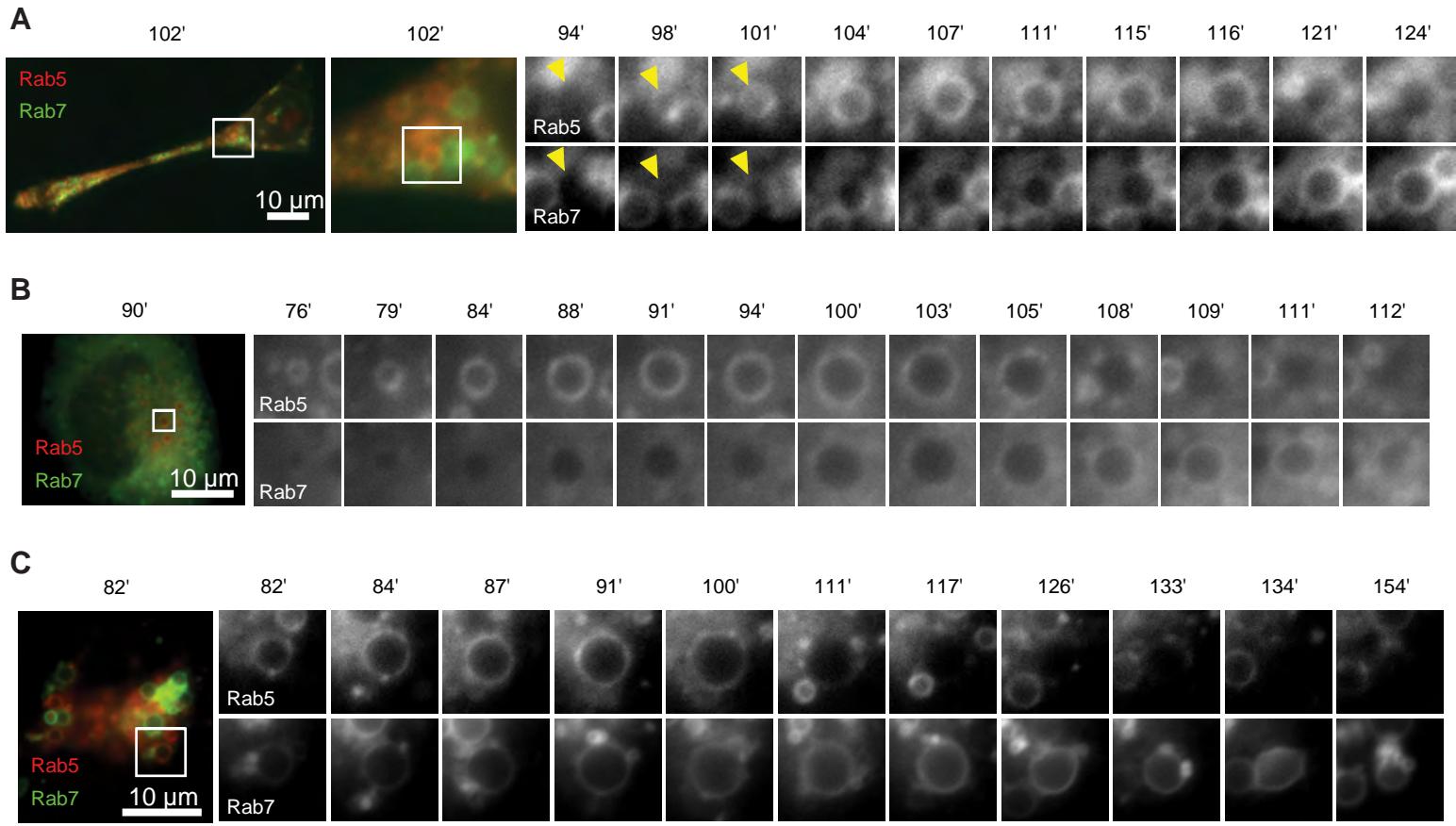


Figure 1, Figure Supplement 3. Enlarged endosomes that undergo Rab conversion can be induced in different cell types. Cells were transiently transfected with mApple-Rab5 and GFP-Rab7, and treated 20 min with nigericin followed by recovery and time-lapse microscopy. (A) HEK293 cells, (B) Neuro2A cells, (C) Cos-1 cells. Images were selected to show Rab conversion in the enlarged endosomes.

Figure 2, Figure Supplement 1

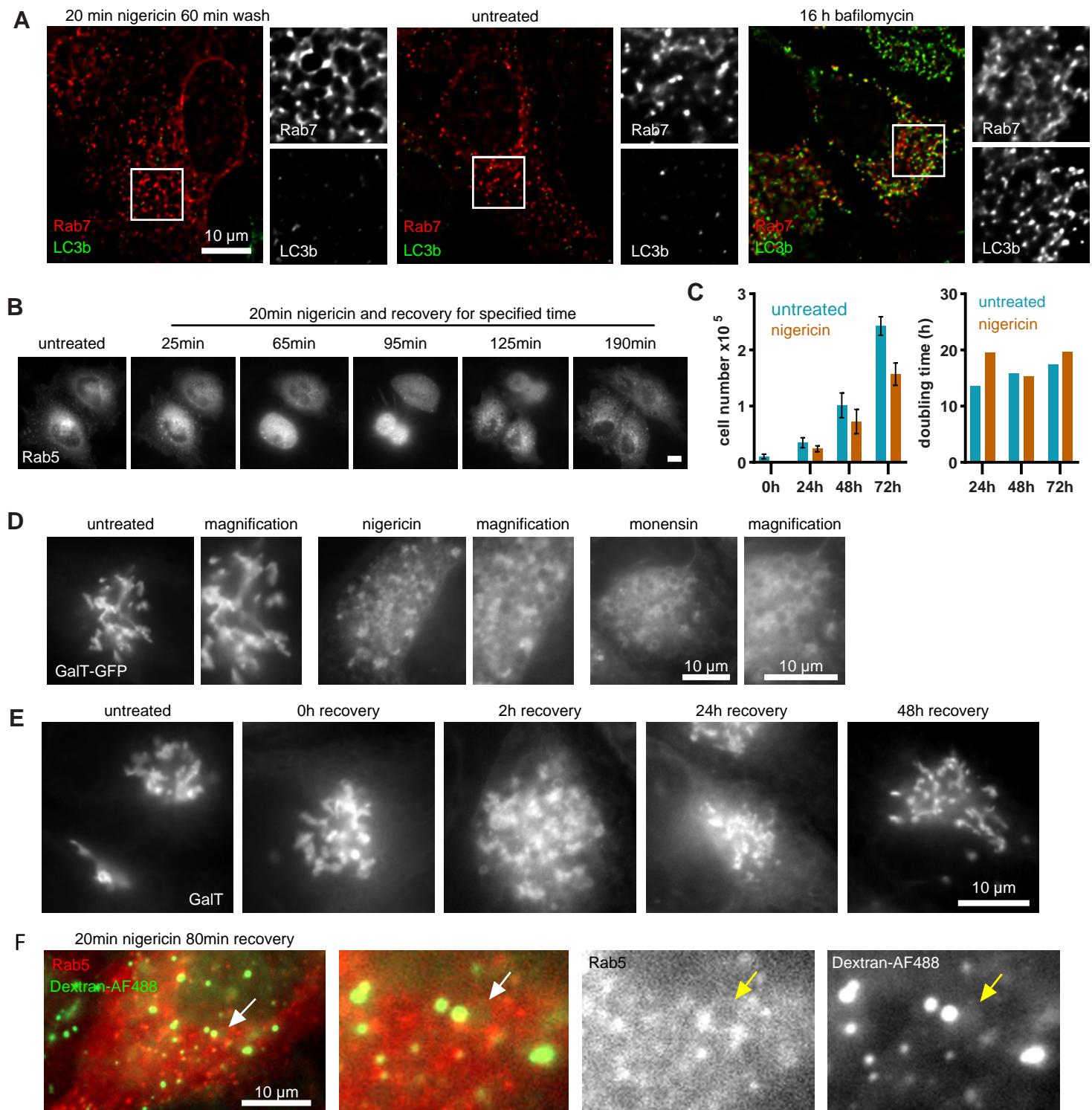


Figure 2, Figure Supplement 1. Cell growth is unaffected by nigericin treatment, which induces reversible Golgi vesiculation. HeLa cells stably expressing mApple-Rab5 (A,B,F) or GalT-GFP (C-E) were treated for 20 min with 10 μ M nigericin or 5 μ M monensin, or left untreated.

(A) Images of cells stained with LC3b antibody to show absence of autophagy induction or presence of LC3B at the enlarged endosomes of nigericin-treated cells. Baflomycin-treated cells are shown here as positive control.

(B) Images to show cells dividing shortly after nigericin treatment, with recovery times specified. Scale bar = 10 μ m.

(C) Cells were plated in 12-well plates at 10^4 cells per well 24 h prior to nigericin treatment, trypsinised and counted at specified recovery times. Triplicate wells were counted per time point per condition. Actual cells numbers and doubling times are presented. Representative graph of three independent experiments. Source data is available in Figure S4C - Source Data 1.

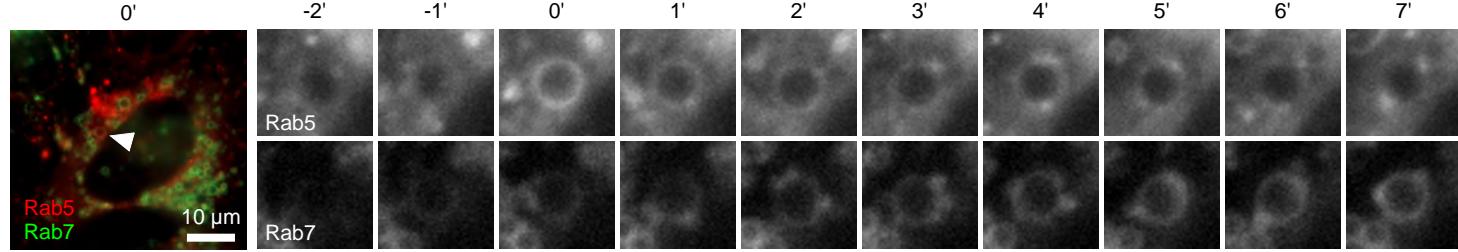
(D) Images to reveal extensive formation of GalT-positive enlarged compartments upon either treatment following 2 h recovery.

(E) Images to show Golgi vesiculation and return to ribbon morphology within 48 h of recovery from nigericin treatment.

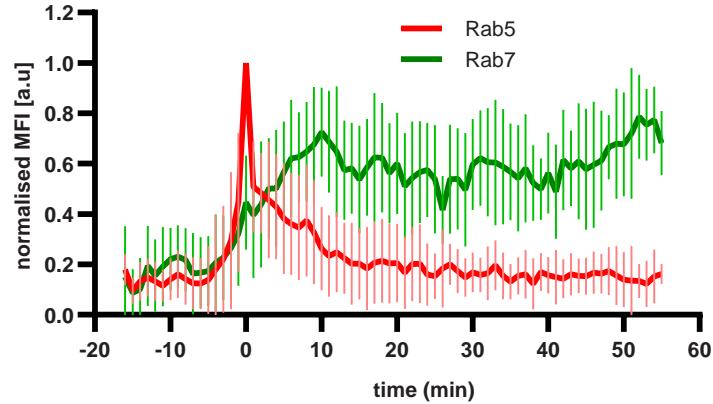
(F) Images of cells incubated with 0.5mg/mL Dextran-AF488 for 65min after nigericin washout to reveal endocytosed dextran presence in the enlarged Rab5-positive endosomes.

Figure 4, Figure Supplement 1

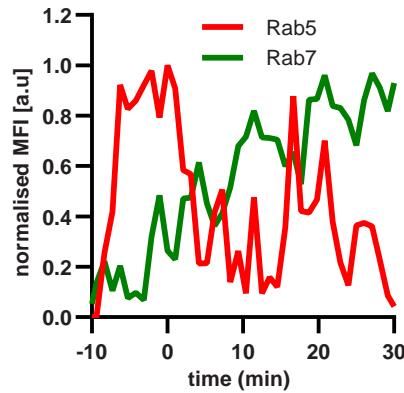
A



B



D



C

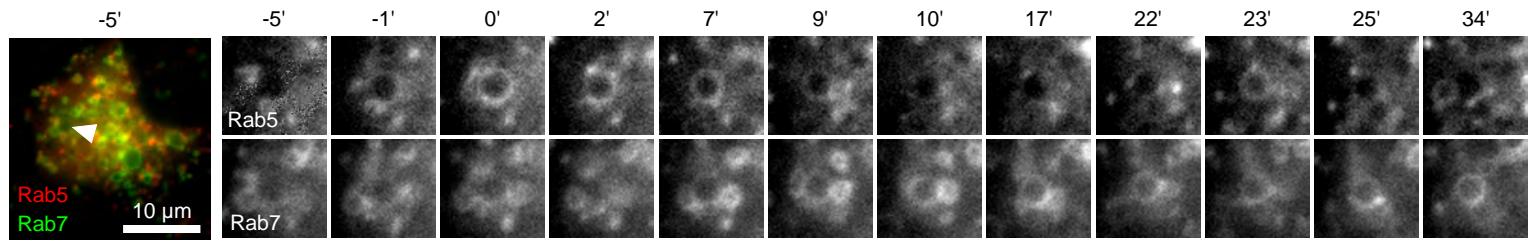


Figure 4, Figure Supplement 1. Enlarged endosomes occasionally display multiple Rab5 peaks and form Rab5 subdomains. HeLa cells, stably expressing mApple-Rab5 and GFP-Rab7 were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A) Time-lapse images of an endosome displaying formation of Rab5 subdomains.

(B) Averaged Rab5 and Rab7 kinetics of 21 endosomes. Error bars represent standard deviation. Representative graph of three independent experiments.

(C-D) Time-lapse images (C) and a corresponding fluorescence quantification plot (D) of an endosome with multiple waves of Rab5 recruitment and a continuous increase in Rab7 levels.

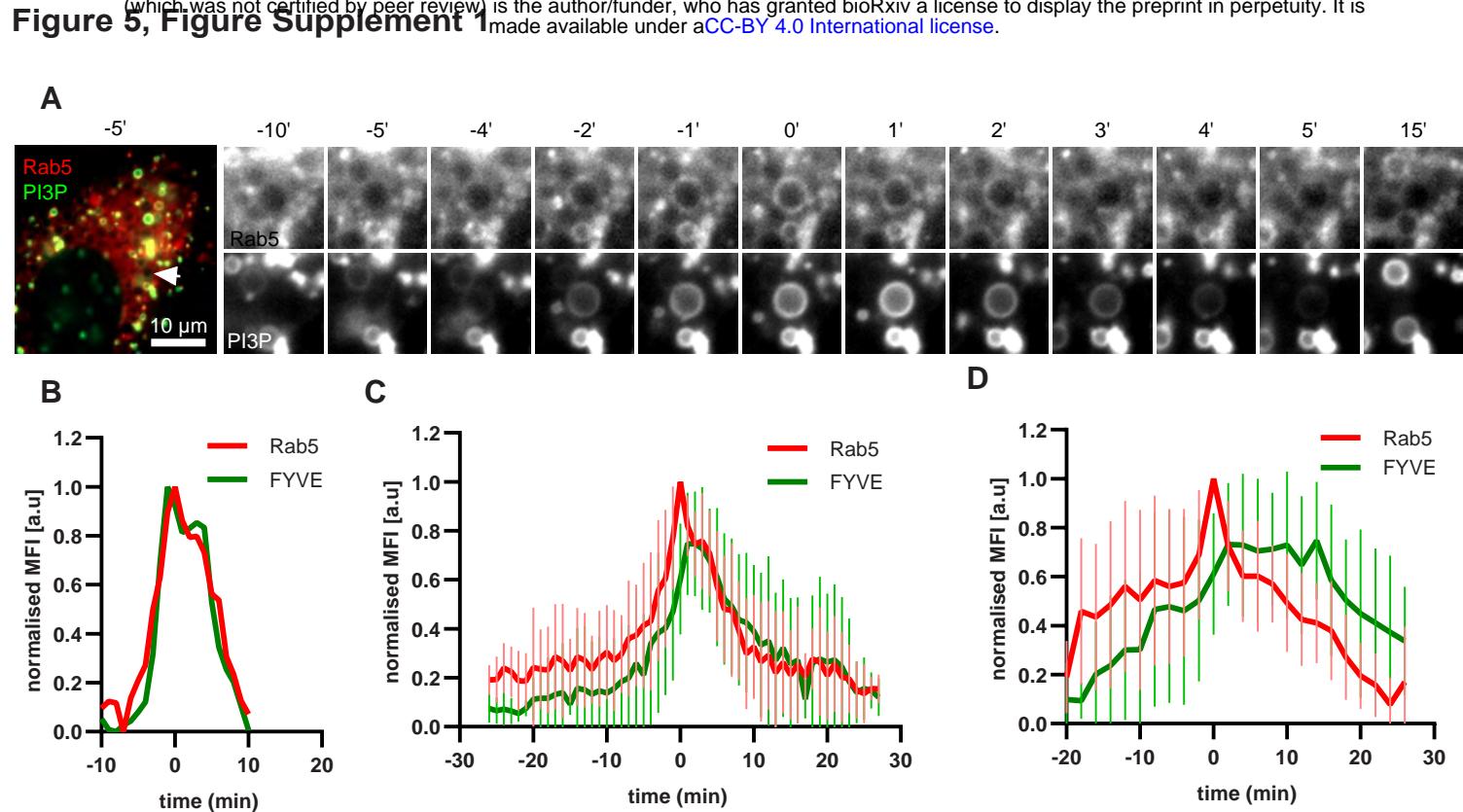


Figure 5, Figure Supplement 1. PI(3)P is recruited to endosomes concomitantly with Rab5.

HeLa cells, stably expressing mApple-Rab5 and transiently transfected with the PI(3)P marker, GFP-FYVE, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

- (A) Time-lapse images of a representative endosome to show transient Rab5 recruitment accompanied by PI(3)P.
- (B) Corresponding graph of normalised mean fluorescence intensity of Rab5 and FYVE at the rim of the endosome in (A) over the time the endosome was detectable.
- (C) Averaged Rab5 and PI(3)P kinetics of 16 endosomes. Error bars represent standard deviation.
- (D) Averaged Rab5 and PI(3)P kinetics of 15 endosomes. Error bars represent standard deviation.
- (C) and (D) represent two independent experiments of a total of three.

Figure 6, Figure Supplement 1

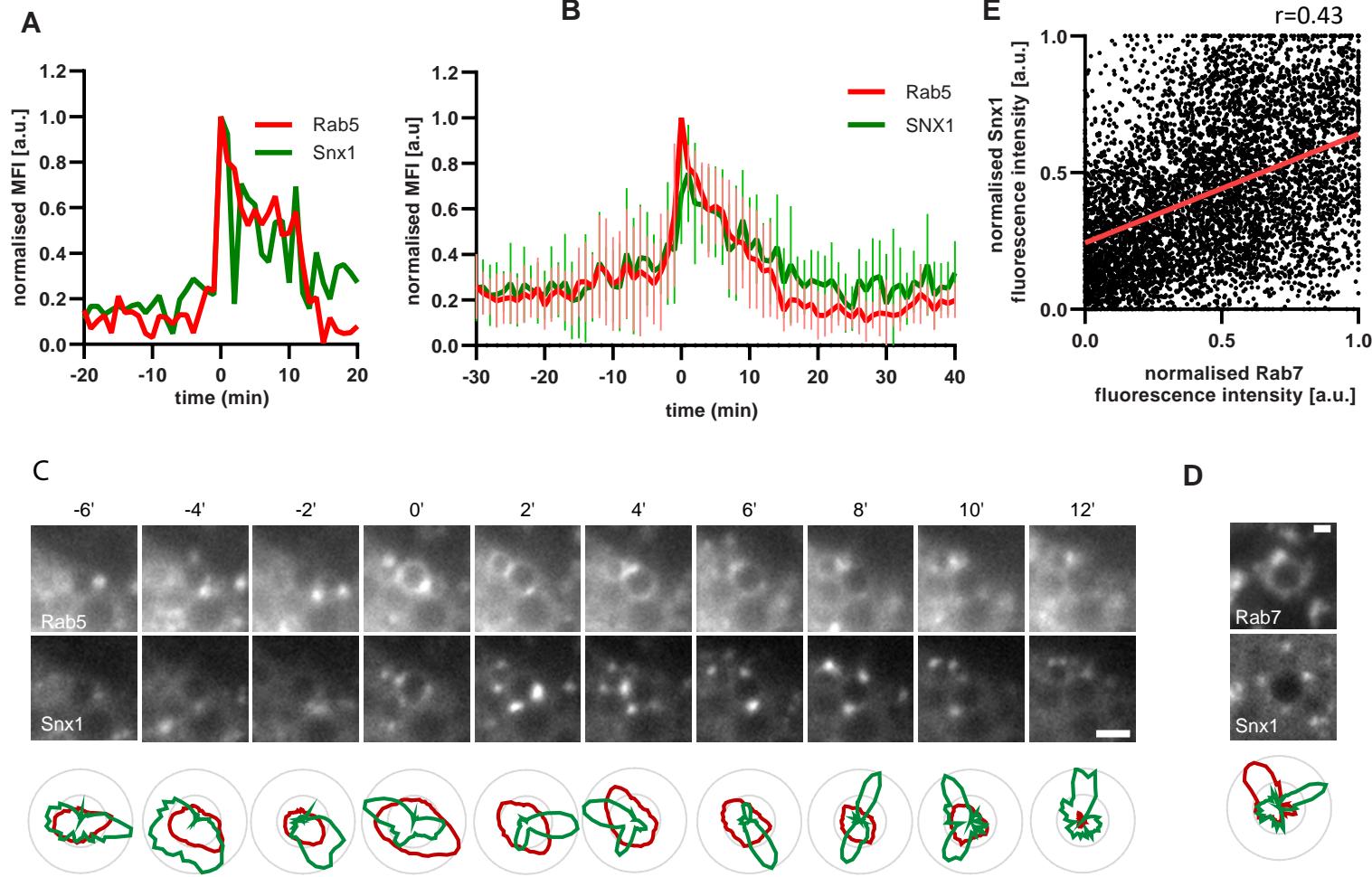


Figure 6, Figure Supplement 1. Dynamic Snx1 recruitment suggest active sorting at the enlarged endosome.

Nigericin was added to HeLa cells for 20 min and washed away, and cells were imaged by time-lapse microscopy, as described in Figure 4A.

(A-E) Cells stably expressing mApple-Rab5 (A-C) or mApple-Rab7 (D-E) and transiently transfected with the Snx1-GFP.

(A) Example graph of normalised mean fluorescence intensity (MFI) of Rab5 and Snx1 at the rim of the endosome over the time the endosome was detectable.

(B) Averaged Rab5 and Snx1 kinetics of 14 endosomes. Error bars represent standard deviation. Representative graph of three independent experiments. Numerical data for all quantified endosomes is available in Figure 6C - Source Data 1.

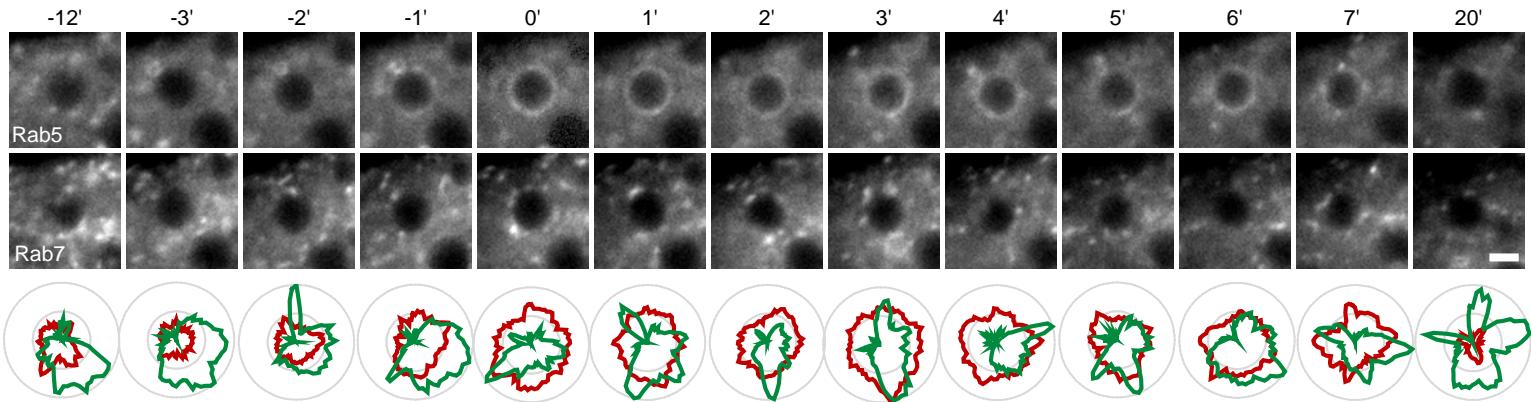
(C) Images and corresponding line profiles of normalised fluorescence intensity of Rab5 and Snx1 along the rim of the maturing endosome at consecutive time points. Rab5 was adjusted for a single maximum and minimum values during the recorded kinetic to highlight its overall signal increase. Snx1 was adjusted for max and min values for each time point to highlight the dynamic nature of Snx1 subdomains. Scale bar = 2 μ m. Numerical data for all quantified endosomes is available in Figure S7C - Source Data 1.

(D) Images of Rab7 and Snx1 at an enlarged endosome and a corresponding line profile of normalised fluorescence intensity along the rim to show co-existence as well as independence of subdomains of the two markers. Scale bar = 1 μ m.

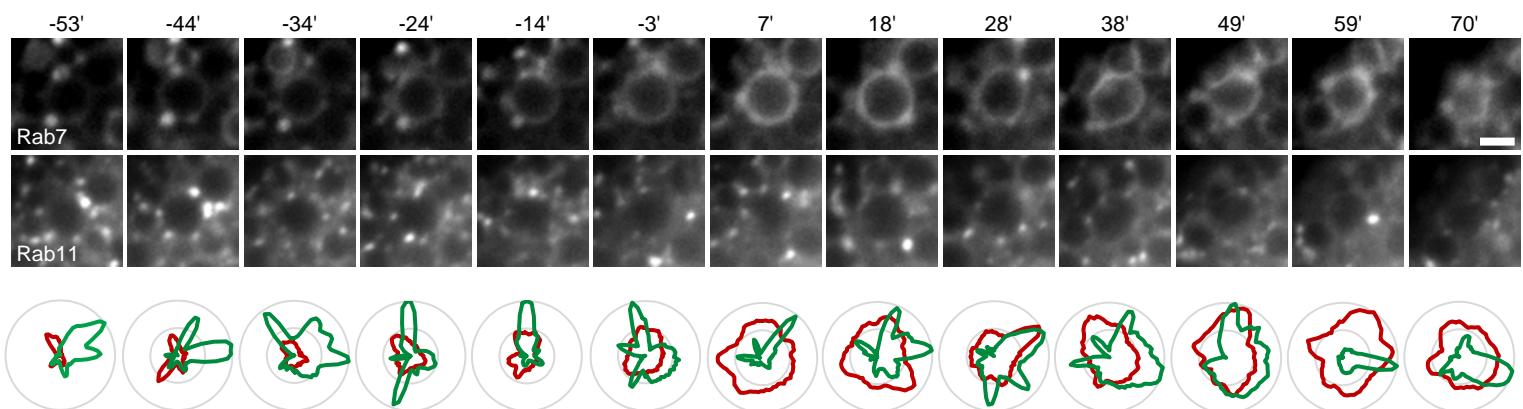
(E) Correlation plot of normalised fluorescence intensity of Rab7 and Snx1 as measured in (E) for 3 endosomes for a total of 96 time points, and a corresponding regression line. Pearson's correlation $r=0.43$. Numerical data for all quantified endosomes is available in Figure S7E - Source Data 1.

Figure 8, Figure Supplement 1

A



B



C

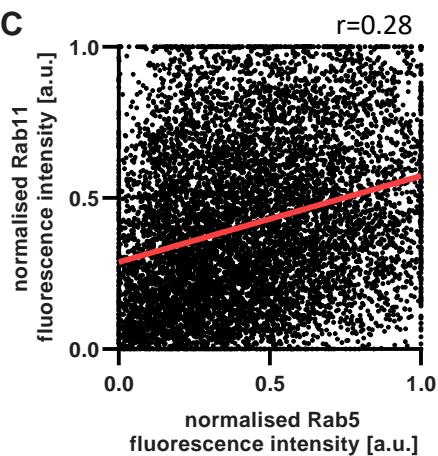


Figure 8, Figure Supplement 1. Rab11 interacts with the maturing endosome independently of Rab5 or Rab7.

HeLa cells, stably expressing mApple-Rab5 (A,C) or mApple-Rab7 (B) and transiently transfected with GFP-Rab11, were treated for 20 min with nigericin, washed and imaged over 3 h, as described in Figure 4A.

(A,B) Images and corresponding line profiles of normalised fluorescence intensity of Rab5 (A) or Rab7 (B) and Rab11 along the rim of the maturing endosome at consecutive time points. Rab5/Rab7 was adjusted for a single maximum and minimum values during the recorded kinetic to highlight its overall signal increase. Rab11 was adjusted for maximum and minimum values for each time point to highlight the dynamic nature of Rab11 interactions with the maturing endosome. Scale bar = 2 μ m.

(C) Correlation plot of normalised fluorescence intensity of Rab5 and Rab11 as measured in Figure 7D for 14 endosomes for a total of 193 time points, and a corresponding regression line. Pearson's correlation $r=0.28$. Numerical data for all analysed endosomes is available in Figure 8D and S8 - Source Data 1 and Figure 8H and S8 - Source Data 1.

Figure 9, Figure Supplement 1

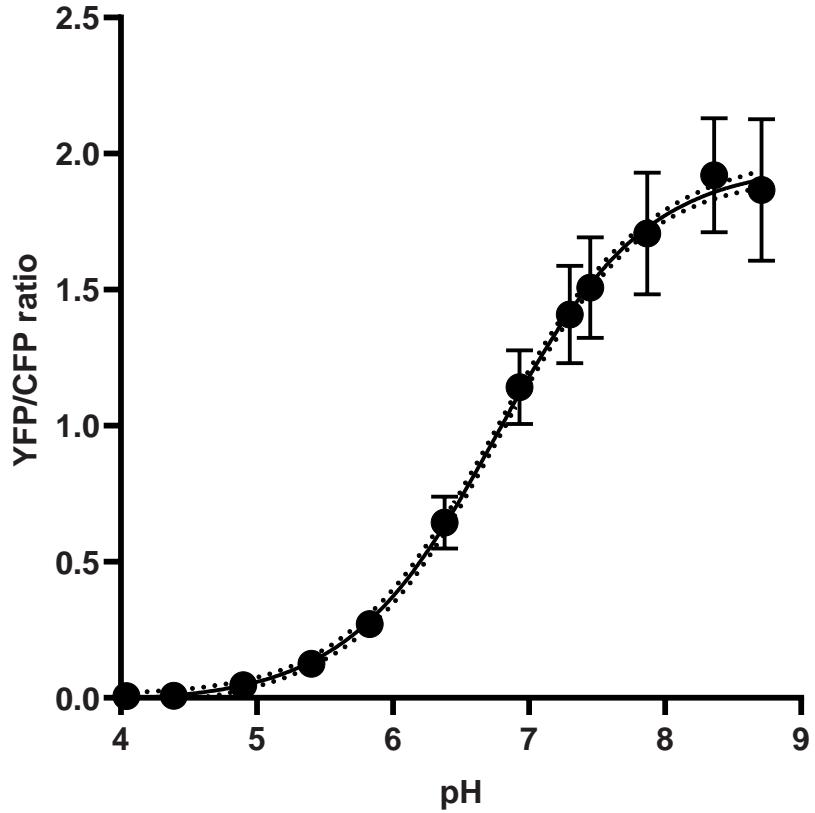
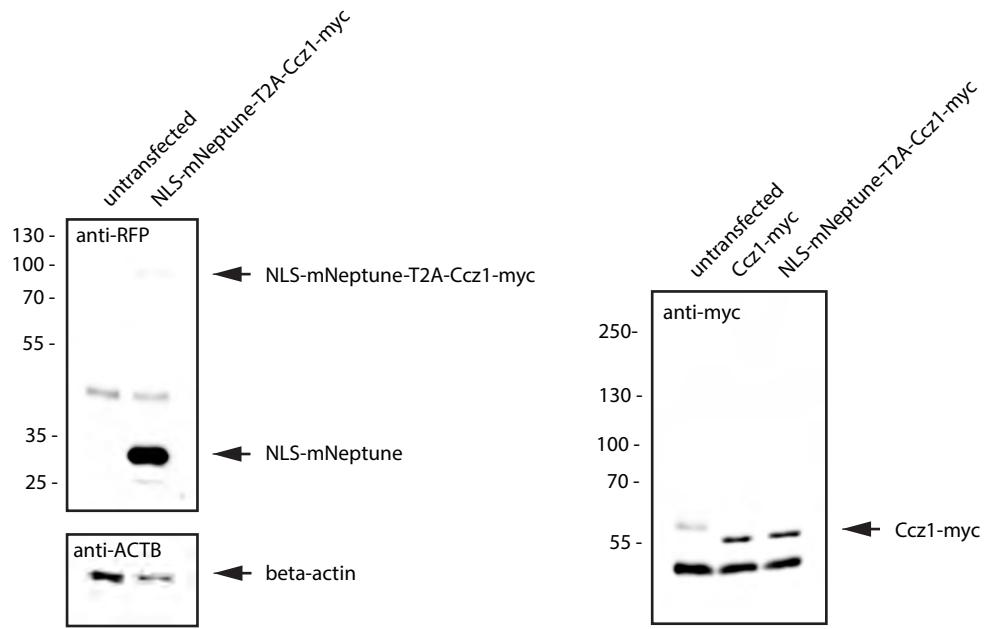


Figure 9, Figure Supplement 1. Galt-pHlemon sensor responds to pH changes in a sigmoidal dose-response manner. HeLa cells were transiently transfected with the ratiometric pH sensor, Galt-pHlemon. Graph to show response of Galt-pHlemon sensor to pH 4.0-9.0 range as displayed by YFP/CFP ratio measurements in cells incubated with calibration buffers of specified pH values as well as the interpolation of sigmoidal dose-response curve. Numerical data for all quantified Golgi ROIs is available in Figure S9 - Source Data 1.

Figure 11, Figure Supplement 2

A



B

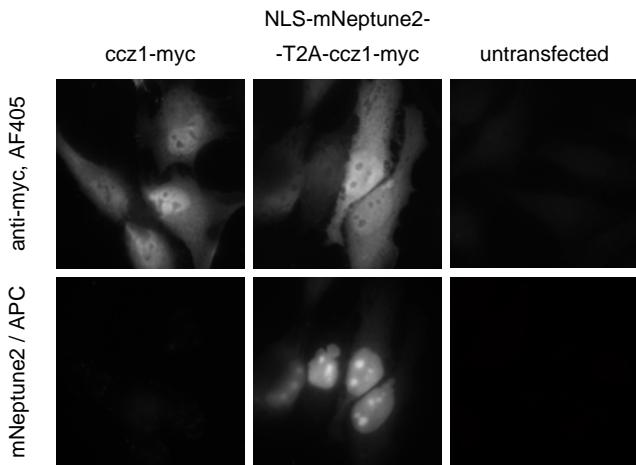


Figure 11, Figure Supplement 2. Validation and characterisation of Ccz1 the rescue construct.

- (A) Western blot of the *ccz1* rescue construct expressing NLS-mNeptune2-T2A-Ccz1-myc to show predominant production of two smaller products, NLS-mNeptune2 and *ccz1*-myc, as visualised by RFP and myc antibodies, respectively. Original uncropped and unformatted images are available in Figure S11A - Source Data 1.
- (B) Immunofluorescence images of NLS-mNeptune2-T2A-Ccz1-myc transiently transfected into HeLa cells to reveal nuclear distribution of the mNeptune2 signal and cytosolic distribution of Ccz1-myc.

Figure 11, Figure Supplement 1

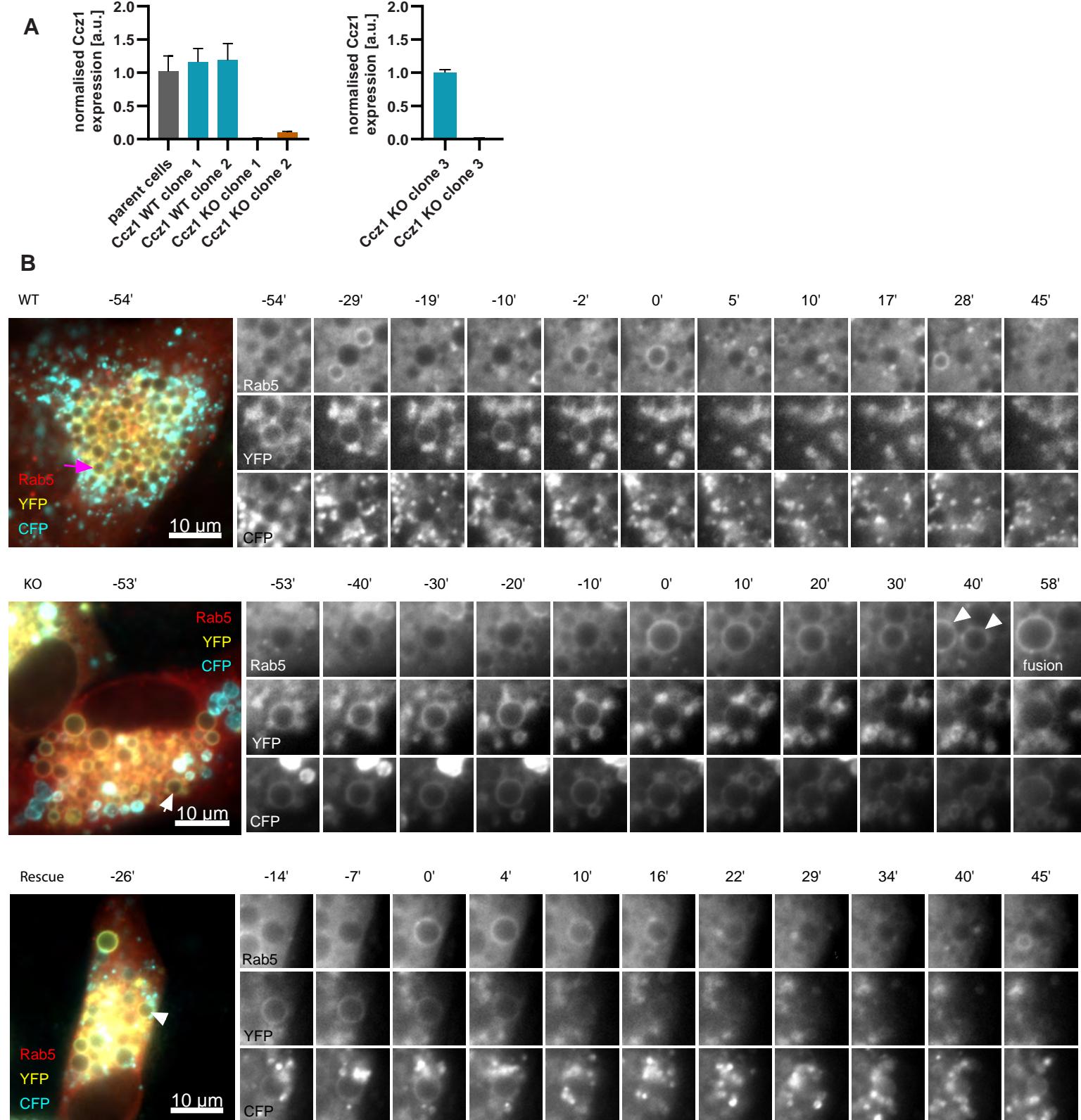


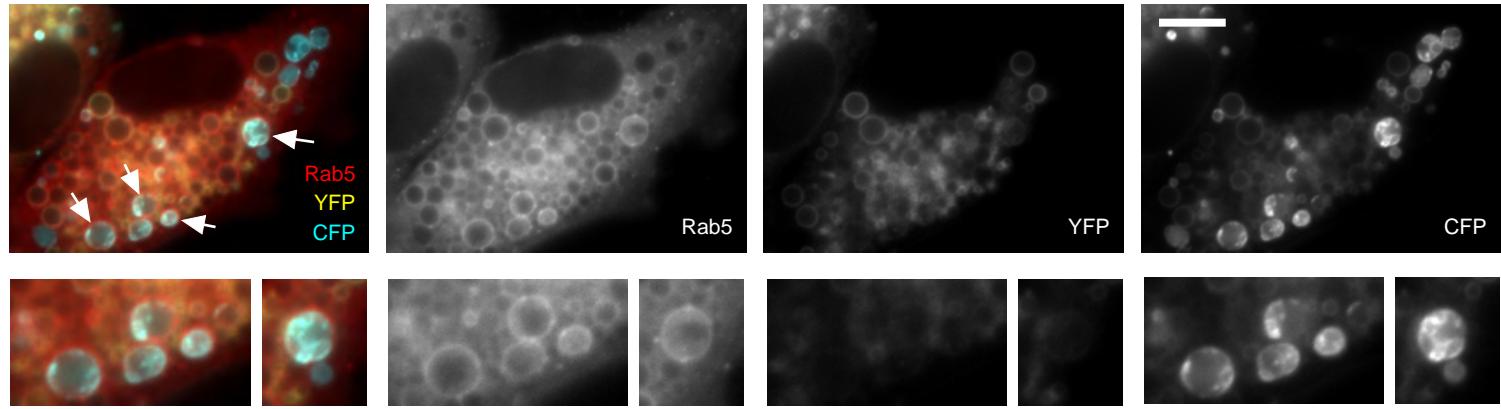
Figure 11, Figure Supplement 1. Validation and characterisation of Ccz1 knockout cell lines.

(A) Ccz1 mRNA expression levels in HeLa cell lines with wild-type (WT) Ccz1 and knocked-out Ccz1 (KO), three clones each, as measured by qRT-PCR and normalised for actin. Raw RT-PCR data is available in Figure S10A - Source Data 1.

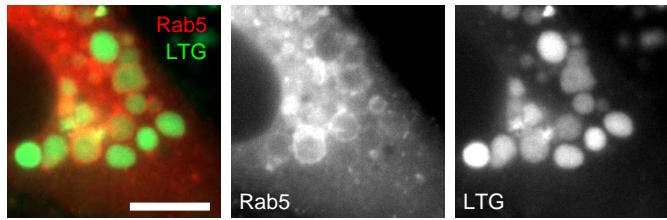
(B) HeLa cell lines with wild-type (WT) Ccz1 and knocked-out Ccz1 (KO) were transiently transfected with mApple-Rab5 and GaT-pHlemon. Ccz1 expression plasmid was co-transfected for 72 h for rescue experiments. Time-lapse images of representative endosomes, corresponding to those quantified in Figure 11B, to show acidification in endosomes recruiting Rab5 in the three cell types.

Figure 12, Figure Supplement 1

A



B



C

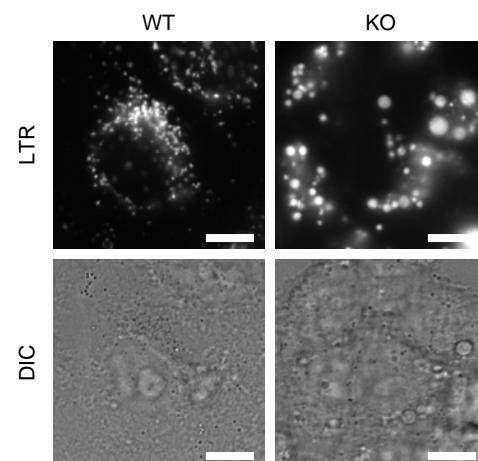


Figure 12, Figure Supplement 1. Characterisation of Ccz1 knockout cell lines.

(A) Images of Ccz1 KO cells pre-treated with nigericin, recorded at 150 min recovery, showing Rab5-positive acidified hybrid compartments (arrows). Cells were transiently transfected with mApple-Rab5 and GalT-pHlemon. Scale bar = 10 μ m.

(B) Images of Ccz1 KO cells pre-treated with nigericin, recorded at 24 h recovery, showing Rab5-positive acidified hybrid compartments, as visualised with Lysotracker Green (LTG). Cells were transiently transfected with mApple-Rab5. Scale bar = 10 μ m.

(C) Images of untreated Ccz1 KO cells stained with Lysotracker Red (LTR) to reveal large, acidified compartments. Scale bar = 10 μ m.