Supplemental Data

Rab32 Regulates Melanosome Transport in *Xenopus* Melanophores by Protein Kinase A Recruitment

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Supplemental Experimental Procedures

Melanophore Culture

Immortalized *Xenopus* melanophores were cultured as described [S1]. For microscopy analysis, we plated melanophores on acid-washed poly-L-lysine-coated coverslips 24–48 hr prior to performing experiments. We induced aggregation and dispersion by incubating melanophores in serum-free media for 60 min, then treating melanophores in serum-free media with either 10 nM melatonin for aggregation or 100 nM MSH for dispersion for 30 min. Cells were rinsed in 0.7× PBS, switched to the opposing hormone for 60 min, and used in experiments. Transfected melanophores were fixed in freshly prepared 4% formaldehyde in 0.7× PBS. To stain the HA epitope in cotransfection experiments, we used a cultured supernatant containing monoclonal antibody 12CA5 that recognizes the HA epitope at a dilution of 1:5. Secondary antibody conjugated with Texas Red was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at a dilution of 1:100. All antibodies were diluted into 1% BSA and 0.1% Triton X-100 in PBS. Cells were scored as aggregated, partially dispersed, or dispersed according to the state of pigment aggregation. Cells scored as fully aggregated had their melanosomes in a dense mass at the center of the cell. Cells scored as partially dispersed had melanosomes that were released a short distance from the central pigment mass. Cells scored as dispersed had melanosomes evenly distributed throughout the cytoplasm.

Melanosome Purification

Cells used in biochemical experiments were rinsed in 0.7× PBS and detached from plates with a plastic cell scraper. Cells were syringe lysed in IMB50 buffer (50 mM imidazol [pH 7.4], 1 mM EGTA, 0.5 mM EDTA, 5 mM magnesium acetate, 175 mM sucrose, and 1 mM DTT) supplemented with protease inhibitors (10 μg/ml chymostatin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin), 150 μg/ml casein, and 1 mM ATP. Melanosome purifications were performed as described [S1]. This fraction was considered pure according to the same electron microscopic and biochemical purity-confirmation criteria set forth by Rogers et al. [S1].

Molecular Biology and Antibodies

The cDNA encoding *Xenopus* Rab32 was described in [S2]. Plasmid pEGFP-C1-*Xenopus* Rab32 (EGFP-*x*Rab32) was cloned into pEGFP-C1 (BD Bioscience Clontech) vector between EcoRI and BamHI restriction sites with the following primers: 5′-GGGGAATTCTATGG CCGGAGAAGGGCG-3′ and 5′-GGGGGATCCTTAACAGCACTGAAA TTTAC-3′. pEGFP-C1-*Xenopus* Rab32T36N (EGFP-*x*Rab32T36N), pEGFP-C1-*Xenopus* Rab32Q82L (EGFP-*x*Rab32Q82L), pEGFP-C1-*Xenopus* Rab32L186P (EGFP-*x*Rab32L186P), pEGFP-C1-*Xenopus* Rab32 lacking COOH-terminal two cysteines (EGFP-*x*Rab32ΔCC), and pET28a-*x*Rab32ΔCC were generated by PCR site-directed mutagenesis. pEGFP-Rab9a (EGFP-Rab9a) was generously provided by Dr. Suzanne Pfeffer. We cloned plasmids mCherry-*Xenopus* Rab32 (mCherry-*x*Rab32), mCherry-*Xenopus* Rab32T36N (mCherry-*x*Rab32T36N), mCherry-*Xenopus* Rab32Q82L (mCherry-*x*Rab32-Q82L), mCherry-*Xenopus* Rab32L186P (mCherry-*x*Rab32L186P), and mCherry-*Xenopus* Rab32ΔCC (mCherry-*x*Rab32ΔCC) into the mCherry [S3] vector between EcoRI and BamHI restriction sites by subcloning from the pEGFP-*x*Rab32 constructs described above. The cDNA encoding *Xenopus* PKA Cα and Cβ were kindly provided by Dr. Angel R. Nebreda, European Molecular Biology Laboratory. Plasmid pEGFP-C1-*Xenopus* PKA Cα (EGFP-*x*PKA Cα) was cloned into the pEGFP-C1 vector in BamHI restriction sites with following primers: 5′-GGGGGATCCATGGGCAACGCGGCTAC-3′ and 5′-GGG GGATCCTTAAAAGTCAGAGAATTCTTTAGCG −3′. Plasmid pEGFP-C2-*Xenopus* PKA Cβ (EGFP-*x*PKA Cβ) was cloned into pEGFP-C2 (BD Bioscience Clontech) vector between EcoRI and BamHI restriction sites with following primers: 5′-GGGGAATTCATGGG AAACGCTGCCACC-3′ and 5′-GGGGGATCCCTAAAAGTCGGCAAA CTCTTTT −3′. The cDNA encoding *Xenopus* PKA RIIα was purchased from Open Biosystems (Huntsville, AL). Plasmid pEGFP-C1-*Xenopus* PKA RIIα (EGFP-*x*PKA RIIα) was cloned into pEGFP-C2 vector in XhoI and BamHI restriction sites with following primers: 5′-GGGCTCGAGATGAGTATAGAAATCCCTGAG-3′ and 5′-GGGGG ATCCTCATGTACTGGGATCAGCAAT-3′. Plasmids pNP210 and pNP211 encode the active and inactive forms of the PKA inhibitor, respectively [S4]. Melanophores were transfected with the FuGENE6 transfection agent according to manufacturer protocols (Roche Diagnostics). Rab32 antibody was raised in rabbits against recombinant His_6-tagged *x*Rab32ΔCC fusion protein purified by Talon affinity chromatography. Rabbit immunization was performed by Proteintech Group. We tested the *Xenopus* Rab32 antibody for crossreactivity to other Rabs by immunoblotting transiently transfected EGFP-Rab27a, EGFP-Rab9a, and EGFP-Rab1a melanophore extracts. No crossreactivity was detected. For western analysis, a 1:5000 dilution of antiserum was used. The anti-PKA catalytic subunit, anti-PKA regulatory Iα, and PKA regulatory IIα antibodies were purchased from Cell Signaling Technology (Cambridge, MA).

cAMP-Agarose Pulldown

Melanophores from 20 10 cm dishes were homogenized in cold homogenization buffer (20 mM HEPES [pH 7.4], 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 1 mM DTT, and 10 μg/ml chymostatin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) with a ball-bearing homogenizer [S5]. We centrifuged the homogenates at 10,000 × *g* for 20 min at 4°C to obtain the supernatant. The supernatant was incubated with cAMP agarose (Biolog, Bremen, Germany) in the presence or absence of 75 mM cAMP at 4°C O/N. The resin was then washed twice with high-salt buffer (10 mM HEPES [pH

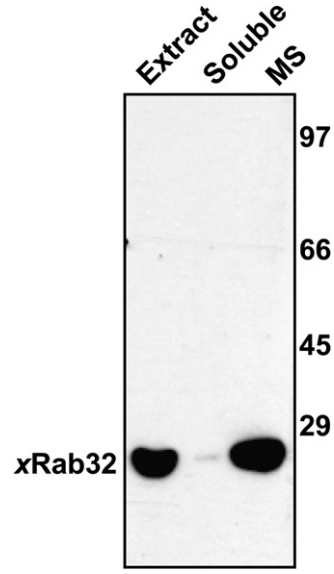

Figure S1. Endogenous *Xenopus* Rab32 Is Localized to Melanosomes

Crude cell extract (extract), soluble proteins (soluble), and purified melanosome fraction (melanosomes) were probed with antibodies specific for *x*Rab32. *x*Rab32 was detected in the cell extract and purified melanosome fractions, but only a small amount of the protein was present in the soluble fraction.

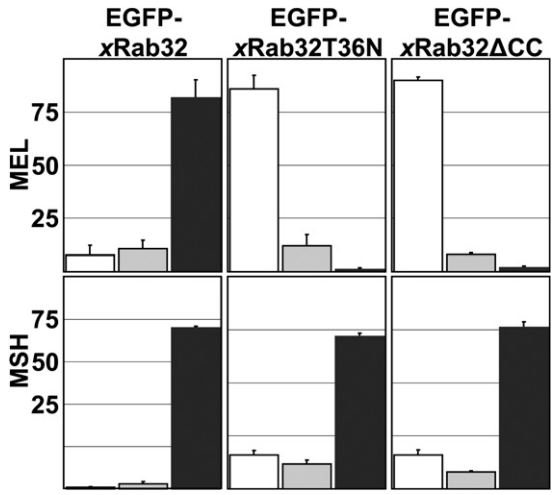

Figure S2. *x*Rab32 Localization to Melanosomes Is Essential for Inhibition of Aggregation

Xenopus melanophores were transiently transfected with EGFP-*x*Rab32T36N or EGFP-*x*Rab32ΔCC. 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. Error bars represent SE. Note that neither of these mutants affected melanosome aggregation or dispersion. The effect of the wild-type *x*Rab32 (data from Figure 5) is reproduced here for comparison.

7.4], 1.5 mM $MgCl_2$, 10 mM KCl, 500 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, and protease inhibitors [10 μg/ml chymostatin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin]), four times with low-salt buffer (same buffer without NaCl) and eluted twice with 75 mM cAMP by rotation for 1 hr at room temperature. The eluted protein was analyzed by western blot with anti-PKA RIIα and anti-Rab32.

Coimmunoprecipitation

Xenopus melanophores were plated on eight 10 cm dishes and cotransfected with pEGFP-xPKA and mCherry-*x*Rab32 with FuGENE6 transfection agent according to manufacturer protocols (Roche Diagnostics). After 48 hr, we harvested cells, resuspended them in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM DTT, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM PMSF, and 10 μg/ml chymostatin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin), and homogenized them by using a 25 gauge syringe needle. Cell extracts were centrifuged at 10,000 × *g* for 20 min. Lysates were precleared with protein A beads for 1 hr at 4°C, then incubated with Rab32 antibodies or preimmune serum prebound to protein A-Sepharose beads (GE Healthcare) overnight at 4°C. The beads were washed with RIPA buffer five times, and proteins were eluted in SDS sample buffer. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. For immunodetection, anti-PKA RIIα and anti-Rab32 were used. We used HRP-conjugated Protein A (Upstate/Millipore), which does not recognize denatured antibodies [S6], to prevent detection of Rab32 antibodies used for pulldown.

Yeast Two-Hybrid Assay

The RIIα AKAP-binding domain (1–45 amino acids) was amplified by PCR from *Xenopus* RIIα cDNA and subcloned into the SalI-BglII sites in the pGAD424 yeast expression vector. This gene encodes an NH_2-terminal GAL4 activation domain fused to the first 45 amino acids of RIIα. *Xenopus* Rab32 lacking two COOH-terminal cysteines was amplified by PCR and subcloned into EcoRI and BamHI sites in the pGBTK7 yeast expression vector, thereby producing a fusion of GAL4 DNA-binding domain with *x*Rab32 ΔCC. *Xenopus* Rab32 mutants, (T36N, Q82L, and L186P) and Rab1a (all also two lacking the COOH-terminal cysteines) were subcloned into the same vector. These constructs were cotransformed with the pGAD424-RIIα 1–45 amino acids into the yeast strain AH109 as described in the BD Matchmaker Library Construction and Screening Kit manual (BD Biosiences Clontech).

Image Acquisition

Images of fixed cells were acquired with an inverted Nikon Eclipse U2000 microscope with a plan-Apo 60 × 1.4 NA objective. Images were captured with a Cool SNAP ES CCD camera (Roper Scientific). Confocal images were taken with an LSM 510 META (Zeiss, Thornwood, NY).

Time-lapse sequences of live cells (Movies S1 and S3) were acquired with an inverted Nikon Eclipse U2000 microscope with a 40 × 1.0 NA oil-immersion objective with bright-field illumination from a 100 W halogen lamp. We directed visible light through a red filter to cut illumination below 695 nm to prevent light-induced pigment dispersion [S7]. Images were captured with an Orca-II ER CCD camera (Hamamatsu Photonics). For fluorescent imaging of live cells (Movie S2), we used an inverted Nikon Eclipse U2000 microscope with a plan-Apo 60 × 1.4 NA objective and a Cool SNAP ES CCD camera (Roper Scientific). Cameras and shutters were driven by Metamorph Version 7 software (Molecular Devices Corporation, Downingtown, PA).

Supplemental References

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