Current Biology, Volume *28*

Supplemental Information

Live Observation of Two Parallel Membrane Degradation Pathways at Axon Terminals

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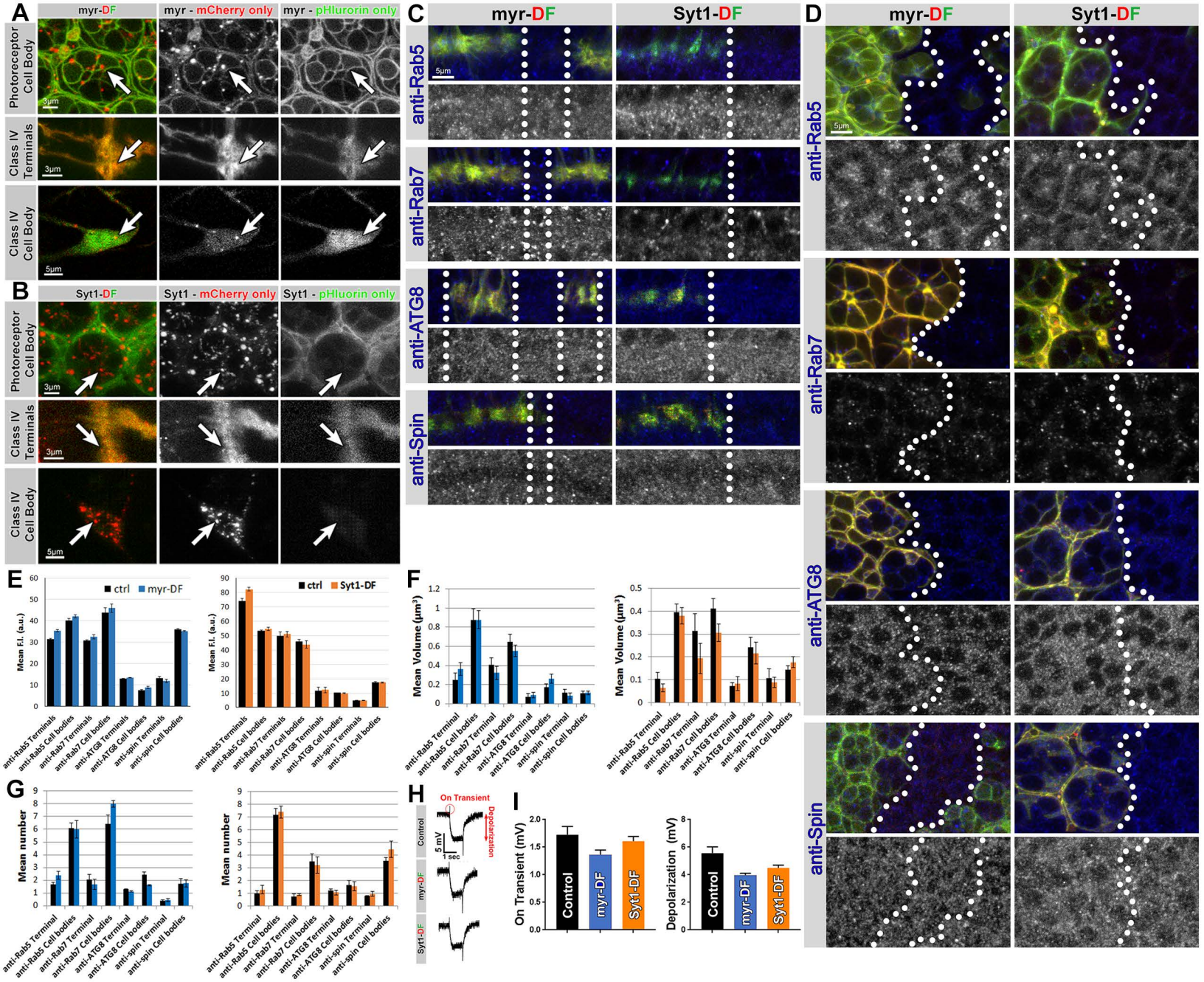


Figure S1. Myr-DF and Syt1-DF probes mark large acidic compartments in photoreceptor cell bodies, class IV da neuron axon terminals and cell bodies, and expression of neither probe affects endolysosomal and autophagic markers or photoreceptor function. Related to Figure 1.

(A and B) Live imaging of myr-DF (A) and Syt1-DF (B) reveal the presence of large acidic compartments in photoreceptor cell bodies at P+40%, class IV dendritic arborization neuron axon terminals and cell bodies. Arrows: examples of acidic (red-only live) compartments. (C and D) Comparison of the levels of endolysosomal and autophagic pathways markers (Rab5, early endosome; Rab7, late endosome/ multivesicular bodies (MVB); ATG8, autophagosome; Spin, lysosome) in mosaic expression of myr-DF or Syt1-DF at P+40% photoreceptor axon terminals (C) and cell bodies (D). The white dotted lines mark clonal boundaries (with and without probe expression). (E-G) Comparison of mean fluorescence (E), mean volume (F) and mean number (G) of endogenous endolysosomal markers in control (black) and probe-expressing [myr-DF (blue), Syt1-DF (orange)] axon terminals and cell bodies. Mean number per axon terminal was quantified, and mean number per ommatidium was quantified for cell bodies. Mean ± SEM, Unpaired t-test, brain n=3 per antibody staining. (H and I) Electroretinogram (ERG) recordings from adult photoreceptors expressing either myr-DF or Syt1-DF. Representative ERG traces (H), and quantifications of on-transient and depolarization amplitudes (I). Mean ± SEM, fly n=10 per genotype.

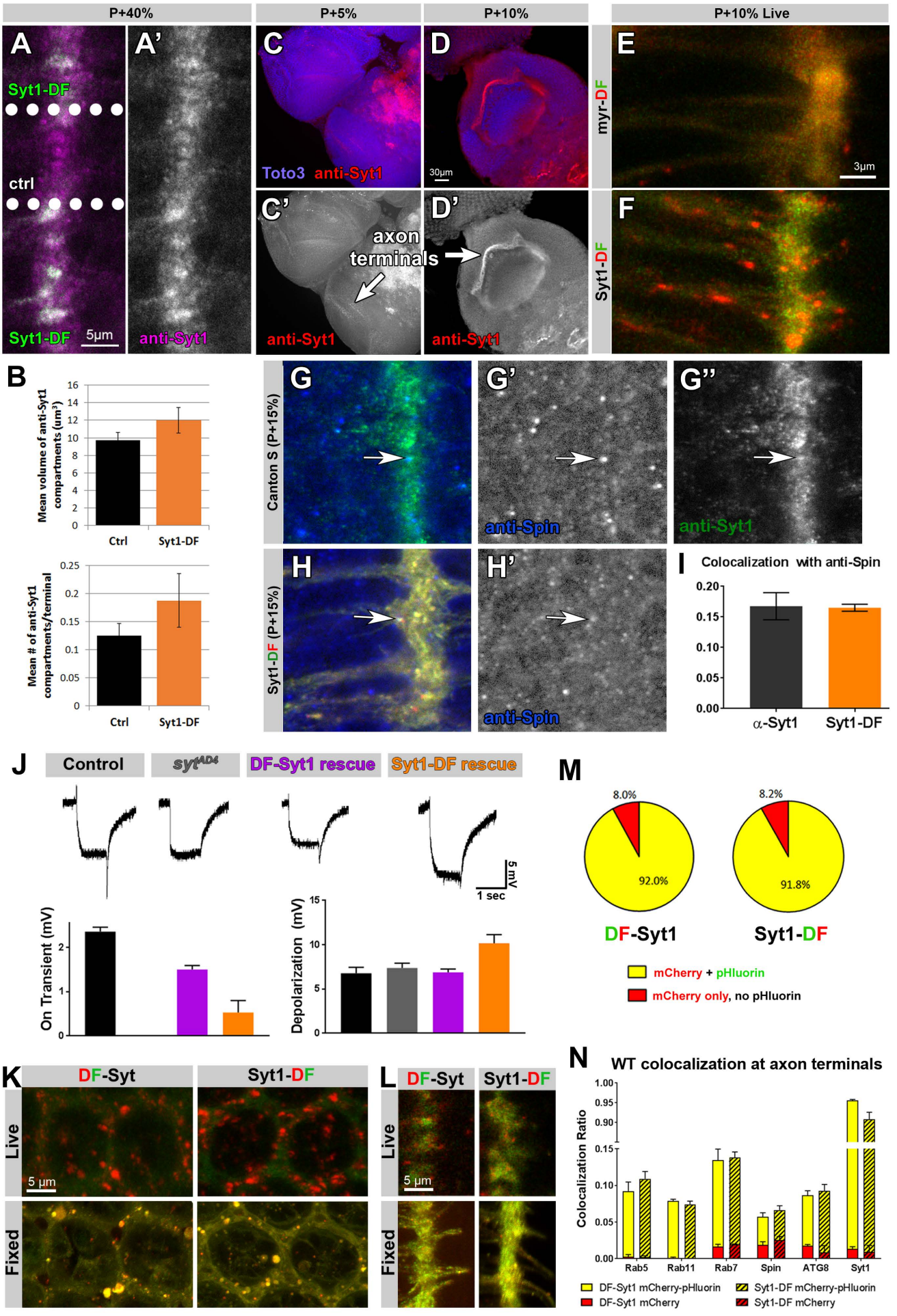

P+40%
P+5%
P+10%
P+10% Live
A
Syt1-DF
ctrl
Syt1-DF
5µm
A'
anti-Syt1
C
Toto3 anti-Syt1
D
30µm
E
myr-DF
3µm
C'
axon terminals
anti-Syt1
D'
anti-Syt1
F
Syt1-DF
B
Mean volume of anti-Syt1 compartments (um³)
Ctrl
Syt1-DF
Mean # of anti-Syt1 compartments/terminal
Ctrl
Syt1-DF
G
Canton S (P+15%)
G'
anti-Spin
G''
anti-Syt1
H
Syt1-DF (P+15%)
H'
anti-Spin
I
Colocalization with anti-Spin
α-Syt1
Syt1-DF
J
Control
syt^AD4
DF-Syt1 rescue
Syt1-DF rescue
5 mV
1 sec
On Transient (mV)
Depolarization (mV)
M
8.0%
92.0%
8.2%
91.8%
DF-Syt1
Syt1-DF
mCherry + pHluorin
mCherry only, no pHluorin
K
DF-Syt
Syt1-DF
Live
5 µm
Fixed
L
DF-Syt
Syt1-DF
Live
5 µm
Fixed
N
WT colocalization at axon terminals
Colocalization Ratio
Rab5
Rab11
Rab7
Spin
ATG8
Syt1
DF-Syt1 mCherry-pHluorin
DF-Syt1 mCherry
Syt1-DF mCherry-pHluorin
Syt1-DF mCherry

Figure S2. Syt1-DF is expressed at endogenous levels and sorted for degradation indistinguishably from endogenous Syt1, and cytoplasmically tagged Syt1-DF and lumenally tagged DF-Syt1 are indistinguishably localized and degraded in the endolysosomal pathway. Related to Figure 1.

(A-B) Antibody labeling with anti-Syt1 in mosaic expression of Syt1-DF in the P+40% photoreceptor axon terminals (A and A') and quantification of mean volume and number of compartments marked by anti-Syt1 (B). Mean ± SEM, brain n=3, Unpaired t-test. The white dotted lines mark clonal boundaries (with and without probe expression). (C-D') Endogenous Syt1 is localized to axon terminals of photoreceptors starting at 10% pupal development (P+10%). (E and F) Live imaging of myr-DF and Syt1-DF probes at P+10% photoreceptor axon terminals. Note that Syt1 protein turnover starts as soon as it is localized to photoreceptor terminals. (G-H') Syt1-DF colocalizes with lysosomal marker Spin equally as the endogenous Syt1 at P+15%. Arrows: colocalization of Syt1 compartments with anti-Spin. (I) Quantification of colocalization of endogenous Syt1 or Syt1-DF with anti-Spin at P+15%. Mean ± SEM, brain n=3 per experimental condition. (J) Rescue experiment of loss of neurotransmission of sytAD4 null mutant photoreceptors using either C- or N-terminally tagged Syt1 expression (Syt1-DF or DF-Syt1) in 1 day old adult flies. Representative ERG traces, and quantification of on-transient and depolarization amplitudes. Fly n=7 per genotype. (K and L) Live and fixed imaging of Syt1-DF and DF-Syt1 in cell bodies (K) and axon terminals (L) of P+40% photoreceptors. (M) Ratio of mCherry-only Syt1-DF and DF-Syt1 compartments at axon terminals after fixation. Brain n=15 per probe. (N) Colocalization of DF-Syt1 (non-striped) and Syt1-DF (striped) compartments with markers of endolysosomal system, autophagy and Syt1 antibody. Shown are ratios for 'yellow-fixed' and 'red-fixed' terminal hub compartments that colocalize with a given antibody divided by the total number of compartments. The 'yellow-fixed' and 'red-fixed' bars are stacked in the bar chart. Mean ± SEM, brain n=3 per antibody staining.

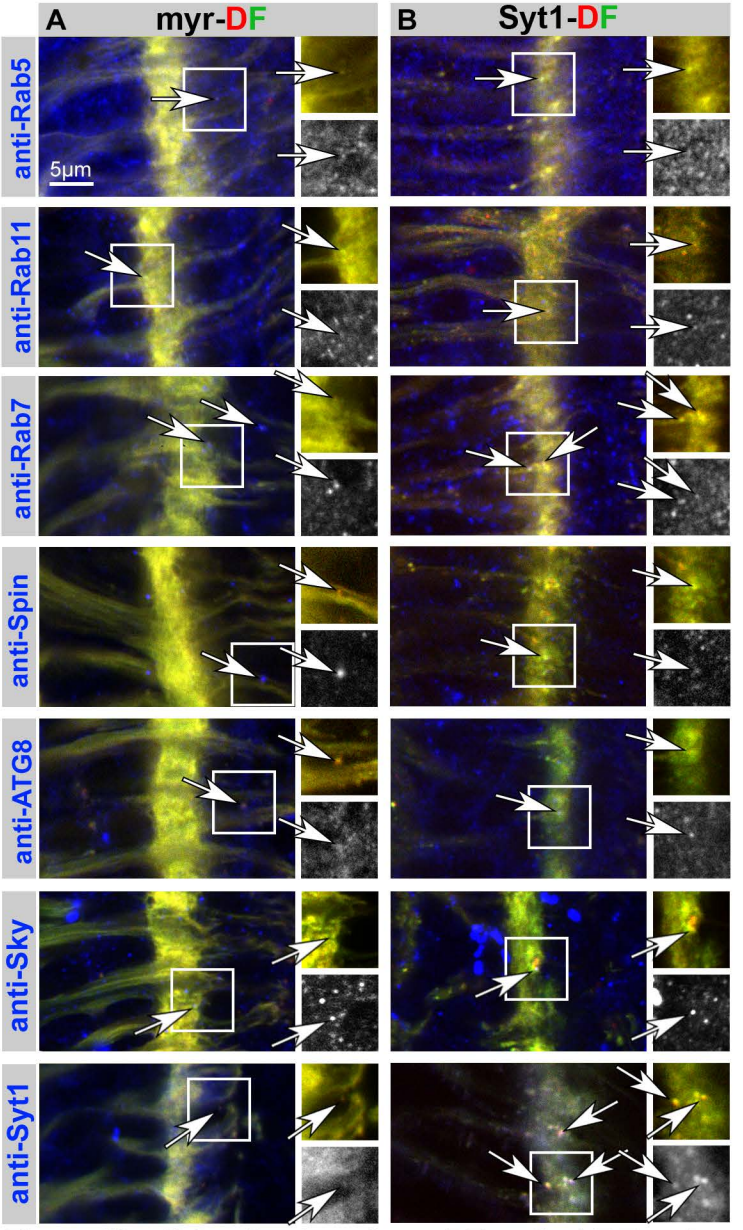
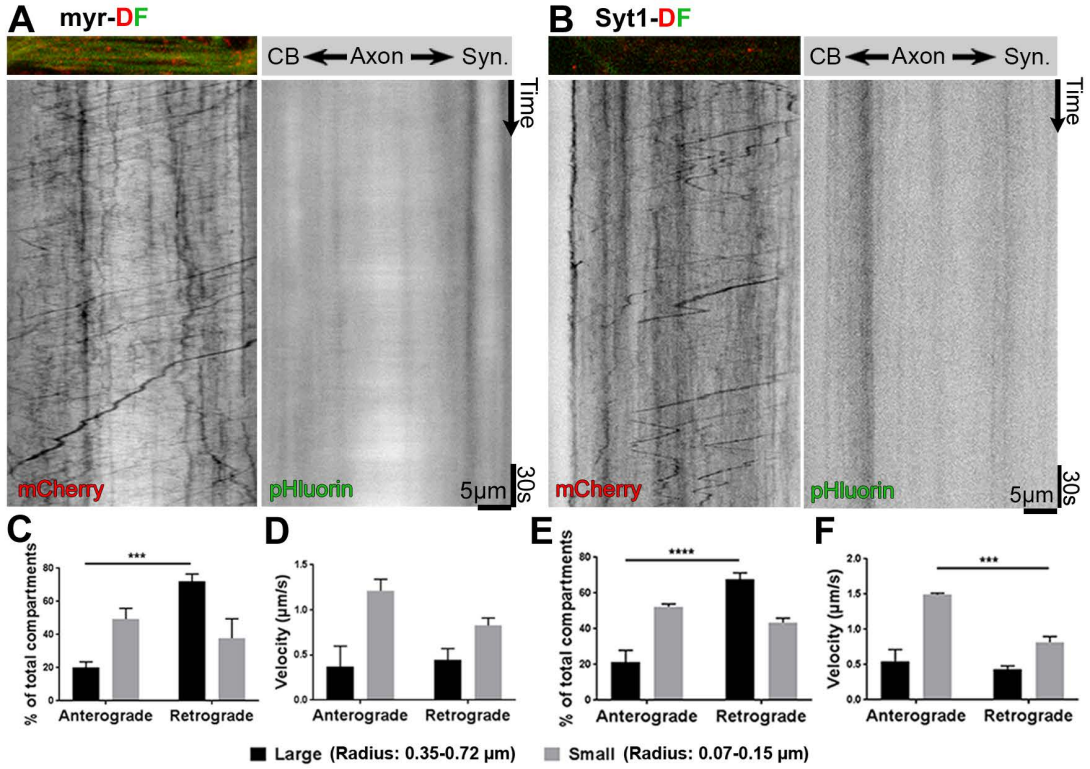
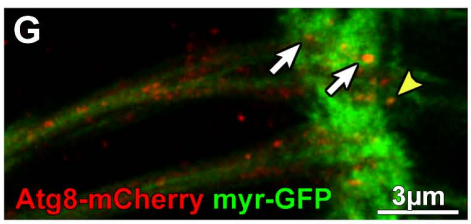


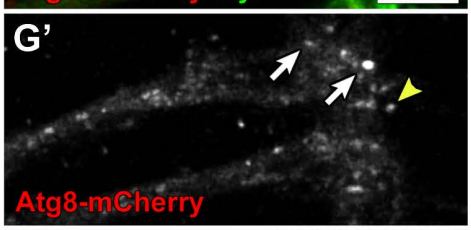
Figure S3. Colocalization of myr-DF or Syt1-DF probes with endolysosomal and autophagosomal markers at axon terminals. Related to Figure 2.

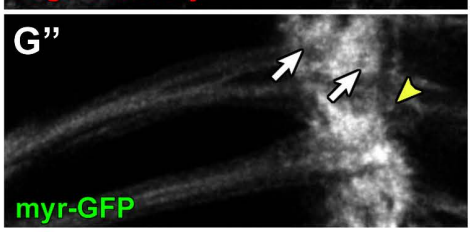
Representative images for colocalization of myr-DF (A) or Syt1-DF (B) at axon terminals with Rab5, Rab11, Rab7, Spinster (Spin), Atg8, Skywalker (Sky) and Synaptotagmin 1 (Syt1). Arrows: examples of colocalization. Single channels are antibody stainings.

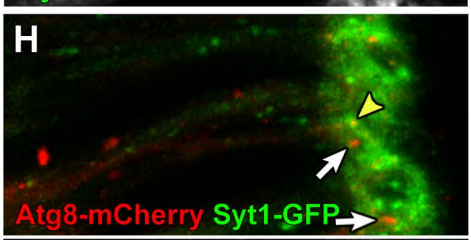

A
myr-DF
CB
Axon
Syn.
Time
mCherry
pHluorin
5μm
30s
B
Syt1-DF
CB
Axon
Syn.
Time
mCherry
pHluorin
5μm
30s

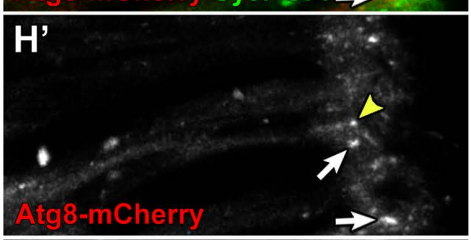
C
% of total compartments
***
Anterograde
Retrograde
D
Velocity (μm/s)
Anterograde
Retrograde
E
% of total compartments
****
Anterograde
Retrograde
F
Velocity (μm/s)
***
Anterograde
Retrograde
Large (Radius: 0.35-0.72 μm)
Small (Radius: 0.07-0.15 μm)

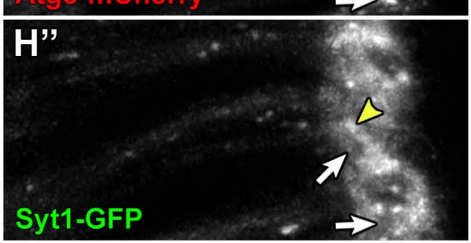

G
Atg8-mCherry myr-GFP
3μm

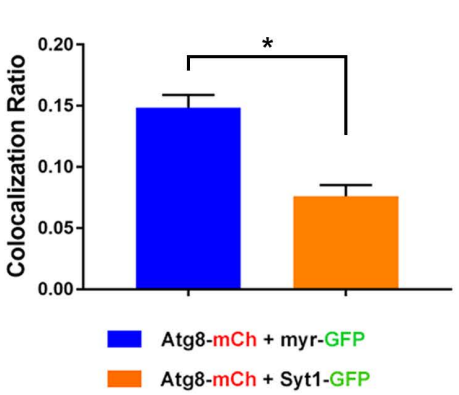

G'
Atg8-mCherry

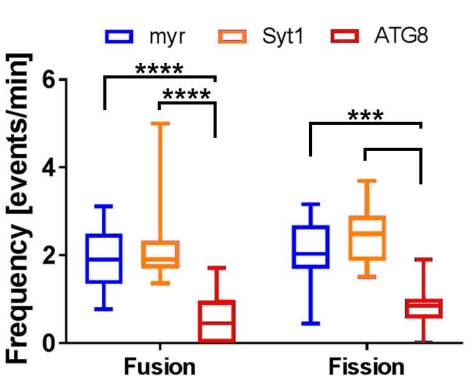

G''
myr-GFP


H
Atg8-mCherry Syt1-GFP


H'
Atg8-mCherry


H''
Syt1-GFP


I
Colocalization Ratio
*
Atg8-mCh + myr-GFP
Atg8-mCh + Syt1-GFP


J
myr
Syt1
ATG8
Frequency [events/min]
****
****
***
Fusion
Fission

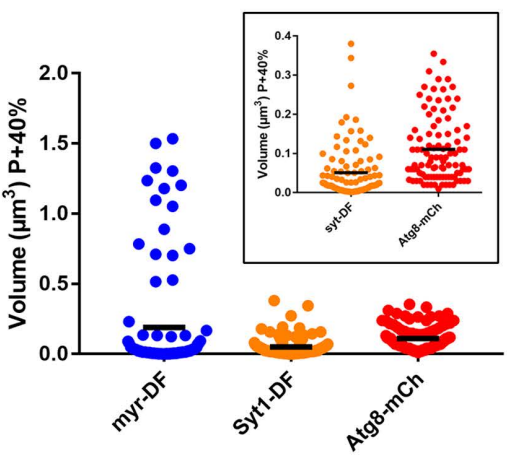

K
Volume (μm3) P+40%
myr-DF
Syt1-DF
Atg8-mCh
syt-DF
Atg8-mCh

Figure S4. Axonal transport dynamics of acidified axonal trafficking vesicles, and characteristics of autophagosomes compared to hub compartments. Related to Figure 3.

(A and B) Kymographs showing axonal transport of myr-DF (A) and Syt1-DF (B) positive compartments. (C and E) Net direction of large or small compartments positive for myr-DF (C) and Syt1-DF (E). Quantification for stationary compartments is not shown. Mean ± SEM, *** $p<0.001$, **** $p<0.0001$, Unpaired t-test, brain n=3 per probe. (D and F) Mean velocity of retrogradely or anterogradely trafficked compartments for myr-DF (D) and Syt1-DF (F). Mean ± SEM, *** $p<0.001$, **** $p<0.0001$, Unpaired t-test, brain n=3 per probe. (G-H'') Live imaging of co-expression of Atg8-mCherry and myr-GFP (G-G'') or Syt1-GFP (H-H'') at P+40% photoreceptor terminals and axons. White arrows: autophagosomes that do not co-localize with myr-GFP or Syt1-GFP positive compartments. Yellow arrowhead: autophagosomes that co-localize with myr-GFP or Syt1-GFP positive compartments. Stacks have been checked in 4D data for co-migration of autophagosomes and Syt1-GFP or myr-GFP positive compartments. (I) Quantification of colocalization ratio between Atg8-mCherry and myr-GFP or Syt1-GFP, Respectively. Mean ± SEM, * $p<0.05$, Unpaired t-test, brain n=3 per co-expression. (J) Fusion and fission frequencies of Atg8-mCherry positive autophagosomes compared to myr-DF and Syt1-DF positive acidified compartments. Box and whiskers plot, showing 5-95 percentiles, hub n=12 to 15 per probe, *** $p<0.001$, **** $p<0.0001$, Unpaired t-test. (K) Volumes of Atg8-mCherry compartments at axon terminals at P+40%, compared with acidified myr-DF and Syt1-DF from Figure 4D. Hub n=100 per probe, black lines indicate mean values. Inset compares volumes of Syt1-DF and Atg8-mCherry compartments.

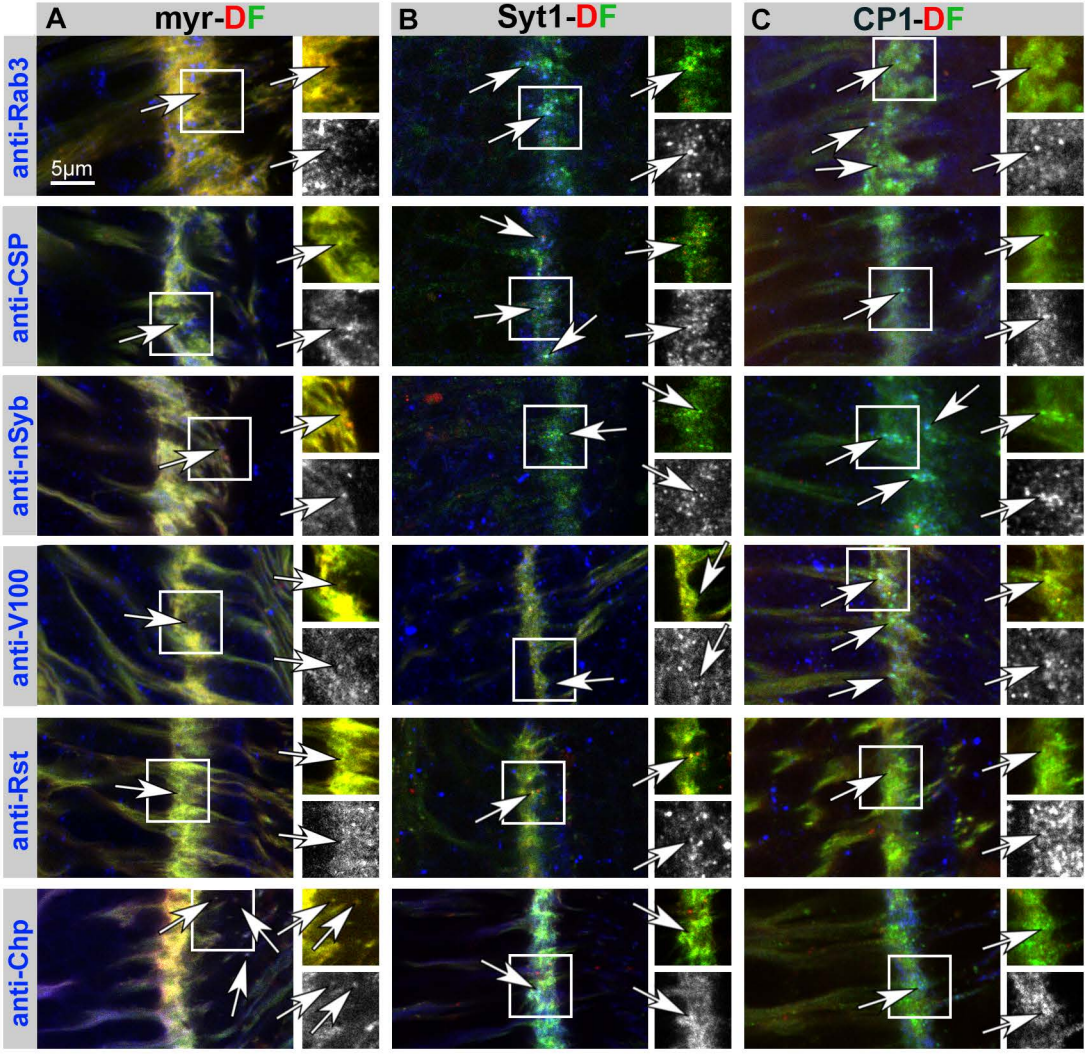


Figure S5. Colocalization of hub compartments at axon terminals with synaptic vesicle proteins and plasma membrane proteins. Related to Figure 4.

Representative images for colocalization of myr-DF (A), Syt1-DF (B), and CP1-DF (C) positive compartments with SV markers (Rab3, Cystein String Proteins (CSP)), SV and endolysosomal markers (neuronal Synaptobrevin (n-Syb) and vesicular ATPase component V100), and two photoreceptor plasma membrane receptors (Chaoptin (Chp) and Roughest (Rst)). Arrows: examples of colocalization. Single channels are antibody stainings.

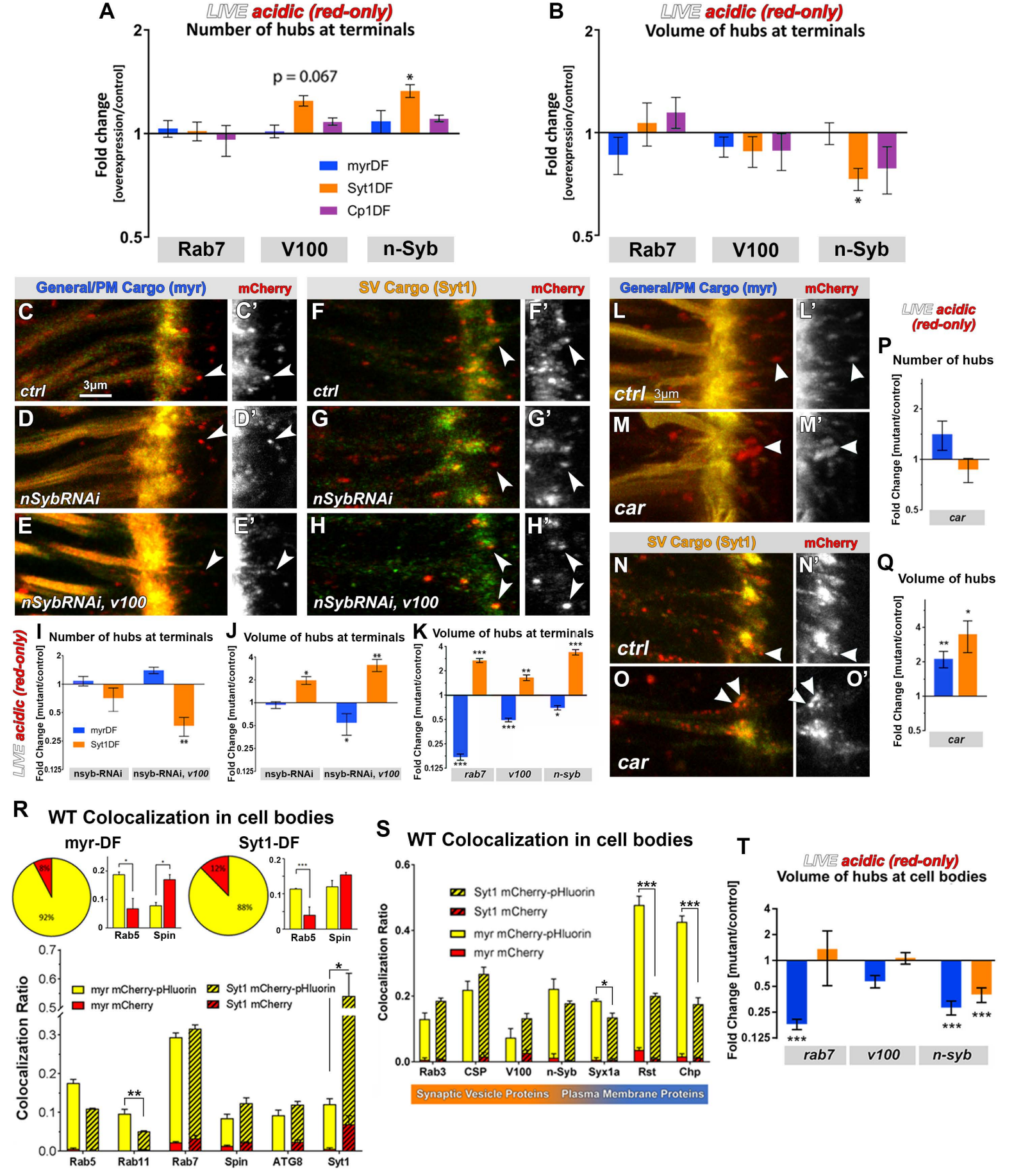

A
LIVE acidic (red-only)
Number of hubs at terminals
Fold change [overexpression/control]
p = 0.067
*
myrDF
Syt1DF
Cp1DF
Rab7
V100
n-Syb
B
LIVE acidic (red-only)
Volume of hubs at terminals
Fold change [overexpression/control]
*
Rab7
V100
n-Syb
General/PM Cargo (myr)
mCherry
SV Cargo (Syt1)
mCherry
C
C'
F
F'
ctrl
3µm
D
D'
G
G'
nSybRNAi
E
E'
H
H"
nSybRNAi, v100
General/PM Cargo (myr)
mCherry
L
L'
ctrl
3µm
M
M'
car
LIVE acidic (red-only)
P
Number of hubs
Fold Change [mutant/control]
car
SV Cargo (Syt1)
mCherry
N
N'
ctrl
O
O'
car
Q
Volume of hubs
Fold Change [mutant/control]
car
LIVE acidic (red-only)
I
Number of hubs at terminals
Fold Change [mutant/control]
myrDF
Syt1DF
nsyb-RNAi
nsyb-RNAi, v100
J
Volume of hubs at terminals
Fold Change [mutant/control]
nsyb-RNAi
nsyb-RNAi, v100
K
Volume of hubs at terminals
Fold Change [mutant/control]
rab7
v100
n-syb
R
WT Colocalization in cell bodies
myr-DF
Syt1-DF
8%
92%
12%
88%
Rab5
Spin
Colocalization Ratio
myr mCherry-pHluorin
myr mCherry
Syt1 mCherry-pHluorin
Syt1 mCherry
Rab5
Rab11
Rab7
Spin
ATG8
Syt1
S
WT Colocalization in cell bodies
Colocalization Ratio
Syt1 mCherry-pHluorin
Syt1 mCherry
myr mCherry-pHluorin
myr mCherry
Rab3
CSP
V100
n-Syb
Syx1a
Rst
Chp
Synaptic Vesicle Proteins
Plasma Membrane Proteins
T
LIVE acidic (red-only)
Volume of hubs at cell bodies
Fold Change [mutant/control]
rab7
v100
n-syb

Figure S6. Live and subcellular characterization of myr-DF and Syt1-DF marked compartments at axon terminals and cell bodies. Related to Figure 5.

(A and B) Relative number (A) and volume (B) of degradative compartments in Rab7, V100 or n-Syb overexpressing axon terminals. Mean ± SEM, * $p<0.05$, Unpaired t-test, brain n=3 per experimental condition. (C-H') Live imaging of myr-DF and Syt1-DF in n-Syb knockdown (n-Syb RNAi) or n-Syb knockdown in v100 mutant background at P+40% photoreceptor axon terminals. Arrowheads: examples of acidic (red-only live) compartments at axon terminals. (I and J) Relative number (I) and volume (J) of degradative compartments from live imaging data shown in A-F'. Mean ± SEM, * $p<0.05$, ** $p<0.01$, Unpaired t-test, brain n=3 per experimental condition. (K) Relative volume of acidic compartments in mutants from live imaging data at axon terminals (Figure 5A-5H'). Mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Kolmogorov-Smirnov test, hub n=240 to 1314, brain n=3 per experimental condition. (L-O') Live imaging of myr-DF and Syt1-DF in car mutant background at P+40% photoreceptor axon terminals. Arrowheads: examples of acidic (red-only live) compartments at axon terminals. (P and Q) Relative number (P) and volume (Q) of acidic compartments in car mutant live imaging data. Mean ± SEM, * $p<0.05$, ** $p<0.01$, Unpaired t-test, brain n=3 per experimental condition. (R) Wildtype colocalization of myr-DF and Syt1-DF compartments in cell bodies. Pie charts show ratios of 'red-fixed' vs 'yellow-fixed' compartments in cell bodies. Bar charts show colocalization ratios of 'yellow-fixed' and 'red-fixed' compartments separately with early endosomal marker Rab5 and lysosomal marker Spin. Bar chart on the bottom shows wildtype colocalization of myr-DF (non-striped) and Syt1-DF (striped) compartments with markers of endolysosomal system, autophagy and Syt1 antibody in cell bodies. Shown are ratios for 'yellow-fixed' and 'red-fixed' compartments that colocalize with a given antibody divided by the total number of compartments. The 'yellow-fixed' and 'red-fixed' bars are stacked in the bar chart. Mean ± SEM, Unpaired t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.01$, brain n=3 to 5. (S) Wildtype colocalization of myr-DF (non-striped) and Syt1-DF (striped) compartments with markers of SV proteins and plasma membrane proteins in cell bodies. Shown are ratios for 'yellow-fixed' and 'red-fixed' compartments that colocalize with a given antibody divided by the total number of compartments. The 'yellow-fixed' and 'red-fixed' bars are stacked in the bar chart. Mean ± SEM, Unpaired t-test, * $p<0.05$, *** $p<0.001$, brain n=3 per antibody staining. (T) Relative volume of acidic compartments in mutants from live imaging data in cell bodies. Mean ± SEM, *** $p<0.001$, Kolmogorov-Smirnov test, hub n=240 to 1314, brain n=3 per experimental condition.

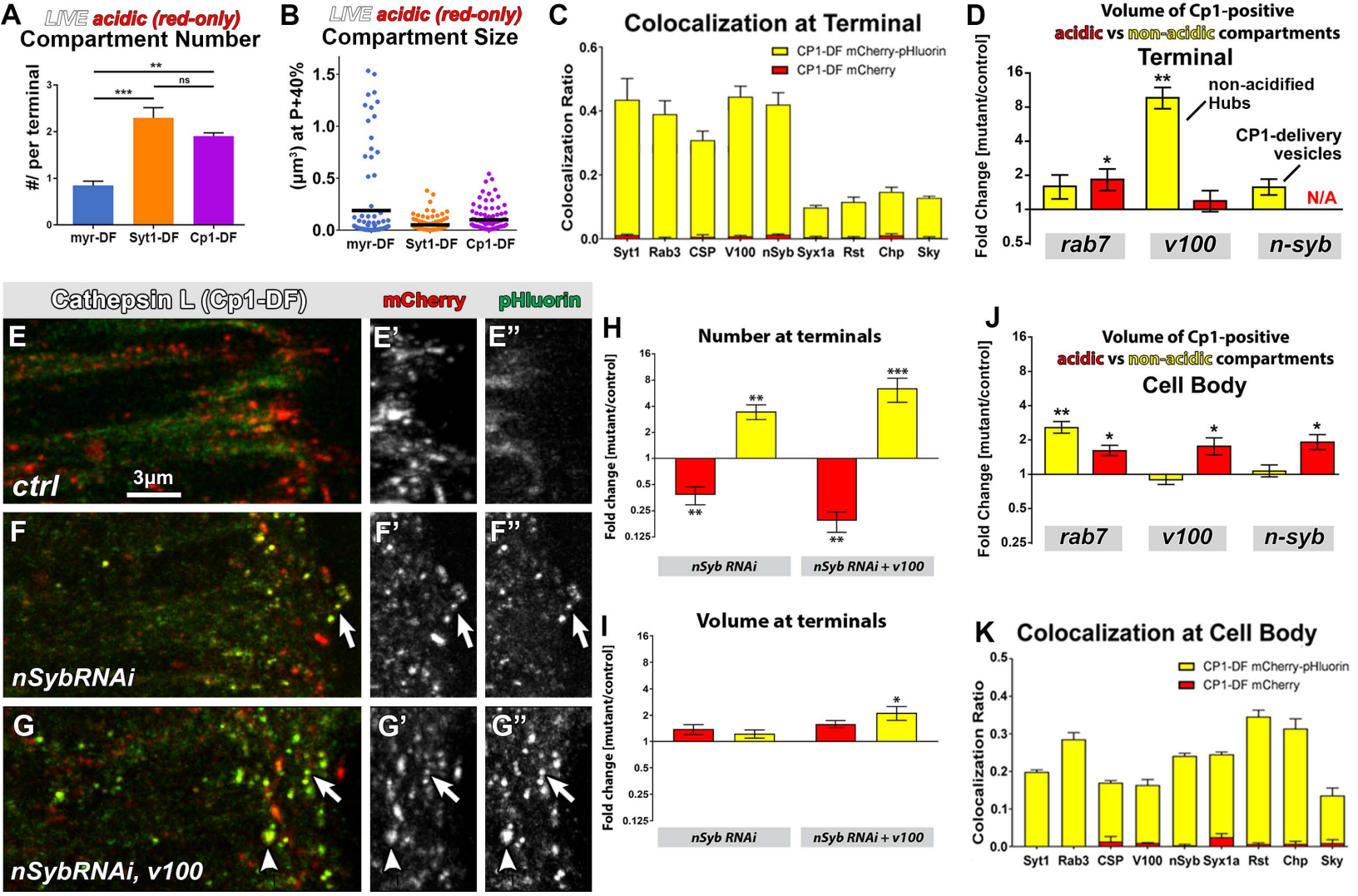


Figure S7. Live and subcellular characterization of CP1-DF marked compartments at axon terminals and cell bodies. Related to Figure 6.

(A and B) Mean number (A) and volume (B) of acidified CP1-DF compartments per axon terminal at P+40% compared to myr-DF and Syt1-DF. n=100 per probe for volume analysis, black lines indicate mean values (B), mean ± SEM (A), **p < 0.01, ***p < 0.001, Unpaired t-test, brain n=3 per probe. (C) Wild-type colocalization of CP1-DF compartments with markers of SV proteins and plasma membrane proteins at axon terminals. Shown are ratios for 'yellow-fixed' and 'red-fixed' terminal compartments that colocalize with a given antibody divided by the total number of compartments. The 'yellow-fixed' and 'red-fixed' bars are stacked in the bar chart. Brain n=3 per antibody staining. (D) Relative volumes of non-acidic (yellow) and acidic (red) compartments containing CP1-DF in rab7, v100 or nsyb mutant backgrounds at axon terminals. Mean ± SEM, * p<0.05, ** p<0.01, Unpaired t-test, brain n=3 per experimental condition. (E-G") Live imaging of CP1-DF in n-Syb knockdown (n-Syb RNAi) or n-Syb knockdown in v100 mutant background at P+40% photoreceptor axon terminals. Arrows: small CP1-delivery vesicles. Arrowheads: large non-acidified hubs. (H and I) Relative number (H) and volume (I) of non-acidic (yellow) and acidic (red) compartments containing CP1-DF in n-Syb knockdown (n-Syb RNAi) or n-Syb knockdown in v100 mutant background at axon terminals. Mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001, Unpaired t-test, brain n=3 per experimental condition. (J) Relative volumes of non-acidic (yellow) and acidic (red) compartments containing CP1-DF in rab7, v100 or nsyb mutant backgrounds in cell bodies. Mean ± SEM, * p<0.05, ** p<0.01, Unpaired t-test, brain n=3 per experimental condition. (K) Wild-type colocalization of CP1-DF compartments with markers of SV proteins and plasma membrane proteins in cell bodies. Shown are ratios for 'yellow-fixed' and 'red-fixed' compartments that colocalize with a given antibody divided by the total number of compartments. The 'yellow-fixed' and 'red-fixed' bars are stacked in the bar chart. Brain n=3 per antibody staining.

Probe, Genotype	Fusion			Fission			Speed			Max. displacement (up to 15 min tracks)		
	Mean [/min]	SEM	Range	Mean [/min]	SEM	Range	Mean [/min]	SEM	Range	Mean [/min]	SEM	Range
myr-DF, Control	1.93	0.21	0.77 - 3.11	2.02	0.24	0.44 - 3.16	5.99	0.51	3.39 - 10.3	2.33	0.22	1.3 - 3.7
myr-DF, *rab7 mutant*		--			--		5.88	0.67	2.73 - 12.4	1.99	0.26	0.9 - 3.6
myr-DF, *v100 mutant*	1.93	0.23	1.04 - 3.36	2.58	0.21	1.19 - 3.82	5.20	0.47	2.37 - 8.60	2.43	0.34	0.7 - 5.2
myr-DF, *nsyb mutant*	1.92	0.24	0.92 - 3.39	2.59	0.27	1.42 - 4.88	5.75	0.42	5.03 - 9.54	2.67	0.47	0.9 - 5.9
Syt1-DF, Control	2.21	0.27	1.35 - 5.00	2.47	0.20	1.50 - 3.69	6.00	0.32	4.48 - 8.47	2.74	0.26	1.2 - 4.1
Syt1-DF, *rab7 mutant*	1.36	0.17	0 - 1.98	1.66	0.15	0.55 - 2.44	5.57	0.49	3.16 - 10.50	2.33	0.15	1.4 - 3.1
Syt1-DF, *v100 mutant*	1.62	0.27	0.44 - 3.57	2.04	0.30	0 - 3.57	5.03	0.42	2.70 - 7.77	1.96	0.33	0.7 - 4.1
Syt1-DF, *nsyb mutant*	1.83	0.17	0.55 - 2.65	2.28	0.29	0.73 - 3.97	6.33	0.46	4.14 - 9.34	2.99	0.50	0.5 - 6.2
ATG8-mCherry-GFP	0.51	0.13	0 - 1.71	0.79	0.11	0 - 1.90	5.53	0.45	3.01 - 8.05	1.99	0.36	0.1 - 5.2

Table S1. Quantitative features of hub compartment dynamics. Related to Figure 3.