The adaptor protein melanophilin regulates dynamic myosin-Va:cargo interaction and dendrite development in melanocytes.

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Abbreviations: HMM, heavy meromyosin; Mlph, melanophilin; Myo-T, GFP fused with the melanocyte-specific tail of myosin-Va; PBFI, pre-bleach fluorescence intensity; R27BD, Rab27 binding domain; smFRAP, single melanosome fluorescence recovery after photo-bleaching.
**Abstract:**

Regulation of organelle transport by the cytoskeleton is fundamental for eukaryotic survival. Cytoskeleton motors are typically modular proteins with conserved motor and diverse cargo binding domains. Motor:cargo interactions are often indirect and mediated by adaptor proteins e.g. Rab GTPases. Rab27a, via effector melanophilin (Mlph), recruits myosin-Va to melanosomes and thereby disperses them into melanocytes dendrites. To better understand how adaptors regulate motor:cargo interaction we used single melanosome fluorescence recovery after photo-bleaching (smFRAP) to characterise the association kinetics between myosin-Va, its adaptors and melanosomes. We found that myosin-Va and Mlph rapidly recovered after photo-bleaching, while Rab27a did not, indicating that myosin-Va and Mlph dynamically associate with melanosomes and Rab27a does not. This suggests that dynamic Rab27a:effector interaction rather than Rab27a melanosome:cytosol cycling regulates myosin-Va:melanosome association. Accordingly a Mlph-Rab27a fusion protein reduced myosin-Va smFRAP, indicating that it stabilised melanosomal myosin-Va. Finally, we tested the functional importance of dynamic myosin-Va:melanosome interaction. We found that while a myosin-Va-Rab27a fusion protein dispersed melanosomes in myosin-Va deficient cells, dendrites were significantly less elongated than in wild-type cells. Given that dendrites are the prime sites of melanosome transfer from melanocytes to keratinocytes we suggest that dynamic myosin-Va:melanosome interaction is important for pigmentation in vivo.
Introduction.

Organelle distribution and transport are fundamental for eukaryotic survival. Motor proteins of the kinesin, dynein and myosin families are essential for organelle transport along microtubule and filamentous (F-) actin tracks, respectively (Hartman et al., 2011, Hirokawa et al., 2009, Kardon and Vale, 2009). In general motor protein heavy chains have a modular structure and are composed of relatively conserved motor domains, that bind to F-actin/MT tracks and generate force through hydrolysis of ATP, coupled to more diverse tails, that link the motor to cargo e.g. organelles (Hartman et al., 2011, Hirokawa et al., 2009, Kardon and Vale, 2009). In some cases motor tails appear to contain lipid binding domains e.g. kinesins, KIF1a and KIF1bβ, and type I myosins contain PH domains, and thus may directly target to organelle membranes via interaction with membrane lipids (Hartman et al., 2011, Kardon and Vale, 2009). However, cargo attachment appears to be more frequently mediated by interaction of the motor tail with cargo binding light chains that are often membrane associated proteins. Among these small GTPase of the Rab family are well-represented (Hutagalung and Novick, 2011, Stenmark, 2009, Hammer and Wu, 2002).

Rabs are the largest family of small Ras-like GTPases (>60 in man). In general Rabs are thought to function as compartment specific molecular switches that regulate transport through the endocytic and secretory pathways of eukaryotes (Barr, 2013, Hutagalung and Novick, 2011, Pfeffer, 2017, Stenmark, 2009). Rabs associate with organelles via interaction of geranygeranyl isoprene moieties, which are covalently post-translationally attached to C-terminus cysteines, with lipids of the cytoplasmic face of organelle membranes. There, active GTP-bound Rabs recruit a diverse group of effector proteins e.g. motor proteins, protein/lipid kinases, and membrane tethering and docking factors from the cytosol that transduce their function(s) in transport. Rab activity is terminated by GTP hydrolysis dependent destabilisation of the conformation of the effector binding ‘switch regions’. This reduces Rab:effector interaction affinity resulting in release of effectors from membranes. General models consider that inactive GDP-bound Rabs are then extracted from membranes into the cytosol by Rab GDI (GDP dissociation inhibitor) and recycled for use in further
rounds of transport. This suggests that the coupled GTP/GDP binding and membrane/cytosol cycles of Rab GTPases could control the transport function of motor proteins by regulating their association with cargo.

Type V myosins, such as myosin-Va, have highly conserved roles in organelle transport in eukaryotes and a number of Rabs recruit them to cargo (Lindsay et al., 2013). For example in yeast direct interaction of Myo2 with ypt31/32 (Rab11 in yeast), and ypt11, drives the polarised delivery of secretory vesicles and mitochondria, respectively, to the growing bud (Chernyakov et al., 2013, Lipatova et al., 2008). In mammalian neurones myosin-Vb, one of 3 type V myosins, is recruited to recycling endosomes by Rab11 and its effector FIP2 and regulates AMPA receptor trafficking and dendritic spine growth that is important in memory and learning (Wang et al., 2008).

Myosin-Va, like other type V motors, is comprised of a dimer of motor heavy chains each of which consists of four domains; motor (head), lever arm, dimerisation and globular tail (Hammer and Sellers, 2012, Trybus, 2008). The motor is conserved among myosins and contains F-actin binding and ATPase activities. ATP binding and hydrolysis regulates interaction with F-actin and generates a conformational change (or power-stroke) necessary for movement. The power-stroke is amplified by the long (24nm) light chain/calmodulin binding lever arm, resulting in detachment and reattachment of the partner motor domain and displacement of the dimer towards the plus end of the track. Dimerisation is achieved by α-helical coiled-coil interaction and the tail and alternatively spliced region mediate cargo binding and regulation of motor activity through direct intramolecular (head-tail) interaction with the motor (Krementsov et al., 2004, Li et al., 2004, Wang et al., 2004). A series of elegant in vitro studies using single molecule imaging, X-ray crystallography, electron microscopy and rapid acquisition atomic force microscopy indicate MyoVa is a processive motor undertaking multiple (10s-100s) catalytic cycles coupled to mechanical changes that move the motor along F-actin with 36nm steps, corresponding to the helical repeat distance of the filament. These studies
indicate that MyoVa moves at 250-450nm/sec in a hand-over-hand fashion with each head alternating in the lead or trailing position (Hammer and Sellers, 2012).

In melanocytes an alternatively spliced myosin-Va isoform plays an essential role in transporting pigment-filled melanosomes into a peripheral network of cell extensions, termed dendrites, which may contact up to 40 keratinocytes. From there melanosomes are transferred to adjacent keratinocytes. This process is fundamental for pigmentation of skin and hair of mammals (Van Den Bossche et al., 2006, Wu and Hammer, 2014). Consistent with this, deficiency of myosin-Va results in defects in melanosome transport within melanocytes and their transfer to keratinocytes (Van Gele et al., 2009). Recruitment of myosin-Va to melanosomes is dependent upon Rab27a and its effector melanophilin (hereafter Mlph) (Fukuda et al., 2002, Strom et al., 2002, Wu et al., 2002b, Nagashima et al., 2002). Melanocyte-spliced myosin-Va contains exon F in the C-terminus portion of the dimerization domain that allows Mlph to recruit it to melanosomes (Wu et al., 2002a). Mlph has a modular structure comprising; 1) an N-terminus Rab27 binding domain that is conserved among most Rab27 effector proteins and 2) a C-terminus myosin-Va and F-actin binding domains that are conserved with myosin-VIIa Rab interacting protein (MyRIP), but not other Rab27 effectors (Fukuda, 2013, Hume et al., 2006, Kuroda et al., 2003). Thus Mlph plays a vital role in allowing Rab27a to recruit myosin-Va to melanosomes indirectly. However, given that models of Rab function predict that the GTPase activity and hypothetical membrane/cytosol cycling activity of Rab27a could regulate motor:cargo attachment, the basis of the requirement for Mlph in myosin-Va recruitment, beyond providing a binding site for melanocyte-spliced myosin-Va, is unclear.

In this study we used single melanosome (sm)FRAP to directly examine the kinetics of the interaction between myosin-Va and a model cargo; the melanosome in melanocytes. We found that myosin-Va and Mlph dynamically associate with melanosomes while Rab27a was less dynamic. Using a Mlph-Rab27a fusion protein (Mlph-Rab) we show that dynamic Rab27a:Mlph interaction in part regulates myosin:melanosome interaction. Finally using a myosin-Va-Rab27a fusion protein (Myo-Rab) we
show that recycling of myosin-Va during melanosome transport is important for the elongated shape of melanocytes. These data highlight a novel and unexpected aspect of Mlph function in regulating the kinetics of myosin-Va-organelle interaction, and indicate that the dynamics of cargo:cytoskeleton interactions can influence cell structure and transport at the level of organelle populations.

Results.

**smFRAP indicates rapid turnover of melanosome associated myosin-Va.**

To investigate the mechanism of myosin-Va dependent organelle transport and the role of adaptors in regulating the motor:cargo association we used single melanosome FRAP (hereafter smFRAP) to characterise the interaction between myosin-Va and melanosomes in melanocytes. To do this we transiently expressed a GFP tagged version of the melanosome binding myosin-Va tail [hereafter Myo-T] in wild-type melanocytes (melan-a) (Figure 1A). We then used confocal microscopy to record the distribution and dynamics of Myo-T and melanosomes and their interaction in living melanocytes. Consistent with previous studies we found that puncta of Myo-T associated with pigmented melanosomes (Figure 1B arrows) (Hume et al., 2001, Wu et al., 1998).

We then photo-bleached Myo-T fluorescence associated with well-separated melanosomes and recorded the subsequent recovery of fluorescence on individual organelles (Figure 1). We observed rapid, partial recovery of melanosomal Myo-T fluorescence after photo-bleaching (Figure 1B-C; Movie 1; mean $t_{1/2}$ of recovery (s) = 33.12 +/- 10.09; mean recovery plateau (% of pre-bleach fluorescence intensity (PBFI)) = 67.86 +/- 8.775; mean maximum recovery (% PBFI) 82.29 +/- 14.05). These data suggest that myosin-Va dynamically associates with melanosomes. Similar results were obtained from studies using Myo-T and full-length GFP-myosin-Va (Myo-FL) transiently expressed in myosin-Va deficient (melan-d) cells (the latter at rescuing i.e. functionally relevant levels) (Figure S1; Movies 2 and 3; mean $t_{1/2}$ rec (s) = 24.69 +/- 12.6 (Myo-T), 14.11 +/- 6.626 (Myo-FL); mean recovery
plateau (% PBFl) = 33.85 +/- 15.69 (Myo-T), 38.8 +/- 21.91 (Myo-FL); mean maximum recovery (% PBFl) = 44.48 +/- 22.89 check this (Myo-T), 53.63 +/- 14.82 (Myo-FL)). One possible explanation for higher recovery of Myo-T in melan-a versus melan-d cells is that interaction with endogenous myosin-Va enhances the level of recovery in melan-a cells.

**Rab27a stably associates with melanosomes in melanocytes.**

To probe the role of cargo adaptors in regulating the turnover of myosin-Va we investigated the kinetics of the association of Rab27a with melanosomes. GTP-bound/active Rab27a was previously shown to recruit myosin-Va to melanosomes via its effector Mlph (Fukuda, 2013, Hammer and Sellers, 2012, Hume and Seabra, 2011). As outlined above Rab GTPases are proposed to couple cycles of GTP hydrolysis/GDP-GTP exchange with membrane association and disassociation, thus regulating the association of their effectors e.g. Mlph/myosin-Va, with membranes (Hutagalung and Novick, 2011, Stenmark, 2009). Therefore, we hypothesised that the dynamic interaction of myosin-Va with the melanosome membrane might be due to a similarly dynamic Rab27a:melanosome interaction. To test this we transiently expressed GFP-Rab27a in wild-type (melan-a) and Rab27a null (melan-ash) melanocytes. As previously reported we observed that GFP-Rab27a co-distributed with melanosomes in melanocytes and restored peripheral melanosome distribution in Rab27a deficient cells confirming the functionality of GFP-Rab27a (Figure 2A and B) (Bahadoran et al., 2001, Hume et al., 2001, Wu et al., 2001).

Using smFRAP analysis in both cell types we observed significantly lower levels of recovery of GFP-Rab27a to individual melanosomes compared with Myo-T expressed in wild-type (melan-a), suggesting that Rab27a more stably associates with melanosomes compared with myosin-Va (Figure 2A, B, E; Movies 4 and 5). The low level of Rab27a recovery in smFRAP experiments prevented reliable modelling of the data to a single exponential function and determination of the half-time and plateau of recovery. Therefore, we report only mean maximum recovery (%PBFl) for each
population as a measure of FRAP in these experiments (melan-a = 13.42 +/- 4.27 and melan-ash = 26.44 +/- 10.65; Figure S2A).

To investigate whether interaction with effectors stabilizes melanosomal Rab27a we tested the recovery of; 1) GFP-Rab27a in Mlph deficient cells (melan-In), and 2) effector interaction deficient-melanosome targeted mutant GFP-Rab27a^{SF1F4} in wild-type (melan-a) cells (Tarafder et al., 2011). Consistent with the other smFRAP experiments using GFP-Rab27a (Figure 2A-B) we observed levels of fluorescence recovery that were significantly lower compared with Myo-T (Figure 1B-C, 2C-E, S2A; Movies 6 and 7; mean maximum recovery (%PBFI) = 17.11 +/- 9.746% and 20.55 +/- 9.484% for GFP-Rab27a in melan-In and the GFP-Rab27a^{SF1F4} mutant in melan-a). This indicates that effectors do not significantly stabilise melanosomal Rab27a, that Rab27a more stably associates with melanosomes than myosin-Va, and that dynamic interaction between myosin-Va and melanosomes is not controlled directly by turnover of melanosomal Rab27a.

**Mlph dynamically associates with melanosomes in melanocytes.**

As indicated above, Mlph directly interacts with myosin-Va and Rab27a, thus allowing attachment of myosin-Va to melanosomes (Fukuda et al., 2002, Strom et al., 2002, Wu et al., 2002b). Dynamic interaction of Mlph with either (or both) of these partners could therefore provide the basis of myosin-Va turnover at the melanosome membrane. To examine this further we used confocal microscopy to investigate the association between Mlph and melanosomes in living melanocytes. In line with the results of previous studies we found that in wild-type (melan-a), Mlph null (melan-In) and myosin-Va null (melan-d) cells, transiently expressed GFP-Mlph was distributed throughout the cytoplasm and was enriched in puncta adjacent to melanosomes seen by phase contrast (Figure 3A-C, red arrows in high magnification images). This confirms that GFP-Mlph associates with melanosomes in a myosin-Va independent manner (Wu et al., 2002b).
smFRAP experiments in wild-type (melan-a) cells showed that GFP-Mlph, like Myo-T, but not Rab27a, rapidly recovered on bleached melanosomes (Figure 1, 3A, D, S2 and Movie 8; mean t1/2 of recovery (s) = 12.67 +/- 11.46; mean recovery plateau (%PBFI) = 73.2 +/- 14.31; mean maximum recovery (%PBFI) = 82.26 +/- 13.69). Similar results were seen in smFRAP studies of GFP-Mlph in Mlph null (melan-ln) and myosin-Va null (melan-d) cells (Figure 3B-D, S2 and Movies 9 and 10; mean t1/2 of recovery (s) = 6.076 +/- 3.369 and 5.396 +/- 2.895; and mean recovery plateau (%PBFI) = 70.3 +/- 10.13 and 65.25 +/- 4.525; mean maximum recovery (%PBFI) = 98.88 +/- 12.72 and 86.69 +/- 14.65). Importantly smFRAP studies of Mlph in Mlph null (melan-ln) cells were performed in cells in which GFP-Mlph rescued melanosome clustering, indicating that these experiments investigated the activity of functionally relevant levels of GFP-Mlph. In summary these data indicate that Mlph, like myosin-Va, turns over more rapidly than Rab27a at the melanosome membrane, and suggest that Mlph interacts dynamically with Rab27a.

**Dynamic Mlph:Rab27a interaction is a dominant factor regulating the myosin-Va:melanosome association.**

Thus far our data suggest that dynamic myosin-Va:melanosome association may be regulated by dynamic Rab27a:Mlph and/or Mlph:myosin-Va interaction. Consistent with this possibility the overall profile of recovery after bleaching for Mlph and Myo-T in wild-type (melan-a) cells were more similar to one another than to Rab27a (Figure 1-3, S2). To investigate this further we generated a vector allowing expression of an mCherry tagged Mlph-Rab27a chimera which contains the C-terminus myosin-Va and F-actin binding domains (but not the N-terminus Rab27a binding domain) of Mlph fused at the N-terminus of the Rab27a$^{SF14}$ mutant (Figure 4A). As confirmed above this mutant targets efficiently to melanosomes but does not interact with effectors (Figure 2D) (Tarafder et al., 2011). Thus it can be used to target proteins that are fused to it to melanosomes. We then co-expressed Mlph-Rab27a with Myo-T in Mlph -/- (melan-ln) melanocytes, and used
smFRAP to measure the dynamics of Myo-T:melanosome interaction. Parallel smFRAP experiments were carried out using mCherry alone and mCherry-Mlph.

In line with previous studies we observed that mCherry-Mlph and mCherry-Mlph-Rab27a, but not mCherry alone, distributed in a punctate cytoplasmic pattern and that these puncta often co-localised with pigmented melanosomes, indicating that both proteins targeted to melanosomes (Figure 4B; S3A-B). We also saw that expression of mCherry-Mlph and mCherry-Mlph-Rab27a rescued perinuclear melanosome clustering in Mlph +/- (melan-In) cells in the majority of expressing cells, confirming that they were expressed at functionally relevant levels (Figure 4B-C, S3D; mean pigment area (% total); mCherry = 22.96 +/- 6.855, mCherry-Mlph = 41.62 +/- 30.98, mCherry-Mlph-Rab27a = 83.05 +/- 13.13). Consistent with previous reports showing that high levels of Mlph expression disrupt melanosome dispersion, due to mis-targeting of Mlph to peripheral F-actin, the efficiency of rescue by mCherry-Mlph (but not mCherry-Mlph-Rab27a) correlated inversely with protein expression level (Figure 4C; linear regression gradient and correlation coefficient R², mCherry = -0.0326 and 0.025, mCherry-Mlph = -0.1736 and 0.1193 and mCherry-Mlph-Rab27a = -0.5156 and 0.4849) (Hume et al., 2006). Finally, we observed that Myo-T distributed in a melanosome associated punctate pattern when co-expressed with Mlph containing fusion proteins, but not mCherry alone, further confirming that both proteins are competent in recruiting myosin-Va to melanosomes, (Figure 4B; S3A-B).

smFRAP revealed that, when recruited to melanosomes by mCherry-Mlph, Myo-T turned over at a similar rate and extent to that seen for GFP-Mlph and Myo-T alone in wild-type (melan-a) cells (Figures 1, 3, 4B+D, S2; Movie 11; mean t1/2 of recovery (s) = 16.81 +/- 13.01 and mean recovery plateau (%PBFI) = 58.17 +/- 15.69 % PBFI and mean maximum recovery (% PBFI) = 73.76 +/- 18.44). In contrast, when recruited by Mlph-Rab27a, although Myo-T recovered to a greater extent than Rab27a, it did so with slower kinetics and to a lesser extent than when recruited by Mlph (Figures 4B+D, S2; movie 12). Similar to Rab27a several fluorescence recovery profiles for Myo-T co-
expressed with Mlph-Rab27a could not be modelled using a single exponential function (Figure S2; Mean t1/2 of recovery (s) = 61.02 +/- 24.93, mean recovery plateau (% PBFI) = 42.77 +/- 15.39 and mean maximum recovery (% PBFI) = 46.81 +/- 15.36). smFRAP analysis of the Mlph-Rab27a fusion protein itself (tagged with GFP i.e. GFP-Mlph-Rab27a) revealed that it recovered to a similar extent (no significant difference) to Myo-T co-expressed with Mlph-Rab27a (Figure S4, S2, Movie 13; Mean t1/2 of recovery (s) = 46.37 +/- 23.33, mean recovery plateau (% PBFI) = 35.31 +/- 18.39 and; mean maximum recovery (% PBFI) = 39.63 +/- 17.26). These observations indicate that dynamic Mlph:Rab27a interaction may be the dominant factor regulating myosin-Va turnover at the melanosome membrane.

**Perturbation of dynamic interaction with melanosomes reduces the functional efficiency of myosin-Va in transport and melanocytes dendrite formation.**

To test the functional importance of the dynamic association of myosin-Va with melanosomes we generated a vector that expresses a fusion protein (Myo-Rab) that can more stably target active myosin-Va to melanosomes. This protein comprises a constitutively active, dimer-forming HMM (heavy meromyosin) fragment of myosin-Va (i.e. containing the motor, lever and dimerization domains, but lacking the melanosome binding tail) fused to the N-terminus of the melanosome-targeted Rab27aS144F mutant (Tarafder et al., 2011) (Figure 5A). We then compared the rescue of melanosome transport defects in myosin-Va null (melan-d) cells (a read-out of myosin-Va function) expressing Myo-Rab and wild-type myosin-Va.

Using confocal microscopy we observed that Myo-Rab localised to, and dispersed melanosomes to a significantly greater extent than GFP alone, but to a somewhat lesser extent (not significant) than Myo-FL (Figure 5B-C (arrows in B highlight motor:melanosome association); mean pigment area (% total); Myo-FL = 80.64 +/- 9.104, Myo-Rab = 75.52 +/- 10.83, GFP = 21.04 +/- 3.37). We also noted that Myo-Rab accumulated strongly in the F-actin-rich periphery of melanocytes (i.e. beyond the melanosome-filled area, Figure 5B box 2) and that cells expressing this fusion were significantly less
elongated and more rounded than those expressing GFP-myosin-Va (Figure 5B, D; circularity (AU); Myo-FL = 0.24 +/- 0.085, Myo-Rab = 0.57 +/- 0.16, GFP = 0.25 +/- 0.11). Observation of F-actin distribution did not reveal striking differences between cells expressing the different myosin-Va proteins (Figure 5). Quantitative analysis of F-actin and myosin-Va distribution in melan-d cells in which x/y cell shape was normalised by culture on disk-shaped micro-patterns confirmed that F-actin distribution is similar in Myo-FL and Myo-Rab expressing cells and the peripheral accumulation of Myo-Rab (Figure S5) (Evans et al., 2014). Expression of Myo-Rab in Rab27a and Mlph deficient melanocytes gave similar results, suggesting that dynamic interaction with cargo enhances myosin-Va function in melanosome dispersion and dendrite development (Figure S6).

An alternative (or additional) mechanistic basis for these observations is that the function of Myo-Rab is compromised by the constitutive activity of its myosin-Va component, a consequence of the replacement of the Myo-T with Rab27aSF1F4 in this chimera. Previous studies have shown that, in the absence of cargo, Myo-T may interact with, and inhibit the motor/ATPase ‘head’ domain of myosin-Va, and in yeast this regulatory interaction is important for type V myosin (Myo2) function in organelle inheritance (Donovan and Bretscher, 2015, Krementsov et al., 2004, Li et al., 2004, Wang et al., 2004). To determine the extent that loss of myosin-Va head-tail regulation contributes to the difference in function of Myo-Rab versus Myo-FL we generated another active myosin fusion protein, Myo-Mlph, in which the Rab27aSF1F4 portion of Myo-Rab was replaced with the Rab27 binding domain (R27BD) of Mlph (Figure 5A). smFRAP studies revealed that Mlph-R27BD alone turns over rapidly on melanosomes like wild-type Mlph (Figure S6; Movie 14). Thus the Myo-Mlph allows active dimeric myosin-Va to dynamically associate with melanosomes via interaction with endogenous Rab27a. When expressed in myosin-Va +/- (melan-d) cells we found that Myo-Mlph localised to, and dispersed melanosomes to a similar extent to Myo-Rab, but did not affect the dendrite development (Figure 5B-D; mean pigment area (% total) = 75.61 +/- 10.29; circularity (AU) = 0.23 +/- 0.069). Using smFRAP we confirmed that Myo-Rab associated with melanosomes stably similar to Rab27a (mean maximum recovery (% total) = 31 +/- 8.715) (Figure S7, Movie 15). These
observations suggest that regulation of myosin-Va, although not essential, is important for optimal function in melanosome dispersion and that dynamic myosin-Va:melanosomes association is important for dendrite development.

**Discussion.**

Here we investigated how adaptor proteins regulate motor:cargo interactions and transport using as a model melanosomes (cargo) in melanocytes, myosin-Va (motor) and Rab27a and melanophilin (cargo adaptors). Our novel findings are four-fold.

Firstly, using smFRAP we discovered that myosin-Va dynamically associates with melanosomes in mouse melanocytes (Figure 1). To our knowledge no previous study has investigated the kinetics of association of myosin-Va with cargo in mammalian cells. In fission yeast FRAP revealed that type V myosin (myo51) turned over rapidly (t1/2 = 6.7 +/- 1.4s) at the contractile ring during cytokinesis (Wang et al., 2014). FRAP studies in HeLa cell revealed that myosin-VI turned over rapidly on endosomes (t1/2 ~ 15 s) (Bond et al., 2012). With our data this suggests that rapid turnover at sites of action may be conserved facet of non-muscle myosins. However, while endosomes and contractile rings are short-lived structures that might be expected to rapidly exchange their contents and distribution as part of their function, melanosome are not. Melanosome biogenesis takes several days in vitro and mature melanosomes can persist in cells for several days (Wasmeier et al., 2006, Sviderskaya et al., 1995). This suggests that myosin-Va-dependent melanosome dispersion is likely to be comprised of multiple short episodes of Mlph:mosin-Va:melanosome interactions and movements that guide melanosomes collectively towards, and into peripheral dendrites.

Secondly, we found that dynamic Mlph:Rab27a interaction, and not Rab27a:melanosome interaction, is the dominant factor regulating dynamic myosin-Va:melanosomes interaction. These findings are consistent with FRAP data showing low turnover of Rab27a on secretory granules in PC12 cells, HUVECs, and melanocytes, and higher turnover of effector Rabphilin in PC12 cells.
(recovery Rab27a = 37.8% (PC12), none detected (HUVEC), 15% (melanocyte); Rabphilin-3a = 66%, t1/2 = 15.4 s) (Handley and Burgoyne, 2008, Handley et al., 2007, Jordens et al., 2006, Kiskin et al., 2010). Our results also agree with non-FRAP studies showing a) low affinity of Mlph:Rab27a interaction relative to other effectors (Kd = 112 nM (Mlph) versus 13.4 nM (Sytl2-a)), b) relatively low intrinsic GTPase activity (~30-fold lower than Rab5a) of Rab27a in vitro, and c) stable membrane association of GDP bound Rab27a in platelets 43-45. Overall these data suggest that Rab27a in melanocytes (like platelets) is mainly GTP-bound and that, in some cases, Rab-GTP:effector interaction stability governs effector recruitment to membranes rather than GTP/GDP status and membrane/cytosol cycling of Rab, as proposed by general models of Rab function (Hutagalung and Novick, 2011, Pfeffer, 2017, Stenmark, 2009). Consistent with this smFRAP studies revealed that the R27BD of the high affinity effector Sytl2-a turned over to a significantly lower extent than Mlph on melanosome membranes (Figures S2 and S8; mean maximum recovery (% PBFI) = 30.34% +/- 11.13%). Unfortunately we were unable to use smFRAP to confirm the GTP/GDP status of Rab27a, as we found that GTP-/GDP-locked Rab27a mutants (Q78L and T23N) did not obviously associate with melanosomes (Figure S9). Other studies have reported similar findings (Bahadoran et al., 2003, Hume et al., 2001, Ishida et al., 2014).

Thirdly, we discovered that disruption of head-tail regulation of myosin-Va in melanocytes (as in Myo-Rab and Myo-Mlph) partially reduced its efficiency in melanosome dispersion and resulted in accumulation of the protein in the peripheral cytoplasm (Figure 5, S5). This is consistent with data from yeast showing that disruption of head-tail regulation of the type V myosin myo2 caused defects in organelle inheritance, accumulation of myo2 in the daughter bud tip and reduced proliferation (Donovan and Bretscher, 2015). A possible mechanistic explanation for the reduced function of Myo-Rab and Myo-Mlph is that in Myo-FL the head-tail regulation ensures that motors are only active when associated with cargo. Thus when the Myo-FL and cargo uncouple then Myo-FL inactivates, detaches from F-actin and may be recycled for further rounds of transport. On the other hand active myosin-Va (Myo-Rab and Myo-Mlph) can move towards the +/-barbed ends of F-actin and remain
there even without cargo. Consistent with this both active myosin-Va fusions tested here (Myo-Rab and Myo-Mlph) accumulated in the peripheral cytoplasm, a region of melanocytes previously found to be enriched in F-actin-rich, filopod-like structures and active myosin-Va proteins (Kapitein et al., 2013, Robinson et al., 2017). It is likely that this peripheral accumulation reduces the efficiency of initial myosin-Va:melanosome association, bearing in mind that melanosomes in myosin-Va deficient cells accumulate in the perinuclear cytoplasm, and also precludes myosin-Va recycling.

Fourthly, we found that dynamic myosin-Va:melanosome interaction is required for the development of elongated, pigment-filled dendrites. Dendrites are a hallmark of melanocytes and are thought to be the prime sites of melanosome transfer to keratinocytes. Specifically, we observed that expression of Myo-Rab in myosin-Va -/- (melan-d) cells significantly increased cell roundness compared with Myo-Mlph and Myo-FL. One possible explanation for this may lie in the observation that Myo-Rab (and Rab27a) appears more uniformly distributed over the surface of melanosomes, compared with Myo-Mlph (and Mlph/myosin-Va), which often localise in spots adjacent to melanosomes (Figure S3C, 5B). We suggest that spots or clusters of Mlph/myosin-Va might allow coordination of the activity of individual motors and directed movement of melanosomes along F-actin bundles towards dendrite tips by ensuring that melanosomes remain associated with local AF bundle for longer than those where motors act alone. In contrast the uniformly distributed Myo-Rab might allow melanosomes to engage a larger number of more randomly oriented F-actin for shorter durations, thereby undertaking less directed transport (Figure 5E). Consistent with this recent single molecule studies have revealed that small teams of myosin-Vc can more efficiently transport cargo along F-actin bundles compared with individual motors on individual filaments(Krementsova et al., 2017). Related to this it is possible that clustering of Mlph/myosin-Va might also facilitate the clustering of F-actin and the formation of filament bundles. Supportive of this the C-terminus of Mlph can interact with actin and expression of active dimeric myosin-Va can bundle F-actin during the formation of filopodia-like structures in culture (Kapitein et al., 2013, Robinson et al., 2017, Kuroda et al., 2003). Alternatively, other Mlph/myosin-Va-tail interacting proteins e.g. end binding
protein 1 (EB1) might contribute to dendrite development by allowing interaction with cytoskeleton elements such as microtubules and intermediate filaments that are present in melanocyte dendrites (Hume et al., 2007, Wu et al., 2005). A further possibility is that a non-melanosomal pool of Mlph/myosin-Va, or interacting partners, contributes to dendrite development. Thus in Myo-Rab expressing cells the activity of the non-melanosomal pool would be altered, thereby reducing dendrites development.

Finally our finding that Myo-Rab can rescue melanosome dispersion in vitro with relatively high efficiency is surprising given the differences in turnover of this protein compared with Myo-FL and the loss of the contribution of Mlph and potentially other interacting proteins. Given the importance of dendrites in melanosome transfer from melanocytes to keratinocytes we suggest that our results using melanosome dispersion after transient over-expression of myosin-Va proteins to measure function may under-estimate the in vivo significance of dynamic turnover of myosin-Va. In vivo protein expression levels are likely to be lower than here, meaning that recycling is more important for myosin-Va function, and the formation of dendrites are critical for transfer of pigment to keratinocytes. Consistent with this, expression of physiological levels of head-tail mutants by modification of the MYO2 gene in yeast resulted in strong reductions in proliferation (Donovan and Bretscher, 2015).

**Methods**

**Plasmid and virus constructs.** pEGFPC3-Rab27a, pEGFPC3-Rab27a<sup>ST</sup>, pEGFPC3-Mlph, pEGFPC3-myosin-Va-tail (MSGTA – here Myo-T encoding aa1277-1877 of the murine myosin-Va encoded by transcript XM_006510828.1) were previously described (Hume et al., 2001, Strom et al., 2002, Tarafder et al., 2011). pENTRmCherryC2-Mlph was made by sub-cloning the full murine Mlph coding sequence from pEGFPC3-Mlph into pENTRmCherryC2 supplied by Jose Ramalho (Nova Universidad
de Lisboa, Lisboa, Portugal). pENTRmCherryC2-MlphΔRBD-Rab27aSF1F4 encoding a fusion protein comprising from the N-terminus; mCherry, the C-terminus myosin-Va- and actin-binding domains (and lacking the N-terminus Rab27a binding domain) fused to the N-terminus of melanosome targeted, but non-functional, Rab27aSF1F4 mutant, was generated by sequential sub-cloning of coding sequence for MlphΔRBD (amino acids 150-590 of murine Mlph; NM_053015) and Rab27aSF1F4 into pENTRmCherryC2. pENTR-GFPC2-myosin-Va(HMM)-Rab27aSF1F4 was generated using a similar sequential sub-cloning approach [myosin-Va (HMM) corresponds to the sequence coding the XM_006510828.1 motor, lever and dimerization domains of myosin-Va heavy chain but lacking the melanocyte specific, Mlph-binding, exons D and F and cargo binding tail (amino acids 1-1300 of the murine protein; XM_006510828.1]. The generation of adenoviruses allowing expression of the GFP alone, GFP-myosin-Va and GFP-myosin-Va(HMM)-Rab27aSF1F4 in melanocytes was as previously described (Hume et al., 2006).

Cell culture and transfection.

Cultures of immortal melan-a (wild-type), melan-d1 (myosin-Va null), melan-ln (Mlph null) and melan-ash (Rab27a null) melanocytes were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin G, and 100 mg/ml streptomycin, 200 nM phorbol 12-myristate 13-acetate, (all Sigma-Aldrich, Poole, United Kingdom), at 37°C with 10% CO2 as described previously (Evans et al., 2014). For live cell experiments and functional studies cells were plated onto 35mm diameter glass bottomed petri dishes (Matek P35G-1.5-20-C) (5x10^4 cells/dish) and 13mm glass cover-slips (1x10^4 cells/coverlip), respectively, and the next day transfected with plasmids or infected with adenovirus, allowing expression of EGFP fusion proteins. Transfection was using FuGene 6 (Promega, UK) as previously described (Hume et al., 2006) and cells were imaged fixed or alive 48 hours later. For live cell experiments growth medium was replaced with L-15 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G, and 100mg/ml streptomycin.
Fluorescence microscopy.

Cells for immunofluorescence were paraformaldehyde fixed, stained and fluorescence and bright-field images showing GFP fusion protein and melanosome distribution, respectively. Images were captured using Axiovision 4.8 software associated with a Zeiss Axiovert 100S inverted microscope fitted with a 40x 1.4NA oil immersion Apochromat objective lens and an Axiocam MR-3 CCD camera, as previously described (Robinson et al., 2017). Antibodies used were mouse monoclonal anti-GFP (Roche 11814460001; 1:300) and goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 568 labelled (Molecular Probes A-11004; 1:500).

For live cell/single melanosome FRAP (smFRAP) experiments, after medium change, cells were transferred to the stage of a Zeiss LSM710 confocal microscope fitted with a 63x 1.4NA oil immersion Apochromat lens within an environmental chamber (37°C). Cells were imaged using 488nm Argon and 561nm HeNe lasers to visualise GFP and mCherry, respectively, and melanosomes were imaged using transmitted light simultaneously with GFP. All images presented here are single sections in the z-plane. For FRAP sequences images were acquired every 1.56 (for GFP-Rab27a) or 1.94 (for GFP-Mlph and Myo-T) seconds. EGFP fusion proteins were excited using 488nm Argon laser set to 2% power with pin-hole set to 1 airy unit. To facilitate downstream FRAP analysis circular bleach regions were defined around single melanosomes well separated from other organelles in thin flat areas of melanocyte cytoplasm in cells which exhibited high signal:noise ratio (melanosome-associated:cytosolic fluorescent protein) and, where appropriate, in cells in which EGFP expression restored dispersed melanosome distribution (indicating physiologically relevant levels of expression). Bleaching of GFP was carried out by scanning these regions five times using the 488nm laser at 100%. For each FRAP series 5 images were acquired prior to bleaching, to establish the 100% level of fluorescence signal associated with each melanosome and then up to 200 images were acquired after bleaching to monitor recovery of melanosome associated fluorescence.

Image analysis.
To determine the rate of fluorescence recovery in bleached melanosomes image sequences were exported from Zen 2011 software and imported to VOLOCITY 6 image analysis software (Improvision) that allows automatic particle tracking. Melanosomes visible in transmitted light images were defined using intensity and size/area filters and then tracked using the shortest path tracking model within VOLOCITY 6 and EGFP signal associated with individual melanosomes over time was extracted as previously described (Hume et al., 2011). Signal associated with bleached melanosomes was then normalised by comparison with non-bleached melanosomes in the same cell and converted to percentage of maximum/pre-bleach signal. To determine the extent (plateau) and kinetics (t1/2) of fluorescence recovery experiment using Mlph and Myo-T, post-bleach data were imported into GraphPad Prism 7 software and each record was fitted to a single exponential function using the non-linear regression curve fit facility. For all data the maximum fluorescence recovery was determined using Graphpad Prism 7 software. Measurement of the function of experimental myosin-Va fusion proteins in melanosome transport (Figure 5) was based on manual measurement of the proportion of cell area occupied by pigmented melanosomes as previously described (Hume et al., 2006). Cell circularity was determined using the formula $4\pi A/P^2$; where $A =$ cell area, and $P =$ cell perimeter.
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Figure 1

A

Myosin-Va

Lever (IQ)

Coiled coil

Globular tail

C

GFP-Myo-T

N

GFP

DF

Globular tail

C

B

GFP-Myo-T

Phase (melanosomes)

Phase inverted

Myo-T/melanosomes

-10s

0s

+25s

+55s

+105s

+155s

0

195s

C

$\tau_{1/2} = 33.12 \pm 10.09$ s

recovery plateau = 67.86 $\pm$ 8.775 %

maximum recovery = 82.29 $\pm$ 14.05 %

$n = 9$

Fluorescence intensity (% maximum)

time [s]
Figure 1. smFRAP analysis of the turnover of melanosome associated Myo-T in melanocytes. Wild-type (melan-a) cells were cultured in glass-bottomed dishes, transfected with a Myo-T expression vector, and the dynamics of the association of Myo-T with melanosomes was investigated using smFRAP analysis (see materials and methods). (A) A schematic representation of the structure of experimental proteins. D and F indicate the position of the Mlph interacting motif encoded by alternatively spliced exons. (B) (Upper panels) Example images showing of the distribution of Myo-T and melanosomes and their co-incidence in living cells. Scale bar = 20 μm. White boxes indicate the parts of cells that were subjected to smFRAP analysis and are shown in the high magnification images below. (Lower panels) images taken from example smFRAP series captured at the indicated time relative to photo-bleaching (t=0). Coloured arrows in these images highlight the position of single melanosomes that were selectively photo-bleached over time (See Movie 1). (C) A line plot showing the average fluorescence intensity associated with photo-bleached melanosomes over time. Error bars are standard deviation. n= 9 melanosomes analysed.
Figure 2 continued

D) GFP-Rab27a
SF1F4

Wild-type

Phase
(melanosomes)

Phase
inverted

Rab27aSF1F4/melanosomes

E) Cell line (protein)

- wild-type
- Rab27a -/-
- melanophilin -/- (Rab27a)
- wild-type (Rab27aSF1F4)

Fluorescence intensity (% maximum)

0 20 40 60 80 100

0 50 100 150 200

time [s]
Figure 2. smFRAP analysis of the turnover of melanosome associated Rab27a in melanocytes.

Melanocytes were cultured in glass-bottomed dishes, transfected with GFP-Rab27a (A-C) (or GFP-Rab27a$^{SF1F4}$ (D)) expression vectors, and the dynamics of the association of GFP-Rab27a with melanosomes was investigated using confocal FRAP analysis (see materials and methods). (A-D) (Upper panels) Example images showing of the distribution of GFP fusion protein and melanosomes in living wild-type (melan-a) (A and D), Rab27a -/- (melan-ash) (B) and Mlph -/- (melan-In) (C) cells, respectively. White boxes indicate the parts of cells that were subjected to FRAP analysis and are shown in the high magnification images below. Scale bars = 20 µm. Lower panels are high magnification images taken from example FRAP series captured at the indicated time relative to photo-bleaching (t=0). Coloured arrows highlight the position over time of melanosomes that were selectively photo-bleached. (E) A line plot showing the average fluorescence intensity associated with photo-bleached melanosomes over time. n = 6 (A), 7 (B), 6 (C) and 16 (D) melanosomes analysed, respectively.
Figure 3

A  GFP-MIph  Phase (melanosomes)  Phase inverted  MIph/melanosomes

Wild-type

-8s  0s  +8s  +24s  +56s  +87s

MIph/melanosomes

B  MIph -/-  MIph/melanosomes

+10s  0s  +6s  +42s  +14.7s  +24.1s

C  Myosin-Va -/-  MIph/melanosomes

+157s  0s  +47s  +45s  +24s  +78.5s

D  Fluorescence intensity (% maximum)

time [s]

green  wild-type  blue  MIph -/-  red  myosin-Va -/-
Figure 3. smFRAP analysis of the turnover of melanosome associated Mlph in melanocytes.

Melanocytes were cultured in glass-bottomed dishes, transfected with a vector expressing GFP-Mlph, and the dynamics of the association of GFP-Mlph with melanosomes was investigated using confocal FRAP analysis (see materials and methods). (A-C) (Upper panels) are example images showing of the distribution of GFP-Mlph and melanosomes in living wild-type (melan-a) (A), Mlph +/- (melan-ln) (B) and myosin-Va +/- (melan-d) (C) cells, respectively. Lower panels are high magnification images taken from example FRAP series captured at the indicated time relative to photo-bleaching (t=0). Red arrows highlight the position over time of melanosomes that were selectively photo-bleached. White boxes in the upper panels indicate the parts of cells that were subjected to FRAP analysis and are shown in the high magnification images below. Scale bars = 20 μm. (D) is a line plot showing the fluorescence intensity associated with photo-bleached melanosomes over time. n = 10 (A), 5 (B) and 5 (C) melanosomes analysed, respectively.
Figure 4

A

| Mlph | N | R27BD | EF | GT | AH | C |
|------|---|-------|----|----|----|---|
| mCherry-Mlph | mCherry | | | | | |
| mCherry-Mlph-Rab27a | mCherry | | | | Rab27aSF1F4 | |

B

| GFP-MVa-tail | mCherry (melanosomes) | Phase inverted | mCherry | melanosomes |
|------------|-----------------------|----------------|---------|-------------|
| mCherry    |                       |                |         |             |
| Mlph       |                       |                |         |             |
| Mlph-Δ (melan-in) |               |                |         |             |
| Mlph-Rab   |                       |                |         |             |

C

[Pigment area (% total) vs Mean fluorescence intensity (AU) graph]

D

[Fluorescence intensity (% maximum) vs time [s] graph]
Figure 4. smFRAP analysis of the turnover of melanosome associated Myo-T in Mlph -/- melanocytes co-expressing Mlph or Mlph-Rab27a. Mlph -/- (melan-In) melanocytes were cultured in glass-bottomed dishes, transfected with vectors allowing expression of GFP-Myo-T in combination with mCherry alone (B), mCherry-Mlph (C) or mCherry-Mlph-Rab27a (D), and the dynamics of association of Myo-T with melanosomes was investigated using confocal FRAP analysis (see materials and methods). (A) A schematic representation of the structure of experimental proteins. R27BD = Rab27 binding domain, GT and EF = myosin-Va globular tail and exon F binding domains AH = amphipathic helix/actin binding domain. (B) Upper panels of each protein group are example images showing of the distribution (or co-distribution) of GFP-myosin-Va-tail, mCherry and melanosomes in living Mlph -/- cells prior to FRAP studies. Lower panels (Mlph and Mlph-Rab groups) are high magnification images taken from example FRAP series captured at the indicated time relative to photo-bleaching (t=0). Coloured arrows highlight the position over time of melanosomes that were selectively photo-bleached. White boxes in the upper panels indicate the parts of cells that were subjected to FRAP analysis and are shown in the high magnification images below (and inset in Figure S3). Scale bars = 20 μm. (C) A scatter plot showing the relationship between expression level and functional efficiency of mCherry-Mlph and mCherry-Mlph-Rab27a, as reported by mean cellular fluorescence intensity (AU = arbitrary units) and pigment area (% total cell area). Lines of best fit are shown in red (Mlph-Rab), black (Mlph) and dotted (mCherry). (D) A line plot showing the fluorescence intensity associated with photo-bleached melanosomes over time. The vertical bars above each plot indicate the standard deviation. n = 9 (Mlph) and 7 (Mlph-Rab) melanosomes analysed, respectively.
Figure 5

A

[Diagram showing the structure of Myosin-Va, GFP-Myo-FL, GFP-Myo-Rab, and GFP-Myo-Mlph, with annotations for the lever, coiled coil, globular tail, and specific domains marked.]

B

[Images showing phase/melanosome and F-actin staining for GFP, Myosin-Va, Myo-Rab, and Myo-Mlph, with rescue and GFP/F-actin/melanosome images presented for Myo-Mlph.]

C

[Graph showing rescue data for pigment area (% of total) with rescue indicated by symbols.]

D

[Graph showing circularity with circularity range and significance indicated by asterisks.]

E

[Diagram illustrating the distribution of actin and melanosome with arrows indicating movement and direction, with text: "Motors spread over the melanosome surface -> less directed movement and dendrite formation." and "Motor clustering -> a) team work b) actin bundling -> more directed movement and dendrite formation." ]
Figure 5. Fusion of active myosin-Va with melanosome associated Rab27aSF1F4 reduces its functional efficiency and affects dendrite development. Myosin-Va -/- (melan-d) cells were infected with adenoviruses expressing the indicated GFP fusion proteins, fixed and stained for immunofluorescence (see materials and methods). (A) A schematic representation of the structure of experimental proteins. F indicates the position of the Mlph interacting motif encoded by alternatively spliced exon F. (B) Representative images of showing the distribution of each GFP fusion and melanosomes (bright-field) in melanocytes (scale bar = 20 μm). (C-D) Scatter plots showing the melanosome dispersion (C) and circularity (D) of individual melanocytes expressing the indicated proteins. Horizontal bars show the median and 25th and 75th percentile of the each population. **** indicates the significance of differences in populations of data (p=<0.0001) as determined by one-way ANOVA. No other significant differences were observed. Data are from one of three independent experiments and are representative of the results of all experiments. Number of cells analysed; GFP = 14, myosin-Va = 25, Myo-Rab = 26, Myo-Mlph = 20. (E) A model indicating how the different arrangement of motors in Myo-Rab versus Myo-FL/Myo-Mlph might result in differences in dendrite formation.