

CK1 activates minus-end directed transport of membrane organelles along microtubules.

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Abstract.

Microtubule (MT) -based organelle transport is driven by MT motor proteins that move cargoes towards MT minus-ends clustered in the cell center (dyneins), or plus-ends extended to the periphery (kinesins). Cells are able to rapidly switch the direction of transport in response to external cues, but the signaling events that control switching remain poorly understood. Here, we examined the signaling mechanism responsible for the rapid activation of dynein-dependent MT minus-end directed pigment granule movement in *Xenopus* melanophores (pigment aggregation). We found that along with the previously identified Protein Phosphatase 2A (PP2A), pigment aggregation signaling also involved Casein Kinase 1 ϵ (CK1 ϵ), that both enzymes were bound to pigment granules, and that their activities were increased during pigment aggregation. Furthermore, we found that CK1 ϵ functioned downstream of PP2A in the pigment aggregation signaling pathway. Finally, we discovered that stimulation of pigment aggregation increased phosphorylation of Dynein Intermediate Chain (DIC), and this increase was partially suppressed by CK1 ϵ inhibition. We propose that signal transduction during pigment aggregation involves successive activation of PP2A and CK1 ϵ and CK1 ϵ -dependent phosphorylation of DIC, which stimulates dynein motor activity and increases minus-end directed runs of pigment granules.

Introduction.

Intracellular transport is a key mechanism responsible for spatial organization of the cytoplasm and the delivery of organelles and particles to their destinations inside the cell (Lane and Allan, 1998; Allan and Schroer, 1999; Guzik and Goldstein, 2004). The driving force for intracellular transport is provided by molecular motors bound to the surface of cargo organelles (Allan and Schroer, 1999; Vale, 2003). Molecular motors recognize the polarity of cytoskeletal transport tracks, MTs or actin filaments, and move specifically to their plus- or minus-ends (Allan and Schroer, 1999; Vale, 2003). MT-dependent motors include kinesins, which generally support transport to the MT plus-ends (Goldstein, 2001; Hirokawa et al., 2009), and dyneins, which are exclusively minus-end directed (King, 2000; Vallee et al., 2004). Kinesins and dyneins are usually bound to the surface of the same cargo organelles and therefore MT-based movement is bi-directional and involves stochastic transitions between three states, uninterrupted displacements to the minus- or plus-ends, respectively, and pauses (Gross, 2004; Welte, 2004). The direction of the net movement is controlled by intracellular signals, which change the lengths of MT runs in one or both directions (Gross, 2004; Welte, 2004). While the changes of run length reflect regulation of motor proteins, the signaling mechanisms responsible for modulation of dynein and kinesin activities remain largely unknown.

An excellent model system for studies of regulation of MT motors by signaling events is *Xenopus* melanophores (Nascimento et al., 2003; Aspengren et al., 2009). The major function of these cells is fast and synchronous redistribution of thousands of membrane-bounded pigment granules, which accumulate in the cell center (pigment aggregation) or uniformly distribute throughout the cytoplasm (pigment dispersion) (Nascimento et al., 2003; Aspengren et al., 2009). During aggregation and dispersion, pigment granules are moved by MT motors dynein and kinesin-2 along cytoplasmic MTs organized into a polarized radial array (minus-ends at the cell center, plus-ends at the periphery) (Tuma et al., 1998). Pigment aggregation involves a sudden dramatic increase in the length of dynein-dependent MT minus-end runs and a simultaneous decrease in the length of plus-end runs generated by kinesin-2 (Gross et al., 2002; Zaliapin et al., 2005). These changes cause rapid movement of pigment granules to the cell center. Pigment dispersion involves opposite changes in the lengths of MT plus- and minus-end MT runs, which result in the redistribution of pigment granules to the cell periphery (Gross et al., 2002; Zaliapin et al., 2005).

Pigment aggregation and dispersion are regulated by a signaling pathway that involves changes of the cytoplasmic levels of the second messenger cAMP and the activity of Protein Kinase A (PKA) (Nascimento et al., 2003; Kashina and Rodionov, 2005; Aspengren et al., 2009). An increase in cAMP levels stimulates PKA activity and causes pigment dispersion, whereas a decrease in cAMP levels inhibits PKA activity and induces aggregation (Nascimento et al., 2003; Kashina and Rodionov, 2005; Aspengren et al., 2009). Pigment aggregation also requires the activity of Protein Phosphatase 2A (PP2A) (Reilein et al., 1998; Kashina and Rodionov, 2005). PKA and PP2A control pigment aggregation and dispersion by modifying the lengths of MT plus- and minus-end runs of individual pigment granules (Rodionov et al., 2003; Kashina and Rodionov, 2005).

Therefore, PKA and PP2A directly or indirectly regulate kinesin-2 and dynein activities that generate pigment granule movement along MTs. However, the signaling mechanisms that link PKA and PP2A activities to the regulation of MT motors remain unknown.

In this study, we examined the signaling pathway responsible for the activation of MT minus-end directed MT movement of pigment granules during pigment aggregation. We hypothesized that along with the activity of PP2A, this pathway involved Casein Kinase 1 (CK1), which has been shown to work together with PP2A in several signaling cascades (Porter and Sale, 2000; Vielhaber and Virshup, 2001; Virshup et al., 2007; MacDonald et al., 2009). We found that melanophores expressed several CK1 isoforms, but only CK1 ϵ was bound to pigment granules. In full agreement with our hypothesis, we found that CK1 inhibitors prevented pigment aggregation in cells with dispersed pigment granules and induced pigment dispersion in melanophores with aggregated pigment granules. The activity of pigment granule-associated CK1 ϵ increased upon stimulation of pigment aggregation and this increase was partially blocked by inhibition of PP2A activity. Stimulation of pigment granule aggregation caused an increase in the phosphorylation of dynein intermediate chain (DIC), and inhibition of CK1 ϵ activity partially prevented this increase. Based on these results, we propose a model for a signal transduction pathway for pigment granule aggregation in which a drop in cAMP levels and down-regulation of PKA activity increases the activities of PP2A and CK1 ϵ , followed by CK1 ϵ -dependent phosphorylation of DIC that stimulates dynein motor activity and results in minus-end directed MT movement of pigment granules.

Materials and Methods.

Cell culture.

Xenopus melanophores (Kashina et al., 2004) were cultured in *Xenopus* tissue culture medium (70% L15 medium supplemented with antibiotics, 20% fetal bovine serum, and 5 µg/ml insulin). To induce pigment aggregation or dispersion, cells were placed in serum-free media 1 hr before hormone addition. Aggregation or dispersion was induced by treatment with 10^{-8} M melatonin or melanocyte-stimulating hormone (MSH), respectively. Cells with a reduced amount of melanin in their pigment granules were obtained as described by Rogers and Gelfand (Rogers and Gelfand, 1998).

To quantify aggregation or dispersion responses, melanophores were treated with melatonin or MSH for 20 min and fixed with formaldehyde. The number of cells with aggregated, partially aggregated, or dispersed pigment granules was determined by counting cells in each category by phase-contrast microscopy, as described previously (Kashina et al., 2004).

Treatment of melanophores with CK1 inhibitors.

Cells were incubated with the CK1 inhibitor IC261 (EMD Chemicals, Inc., Gibbstown, NJ) at concentrations 25-100 µM for 10 min. During the IC261 treatment, cytoplasmic microtubules were stabilized with Taxol (0.1 µM) to prevent IC261-induced MT depolymerization. The CK1 inhibitor D4476 (150 µM) was applied to cells for 2h in a mixture with Fugene 6 (Roche Applied Science, Indianapolis, IN) (Rena et al., 2004) to promote solubility.

Immunoblotting.

Immunoblotting was performed as described previously (Kashina et al., 2004). Primary antibodies were mouse monoclonal antibodies specific for CK1ε (BD Biosciences, San Jose, CA), CK1α (Cell Signaling Technology, Danvers, MA), the PP2A catalytic subunit (Millipore Corp., Billerica, MA), or DIC (74.1, Covance, Princeton, NJ) or a goat polyclonal affinity purified antibody raised against CK1δ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive bands were detected with SuperSignal West Femto maximum-sensitivity substrate (Thermo Scientific, Rockford, IL).

CK1ε DNA constructs and cell transfection.

The constitutively active CK1ε mutant MM2 (Cegielska et al., 1998) was a gift from Dr. David Virshup. To exclude the possibility of MM2 regulation by PKA, an additional mutation (S377A) was introduced into the potential inhibitory PKA phosphorylation site (Giamas et al., 2007) using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Wild type and constitutively active CK1ε were subcloned into the pEGFP-C1 vector (Clontech). Transfection of melanophores with GFP-tagged wild type or constitutively active CK1ε was performed using the Lipofectamine 2000 reagent

according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). The expression levels of the wild type and mutant CK1 ϵ , and GFP were compared by measuring the GFP fluorescence within cell outlines. Expression levels of endogenous CK1 ϵ and wild type GFP-CK1 ϵ were compared using quantitative immunoblotting. Protein bands corresponding to CK1 ϵ and GFP-CK1 ϵ , were revealed by immunostaining with a mouse monoclonal CK1 ϵ antibody (BD Biosciences, San Jose, CA) and IRDye800-conjugated affinity-purified anti-mouse immunoglobulin G (Rockland Immunochemicals, Gilbertsville, PA), and the intensity of the infrared signal was quantified with the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The results of quantification were normalized for the fraction of cells expressing GFP-CK1 ϵ , which was determined by counting the number of GFP-CK1 ϵ -expressing cells using a fluorescence microscope.

Fluorescence staining of lysosomes.

For fluorescence staining of lysosomes, melanophores were incubated with LysoTracker Yellow (0.5 μ M; Invitrogen Corp., Carlsbad, CA) for 30 min at room temperature. LysoTracker solution was replaced with fresh tissue culture medium and cells were observed using a fluorescence microscope.

Image acquisition and analysis.

Fluorescence images of cells were acquired using a Nikon Eclipse TE300 inverted microscope equipped with a Plan x100 1.25 NA objective lens using an Andor iXon EM-CCD camera (Andor Technology, Windsor, CT) driven by Metamorph image acquisition and analysis software (Molecular Dynamics, Downingtown, PA).

The movement of individual pigment granules was recorded and analyzed as described previously (Zaliapin et al., 2005). The data were collected 10 min after the stimulation of cells with melatonin or MSH.

Isolation of pigment granules and measurement of PP2A and CK1 activities in pigment granule preparations.

Pigment granules were purified as described previously (Kashina et al., 2004). Preparations of pigment granules isolated from cells with aggregated or dispersed granules were normalized by melanin content (Ozeki et al., 1996). PP2A activity was measured by estimating the release of inorganic phosphate from the PP2A substrate peptide using the Ser/Thr phosphatase assay system (Promega Corp., Madison, WI). CK1 activity was determined using the Casein Kinase I Activity Assay Kit (Sigma Chemical Co., St. Louis, MO) by measuring the phosphorylation with γ -³²P-ATP of the CK1 substrate peptide derived from the CK1 phosphorylation site at Ser10 of rabbit muscle glycogen synthase. The phosphorylated substrate was separated from the radioactive reagent by absorption on P81 cellulose phosphate paper squares. After extensive washing with 0.5% phosphoric acid, ethanol, and acetone, the radioactivity absorbed on the paper was counted using a scintillation counter.

Phosphorylation of dynein *in vivo* and *in vitro*.

To determine the levels of phosphorylation of dynein subunits in cells stimulated to aggregate or disperse pigment granules or treated with a CK1 inhibitor, melanophores were transfected with mouse DIC2 DNA subcloned into the pEGFP-C1 vector. After overnight culturing in phosphate-free L15 medium (United States Biological, Swampscott, MA), cells were incubated for 1h in the presence of ^{32}P i (22 $\mu\text{Ci}/\text{cm}^2$ of cell monolayer; PerkinElmer Inc., Shelton, CT), stimulated with melatonin or MSH for aggregation or dispersion of pigment granules, or treated with IC261 (100 μM) before melatonin stimulation to inhibit CK1 activity, and lysed with a Triton X-100-containing buffer as described previously (Kashina et al., 2004). Dynein was immunoprecipitated from cell lysates with an anti-GFP antibody (Abcam Inc., Cambridge, MA) immobilized on Protein A Agarose (Millipore Inc., Billerica, MA). Dynein immunoprecipitates were examined using SDS-PAGE and autoradiography. To quantify the levels of phosphorylation of GFP-DIC, autoradiograms were scanned and integrated grey scale densities of GFP-DIC bands were measured using Metamorph software.

For the measurement of the phosphorylation of DIC by CK1 *in vitro*, dynein was immunoprecipitated with the anti-GFP antibody from extracts of melanophores expressing GFP-DIC treated with MSH to disperse pigment granules. Immunoprecipitates were incubated with recombinant CK1 (100 u/ μl ; Sigma Chemical Co., St. Louis, MO) in the presence of $\gamma\text{-}^{32}\text{P}$ -ATP (PerkinElmer Inc., Shelton, CT), and examined using SDS-PAGE and autoradiography.

Results.

Several CK1 isoforms are expressed in melanophores but only CK1 ϵ is associated with pigment granules.

To examine the possibility that CK1 is involved in pigment granule transport in melanophores, we asked whether melanophores expressed CK1. We used immunoblotting to probe whole cell extracts and preparations of purified pigment granules with antibodies raised against major CK1 isoforms. We found that antibodies specific for CK1 α , CK1 δ , and CK1 ϵ isoforms recognized relevant protein bands in whole cell extracts (Fig. 1A). Remarkably, only the CK1 ϵ -specific antibody, but not antibodies to the other two isoforms, reacted with preparations of purified pigment granules (Fig. 1A). This result indicated that several CK1 isoforms were expressed in melanophores but only the CK1 ϵ was bound to purified pigment granules. This is the expected localization for a signaling enzyme that regulates activities of granule-bound MT motors (Kashina et al., 2004; Kashina and Rodionov, 2005).

To confirm the binding of CK1 ϵ to pigment granules, we tested whether CK1 ϵ was associated with pigment granules in cells using a CK1 ϵ -GFP fusion. We found that CK1 ϵ -GFP was localized in fluorescent dots that accumulated in the cell center or distributed homogeneously throughout the cytoplasm in response to treatment with the hormones that induced pigment aggregation (melatonin) or dispersion (Melanocyte-Stimulating Hormone; MSH), and therefore behaved similarly to pigment granules (Fig. 1B). Control experiments showed that melatonin and MSH did not induce significant changes in the distribution of other membrane organelles, such as lysosomes (Fig. 1B). We conclude that the CK1 isoform CK1 ϵ is bound to pigment granules in melanophores and therefore is a good candidate for regulating MT-based pigment granule transport.

CK1 activity is required for the induction and maintenance of pigment granule aggregation.

To directly test whether CK1 activity is involved in the regulation of pigment granule transport, we examined melatonin-induced aggregation and MSH-induced dispersion of pigment granules in melanophores treated with two chemically distinct CK1 inhibitors. D4476 is a broad-specificity CK1 inhibitor (Rena et al., 2004), and IC261 is a selective inhibitor of CK1 ϵ and CK1 δ (Mashhoon et al., 2000). Since we found that IC261 partially depolymerized cytoplasmic MTs in melanophores, which could interfere with MT-based transport, we pre-treated cells with the MT-stabilizing drug Paclitaxel (Taxol) at 0.1 μ M. Control experiments showed that 0.1 μ M Taxol prevented MT depolymerization induced by IC261, but did not significantly affect the density or distribution of cytoplasmic MTs (data not shown).

We found that D4476 and IC261 both markedly inhibited melatonin-induced pigment aggregation as evidenced from the increase in the number of cells that completely or partially failed to aggregate pigment granules (Fig. 2A and B). In agreement with our previously published data (Lomakin et al., 2009), treatment of cells with Taxol alone

partially inhibited pigment granule aggregation (Fig. 2B). IC261 further enhanced this inhibition in a dose-dependent manner (Fig. 2B). At the highest tested concentration of IC261 (100 μ M) more than 80% of melanophores completely failed to aggregate pigment granules in response to melatonin treatment. In contrast to aggregation, neither D4476 nor IC261 significantly affected MSH-induced dispersion of pigment granules (Fig. 2C, and data not shown). Remarkably, IC261 treatment of the melatonin-stimulated melanophores with aggregated pigment granules induced rapid granule dispersion (Fig. 2D). We conclude that CK1 activity is essential for both the induction and maintenance of pigment granule aggregation.

Pigment aggregation stimuli increase the activity of pigment granule-bound CK1 ϵ .

The results of our experiments showed that CK1 ϵ was bound to pigment granules, and that CK1 activity was required for granule aggregation. To determine whether the pigment granule-bound CK1 ϵ was constitutively active or was stimulated by granule aggregation signals, we measured the enzymatic activity of CK1 in the preparations of pigment granules isolated from melanophores treated with melatonin or MSH, to aggregate or disperse pigment granules, respectively. We found that CK1 activity was \sim 2-fold higher in preparations of pigment granules isolated from melatonin-treated cells, as compared to MSH-treated cells (Fig. 3, A). We conclude that CK1 ϵ bound to pigment granules is activated during pigment aggregation.

Activation of granule-bound CK1 ϵ requires PP2A.

Our data identified CK1 ϵ as the pigment granule-bound CK1 isoform and showed that its activity increased during granule aggregation. Therefore, our results strongly suggested that pigment granule aggregation signals stimulated the activity of CK1 ϵ .

CK1 ϵ activation is known to involve the dephosphorylation of amino acid residues in the C-terminal autophosphorylation inhibitory domain of the CK1 ϵ molecule (Gietzen and Virshup, 1999; Knippschild et al., 2005; Virshup et al., 2007). CK1 ϵ dephosphorylation can be catalyzed by PP2A, which has been shown to activate CK1 ϵ *in vitro* (Gietzen and Virshup, 1999). Furthermore, it has been demonstrated that PP2A activity is essential for aggregation of pigment granules in melanophores (Reilein et al., 1998). We therefore hypothesized that activation of pigment granule-associated CK1 ϵ required PP2A activity.

To test this hypothesis, we first examined whether PP2A co-localized with CK1 ϵ on pigment granules, as would be expected for signaling enzymes involved in the same signal transduction pathway. We also investigated whether activity of granule-bound PP2A was elevated in cells stimulated to aggregate pigment granules. We performed Western blotting of granule preparations using an antibody against the catalytic subunit of PP2A, and measured PP2A activity in preparations of purified pigment granules isolated from cells stimulated to aggregate or disperse pigment. We found PP2A present in the pigment granule preparations (Fig. 1A, bottom). We also found that PP2A activity was \sim 4 times higher in pigment granule preparations from cells with aggregated versus dispersed granules (Fig. 3B). Therefore, highly active PP2A co-purified with pigment granules.

We next tested whether CK1 ϵ functions downstream of PP2A in pigment granule aggregation. We examined whether suppression of granule aggregation in cells with inhibited PP2A activity could be rescued by overexpression of a constitutively active CK1 ϵ mutant (Swiatek et al., 2004). Such a CK1 ϵ mutant was generated by replacing Ser and Thr amino acid residues with Ala in the inhibitory autophosphorylation sites (Swiatek et al., 2004). We compared the inhibitory effect of the highly specific PP2A inhibitor okadaic acid on pigment granule aggregation in cells expressing this mutant versus cells expressing wild type CK1 ϵ at similar levels (Fig. 4B), which were significantly lower than the level of expression of endogenous CK1 ϵ (the ratio of expression levels of wild-type to endogenous CK1 ϵ was 0.14 ± 0.03 ; mean \pm SEM). To identify transfected cells, wild type and mutant CK1 ϵ were expressed as GFP fusions. We found that okadaic acid dramatically inhibited pigment granule aggregation in control non-transfected cells or cells expressing GFP, as well as in wild type CK1 ϵ -expressing cells (Fig. 4A). This result was consistent with published data that showed that PP2A activity was required for pigment granule aggregation (Reilein et al., 1998). Remarkably, in cells expressing the constitutively active CK1 ϵ mutant the fraction of melanophores with aggregated pigment granules was markedly increased. This effect was statistically significant (the p-value for non-transfected and the constitutively active CK1 ϵ -expressing cells was 0.00061 compared to 0.503 and 0.497 for cells expressing GFP or wild type CK1 ϵ). Thus, expression of the constitutively active CK1 ϵ mutant partially rescued the inhibition of pigment aggregation induced by okadaic acid. This indicates that CK1 ϵ acts downstream of PP2A in the granule aggregation signaling pathway.

Finally, we directly examined whether pigment granule-bound CK1 ϵ was regulated by PP2A, by comparing CK1 activities in preparations of pigment granules isolated from control and okadaic acid-treated cells. We found that the activity of CK1 was significantly reduced in the granule preparations isolated from cells treated with okadaic acid (Fig. 3B, Mel+OA, and Mel). Therefore, suppression of PP2A activity markedly inhibited the activity of CK1 ϵ bound to pigment granules. Taken together, our data indicate that pigment granule aggregation signals increase PP2A activity and that PP2A stimulates the activity of CK1 ϵ during granule aggregation.

CK1 regulates the length of MT minus-end runs of pigment granules.

Our results indicated that CK1 inhibitors dramatically suppressed pigment granule aggregation. This aggregation involves granule runs towards the MT minus-ends focused at the cell center (Rodionov et al., 2003; Kashina and Rodionov, 2005). Thus, CK1 inhibitors likely suppressed pigment aggregation by reducing either the length or the velocity of minus-end runs of pigment granules. To test how the inhibition of CK1 activity affected MT minus-end runs, we tracked individual granules in cells treated with the CK1 ϵ inhibitor IC261 at 25 μ M. At this concentration, IC261 reduced the rate of pigment granule aggregation but did not block it completely (Fig. 2B). This allowed us to measure the velocity and length of bi-directional granule movements. Comparison of granule movement parameters between the IC261-treated and control untreated cells showed that partial inhibition of CK1 activity reduced the average length of minus-end

runs of pigment granules to 69% of the control length, without changing the average velocity of movement (Table 1). The length of plus-end runs was reduced to some extent as well (Table 1), which is consistent with the observation that transport in either direction requires the activity of opposite-polar motor proteins (Ally et al., 2009). Thus, the inhibition of CK1 activity suppressed pigment aggregation by shortening the length of minus-end granule runs. We conclude that CK1 activity is required to increase the length of minus-end runs of pigment granules during their aggregation.

CK1 phosphorylates DIC *in vivo* and *in vitro*.

It is known that MT minus-end runs of pigment granules are generated by cytoplasmic dynein, and that dynein activity increases during granule aggregation (Nilsson and Wallin, 1997; Gross et al., 2002; Rodionov et al., 2003; Zaliapin et al., 2005). Our data showed that the increase in the length of minus-end runs required the activity of CK1. Therefore, we assumed that CK1 was involved in the stimulation of dynein activity. While dynein regulation remains a mystery, the existing experimental data suggest that the regulation mechanism involves reversible phosphorylation of dynein subunits (Reilein et al., 2001). We therefore performed experiments to determine whether the phosphorylation levels of dynein subunits changed after the stimulation of pigment aggregation, and whether inhibition of CK1 activity prevented these changes.

To detect changes in the levels of phosphorylation of dynein subunits, we compared the patterns of protein phosphorylation in dynein immunoprecipitates prepared from cells stimulated to aggregate or disperse pigment granules. We incubated melanophores overexpressing GFP-DIC or control non-transfected melanophores with ^{32}P i, stimulated cells with melatonin or MSH to induce granule aggregation or dispersion, and immunoprecipitated dynein from total extracts of stimulated cells using an anti-GFP antibody. Autoradiography indicated that ^{32}P incorporated predominantly into the GFP-DIC (Fig. 5A, middle) identified on the basis of electrophoretic mobility and immunoblotting with the anti-DIC antibody 74.1 (data not shown). This incorporation was specific because it was not observed in immunoprecipitates prepared from control non-transfected cells (Supplemental Figure 1). Incorporation of ^{32}P into the endogenous DIC could not be detected because the immunoblotting data indicated that its amounts in the GFP immunoprecipitates were small. Remarkably, ^{32}P incorporation into GFP-DIC was significantly higher in the melatonin- than MSH-stimulated cells (Fig. 5A). Furthermore, the levels of ^{32}P incorporation into GFP-DIC were also reduced in dynein immunoprecipitates obtained from melatonin-stimulated cells pre-treated with the CK1 ϵ inhibitor IC261 (Fig. 5A). Quantification of the ^{32}P signal showed that the levels of ^{32}P incorporation into GFP-DIC in cells treated with MSH and IC261 were $61.0 \pm 13.0\%$ and $80.8 \pm 8.5\%$ (mean \pm SEM; $n=3$) of the levels in melatonin-treated cells. Pre-treatment of cells with the broad-specificity CK1 inhibitor D4476 also reduced ^{32}P incorporation into GFP-DIC (Supplemental Figure 2). Thus, our results indicate that granule aggregation signals increase the phosphorylation of DIC and that this increase is diminished upon inhibition of CK1 ϵ activity, suggesting that CK1 ϵ directly or indirectly regulates DIC phosphorylation.

To examine whether CK1 could directly phosphorylate DIC, we immunoprecipitated dynein from extracts of GFP-DIC-expressing melanophores stimulated to disperse pigment granules, and incubated the immunoprecipitates with ^{32}P -ATP in the presence or absence of recombinant CK1. We found that ^{32}P incorporated predominantly into the GFP-DIC and that this incorporation did not occur in the absence of CK1 (Fig. 5B). Thus, DIC was the major substrate of CK1 in dynein immunoprecipitates. We conclude that DIC might be directly phosphorylated by CK1 during pigment granule aggregation.

Discussion.

In this study, we identified CK1 as a key regulator of MT minus-end directed MT pigment granule transport in *Xenopus* melanophores. This conclusion is based on several lines of experimental evidence. First, the CK1 isoform CK1 ϵ was bound to pigment granules and therefore was properly localized to regulate dynein. Second, the activity of pigment granule-bound CK1 was significantly increased in cells stimulated to aggregate granules, as would be expected for a signaling enzyme regulating minus-end directed granule transport. Finally, the aggregation of pigment granules was markedly suppressed by treatment of melanophores with CK inhibitors, which confirmed that CK1 activity was essential for minus-end directed MT transport. Taken together, these results show that CK1 regulates minus-end directed MT transport of pigment granules in melanophores.

Our data are consistent with the results of studies in yeast and mammalian cells, which link CK1 to transport of membrane organelles. In yeast cells, knockout of CK1 blocks the movement of membrane vesicles containing uracil permease from the plasma membrane to the vacuole (Marchal et al., 2002). In mammalian cells, CK1 isoforms interact with endomembrane compartments such as the ER, Golgi apparatus, and transport vesicles (Gross and Anderson, 1998; Behrend et al., 2000; Faundez and Kelly, 2000; Milne et al., 2001; Yu and Roth, 2002), and are involved in synaptic vesicle exocytosis (Kreutz et al., 1997; Dubois et al., 2001). Therefore experimental evidence indicates that CK1 participates in transport of membrane organelles. However, the mechanism by which CK1 participates in membrane organelle transport was not elucidated in these studies. Our work for the first time reveals a specific function of CK1 in regulating dynein-based transport of membrane organelles.

Further, we found that PP2A was bound to pigment granules along with CK1 ϵ , and that this phosphatase functioned upstream of CK1 in the pigment aggregation signaling pathway. We also found that the increase of CK1 activity observed during granule aggregation was suppressed by okadaic acid, a highly specific PP2A inhibitor. Our data are consistent with the results of *in vivo* studies that suggest that the PP2A-induced stimulation of CK1 activity is involved in other major signaling pathways, including canonical Wnt signaling (Swiatek et al., 2004), chromosome cohesion (Ishiguro et al. 2010) and flagellar motility (Wirschell *et al.*, 2007). Our data are also consistent with *in vitro* experiments that show that PP2A activates CK1 isoforms, such as the granule-bound CK1 ϵ , by the direct dephosphorylation of inhibitory autophosphorylation sites (DeMaggio et al., 1992; Fish et al., 1995; Graves and Roach, 1995; Zhai et al., 1995; Swiatek et al., 2004). Therefore in melanophores activation of CK1 during aggregation of pigment granules likely involves a dephosphorylation step catalyzed by PP2A.

Signal transduction during granule aggregation that induces increase of CK1 activity implicates the activation PP2A. This conclusion is based on our data that show that signaling during granule aggregation enhances activity of PP2A bound to pigment granules. The major mechanism of PP2A regulation implicates phosphorylation of amino acid residues in the conserved C-terminal part of the PP2A catalytic subunit, which inhibits its enzymatic activity (Goldberg, 1999; Lechward et al., 2001; Sontag, 2001).

Reactivation occurs through the unique ability of PP2A to dephosphorylate itself. (Goldberg, 1999; Lechward et al., 2001; Sontag, 2001). The autodephosphorylation reaction occurs at a steady rate and therefore intracellular signals regulate PP2A activity indirectly through control over activities of protein kinases responsible for PP2A inactivation (Goldberg, 1999; Lechward et al., 2001; Sontag, 2001). Thus, in melanophores aggregation signals likely activate PP2A by suppressing activities of inhibitory protein kinase(s). An attractive possibility is that PP2A is regulated by a direct phosphorylation by PKA and that PP2A activation results from the reduction of PKA activity caused by a drop in cAMP level during granule aggregation.

Our data show that the activation of minus-end movement of pigment granules driven by cytoplasmic dynein correlates with the increase in phosphorylation of the granule-bound DIC, and that CK1 inhibition prevents this phosphorylation. Our data also demonstrate that recombinant CK1 phosphorylates DIC *in vitro*. It is therefore possible that CK1 stimulates dynein motor activity via the direct phosphorylation of DIC. In support of this possibility, studies in *Chlamydomonas* indicate that CK1 regulates flagellar motility through the phosphorylation of a 138 kDa intermediate chain of axonemal dynein (Yang and Sale, 2000; Gokhale et al., 2009). However, partial suppression of DIC phosphorylation in the presence of high concentrations of a selective CK1 inhibitor observed in our experiments indicates that dynein regulation is likely complex and might involve other protein kinases that cooperate with CK1 in phosphorylating DIC, leading to dynein activation. Nonetheless our data show that CK1 is essential for dynein activation and suggest that the role of CK1 in this process is in phosphorylation of DIC.

Based on our results, we propose a model for the activation of minus-end directed transport of pigment granules along MTs in melanophores (Fig. 6). We suggest that stimulation involves sequential changes in the activities of PKA, PP2A and CK1 ϵ bound to pigment granules. First, a drop in cytoplasmic cAMP levels caused by melatonin treatment reduces the activity of PKA, which allows PP2A to dephosphorylate and activate itself. Second, PP2A dephosphorylates amino acid residues in the inhibitory autophosphorylation sites of CK1 ϵ , which increases CK1 ϵ kinase activity. Finally, CK1 ϵ induces phosphorylation of DIC, which is required to stimulate dynein activity. This leads to an increase in the length of minus-end runs, which results in efficient pigment granule aggregation. Testing predictions of our model is an exciting new direction for the future research.

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References.

- Allan, V.J., and Schroer, T.A. (1999). Membrane motors. *Curr Opin Cell Biol* 11, 476-482.
- Ally, S., Larson, A.G., Barlan, K., Rice, S.E., and Gelfand, V.I. (2009). Opposite-polarity motors activate one another to trigger cargo transport in live cells. *J Cell Biol* 187, 1071-1082.
- Aspengren, S., Hedberg, D., Skold, H.N., and Wallin, M. (2009). New insights into melanosome transport in vertebrate pigment cells. *Int Rev Cell Mol Biol* 272, 245-302.
- Behrend, L., Stoter, M., Kurth, M., Rutter, G., Heukeshoven, J., Deppert, W., and Knippschild, U. (2000). Interaction of casein kinase 1 delta (CK1delta) with post-Golgi structures, microtubules and the spindle apparatus. *Eur J Cell Biol* 79, 240-251.
- Cegielska, A., Gietzen, K.F., Rivers, A., and Virshup, D.M. (1998). Autoinhibition of casein kinase I epsilon (CKI epsilon) is relieved by protein phosphatases and limited proteolysis. *J Biol Chem* 273, 1357-1364.
- DeMaggio, A.J., Lindberg, R.A., Hunter, T., and Hoekstra, M.F. (1992). The budding yeast HRR25 gene product is a casein kinase I isoform. *Proc Natl Acad Sci U S A* 89, 7008-7012.
- Dubois, T., Kerai, P., Zemlickova, E., Howell, S., Jackson, T.R., Venkateswarlu, K., Cullen, P.J., Theibert, A.B., Larose, L., Roach, P.J., and Aitken, A. (2001). Casein kinase I associates with members of the centaurin-alpha family of phosphatidylinositol 3,4,5-trisphosphate-binding proteins. *J Biol Chem* 276, 18757-18764.
- Faundez, V.V., and Kelly, R.B. (2000). The AP-3 complex required for endosomal synaptic vesicle biogenesis is associated with a casein kinase Ialpha-like isoform. *Mol Biol Cell* 11, 2591-2604.
- Fish, K.J., Cegielska, A., Getman, M.E., Landes, G.M., and Virshup, D.M. (1995). Isolation and characterization of human casein kinase I epsilon (CKI), a novel member of the CKI gene family. *J Biol Chem* 270, 14875-14883.
- Giamas, G., Hirner, H., Shoshiashvili, L., Grothey, A., Gessert, S., Kuhl, M., Henne-Bruns, D., Vorgias, C.E., and Knippschild, U. (2007). Phosphorylation of CK1delta: identification of Ser370 as the major phosphorylation site targeted by PKA in vitro and in vivo. *Biochem J* 406, 389-398.
- Gietzen, K.F., and Virshup, D.M. (1999). Identification of inhibitory autophosphorylation sites in casein kinase I epsilon. *J Biol Chem* 274, 32063-32070.
- Gokhale, A., Wirschell, M., and Sale, W.S. (2009). Regulation of dynein-driven

- microtubule sliding by the axonemal protein kinase CK1 in *Chlamydomonas* flagella. *J Cell Biol* *186*, 817-824.
- Goldberg, Y. (1999). Protein phosphatase 2A: who shall regulate the regulator? *Biochem Pharmacol* *57*, 321-328.
- Goldstein, L.S. (2001). Kinesin molecular motors: transport pathways, receptors, and human disease. *Proc Natl Acad Sci U S A* *98*, 6999-7003.
- Graves, P.R., and Roach, P.J. (1995). Role of COOH-terminal phosphorylation in the regulation of casein kinase I delta. *J Biol Chem* *270*, 21689-21694.
- Gross, S.D., and Anderson, R.A. (1998). Casein kinase I: spatial organization and positioning of a multifunctional protein kinase family. *Cell Signal* *10*, 699-711.
- Gross, S.P. (2004). Hither and yon: a review of bi-directional microtubule-based transport. *Phys Biol* *1*, R1-11.
- Gross, S.P., Tuma, M.C., Deacon, S.W., Serpinskaya, A.S., Reilein, A.R., and Gelfand, V.I. (2002). Interactions and regulation of molecular motors in *Xenopus melanophores*. *J Cell Biol* *156*, 855-865.
- Guzik, B.W., and Goldstein, L.S. (2004). Microtubule-dependent transport in neurons: steps towards an understanding of regulation, function and dysfunction. *Curr Opin Cell Biol* *16*, 443-450.
- Hirokawa, N., Noda, Y., Tanaka, Y., and Niwa, S. (2009). Kinesin superfamily motor proteins and intracellular transport. *Nat Rev Mol Cell Biol* *10*, 682-696.
- Ishiguro, T., Tanaka, K., Sakuno, T., and Watanabe, Y. (2010). Shugoshin-PP2A counteracts casein-kinase-1-dependent cleavage of Rec8 by separase. *Nat Cell Biol* *12*, 500-506.
- Kashina, A., and Rodionov, V. (2005). Intracellular organelle transport: few motors, many signals. *Trends Cell Biol* *15*, 396-398.
- Kashina, A.S., Semenova, I.V., Ivanov, P.A., Potekhina, E.S., Zaliapin, I., and Rodionov, V.I. (2004). Protein kinase A, which regulates intracellular transport, forms complexes with molecular motors on organelles. *Curr Biol* *14*, 1877-1881.
- King, S.M. (2000). The dynein microtubule motor. *Biochim Biophys Acta* *1496*, 60-75.
- Knippschild, U., Gocht, A., Wolff, S., Huber, N., Lohler, J., and Stoter, M. (2005). The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. *Cell Signal* *17*, 675-689.

Kreutz, M.R., Bockers, T.M., Sabel, B.A., Hulser, E., Stricker, R., and Reiser, G. (1997). Expression and subcellular localization of p42IP4/centaurin-alpha, a brain-specific, high-affinity receptor for inositol 1,3,4,5-tetrakisphosphate and phosphatidylinositol 3,4,5-trisphosphate in rat brain. *Eur J Neurosci* *9*, 2110-2124.

Lane, J., and Allan, V. (1998). Microtubule-based membrane movement. *Biochim Biophys Acta* *1376*, 27-55.

Lechward, K., Awotunde, O.S., Swiatek, W., and Muszynska, G. (2001). Protein phosphatase 2A: variety of forms and diversity of functions. *Acta Biochim Pol* *48*, 921-933.

Lomakin, A.J., Semenova, I., Zaliapin, I., Kraikivski, P., Nadezhdina, E., Slepchenko, B.M., Akhmanova, A., and Rodionov, V. (2009). CLIP-170-dependent capture of membrane organelles by microtubules initiates minus-end directed transport. *Dev Cell* *17*, 323-333.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* *17*, 9-26.

Marchal, C., Dupre, S., and Urban-Grimal, D. (2002). Casein kinase I controls a late step in the endocytic trafficking of yeast uracil permease. *J Cell Sci* *115*, 217-226.

Mashhoon, N., DeMaggio, A.J., Tereshko, V., Bergmeier, S.C., Egli, M., Hoekstra, M.F., and Kuret, J. (2000). Crystal structure of a conformation-selective casein kinase-1 inhibitor. *J Biol Chem* *275*, 20052-20060.

Milne, D.M., Looby, P., and Meek, D.W. (2001). Catalytic activity of protein kinase CK1 delta (casein kinase 1delta) is essential for its normal subcellular localization. *Exp Cell Res* *263*, 43-54.

Nascimento, A.A., Roland, J.T., and Gelfand, V.I. (2003). Pigment cells: a model for the study of organelle transport. *Annu Rev Cell Dev Biol* *19*, 469-491.

Nilsson, H., and Wallin, M. (1997). Evidence for several roles of dynein in pigment transport in melanophores. *Cell Motil Cytoskeleton* *38*, 397-409.

Ozeki, H., Ito, S., Wakamatsu, K., and Thody, A.J. (1996). Spectrophotometric characterization of eumelanin and pheomelanin in hair. *Pigment Cell Res* *9*, 265-270.

Porter, M.E., and Sale, W.S. (2000). The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility. *J Cell Biol* *151*, F37-42.

Reilein, A.R., Rogers, S.L., Tuma, M.C., and Gelfand, V.I. (2001). Regulation of molecular motor proteins. *Int Rev Cytol* *204*, 179-238.

- Reilein, A.R., Tint, I.S., Peunova, N.I., Enikolopov, G.N., and Gelfand, V.I. (1998). Regulation of organelle movement in melanophores by protein kinase A (PKA), protein kinase C (PKC), and protein phosphatase 2A (PP2A). *J Cell Biol* 142, 803-813.
- Rena, G., Bain, J., Elliott, M., and Cohen, P. (2004). D4476, a cell-permeant inhibitor of CK1, suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO Rep* 5, 60-65.
- Rodionov, V., Yi, J., Kashina, A., Oladipo, A., and Gross, S.P. (2003). Switching between microtubule- and actin-based transport systems in melanophores is controlled by cAMP levels. *Curr Biol* 13, 1837-1847.
- Rogers, S.L., and Gelfand, V.I. (1998). Myosin cooperates with microtubule motors during organelle transport in melanophores. *Curr Biol* 8, 161-164.
- Sontag, E. (2001). Protein phosphatase 2A: the Trojan Horse of cellular signaling. *Cell Signal* 13, 7-16.
- Swiatek, W., Tsai, I.C., Klimowski, L., Pepler, A., Barnette, J., Yost, H.J., and Virshup, D.M. (2004). Regulation of casein kinase I epsilon activity by Wnt signaling. *J Biol Chem* 279, 13011-13017.
- Tuma, M.C., Zill, A., Le Bot, N., Vernos, I., and Gelfand, V. (1998). Heterotrimeric kinesin II is the microtubule motor protein responsible for pigment dispersion in *Xenopus* melanophores. *J Cell Biol* 143, 1547-1558.
- Vale, R.D. (2003). The molecular motor toolbox for intracellular transport. *Cell* 112, 467-480.
- Vallee, R.B., Williams, J.C., Varma, D., and Barnhart, L.E. (2004). Dynein: An ancient motor protein involved in multiple modes of transport. *J Neurobiol* 58, 189-200.
- Vielhaber, E., and Virshup, D.M. (2001). Casein kinase I: from obscurity to center stage. *IUBMB Life* 51, 73-78.
- Virshup, D.M., Eide, E.J., Forger, D.B., Gallego, M., and Harnish, E.V. (2007). Reversible protein phosphorylation regulates circadian rhythms. *Cold Spring Harb Symp Quant Biol* 72, 413-420.
- Welte, M.A. (2004). Bidirectional transport along microtubules. *Curr Biol* 14, R525-537.
- Wirschell, M., Hendrickson, T., and Sale, W.S. (2007). Keeping an eye on I1: I1 dynein as a model for flagellar dynein assembly and regulation. *Cell Motil Cytoskeleton* 64, 569-579.

Yang, P., and Sale, W.S. (2000). Casein kinase I is anchored on axonemal doublet microtubules and regulates flagellar dynein phosphorylation and activity. *J Biol Chem* 275, 18905-18912.

Yu, S., and Roth, M.G. (2002). Casein kinase I regulates membrane binding by ARF GAP1. *Mol Biol Cell* 13, 2559-2570.

Zaliapin, I., Semenova, I., Kashina, A., and Rodionov, V. (2005). Multiscale trend analysis of microtubule transport in melanophores. *Biophys J* 88, 4008-4016.

Zhai, L., Graves, P.R., Robinson, L.C., Italiano, M., Culbertson, M.R., Rowles, J., Cobb, M.H., DePaoli-Roach, A.A., and Roach, P.J. (1995). Casein kinase I gamma subfamily. Molecular cloning, expression, and characterization of three mammalian isoforms and complementation of defects in the *Saccharomyces cerevisiae* YCK genes. *J Biol Chem* 270, 12717-12724.

Figure Legends.

Figure 1. CK1 ϵ and PP2A are bound to pigment granules. (A) Immunoblots of cell extracts (left lanes) or preparations of pigment granules (right lanes) probed for CK1 isoforms (top) or the catalytic subunit of PP2A (bottom). PP2A and CK1 ϵ , but not other CK1 isoforms are enriched in preparations of purified pigment granules. (B) Fluorescence images of melanophores expressing GFP-CK1 ϵ (top row) or stained with LysoTracker (bottom row) sequentially treated with melatonin (left column) and MSH (right column) to induce aggregation and dispersion of pigment granules. GFP-CK1 ϵ is localized to fluorescent dots which accumulate in the cell center or redisperse throughout the cytoplasm after treatment with melatonin and MSH, respectively, as would be expected from pigment granules, whereas the distribution of lysosomes does not change significantly in response to hormones. The scale bar is 20 μ m.

Figure 2. CK1 activity is required for pigment granule aggregation and maintenance of the aggregated state. Quantification of responses of pigment granules to pigment aggregating or dispersing hormones melatonin (A, B) or MSH (C) in cells pre-treated with various concentrations of a broad-specificity CK1 inhibitor D4476, or the CK1 δ and CK1 ϵ specific inhibitor IC261; (D) responses of pigment granules to IC261 in cells with pigment aggregated with melatonin. The data are expressed as the percentages of cells with aggregated (white bars), partially aggregated (gray bars), or dispersed (black bars) granules. In panel B, large fraction of cells with dispersed pigment granules among IC261-untreated melanophores (0 μ M) is explained by reduced rate of pigment aggregation in the presence of Taxol, which was used in this experiment to prevent MT depolymerization induced by IC261. D4476 and IC261 treatments inhibit melatonin-induced aggregation, but not MSH-induced dispersion of pigment granules; IC261 causes pigment dispersion in melatonin-treated cells with aggregated granules.

Figure 3. Enzymatic activities of the pigment granule-bound CK1 and PP2A are increased upon pigment aggregation. Activities of CK1 (A) or PP2A (B) measured in preparations of pigment granules isolated from melanophores stimulated with melatonin (Mel) or MSH (MSH), to aggregate or disperse pigment granules, respectively. The bar labeled Mel+OA (panel A) shows the data for melatonin-stimulated cells pre-treated with the PP2A inhibitor okadaic acid. Average values determined for melatonin-treated cells are set at 100%. PP2A and CK1 activities are significantly higher in preparations of pigment granules obtained from melatonin- than MSH-stimulated cells, which shows that the activities of both enzymes increase during granule aggregation; okadaic acid treatment reduces the activity of CK1, indicating that CK1 activity is stimulated by PP2A.

Figure 4. Overexpression of a constitutively active CK1 ϵ mutant partially rescues the inhibition of pigment granule aggregation by the PP2A inhibitor okadaic acid. (A) Quantification of response to melatonin of non-transfected melanophores, or melanophores expressing GFP, wild typeGFP-CK1 ϵ or the mutant GFP-CK1 ϵ with constitutively high enzymatic activity; prior to stimulation with melatonin cells were treated with PP2A inhibitor okadaic acid (OA). The data are expressed as the percentage

of cells with aggregated (white bars), partially aggregated (gray bars), or dispersed (black bars) pigment. OA treatment significantly inhibits aggregation of pigment granules in non-transfected melanophores and in cells expressing GFP or wild type GFP-CK1 ϵ . Expression of the constitutively active CK1 ϵ mutant partially rescues OA-induced inhibition of pigment granule aggregation as evidenced from the increased fraction of cells with aggregated granules. (B) Comparison of the levels of expression of GFP and CK1 ϵ constructs; wild type and the constitutively active mutant CK1 ϵ are expressed at approximately the same levels, whereas GFP is expressed at significantly higher levels compared to CK1 ϵ constructs.

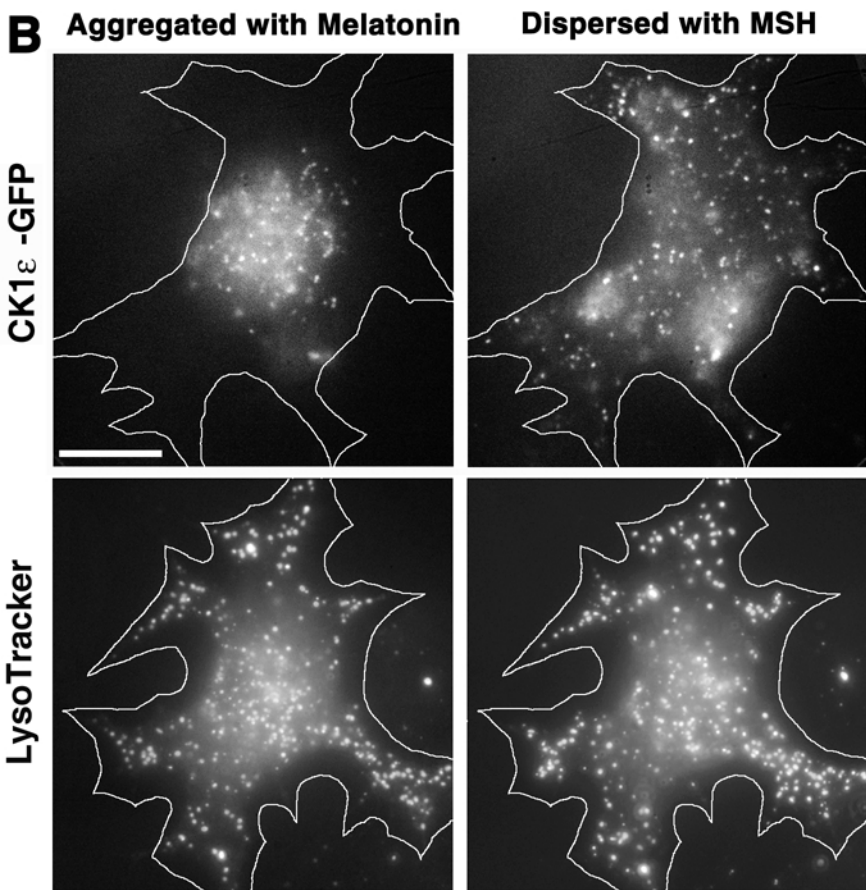
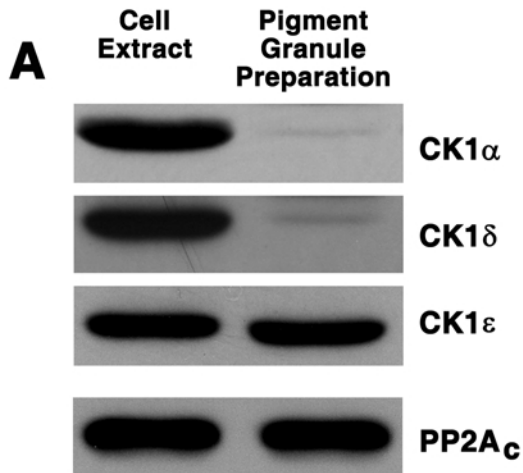
Figure 5. CK1 phosphorylates DIC *in vivo* and *in vitro*. (A) Immunoprecipitation of dynein with GFP antibody from extracts of GFP-DIC-expressing melanophores metabolically labeled with ^{32}P ; left panel, Coomassie-stained gel; right panel, autoradiograph; MSH, Mel, and Mel+IC261 on top of each panel indicate extracts of cells treated with MSH (to induce pigment dispersion), melatonin (to induce aggregation) or IC261 and melatonin (to inhibit CK1 activity prior to induction of aggregation), respectively; M, lanes with molecular weight markers. In immunoprecipitates, ^{32}P incorporates predominantly into GFP-DIC, this incorporation is increased in cells stimulated to aggregate pigment granules, and this increase is diminished by inhibition of CK1 activity. (B) *In vitro* phosphorylation by CK1 of dynein immunoprecipitated with an anti-GFP antibody from the extracts of GFP-DIC expressing cells; left, Coomassie-stained gel of immunoprecipitate; right, autoradiograph of immunoprecipitate incubated with γ - ^{32}P -ATP in the presence (left) or absence (right) of recombinant CK1; DHC, GFP-DIC, Ab, and CK1 indicate positions of dynein heavy chain, GFP-DIC fusion protein, antibody heavy and light chains, and recombinant CK1, respectively. CK1 predominantly phosphorylates the protein with electrophoretic mobility similar to GFP-DIC.

Figure 6. Model for the activation of MT minus-end directed transport of pigment granules during pigment aggregation in melanophores. Melatonin decreases intracellular levels of cAMP, which in turn reduces the activity of PKA. PKA inactivation relieves inhibition of PP2A, which then dephosphorylates and activates CK1 ϵ . CK1 ϵ phosphorylates DIC, and this phosphorylation stimulates dynein activity and increases the length of MT minus-end directed runs of pigment granules, leading to their aggregation in the cell center.

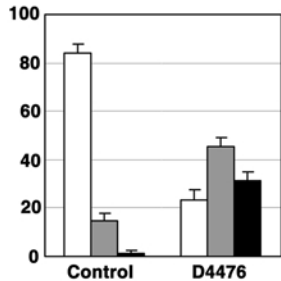
Supplemental Figure 1. Specificity of ^{32}P incorporation into GFP-DIC. Autoradiograph of the GFP antibody immunoprecipitates prepared using extracts of melanophores metabolically labeled with ^{32}P ; left lane, extract of cells expressing GFP-DIC; right lane, extract of control non-transfected melanophores. ^{32}P incorporates into the GFP-DIC band immunoprecipitated from extracts of GFP-DIC-expressing but not control non-transfected cells.

Supplemental Figure 2. Broad specificity CK1 inhibitor D4476 inhibits phosphorylation of DIC. Immunoprecipitation of dynein with GFP antibody from extracts of GFP-DIC-expressing melanophores metabolically labeled with ^{32}P ; left panel, Coomassie-stained gel; right panel, autoradiograph; Mel, MSH, and Mel+D4476 on top of each panel

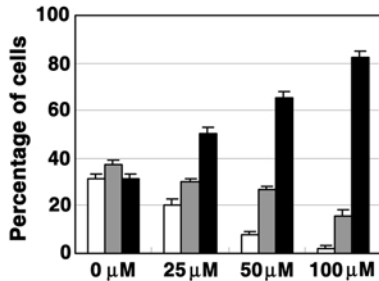
indicate extracts of cells treated with melatonin (to induce aggregation; A) MSH (to induce pigment dispersion; D), or D4476 and melatonin (to inhibit CK1 activity prior to induction of aggregation; Mel+D4476), respectively. In immunoprecipitates, ³²P incorporates predominantly into GFP-DIC, and this incorporation is diminished by inhibition of CK1 activity with D4476.



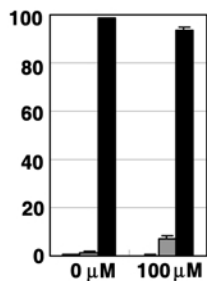
A D4476-treated cells
Aggregation with melatonin



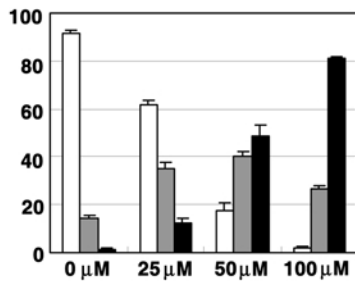
B IC261-treated cells
Aggregation with melatonin



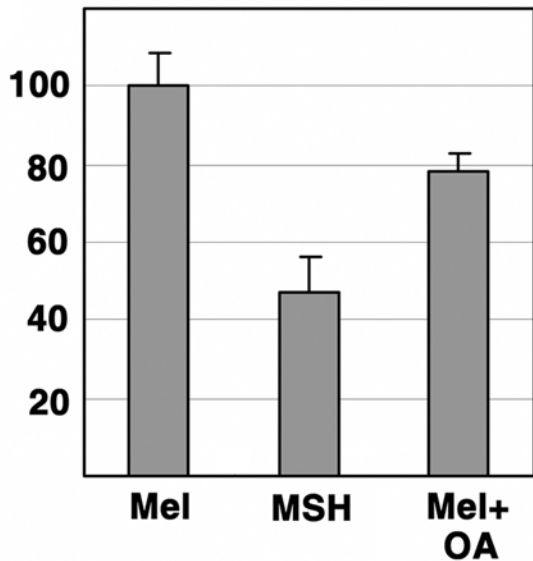
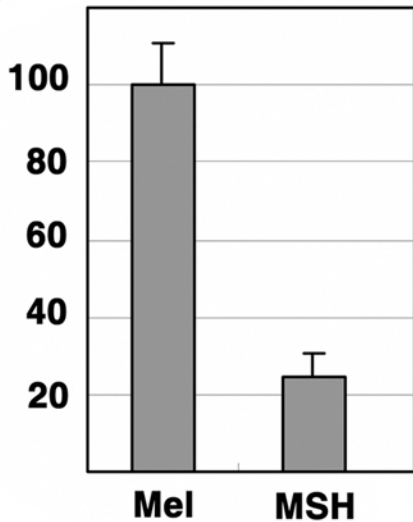
C IC261-treated cells
Dispersion with MSH

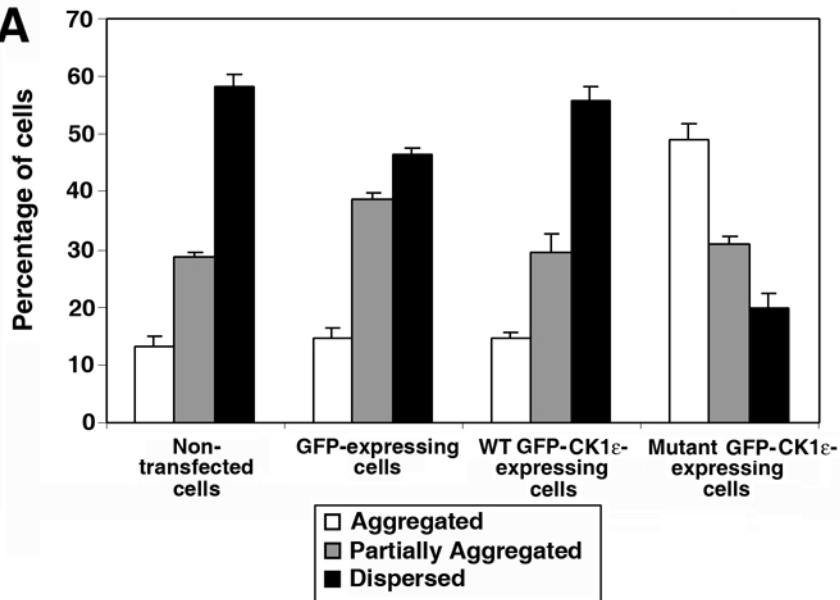
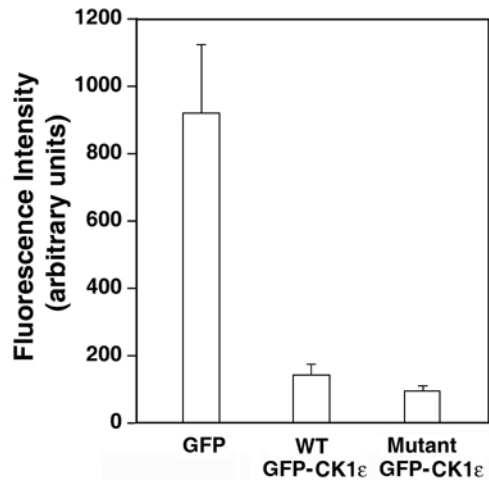


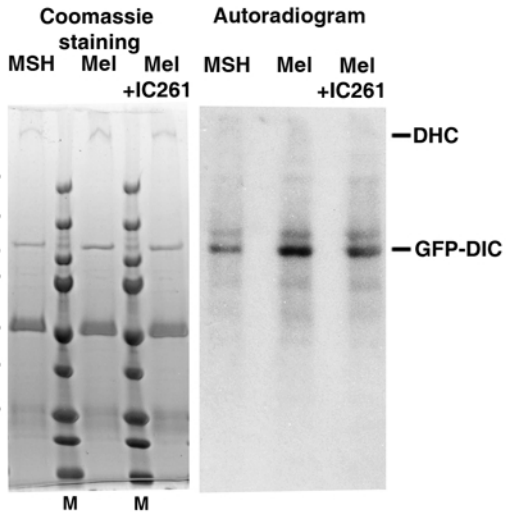
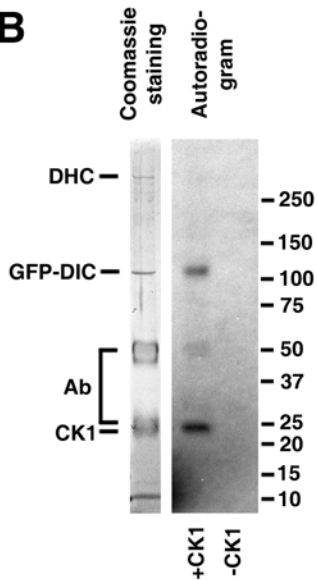
D Melatonin-aggregated cells
Treatment with IC261



□ Aggregated ■ Partially Aggregated ■ Dispersed

A**CK1 Activity (%)****B****PP2A Activity (%)**

A**B**

A**B**

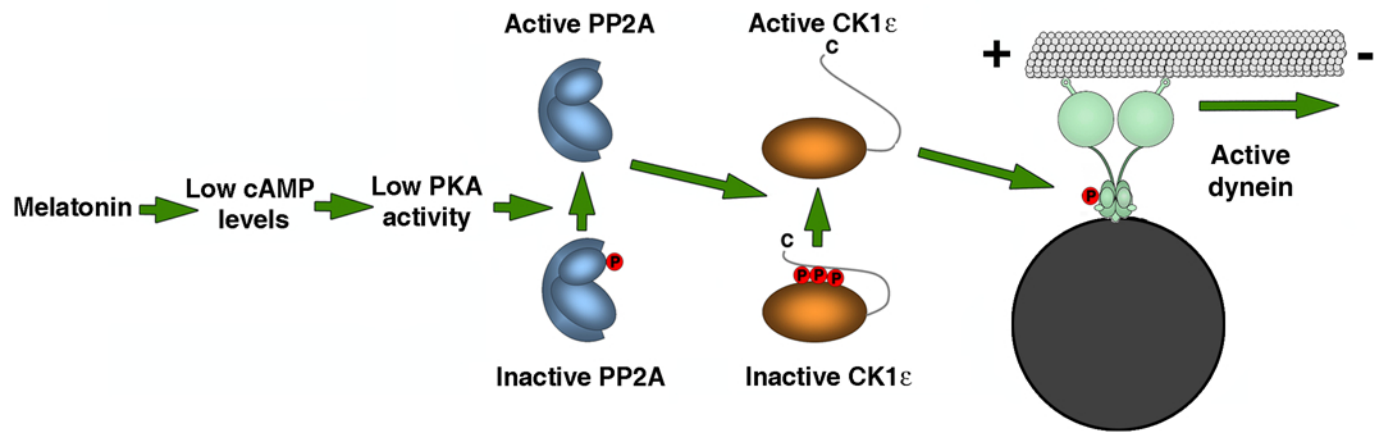


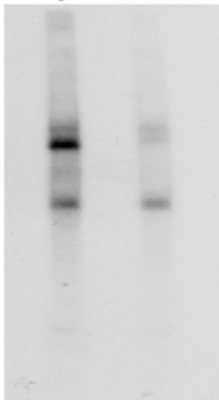
Table 1. Parameters of bidirectional movement of single melanosomes along MTs during pigment aggregation in melanophores treated with CK1 inhibitor IC261.

	Control	IC261 (25 μM)
Velocity of minus-end runs (nm/s)	348.8 \pm 7.4	327.7 \pm 6.8
Length of minus-end runs (nm)	134.4 \pm 9.1	92.5 \pm 5.4
Number of examined minus-end runs	1306	1606
Velocity of plus-end runs (nm/s)	296.1 \pm 9.0	312.1 \pm 8.4
Length of plus-end runs (nm)	42.4 \pm 1.8	34.7 \pm 1.1
Number of examined plus-end runs	1037	1341
Duration of pauses (s)	0.64 \pm 0.2	0.65 \pm 0.2
Number of examined pauses	428	499
Number of examined trajectories	76	85

Numbers indicate average \pm SEM.

GFP-DIC-transfected

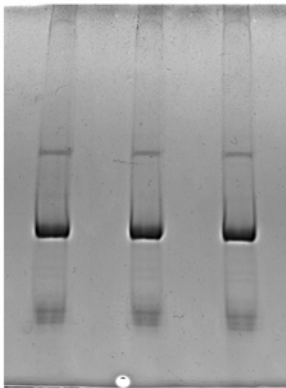
Non-transfected



**Coomassie
staining**

Mel MSH Mel
 + D4476

250 -
150 -
100 -
75 -
50 -
37 -
25 -



Autoradiogram

Mel MSH Mel
 + D4476

— GFP-DIC

