Supplemental Materials

Molecular Biology of the Cell

Robinson et al.

Supplementary information.

Figure S1. Rapid partial recovery of melanosome associated Myo-T and Myo-FL after photobleaching in myosin-Va -/- cells. Myosin-Va -/- (melan-d cells) were cultured in glass-bottomed dishes, infected with adenoviruses expressing MVa-tail (A) or MVa-FL (B) and the dynamics of their association with melanosomes was investigated using confocal FRAP analysis (see materials and methods). (A, B) Upper panels are images showing the distribution of GFP-myosin-Va and melanosomes and their co-incidence in living cells. Scale bars = 20 μm. White boxes indicate the regions shown in the high magnification images below. Lower panels images taken from example FRAP series captured at the indicated time relative to photo-bleaching (t = 0) (See movies 2 and 3). Yellow arrows highlight the position of the photo-bleached melanosome over time. (C) A line plot showing the average fluorescence intensity associated with photo-bleached melanosomes over time. n= 11 (Myo-T) and 8 (Myo-FL) melanosomes analysed (supports Figure 1).

Figure S1

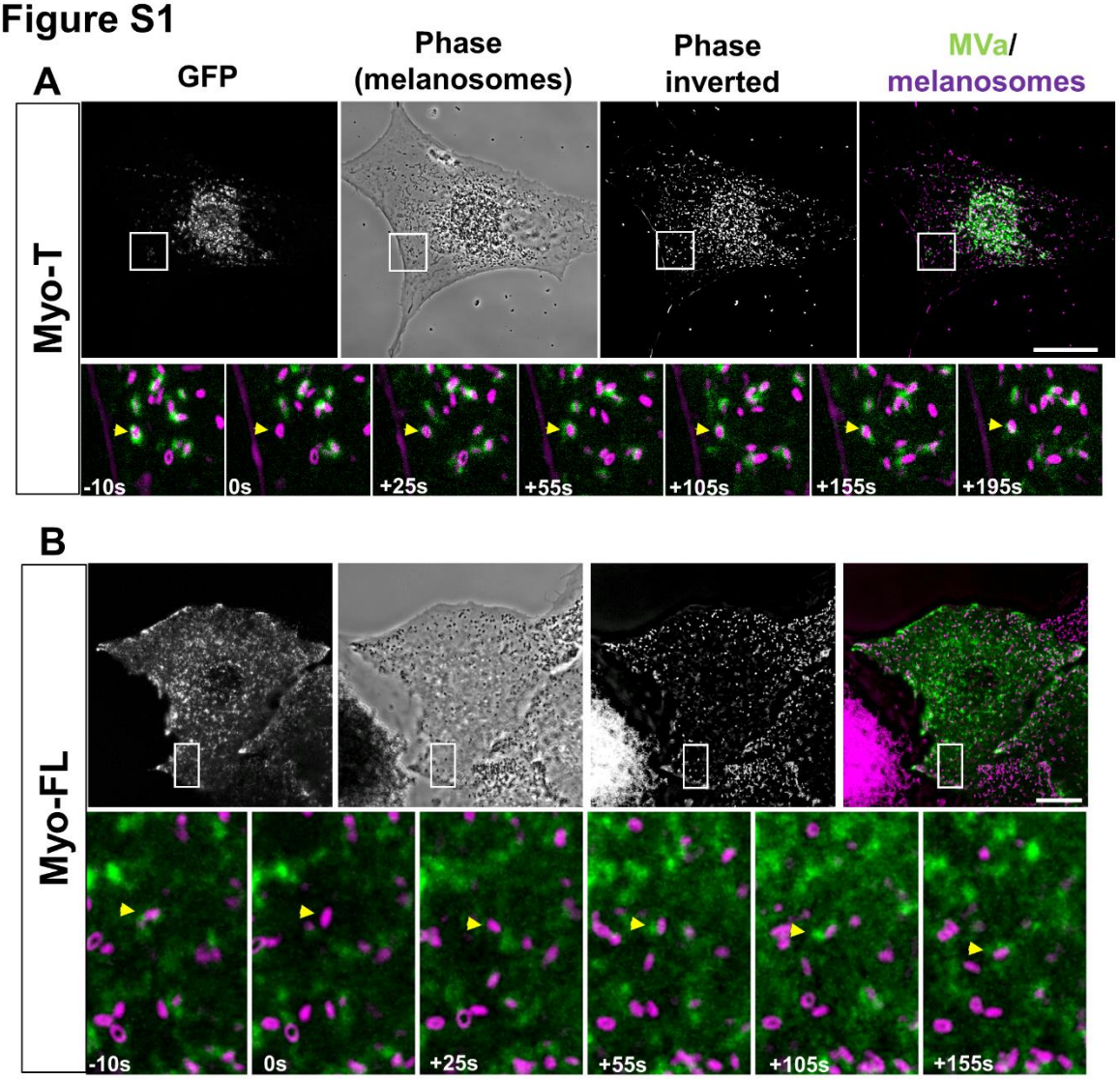
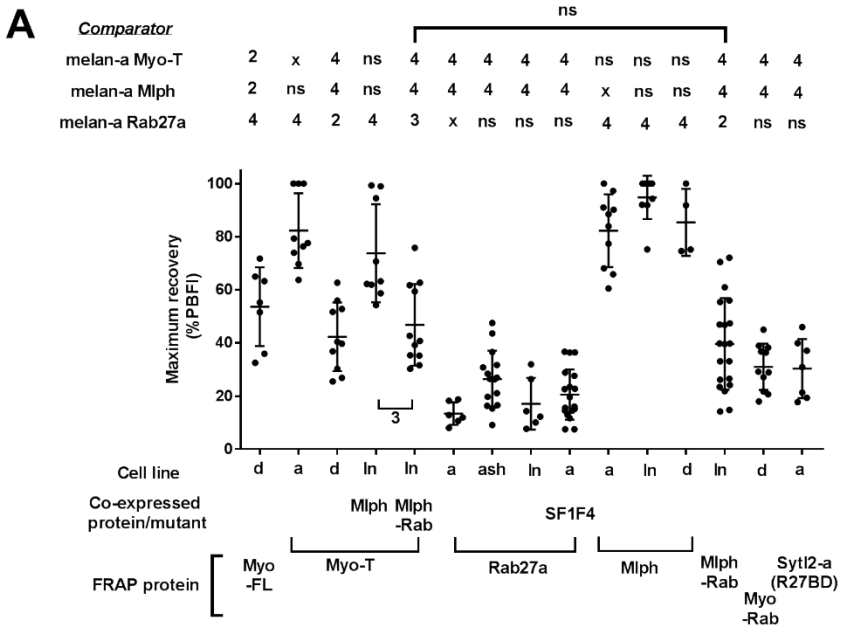
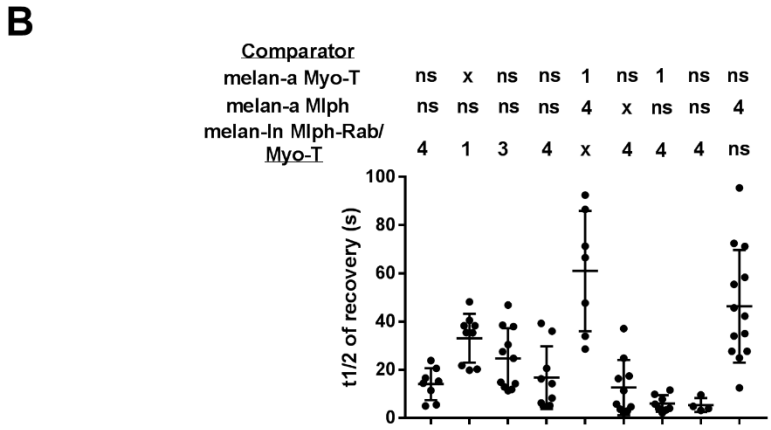

A
GFP
Phase (melanosomes)
Phase inverted
MVa/ melanosomes
Myo-T
-10s
0s
+25s
+55s
+105s
+155s
+195s
B
Myo-FL
-10s
0s
+25s
+55s
+105s
+155s
C
Fluorescence intensity (% maximum)
100
80
60
40
20
0
0
50
100
150
200
time [s]
Myo-FL
Myo-T

Figure S2. Comparison of smFRAP recovery parameters for myosin-Va and adaptors (Mlph and Rab27) in melanocytes. (A-C) Scatter-plots showing the indicated recovery parameters for the indicated proteins/cell line combinations. For cells lines a = melan-a/wild-type, ln = melan-ln/Mlph -/- and d = melan-d/myosin-Va -/-. 4, 3, 2, 1, ns and x indicate the significance of differences in populations of data of p = <0.0001, p = <0.001, p = <0.01, p = <0.05, no significance and no test as determined by one-way ANOVA (supports Figures 1-4).

Figure S2


A
Comparator
ns
melan-a Myo-T 2 x 4 ns 4 4 4 4 4 ns ns ns 4 4 4
melan-a Mlph 2 ns 4 ns 4 4 4 4 4 x ns ns 4 4 4
melan-a Rab27a 4 4 2 4 3 x ns ns ns 4 4 4 2 ns ns
Maximum recovery (%PBFI)
100
80
60
40
20
0
3
Cell line d a d ln ln a ash ln a a ln d ln d a
Co-expressed protein/mutant
Mlph Mlph -Rab
SF1F4
FRAP protein
Myo -FL
Myo-T
Rab27a
Mlph
Mlph -Rab
Sytl2-a (R27BD)
Myo -Rab


B
Comparator
melan-a Myo-T ns x ns ns 1 ns 1 ns ns
melan-a Mlph ns ns ns ns 4 x ns ns 4
melan-ln Mlph-Rab/ Myo-T 4 1 3 4 x 4 4 4 ns
t1/2 of recovery (s)
100
80
60
40
20
0

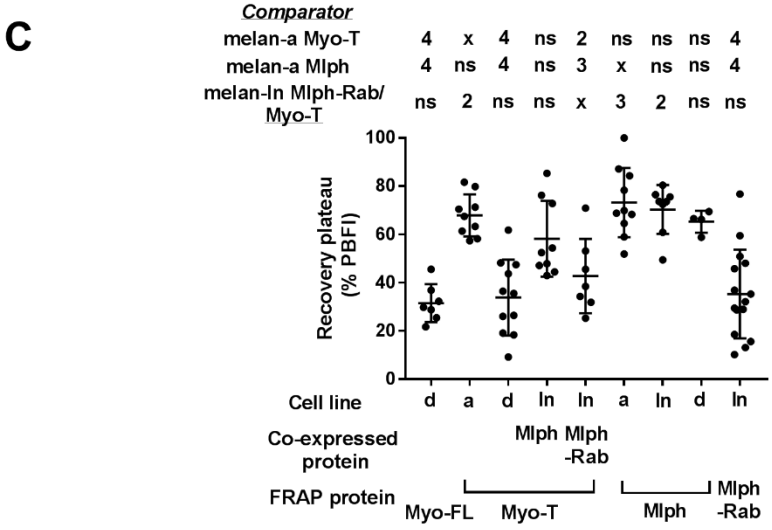

C
Comparator
melan-a Myo-T 4 x 4 ns 2 ns ns ns 4
melan-a Mlph 4 ns 4 ns 3 x ns ns 4
melan-ln Mlph-Rab/ Myo-T ns 2 ns ns x 3 2 ns ns
Recovery plateau (% PBFI)
100
80
60
40
20
0
Cell line d a d ln ln a ln d ln
Co-expressed protein
Mlph Mlph -Rab
FRAP protein
Myo-FL
Myo-T
Mlph
Mlph -Rab

Figure S3. Co-localisation of experimental proteins with melanosomes. (A - B) mCherry-Mlph/Mlph-Rab27a and GFP-MVa-tail with melanosomes in melan-ln cells (supports Figure 4). Images are high magnification views of the boxed regions in Figure 4B-C showing the distribution of MVa-tail (GFP), mCherry-Mlph/Mlph-Rab27a, melanosomes (inverted) and their colocalisation. Coloured arrows highlight regions of co-localisation (C) MVa-tail in melan-a/wild-type and Rab27a/Mlph in melan-ln/Mlph -/- melanocytes (supports Figures 1-3). Images are high magnification views of the boxes regions shown in figures 1A, 2C, and 3B showing the merge and individual GFP and inverted LUT melanosomes in pre-bleach images. Scale bars = 2 μm.

Figure S3

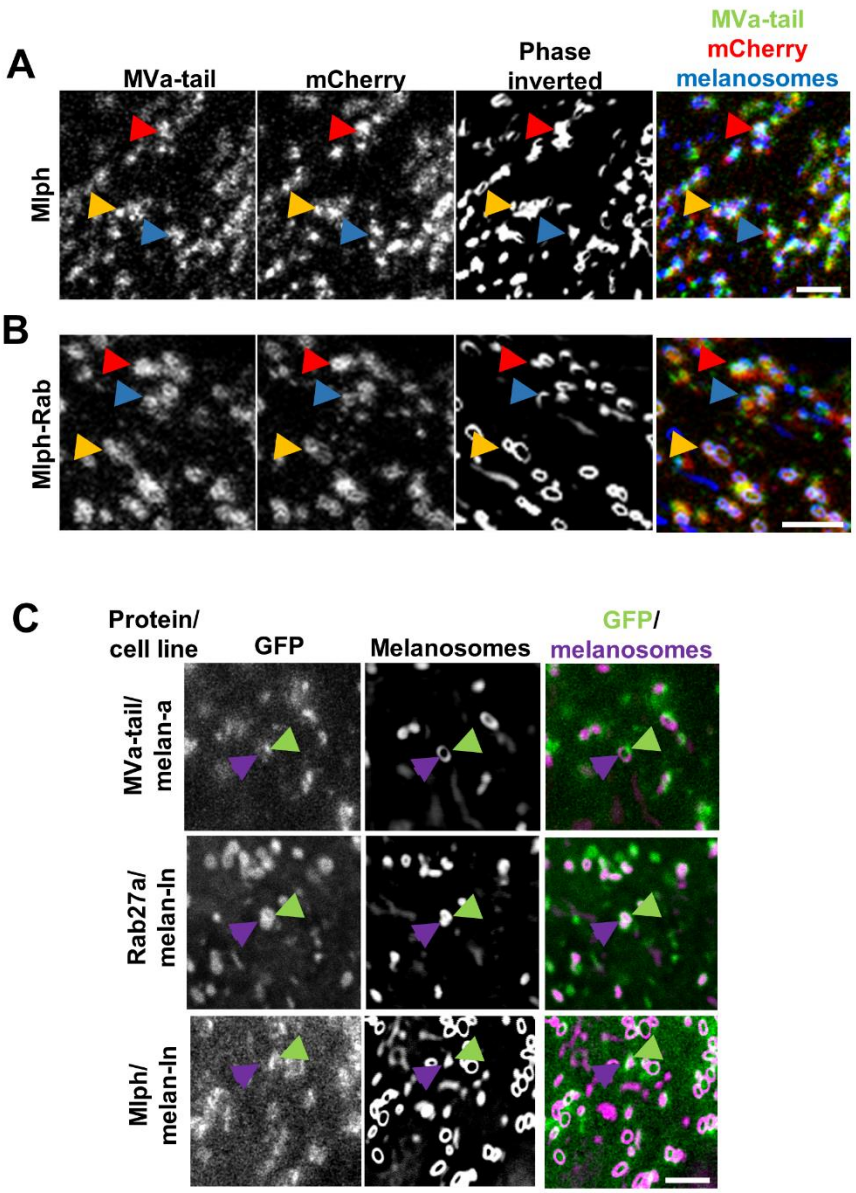

A
MVa-tail
mCherry
Phase inverted
MVa-tail
mCherry
melanosomes
Mlph
B
Mlph-Rab
C
Protein/ cell line
GFP
Melanosomes
GFP/ melanosomes
MVa-tail/ melan-a
Rab27a/ melan-In
Mlph/ melan-In

Figure S4. Localisation and smFRAP data showing the extent of turnover of melanosome associated GFP-Mlph-Rab27a in melanocytes. Mlph deficient (melan-ln) melanocytes were cultured in glass-bottomed dishes, transfected with a plasmid vector expressing GFP-Mlph-Rab27a, and the dynamics of the association of GFP-Mlph-Rab27a with melanosomes was investigated using smFRAP (see materials and methods). (A) A schematic representation of the structure of GFP-Mlph-Rab27a. (B) The upper panels are images showing the distribution of GFP-Mlph- Rab27a, melanosomes and their co-localisation in living cells. White boxes indicate the parts of the cell that were subjected to smFRAP analysis and are shown in the high magnification images below. Scale bar = 20 μm. Lower panels are images taken from an example FRAP series captured at the indicated time relative to photo-bleaching (t=0). Arrows highlight the position over time of the melanosome that was selectively photo-bleached (see Movie 13). (C) A line plot showing the fluorescence intensity associated with photo-bleached melanosomes over time. The vertical bars indicate standard deviation. n = 20 melanosomes (supports Figure 4).

Figure S4

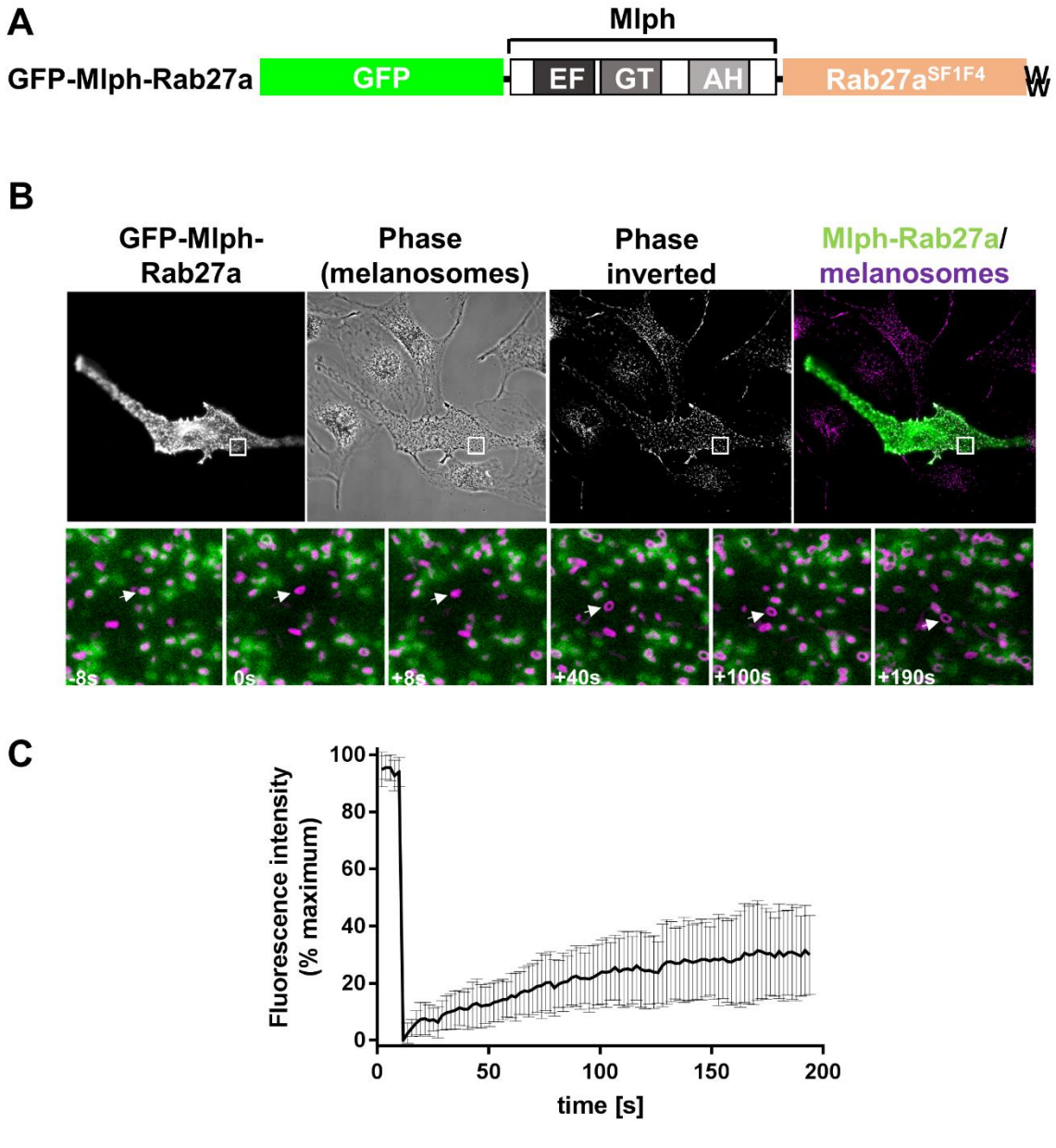

A
Mlph
GFP-Mlph-Rab27a
GFP
EF
GT
AH
Rab27a^SF1F4
B
GFP-Mlph-Rab27a
Phase (melanosomes)
Phase inverted
Mlph-Rab27a/ melanosomes
-8s
0s
+8s
+40s
+100s
+190s
C
Fluorescence intensity (% maximum)
100
80
60
40
20
0
0
50
100
150
200
time [s]

Figure S5. Analysis of the distribution of F-actin and motor protein in myosin-Va -/- melanocytes expressing wild-type myosin-Va, Myo-Rab or GFP. Myosin-Va deficient (melan-d) melanocytes were infected with adenoviruses expressing the indicated proteins, plated on micro-patterned (disk-shape) substrates, fixed and stained for confocal immunofluorescence microscopy (as described previously Evans 2014). (A) Probability maps showing the distribution of F-actin and expressed protein (upper and lower panels) in each population of cells. Data are displayed using the fire look up table LUT. The white circle and bracketed value in the bottom right-hand corner of each image indicate the border of the micropattern (diameter = 46 μm) and the size of each population analyzed, respectively. (B) Radial profile plots showing the distribution of F-actin and expressed protein along the average cell radius for each population. (C) A scatterplot showing the pigment dispersion distance (PDD) for each cell (C) in each population of cells. Horizontal bars indicate the median and 25th and 75th percentiles of each population. The statistical significance of differences in PDD values between populations as determined by one-way ANOVA are indicated above (supports Figure 5).

Figure S5

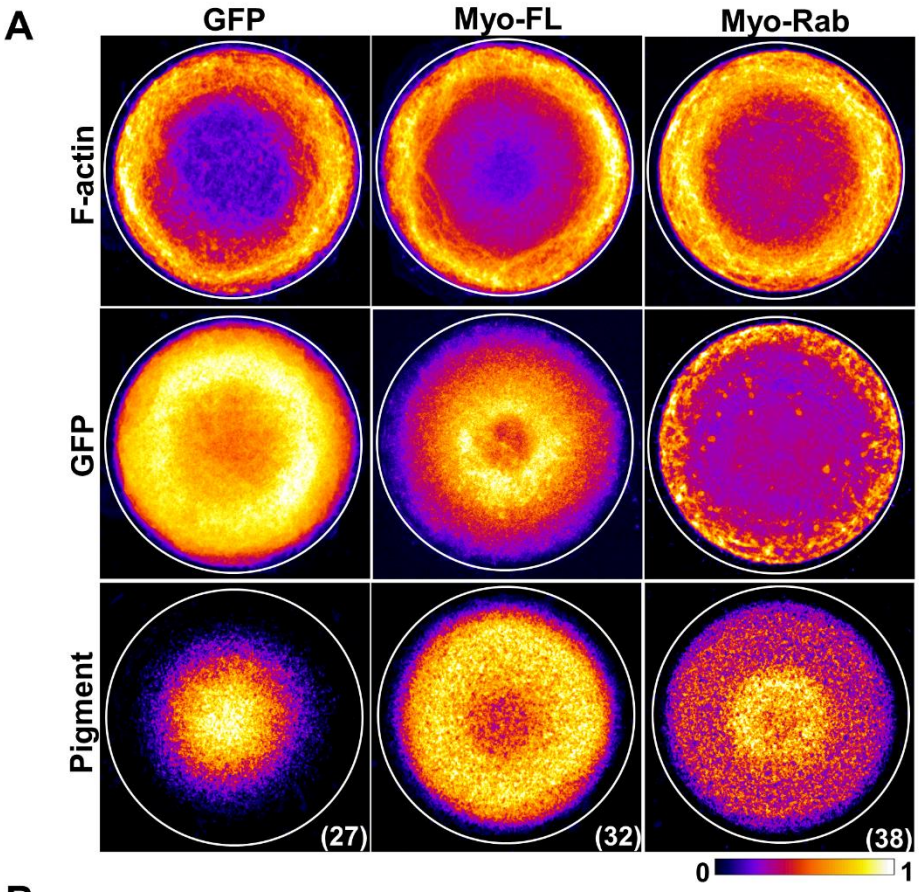

A
GFP
Myo-FL
Myo-Rab
F-actin
GFP
Pigment
(27)
(32)
(38)
0
1
B
F-actin
GFP
Myo-FL
Myo-Rab
GFP
% Signal
Radius [µm]
Radius [µm]
C
Pigment dispersion
Pigment dispersion distance (µm)
Myo-FL
Myo-Rab
GFP

Figure S6. Representative images showing the effect of expression of active myosin-Va fusions on melanosome distribution and cell shape in Mlph and Rab27a deficient melanocytes. Mlph -/- (melan-ln) and Rab27a -/- (melan-ash) cells were infected with adenovirus vectors expressing the indicated GFP fusion proteins, fixed and stained for immunofluorescence (see materials and methods). Representative images of showing the distribution of each protein and melanosomes (bright-field) in melanocytes are presented (scale bar = 30μm) (supports Figure 5).

Figure S6

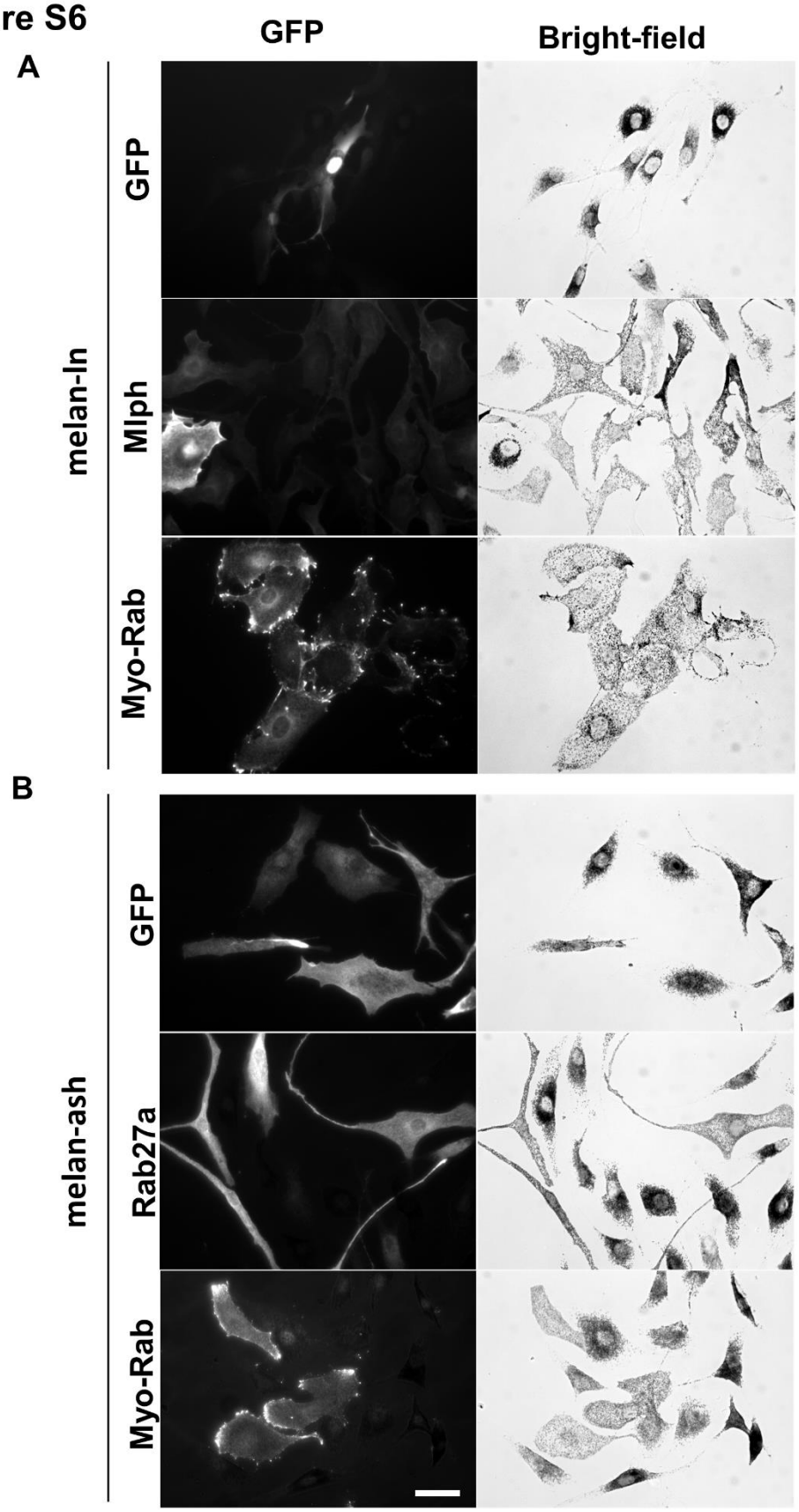

GFP
Bright-field
A
melan-ln
GFP
Mlph
Myo-Rab
B
melan-ash
GFP
Rab27a
Myo-Rab

Figure S7. Localisation and smFRAP data showing rapid turnover of melanosome associated Mlph (R27BD) and Myo-Rab in melanocytes. Wild-type (melan-a) and myosin-Va deficient (melan-d) cells were cultured in glass-bottomed dishes, transfected with vectors expressing GFP-Mlph (R27BD) (A) or Myo-Rab (B, C), and the dynamics of the association of the protein with melanosomes was investigated using confocal FRAP analysis (see materials and methods). (A and B) Upper panels are images showing of the distribution of expressed protein, melanosomes and their co-localisation in living cells. White boxes indicate the parts of the cell that were subjected to FRAP analysis and are shown in the high magnification images below. Scale bar = 20 μm. Lower panels are images taken from an example FRAP series captured at the indicated time relative to photo-bleaching (t=0). Arrows highlight the position over time of melanosomes that were selectively photo-bleached. (C) A line plot showing the fluorescence intensity associated with photo-bleached melanosomes over time in cells expressing Myo-Rab. The vertical bars indicate standard deviation. n = 11 melanosomes. (supports Figure 5; see Movies 14 and 15)

Figure S7

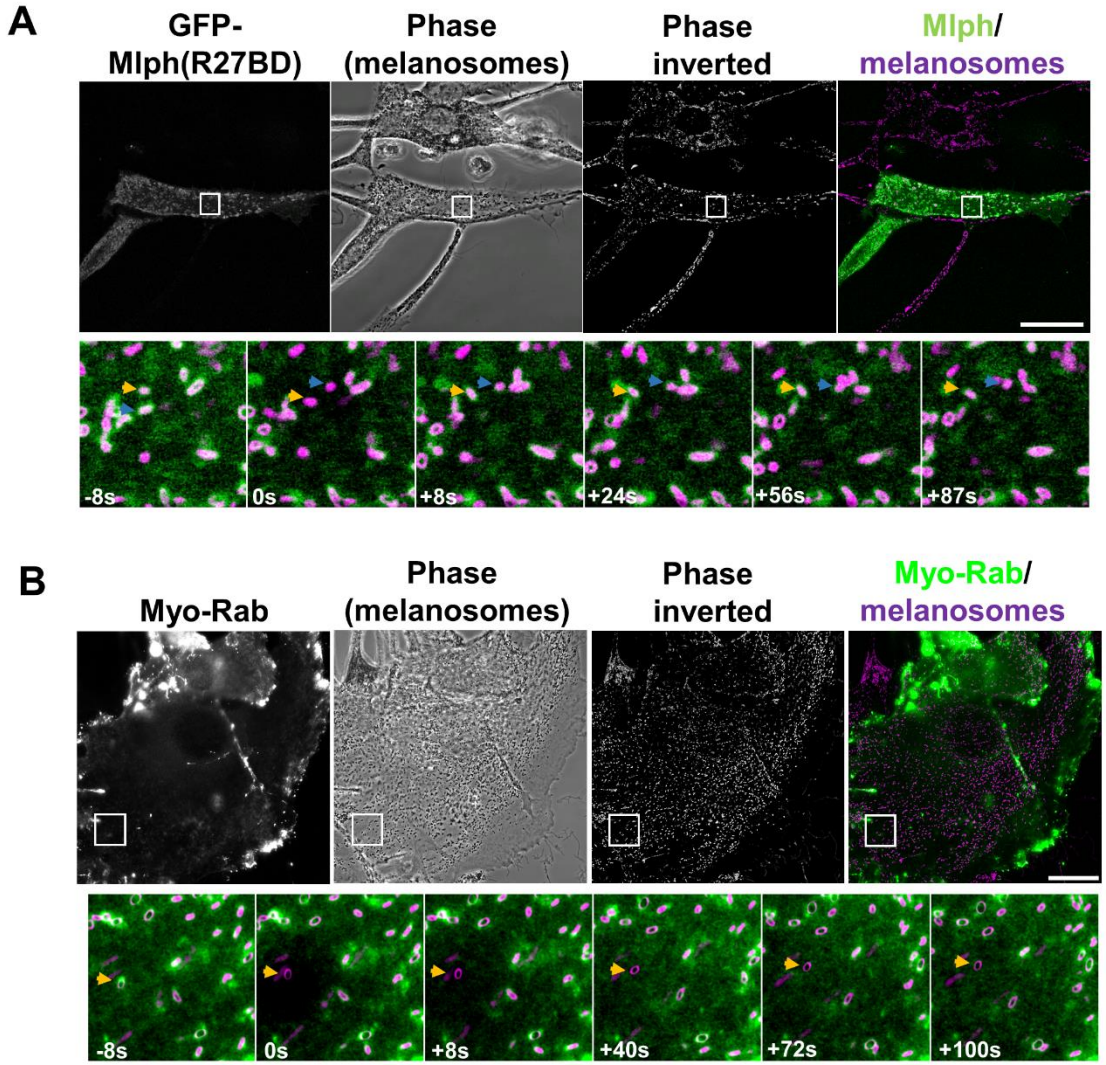
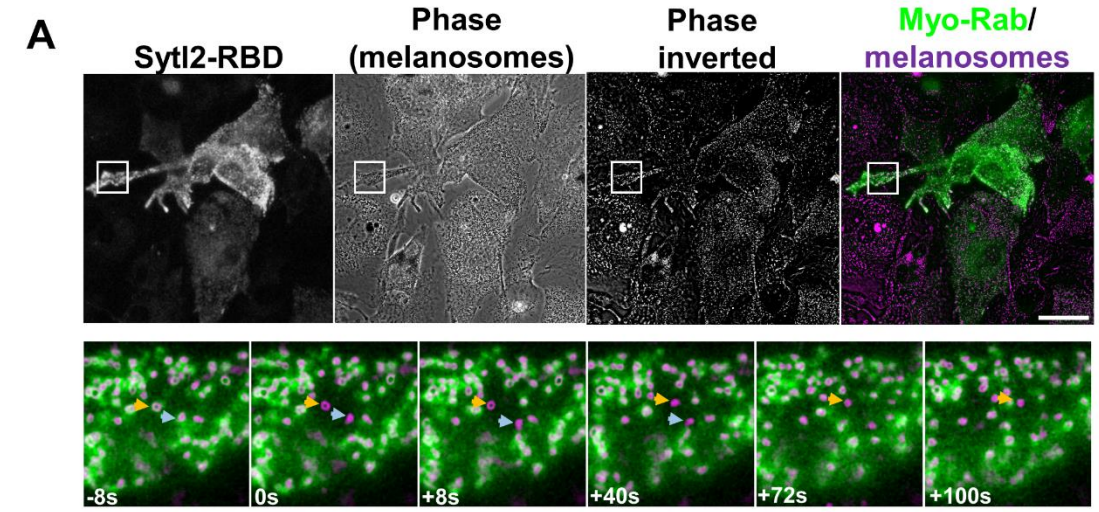

A
GFP-
Mlph(R27BD)
Phase
(melanosomes)
Phase
inverted
Mlph/
melanosomes
-8s
0s
+8s
+24s
+56s
+87s
B
Myo-Rab
Phase
(melanosomes)
Phase
inverted
Myo-Rab/
melanosomes
-8s
0s
+8s
+40s
+72s
+100s
C
Fluorescence intensity
(% maximum)
100
80
60
40
20
0
0
50
100
150
time [s]

Figure S8. Localisation and smFRAP data showing turnover of melanosome associated Sytl2 (R27BD) in melanocytes. Wild-type (melan-a) cells were cultured in glass-bottomed dishes, transfected with plasmid vector allowing expression of GFP-Sytl2 (R27BD), and the dynamics of its association with melanosomes was investigated using confocal FRAP analysis (see materials and methods). (A) Upper panels are images showing of the distribution of fusion protein, melanosomes and their co-localisation in living cells. White boxes indicate the parts of the cell that were subjected to FRAP analysis and are shown in the high magnification images below. Scale bar = 20 μm. Lower panels are images taken from an example FRAP series captured at the indicated time relative to photo-bleaching (t=0) (see Movie 16). Arrows highlight the position over time of melanosomes that were selectively photo-bleached. (B) A line plot showing the fluorescence intensity associated with photo-bleached melanosomes over time. The vertical bars indicate standard deviation. n = 7 melanosomes (supports Figure 3).

Figure S8


A
Sytl2-RBD
Phase (melanosomes)
Phase inverted
Myo-Rab/ melanosomes
-8s
0s
+8s
+40s
+72s
+100s

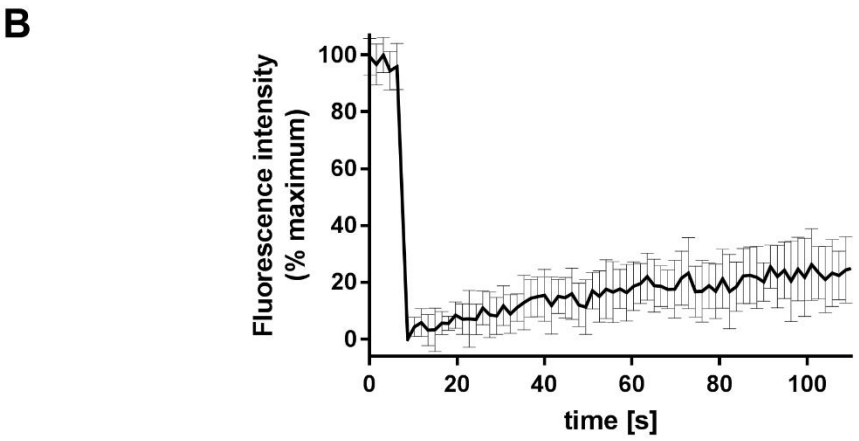
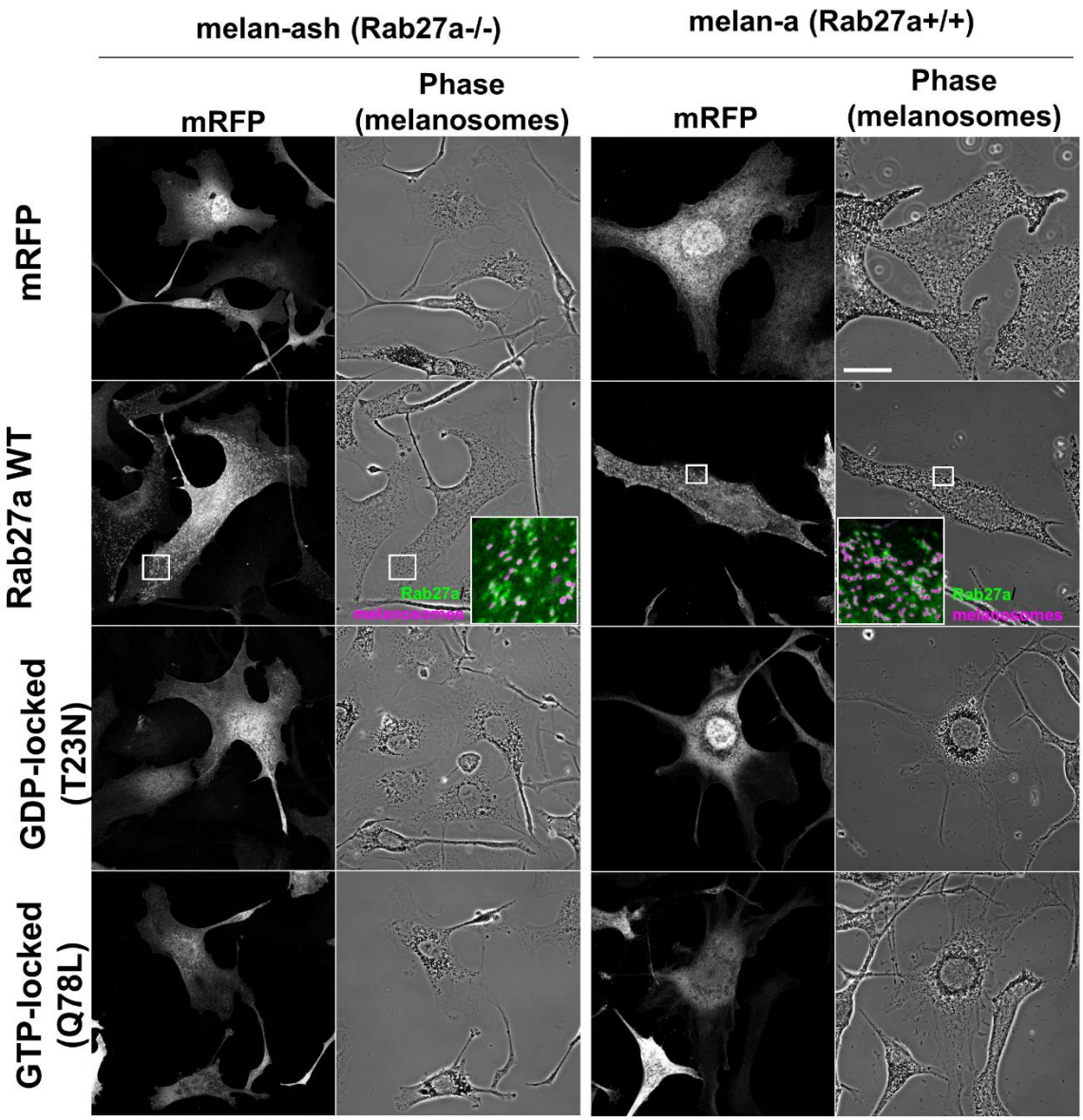

B
Fluorescence intensity (% maximum)
100
80
60
40
20
0
0
20
40
60
80
100
time [s]

Figure S9. Representative images showing the distribution of GDP- and GTP-bound Rab27a mutants and their effect on melanosome distribution in Rab27a deficient melanocytes. Rab27a -/- (melan-ash) and wild-type (melan-a) cells were infected with adenovirus vectors expressing the indicated mRFP fusion proteins, fixed and the distribution of fluorescence protein and melanosomes recorded using a confocal microscope (see materials and methods). Representative images of showing the distribution of each protein and melanosomes (phase) in melanocytes are presented (scale bar = 20μm; supports Figure 2).

Figure S9


melan-ash (Rab27a-/-)
melan-a (Rab27a+/+)
mRFP
Phase (melanosomes)
mRFP
Phase (melanosomes)
mRFP
Rab27a WT
GDP-locked (T23N)
GTP-locked (Q78L)
Rab27a/ melanosomes
Rab27a/ melanosomes

Movie Legends.

All movies are confocal FRAP series (acquired as described in Experimental Procedures) showing melanosomes (false-coloured magenta) and expressed proteins (green). Movie playback rate is 10 frames s^{-1}. Arrows indicate melanosomes where GFP was photo-bleached.

Movie 1. MVa-tail expressed in wild-type (melan-a) cells (supports Figure 1).

Movie 2. MVa-tail expressed in myosin-Va -/- (melan-d) cells (supports Figure S1A).

Movie 3. MVa-FL expressed in myosin-Va -/- (melan-d) cells (supports Figure S1C).

Movie 4. Rab27a expressed in wild-type (melan-a) cells (supports Figure 2A).

Movie 5. Rab27a expressed in Rab27a -/- (melan-ash) cells (supports Figure 2B).

Movie 6. Rab27a expressed in Mlph -/- (melan-ln) cells (supports Figure 2C).

Movie 7. Rab27aSF1F4 expressed in wild-type (melan-a) cells (supports Figure 2D).

Movie 8. Mlph expressed in wild-type (melan-a) cells (supports Figure 3A).

Movie 9. Mlph expressed in Mlph -/- (melan-ln) cells (supports Figure 3B).

Movie 10. Mlph expressed in myosin-Va -/- (melan-d) cells (supports Figure 3C).

Movie 11. MVa-tail co-expressed with mCherry-Mlph expressed in Mlph -/- (melan-ln) cells (supports Figure 4B).

Movie 12. MVa-tail co-expressed with mCherry-Mlph-Rab27a^{SF1F4} expressed in Mlph -/- (melan-ln) cells (supports Figure 4C).

Movie 13. GFP-Mlph- Rab27a^{SF1F4} expressed in Mlph -/- (melan-ln) cells (supports Figure S4).

Movie 14. Mlph R27BD expressed in wild-type (melan-a) cells (supports Figure S5).

Movie 15. Myo-Rab expressed in myosin-Va -/- (melan-d) cells (supports Figures 5 and S7).

Movie 16. Sytl2 (R27BD) expressed in wild-type (melan-a) cells (supports Figures S8).