A Family of Rab27-binding Proteins

MELANOPHILIN LINKS Rab27a AND MYOSIN Va FUNCTION IN MELANOSOME TRANSPORT

The Rab27a GTPase regulates diverse processes involving lysosome-related organelles, including melanosome motility in melanocytes, and lytic granule release in cytotoxic T lymphocytes. Toward an understanding of Rab27a function, we searched for proteins that interact with Rab27a(GTP) using the yeast two-hybrid system and identified JFC1/Slp1, a protein of unknown function. JFC1/Slp1 and related proteins, including melanophilin, contain a conserved amino-terminal domain similar to the Rab3a-binding domain of Rabphilin-3. We used several methods to demonstrate that this conserved amino-terminal domain is a Rab27a-binding domain. We show that this domain interacts directly, and in a GTP-dependent manner with Rab27a. Furthermore, overexpression of this domain in melanocytes results in perinuclear clustering of melanosomes, suggesting that this region is sufficient for interaction with, and perturbation of function of, Rab27a in a physiological context. Thus, we identified a novel family of Rab27-binding proteins. We also show that melanophilin associates with Rab27a and myosin Va on melanosomes in melanocytes, and present evidence that a domain within the carboxy-terminal region of melanophilin interacts with the carboxy-terminal tail of the melanocyte-specific splice isoform of myosin Va. Thus, melanophilin can associate simultaneously with activated Rab27a and myosin Va via distinct regions, and serve as a linker between these proteins.

A large family of Ras-like GTPases, termed Rab in mammals and Ypt/Sec4 in yeast, are important regulators of organelle identity and vesicular transport (1–3). Precisely how Rabs regulate membrane transport remains unclear, however, a common mechanism appears to be the ability to regulate the membrane recruitment of a diverse group of “effector” molecules when activated by conformational changes created by GTP-binding.

Loss of function mutations in one of these Rab proteins, Rab27a, leads to Griscelli syndrome, a combination of partial albinism and severe immunodeficiency with hemophagocytic syndrome (3, 4). The clinical picture appears to be a manifestation of defects in two specialized lysosome-related organelles, the melanosome in melanocytes and lytic granules in cytotoxic T-lymphocytes (CTL).1

Melanosomes are normally evenly distributed throughout the cytoplasm and peripheral dendrites in skin melanocytes. Current models suggest that this steady-state distribution is achieved through coupling of microtubule-driven transport to the periphery from the perinuclear site of synthesis, followed by actin-mediated retention of melanosomes at the periphery (5, 6). In contrast, melanosomes within melanocytes isolated from Griscelli syndrome patients, and from the respective mouse model ashen (Rab27a<sup>ash</sup>), display a striking redistribution pattern with the majority of them present in the perinuclear area (7–10). Two other mouse mutants display similar coat color dilution and cellular phenotype. One is the dilute (Mylph<sup>d</sup>) mouse caused by mutations in the unconventional myosin, myosin Va, and the other is leaden (Mlhph<sup>le</sup>), whose gene has been identified recently by Jenkins and co-workers (11, 12) and termed melanophilin (Mlph, also known as Sla2-a). The similarities between all three phenotypes suggested that the three proteins are active in a common pathway in melanosome transport, most likely in tethering melanosomes to the peripheral actin meshwork. Several recent observations are consistent with this hypothesis. First, all three mutants are suppressed by the dilute suppressor mutation (13). Second, myosin Va is co-immunoprecipitated with Rab27a in melanocyte extracts (9). Third, association of myosin Va with melanosomes is greatly reduced in cultured melanocytes in the absence of either Rab27a (ashen) or Mlph (leaden) (9, 10, 14, 15). In contrast, Rab27a is correctly localized to melanosomes in cultured melanocytes in the absence of either Mlhph (leaden) or myosin Va (dilute) (9, 14, 15). Together, these observations indicate that targeting of Rab27a is independent of myosin Va and Mlhph, and that Rab27a may act together with or via Mlhph to allow the recruitment of myosin Va to melanosomes, thereby allowing their retention in the peripheral actin cytoskeleton. Further support for this order of events comes from the finding that Mlhph contains an amino terminus 150-amino acid domain which is similar to the Rab-binding domain of the Rab3a effector Rabphilin-3 (12, 16), suggesting it may directly interact with Rab27a.

In CTLs, the Rab27a mutation leads to a late block in secretion of lytic granules into the immunological synapse that forms between an activated T-cell and its target cell (17, 18). Although the loss of CTL activity is apparently not deleterious...
in laboratory mice, it may underlie the lethal hemophagocytic syndrome in Griscelli syndrome patients (4). Interestingly, dilute and leaden mice do not exhibit CTL defects, suggesting that the Rab27a function is mediated by different sets of effectors in melanocytes and CTLs (14, 18). This hypothesis is supported by the observation that Mlp and myosin Va are not detectably expressed in CTLs (14).

In this paper, we present evidence that Mlp and related proteins constitute a family of Rab27-binding proteins. Furthermore, we show that Mlp is a Rab27 effector protein as it is able to bridge activated Rab27a and myosin Va by binding to both molecules via distinct regions at the amino terminus and central region of the molecule, respectively.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—cDNAs for human Rab13, 18, -35, and rat Rab38 were EcoRI/SalI subcloned in pBTM116 (a generous gift of S. Fields, University of Washington) following reverse transcriptase-PCR from human RNA or rat RNA with the Titan reverse transcriptase-PCR kit (Roche Molecular Biochemicals) using the following primers: Rab13, 5′-GAGAACATGGCCAAAGCCTACG and 5′-AGATCGACCGCAAGACCGTGC and 5′-AGCTGGTTCCAGACGACTACG-3′; Rab18, 5′-GAGAATCGACCGCAAGACCGTGC and 5′-AGCTGGTTCCAGACGACTACG-3′; Rab38, 5′-GAGAATCGACCGCAAGACCGTGC and 5′-AGCTGGTTCCAGACGACTACG-3′. DNA from pET14b-Rat Rab27a and pET14b-Rab5a (21) was amplified from pET14b-Rat Rab27a and pET14b-Rab5a following PCR amplification from pET14b-Mlp (1-150, 5′-GGGAATTCGGATCCGCTCTCGAGGTGCTGGAACCACTGAT; antisense-590, 5′-CCGGCTCGAGCCCAGAGGGTAAAGGGC; antisense-400, 5′-GGCCGAATTCATGCCCC-3′) (see below). pCMV-MYC, a mammalian expression vector allowing the protein samples were subjected to SDS-PAGE, and proteins were separated by electrophoresis on a 15% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and incubated with the appropriate primary antibody for 1 hr, extensively washed, incubated for 1 hr at room temperature with 2 bed volumes of buffer B (600 mM imidazole, 400 mM NaCl). Eluted Rab was then diluted 4-fold in buffer C (50 mM Tris-HCl, pH 7.5, 180 mM NaCl, and 5 mM MgCl2) for 20 min at room temperature. Then the Rab was washed twice with buffer C before the bound nucleotide was eluted by washing with 1 M guanidine-HCl, and followed by two washes with ice-cold buffer A. The immobilized His-Rab was nucleotide loaded by incubation for 10 min on ice in 200 μl of buffer A supplemented with 20 μM of either GTPyS or GDP. The beads were then washed rapidly, three times with buffer A, before the Rab was eluted from the N-agarose beads by incubating for 15 min at room temperature with 2 bed volumes of buffer B (600 mM imidazole, 400 mM NaCl). Eluted Rab was then diluted 4-fold in buffer C (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM Nonidet P-40, and 0.5 mM GTPγS or GDP), and incubated with GST fusion proteins in the presence of 20 μM of glutathione-Sepharose beads for 20 min at room temperature with gentle agitation. The beads were then washed 4 times in buffer C containing the appropriate nucleotide, and bound proteins were eluted by incubating for 15 min at room temperature with 2 bed volumes of 20 μM glutathione. The eluates were analyzed by immunoblotting using either anti-Rab27a or anti-Rab1a antibodies.

**Immunoblotting**—Protein samples were subjected to SDS-PAGE, and were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk and incubated with PBS (0.2%) + 2% Tween 20 with the appropriate primary antibody for 1 hr, extensively washed, incubated for 30 min with 1:5000 dilution of appropriate horseradish peroxidase-conjugated secondary antibody (Dako), and were washed as before. Bound antibody was detected using the Western Supersignal (Pierce). Blots were calibrated with prestained molecular weight standards (Bio-Rad).
and quenched by incubation in 50 mM NH₄Cl for 10 min. Fixed cells were transfection. Excess fixative was removed by extensive washing in PBS were fixed for 15 min in 3% paraformaldehyde in PBS 24 h after instructions.

according to manufacturer’s instructions.

Immunofluorescence—Coverslip-grown cells for immunofluorescence were fixed for 15 min in 3% paraformaldehyde in PBS 24 h after transfection. Excess fixative was removed by extensive washing in PBS and quenched by incubation in 50 mM NH₄Cl for 10 min. Fixed cells were then incubated with diluted primary antibody for 30 min, washed extensively, incubated for 30 min with appropriate Alexa 488- and/or Alexa 568-conjugated secondary antibodies (Molecular Probes), washed as before, and were mounted in ImmunoFluor medium (ICN). All antibody incubations and washes used 1 × PBS, 0.5% bovine serum albumin, 0.05% saponin. Cells were observed using a Leica DM-IRBE confocal microscope, and images were processed using Leica TCS-NT software associated with the microscope and Adobe Photoshop 5.0 software. All images presented are single sections in the z-plane. To evaluate pigment distribution following overexpression of Rab27a, the area of the cytoplasm occupied by pigment granules was compared with the total area of the cytoplasm. Cells in which the area occupied by 90% of the granules was less than or equal to 50% of the total were defined as “aggregated” in the legend to Fig. 2. Simultaneous immunofluorescence using two rabbit polyclonal antibodies was performed and controlled as described previously (14).

Immunoprecipitation—HEK293T cells were grown on 10-cm dishes—Cell Culture and Transfection—Melanocyte cell lines, melan-a and melan-c cells, were cultured as described previously (9). HEK293T cells (generous gift of Charles Coutelle, Imperial College) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 units/ml streptomycin at 37 °C with 5% CO₂. All transfections used the liposomal transfection reagent FuGENE 6 (Roche Molecular Biochemicals) according to manufacturer’s instructions.

**RESULTS**

A Family of Rab27a-binding Proteins—In an effort to understand the molecular mechanisms underlying Rab27a function, we used the yeast two-hybrid system to search for candidate Rab27a-binding proteins. To this end, we used a putative GTPase-deficient mutant version of Rab27a, Rab27aQ78L, to select for proteins that bind preferentially to the active Rab27a(GTP) form. We first screened a human keratinocyte library. Of 2 × 10⁷ plated colonies, we isolated 10 His +/β-Gal+ colonies. Two of these colonies yielded an 1.8-kb band when PCR-amplified using oligonucleotides flanking the multiple cloning site of pACTII, and were identified by sequencing as a previously isolated protein known as JFC1 in humans and Slp1 in mice (31, 32). Because of the low number of positive colonies, we followed the same procedure to screen a human leukocyte library. Of 2.6 × 10⁶ mated colonies, we isolated 45 His +/β-Gal+ colonies. Thirty of these colonies yielded a 1.9-kb band when PCR-amplified, and were also identified by sequencing as JFC1/Slp1.

BLAST analysis revealed that JFC1/Slp1 is related to Rabphilin-3, a Rab3a effector. The structural homology encompasses two areas, the amino terminus corresponding to the Rab-interacting domain in Rabphilin-3, and the carboxyl terminus within which both proteins contain two tandem C2 domains, C2A and C2B. Previous studies indicated that the amino-terminal conserved domain is shared by a number of proteins, including Slp2-a, Slp-3a, granophilin (Gnp), also called Slp4), and Mph (12, 16, 33). Phylogenetic analysis suggested that Rabphilin-3 and JFC1/Slp1 formed two distinct branches (16). The Rabphilin branch of the family includes proteins such as RIM and Noc2, all known to bind Rab3 proteins. Therefore we hypothesized that the JFC1/Slp1 branch of this family of proteins could represent Rab27-binding proteins. To test this idea, we first analyzed the interaction of Rab27aQ78L with amino acids 1–200 of JFC1/Slp1 by the yeast two-hybrid assay, and observed that the interaction was as effective as with the full-length JFC1/Slp1 isolated originally (data not shown). We then isolated cDNAs encompassing the putative Rab-interacting domain (amino-terminal 150 amino acids approximately) for several JFC1/Slp1-related proteins, and tested them in the same assay. Equivalent amino-terminal fragments from the related proteins Slp2-a, Gnp, and Mph all showed similar patterns of interactions with the different Rab baits tested (Table I). These fragments interacted strongly with Rab27a wild type, Rab27aQ78L mutant, Rab27b wild type, and Rab27bQ78L mutant. There was little or no interaction with the dominant-negative mutants Rab27aT23N and Rab27bN133I, which are predicted to remain preferentially in the GDP-bound form or nucleotide-free (9). In addition, we detected a weak interaction between Rab8a and Gnp, but all other Rabbs tested (3a, 5a, 8a, 13, 18, 35, and 38) gave no interaction. All the LexA-Rab fusion proteins were expressed to similar levels except Rab27 proteins harboring the Q78L mutation, which were expressed at reduced levels (data not shown). As a positive control, we observed interaction of Rabs3a, -5a, -8a, and -35 with Rab Escort Protein-1, a general Rab-binding protein (data not shown) (34). To test the interac-

| TABLE I Binding specificity of JFC1/Slp1 and related proteins to Rab GTPases using the yeast two-hybrid system |
| Rab27a wt | +/+/+ | +/+/+ | +/+/+ | - |
| Rab27a TN | +/+ | +/+ | +/+ | - |
| Rab27a QL | +/+ | +/+ | +/+ | - |
| Rab27b QL | +/+ | +/+ | +/+ | - |
| Rab3a | - | - | - | - |
| Rab5a | - | - | - | - |
| Rab8a | - | - | - | - |
| Rab13 | - | - | - | - |
| Rab27a QL | +/+ | +/+ | +/+ | - |
| Rab35 | - | - | - | - |
| Rab38 | - | - | - | - |
| pBTM alone | - | - | - | - |

* ND, not determined.

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assay. This in vitro study suggests that the JFC1/Slp1 family of proteins bind directly and specifically to the active form of Rab27a, and can thus be classified as Rab27(GTP)-binding proteins.

Overexpression of the Rab27a-binding Domain in Melanocytes Result in Perinuclear Clustering of Melanosomes—We had previously observed that transient transfection of dominant-negative Rab27aT23N and Rab27aN133I mutants in cultured melanocytes led to significant clustering of melanosomes in the perinuclear area (9). This is similar to the steady-state distribution of melanosomes in ashen melanocytes, which lack the Rab27a protein (7–10), indicating that overexpression of these constructs perturbs the function of endogenous Rab27a. We reasoned that if the observed interactions between Rab27a and JFC1/Slp1, Mlph, and related proteins were physiologically relevant, then the transient overexpression of the Rab27-binding domains of these proteins in melanocytes would have a dominant-negative effect as they would perturb the association of Rab27a with its endogenous effectors. Indeed as shown in Fig. 2, overexpression of EGFP-Mlph-(1–150) (panels C and D), EGFP-JFC1/Slp1-(1–150) (panels E and F), EGFP-Slp2a-(1–133) (panels G and H), and EGFP-Gnph-(1–150) (panels I and J), but not EGFP alone (panels A and B), resulted in aggregation of melanosomes close to the nucleus in transfected cells. Altogether, these results strongly suggest that the JFC1/Slp1 family of proteins are specific binding partners of Rab27 proteins.

Melanophilin Co-localizes with Melanosome-associated Rab27a and Myosin Va in Melanocytes—Recent work suggested that Mlph plays an important role in melanosome transport in melanocytes (12). We decided to further investigate the role of Mlph in these cells by studying the interactions with its partners, Rab27a and myosin Va. We first examined the localization of both transiently overexpressed Myc-tagged Mlph and endogenous Mlph in melan-c melanocytes. We observed in both cases that the protein was associated primarily with punctate structures ~0.5 μm in diameter located throughout the peripheral dendrites of these cells (Fig. 3, panels A, D, H, and K). Co-staining of melan-c cells with antibodies directed against Rab27a (Fig. 3, panels B and G) and myosin Va (Fig. 3, panel E and J) shows striking co-localization of Mlph with these proteins, confirming that these structures are indeed melanosomes. In addition, we observed that all three proteins were associated with other less defined structures in the body of the cell close to the nucleus.

Melanophilin Interacts with Rab27a and Myosin Va via Independent Domains—Our previous work indicated that myosin Va is present in a complex with Rab27a in melanocytes (9). However, we have been unable to demonstrate a direct interaction between them using the yeast two-hybrid system or GST pull-down assays (data not shown). Given the similarity of the phenotype of melanocytes from mutant mice lacking each of these proteins, and that all three proteins co-localize on melanosomes, we decided to test whether Mlph might bridge the myosin Va-Rab27a interaction. Using the yeast two-hybrid assay, we first observed a positive interaction between full-length Mlph (1–590 amino acids) and a carboxyl-terminal 621-amino acid fragment of myosin Va. This myosin Va fragment included melanocyte-specific exons D and F and the globular tail (36), which are sufficient to target GFP to melanosomes (6). We then used a series of truncation mutants of Mlph to map more precisely the region of Mlph required for interaction with the carboxyl terminus of myosin Va (Fig. 4). We observed that full-length Mlph and the truncations 1–400, 150–590, 367–590, 367–467, and 367–400 interacted with the tail of myosin Va, whereas truncations 1–266, 150–338, and 467–590 did not.
Our results suggest that amino acids 367–400 of Mlph comprise a minimal determinant of myosin Va-binding domain (Fig. 4).

We next analyzed the ability of these constructs to interact with Rab27a using the same assay (Fig. 4). We found that constructs encoding 1–266, 1–400, and full-length Mlph interacted strongly with Rab27a wild type and Rab27aQ78L, but not Rab27aT23N. In contrast, we observed no interaction between constructs encoding 150–590 amino acids of Mlph and Rab27a wild type or mutants. In summary, these results strongly support the possibility that Mlph acts as a linker bridging Rab27a and the carboxyl terminus of myosin Va via non-overlapping regions.

To confirm the yeast two-hybrid observations, we investigated whether Rab27a, Mlph, and the carboxyl terminus of myosin Va could form a tripartite complex in mammalian cells. We transfected HEK293 cells with combinations of plasmids encoding Rab27a, Myc-tagged Mlph, and EGFP-tagged myosin Va carboxyl terminus (EGFP-MVaMSGTA) and precipitated Rab27a using specific antibodies, and then analyzed immunoprecipitates by immunoblotting (Fig. 5). We found that fusion proteins containing Mlph-(1–150), Mlph-(1–400), full-length Mlph, and full-length JFC1/Slp1 were precipitated with anti-Rab27a antibodies, whereas those containing amino acids 150–400 and 150–590 were not (Fig. 5, middle panels). In the experiment shown, the signal obtained for Mlph-(1–200) was weak and could only be revealed after long exposures of the film (data not shown). Also, we noted that full-length Mlph was proteolyzed during the protocol, however, this did not prevent its precipitation. We found that myosin Va could be precipitated only from extracts containing full-length Mlph or Mlph-(1–400), but not from those containing JFC1/Slp1 or Mlph-(1–200) (Fig. 5, upper panels). These results highlight the specificity of the Mlph-myosin Va interaction, and further suggest that the Rab27- and myosin Va-interacting domains are non-overlapping in Mlph. In summary, these results indicate that Mlph may act as a linker mediating the recruitment of myosin Va to Rab27a-containing melanosomes in melanocytes, thereby mediating their peripheral retention. Additionally, they suggest that the 1–400-amino acid fragment of Mlph represents a minimal region that binds both Rab27a (via 1–150) and myosin Va (via 367–400).

**DISCUSSION**

We report here the identification of a novel family of proteins that act as Rab27-binding proteins. This family of proteins includes Mlph involved in melanosome transport, Gnh involved in insulin secretion from pancreatic β-cells, and a variety of other proteins of unknown function (JFC1/Slp1, Slp2a, Slp3a, and Slac2-b). This finding supports our recent suggestion that Rab27a acts through different sets of effectors in different cell types (14).
and identified one protein, JFC1/Slp1. This protein was recently isolated by Babior and co-workers (31) who identified the human version in a yeast two-hybrid screen as a p67phox-interacting protein, showed that it binds phosphatidylinositides, and named it JFC1. Also, Mikoshiba and co-workers (32) independently cloned the mouse version as one of three novel proteins containing tandem C2 domains related to synaptotagmin, and called it synaptotagmin-like protein-1 (Slp1). The results of

**FIG. 3.** Endogenous and overexpressed melanophilin co-localize with melanosomal proteins Rab27a and myosin Va in melan-c melanocytes. Panels A–F, melan-c melanocytes were transfected with pCMVMYC-Mlph, fixed, and stained with anti-Myc monoclonal antibodies detected using Alexa 488-labeled goat anti-mouse antibodies (panels A and D), and either anti-Rab27a (panel B) or anti-myosin Va (Panel E) rabbit polyclonal antibodies detected using Alexa 568-labeled goat anti-rabbit antibodies. Panels G–L, melan-c melanocytes were stained with anti-Rab27a (panel G) or anti-myosin Va (panel J) rabbit polyclonal antibodies detected using Alexa 488-labeled goat anti-rabbit antibodies, and anti-Mlph (H and K) rabbit polyclonal antibodies detected using Alexa 568-labeled goat anti-rabbit antibodies. See “Experimental Procedures” for more details. Panels C, F, I, and L show the extent of overlap of the green and red signals in each case. Bars = 20 μm.
the former study suggested that JFC1/Slp1 is involved in the
activation of NADPH oxidase in neutrophils, and the present
study raises the interesting possibility that Rab27a is involved
in the same process.

We demonstrate here that full-length JFC1/Slp1 and Mlph
interact with Rab27a, and furthermore, that the amino-termi-
nal conserved domain in JFC1/Slp1, Mlph, and other Slps is a
Rab27-interacting domain. We reached this conclusion using a
variety of methods, including an in vitro binding assay using
recombinant proteins, a yeast two-hybrid assay, a melanocyte-
based assay scoring for dominant-negative effects, and a pre-
cipitation assay following transient transfection. In a very re-
cent study, Mikoshiba and co-workers (38) performed a
thorough analysis of the Rab binding specificity of six members
of the JFC1/Slp1 family in vitro and reported a similar conclu-
sion. Independently, Izumi and co-workers (39) recently iden-
tified Rab27a as a binding protein for Gnph, a member of this
family that is expressed primarily in endocrine tissues, such as
pancreatic \( \beta \)-cells and the pituitary. Rab27a and Gnph co-
localize to the limiting membrane of insulin-containing dense
granules in pancreatic \( \beta \)-cells, and overexpression of wild-type
Rab27a and Rab27aQ78L mutant significantly enhanced high

![Fig. 4. Yeast two-hybrid screening defines distinct regions of melanophilin involved in binding of Rab27a and the carboxyl terminus of myosin Va.](image)

![Fig. 5. Formation of a trimeric complex containing Rab27a, melanophilin, and myosin Va in transiently transfected cells.](image)
K⁺-induced insulin secretion, suggesting their involvement in regulated insulin exocytosis. Our results confirm these recent results given the different experimental approaches used, and extend them by demonstrating through the use of purified recombinant proteins that the interaction between Rab27a and this family of proteins is direct and GTP-dependent.

In melanocytes, it is now well established that Rab27a regulates melanosomal motility in concert with Mlph and myosin Va (see Introduction). Mutations in all three genes in mice result in similar perinuclear clustering of melanosomes in melanocytes, and consequently a coat color dilution because of deficient melanocyte/keratinocyte melanosome transfer. Previous studies have suggested an order of events where Rab27a, but not Mlph or myosin Va, acts as a linker to bridge the interaction between activated Rab5, for example, interacts with a variety of effectors that are Rab27a is acting sequentially with several effector proteins. Rab5, for instance, interacts with a variety of effectors that are structurally and functionally unrelated to orchestrate a series of events that regulate early endosome dynamics (1). Future experiments should elucidate the mechanism of action of Rab27a in the different cell types.

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A Family of Rab27-binding Proteins: MELANOPHILIN LINKS Rab27a AND MYOSIN Va FUNCTION IN MELANOSOME TRANSPORT

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