Supporting Materials and Methods

General Methods and Strains

All *C. elegans* strains were derived originally from the wild-type Bristol strain N2. Worm cultures, genetic crosses, and other *C. elegans* husbandry were performed according to standard protocols (74). The following mutant strains were used in this study: *dpy-23(e840), toca-1(tm2056), toca-2(ng11), cup-5(ar465), wve-1(ne350), rme-1(b1045), snx-3(tm1595), vps-35(hu68), vps-26(tm1523), vps-29(tm1320), wsp-1(gm324), cwn-1(ok546),* and *egl-20(n585).*

Plasmid Construction

To construct GFP or RFP fusion transgenes for expression in the worm intestine, Gateway destination vectors were used. Each vector uses the promoter region of the intestine-specific gene *vha-6* cloned into the C. elegans pPD117.01 vector, followed by GFP, mCherry, or tagRFP coding sequences, a Gateway cassette (Invitrogen, Carlsbad, CA), *let-858* 3' UTR sequences, and the *unc-119* gene of *C. briggsae*. The genomic or cDNA sequences of *C. elegans toca-1a, toca-2, wve-1, tgn-38 T12G3.7, cdc-42, snx-3, vps-35* genes were cloned individually into entry vector pDONR221. Mutant forms of the coding regions were obtained using site-directed mutagenesis using the Quikchange II kit (Stratagene). Coding regions were transferred into P*vha-6*-GFP or P*vha-6*-RFP vectors by LR reaction (Invitrogen). To drive expression of GFP-CDC-42(G12V) in cells that normally express *egl-20*, the promoter region of the *egl-20* gene (1903 bp) was cloned into *C. elegans* vector pPD117.01 vector with Gateway cassette (Invitrogen, Carlsbad, CA), and *let-858* 3' UTR sequences, followed by the *unc-119* gene of *C. briggsae*.

Protein Expression, Pulldown Assays, and Western Analysis

N-terminally hemagglutinin (HA)-tagged proteins, WVE-1 and CDC-42(G12V), were synthesized *in vitro* using the TNT-coupled transcription-translation system (Promega, Madison, WI) using DNA templates pcDNA3.1–2xHA-WVE-1 and pcDNA3.1–2xHA-CDC-42(G12V), respectively (1.6 ug/each 50-ul reaction). The reaction cocktail was incubated at 30°C for 90 min. Control glutathione *S*-transferase (pGEX-2T), GST-TOCA-1 (SH3 or HR1 domains), GST-TOCA-2 (SH3, SH3(I413S), HR1, or HR1(W585K) domains) fusion proteins were expressed in the *ArcticExpress* strain of *Escherichia coli* (Stratagene, La Jolla, CA). Bacterial pellets were lysed in 10 ml B-PER Bacterial Protein Extraction Reagent (Pierce, Rockford, ILL) with Complete Protease Inhibitor Cocktail Tablets (Roche, Indianapolis, IN). Extracts were cleared by centrifugation, and supernatants were incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia, Piscataway, NJ) at 4°C overnight. Beads were then washed six times with cold STET buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20). In vitro–synthesized HA-tagged protein (10 ul TNT mix diluted in 500 ul STET) was added to the beads and allowed to bind at 4°C for 2 h. After six additional washes in STET the proteins were eluted by boiling in 30 ul 2X-SDS-PAGE sample buffer. Eluted proteins were separated on SDS-PAGE (10% polyacrylamide), blotted to nitrocellulose, visualized with Ponceau S and scanned, washed to remove Ponceau staining, and probed with anti-HA (16B12) antibodies and HA-conjugated secondary antibodies (Pierce). Detection was achieved using Super Signal West Pico detection system (Pierce).

For immunoblotting of whole worm lysates, lysates were prepared from equal numbers of hand-picked adults by incubation at 37°C in Laemmli sampling buffer and subjected to immunoblotting using rabbit goat anti-GFP antibody directly conjugated to HRP (RDI-GRNFP3abg-HRP).

Supplemental Figure Legends

Figure S1. The steady-state level of retrograde cargo MIG-14-GFP, but not other recycling cargos, is strongly reduced in *toca-1(tm2056);toca-2(ng11)* double mutants. Confocal images of intact living animals expressing GFP tagged recycling cargo proteins in the intestinal epithelial cells are shown. (a-d) MIG-14-GFP levels are reduced in *toca-1(tm2056);toca-2(ng11)* double mutants, but not *toca-1(tm2056)* or toca-*2(ng11)* single mutants. (e-h, i-l) Both hTfR-GFP and hTAC-GFP levels and distribution are unchanged in *toca* mutants. (m) Quantification of MIG-14-GFP puncta intensity of genotypes as noted. *P<0.001. Large arrowheads mark the intestinal lumen (enclosed by apical membrane). Small arrowheads mark lateral membranes where cells meet side-to-side or end-to-end. About one cell-length of the intestine is shown in each panel. The scale BARs represents 10μm.

Figure S2. MIG-14-GFP is missorted into the degradative pathway after endocytosis in the intestinal epithelia of *toca-1; toca-2* double mutants or after RNAi-mediated knockdown of *cdc-42, par-6, pkc-3* or *wve-1*. (a) Quantification of MIG-14-GFP average integrated intensity, in arbitrary units, for the indicated genotypes, as shown in Fig. 1. *P<0.001. (b) Anti-GFP and anti-actin Western blot analysis for animals of the indicated genotypes, as shown in Fig. 1. (c-g") Spinning Disc confocal images were acquired in intact living animals expressing GFP-tagged MIG-14/Wls and RFP-tagged RAB-7 in the intestine. About one cell-length of the intestine is shown in each panel. Control is shown in (c-c"), while *toca-1(tm2056); toca-2(ng11)* double mutants (d-d"), *cdc-42* RNAi (e-e"), *pkc-3* RNAi (f-f"), or *wve-1* RNAi (g-g") animals. In each set of images autofluorescent lysosome-like organelles can be seen in blue. GFP appears only in the green channel, and RFP appears only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bone fide GFP or RFP signals, respectively. The scale BAR represents 10μm.

Figure S3. Analysis of TOCA-2 HR1 and SH3 domain binding to CDC-42 and WVE-1. (a-b) *in vitro* translated HA-tagged proteins, CDC-42(G12V) or WVE-1, were incubated with immobilized recombinant proteins: GST-only, GST-HR1, or GST-HR1(I413S) domains of TOCA-2 (a), or GST-only, GST-SH3, or GST-SH3(W585K) domains of TOCA-2 (b). After washing, bound proteins were eluted by boiling and analyzed by Western blot with anti-HA antibody. Input lanes represents 20% of the original HA-tagged proteins. Lower panels show total GST fusion bait proteins visualized by Ponceau S staining prior to antibody probing.

Figure S4. MIG-14-GFP levels are reduced in *rme-1* and *gex-3* mutants but are unchanged in *wsp-1/WASP* mutants. (a-b, d-e, g-h) Confocal images of intestine-specific MIG-14-GFP fluorescence for the indicated genotypes. (c, f, i) Quantification of MIG-14-GFP puncta intensity (arbitrary units). *P<0.001, The scale BARs represents

10μm.

Figure S5. TOCA-1 and TOCA-2 morphology changes upon loss of RME-1 or CDC-42 and TOCA-1 and TOCA-2 are not enriched on the early endosomes or Golgi. In (a-a", b-b") confocal images were acquired in intact living animals expressing GFP tagged TOCA-1 or TOCA-2 specifically in the intestinal epithelial cells. Note the increase in puncta size in *rme-1* mutant (a'-b') and *cdc-42(RNAi)* (a"-b") animals. Quantification of puncta area is shown in (c-d) *P<0.001. Spinning Disc confocal images were acquired in intact living animals expressing GFP-tagged TOCA-1 (a-a", c-c") or TOCA-2 (b-b") and RFP-tagged RAB-5 (a', b'), an early endosome marker, or RAB-6.2 (a'-a", b'-b"), a Golgi marker. Arrowheads indicate TOCA protein labeled puncta. Arrows indicate RAB-5 or RAB-6.2 labeled puncta. In each set of images autofluorescent lysosome-like organelles can be seen in blue. GFP appears only in the green channel, and RFP appears only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bone fide GFP or RFP signals, respectively. The scale BARs represent 10μm. Insets are magnified 3X.

Figure S6. SNX-3 is enriched on early endosomes but not recycling endosomes. Spinning Disc confocal images acquired in intact living animals expressing green and red marker proteins. The early endosome marker GFP- RAB-5 (a-a"), apical recycling endosome marker GFP-RAB-11 (b-b"), or basolateral recycling endosome marker GFP-RME-1, were co-expressed in the intestinal epithelial cells with RFP-tagged SNX-3 (a', b', c'). In each set of images autofluorescent lysosome-like organelles can be seen in blue. GFP appears only in the green channel, and RFP appears only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bone fide GFP or RFP signals, respectively. The scale BARs represents 10μm.

Figure S7. Retromer mutants strongly affect early endosome morphology. (a-b) Quantification of puncta number and puncta area of confocal images of *C. elegans* intestinal cells expressing early endosome marker GFP-RAB-5. *P<0.001. (c) Western blot of GFP-RAB-5 (anti-GFP) and actin from lysates of wild-type and *vps-29* mutant animals. Note that GFP-RAB-5 levels are unchanged in *vps-29* mutants.

Figure S8. *vps-35* mutants do not affect the morphology of TOCA protein labeled endosomes. (a-d) Confocal images of intestinally expressed TOCA-1-GFP or TOCA-2-GFP in WT and *vps-35* retromer mutant animals. (e-f) Quantification of puncta area. GFP-tagged TOCA-1 and TOCA-2-GFP morphology is unaffected in *vps-35* retromer mutants. The scale BARs represents 10μm.

Figure S9. Loss of VPS-26 or SNX-3, but not TOCA-1/2, CDC-42, PKC-3, PAR-6, or WVE-1 disrupts the intracellular distribution of retromer component GFP-VPS-35. (a-i) Confocal images of *C. elegans* intestinal cells expressing GFP-VPS-35 in the indicated genetic backgrounds. Note that GFP-VPS-35 appeared abnormal, losing its normally punctate localization, in *snx-3* or *vps-26* mutant worms. In *toca-1/2* double mutants, or *cdc-42, pkc-3*, *par-6,* or *wve-1* RNAi animals, GFP-VPS-35 appeared unperturbed. The scale BARs represent 10μm. (j) Quantification of GFP-VPS-35 puncta intensity. L4440 = empty vector control. *P<0.001

Figure S10. Conserved features of TGN38-family proteins. (a) Alignment of TGN38 protein sequences from multiple species show sequence conservation is mainly restricted to the intracellular domain. Predicted transmembrane domain is underlined in black. The endocytosis signal is underlined in red. (b) The extracellular domains of TGN38 proteins contain repeated sequences, although the length and number of repeats differ. (c) Quantification of TGN-38-GFP as in Fig. 6. L4440 = empty vector control. *P<0.001.

Figure S11. Abnormal sensory neuron ALM polarity upon disruption of retrograde recycling. Images are shown of the ALM mechanosensory neuron expressing a MEC-4-GFP marker in young adult animals of the indicated genotype. Normally this neuron is monopolar and extends its neurite to the anterior (left). (a) Wild-type, (b) *toca-1; toca-2* double mutant, (c) *cdc-42(RNAi)*. Arrowheads indicate ALM cell body. Thin arrows indicate abnormal posterior directed neurites.

Figure S12. Loss of ARP-2 or DYN-1 blocks the endocytosis of MIG-14-GFP. Confocal images of intestine-specific MIG-14-GFP localization in *arp-2* RNAi (b,e) or *dyn-1* RNAi (c,f) animals compared to control (a,d). In *arp-2* RNAi (b,e) or *dyn-1* RNAi (c,f) MIG-14-GFP was trapped at the basolateral plasma membrane. Arrowheads indicate MIG-14-GFP puncta, representing Golgi ministacks and endosomes. Arrows indicate MIG-14-GFP trapped at the basolateral plasma membrane. (a-c) show the top focal plane (basolateral surface). (d-f) show the middle focal plane (cross section). The scale BAR represents 10μm.

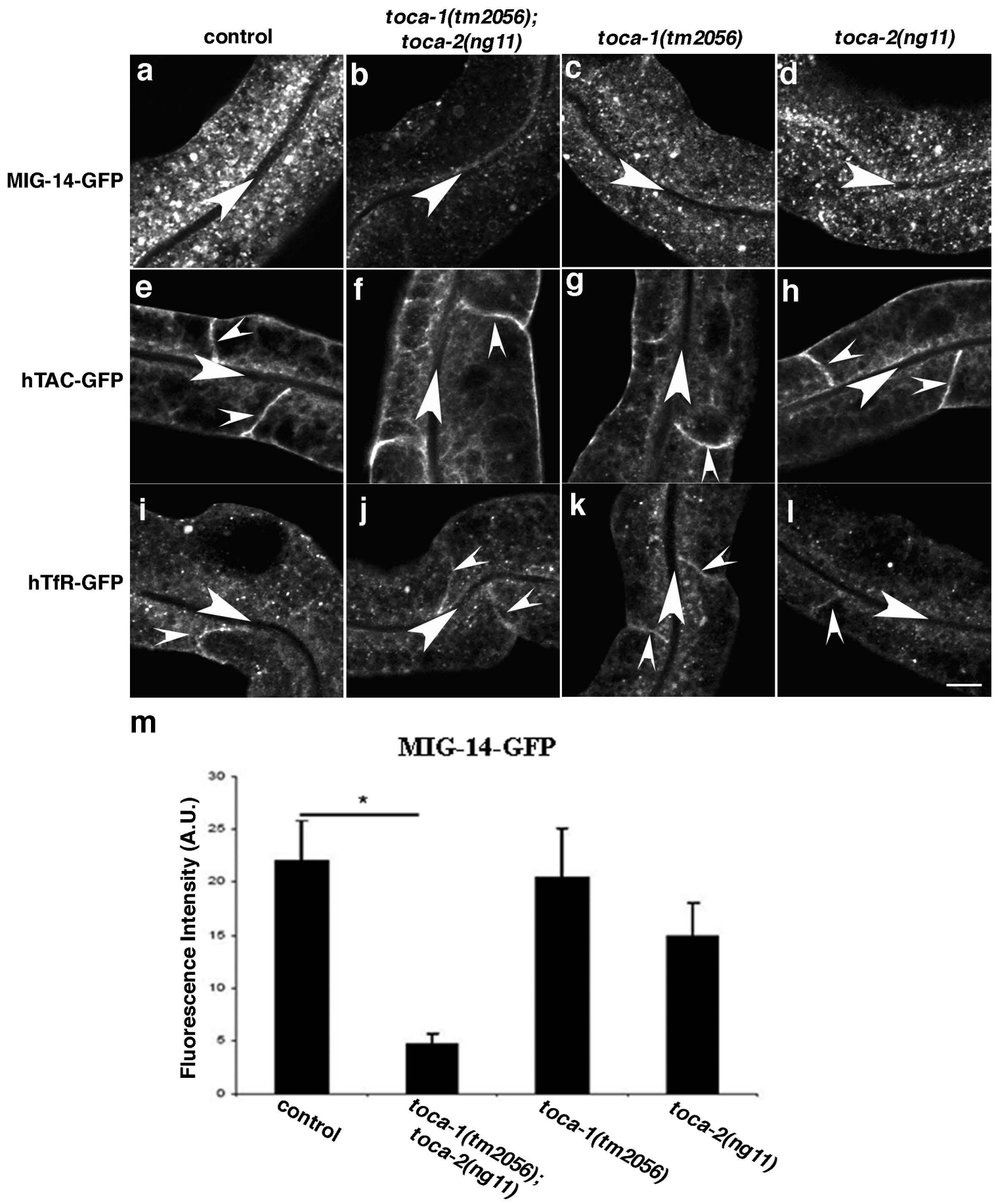

control
toca-1(tm2056); toca-2(ng11)
toca-1(tm2056)
toca-2(ng11)
MIG-14-GFP
hTAC-GFP
hTfR-GFP
a
b
c
d
e
f
g
h
i
j
k
l
m
MIG-14-GFP
Fluorescence Intensity (A.U.)
30
25
20
15
10
5
0
*
control
toca-1(tm2056); toca-2(ng11)
toca-1(tm2056)
toca-2(ng11)

Fig. S1

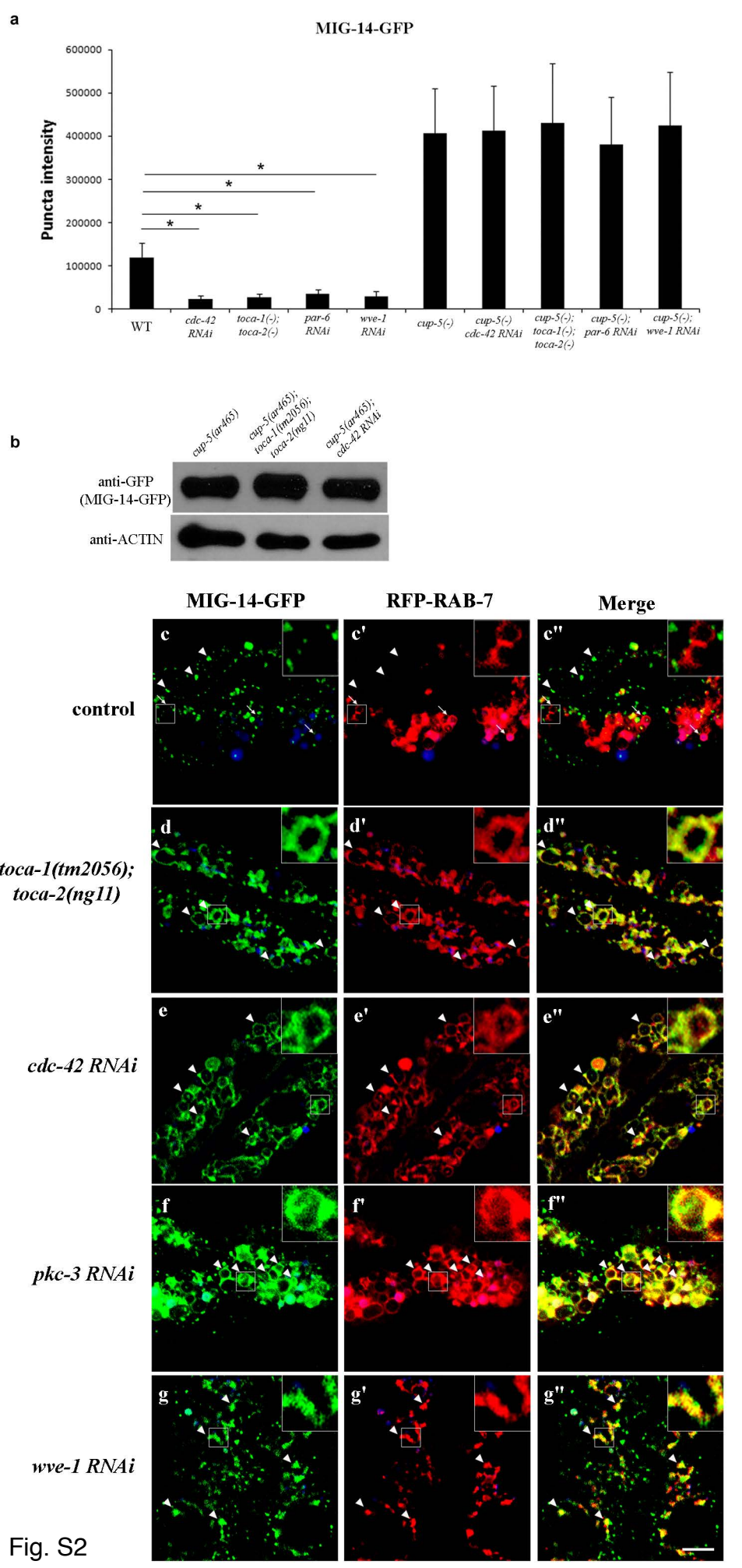
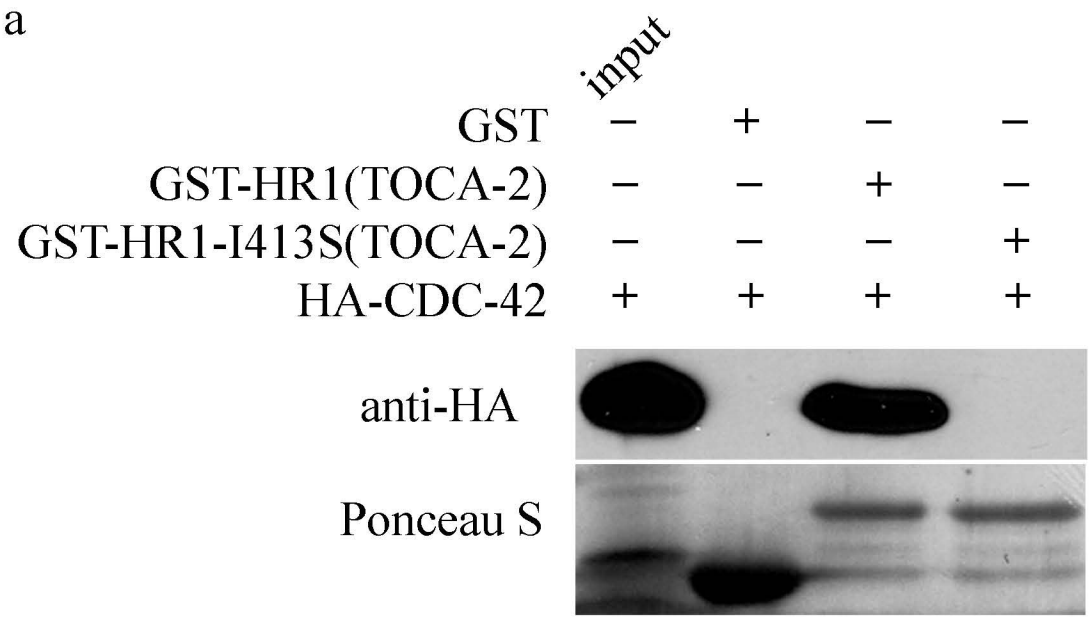

a
MIG-14-GFP
Puncta intensity
600000
500000
400000
300000
200000
100000
0
*
*
*
*
WT
cdc-42 RNAi
toca-1(-); toca-2(-)
par-6 RNAi
wve-1 RNAi
cup-5(-)
cup-5(-) cdc-42 RNAi
cup-5(-); toca-1(-); toca-2(-)
cup-5(-); par-6 RNAi
cup-5(-); wve-1 RNAi
b
cup-5(ar465)
cup-5(ar465); toca-1(tm2056); toca-2(ng11)
cup-5(ar465); cdc-42 RNAi
anti-GFP (MIG-14-GFP)
anti-ACTIN
MIG-14-GFP
RFP-RAB-7
Merge
control
toca-1(tm2056); toca-2(ng11)
cdc-42 RNAi
pkc-3 RNAi
wve-1 RNAi
c
c'
c''
d
d'
d''
e
e'
e''
f
f'
f''
g
g'
g''

Fig. S2

a


input
GST − + − −
GST-HR1(TOCA-2) − − + −
GST-HR1-I413S(TOCA-2) − − − +
HA-CDC-42 + + + +
anti-HA
Ponceau S

b

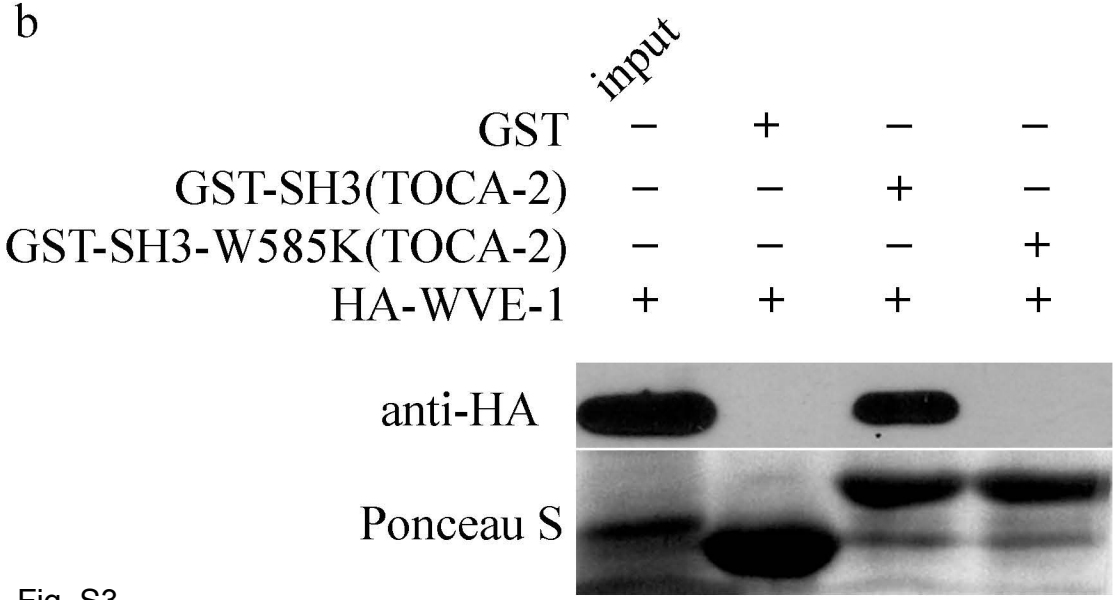

input
GST − + − −
GST-SH3(TOCA-2) − − + −
GST-SH3-W585K(TOCA-2) − − − +
HA-WVE-1 + + + +
anti-HA
Ponceau S

Fig. S3

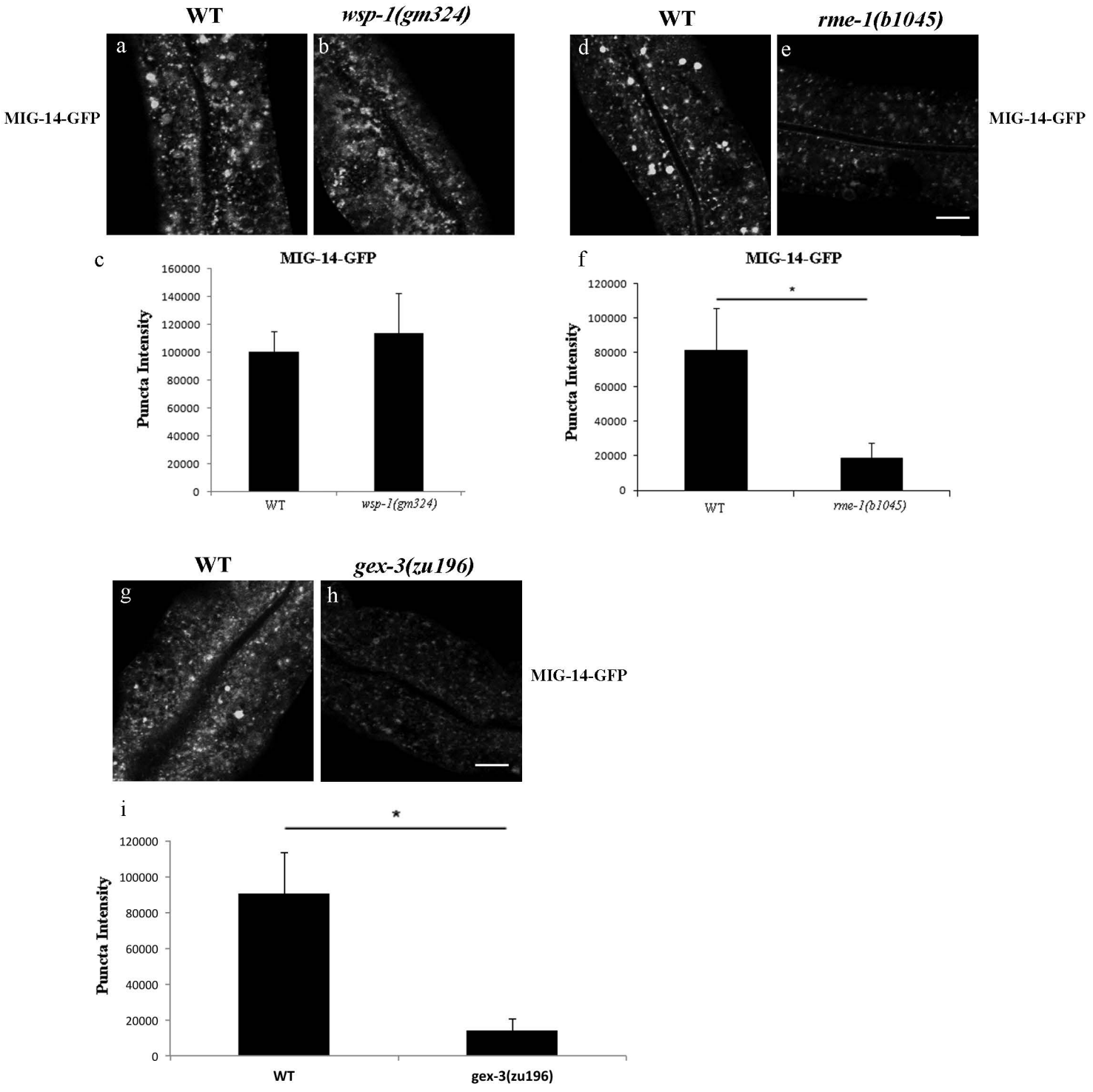

WT
wsp-1(gm324)
a
b
MIG-14-GFP
WT
rme-1(b1045)
d
e
MIG-14-GFP
c
MIG-14-GFP
Puncta Intensity
160000
140000
120000
100000
80000
60000
40000
20000
0
WT
wsp-1(gm324)
f
MIG-14-GFP
Puncta Intensity
120000
100000
80000
60000
40000
20000
0
*
WT
rme-1(b1045)
WT
gex-3(zu196)
g
h
MIG-14-GFP
i
Puncta Intensity
120000
100000
80000
60000
40000
20000
0
*
WT
gex-3(zu196)

Fig. S4

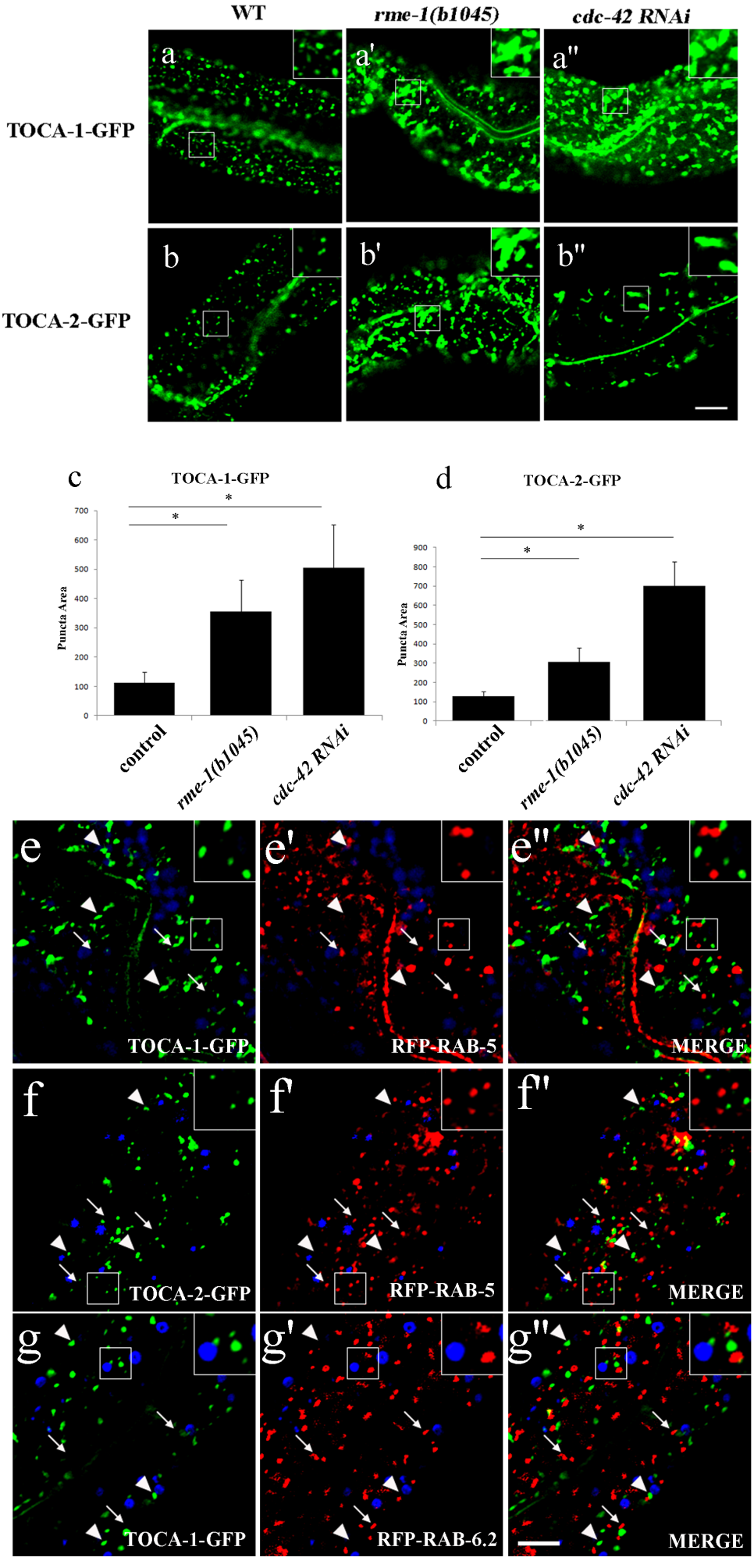

WT
rme-1(b1045)
cdc-42 RNAi
TOCA-1-GFP
TOCA-2-GFP
a
a'
a''
b
b'
b''
c
TOCA-1-GFP
Puncta Area
control
rme-1(b1045)
cdc-42 RNAi
d
TOCA-2-GFP
Puncta Area
control
rme-1(b1045)
cdc-42 RNAi
e
e'
e''
TOCA-1-GFP
RFP-RAB-5
MERGE
f
f'
f''
TOCA-2-GFP
RFP-RAB-5
MERGE
g
g'
g''
TOCA-1-GFP
RFP-RAB-6.2
MERGE

Fig. S5

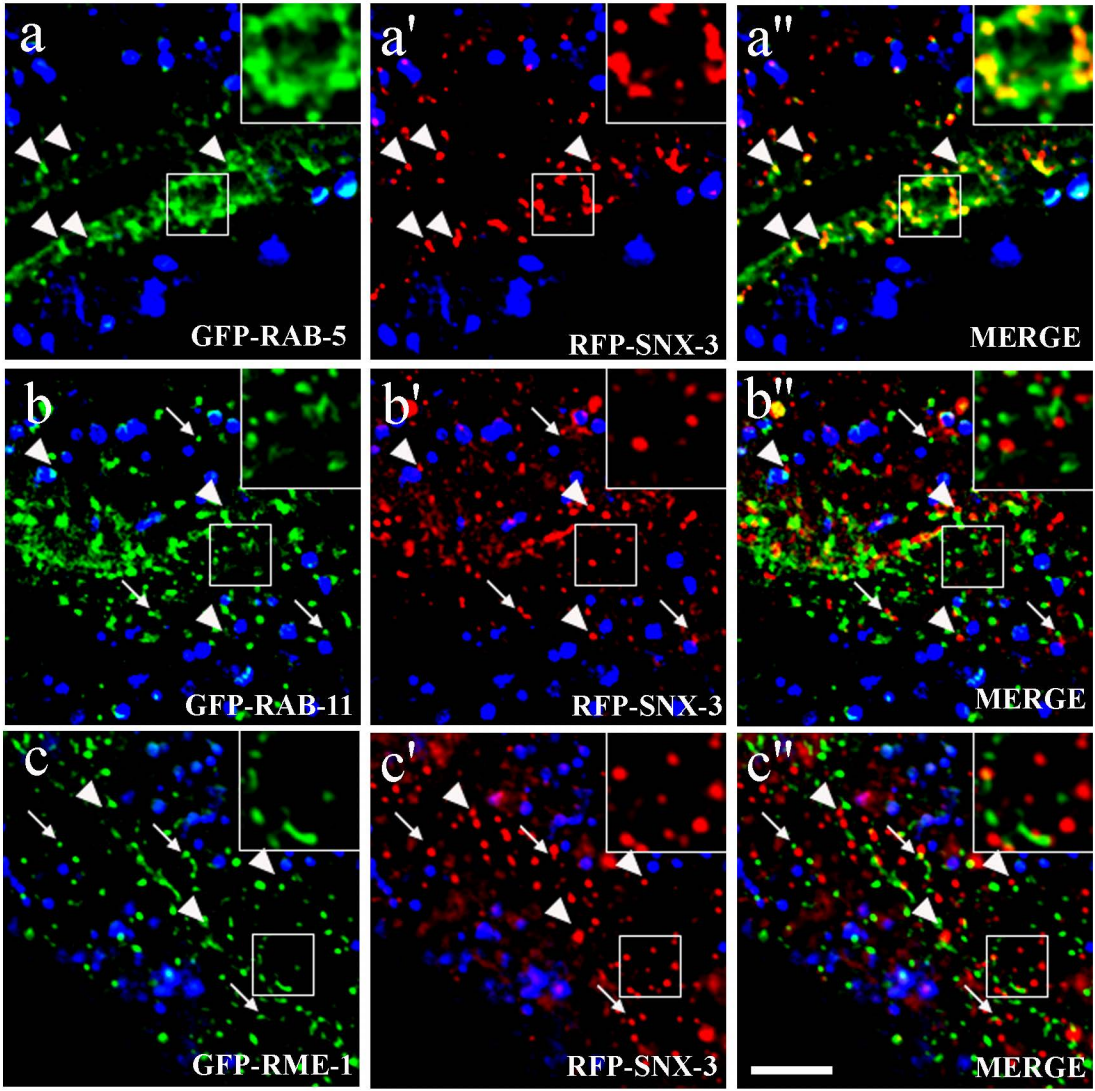
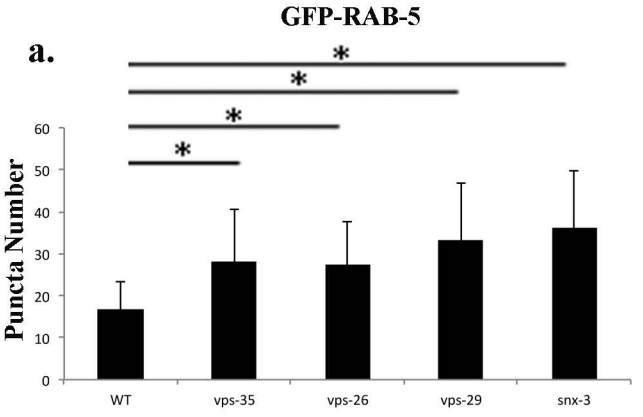
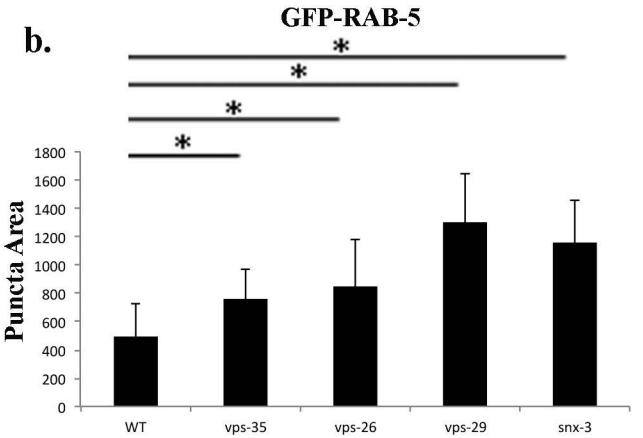

a
GFP-RAB-5
a'
RFP-SNX-3
a''
MERGE
b
GFP-RAB-11
b'
RFP-SNX-3
b''
MERGE
c
GFP-RME-1
c'
RFP-SNX-3
c''
MERGE

Fig. S6


GFP-RAB-5
a.
*
*
*
*
Puncta Number
60
50
40
30
20
10
0
WT
vps-35
vps-26
vps-29
snx-3


GFP-RAB-5
b.
*
*
*
*
Puncta Area
1800
1600
1400
1200
1000
800
600
400
200
0
WT
vps-35
vps-26
vps-29
snx-3

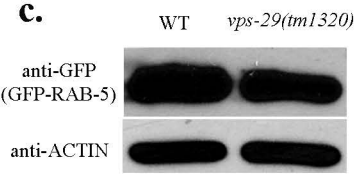
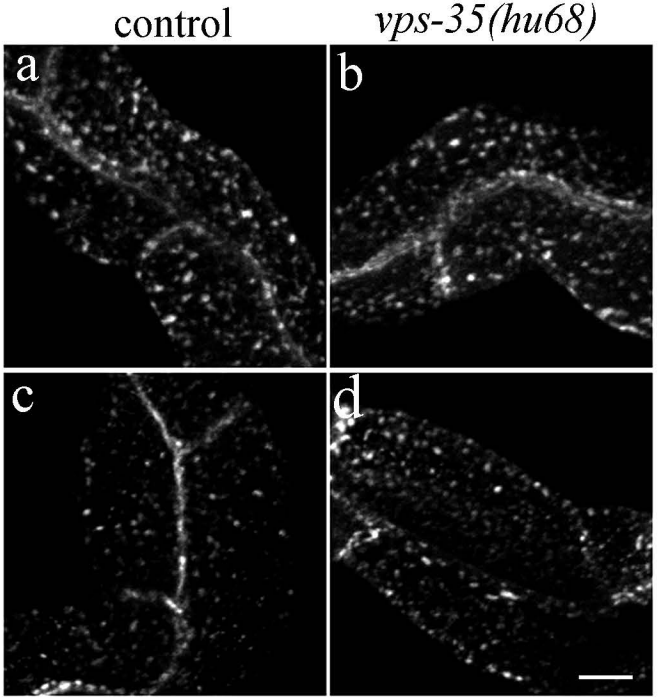
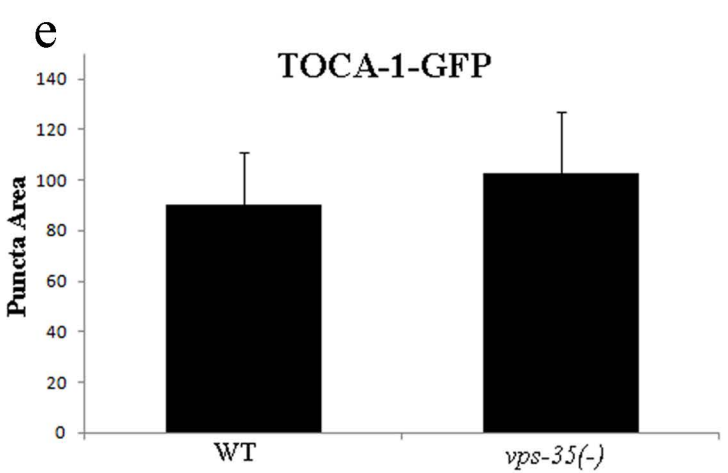
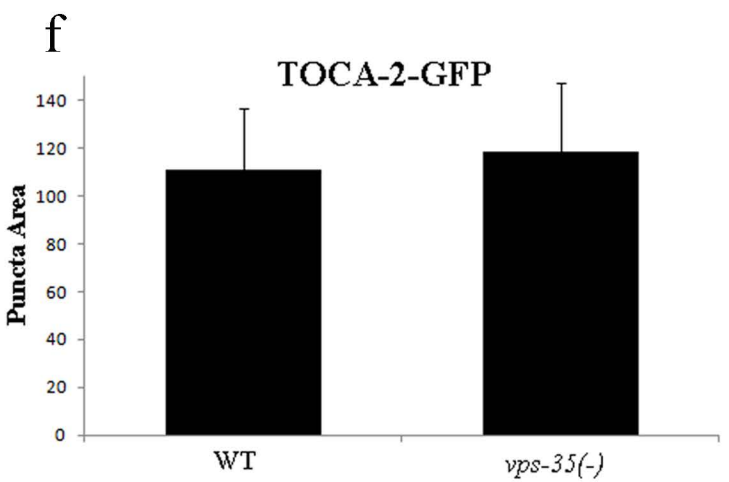

c.
WT
vps-29(tm1320)
anti-GFP
(GFP-RAB-5)
anti-ACTIN

Fig. S7

Fig. S8


control
vps-35(hu68)
a
b
c
d


e
TOCA-1-GFP
Puncta Area
140
120
100
80
60
40
20
0
WT
vps-35(-)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TOCA-2-GFP
Puncta Area
140
120
100
80
60
40
20
0
WT
vps-35(-)

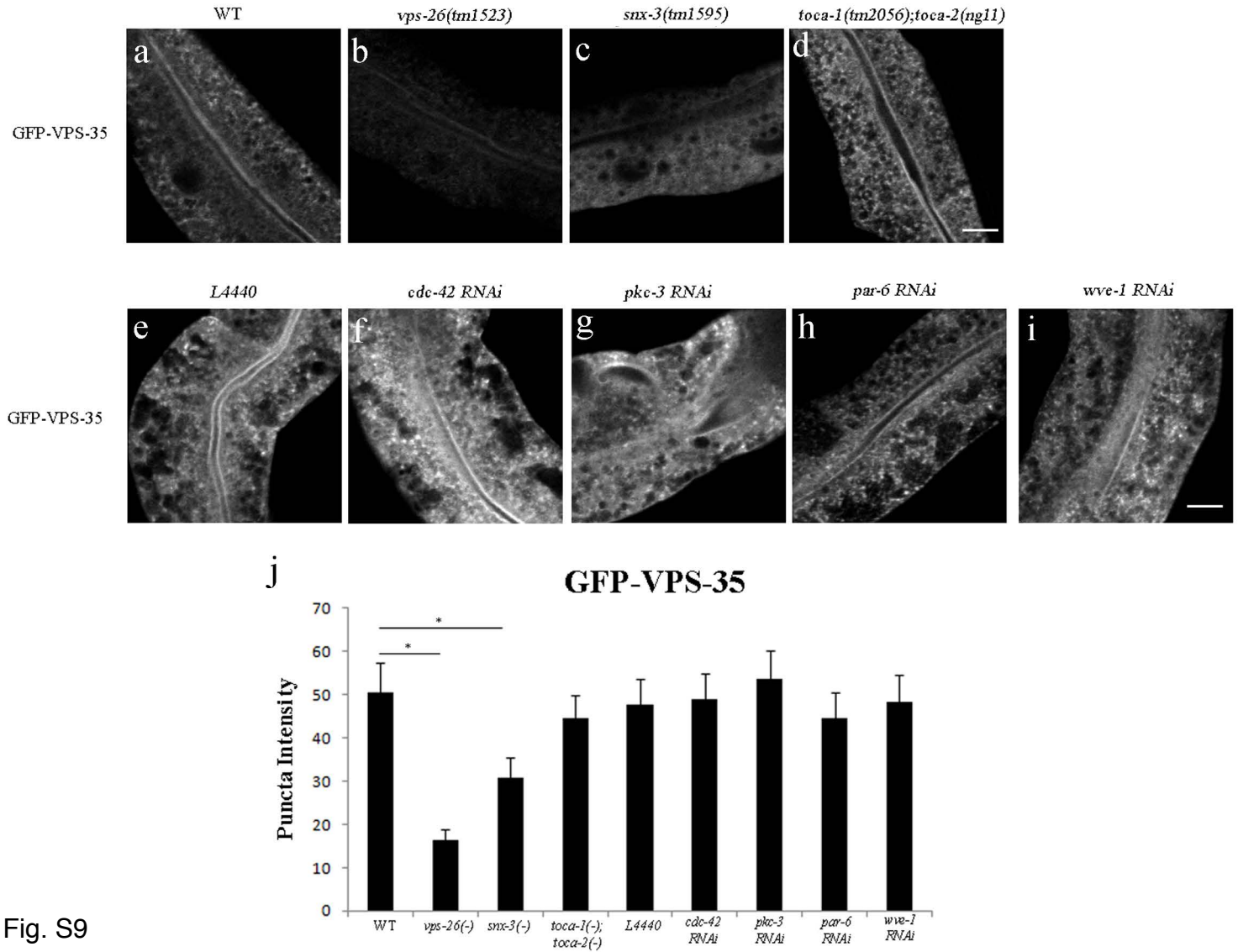

WT
vps-26(tm1523)
snx-3(tm1595)
toca-1(tm2056);toca-2(ng11)
a
b
c
d
GFP-VPS-35
L4440
cdc-42 RNAi
pkc-3 RNAi
par-6 RNAi
wve-1 RNAi
e
f
g
h
i
GFP-VPS-35
j
GFP-VPS-35
Puncta Intensity
70
60
50
40
30
20
10
0
*
*
WT
vps-26(-)
snx-3(-)
toca-1(-); toca-2(-)
L4440
cdc-42 RNAi
pkc-3 RNAi
par-6 RNAi
wve-1 RNAi

Fig. S9

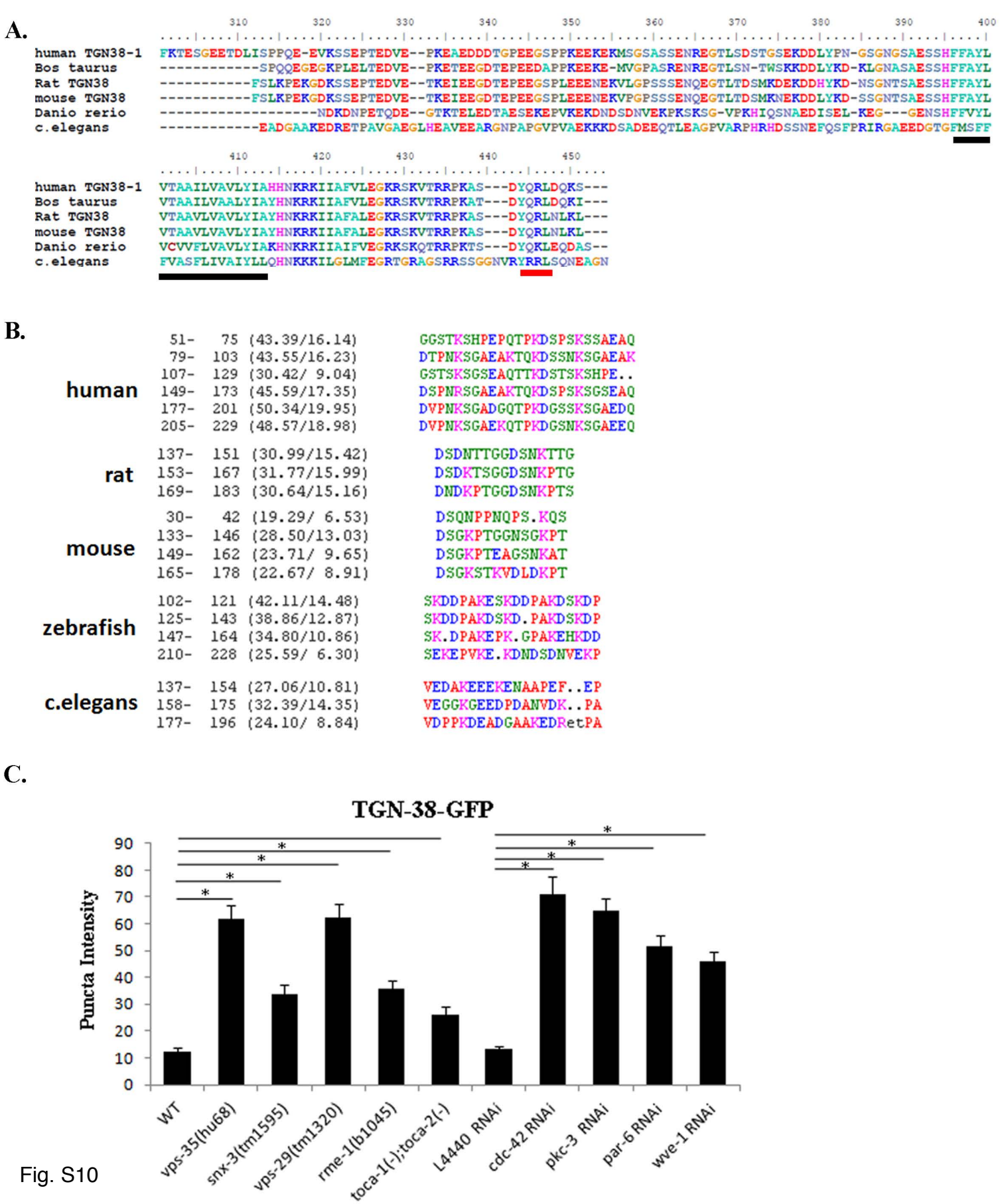
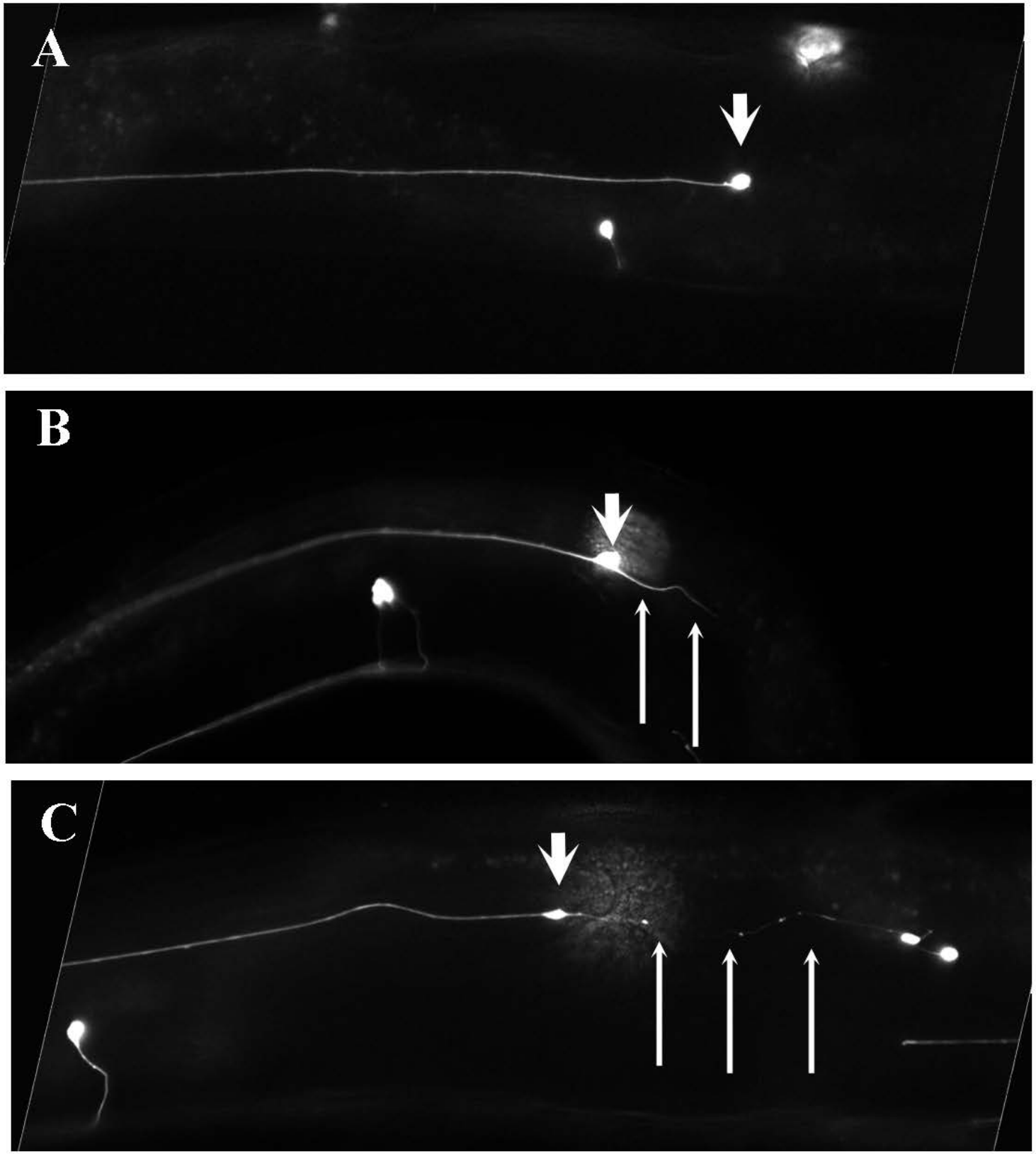

A.
human TGN38-1
Bos taurus
Rat TGN38
mouse TGN38
Danio rerio
c.elegans
B.
human
rat
mouse
zebrafish
c.elegans
C.
TGN-38-GFP
Puncta Intensity
WT
vps-35(hu68)
snx-3(tm1595)
vps-29(tm1320)
rme-1(b1045)
toca-1(-);toca-2(-)
L4440 RNAi
cdc-42 RNAi
pkc-3 RNAi
par-6 RNAi
wve-1 RNAi

Fig. S10



control

toca-1(tm2056); toca-2(ng11)

cdc-42(RNAi)

Fig. S11

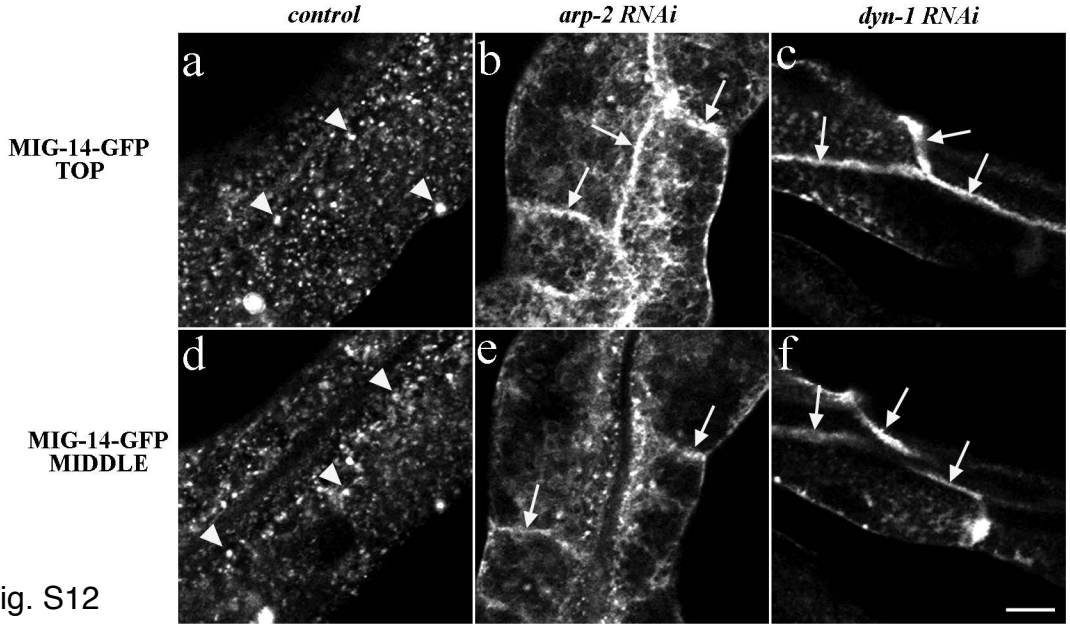

control
arp-2 RNAi
dyn-1 RNAi
MIG-14-GFP
TOP
MIG-14-GFP
MIDDLE
a
b
c
d
e
f

Fig. S12

Genotype	ALM abnormal n=100	ALM abnormal n=100
	vps-29(+)	*vps-29(tm1320)*
+	0	0
toca-1(tm2056); toca-2(ng11)	6	24
cdc-42 RNAi	6	15
par-6 RNAi	5	12
wve-1 RNAi	15	25
vps-35(hu68)	26	ND

Supplemental Table 1. The TOCA, CDC-42, PAR-6, and WVE-1 proteins are required for sensory neuron ALM polarity. 100 animals expressing a MEC-4-GFP marker, that labels the six mechanosensory touch neurons, were assayed for ALM neuron polarity in otherwise wild-type animals, or in the noted genotypes. ND = not done

Genotype	ALM abnormal n=100
cwn-1(ok546)	1
egl-20(n585)	0
cwn-1(ok546);egl-20(n585)	39
egl-20p-GFP-CDC-42(G12V)	0
cwn-1(ok546);egl-20p-GFP-CDC-42(G12V)	16

Supplemental Table 2. CDC-42 is required in WNT secreting cells. CDC-42(G12V) was expressed in a group of WNT ligand secreting cells using the *egl-20* promoter, which does not express in WNT receiving cells such as ALM. 100 animals expressing a MEC-4-GFP marker, that labels the six mechanosensory touch neurons, were assayed for ALM neuron polarity in the noted genotypes.