Molecular Biology of the Cell
Vol. 21, 2412–2424, July 15, 2010

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

**Anne-Sophie Bach,* Sandrine Enjalbert,* Franck Comunale,* Stéphane Bodin,* Nicolas Vitale,† Sophie Charrasse,*‡ and Cécile Gauthier-Rouvière*‡**

*Universités Montpellier 2 et 1, Centre de Recherche en Biochimie Macromoléculaire, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5237, Institut Fédératif de Recherche 122 1919 Route de Mende, 34293 Montpellier, France; and †Université de Strasbourg, Institut des Neurosciences Cellulaires et Intégratives, Centre National de la Recherche Scientifique Unité Propre de Recherche-3212, 5 Rue Blaise Pascal, 67084 Strasbourg, France

Submitted December 22, 2009; Revised April 26, 2010; Accepted May 19, 2010
Monitoring Editor: Jonathan Chernoff

**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 (ARF)6 GTPase has been shown to bind to Trio and to regulate Rac1 activity. Moreover, Loner/GEP$_{100}$/BRAG2, a GEF of ARF6, has been involved in mammalian and *Drosophila* myoblast fusion, but the specific role of ARF6 has been not 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*., 2008). 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 $Ca^{2+}$-dependent adhesion

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–12–1063) on May 26, 2010.

‡ These authors contributed equally to this work.

Address correspondence to: Cécile Gauthier-Rouvière (e-mail: cecile.gauthier@crbm.cnrs.fr).

Abbreviations used: DM, differentiation medium; GEF, guanine nucleotide exchange factor; GM, growth medium; Luci, luciferase; MHCd, myosin heavy chain developmental; PA, phosphatidic acid; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; PM, plasma membrane.

molecules, is one of such proteins involved in vertebrate myoblast fusion (Zeschnigk *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)6 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.*, 2001; Vitale *et al.*, 2002; D'Souza-Schorey and Chavrier, 2006; Gillingham and Munro, 2007). The resulting generation of phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)$P_2$] 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 Kalirin, 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, coimmunoprecipitation 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)$P_2$ level are important ARF6 downstream players during myoblast fusion. These results demonstrate that ARF6 is involved in myoblast fusion 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 medium 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. Calcimycin (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 at100 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 piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% IGEPAL CA-630, 1 mM EDTA, 1 mM ortho-vanadate, and protease inhibitor 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), troponin T (1:1000) and myosin (1:2000) (Sigma-Aldrich), myogenin (1:500; BD Pharmingen, San Diego, CA), α-tubulin (1:100), 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 GGA3 fused to glutathione transferase ([GST]; GST-GGA3; 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-CRIB) (Cytoskeleton, Denver, CO) bound to beads. ARF6 and Rac1 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 2 g/l gelatin and 0.25% Triton X-100. Sections were then incubated with anti-myosin heavy chain developmental (MHCd) primary antibody (1:20; Novocastra, Newcastle, United Kingdom) at 4°C overnight and revealed by incubation with Alexa 546-conjugated mouse immunoglobulin G. DNA was stained with Hoechst (0.1 μg/ml; Sigma-Aldrich). Active ARF and Rac proteins were detected through binding to ARF binding domain of ARHGAP10 fused to glutathione transferase (GST-ARHGAP10) and GST-PAK CRIB, 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 GATCCG**GTTGAAGCTGGGCCAATCG**ttcaagaga**CGATTGGCCCAGCTTC-AAC**CTTTTTTACGCGTG (top) and AATTCACGCGTAAAAAAG**GTT-GAAGCTGGGCCAATCG**tctcttgaa**CGATTGGCCCAGCTTCAAC**CG (bottom) were inserted into RNAi-Ready pSIREN-RetroQ and RNAi-Ready pSIREN-RetroQ-ZsGreen vectors (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 **ATCCTCATCTTCGCCAACA** and **TGTTGGCGAAGAT-GAGGAT** (oligonucleotides 865-873 of the ARF6 sequence) for the top strand. Retrovirus production in Phoenix cells and infection was performed as described previously (Fortier *et al.*, 2008). Stably transfected C2C12 cells with *ARF6* shRNA1 or 2 in pSIREN-RetroQ were selected in medium containing Puro (1 μg/ml), whereas cells transfected with *ARF6* shRNA1 or 2 in pSIREN-RetroQ-ZsGreen were sorted by fluorescence-activated cell sorting. Different clones were isolated by limited dilution. ARF6 inhibition was assessed in 10 random clones and a pool 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 Thermanox 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)-PLCδ-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 (Baecker, 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-PLCδ, PM-FKB, FKBP-5-ptase, or PLD1 by using Lipofectamine 2000 (Invitrogen) were fixed in 3.2% paraformaldehyde after 2–3 d in DM. After 5-min permeabilization 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, Montluçon, 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)$P_2$ Detection

Cellular PI(4,5)$P_2$ levels were measured after lipid extraction in parental, *Luci* shRNA, and *ARF6* shRNA C2C12 myoblasts at different times of differentiation by using the PI(4,5)$P_2$ Mass ELISA kit (Echelon Biosciences, Slat Lake City, UT). Proteins were extracted from the supernatant that is normally discarded after neutral lipids extraction (Wessel and Flugge, 1984). PI(4,5)$P_2$ 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

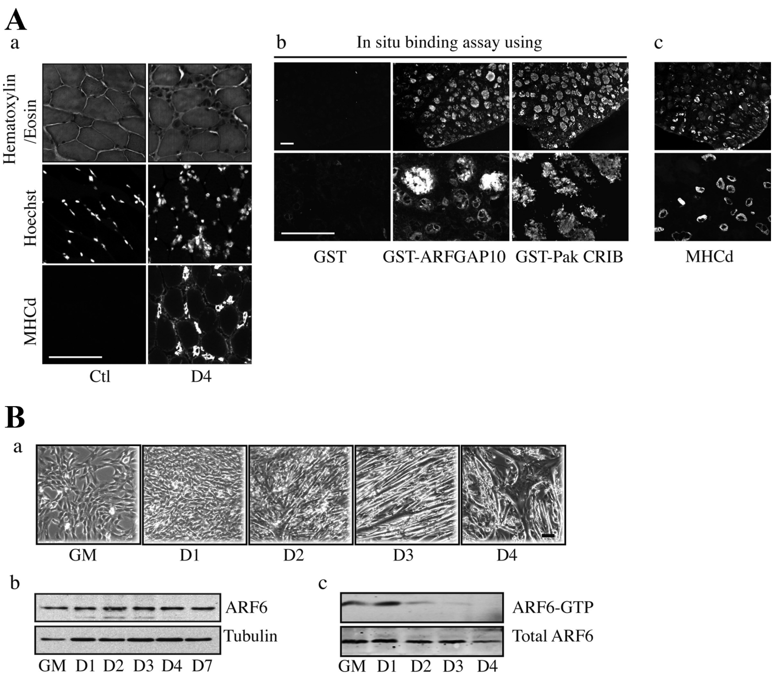
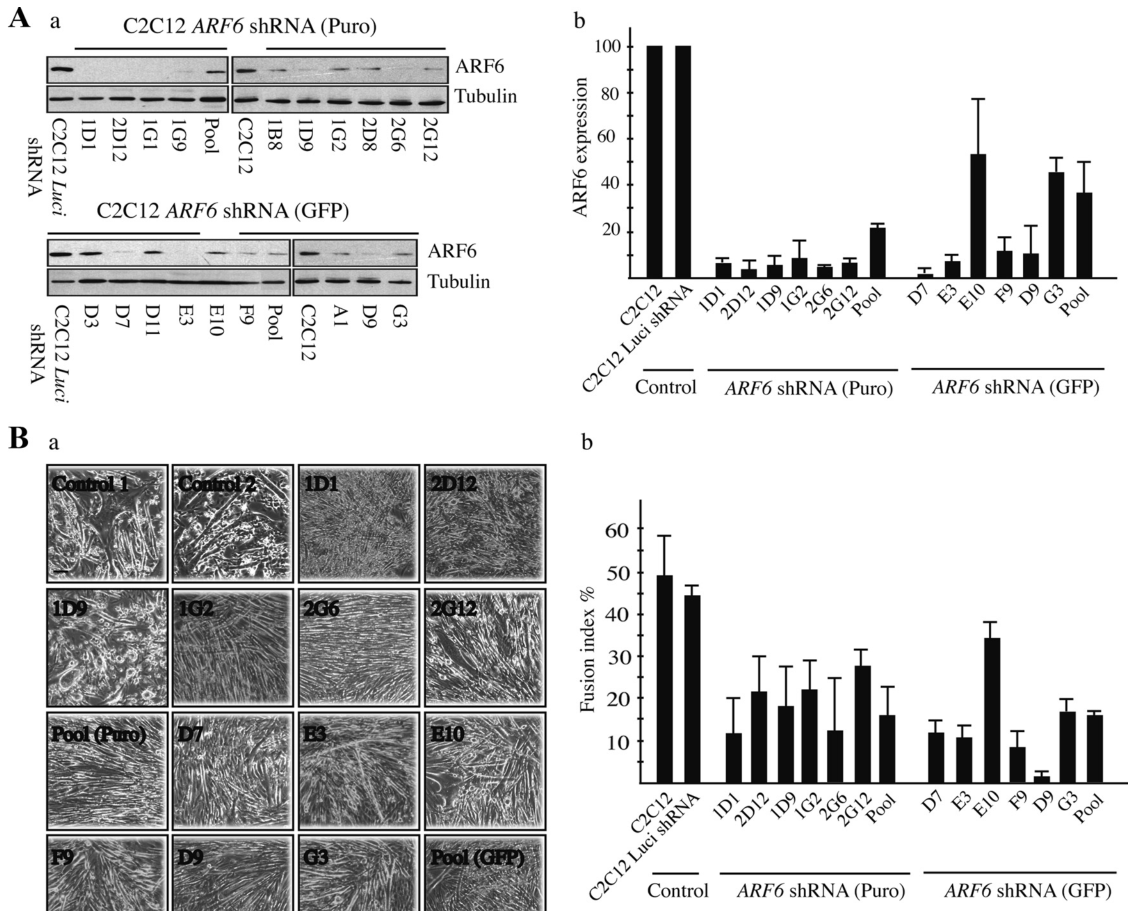



**Figure 1.** ARF6 activity during myoblast fusion. (A) ARF and Rac1/Cdc42Hs are activated during muscle regeneration. a, serial sections obtained from control (Ctl) (left) and injured tibialis anterior muscles 4 d after notexin injection (right) were stained with hematoxylin and eosin (top), with Hoechst dye to visualize DNA (middle) or incubated with anti-MHCd antibody (indirect immunofluorescence) (bottom and c). Bar, 50 μm. b, serial sections obtained from injured tibialis anterior muscles 4 d after notexin injection were incubated with GST alone (left), GST-ARHGAP10 (middle), or GST-Pak CRIB (right) for detection of endogenous ARF and Rac/Cdc42 activities. Bottom, higher magnification of the top images. At least three injured muscles were analyzed for each condition. Bar, 50 μM. c, expression of MHCd in injured muscles. (B) ARF6 is activated at the onset of myoblast fusion. a, phase contrast images of C2C12 myoblasts and myotubes at different times during differentiation; GM, growth medium; D1–D4, days in differentiation medium (DM). Bar, 30 μm. b, protein extracts (50 μg/well) from C2C12 myoblasts, collected at the indicated times, were immunoblotted for the assessment of ARF6 and α-tubulin expression. (C) Level of GTP-bound ARF6 was measured using GST-ARHGAP10 in lysates obtained from C2C12 myoblasts in GM or in DM and collected at the indicated times. ARF6 was detected by immunoblotting.

observed, GST-ARHGAP10 strongly associated with regenerating fibers (Figure 1Ab). Because ARF6, but not ARF1, activates Rac1 (Santy 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.



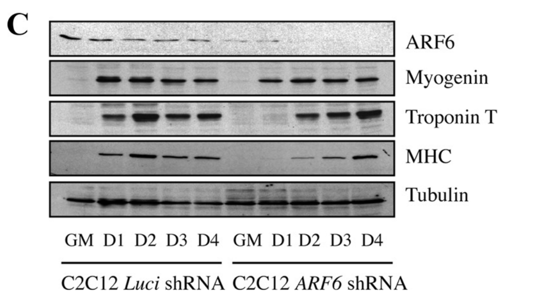




**Figure 2.** Inhibition of *ARF6* expression by RNA interference impairs myotube formation. (A) a, *Luci* and *ARF6* shRNAs were delivered in C2C12 myoblasts by retroviral infection. After selection, a pool and 10 clones of resistant cells were analyzed for the expression of ARF6 and $\alpha$-tubulin 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 $\alpha$-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 $\mu$m. 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 $\mu$g) 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 $\alpha$-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

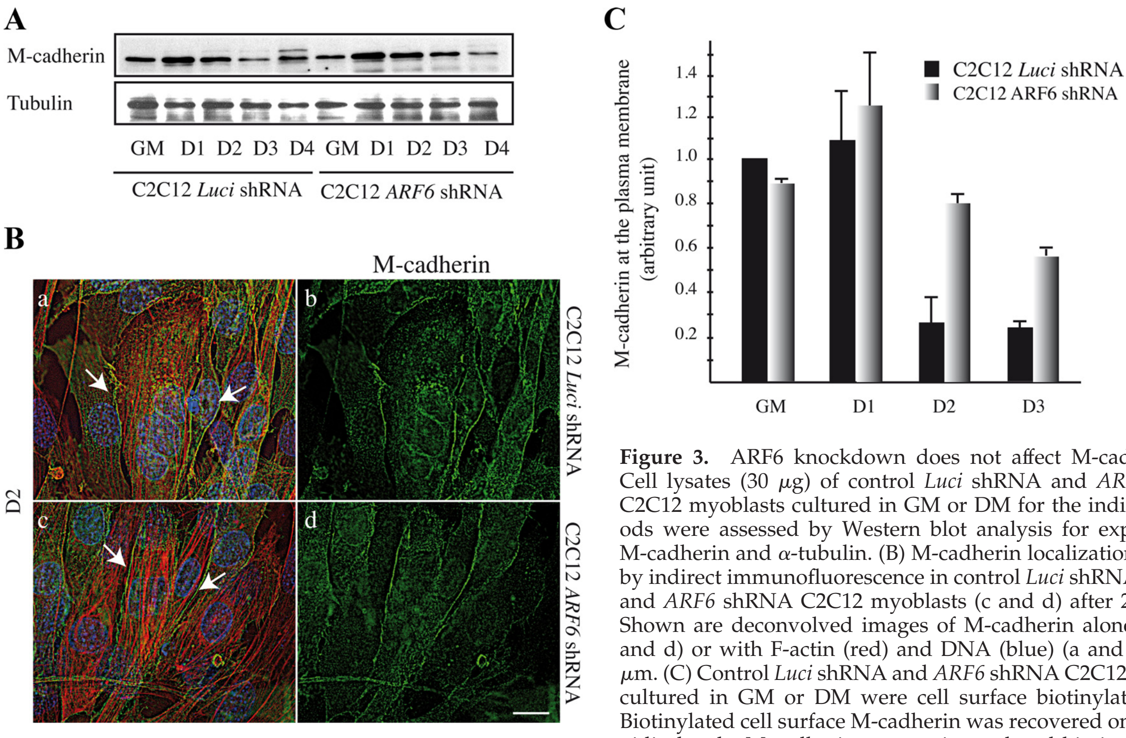



**Figure 3.** ARF6 knockdown does not affect M-cadherin. (A) Cell lysates (30 $\mu$g) of control *Luci* shRNA and *ARF6* shRNA C2C12 myoblasts cultured in GM or DM for the indicated periods were assessed by Western blot analysis for expression of M-cadherin and $\alpha$-tubulin. (B) M-cadherin localization 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 $\mu$m. (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.

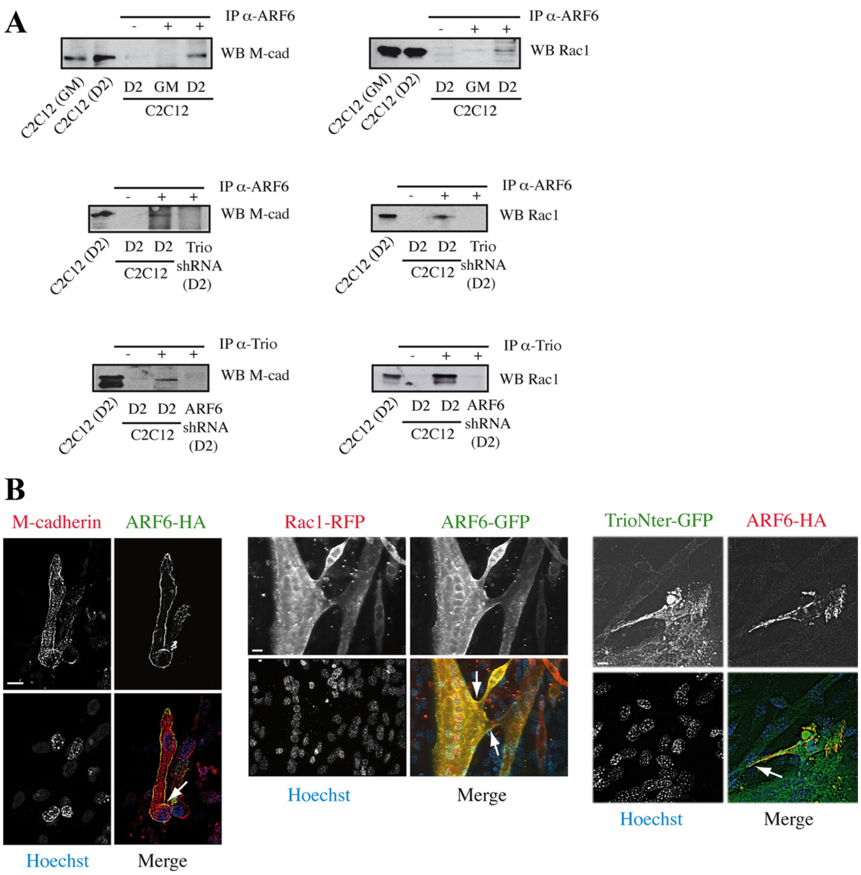



**Figure 4.** ARF6 and Rac1 are complexed with M-cadherin at the onset of myoblast fusion. (A) Cell lysates of control, *ARF6* shRNA or *Trio* shRNA C2C12 myoblasts cultured in GM or after 2 d in DM (D2) were immunoprecipitated (+) or not (−) using anti-ARF6 or anti-Trio antibodies and immunoblotted for the presence of endogenous M-cadherin (M-cad) or Rac1. (B) C2C12 myoblasts were either transfected with HA- or GFP-ARF6, RFP-Rac1 or GFP-TrioNter. HA tag and M-cadherin were revealed by indirect immunofluorescence after 3 d in DM. Stacks of images were deconvolved using the Huygens System image restoration software. Arrows show colocalization at regions likely to be fusion sites. Bar, 10 $\mu$m.

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). M-cadherin, Rac1, and Trio-Nter colocalized with ARF6 at regions likely to be fusion sites (Figure 4B, arrows).

### *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 5Ac′). In contrast, in *ARF6* shRNA myoblasts, the formation of these membrane morphological modifications was reduced (Figure 5Af′). 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 myoblasts and myotubes and accumulate at the fusion zone (arrow in Figure 4Bb), 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

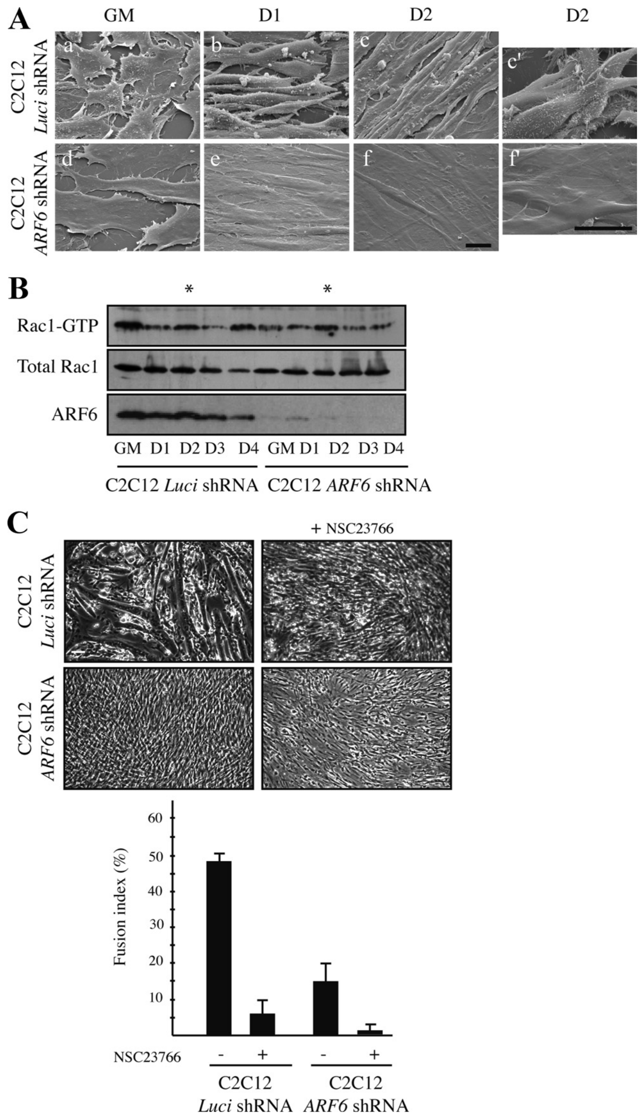



**Figure 5.** Rac1 activity is maintained in *ARF6* knockdown myoblasts. (A) Scanning electron micrographs of parental and *ARF6* shRNA C2C12 myoblasts cultured in GM or DM for the indicated periods. Bar, 10 $\mu$m. (B) GTP-bound Rac1 was measured in lysates obtained from control *Luci* shRNA and *ARF6 shRNA* C2C12 myoblasts in GM or DM. Rac1 and ARF6 were detected by Western blot analysis. Asterisks represent the increase of active Rac1 at the onset of myoblast fusion. Data are representative of four independent experiments. (C) Phase contrast images of *Luci* shRNA and *ARF6* shRNA myoblasts treated or not with the Rac1 inhibitor NSC23766 and cultured in DM for 3 d. The histogram represents the fusion index calculated from three independent experiments. Bar, 30 $\mu$m.

cells 4 h after DM addition to avoid an effect on myogenesis induction (Meriane *et al.*, 2000), and fusion index was analyzed. In *ARF6* shRNA C2C12 myoblasts, NSC23766 completely inhibited myoblast fusion (Figure 5C). Together, these data suggest that the defect in myoblast fusion in ARF6 shRNA myoblasts is not due to an inhibition of Rac1 activity.

### *PLD1 Activation Is Essential for Myoblast Fusion and Requires ARF6*

In addition to Rac1, ARF6 also activates PLD1 in vitro (Massenburg *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.

### *ARF6 Silencing Decreases PI(4,5)$P_2$ Level during Myogenesis*

ARF6 also directly activates PIP5K in vitro to generate PI(4,5)$P_2$ (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)$P_2$ in *Luci* shRNA and *ARF6* shRNA C2C12 myoblasts (Figure 7A). At D1 after the switch to DM, the amount of PI(4,5)$P_2$ increased in control cells but not in *ARF6* shRNA cells. To investigate the localization of PI(4,5)$P_2$, C2C12 myoblasts were transfected with the construct PH-PLC$\delta$-GFP that specifically binds to PI(4,5)$P_2$ (Holz *et al.*, 2000). In agreement with a previous report, PI(4,5)$P_2$ was essentially detected at the PM and concentrated in fluorescent structures that correlated with dynamic ruffles and dorsal membrane projections (Nowak *et al.*, 2009). Moreover, PI(4,5)$P_2$ accumulated at myoblast–myotube and myotube–myotube contacts where it colocalized with M-cadherin (Figure 7B, arrows). A decrease in the accumulation of PH-PLC$\delta$-GFP was observed in *ARF6* shRNA myoblasts (Figure 7C). Moreover, PI(4,5)$P_2$ accumulation at cell–cell contacts between myotubes was followed by their fusion (Supplemental Videos 3 and 4). These data indicate that the increase of PI(4,5)$P_2$ occurring during myoblast differentiation requires ARF6. Furthermore they suggest that PI(4,5)$P_2$ accumulation at cell–cell contacts could participate in the fusion process. To

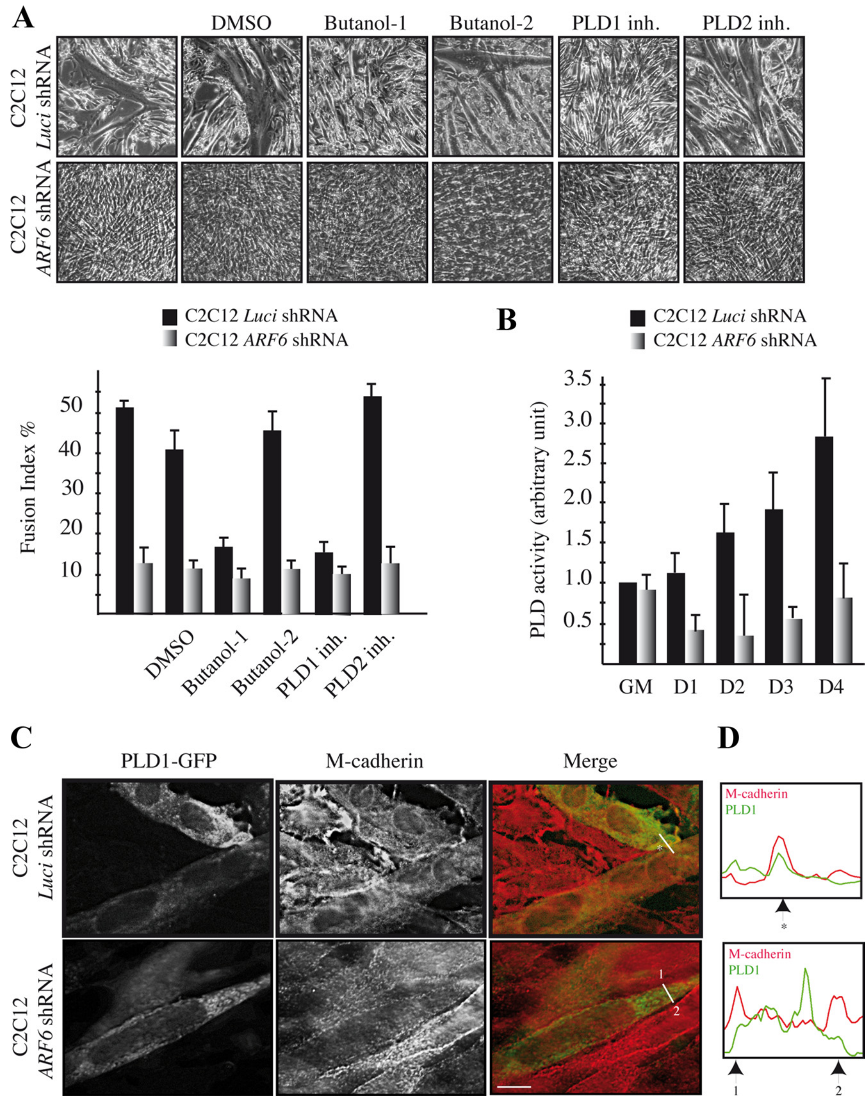



**Figure 6.** PLD activity is decreased in *ARF6* knockdown myoblasts. (A) *Luci* shRNA and *ARF6* shRNA C2C12 myoblasts were cultured in DM with DMSO alone or 0.4% of 1-butanol or butanol-2, or 10 $\mu$M PLD1 or PLD2 inhibitors for 4 d. Phase contrast images are shown and the histogram represents the fusion index calculated from three independent experiments. (B) *Luci shRNA* and *ARF6* shRNA C2C12 myoblasts in GM or cultured in DM as indicated were assessed for relative PLD activity and normalized to protein content. At least three independent experiments were analyzed. (C) M-cadherin expression was assessed by indirect immunofluorescence in *Luci shRNA* and *ARF6* shRNA C2C12 myoblasts transfected with GFP-PLD1 and cultured in DM for 2 d. (D) Colocalization of PLD1 and M-cadherin signals was analyzed along the line shown in the "merge" panels in C by line scan (MetaMorph software).

confirm this point, we treated C2C12 myoblasts with agents that reduce (calcimycin, LiCl) or mask (neomycin) PI(4,5)$P_2$ (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)$P_2$ reduction on myoblast fusion, we generated by retroviral infection stable C2C12 cell lines in which the expression of the cadherin-associated *PIP5KIγ* 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 *PIP5KIγ* shRNA cells in comparison with controls (Supplemental Figure 3). Finally, we examined whether PI(4,5)$P_2$ reduction affected Rac1 and Trio-Nter localization at cell–cell contacts of myoblasts at fusion time. PI(4,5)$P_2$ at the PM was depleted by rapamycin-induced PM translocation of a type IV phosphoinositide 5-phosphatase (5-ptase) (Varnai *et al*., 2006) after heterodimerization of PM-FRB-monomeric (m)RFP (the FRB domain of mammalian target of rapamycin [mTOR] binds to FKBP and is a rapamycin target) with mRFP-FKBP-5-ptase. The efficiency of PI(4,5)$P_2$ reduction was monitored by analyzing the dissociation of PH-PLCδ-GFP from the PM. In C2C12 myoblasts cotransfected with PM-FRB-mRFP, mRFP-FKBP-5-ptase, and PH-PLCδ-GFP, PH-PLCδ-GFP was no more accumulated at cell contacts after rapamycin addition compared with control myoblasts (Figure 8B), indicating a loss of PI(4,5)$P_2$ at the PM. Then, C2C12 myoblasts were cotransfected with PM-FRB-mRFP, mRFP-FKBP-5-ptase 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 and f to g and h) but did not affect it in nontransfected cells (data not shown). Together, these data suggest that PI(4,5)$P_2$ 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

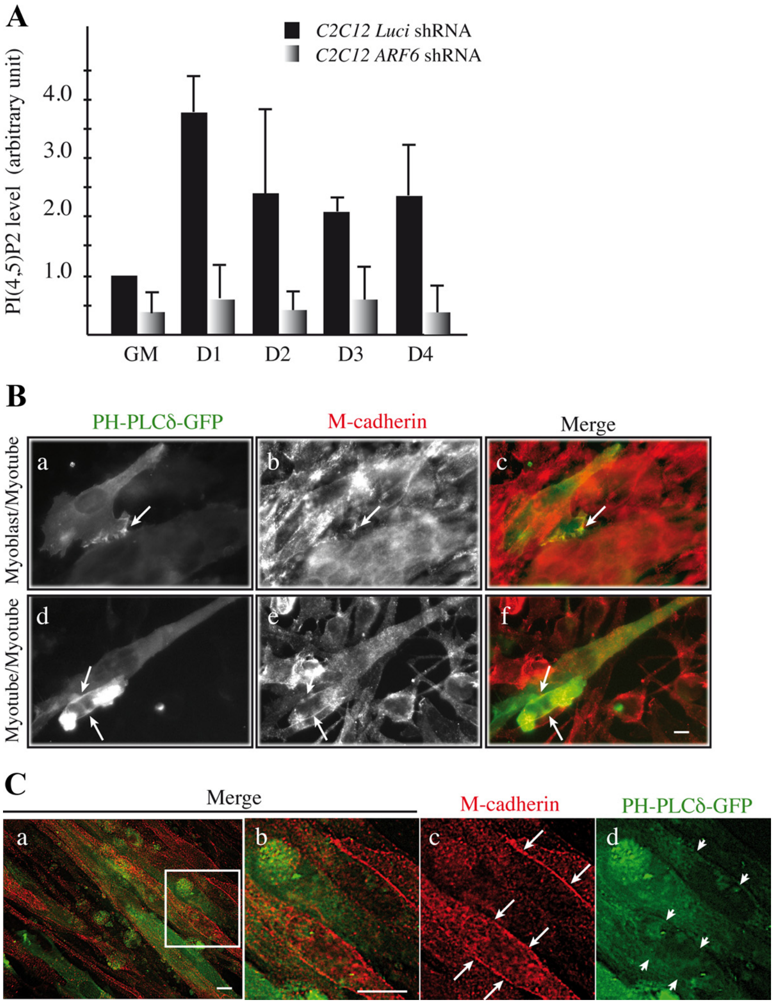
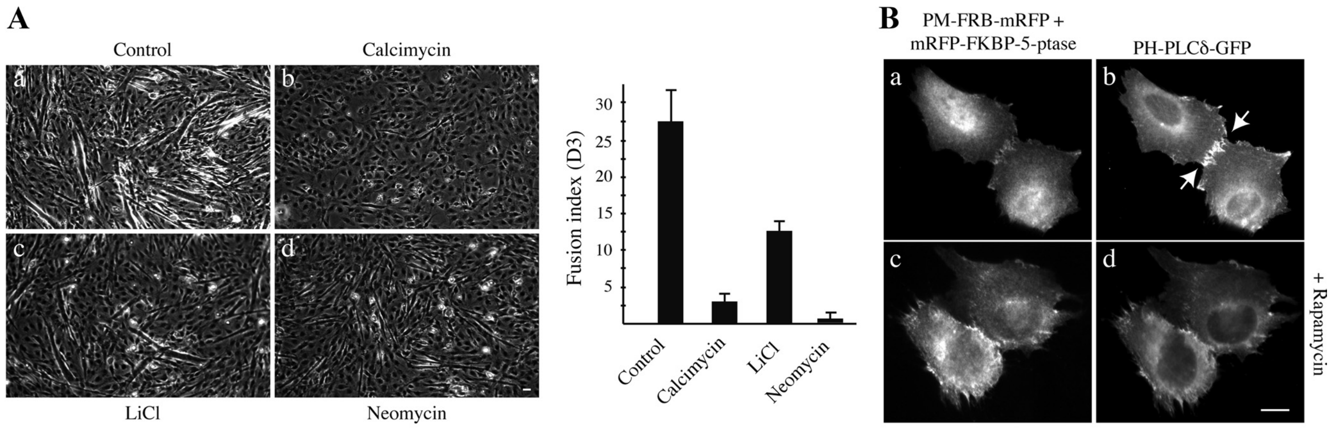



**Figure 7.** $PI(4,5)P_2$ level is reduced in *ARF6* knockdown myoblasts. (A) *Luci* shRNA and *ARF6* shRNA C2C12 myoblasts cultured in GM or at different time points after DM addition (D1–D4) were analyzed for $PI(4,5)P_2$ content. The histogram shows the amount of $PI(4,5)P_2$ normalized to the total amount of protein in the lysate. At least three independent experiments were analyzed. (B) C2C12 myoblasts were transfected with PH-PLCδ-GFP (a and d), cultured in DM for 2 d and M-cadherin expression was evaluated by indirect immunofluorescence (b and e). M-cadherin and $PI(4,5)P_2$ colocalize (arrows) at myoblast/myotube (a to c) and myotube/myotube (panels d to f) cell–cell contacts. Bar, 10 μm. (C) C2C12 *ARF6* shRNA myoblasts were transfected with PH-PLCδ-GFP (green in a, b, and d), cultured in DM for 2 d, and M-cadherin expression was assessed by indirect immunofluorescence (red in a–c). b–d correspond to the selected area in a. Shown are deconvolved images. Bar, 10 μm.

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 $PI(4,5)P_2$ 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 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-Diaz *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.*,



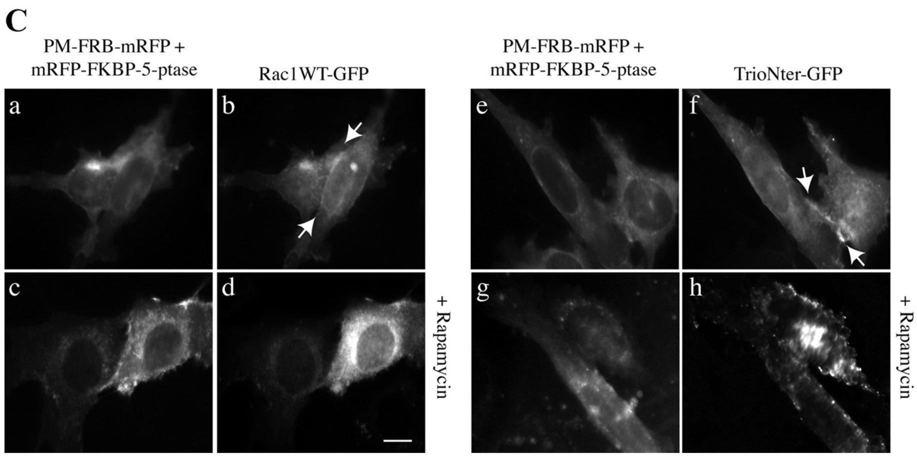




**Figure 8.** $PI(4,5)P_2$ reduction inhibits myoblast fusion. (A) C2C12 myoblasts were cultured in DM complemented with agents that reduce (calcimycin and LiCl) or mask (neomycin) $PI(4,5)P_2$, and fusion was analyzed after 3 d. Treatment times were as follows: calcimycin for 24 h and LiCl and neomycin 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 $\mu$m. (B) C2C12 myoblasts were transfected with the PH-PLCδ-GFP plasmid to monitor $PI(4,5)P_2$ reduction together with the membrane-targeted FRB fragment of mTOR (PM-FRB-mRFP) and cytosolic 5-ptase fused to FKBP (mRFP-FKBP-5-ptase). Heterodimerization of PM-FRB with FKBP-5-ptase upon rapamycin addition causes PM recruitment of the enzyme and rapid dephosphorylation of $PI(4,5)P_2$. Addition of 100 nM rapamycin induces translocation of the 5-ptase to the membrane (c), causing a loss of PH-PLCδ-GFP localization (d) compared with untreated cells (a and b). Bar, 10 $\mu$m. (C) C2C12 myoblasts were transfected with PM-FRB-mRFP together with mRFP-FKBP-5-ptase 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 $\mu$m.

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. PLD hydrolyzes phosphatidylcholine to form choline and PA (Cockcroft *et al*., 1994; Exton, 1998). PA is well known to promote endocytosis, exocytosis, and secretion and to increase the rate of vesicle fusion (Donaldson, 2009). PA is a bioactive lipid that functions in signal transduction events, such as activation of a phosphoinositide kinase to produce $PI(4,5)P_2$, or as a lipid-binding partner to several proteins (Jenkins and Frohman, 2005). Due to their conical-shaped lipids, PA and/or its metabolites diacylglycerol and lysophosphatidate modulate membrane curvature and may function in membranes fusion (Lundmark *et al*., 2008). Moreover, ARF6 and PA activate PIP5K and generate $PI(4,5)P_2$ at PM, which controls dynamic membrane function and cell shape (Raucher *et al*., 2000; Brown *et al*., 2001). Interestingly, we observed that $PI(4,5)P_2$ accumulates at cell–cell contact sites before myoblast fusion. Moreover, $PI(4,5)P_2$ level and accumulation at cell–cell contacts is decreased in *ARF6* knockdown C2C12 myoblasts and $PI(4,5)P_2$ reduction decreases myoblast fusion. This defect in $PI(4,5)P_2$ 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 $PI(4,5)P_2$-enriched domains (Skowronek *et al*., 2004; Macia *et al*., 2008). We observed that $PI(4,5)P_2$ reduction at the PM impaired Rac1 and Trio recruitment at regions likely to be fusion sites. $PI(4,5)P_2$ 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). $PI(4,5)P_2$ also might recruit $Ca^{2+}$-dependent activator protein for secretion, which enables exocytosis of dense core vesicles in neuroendocrine cells (Grishanin *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 PIP5K$\gamma$ at sites of N-cadherin ligation resulting in PI(4,5)$P_2$ 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 participates in PI(4,5)$P_2$ 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)$P_2$ production. All these factors participate in myoblast fusion through the reorganization of the actin cytoskeleton and of PM dynamics.

## ACKNOWLEDGMENTS

We thank the Imaging facility (http://www.mri.cnrs.fr/). We are grateful to Anne Debant (CNRS) for the Trio-Nter construct; Michel Franco (CNRS) and Philippe Chavrier (CNRS) for the ARF6 constructs; H. Alex Brown (Vanderbilt University) and Craig W. Lindsley (Vanderbilt University) for the PLD1 and PLD2 inhibitors; and Tamas Balla (National Institutes of Health) for PM-FRB-mRFP and mRFP-FKBP-5-ptase constructs. This work was supported by the Agence Nationale de la Recherche, the Association Française contre les Myopathies and the Ligue Nationale contre le Cancer (Equipe labelisée LIGUE 2008). C.G.R. was supported by WASP-interacting protein and S. E. was supported by Agence Nationale de la Recherche.

## REFERENCES

Baecker, V., and Travo, P. (2006). Cell Image Analyzer-a visual scripting interface for ImageJ and its usage at the microscope facility Montpellier RIO Imaging. Proc. ImageJ User Developer Conf. *1*, 105–110.

Béglé, A., Tryoen-Toth, P., de Barry, J., Bader, M., and Vitale, N. (2009). ARF6 regulates the synthesis of fusiogenic lipids for calcium-regulated exocytosis in neuroendocrine cells. J. Biol. Chem. *284*, 4836–4845.

Berger, S., Schafer, G., Kesper, D. A., Holz, A., Eriksson, T., Palmer, R. H., Beck, L., Klambt, C., Renkawitz-Pohl, R., and Onel, S. F. (2008). WASP and SCAR have distinct roles in activating the Arp2/3 complex during myoblast fusion. J. Cell Sci. *121*, 1303–1313.

Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T., and Donaldson, J. G. (2001). Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. J. Cell Biol. *154*, 1007–1017.

Bryan, B. A., Mitchell, D. C., Zhao, L., Ma, W., Stafford, L. J., Teng, B. B., and Liu, M. (2005). Modulation of muscle regeneration, myogenesis, and adipogenesis by the Rho family guanine nucleotide exchange factor GEFT. Mol. Cell Biol. *25*, 11089–11101.

Buckingham, M. (2006). Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr. Opin. Genet. Dev. *16*, 525–532.

Causeret, F., Hidalgo-Sanchez, N., Fort, P., Backer, S., Popoff, M. R., Gauthier-Rouviere, C., and Bloch-Gallego, E. (2004). Distinct roles of Rac1/Cdc42 and Rho/Rock for axon outgrowth and nucleokinesis of precerebellar neurons toward netrin 1. Development *131*, 2841–2852.

Charrasse, S., Comunale, F., Fortier, M., Portales-Casamar, E., Debant, A., and Gauthier-Rouviere, C. (2007). M-cadherin activates Rac1 GTPase through the Rho-GEF trio during myoblast fusion. Mol. Biol. Cell *18*, 1734–1743.

Charrasse, S., Comunale, F., Grumbach, Y., Poulat, F., Blangy, A., and Gauthier-Rouviere, C. (2006). RhoA GTPase regulates M-cadherin activity and myoblast fusion. Mol. Biol. Cell *17*, 749–759.

Charrasse, S., Meriane, M., Comunale, F., Blangy, A., and Gauthier-Rouviere, C. (2002). N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. J. Cell Biol. *158*, 953–965.

Chen, E. H., and Olson, E. N. (2005). Unveiling the mechanisms of cell-cell fusion. Science *308*, 369–373.

Chen, E. H., Pryce, B. A., Tzeng, J. A., Gonzalez, G. A., and Olson, E. N. (2003). Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector ARF6. Cell *114*, 751–762.

Cifuentes-Diaz, C., Nicolet, M., Alameddine, H., Goudou, D., Dehaupas, M., Rieger, F., and Mege, R. M. (1995). M-cadherin localization in developing adult and regenerating mouse skeletal muscle: possible involvement in secondary myogenesis. Mech. Dev. *50*, 85–97.

Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994). Phospholipase D: a downstream effector of ARF in granulocytes. Science *263*, 523–526.

D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. Nat. Rev. Mol. Cell Biol. *7*, 347–358.

Doberstein, S. K., Fetter, R. D., Mehta, A. Y., and Goodman, C. S. (1997). Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. J. Cell Biol. *136*, 1249–1261.

Donaldson, J. G. (2003). Multiple roles for Arf 6, sorting, structuring, and signaling at the plasma membrane. J. Biol. Chem. *278*, 41573–41576.

Donaldson, J. G. (2008). Arfs and membrane lipids: sensing, generating and responding to membrane curvature. Biochem. J. *414*, e1–e2.

Donaldson, J. G. (2009). Phospholipase D in endocytosis and endosomal recycling pathways. Biochim. Biophys. Acta *1791*, 845–849.

Dyer, N., Rebollo, E., Dominguez, P., Elkhatib, N., Chavrier, P., Daviet, L., Gonzalez, C., and Gonzalez-Gaitan, M. (2007). Spermatocyte cytokinesis requires rapid membrane addition mediated by ARF6 on central spindle recycling endosomes. Development *134*, 4437–4447.

El Sayegh, T. Y., Arora, P. D., Ling, K., Laschinger, C., Janmey, P. A., Anderson, R. A., and McCulloch, C. A. (2007). Phosphatidylinositol-4,5 bisphosphate produced by PIP5KIgamma regulates gelsolin, actin assembly, and adhesion strength of N-cadherin junctions. Mol. Biol. Cell *18*, 3026–3038.

Erickson, M. R., Galletta, B. J., and Abmayr, S. M. (1997). Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. J. Cell Biol. *138*, 589–603.

Estrach, S., Schmidt, S., Diriong, S., Penna, A., Blangy, A., Fort, P., and Debant, A. (2002). The Human Rho-GEF trio and its target GTPase RhoG are involved in the NGF pathway, leading to neurite outgrowth. Curr. Biol. *12*, 307–312.

Exton, J. H. (1998). Phospholipase D. Biochim. Biophys. Acta *1436*, 105–115.

Fernandes, J. J., Atreya, K. B., Desai, K. M., Hall, R. E., Patel, M. D., Desai, A. A., Benham, A. E., Mable, J. L., and Straessle, J. L. (2005). A dominant negative form of Rac1 affects myogenesis of adult thoracic muscles in *Drosophila*. Dev. Biol. *285*, 11–27.

Fortier, M., Comunale, F., Kucharczak, J., Blangy, A., Charrasse, S., and Gauthier-Rouviere, C. (2008). RhoE controls myoblast alignment prior fusion through RhoA and ROCK. Cell Death Differ. *15*, 1221–1231.

Gabev, E., Kasianowicz, J., Abbott, T., and McLaughlin, S. (1989). Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP2). Biochim. Biophys. Acta *979*, 105–112.

Gao, Y., Dickerson, J. B., Guo, F., Zheng, J., and Zheng, Y. (2004). Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc. Natl. Acad. Sci. USA *101*, 7618–7623.

Gillingham, A. K., and Munro, S. (2007). The small G proteins of the Arf family and their regulators. Annu. Rev. Cell Dev. Biol. *23*, 579–611.

Griffin, H. D., and Hawthorne, J. N. (1978). Calcium-activated hydrolysis of phosphatidyl-myo-inositol 4-phosphate and phosphatidyl-myo-inositol 4,5-bisphosphate in guinea-pig synaptosomes. Biochem. J. *176*, 541–552.

Grishanin, R. N., Kowalchyk, J. A., Klenchin, V. A., Ann, K., Earles, C. A., Chapman, E. R., Gerona, R. R., and Martin, T. F. (2004). CAPS acts at a prefusion step in dense-core vesicle exocytosis as a PIP2 binding protein. Neuron *43*, 551–562.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L., and Dickson, B. J. (2002). Rac function and regulation during *Drosophila* development. Nature *416*, 438–442.

Hall, A. (1998). G proteins and small GTPases: distant relatives keep in touch. Science *280*, 2074–2075.

Hallcher, L. M., and Sherman, W. R. (1980). The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. J. Biol. Chem. *255*, 10896–10901.

Hiroi, T., Someya, A., Thompson, W., Moss, J., and Vaughan, M. (2006). GEP100/BRAG 2, activator of ADP-ribosylation factor 6 for regulation of cell adhesion and actin cytoskeleton via E-cadherin and alpha-catenin. Proc. Natl. Acad. Sci. USA *103*, 10672–10677.

Hollnagel, A., Grund, C., Franke, W. W., and Arnold, H. H. (2002). The cell adhesion molecule M-cadherin is not essential for muscle development and regeneration. Mol. Cell. Biol. *22*, 4760–4770.

Holz, R. W., Hlubek, M. D., Sorensen, S. D., Fisher, S. K., Balla, T., Ozaki, S., Prestwich, G. D., Stuenkel, E. L., and Bittner, M. A. (2000). A pleckstrin homology domain specific for phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis. J. Biol. Chem. *275*, 17878–17885.

Honda, A., *et al*. (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell *99*, 521–532.

Hornberger, T. A., Chu, W. K., Mak, Y. W., Hsiung, J. W., Huang, S. A., and Chien, S. (2006). The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proc. Natl. Acad. Sci. USA *103*, 4741–4746.

Horsley, V., and Pavlath, G. K. (2004). Forming a multinucleated cell: molecules that regulate myoblast fusion. Cells Tissues Organs *176*, 67–78.

Jenkins, G. M., and Frohman, M. A. (2005). Phospholipase D: a lipid centric review. Cell. Mol. Life Sci. *62*, 2305–2316.

Kesper, D. A., Stute, C., Buttgereit, D., Kreiskother, N., Vishnu, S., Fischbach, K. F., and Renkawitz-Pohl, R. (2007). Myoblast fusion in *Drosophila melanogaster* is mediated through a fusion-restricted myogenic-adhesive structure (FuRMAS). Dev. Dyn. *236*, 404–415.

Kim, S., Shilagardi, K., Zhang, S., Hong, S. N., Sens, K. L., Bo, J., Gonzalez, G. A., and Chen, E. H. (2007). A critical function for the actin cytoskeleton in targeted exocytosis of prefusion vesicles during myoblast fusion. Dev. Cell *12*, 571–586.

Knudsen, K. A., and Horwitz, A. F. (1977). Tandem events in myoblast fusion. Dev. Biol. *58*, 328–338.

Komati, H., Naro, F., Mebarek, S., De Arcangelis, V., Adamo, S., Lagarde, M., Prigent, A. F., and Nemoz, G. (2005). Phospholipase D is involved in myogenic differentiation through remodeling of actin cytoskeleton. Mol. Biol. Cell *16*, 1232–1244.

Koo, T. H., Eipper, B. A., and Donaldson, J. G. (2007). Arf6 recruits the Rac GEF Kalirin to the plasma membrane facilitating Rac activation. BMC Cell Biol. *8*, 29.

Krauss, R. S. (2007). Evolutionary conservation in myoblast fusion. Nat. Genet. *39*, 704–705.

Krauss, R. S., Cole, F., Gaio, U., Takaesu, G., Zhang, W., and Kang, J. S. (2005). Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact. J. Cell Sci. *118*, 2355–2362.

Laurin, M., Fradet, N., Blangy, A., Hall, A., Vuori, K., and Cote, J. F. (2008). The atypical Rac activator Dock180 (Dock1) regulates myoblast fusion in vivo. Proc. Natl. Acad. Sci. USA *105*, 15446–15451.

Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D., and Caroni, P. (2000). GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J. Cell Biol. *149*, 1455–1472.

Ling, K., Bairstow, S. F., Carbonara, C., Turbin, D. A., Huntsman, D. G., and Anderson, R. A. (2007). Type Igamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin. J. Cell Biol. *176*, 343–353.

Lundmark, R., Doherty, G. J., Vallis, Y., Peter, B. J., and McMahon, H. T. (2008). Arf family GTP loading is activated by, and generates, positive membrane curvature. Biochem. J. *414*, 189–194.

Luo, L., Liao, Y. J., Jan, L. Y., and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. *8*, 1787–1802.

Macia, E., Partisani, M., Favard, C., Mortier, E., Zimmermann, P., Carlier, M. F., Gounon, P., Luton, F., and Franco, M. (2008). The pleckstrin homology domain of the Arf6-specific exchange factor EFA6 localizes to the plasma membrane by interacting with phosphatidylinositol 4,5-bisphosphate and F-actin. J. Biol. Chem. *283*, 19836–19844.

Mary, S., Charrasse, S., Meriane, M., Comunale, F., Travo, P., Blangy, A., and Gauthier-Rouviere, C. (2002). Biogenesis of N-cadherin-dependent cell-cell contacts in living fibroblasts is a microtubule-dependent kinesin-driven mechanism. Mol. Biol. Cell *13*, 285–301.

Massarwa, R., Carmon, S., Shilo, B. Z., and Schejter, E. D. (2007). WIP/WASp-based actin-polymerization machinery is essential for myoblast fusion in *Drosophila*. Dev. Cell *12*, 557–569.

Massenburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J., and Vaughan, M. (1994). Activation of rat brain phospholipase D by ADP-ribosylation factors 1,5, and *6*, separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. Proc. Natl. Acad. Sci. USA *91*, 11718–11722.

Mebarek, S., Komati, H., Naro, F., Zeiller, C., Alvisi, M., Lagarde, M., Prigent, A. F., and Nemoz, G. (2007). Inhibition of de novo ceramide synthesis up-regulates phospholipase D and enhances myogenic differentiation. J. Cell Sci. *120*, 407–416.

Melendez, A. J., Harnett, M. M., and Allen, J. M. (2001). Crosstalk between ARF6 and protein kinase Calpha in Fc(gamma)RI-mediated activation of phospholipase D1. Curr. Biol. *11*, 869–874.

Menon, S. D., and Chia, W. (2007). Actin on multiple fronts to generate a muscle fiber. Dev. Cell *12*, 479–481.

Meriane, M., Roux, P., Primig, M., Fort, P., and Gauthier-Rouviere, C. (2000). Critical activities of Rac1 and Cdc42Hs in skeletal myogenesis: antagonistic effects of JNK and p38 pathways. Mol. Biol. Cell *11*, 2513–2528.

Moore, C. A., Parkin, C. A., Bidet, Y., and Ingham, P. W. (2007). A role for the Myoblast city homologues Dock1 and Dock5 and the adaptor proteins Crk and Crk-like in zebrafish myoblast fusion. Development *134*, 3145–3153.

Moore, R., and Walsh, F. S. (1993). The cell adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal muscle. Development *117*, 1409–1420.

Moraczewski, J., Archacka, K., Brzoska, E., Ciemerych, M. A., Grabowska, I., Janczyk-Ilach, K., Streminska, W., and Zimowska, M. (2008). From planarians to mammals—the many faces of regeneration. Int. J. Dev. Biol. *52*, 219–227.

Myers, K. R., and Casanova, J. E. (2008). Regulation of actin cytoskeleton dynamics by Arf-family GTPases. Trends Cell Biol. *18*, 184–192.

Nolan, K. M., Barrett, K., Lu, Y., Hu, K. Q., Vincent, S., and Settleman, J. (1998). Myoblast city, the *Drosophila* homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes. Genes Dev. *12*, 3337–3342.

Nowak, S. J., Nahirney, P. C., Hadjantonakis, A. K., and Baylies, M. K. (2009). Nap1-mediated actin remodeling is essential for mammalian myoblast fusion. J. Cell Sci. *122*, 3282–3293.

O'Brien, S. P., Seipel, K., Medley, Q. G., Bronson, R., Segal, R., and Streuli, M. (2000). Skeletal muscle deformity and neuronal disorder in Trio exchange factor-deficient mouse embryos. Proc. Natl. Acad. Sci. USA *97*, 12074–12078.

Pajcini, K. V., Pomerantz, J. H., Alkan, O., Doyonnas, R., and Blau, H. M. (2008). Myoblasts and macrophages share molecular components that contribute to cell-cell fusion. J. Cell Biol. *180*, 1005–1019.

Palacios, F., Price, L., Schweitzer, J., Collard, J. G., and D'Souza-Schorey, C. (2001). An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. EMBO J. *20*, 4973–4986.

Palacios, F., Schweitzer, J. K., Boshans, R. L., and D'Souza-Schorey, C. (2002). ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat. Cell Biol. *4*, 929–936.

Peckham, M. (2008). Engineering a multi-nucleated myotube, the role of the actin cytoskeleton. J. Microsc. *231*, 486–493.

Radhakrishna, H., Al-Awar, O., Khachikian, Z., and Donaldson, J. G. (1999). ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. J. Cell Sci. *112*, 855–866.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P., and Meyer, T. (2000). Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. Cell *100*, 221–228.

Richardson, B. E., Beckett, K., Nowak, S. J., and Baylies, M. K. (2007). SCAR/WAVE and Arp2/3 are crucial for cytoskeletal remodeling at the site of myoblast fusion. Development *134*, 4357–4367.

Richardson, B. E., Nowak, S. J., and Baylies, M. K. (2008). Myoblast fusion in fly and vertebrates: new genes, new processes and new perspectives. Traffic *9*, 1050–1059.

Rose, O., Rohwedel, J., Reinhardt, S., Bachmann, M., Cramer, M., Rotter, M., Wobus, A., and Starzinski-Powitz, A. (1994). Expression of M-cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cells in culture. Dev. Dyn. *201*, 245–259.

Santy, L. C., and Casanova, J. E. (2001). Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. J. Cell Biol. *154*, 599–610.

Santy, L. C., Ravichandran, K. S., and Casanova, J. E. (2005). The DOCK180/Elmo complex couples ARNO-mediated Arf6 activation to the downstream activation of Rac1. Curr. Biol. *15*, 1749–1754.

Scott, S. A., Selvy, P. E., Buck, J. R., Cho, H. P., Criswell, T. L., Thomas, A. L., Armstrong, M. D., Arteaga, C. L., Lindsley, C. W., and Brown, H. A. (2009). Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. Nat. Chem. Biol. *5*, 108–117.

Skowronek, K. R., Guo, F., Zheng, Y., and Nassar, N. (2004). The C-terminal basic tail of RhoG assists the guanine nucleotide exchange factor trio in binding to phospholipids. J. Biol. Chem. *279*, 37895–37907.

Sohn, R. L., Huang, P., Kawahara, G., Mitchell, M., Guyon, J., Kalluri, R., Kunkel, L. M., and Gussoni, E. (2009). A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. Proc. Natl. Acad. Sci. USA *106*, 9274–9279.

Srinivas, B. P., Woo, J., Leong, W. Y., and Roy, S. (2007). A conserved molecular pathway mediates myoblast fusion in insects and vertebrates. Nat. Genet. *39*, 781–786.

Suzuki, T., *et al*. (2006). Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development. Mol. Cell. Biol. *26*, 6149–6156.

Swailes, N. T., Colegrave, M., Knight, P. J., and Peckham, M. (2006). Non-muscle myosins 2A and 2B drive changes in cell morphology that occur as myoblasts align and fuse. J. Cell Sci. *119*, 3561–3570.

Swailes, N. T., Knight, P. J., and Peckham, M. (2004). Actin filament organization in aligned prefusion myoblasts. J. Anat. *205*, 381–391.

Tall, E. G., Spector, I., Pentyala, S. N., Bitter, I., and Rebecchi, M. J. (2000). Dynamics of phosphatidylinositol 4,5-bisphosphate in actin-rich structures. Curr. Biol. *10*, 743–746.

Taylor, M. V. (2003). Muscle differentiation: signalling cell fusion. Curr. Biol. *13*, R964–R966.

Taylor, M. V. (2006). Comparison of muscle development in *Drosophila* and vertebrates. In: Muscle Development in Drosophila, ed. H. Sink, Georgetown, TX: Landes/Eurekah Publishing.

Varnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. (2006). Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. J. Cell Biol. *175*, 377–382.

Vasyutina, E., Martarelli, B., Brakebusch, C., Wende, H., and Birchmeier, C. (2009). The small G-proteins Rac1 and Cdc42 are essential for myoblast fusion in the mouse. Proc. Natl. Acad. Sci. USA *106*, 8935–8940.

Vitale, N., Chasserot-Golaz, S., Bailly, Y., Morinaga, N., Frohman, M. A., and Bader, M. F. (2002). Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. J. Cell Biol. *159*, 79–89.

Wessel, D., and Flugge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. *138*, 141–143.

Yoon, M. S., and Chen, J. (2008). PLD regulates myoblast differentiation through the mTOR-IGF2 pathway. J. Cell Sci. *121*, 282–289.

Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M., and Starzinski-Powitz, A. (1995). Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. J. Cell Sci. *108*, 2973–2981.

Zhang, Q., Calafat, J., Janssen, H., and Greenberg, S. (1999). ARF6 is required for growth factor- and rac-mediated membrane ruffling in macrophages at a stage distal to rac membrane targeting. Mol. Cell Biol. *19*, 8158–8168.