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Rab32 Regulates Melanosome Transport in *Xenopus* Melanophores by Protein Kinase A (Cβ/RIIα) Recruitment

Minjong Park[1], **Anna S. Serpinskaya**[1], **Nancy Papalopulu**[2], and **Vladimir I. Gelfand**[1,*]

1*Department of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611*

2*Faculty of Life Sciences, The University of Manchester, Manchester, M13 9PT, UK*

Summary

Intracellular transport is essential for cytoplasm organization, but mechanisms regulating transport are mostly unknown. In *Xenopus* melanophores, melanosome transport is regulated by cAMP-dependent protein kinase A (PKA) [1]. Melanosome aggregation is triggered by melatonin, whereas dispersion is induced by melanocyte-stimulating hormone (MSH) [2]. The action of hormones is mediated by cAMP: high cAMP in MSH-treated cells stimulates PKA while low cAMP in melatonin-treated cells inhibits it. PKA activity is typically restricted to specific cell compartments by A-kinase anchoring proteins (AKAP) [3]. Recently, Rab32 has been implicated in protein trafficking to melanosomes [4] and shown to function as an AKAP on mitochondria [5]. Here we tested the hypothesis that Rab32 is involved in regulation of melanosome transport by PKA. We demonstrated that Rab32 is localized to the surface of melanosomes in a GTP-dependent manner and binds to the regulatory subunit RIIα of PKA. Both RIIα and Cβ subunits of PKA are required for transport regulation and are recruited to melanosomes by Rab32. Overexpression of wild type Rab32, but not mutants unable to bind PKA or melanosomes, inhibits melanosome aggregation by melatonin. Therefore, in melanophores Rab32 is a melanosome-specific AKAP that is essential for regulation of melanosome transport.

RESULTS

Identification of the PKA Isoform That Is Localized to Melanosomes and Involved in the Regulation of Melanosome Transport

Melanosome transport in *Xenopus* melanophores is tightly regulated by PKA, and PKA is associated with melanosomes [1,6]. PKA holoenzyme consists of two catalytic and two regulatory subunits [7]. In *Xenopus*, catalytic subunits Cα and Cβ [8] and regulatory subunits RIα and RIIα have been characterized thus far. Recently, Kashina et al. have shown that PKA RIIα is localized to melanosomes in *Xenopus* melanophores [6]. We first sought to determine if a catalytic subunit of PKA forms a complex with RIIα on melanosomes. Western blotting with antibodies against PKA C and PKA Rllα detected bands of molecular masses consistent to each protein in the cell extract and in the melanosome fraction (Fig. 1A). However, PKA RIα was found only in the extract (Fig. 1A), but not on melanosomes. Thus, Rllα and C are present on melanosomes, whereas RIα is not.

*For Correspondence: Vladimir I. Gelfand, Department of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave., Chicago, IL 60611. Phone (312) 503-0530. Fax (312) 503-2696. E-mail: vgelfand@northwestern.edu.

Available PKA C antibodies do not discriminate between the isoforms of the catalytic subunits, Cα and Cβ. We therefore sought to distinguish between isoforms using a functional assay. We overexpressed pEGFP-tagged Cα or Cβ in melanophores and treated cells with melatonin or MSH. Overexpression of the EGFP-Cα did not affect the ability of cells to aggregate or disperse melanosomes (Fig. 1C). However, EGFP-Cβ overexpression resulted in a complete block of aggregation (Fig. 1B, 1C and Movie 1). Thus, the overexpression studies demonstrate that *x*PKA Cβ is the catalytic subunit that regulates melanosome transport. Collectively, the biochemical and overexpression data demonstrate that the Cβ/RIIα complex is localized to melanosomes and regulates their transport.

Colocalization of Rab32 with Melanosomes

Localization of PKA to specific compartments is typically mediated by AKAPs. In a search for AKAPs that mediate melanosomal targeting of PKA, we noticed that one of the Rab family of Ras-like GTPases (Rab32) was reported to function as an AKAP in human fibroblasts [5]. Although in fibroblasts Rab32 is localized to mitochondria, Rab32 is involved in the biogenesis of melanosomes in human melanocytes [4] and is highly expressed in melanocytes [9]. In *Xenopus*, Rab32 is highly expressed in the pigment epithelium of the retina and a clone for *Xenopus* Rab32 (*x*Rab32) has been isolated in a functional screen, based on its ability to cause abnormal pigmentation under conditions of overexpression in the embryonic ectoderm [10]. Based on these reports, we wanted to determine if *x*Rab32 functions as a melanosomal AKAP and determine its role in the regulation of melanosome transport.

We first examined the subcellular localization of *x*Rab32 in *Xenopus* melanophores using fluorescent microscopy and biochemical techniques. *x*Rab32 was tagged with mCherry or EGFP and transiently expressed in melanophores. Both mCherry- and EGFP-*x*Rab32 form a distinct punctate pattern in the cytoplasm (Fig. 2, left panel). At higher magnification, most of the puncta that are localized in the focal plane of the microscope are composed of a fluorescent ring encircling a dark inner core (Fig. 2, left panel inset). Comparison of the fluorescence pattern with the distribution of melanosomes, as seen using bright-field microscopy, demonstrates (Fig. 2, middle panel) that the majority of the punctate structures correspond to melanosomes (Fig. 2, right panel). In addition, mCherry-*x*Rab32 is localized on the surface of smaller vesicles that neither have a dark inner core nor contain melanin (Fig. 2 and Movie 2). Time-lapse microscopy of the dynamics of mCherry-*x*Rab32 demonstrates that most of the time *x*Rab32 remains associated with melanosomes (Movie 2). Occasionally, round or elongated vesicular structures budding and fusing with melanosomes were observed in the cytoplasm (Movie 2).

To demonstrate that endogenous Rab32 is expressed in *Xenopus* melanophores and is bound to melanosomes, we raised an antibody specific to *x*Rab32. This antibody detects a single band with a molecular weight of 27 kD on Western blots of melanophore extracts (Fig. S1). The same 27 kD band is detected in the purified melanosome fraction (Fig. S1). It is interesting to note, that the majority of Rab32 in melanophores is associated with melanosomes and that these cells have very little soluble Rab32. Therefore, the distribution of mCherry-*x*Rab32 faithfully reproduces the distribution of the endogenous protein.

In addition to the wild-type protein, we examined the subcellular localization of Q82L and T36N mutants of *x*Rab32, which in human Rab32 have been reported to have defects in GTP hydrolysis and GTP binding, respectively [5]. mCherry-*x*Rab32T36N, mimicking the GDP-bound state of the protein, is not localized to melanosomes. Instead, it was predominantly concentrated in the perinuclear area and in the cytoplasm (Fig 3A). In contrast, a constitutively active mCherry-*x*Rab32Q82L was localized to melanosomes (Fig 3B). We also generated *x*Rab32 lacking two COOH-terminal cysteine residues (*x*Rab32ΔCC) that are normally prenylated and required for the targeting of Rabs to the membrane [11]. This mutant did not

localize to melanosomes (Fig 3D). Together these results suggest that *x*Rab32 protein is localized to the membrane of melanosomes in a GTP-dependent manner using the COOH-terminal cysteines.

Xenopus Rab32 Is an A-Kinase Anchoring Protein

Human Rab32 is known to bind PKA [5]. In *Xenopus* melanophores, pigment movement is regulated by PKA [1], and both PKA [6] and *x*Rab32 (see above) are localized to melanosomes. Therefore, it is logical to suggest that *x*Rab32 is involved in targeting PKA to melanosomes. To demonstrate that *x*Rab32 is an AKAP, we examined the binding of PKA RIIα to *x*Rab32 using a cAMP agarose pull-down assay and immunoprecipitation. As expected, the regulatory subunit RIIα of PKA bind to the cAMP-agarose and this binding is abolished by the addition of free cAMP. The same binding pattern is detected for Rab32 (Fig. 4A). This result indicates that the two proteins probably form a complex and that *x*Rab32 could bind to the column via RIIα. To show that Rab32 indeed binds PKA RIIα, we performed a coimmunoprecipitation assay. We cotransfected mCherry-*x*Rab32 and EGFP-*x*PKA RIIα into *Xenopus* melanophores and pulled down mCherry-*x*Rab32 using anti-Rab32 antibody. Precipitates were probed with a PKA RIIα antibody and HRP-Protein A [12] to avoid detection of the Rab32 antibody used in the pull-down). Fig. 4B shows that the Rab32-PKA RIIα complex is present only in Rab32 precipitates and not in preimmune precipitates (Fig. 4B).

An additional test for *x*Rab32-RIIα was performed using a yeast two-hybrid assay. To avoid membrane binding by xRab32 expressed in yeast, the assay was performed using xRab32 lacking two COOH-terminal cysteine residues required for prenylation and membrane localization. Fig. 4C demonstrates that *Xenopus* Rab32 binds to *Xenopus* RIIα, whereas Rab1a, used as a control, does not. Furthermore, both Q82L and T36N mutants of *x*Rab32 that mimic the GTP- and GDP-bound states of the protein [5], were able to interact with *x*PKA RIIα (Fig. 4C). On the other hand, mutation L186P, known to inhibit PKA binding in mammalian cells, prevented the interaction of xRab32 with RIIα (Fig. 4C).

Rab32 is Required for Regulation of Melanosome Transport

To test the role of *x*Rab32 in melanosome transport, we overexpressed pEGFP-*x*Rab32 and induced melanosome aggregation or dispersion by melatonin or MSH, respectively. As a control, we transfected cells with pEGFP-Rab9, a Rab protein that is localized to late endosomes [13]. Strikingly, overexpression of the pEGFP-*x*Rab32 completely prevented melanosome aggregation by melatonin, while Rab9 overexpression had no effect (Fig. 5A, 5B, and Movie 3). Time-lapse analysis shows that overexpression of *x*Rab32 does not stop melanosome movement, but abolishes the bias of movement toward the cell center. This demonstrates that Rab32 plays a role in regulation of transport, rather than on movement itself.

To elucidate whether *x*Rab32 localization is important to its effect on regulation, we tested the effect of the mutant EGFP-*x*Rab32ΔCC that is unable to bind to melanosomes (Fig. 3D) but capable of PKA binding (Fig. 4B). Overexpression of this mutant did not affect the cell response to melatonin or MSH (Fig. S2). To determine, if Rab32-PKA binding is essential for its effects on melanosome behavior, we overexpressed a mutant *x*Rab32L186P that does not interact with PKA (Fig. 5B). Fluorescent microscopy shows, that similarly to the wild-type protein, mCherry-*x*Rab32L186P binds to melanosomes forming a fluorescent halo around a dark melanin core (Fig. 3C). As expected, this mutant does not inhibit melanosome aggregation Finally, if the impact of Rab32 on regulation is explained by PKA recruitment, inhibition of PKA should induce pigment aggregation even in cells overexpressing Rab32 because PKA functions downstream of Rab32. Indeed, an inhibitor of PKA, PKI, overexpressed in melanophores together with *x*Rab32 induced pigment aggregation (Fig. 5C). Thus, overexpression experiments demonstrate that *x*Rab32 is involved in the regulation of

melanosome transport, and that its localization to melanosomes and binding to PKA are required for this function.

Conclusion

Melanosome transport in melanophores is regulated by PKA. PKA also regulates many other cellular functions and therefore PKA activation is restricted to specific areas of the cell. This is typically accomplished by AKAPs [3,14]. In this report, we showed that *Xenopus* Rab32 is associated with melanosomes and links PKA to these organelles.

In other organisms, orthologues of *x*Rab32 are localized to pigment organelles and function in their biogenesis. Human and mouse Rab32 to localize to melanosomes, and the level of Rab32 correlats to pigment production [4,9]. A *Drosophila* Rab32 homolog, Rab-RP1, is localized to pigment granules in the eye and its mutation causes eye color defects [15,16].

Although in many cell types Rab32 (like many other Rabs) functions in protein trafficking, *Xenopus* Rab32 has a second important function – to link PKA to the surface of melanosomes, organelles that are regulated by PKA, ensuring the spatial specificity of PKA signaling. Recent studies exploring the mechanism of PKA in melanosome transport demonstrated that a regulatory subunit of PKA, PKA-RIIα, is present on melanosomes [6]. Additionally, other signaling molecules, involved in the regulation of melanosome movement downstream of PKA are present on the surface of melanosomes [17] but the molecular mechanisms of such spatial restriction are not known. We demonstrated here that *x*Rab32 is a key component of the signaling cascade regulating melanosome transport by linking PKA to the melanosome surface. These data agree well with the results by Scott and colleagues demonstrating that human Rab32 functions as an AKAP on mitochondria [5].

Rab proteins are known to recruit motor proteins to cargo either directly or through adapter proteins [18]. Therefore, in order to better understand the role of Rab32, it will be interesting to identify other interacting partners for *x*Rab32 and to test if it can interact with molecular motors that move melanosomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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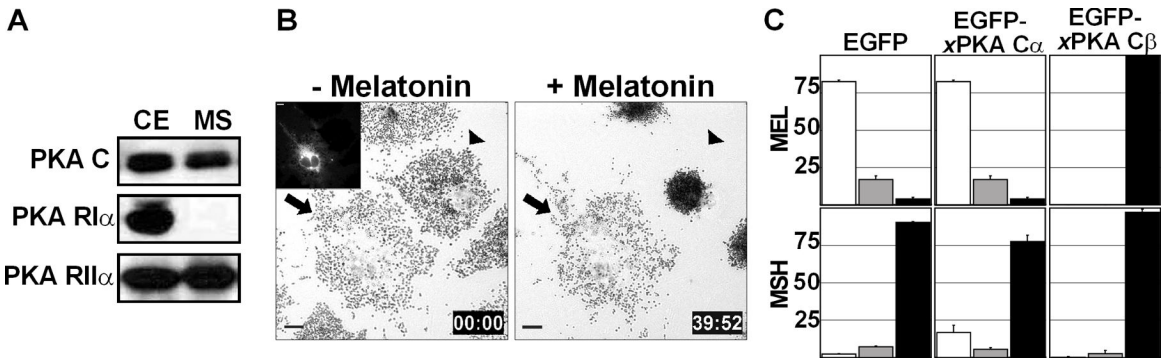

Figure 1. PKA Cβ/RIIα Regulates Melanosome Transport

(A) PKA C and RIIα are localized to melanosomes. *Xenopus* melanophore extract (CE) and purified melanosome fraction (MS) were probed with antibodies against PKA subunit isoforms. PKA C and RIIα, but not RIα, are present in melanosome fraction. (B) PKA Cβ overexpression blocks melanosome aggregation by melatonin. The left panel is a bright-field image showing melanosome distribution before melatonin stimulation. The black arrow indicates a cell expressing EGFP-xPKA Cβ (inset) and the black arrowhead indicates a control cell. The right panel shows the distribution of melanosomes after 40 minutes of melatonin stimulation. EGFP-xPKA Cβ overexpression in a transfected cell (black arrow) completely blocks pigment aggregation. Bars, 10 μm. This figure represents two frames from Supplementary Movie 1.

(C) *Xenopus* melanophores were transiently transfected with EGFP, EGFP-xPKA Cα, or EGFP-xPKA Cβ. Transfected cells were treated with melatonin or MSH and scored into three groups (aggregated, partially dispersed, and dispersed). In each experiment, aggregated, partially dispersed, and dispersed cells are shown as white, gray, and black bars, respectively. n=100 for each condition. The experiment was repeated three times.

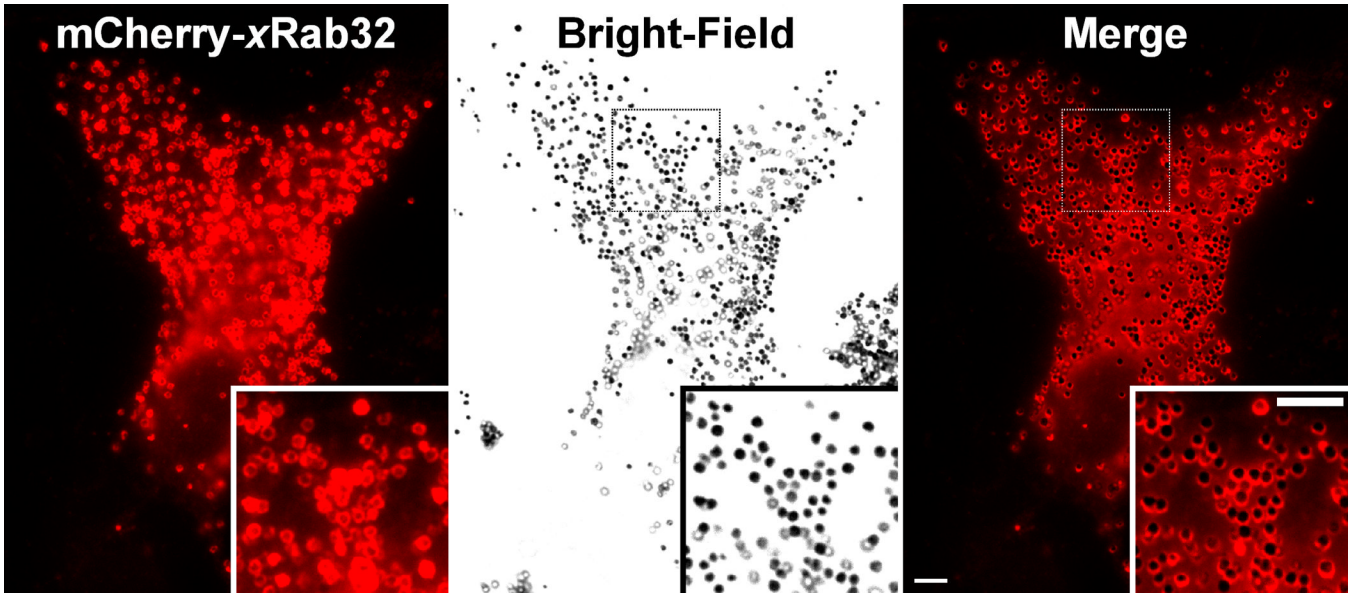

Figure 2. Rab32 is Localized to Melanosomes

mCherry-*x*Rab32 is localized to melanosomes. *Xenopus* melanophores were transfected with mCherry-*x*Rab32 for 24 hr. The left panel shows the distribution of the mCherry-*x*Rab32 fusion protein, the middle panel shows in bright-field distribution of melanosomes, and the right panel shows the bright field image merged with the distribution of mCherry-*x*Rab32 fusion proteins. Melanosomes are decorated with mCherry-*x*Rab32 (right panel inset), indicating that the mCherry-*x*Rab32 fusion protein is localized to melanosomes. Bars, 5 μm.

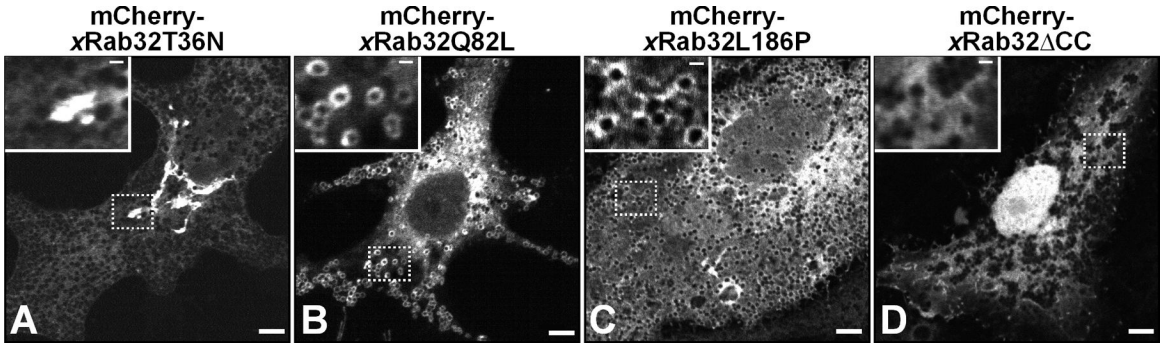

Figure 3. Localization of *x*Rab32 mutants

Xenopus melanophores were transfected with mCherry-*x*Rab32T36N (mimicking the GDP-bound state of the protein) (A), mCherry-*x*Rab32Q82L (mimicking the GTP-bound state of the protein) (B), mCherry-*x*Rab32L186P (a mutant defective in PKA binding) (C), and mCherry-*x*Rab32ΔCC (lacking C-terminal cysteines) (D) for 24 hr. mCherry-*x*Rab32Q82L and mCherry-*x*Rab32L186P are localized to melanosomes (B and C insets, respectively), whereas, mCherry-*x*Rab32T36N and mCherry-*x*Rab32ΔCC are not (A and D inset, respectively). Bars, 5 μm in main images and 1 μm in insets.

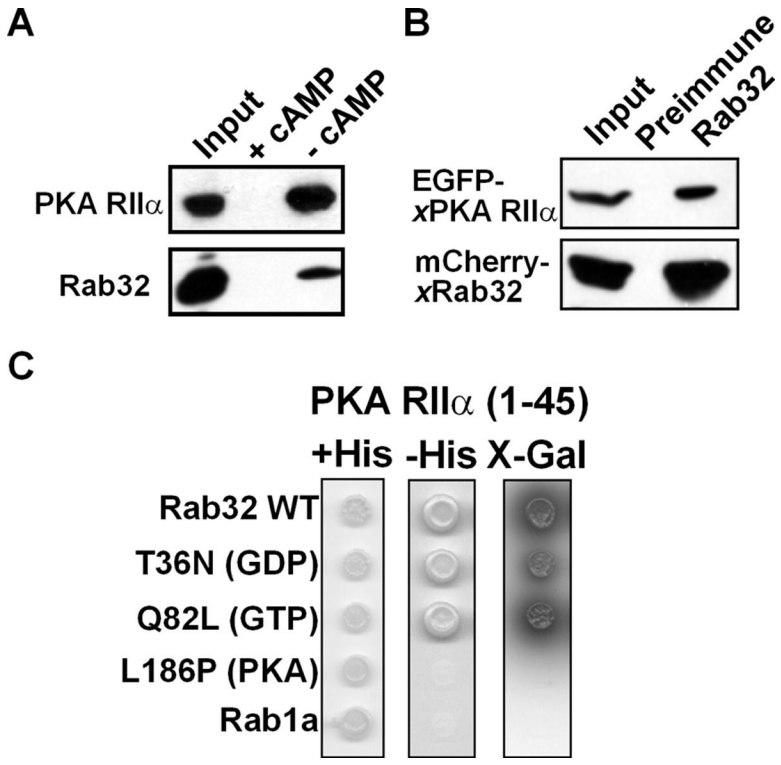

Figure 4. *Xenopus* Rab32 is an A-Kinase Anchoring Protein

(A) PKA RIIα binds *Xenopus* Rab32. *Xenopus* melanophore extracts were incubated with cAMP-agarose resin in the presence of 75 mM cAMP (+ cAMP) or in the absence cAMP (- cAMP). cAMP-agarose resin was washed and eluted with 75mM cAMP. The Western blot was performed using PKA RIIα and *x*Rab32 antibodies. The cAMP agarose resin binds RIIα and also pulls down *x*Rab32. This interaction is abolished in the presence of 75 mM cAMP.

(B) Rab32 binds to PKA RIIα *in vivo*. Extracts from cells coexpressing EGFP-*x*PKA RIIα and mCherry-*x*Rab32 were immunoprecipitated with an anti-Rab32 antibody or preimmune IgG. Precipitates were probed using anti-PKA RIIα antibody or anti-Rab32 and developed using HRP-protein A. Note that Rab32 antibody but not the preimmune IgG pulls down PKA RIIα. Inputs are 5% of cell extracts from sample.

(C) Two-hybrid analysis of Rab32-RIIα binding. *x*Rab32 bait constructs (lacking C-terminal cysteins to prevent membrane binding) were tested against the indicated prey constructs in the yeast two-hybrid system for the ability to grow on minimal media in the presence (+His), or absence (-His) of histidine. The Rab32- PKA RIIα interaction was also tested with high-stringency (SD/-Trp/-Leu/-His/-Ade/X-α-Gal) plates. Growth on minimal media in the absence of histidine or α-galactosidase activity represents a positive interaction.

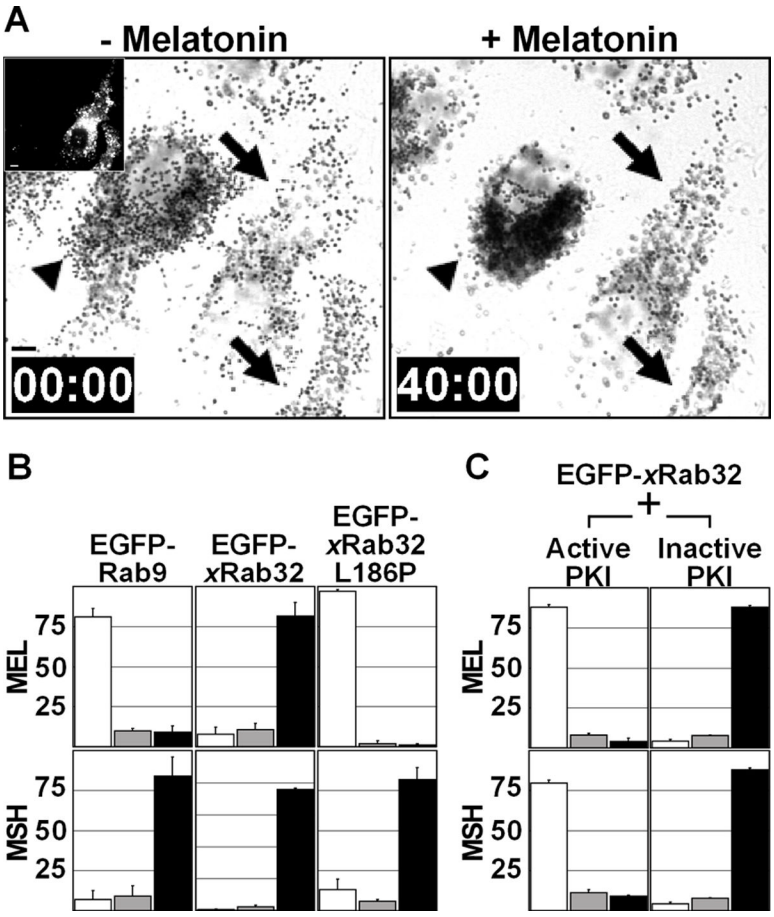

Figure 5. *x*Rab32 is Involved in Regulation of Melanosome Transport

(A) *x*Rab32 blocks melanosome aggregation by melatonin. The left panel is a bright-field image showing melanosome distribution before melatonin stimulation. The black arrows indicate EGFP-*x*Rab32 transfected cells (inset) and the black arrowhead indicates a control cell. The right panel shows the distribution of melanosomes after 40 minutes of melatonin stimulation. EGFP-*x*Rab32 overexpression in transfected cells (black arrows) completely blocks pigment aggregation by melatonin. Bars, 10 μm. This figure represents frames from Supplementary Movie 3.

(B) Recruitment of PKA by *x*Rab32 to melanosomes is essential for inhibition. *Xenopus* melanophores were transiently transfected with EGFP-Rab9, EGFP-*x*Rab32, or EGFP *x*Rab32L186P. Transfected cells were treated with melatonin or MSH and scored into three groups (aggregated, partially dispersed, and dispersed). In each experiment, aggregated, partially dispersed, and dispersed cells are shown as white, gray, and black bars, respectively. n=100 for each condition. The experiment was repeated three times.

(C) *x*Rab32 regulates melanosome movement upstream of PKA.

Xenopus melanophores were cotransfected with EGFP-*x*Rab32 and constructs pNP210 or pNP211 encoding HA-epitope tagged active or inactive PKA inhibitor, PKI, respectively. Transfected cells were treated with melatonin or MSH, fixed, immunostained for HA and scored into three groups (aggregated, partially dispersed, and dispersed). In each experiment, aggregated, partially dispersed, and dispersed cells are shown as white, gray, and black bars, respectively. n=100 for each condition. The experiment was repeated three times.