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**ADP-Ribosylation Factor 6 Regulates Mammalian Myoblast Fusion through Phospholipase D1 and Phosphatidylinositol 4,5-Bisphosphate Signaling Pathways**

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Myoblast fusion is an essential step during myoblast differentiation that remains poorly understood. M-cadherin-dependent pathways that signal through Rac1 GTPase activation via the Rho-guanine nucleotide exchange factor (GEF) Trio are important for myoblast fusion. The ADP-ribosylation factor (ARF6 GTPase has been shown to bind to Trio and to regulate Rac1 activity. Moreover, Loner/GEP100/BRAG2, a GEF of ARF6, has been involved in mammalian and Drosophila myoblast fusion, but the specific role of ARF6 has not been fully analyzed. Here, we show that ARF6 activity is increased at the time of myoblast fusion and is required for its implementation in mouse C2C12 myoblasts. Specifically, at the onset of myoblast fusion, ARF6 is associated with the multiproteic complex that contains M-cadherin, Trio, and Rac1 and accumulates at sites of myoblast fusion. ARF6 silencing inhibits the association of Trio and Rac1 with M-cadherin. Moreover, we demonstrate that ARF6 regulates myoblast fusion through phospholipase D (PLD) activation and phosphatidylinositol 4,5-bis-phosphate production. Together, these data indicate that ARF6 is a critical regulator of C2C12 myoblast fusion and participates in the regulation of PLD activities that trigger both phospholipids production and actin cytoskeleton reorganization at fusion sites.

INTRODUCTION

Myoblast fusion is an essential process for the development and maintenance of skeletal muscle tissue (Chen and Olson, 2005; Buckingham, 2006). Moreover, during muscle regeneration, satellite cells, which are quiescent muscle precursor cells, become activated and proliferate, differentiate, and finally fuse with existing muscle fibers and with other satellite cells to restore normal tissue architecture (Buckingham, 2006; Moraczewski et al., 2005). Therefore, to fight skeletal muscle diseases and skeletal muscle wasting due to aging or chemotherapy, it is crucial to identify the different cellular mechanisms governing cell fusion.

Myoblast fusion is an ordered set of specific cellular events: cell migration; attraction; recognition; adhesion; alignment; formation of prefusion complexes; transient electron-dense plaque formation; membrane breakdown; and, as a result, fusion of the lipid bilayers (Knudsen and Horwitz, 1977; Doberstein et al., 1997; Swailes et al., 2004, 2006; Peckham, 2008). These distinct phases share common ultrastructural features and some molecular players in Drosophila and vertebrates (Taylor, 2006; Srinivas et al., 2007; Richardson et al., 2008). Genetic approaches in Drosophila melanogaster and Caenorhabditis elegans as well as mouse models and mammalian myoblast cell lines represent valuable tools for the identification of the involved molecular components. Indeed, they allowed the determination of the major role played by cell surface proteins, components of the cytoskeleton, cell membrane, and signal transduction cascades in myoblast fusion (Taylor, 2003; Horsley and Pavlath, 2004; Bryan et al., 2005; Krauss, 2007). So far most of the identified pathways involved in myoblast fusion converge on actin cytoskeletal rearrangement (Menon and Chia, 2007).

Whereas most of the intracellular components of the network involved in myoblast fusion seem to be conserved between flies and vertebrates, the initial recognition and adhesion steps might occur through different sets of transmembrane receptors (Kesper et al., 2007; Srinivas et al., 2007). Specifically, no homologues of the Drosophila membrane proteins Blow, Duf/kirre, and Rols were found in vertebrates (Kesper et al., 2007), where other families of proteins involved in cell–cell contact were identified (Krauss et al., 2005; Sohn et al., 2009). For example, M-cadherin, which belongs to the Cadherin family of Ca2+-dependent adhesion
molecules, is one of such proteins involved in vertebrate myoblast fusion (Zeschник et al., 1995; Charrasse et al., 2006). This role was demonstrated in cultured myoblasts, whereas M-cadherin-deficient mice do not show defects in skeletal muscle development, probably because of compensation by other cadherins, in particularly N-cadherin (Hollnagel et al., 2002). M-cadherin is found predominantly in developing skeletal muscles and is highly expressed during secondary myogenesis. In mature skeletal muscle, M-cadherin is detected in satellite cells and at the sarcolemma of myofibers underlying satellite cells (Moore and Walsh, 1993; Rose et al., 1994; Cifuentes-Diaz et al., 1995).

Recently, we have described a crucial signaling pathway involved in mammalian myoblast fusion that implicates M-cadherin-dependent adhesion and Rac1 GTPase activation via the Rho-guanine nucleotide exchange factor (GEF) Trio (Charrasse et al., 2007). Many studies in Drosophila indicate that Rac1 is a major regulator of myoblast fusion (Luo et al., 1994; Erickson et al., 1997; Nolan et al., 1998; Hakeda-Suzuki et al., 2002; Fernandes et al., 2005). Rac1 seem to be essential for myoblast fusion also in vertebrates and Danio rerio (Charrasse et al., 2007; Moore et al., 2007; Srinivas et al., 2007; Vasyutina et al., 2009). To date, DOCK180/Mbc and Trio, two GEFs for Rac1, have been described to act upstream of Rac1 in this process (Erickson et al., 1997; Nolan et al., 1998; O’Brien et al., 2000; Laurin et al., 2008). Moreover, Brag2/Loner, a GEF for the ADP-ribosylation factor (ARF)/GTPase, also is involved in Rac1 regulation through the control of its membrane localization (Chen et al., 2003). However, no defects in myoblast fusion were detected in a Drosophila ARF6 null mutant (Dyer et al., 2007) and ARF6−/− mice give no clue on its possible involvement in myoblast fusion in vivo because ARF6−/− mice dye between mid- and late gestation (Suzuki et al., 2006). In contrast, ARF6 regulates actin cytoskeletal reorganization and phosphoinositide metabolism, two important events for myoblast fusion. ARF6 is the only member of the ARF family to localize at the plasma membrane (PM) where it regulates membrane traffic through its action on phospholipase D (PLD) and phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Brown et al., 1997; Nolan et al., 1998; Hakeda-Suzuki et al., 2002). The resulting generation of phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) at defined membrane sites is important for the changes to the cortical actin structure at PM, for vesicular trafficking, and for membrane curvature, all of which are essential events for myoblast fusion (Donaldson, 2008). Moreover, ARF6 activates Rac1 and interacts with Kallrin, a Trio orthologue, which might regulate its effect on Rac1 (Koo et al., 2007).

We thus decided to determine whether ARF6 is involved in mammalian myoblast fusion. We analyzed ARF6 activity during differentiation of C2C12 myoblasts and during muscle regeneration in mice and we show that ARF6 is activated at the time of myoblast fusion. We demonstrate that inhibition of ARF6 expression by RNA interference impairs myoblast fusion. Moreover, communoprecipitation experiments show that ARF6 is complexed with M-cadherin, Trio, and Rac1 at the time of fusion. In an effort to elucidate the molecular mechanisms involved in the control of myoblast fusion by ARF6, we demonstrate that PLD activity and PI(4,5)P2 level are important ARF6 downstream players during myoblast fusion. These results demonstrate that ARF6 is involved in myoblast fusion process through the regulation of multiple pathways.

**MATERIALS AND METHODS**

**Cell Culture**

C2C12 mouse myoblasts were grown and induced to differentiate as described previously (Charrasse et al., 2006). Stable C2C12-derived cell lines (see below) were cultured under the same conditions in DMEM supplemented with puromycin (1 µg/ml; Puro). The Rac1 inhibitor NSC23766 (Calbiochem, San Diego, CA) and the PLD1 (VU0155069) and PLD2 (VU0285655-1) inhibitors were used at 10 µM (Scott et al., 2009), butanol-1 at 0.4% (Sigma-Aldrich, St. Louis, MO). They were added 12 h after differentiation medium (DM) addition, and inhibitor-containing medium was refreshed every day. Calcinymycin (50 µM for 20 h; Sigma-Aldrich), LiCl (1 mM for 48 h; Sigma-Aldrich), and neomycin (1 mM for 48 h; Sigma-Aldrich) were added to DM. Rapamycin (Sigma-Aldrich) was used at 100 nM for 5 min.

**Gel Electrophoresis and Immunoblotting**

Cell cultured in 100-mm dishes were rinsed in cold phosphate-buffered saline (PBS) and lysed in 10 mM piperezine-N,N′-bis(2-ethanesulfonic acid), pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 0.5% IGEFAL CA-630, 1 mM EDTA, 1 mM ortho-vanadate, and antiprotease cocktail (Sigma-Aldrich). Then, 20–60 µg of protein extracts was resolved on polyacrylamide gels (8, 12, and 15%) and transferred onto Immobilon-P membranes. Membranes were then incubated with monoclonal antibodies against ARF6 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), Rac1 (1:500; BD Biosciences Transduction Laboratories, Erembodegem, Belgium), tropolin (1:1000) and myosin (1:2000) (Sigma-Aldrich), myogenin (1:500; BD Pharmingen, San Diego, CA), α-tubulin (1:1000), and M-cadherin (1:200; NanoTools, Munich, Germany) or with a polyclonal antibody against PLD1 (1:200; Invitrogen, Carlsbad, CA). After washing, membranes were processed as described previously (Charrasse et al., 2002). For protein quantification, the Odyssey system from LI-COR Biosciences (Cambridge, United Kingdom) was used.

**ARF6 and Rac1 GTPases Activity Assay**

C2C12 myoblasts were lysed as described previously (Meriane et al., 2000; Charrasse et al., 2002). Cleared lysates were incubated with either the ARF binding domain of GGAG fused to glutathione transerse (GST; GST-GGAG; Thermo Fisher Scientific, Waltham, MA), or with the Cdc42/Rac interactive binding domain (CRIB) of p21-activated kinase 1 (PAK) fused to GST (GST-PAK-PRIP) (Cytoskeleton, Denver, CO) bound to beads. ARF6 and Rac fractions were revealed by Western blotting.

**Muscle Regeneration Model and In Situ Detection of Active GTPases**

Muscle regeneration was induced as described previously (Fortier et al., 2008). Cross sections (10 µm) of regenerating tibialis anterior muscles and contralateral controls were cut with a cryostat, dried at 40°C for 2 h, and stored at −80°C. Histological appearance of muscle sections was checked by hematoxylin/eosin staining. Immunohistochemistry on cryosections was performed after methanol fixation at −20°C for 3 min followed by incubation in PBS containing 1% gelatin and 1% BSA. Sections were incubated with anti-myosin heavy chain development (MHCd) primary antibody (1:20; Novocastra, Newcastle, United Kingdom) at 4°C overnight and revealed by incubation with Alexa 546-conjugated mouse immunoglobulin G (Gn)P; GnAS stained with Hoechst (0.1 µg/ml; Sigma-Aldrich). Active Rac and Rac proteins were detected through binding to ARF binding domain of ARHGA10 fused to glutathione transerse (GST-ARHGA10P) and GST-PAK CRIP (GST), respectively, with some modifications (Causeret et al., Development 2004). Three animals were examined for each condition tested.

**Establishment of ARF6 Short Hairpin RNA (shRNA)**

**Stable Cell Lines**

shRNA constructs were made using the retroviral vector pSIREN-RetroQ according to the manufacturer’s protocol (BD Biosciences, San Jose, CA). To suppress endogenous ARF6 expression, the annealed double strand oligonucleotides GATCCGTTGAGGCTGGCCCAATCTtgctcgagGATTTGCCACGCTTCC-AACCTTTTTTACGCGTG (top) and AATTCACCGGTATAAAAGGTT- GAAGCTGGCCACACGTttcatcctgagCGATTGGCCCAGCTTC- CGATTGGCCCAGCTTC- (bottom) were inserted into RNAi-Ready pSIREN-RetroQ and RNAi-Ready pSIREN-RetroVir vector (Clontech, Mountain View, CA) to produce ARF6 shRNA1. Bold letters correspond to oligonucleotides 602–620 of the mouse ARF6 cDNA sequence (NM007481). For ARF6 shRNA2, the sequence in bold letters was replaced by ATTCCTACGCTGCCAAA and GTTCCGGGAAAGAGATT- CGATTGGCCCAGCTTC- (bottom)
of ARF6 shRNA1 or 2 pSIREN-RetroQ C2C12 myoblasts, and in nine random clones and a pool of ARF6 shRNA1 pSIREN-RetroQ-ZsGreen C2C12 myoblasts. All experiments presented were performed with at least three random clones used in triplicate. As a control, we used Luciferase shRNA (Luci shRNA) C2C12 cells (Fortier et al., 2008). C2C12 Trio shRNA myoblasts have been described previously (Charrasse et al., 2007).

**Scanning Electron Microscopy**

Parental and ARF6 shRNA C2C12 myoblasts were grown on Thermofax coverslips (Nalge Nunc International, Rochester, NY) either in growth medium (GM) or in DM and processed as described previously (Fortier et al., 2008). For each condition, at least 100 cells were examined.

**Time-Lapse Imaging**

Parental and ARF6 shRNA C2C12 myoblasts were grown to confluence before analysis by time-lapse microscopy. Alternatively, parental and ARF6 shRNA C2C12 myoblasts were transfected with pleckstrin homology (PH)-PLC- green fluorescent protein (GFP). Time-lapse epifluorescence microscopy was performed as described previously (Mary et al., 2002). Exposure time was 800 ms. Images were saved as Tif files and further compiled into QuickTime movies using the Montpellier RIO Imaging Cell Image Analyzer program (Baeker, 2006).

**Fusion Index Determination**

Cells were treated and analyzed as described previously (Fortier et al., 2008).

**Cell Surface Biotinylation**

The presence of M-cadherin at the cell surface was analyzed as described previously (Charrasse et al., 2006). Quantification of at least four independent experiments was performed using the Odyssey system (LI-COR Biosciences).

**Immunoprecipitation**

Cell lysates were obtained as described in Gel Electrophoresis and Immunoblotting. Polyclonal anti-M-cadherin antibody (15 μl; Charrasse et al., 2006), 1 μg of anti-ARF6 antibody, or 1 μg of anti-Trio antibody (mix of C-20 and D-20; Santa Cruz Biotechnology) was incubated with protein A or G (Dynal beads; Invitrogen) at room temperature for 1 h. After washing, 1 mg of protein extract was added for 1 h at room temperature for 1 h. Immunoprecipitates were then analyzed by immunoblotting as described previously (Charrasse et al., 2007).

**Immunohistochemistry**

Cells transfected with plasmids encoding hemagglutinin (HA)-, GFP-, or red fluorescent protein (RFP)-tagged ARF6, Rac1, Trio-Nter, PH-PLC, FKBP-5-pase, or PLD1 by using Lipofectamine 2000 (Invitrogen) were fixed with 0.1% Triton X-100 in PBS, cells were incubated with mouse monoclonal anti-HA (1:2000) or anti-M-cadherin (1:200) antibodies. Antibodies were revealed with Alexa Fluor 546-conjugated or Alexa Fluor 488-conjugated goat anti-mouse antibodies (Molecular Probes, Interchim, Monluçon, France). Cells were analyzed as described previously (Charrasse et al., 2002). Images were taken with PL APO 63× or 40× objectives (numerical aperture 1.32; Leica, Wetzlar, Germany) and a CoolSNAP HQ camera (Photometrics, Tucson, AZ). Stacks of images were captured using a piezo stepper (E662; Physik Instruments, Montrouge, France) with a Z-step of 0.2 μm. Stacks were then restored with the Huygens deconvolution software (Scientific Volume Imaging, Hilversum, The Netherlands) and restored images were viewed in three dimensions with MetaMorph software (Molecular Devices, Sunnyvale, CA).

**PLD Activity Assay**

Endogenous PLD activity was measured with the Amplex Red Phospholipase D Assay kit (Invitrogen) in parental, Luci shRNA and ARF6 shRNA C2C12 myoblasts at different times of differentiation. PLD activity was normalized to the total protein amount (bicinchoninic acid [BCA]; Sigma-Aldrich).

**PI(4,5)P2 Detection**

Cellular PI(4,5)P2 levels were measured after lipid extraction in parental, Luci shRNA, and ARF6 shRNA C2C12 myoblasts at different times of differentiation by using the PL(4,5)P2 Mass ELISA kit (Echelon Biosciences, Salt Lake City, UT). Proteins were extracted from the supernatant that is normally discarded after neutral lipids extraction (Wessel and Flugge, 1984). PL(4,5)P2 levels were normalized to the total protein amount (BCA; Sigma-Aldrich).

**RESULTS**

**ARF6 Is Activated during Myoblast Fusion and Muscle Regeneration**

To analyze whether ARF6 participates in skeletal muscle differentiation in vivo, we examined its activity in a mouse model of muscle regeneration. Skeletal muscle injury was induced by injection of notexin in the tibialis anterior muscle. Regeneration was monitored by histological analysis, DNA staining and assessment of MHCd expression 4 d after injection (Figure 1Aa). Desmin and M-cadherin expression also was assessed to visualize satellite cells (data not shown). To detect ARF1 and ARF6 activity, in situ binding assays using GST-ARHGAP10 were performed on cryosections of regenerating muscle and uninjured controls. Whereas no binding of GST alone was detected by immunoblotting.

![Figure 1](image-url)
observed, GST-ARHGAP10 strongly associated with regenerating fibers (Figure 1Ab). Because ARF6, but not ARF1, activates Rac1 (Sanyt and Casanova, 2001), a major regulator of myogenesis (Luo et al., 2004), we also analyzed Rac1 activity during muscle regeneration by in situ binding assay using GST-PAK CRIB. Activated Rac1/Cdc42 was detected in regenerating fibers (Figure 1Ab) that were visualized also by the MHCd antibody (Figure 1Ac). Uninjured control muscle sections did not show binding to GST-ARHGAP10 and GST-PAK CRIB (data not shown). These data show that activated ARF and Rac1 or Cdc42Hs can be detected only during muscle regeneration.

We next analyzed ARF6 expression level during C2C12 myoblast differentiation. C2C12 cells grown to 80% confluence in GM where shifted to DM for 4 d. One day after the switch to DM (D1), cells aligned before starting to fuse at D2. At D3 and D4, myotubes were clearly formed (Figure 1Ba). Whereas ARF6 protein expression level was constant throughout the differentiation process (Figure 1Bb), ARF6 activity, measured by pull-down assays, was detected only during myoblast fusion (D1 and D2) and was below detection level thereafter (Figure 1Bc). These data show that ARF6 is activated just before and during C2C12 myoblast fusion.

**Figure 2.** Inhibition of ARF6 expression by RNA interference impairs myotube formation. (A) a, Luci and ARF6 shRNAs were delivered in C2C12 myoblasts by retroviral infection. After selection, a pool and 10 clones of resistant cells were analyzed for the expression of ARF6 and α-tubulin by immunoblot analysis. Puro (top) corresponds to stable cell lines (pool and clones) that express ARF6 shRNA1 pSIREN-RetroQ, whereas GFP (bottom) corresponds to the cell lines (pool and clones) that express ARF6 shRNA1 pSIREN-RetroQ-ZsGreen. Parental C2C12 and C2C12 Luci shRNA were used as controls. b, histogram represents the quantification of ARF6 normalized to the amount of α-tubulin in the different clones from three different experiments. (B) a, phase contrast images of parental (control 1), Luci shRNA (control 2), ARF6 shRNA pools and clones described in A, 4 d after DM addition. Bar, 30 μm. b, histogram represents the fusion index in control myoblasts and the indicated ARF6 shRNA pools and clones. The results are representative of three independent experiments. At least 3000 nuclei were counted per experiment. (C) Cell lysates (30 μg) of control Luci shRNA and ARF6 shRNA C2C12 myoblasts cultured in GM or DM for the indicated periods were assessed by Western blot analysis for expression of ARF6, myogenin, troponin T, MHC, and α-tubulin. Results are representative of three independent experiments performed with three different clones.
**ARF6 Is Required for Myoblast Fusion**

To analyze the role of ARF6 during mammalian myogenesis, we generated by retroviral infection stable C2C12 cell lines in which ARF6 expression was inactivated by RNA interference. Two ARF6 shRNAs were used, ARF6 shRNA1 (Figure 2) and ARF6 shRNA2 (Supplemental Figure 1). Various clones expressing either a resistance marker (Puro) or GFP were obtained and analyzed, and in most of them a reduction of 90% of ARF6 protein expression level was observed (Figure 2A and Supplemental Figure 1). As controls, parental and C2C12 myoblasts expressing a Luciferase shRNA were used. We then examined whether ARF6 silencing affected myoblast fusion. Cells were induced to differentiate and myotube formation analyzed after 4 d in DM. Significant reduction in the number of myotubes and in the fusion index was observed in ARF6 shRNA cells (Figure 2B, a and b). Results were comparable independently from the ARF6 shRNA used and also when pools or selected (Puro or GFP) clones were used. Therefore, for the next experiments we used clone 2G6 (Puro; shown in the figures) and clones 2D12 and 1D9 (data not shown) of ARF6 shRNA1. Time-lapse imaging of parental and ARF6 shRNA myoblasts also confirmed that ARF6 knockdown decreased myotube formation whereas proliferation, alignment and elongation were not affected (Supplemental Videos 1 and 2). We also examined, by Western blot analysis, whether ARF6 silencing affected the expression of myogenin and troponin T, two early myogenic markers, and MHC, a protein of the contractile apparatus. We did not detect any significant change in the expression of Myogenin in ARF6 shRNA myoblasts. Expression of troponin T and MHC were slightly delayed (i.e., their expression after DM addition started one day later compared with control myoblasts) but reached similar expression level as in control myoblasts thereafter (Figure 2C). These data show that ARF6 is specifically required for myotube formation but not for myogenesis induction.

**ARF6 Silencing Does Not Affect M-cadherin Expression and Distribution**

We showed previously that M-cadherin is a major regulator of myoblast fusion (Charrasse et al., 2006). In contrast, ARF6 is involved in internalization and recycling of various cell surface receptors, including members of the cadherin family (Palacios et al., 2002). Thus, we analyzed whether ARF6 could regulate myoblast fusion via an effect on M-cadherin expression and/or localization. In control cells, M-cadherin expression increased just before the onset of myoblast fusion (D1), and this feature was not changed by ARF6 silencing (Figure 3A). We then analyzed M-cadherin localization by immunocytochemistry at D2 (Figure 3B). M-cadherin accumulated at cell–cell contacts in both control (Figure 3B, a and b) and ARF6 shRNA C2C12 cells (Figure 3B, c and d). We then used a biotinylation assay to further quantify M-cadherin level at the cell surface. We analyzed total and cell surface biotinylated M-cadherin in Luci shRNA and ARF6 shRNA C2C12 myoblasts cultured in GM or in DM for the indicated times (Figure 3C). No decrease in the level of M-cadherin at the cell surface appeared in ARF6 shRNA cells. These data show that ARF6 silencing does not affect M-cadherin expression and localization.

**M-cadherin Is Associated with Trio, ARF6, and Rac1 during Fusion**

The previous results suggest that the function of ARF6 during myoblast fusion should not involve the control of M-cadherin expression or distribution. However, because ARF6 can activate Rac1, we decided to assess whether ARF6 participates in the M-cadherin signaling pathway involved in producing the phenotype described above. In this experiment, we tested whether ARF6 silencing affects the association between M-cadherin and Rac1 via the Trio scaffold. In addition, M-cadherin at the cell surface appeared in ARF6 shRNA1. The histogram represents the quantification of biotinylated M-cadherin at the plasma membrane normalized to the total amount of M-cadherin calculated from at least three independent experiments.

**Figure 3.** ARF6 knockdown does not affect M-cadherin. (A) Cell lysates (30 μg) of control Luci shRNA and ARF6 shRNA C2C12 myoblasts cultured in GM or DM for the indicated periods were assessed by Western blot analysis for expression of M-cadherin and α-tubulin. (B) M-cadherin localization analyzed by indirect immunofluorescence in control Luci shRNA (a and b) and ARF6 shRNA C2C12 myoblasts (c and d) after 2 d in DM. Shown are deconvolved images of M-cadherin alone (green; b and d) or with F-actin (red) and DNA (blue) (a and c). Bar, 10 μm. (C) Control Luci shRNA and ARF6 shRNA C2C12 myoblasts cultured in GM or DM were cell surface biotinylated at 4°C. Biotinylated cell surface M-cadherin was recovered onto streptavidin beads. M-cadherin content in total and biotinylated fractions was analyzed by immunoblotting. The histogram represents the quantification of biotinylated M-cadherin at the plasma membrane normalized to the total amount of M-cadherin calculated from at least three independent experiments.
in myoblast fusion. To this aim, we first examined the interaction between endogenous ARF6 and M-cadherin in C2C12 myoblasts (Figure 4A). ARF6 was immunoprecipitated from cell extracts of proliferating (GM) or differentiating (at D2 in DM) C2C12 myoblasts. Western blot analysis revealed the association of M-cadherin with ARF6 specifically at the time of fusion. Rac1 also was strongly complexed with ARF6 at D2 and weakly in proliferating cells (Figure 4A, top). We showed previously that the Rho-GEF Trio and Rac1 associate with M-cadherin at the onset of myoblast fusion (Charrasse et al., 2007) and Donaldson’s group reported a direct interaction between ARF6 and the spectrin domain 5 of Trio (Koo et al., 2007). To determine whether Trio is required for M-cadherin/Rac1 and M-cadherin/ARF6 association, ARF6 was immunoprecipitated from Trio shRNA myoblasts (Charrasse et al., 2007). Neither M-cadherin nor Rac1 was found associated with ARF6 in Trio shRNA cells (Figure 4A, middle). Similarly, when Trio was immunoprecipitated from ARF6 shRNA myoblasts, no association between M-cadherin and Trio or M-cadherin and Rac1 was observed (Figure 4A, bottom). Supplemental Figure 2 shows that the total levels of M-cadherin and Rac1 in Trio and ARF6 knockdown cells were unchanged compared with control cells. These data indicate that ARF6, Trio, and Rac1 are complexed with M-cadherin at the onset of myoblast fusion.

We next analyzed the distribution of these proteins during myoblast fusion (Figure 4B). Visualization of ARF6 and Rac1 was performed by expression of HA- or GFP-tagged forms. For Trio, we expressed only the N-terminal portion of the molecule (Trio-Nter, aa 1-1813) because the full-length protein is very difficult to express and Trio-Nter mimics full-length Trio effects on neurite outgrowth (Estrach et al., 2002).

ARF6 Silencing Does Not Inhibit Rac1 Activity
Because ARF6 is known to control membrane dynamics (Donaldson, 2003), we analyzed the morphological modifications occurring during myogenesis in parental and ARF6 shRNA C2C12 myoblasts by using scanning electron microscopy (Figure 5A). C2C12 myoblasts elongated and aligned before fusion (Figure 5A, a–c). C2C12 ARF6 shRNA myoblasts also elongated and aligned, but did not fuse efficiently (Figure 5A, d–f). Control myoblasts were characterized by the presence of many membrane structures such as lamellipodia, ruffles, and microvilli, which were clearly visible at higher magnification (Figure 5A, c). In contrast, in ARF6 shRNA myoblasts, the formation of these membrane morphological modifications was reduced (Figure 5A, f). Rac1 is required for myoblast fusion (Luo et al., 1994; Charrasse et al., 2007), and the observed membrane modifications can be reminiscent of the effects of Rac1 activation (Hall, 1998). Because Rac1 is activated by ARF6 (Santy and Casanova, 2001) and ARF6 and Rac1 are colocalized in myotubes and accumulate at the fusion zone (arrow in Figure 4B), we measured Rac1 activity by pull-down assays in Luci shRNA and ARF6 shRNA C2C12 myoblasts at different times after the shift to DM (Figure 5B). As described previously, Rac1-GTP levels were increased at D2, which corresponded to the onset of the fusion process (Charrasse et al., 2006, 2007) in control cells. Surprisingly, Rac1 activity also increased in C2C12 ARF6 shRNA myoblasts at D2 (*). To confirm this result, NSC23766, a chemical inhibitor of Rac1 (Gao et al., 2004), was added to both Luci and ARF6 shRNA
ARF6 Silencing Decreases PI(4,5)P₂ Level during Myogenesis

ARF6 also directly activates PIP5K in vitro to generate PI(4,5)P₂ (Honda et al., 1999), which regulates a wide range of molecular targets and cellular functions, including the recruitment of PH domain-containing proteins to membranes and the regulation of actin polymerization (Tall et al., 2000), which are fundamental processes during myoblast fusion. Thus, we measured the cellular level of PI(4,5)P₂ in control Luci shRNA C2C12 myoblasts, whereas dimethyl sulfoxide (DMSO), butanol-2, or the PLD2 inhibitor did not show any effect (Figure 6A). Conversely, in ARF6 shRNA C2C12 myoblasts, myotube formation, and fusion index were not further decreased by the addition of butanol-1 or of the PLD1 inhibitor. Then, to determine whether ARF6 was required for PLD activity in myoblasts, we measured PLD activity in cell lysates of Luci shRNA or ARF6 shRNA C2C12 myoblasts cultured in GM or DM for 4 d (Figure 6B). After the switch to DM medium, PLD activity increased in control cells, in line with a previous report (Yoon and Chen, 2008), and decreased in ARF6 shRNA cells (Figure 6B). This reduction was not the consequence of a decrease of PLD1 protein expression (data not shown).

Finally, we analyzed the distribution of GFP-PLD1 during myoblast fusion. PLD1 accumulated at fusion sites of control Luci shRNA C2C12 myoblasts, where it colocalized with M-cadherin (Figure 6C). In contrast, in ARF6 shRNA cells, PLD1 did not accumulate at cell–cell contacts and did not colocalize with M-cadherin. These results are clearly illustrated by the line scan analysis (Figure 6D) that shows PLD1 colocalization with M-cadherin only in control myoblasts. These data indicate that ARF6-dependent PLD1 activity plays a critical role during myoblast fusion.

PLD1 Activation Is Essential for Myoblast Fusion and Requires ARF6

In addition to Rac1, ARF6 also activates PLD1 in vitro (Masesenburg et al., 1994) and in vivo (Melendez et al., 2001; Vitale et al., 2002). Because PLD1 was recently shown to be involved in myoblast differentiation (Yoon and Chen, 2008), we then analyzed the role of PLD1 in myogenesis in Luci shRNA and ARF6 shRNA C2C12 myoblasts. To this aim, cells were incubated with 1-butanol, a primary alcohol that inhibits PLD-catalyzed PA formation, and with VU0155069 and VU0285655-1, which inhibit PLD1 and PLD2, respectively (Scott et al., 2009). Because PLD1 activity is required for myogenesis induction (Yoon and Chen, 2008), 1-butanol was added 24 h after induction of differentiation. As a control, butanol-2, which does not interfere with PLD-catalyzed reactions, was used. Butanol-1 and the PLD1 inhibitor drastically decreased myotube formation and fusion index in Luci shRNA C2C12 myoblasts, whereas dimethyl sulfoxide (DMSO), butanol-2, or the PLD2 inhibitor did not show any effect (Figure 6A). Conversely, in ARF6 shRNA C2C12 myoblasts, myotube formation, and fusion index were not further decreased by the addition of butanol-1 or of the PLD1 inhibitor. Then, to determine whether ARF6 was required for PLD activity in myoblasts, we measured PLD activity in cell lysates of Luci shRNA or ARF6 shRNA C2C12 myoblasts cultured in GM or DM for 4 d (Figure 6B). After the switch to DM medium, PLD activity increased in control cells, in line with a previous report (Yoon and Chen, 2008), and decreased in ARF6 shRNA cells (Figure 6B). This reduction was not the consequence of a decrease of PLD1 protein expression (data not shown).

Finally, we analyzed the distribution of GFP-PLD1 during myoblast fusion. PLD1 accumulated at fusion sites of control Luci shRNA C2C12 myoblasts, where it colocalized with M-cadherin (Figure 6C). In contrast, in ARF6 shRNA cells, PLD1 did not accumulate at cell–cell contacts and did not colocalize with M-cadherin. These results are clearly illustrated by the line scan analysis (Figure 6D) that shows PLD1 colocalization with M-cadherin only in control myoblasts. These data indicate that ARF6-dependent PLD1 activity plays a critical role during myoblast fusion.
confirm this point, we treated C2C12 myoblasts with agents that reduce (calcimycin, LiCl) or mask (neomycin) PI(4,5)P2 (Griffin and Hawthorne, 1978; Hallcher and Sherman, 1980; Gabev et al., 1989; Laux et al., 2000). Cells were induced to differentiate and myoblast fusion analyzed 3 d after. A significant reduction in the number of myotubes and in the fusion index was observed after calcimycin, LiCl, or neomycin addition (Figure 8A). Then, to further dissect the effect of PI(4,5)P2 reduction on myoblast fusion, we generated by retroviral infection stable C2C12 cell lines in which the expression of the cadherin-associated PIP5KI/H9253 was inactivated by RNA interference (El Sayegh et al., 2007; Ling et al., 2007). As controls, C2C12 myoblasts expressing a Luciferase shRNA were used. Cells were induced to differentiate and myotube formation analyzed after 4 d in DM. Significant reduction in the number of myotubes and in the fusion index was observed in PIP5KI shRNA cells compared with controls (Figure 8B), indicating a loss of PI(4,5)P2 at the PM. Then, C2C12 myoblasts were cotransfected with PM-FRB-mRFP, mRFP-FKBP-5-tpase, and PH-PLC6-GFP, PH-PLC6-GFP was no more accumulated at cell contacts after rapamycin addition compared with control myoblasts (Figure 8B), indicating a loss of PI(4,5)P2 at the PM. Then, C2C12 myoblasts were cotransfected with PM-FRB-mRFP, mRFP-FKBP-5-tpase and either Rac1WT-GFP or TrioNter-GFP and cultured in DM for 2 d (Figure 8C). Rapamycin addition impaired Rac1 and Trio-Nter localization at regions likely to be fusion sites (compare a and b to c and d and e to g and h) but did not affect it in nontransfected cells (data not shown). Together, these data suggest that PI(4,5)P2 reduction impairs myoblast fusion and Rac1 and Trio recruitment at contact sites likely to be fusion sites.

DISCUSSION

In this article, we assessed the contribution of ARF6 to myoblast fusion by silencing its expression in C2C12 myoblasts. We demonstrate that ARF6 is involved in the regulation of myoblast fusion and that ARF6, Rac1, and Trio are
associated with M-cadherin in a multicomplex recruited at M-cadherin-dependent cell–cell contacts during myoblast fusion. This association is disrupted upon silencing of ARF6. Moreover, we show that Rac1 activity is not inhibited in ARF6 shRNA myoblasts and that ARF6 controls myoblast fusion through PLD1 activation and PI(4,5)P2 production, which trigger both membrane and actin cytoskeleton remodeling at fusion sites.

ARF6 is a regulator of membrane trafficking, of the cortical actin cytoskeleton and of the recycling endosomal system (Donaldson, 2003). Our results establish that ARF6 is required for myoblast fusion to occur in C2C12 myoblasts. Indeed, inhibition of ARF6 expression impairs myoblast fusion and ARF6 activity increases at the onset of myoblast fusion. We reported previously that Rac1 activity is also activated at the time of fusion and it is required for this process (Charrasse et al., 2007). Surprisingly, ARF6 does not seem to control Rac1 activity at the onset of myoblast fusion, although Rac1 activation by ARF6 was reported in various biological processes (Radhakrishna et al., 1999; Zhang et al., 1999; Santy and Casanova, 2001, 2005; Myers and Casanova, 2008; Béglé et al., 2009). ARF6-mediated recruitment of Rac1 GEFs has been proposed as a mechanism for the regulation of Rac1 activity by ARF6, and Loner/Brag2 and ARF6 were shown to control membrane localization of Rac1 (Chen et al., 2003). Among these Rac GEFs, mbc/DOCK180 and Trio are involved in myoblast fusion (Erickson et al., 1997; Nolan et al., 1998; O’Brien et al., 2000; Charrasse et al., 2007; Laurin et al., 2008; Pajcini et al., 2008). Trio, as its brain-specific homologue Kalirin-5, was shown to directly bind to ARF6, which allows its recruitment to the PM where it might regulate Rac1 activation (Koo et al., 2007). Here, we show that 1) ARF6, Trio, and Rac1 are associated with M-cadherin at the time of fusion; 2) in the absence of Trio, ARF6 is no longer associated with either Rac1 or M-cadherin; and 3) in the absence of ARF6, Rac1, and Trio do not associate with M-cadherin. M-cadherin might thus allow the recruitment of a multiprotein “fusion complex” composed of at least ARF6, Trio, and Rac1. The absence of inhibition of Rac1 activity in myoblasts in which ARF6 has been silenced suggests that ARF6 might be involved in the proper membrane localization of Rac1, as reported previously (Chen et al., 2003). Nevertheless, we cannot rule out that a compensatory mechanism is established or that DOCK180 might be responsible for the continuous Rac1 activity observed after ARF6 silencing. In vivo, DOCK180 has been shown to be involved in primary myogenesis (Laurin et al., 2008), whereas Trio is involved in secondary myogenesis (O’Brien et al., 2000). Moreover, M-cadherin accumulates at the areas of contact between fusing secondary myoblasts and myotubes (Cifuentes-Díaz et al., 1995). We thus think that the M-cadherin/ARF6/Trio/Rac1 complex might play an important role in secondary myogenesis. ARF6 can regulate cell–cell junctions (Palacios et al., 2001, 2002; Charrasse et al., 2006; Hiroi et al., 2007).
2006), and because M-cadherin is important for C2C12 myoblast fusion (Charrasse et al., 2006), we precisely analyzed M-cadherin in ARF6 knockdown myoblast. However, expression and localization of M-cadherin were not altered following ARF6 silencing, suggesting that ARF6 is not essential for the regulation of the function of this adhesive receptor.

Another activity of ARF6 (i.e., the activation of PLD) emerges as a new and important regulator of myoblast fusion. Here, we show that 1) ARF6 knockdown impairs PLD activation normally observed during C2C12 myoblast differentiation; 2) specific PLD1 inhibition prevents myotube formation; and 3) PLD1 colocalizes with M-cadherin at fusion sites. Previous studies have reported that PLD1 is involved in myogenesis and particularly in mTOR signaling (Komati et al., 2005; Hornberger et al., 2006; Mebarek et al., 2007; Yoon and Chen, 2008), and here we provide the first demonstration of a role for PLD1 in myoblast fusion. Moreover, PLD1 level and accumulation at cell–cell contacts is decreased in ARF6 knockdown C2C12 myoblasts and PLD1 reduction decreases myoblast fusion. This defect in PLD1 production might impair the recruitment at fusion sites of PH domain-containing proteins involved in the regulation of myoblast fusion. Trio, PLD, and ARF6 itself are localized at the PM in PL(4,5)P2-enriched domains (Skowronek et al., 2004; Macia et al., 2008). We observed that PL(4,5)P2 reduction at the PM impaired Rac1 and Trio recruitment at regions likely to be fusion sites. PL(4,5)P2 also can recruit and influence the activity of several actin-binding proteins, such as Profilin, CapZ, Gelsolin, Ezrin, or neuronal-Wiskott-Aldrich syndrome protein (N-WASP), that lead to changes in the cortical actin network (Donaldson, 2003). Recently, genetic approaches have demonstrated the role of proteins controlling the actin cytoskeleton in myoblast fusion which was confirmed in C2C12 myoblasts for WASP-interacting protein and N-WASP (Kim et al., 2007; Massarwa et al., 2007; Richardson et al., 2007; Berger et al., 2008). PL(4,5)P2 also might recruit Ca2+-dependent activator protein for secretion.

FKBP-5-pasen upon rapamycin addition causes PM recruitment of the enzyme and rapid dephosphorylation of PL(4,5)P2. Addition of 100 nM rapamycin induces tranlocation of the 5-pase to the membrane (c), causing a loss of PH-PLCδ-GFP localization (d) compared with untreated cells (a and b). Bar, 10 μm. (C) C2C12 myoblasts were transfected with PM-FRB-mRFP together with mRFP-FKBP-5-pase and Rac1WT-GFP (a–d) or TrioNter-GFP (e–h) and cultured in DM for 2 d (a, b, e, and f) before addition of 100 nM rapamycin for 5 min (c, d, g, and h). Bar, 10 μm.

**Figure 8.** PL(4,5)P2 reduction inhibits myoblast fusion. (A) C2C12 myoblasts were cultured in DM complemented with agents that reduce (calcimycin and LiCl) or mask (neomycin) PL(4,5)P2, and fusion was analyzed after 3 d. Treatment times were as follows: calcimycin for 24 h and LiCl and neomycin for 48 h. The histogram represents the fusion index in control and treated myoblasts. The results are representative of three independent experiments. At least 3000 nuclei were counted per experiment. Bar, 20 μm. (B) C2C12 myoblasts were transfected with the PH-PLCδ-GFP plasmid to monitor PL(4,5)P2 reduction together with the membrane-targeted FRB fragment of mTOR (PM-FRB-mRFP) and cytosolic 5-pase fused to FKBP (mRFP-FKBP-5-pase). Heterodimerization of PM-FRB with FKBP-5-pase upon rapamycin addition causes PM recruitment of the enzyme and rapid dephosphorylation of PL(4,5)P2. Addition of 100 nM rapamycin induces translocation of the 5-pase to the membrane, causing a loss of PH-PLCδ-GFP localization (d) compared with untreated cells (a and b). Bar, 10 μm. (C) C2C12 myoblasts were transfected with PM-FRB-mRFP together with mRFP-FKBP-5-pase and Rac1WT-GFP (a–d) or TrioNter-GFP (e–h) and cultured in DM for 2 d (a, b, e, and f) before addition of 100 nM rapamycin for 5 min (c, d, g, and h). Bar, 10 μm.
which enables exocytosis of dense core vesicles in neuroendocrine cells (Grishinòn et al., 2004). It will be interesting to determine whether these proteins also might contribute to the regulated secretion of dense core granules, structures observed as fusion proceeds (Doberstein et al., 1997).

Recruitment and activation of PI(3)K at sites of N-cadherin ligation resulting in PI(4,5)P2 production has been reported previously (El Sayegh et al., 2007). Further studies are required to analyze whether M-cadherin-dependent cell–cell contact formation might also participate in PI(4,5)P2 production through a similar mechanism.

In conclusion, we propose that M-cadherin might be involved both in myoblast recognition and in the induction of localized intracellular signaling pathways leading to ARF6 activation, which induces PLD1 activity and PA and PI(4,5)P2 production. All these factors participate in myoblast fusion through the reorganization of the actin cytoskeleton and of PM dynamics.

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