The Abl-related Gene Tyrosine Kinase Acts through p190RhoGAP to Inhibit Actomyosin Contractility and Regulate Focal Adhesion Dynamics upon Adhesion to Fibronectin[□] [▽]

Justin G. Peacock,^{*†} Ann L. Miller,^{*‡} William D. Bradley,^{*} Olga C. Rodriguez,[§] Donna J. Webb,^{||} and Anthony J. Koleske^{*¶#}

^{*}Department of Molecular Biophysics and Biochemistry, [¶]Department of Neurobiology, and [#]Interdepartmental Neuroscience Program, Yale University, New Haven, CT 06511; [§]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037; and ^{||}Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235

Submitted January 29, 2007; Revised June 8, 2007; Accepted July 13, 2007
Monitoring Editor: Richard Assoian

In migrating cells, actin polymerization promotes protrusion of the leading edge, whereas actomyosin contractility powers net cell body translocation. Although they promote F-actin-dependent protrusions of the cell periphery upon adhesion to fibronectin (FN), Abl family kinases inhibit cell migration on FN. We provide evidence here that the Abl-related gene (Arg/Abl2) kinase inhibits fibroblast migration by attenuating actomyosin contractility and regulating focal adhesion dynamics. *arg*^{−/−} fibroblasts migrate at faster average speeds than wild-type (wt) cells, whereas Arg re-expression in these cells slows migration. Surprisingly, the faster migrating *arg*^{−/−} fibroblasts have more prominent F-actin stress fibers and focal adhesions and exhibit increased actomyosin contractility relative to wt cells. Interestingly, Arg requires distinct functional domains to inhibit focal adhesions and actomyosin contractility. The kinase domain-containing Arg N-terminal half can act through the RhoA inhibitor p190RhoGAP to attenuate stress fiber formation and cell contractility. However, Arg requires both its kinase activity and its cytoskeleton-binding C-terminal half to fully inhibit focal adhesions. Although focal adhesions do not turn over efficiently in the trailing edge of *arg*^{−/−} cells, the increased contractility of *arg*^{−/−} cells tears the adhesions from the substrate, allowing for the faster migration observed in these cells. Together, our data strongly suggest that Arg inhibits cell migration by restricting actomyosin contractility and regulating its coupling to the substrate through focal adhesions.

INTRODUCTION

The precise control of cell migration is essential for proper organismal development, tissue maintenance, and repair.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-01-0075>) on July 25, 2007.

[□] [▽] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

[†] These authors contributed equally to this work.

[‡] Present address: Department of Zoology, University of Wisconsin, Madison, WI.

Address correspondence to: Anthony J. Koleske (anthony.koleske@yale.edu).

Abbreviations used: p190RhoGAP, 190-kD GTPase-activating protein for Rho; Abl, Abelson; Arg, Abelson-related gene; ArgAC-YFP, Arg mutant lacking the F-actin- and microtubule-binding C-terminal half; Arg-YFP, Arg-yellow fluorescent protein; F, filamentous; FA, focal adhesion; FN, fibronectin; ArgKI-YFP, kinase-inactive Arg point mutant; MT, microtubule; OD, optical density; *p190*^{+/+}, *p190rhogap*^{+/+}; *p190*^{−/−}, *p190rhogap*^{−/−}; Rac, Rac1; MLC, regulatory myosin light chain; Rho, RhoA; ROCK, Rho-associated kinase; pMLC, Ser-19 phosphorylated regulatory myosin light chain; wt, wild type.

Cell migration initiates via formation of actin-polymerization-based protrusions at the leading edge (Mitchison and Cramer, 1996; Pollard and Borisy, 2003; Ponti *et al.*, 2004). Integrin receptor clusters within the protrusion bind the extracellular matrix (Jockusch *et al.*, 1995; Lauffenburger and Horwitz, 1996). These adhesions anchor the protrusion and link the cellular F-actin cytoskeleton to the matrix. Myosin-based contraction of this actin network generates traction force to pull the cell body forward and detach adhesions at the cell's trailing edge (Jay *et al.*, 1995; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Ridley *et al.*, 2003; de Rooij *et al.*, 2005; Gupton and Waterman-Storer, 2006). The central goal of cell migration research is to understand how extracellular cues coordinate these events in space and time to achieve carefully controlled migration.

The Rho family GTPases RhoA (Rho), Rac1 (Rac), and Cdc42 regulate cell migration by coordinating cytoskeletal rearrangements with adhesion formation and dissolution (Kaibuchi, 1999; Ridley, 2001). After activation by extracellular cues, Rac and Cdc42 stimulate both actin-polymerization-based protrusions (Pollard and Borisy, 2003; Affolter and Weijer, 2005) and nascent adhesion formation at the leading edge (Nobes and Hall, 1995; Rottner *et al.*, 1999). Rho promotes nascent adhesion maturation into larger focal adhesions and stimulates formation of F-actin stress fibers that anchor to adhesions and transmit contractile forces (Ridley

and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996; Rottner *et al.*, 1999). Although studies using activated or dominant-negative mutants have clearly implicated Rho, Rac, and Cdc42 in these cytoskeletal and adhesion rearrangements, it is largely unclear how the activities of these proteins are controlled by extracellular cues during cell migration.

Abl family tyrosine kinases, including the vertebrate Abl and Arg proteins, are also essential regulators of cell migration and morphogenesis in developing animals (Pendergast, 2002; Woodring *et al.*, 2003; Hernandez *et al.*, 2004a; Koleske, 2006). These kinases translate signals from adhesion, guidance, and growth factor receptors into cytoskeletal rearrangements. Abl and Arg promote the formation of actin-based cell protrusions during integrin-mediated cell attachment and spreading (Woodring *et al.*, 2002, 2004; Miller *et al.*, 2004; Moresco *et al.*, 2005). Based on this observation, Abl family kinases would be expected to promote cell migration on adhesive surfaces. However, Abl family kinases inhibit integrin-mediated cell migration (Kain and Klemke, 2001; this study) and the migratory response of thyroid cancer cells to hepatocyte growth factor (Frasca *et al.*, 2001). How Abl family kinases promote cell edge protrusion and yet inhibit cell migration is a major unresolved issue.

We previously identified the 190-kDa GTPase-activating protein for Rho (p190RhoGAP) as a major Arg substrate in the developing brain (Hernandez *et al.*, 2004b). Integrin receptor engagement induces Arg-dependent phosphorylation of p190RhoGAP (Hernandez *et al.*, 2004b), which triggers p190RhoGAP localization to the cell membrane where it inhibits Rho activity (Bradley *et al.*, 2006). These findings led us to examine a possible role for integrin signaling through Arg, p190RhoGAP, and Rho in regulating cell adhesion and contractility during cell migration.

We provide evidence here that Arg inhibits integrin-mediated cell migration by attenuating cell contractility and regulating focal adhesion dynamics. *arg*^{-/-} fibroblasts migrate faster than wild type (wt) cells, and elevation of Arg levels slows cell migration. Interestingly, *arg*^{-/-} fibroblasts have more prominent F-actin stress fibers and focal adhesions and are hyper-contractile relative to wt cells. Arg re-expression in *arg*^{-/-} cells reduces focal adhesions and stress fibers and attenuates cell contractility. Interestingly, we find that Arg requires different functions to inhibit these processes: the Arg kinase domain-containing N-terminal half can act through p190RhoGAP to attenuate stress fiber formation and contractility, whereas Arg requires both its kinase domain and C-terminal cytoskeleton-binding half to regulate focal adhesions. Although focal adhesions do not turn over normally in *arg*^{-/-} cells, the increased contractility of these cells detaches trailing edge adhesions from the substrate, allowing for the more rapid observed migration. Together, our data strongly suggest that Arg inhibits cell migration by restricting actomyosin contractility and regulating its coupling to the substrate through focal adhesions. We propose a model in which Arg coordinates leading edge protrusion with adhesion and contractility to allow environment sampling during cell migration on adhesive surfaces.

MATERIALS AND METHODS

Constructs

All Arg expression constructs have been previously described (Miller *et al.*, 2004).

Cell Culture and Retroviral Infection

The generation of wt and *arg*^{-/-} fibroblasts was described previously (Koleske *et al.*, 1998; Miller *et al.*, 2004). *p190rhogap*^{+/+} and *p190rhogap*^{-/-}

fibroblasts were a generous gift from Jeff Settleman (Massachusetts General Hospital). The infections of these lines with retroviruses expressing Arg-yellow fluorescent protein (YFP)/ArgΔC-YFP/ArgKI-YFP have been described previously (Miller *et al.*, 2004), and infected cells were selected by slowly increasing puromycin concentration from 0.25 to 1.0 μg/ml. Arg-YFP-expressing cells obtained in this manner expressed Arg-YFP at an overall average of 2–3-fold endogenous levels, but these cells were also fluorescence-activated cell sorted into a low Arg-YFP-expressing population, expressing Arg-YFP at 1.5–2-fold over endogenous levels (*arg*^{-/-} + Arg-YFP [lo] cells). Expression levels of these proteins were determined by quantitative immunoblotting. Cell lysates were prepared, and a dilution series of cell lysates and purified Arg protein was immunoblotted with anti-Arg antibodies and quantitated using a densitometer (Bio-Rad, Hercules, CA). These values were corrected for the percent of cells expressing the Arg/Arg mutant-YFP fusion proteins as determined by fluorescence-activated cell sorting (FACS) or light microscopy. Fibroblasts were transfected with 2.5 μg paxillin-GFP or paxillin-DsRed DNA in a six-well plate or 20 μg paxillin-GFP or paxillin-DsRed DNA in a 10-cm plate using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Single-Cell Migration Assays

wt, *arg*^{-/-} cells, or *arg*^{-/-} cells expressing Arg-YFP, ArgKI-YFP, or ArgΔC-YFP were plated at a subconfluent level on glass coverslips coated with 1 μg/ml fibronectin (FN; Sigma-Aldrich, St. Louis, MO). Phase-contrast images were taken every 3 min for a total of 7 h. Movies were obtained using a Nikon Plan Fluor 20×/0.5 NA objective on a Nikon Eclipse TE200 (Melville, NY) outfitted with a heated Nevtek air stream incubator (Burnsville, VA) and a Hamamatsu ORCA-285 CCD camera (Bridgewater, NJ) driven by MetaMorph software (Universal Imaging, West Chester, PA). Movies were analyzed using MetaMorph software (Universal Imaging) or ImageJ with the *Track Points* function or *Manual Tracking* function, using an identifiable feature of the nucleus to track the cells in each frame. All cells analyzed remained within the field of view, did not more than transiently touch other cells, and did not divide. In several cases, a cell divided or interacted with another cell part way through the movie; for such cells, the analysis was truncated at that point. The *Track Points* data from MetaMorph or ImageJ was exported to Microsoft Excel (Redmond, CA) for analysis. The distances traveled per 3-min interval were determined from the tracking data, and average linear regression velocity (μm/h) was determined.

Immunofluorescence Microscopy

Cells were plated on glass coverslips coated with 1 μg/ml FN as indicated in the figure legends and blocked with 1% bovine serum albumin (BSA; Invitrogen/BRL, Carlsbad, CA) for the times indicated in the figure legends. Cells were rinsed before fixation with PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EDTA, 2 mM MgCl₂, pH 6.9) that was prewarmed to 37°C. Cells were fixed with 4% paraformaldehyde (prewarmed to 37°C) for 20 min at room temperature and then permeabilized with 0.5% or 1% Triton X-100 for 10 min. Cells were stained with antibodies to paxillin (Transduction Laboratories, Lexington, KY; cat. no. 610051), vinculin (Sigma; clone VIN-11-5), α-actinin (Sigma; clone BM-75.2), phospho-Myosin Light Chain 2 (Ser19; pMLC; Cell Signaling, Beverly, MA; cat. no. 3675/3671), pan-Myosin II, heavy chain (MyoII; Abcam, Cambridge, MA; clone C5C.S2), and Alexa 594- or Alexa 488-labeled secondary antibodies (Molecular Probes, Eugene, OR), followed by Alexa 350-phalloidin (Molecular Probes) to visualize the F-actin cytoskeleton. Cells were imaged on a Nikon TE2000-S microscope coupled to a Qimaging Retiga 1300C camera (Burnaby, BC, Canada) driven by Openlab software (Improvision, Lexington, MA) using 40× Nikon PlanApo 1.0 or 100× Nikon PlanFuor 1.3 oil objectives.

Rho Activity Assays

Rho activity assays were performed as previously described (Bradley *et al.*, 2006).

Contractility Assays

Contractility assays were performed as previously described (Arora and McCulloch, 1994). Briefly, 6000 cells were suspended in 100 μl of 40% collagen gel that contained 0.08 mg/ml FN and allowed to harden on siliconized glass coverslips. Collagen gels were surrounded by tissue culture medium. In most cases, the gel diameter was measured under a dissecting microscope after 20 h. The matched *p190*^{+/+} and *p190*^{-/-} cells were less contractile, and therefore gel diameters were recorded at 8 d after plating.

Supplementary Videomicroscopy, Focal Adhesion Rainbow Analysis, and Focal Adhesion Dynamics Quantitation

For time-lapse microscopy, wt or *arg*^{-/-} cells expressing paxillin-GFP or *arg*^{-/-} cells expressing ArgKI-YFP and paxillin-DsRed were adapted to microscopy media (growth media with 10 mM HEPES; Invitrogen/BRL) for ~16 h and plated on glass coverslips coated with 1 μg/ml FN and BSA-

blocked. Cells were imaged beginning at 2 h after plating using a Nikon TE2000-S microscope coupled to a Qimaging Retiga 1300C camera driven by Openlab software using 40× Nikon PlanApo 40×/1.0 NA or Nikon PlanFuor Oil 100×/1.3 NA objectives. Cells were maintained at 37°C during imaging with an in-line flow heater and a heated chamber (Warner Instruments, Hamden, CT). Movies at 40× were up to 30 min long with frames taken every minute. Focal adhesion (FA) formation and disassembly rate constants for the wt and *arg*^{-/-} cells expressing paxillin-GFP were determined. Briefly, the paxillin-GFP intensities for 12–20 FAs from four to five cells were quantified and plotted as the logarithm of the normalized FA fluorescence relative to the original FA fluorescence as a function of time (Webb *et al.*, 2002).

Quantitation of FAs and Stress Fibers

FA densities were quantified using NIH ImageJ software. Images were taken of cells stained for paxillin at 40× magnification. The TIFF images were converted to 8-bit grayscale, calibrated in ImageJ to optical density (OD) values, and the foreground/background colors were inverted. The threshold on the image was adjusted until the paxillin fluorescence was excluded from the threshold limits. Membrane extensions were individually outlined and measured using the freehand selection tool. The ImageJ *analyze particles* function was used to select and measure areas of paxillin fluorescence within the freehand selection. The particle areas for each selection were summed and divided by the total freehand selection area to obtain the FA density at the periphery. The individual selection densities were averaged for each cell to obtain the FA density values per cell.

For stress fiber quantitation, 40× magnification images were processed as for FA images. We used the ImageJ *threshold* tool separately to determine an OD limit that encapsulated most of the visible F-actin bundles, eliminating background fluorescence. For each peripheral extension, a line selection perpendicular to the extension axis was made (see Figure 2F). The ImageJ *plot profile* function was used to obtain the average pixel intensities at several intervals along the line selection. We then calculated the density ratio as the distance along the line occupied by pixels with average OD values greater than the threshold limit divided by the total line distance. The peripheral stress fiber density entering a cell extension per cell was determined from the density ratio averages for all the extensions in a cell.

Statistical Analysis

We used ANOVA ($\alpha = 0.05$) to compare the multiple groups of data from cells of different genotypes. We performed a post hoc Student-Newman-Keuls test to determine the statistical significance of the differences between these groups. This test is the most stringent and appropriate to compare two or more unpaired groups.

RESULTS

Arg Inhibits Cell Migration

We used a wound-healing assay to examine how Arg expression affects cell migration. Confluent monolayers of Swiss3T3 cells expressing YFP or Arg-YFP were scrape-wounded, and the resulting migration of the cells into the “wound” was monitored by time-lapse videomicroscopy. Control YFP-expressing cells migrate normally into the wound (Supplementary Figure S1A, and Supplementary Video SV1), with 100% of the YFP-positive cells initially at the wound-edge crossing into the wound (Supplementary Figure S1C). Infection with Arg-YFP virus yielded a mixed cell population, in which only a subset express Arg-YFP. Notably, Arg-YFP-expressing cells are highly dynamic, sending out frequent lamellipodial protrusions and retractions, but only 45% percent of the Arg-YFP-expressing cells initially at the wound-edge enter the wound (Supplementary Figure S1, B and C, and Supplementary Video SV2). These observations indicate that Arg overexpression can inhibit cell migration during wound healing.

arg^{-/-} Fibroblasts Migrate Aberrantly, but Faster than wt Fibroblasts

We used time-lapse videomicroscopy to analyze the migration phenotypes of wt fibroblasts expressing YFP and *arg*^{-/-} fibroblasts expressing either YFP or a functional Arg-YFP fusion (Miller *et al.*, 2004). We examined the cells as they migrated on coverslips coated with 1 μ g/ml FN over a 7-h filming period.

Migrating wt mouse embryo fibroblasts assume a polarized shape, with lamellipodial extension at the leading edge and tail retraction at the trailing edge (Figure 1A). Control wt fibroblasts maintain their polarity as they move and change direction by pausing, extending additional lamellipodia, and repolarizing in the new direction of movement (Supplementary Video SV3). In contrast, *arg*^{-/-} fibroblasts have less distinct leading and trailing edges and migrate by “snapping” forward in large abrupt steps (Figure 1B; Supplementary Videos SV4 and SV5). The snaps result from *arg*^{-/-} cells suddenly tearing and leaving behind their trailing edge (Figure 1B), which in some cases represents a sizeable part of the cell (Figure 1C; Supplementary Video SV5).

We monitored the migration path, step size, and speed by tracking a single nucleolus in each cell (Figure 1, D and E). Large migration steps (2–7.5 μ m/interval) are observed at increased frequencies in *arg*^{-/-} + YFP cells compared with wt + YFP cells, and only *arg*^{-/-} + YFP cells make longer snap-like steps ≥ 8 μ m/interval (Figure 1F). Importantly, *arg*^{-/-} + YFP fibroblasts migrate 62% faster than wt + YFP cells (Figure 1G).

Re-expression of Arg-YFP in *arg*^{-/-} cells (*arg*^{-/-} + Arg-YFP cells) leads to a dramatic reduction in step size and migration speed compared with control YFP-expressing *arg*^{-/-} cells (Figure 1, F and G). In fact, *arg*^{-/-} + Arg-YFP cells migrate with smaller step sizes than wt + YFP cells. This reduced step size may result from the average 2–3-fold elevation of Arg-YFP over wt endogenous levels in these cells (Miller *et al.*, 2004). *arg*^{-/-} + Arg-YFP cells also migrate 29% slower than wt + YFP cells (Figure 1G). To determine whether this decreased migration speed resulted from Arg-YFP overexpression, we purified *arg*^{-/-} cells expressing more uniform low levels of Arg-YFP expression by FACS. These *arg*^{-/-} + Arg-YFP (lo) cells express Arg-YFP at 1.5–2-fold over endogenous Arg levels (Quantitative blotting of Arg and Arg mutant expression levels for all cells used is shown in Supplementary Figure S2). These cells migrate with speeds that are indistinguishable from wt + YFP cells (Supplementary Figure S3). Together, these data indicate that cell migration, step size, and average migration velocity become reduced as Arg levels increase in cells.

Arg Inhibits Focal Adhesions and Stress Fibers

The unusual migratory behavior and increased speed of *arg*^{-/-} fibroblasts suggested they might have altered adhesive and/or contractile systems. We examined the size, density, and distribution of focal adhesions (FAs) and stress fibers in wt and *arg*^{-/-} fibroblasts plated for 2 h on glass coverslips coated with 1 μ g/ml FN. Paxillin-positive FAs are significantly larger and more dense in *arg*^{-/-} fibroblasts than in wt cells (compare Figure 2, A and B). Staining for the FA protein vinculin yields similar differences between the two cell types (data not shown). FA staining is significantly reduced in both cell types plated on uncoated glass coverslips, indicating that integrin-mediated engagement is required for FA formation (data not shown).

We examined whether Arg-YFP re-expression in *arg*^{-/-} cells could inhibit their increased FAs and stress fibers. As expected, control YFP-expressing *arg*^{-/-} fibroblasts have large paxillin-positive FAs and prominent F-actin stress fibers (Figure 2C). When expressed in *arg*^{-/-} fibroblasts, Arg-YFP localizes to the cell periphery where it is associated with F-actin-rich structures (Figure 2D). We noted that the concentration of Arg-YFP to the cell periphery and its colocalization with F-actin was reduced somewhat in these fi-

Figure 1. Arg inhibits cell migration on FN. (A–C) Individual frames from time-lapse movies of wt (A; see Supplementary Video SV3) or *arg*^{-/-} (B and C; see Supplementary Videos SV4 and SV5) cells migrating on coverslips coated with 1 $\mu\text{g/ml}$ FN. Elapsed time, hour:minute. *arg*^{-/-} cells migrate in “snaps” as they suddenly detach and lurch forward. The arrow in B indicates the location of the *arg*^{-/-} cell’s trailing edge in the previous time-lapse image. Note the large distance traveled by the *arg*^{-/-} cell during this time period. The asterisk in C indicates where the *arg*^{-/-} cell tore apart during the preceding interval. (D and E) Individual frames from time-lapse movies of wt (D; see Supplementary Information, Supplementary Video SV3) or *arg*^{-/-} (E; see Supplementary Information, Supplementary Video SV4) cells migrating on coverslips coated with 1 $\mu\text{g/ml}$ FN. Elapsed time, hour:minute. The white lines indicate the migration paths of the cells, with a star indicating the start of the path and the arrowhead representing the end of the path. Note the larger distance traveled by the *arg*^{-/-} cell compared with the wt cell during the same time period. The arrow in B indicates *arg*^{-/-} cell fragment remaining after the cell “snapped” forward. Bar, (A–E) 20 μm (bottom left corner). (F) Plot of distribution of migration step sizes per 3-min interval in wt + YFP, *arg*^{-/-} + YFP, *arg*^{-/-} + Arg-YFP, *arg*^{-/-} + Arg Δ C-YFP, and *arg*^{-/-} + ArgKI-YFP cells. Note the increased frequency of large migration steps in *arg*^{-/-} + YFP cells. wt + YFP, *n* = 18 cells; *arg*^{-/-} + YFP, *n* = 21 cells; *arg*^{-/-} + Arg-YFP, *n* = 22 cells; *arg*^{-/-} + Arg Δ C-YFP, *n* = 20 cells; and *arg*^{-/-} + ArgKI-YFP, *n* = 23 cells. Parametric ANOVA between data for all cell types, *p* < 0.0001. All pairwise comparisons except for *arg*^{-/-} + Arg-YFP versus *arg*^{-/-} + ArgKI-YFP are significantly different from each other by post hoc Student-Newman-Keuls test (*p* < 0.05). (G) The average migration speed of wt + YFP, *arg*^{-/-} + YFP, *arg*^{-/-} + Arg-YFP, *arg*^{-/-} + Arg Δ C-YFP, and *arg*^{-/-} + ArgKI-YFP cells on FN coverslips was monitored over 7 h. wt + YFP, *n* = 18 cells; *arg*^{-/-} + YFP, *n* = 21 cells; *arg*^{-/-} + Arg-YFP, *n* = 22 cells; *arg*^{-/-} + Arg Δ C-YFP, *n* = 20 cells; and *arg*^{-/-} + ArgKI-YFP, *n* = 23 cells. ANOVA between data for all cell types, *p* < 0.0001. *post hoc Student-Newman-Keuls, *p* < 0.05.

broblasts, plated on 1 $\mu\text{g/ml}$ FN, as compared with the same fibroblasts plated on 10 $\mu\text{g/ml}$ FN (as reported in Miller *et al.*, 2004). Interestingly, Arg-YFP re-expression in *arg*^{-/-} fibroblasts reduces both FAs and stress fibers and this correlates with the level of Arg-YFP expression (Figure 2D).

We used ImageJ software to quantitate the FA marker (paxillin) and F-actin stress fiber density at the cell periphery (Figure 2, E–H; see Materials and Methods for details). Cells were chosen randomly by identifying YFP-expressing cells that were not contacting other cells. Paxillin-staining density at the cell periphery is increased 2.6-fold in *arg*^{-/-} + YFP cells relative to wt + YFP cells (Figure 2G). wt + YFP cells

have a prominent F-actin “belt” around the center of the cell (Figure 2A). This cortical F-actin network is less pronounced in *arg*^{-/-} cells, and instead prominent F-actin stress fibers extend tangentially like “tent ropes” across the cell and terminate in the FAs (Figure 2B). Quantitation revealed that the stress fibers are 1.8-fold more dense in *arg*^{-/-} + YFP cells than in wt + YFP cells (Figure 2H). Peripheral FAs or stress fibers were not altered in *abl*^{-/-} fibroblasts (data not shown), indicating that the loss of Abl function does not significantly influence these structures.

Quantitation confirmed that FA and stress fiber size correlate inversely with Arg expression levels (Figure 2, G and

Figure 2. Arg expression inhibits FAs and stress fibers. (A and B) wt (A) or *arg*^{-/-} (B) fibroblasts were plated on FN-coated coverslips, fixed, and stained with Alexa350-phalloidin to visualize F-actin and with anti-paxillin antibodies to visualize focal adhesions (FAs). Top row images were obtained with a 40× objective lens, and bottom row images were obtained with a 100× objective lens and are magnified images of the top row's boxed regions. Merged image (merge) shows F-actin (red) and paxillin (green). Bar, 10 μm. (C and D) *arg*^{-/-} + YFP (C) or *arg*^{-/-} + Arg-YFP (D) fibroblasts were plated on FN-coated coverslips, fixed, and stained with Alexa350-phalloidin to visualize F-actin and with anti-paxillin antibodies to visualize FAs. In D, *arg*^{-/-} cells expressing no (*), low (**), or high (***) levels of Arg-YFP are indicated on the YFP channel to the far left. Images were obtained with a 20× objective lens. Merged image (merge) shows F-actin (red) and paxillin (green). Bar, 10 μm. (E) Example FA quantitation diagram of *arg*^{-/-} cell from B. The original illustrates an inverted image of focal adhesion staining. Peripheral FA density was determined in areas that protrude from the cell body in threshold-adjusted images (see highlighted area in Threshold image). For E and F, see Materials and Methods for details. (F) Example stress fiber quantitation diagram of *arg*^{-/-} cell from B. The original illustrates an inverted F-actin-staining image. Lines were drawn perpendicular to the stress fiber orientation, and plot profile measurements allowed for determination of stress fiber densities (see Line Profile image). (G and H) Quantitation of peripheral FA density (G) and peripheral stress fiber density (H) in wt + YFP, *arg*^{-/-} + YFP and *arg*^{-/-} + Arg-YFP cells. Mean ± SE; n = 20 cells for all genotypes. ANOVA between data for all genotypic classes (G and H) p < 0.0001. *post hoc Student-Newman-Keuls, p < 0.05.

H). Focal adhesions and stress fibers are most prominent in *arg*^{-/-} + YFP cells, reduced in wt + YFP cells, and reduced further in *arg*^{-/-} + Arg-YFP cells that express slightly

elevated levels of Arg-YFP (Figure 2, G and H). Therefore, increasing Arg expression levels lead to decreased size and density of FAs and stress fibers.

							Effect when re-expressed in <i>arg</i> ^{-/-} cells:				
							focal	stress	Rho	cell	cell
							adhesions	fibers	activity	contractility	migration
Arg	<div><div>3</div><div>2</div><div>Kinase</div><div></div><div>F</div><div>MT</div><div>F</div></div>						↓↓↓	↓↓↓	↓	↓↓	↓
	K317R										
ArgKI	<div><div>3</div><div>2</div><div>Kinase</div><div></div><div>F</div><div>MT</div><div>F</div></div>						↓↓	—	—	↓	↓
ArgΔC	<div><div>3</div><div>2</div><div>Kinase</div></div>						↓	↓↓↓	↓	↓↓	↓

Table 1. Diagram of Arg and Arg mutants used in these assays and summary of their effects. The Arg N-terminal half contains SH3 (3), SH2 (2) and kinase domains. The Arg C-terminal half contains two F-actin-binding domains (F) and a microtubule-binding domain (MT). ArgΔC lacks the C-terminus. ArgKI has a point mutation rendering it kinase-inactive. The relative effects of Arg, ArgΔC, or ArgKI on focal adhesions, stress fibers, cell contractility, and cell migration are shown. “—” = Not significantly different from *arg*^{-/-} cells.

Figure 3. Arg regulates focal adhesions and stress fibers via distinct domains. (A–C) *arg*^{-/-} fibroblasts expressing YFP (A), ArgΔC-YFP (B), or ArgKI-YFP (C) were plated on FN-coated coverslips and fixed and stained for F-actin and paxillin. Images were obtained with a 20× objective lens. Merged image (merge) shows YFP (blue), F-actin (red), and paxillin (green). Bar, 10 μm. (D and E) Peripheral FA and stress fiber density quantitation in *arg*^{-/-} cells + Arg- or Arg mutant-YFP. Mean ± SE; 20 cells were analyzed for each genotype and both quantitations. ANOVA between data for all genotypes, $p < 0.0001$; *post hoc Student-Newman-Keuls, $p < 0.05$.

Arg Requires Different Functional Domains to Inhibit Focal Adhesions versus Stress Fibers

Arg is a multifunctional protein with an N-terminal half containing SH3, SH2, and kinase domains (Kruh *et al.*, 1990) and a C-terminal half containing several proline-rich motifs (Wang *et al.*, 1996), two F-actin-binding domains (Wang *et al.*, 2001), and a microtubule-binding domain (Miller *et al.*, 2004; Table 1). We used Arg mutants to demonstrate that distinct Arg functions are required to inhibit stress fibers versus FAs.

Expression of an Arg mutant lacking the F-actin- and microtubule-binding C-terminal half (ArgΔC-YFP; Table 1), but containing Arg kinase activity in *arg*^{-/-} cells potently inhibits F-actin stress fibers in the central region of the membrane protrusion, leaving some residual peripheral stress fibers and actin network intact (Figure 3, B and E). Quantitation revealed that ArgΔC-YFP inhibits stress fibers to a similar extent as full-length Arg-YFP, decreasing stress fibers 2.2-fold relative to *arg*^{-/-} + YFP cells (Figure 3E). Although smaller than in *arg*^{-/-} + YFP cells, FAs in *arg*^{-/-} + ArgΔC-YFP cells are not fully inhibited (Figure 3, A and B). FA quantitation actually shows that FA peripheral density is slightly higher in *arg*^{-/-} + ArgΔC-YFP cells relative to wt cells (Figures 2G and 3D). The stress fiber and FA phenotypes in *arg*^{-/-} + ArgΔC-YFP cells suggest that Arg kinase activity can inhibit stress fiber formation with only partial effects on FAs.

A kinase-inactive Arg point mutant (ArgKI-YFP; Table 1) results in significantly less dense paxillin staining in peripheral FAs relative to *arg*^{-/-} + YFP and *arg*^{-/-} + ArgΔC-YFP cells (2.4-fold reduction relative to *arg*^{-/-} + YFP cells),

but has only modest effects on the exaggerated F-actin stress fiber bundles observed in *arg*^{-/-} + YFP cells (Figure 3, A, C, D, and E). ArgKI-YFP also reduces vinculin staining at FAs (data not shown). The finding that ArgKI-YFP expression leads to reduced paxillin and vinculin staining indicates that Arg can act via a kinase-independent mechanism to alter FA dynamics and/or composition.

In summary, comparing the differential effects of Arg mutants on FAs and stress fibers indicates that Arg requires distinct functional domains to inhibit FAs and stress fibers (Table 1). Arg requires its kinase domain to inhibit stress fiber formation, and this function does not require the Arg C-terminal half. Arg requires both its kinase activity and C-terminal half to fully inhibit focal adhesions.

Arg Kinase Activity Acts through p190RhoGAP to Inhibit Rho and Rho-induced Stress Fibers

In its active form, Rho promotes FA and stress fiber formation (Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996; Rottner *et al.*, 1999). We hypothesized that the more prominent FAs and stress fibers observed in *arg*^{-/-} cells (Figure 2B), might result from hyperactive Rho signaling. We have previously shown that Arg is required for inhibition of Rho after integrin-mediated adhesion to FN (Bradley *et al.*, 2006). Re-expression of Arg restores adhesion-dependent inhibition of Rho to *arg*^{-/-} cells (Bradley *et al.*, 2006). Re-expression of an Arg mutant lacking the C-terminal F-actin- and microtubule-binding domains (ArgΔC-YFP) in *arg*^{-/-} cells restores adhesion-dependent Rho inhibition at 10 and 20 min; however, active Rho levels are slightly higher than

Figure 4. Arg inhibits stress fibers through p190RhoGAP. (A and B) Arg kinase activity is required for adhesion-dependent Rho inhibition. (A) Relative Rho activity plotted as a function of time. Rho activity was assessed in wt (blue diamonds), $arg^{-/-}$ + YFP (red squares), $arg^{-/-}$ + Arg Δ C-YFP (green triangles), and $arg^{-/-}$ + ArgKI-YFP (purple $\times$) cells in suspension (0 min) or plated on FN for 10, 20, 30, or 60 min. Relative Rho activity indicates Rho activity at each time point normalized to the zero time point for each cell type. Mean $\pm$ SE; $n \geq 3$. Analysis of variance between all cell types: 10-min time point, $p = 0.0008$; 20-min time point, $p = 0.0498$; 30-min time point, $p = 0.0020$; and 60-min time point, $p = 0.1283$. The following are significantly different as indicated by pairwise post hoc Fisher's PLSD test ($p < 0.05$): 10 min, WT and YFP, WT and ArgKI-YFP, YFP and Arg Δ C-YFP, Arg Δ C-YFP and ArgKI-YFP; 20 min, WT and YFP, WT and ArgKI-YFP, Arg Δ C-YFP and ArgKI-YFP; 30 min, WT and YFP, WT and ArgKI-YFP, WT and Arg Δ C-YFP; and 60 min, WT and YFP. (B) Total Rho levels were determined for each cell type at each time point to ensure equal loading for the assay; 75 μ g of total protein extract was immunoblotted for RhoA. (C–F) $p190^{rhogap+/+}$ ($p190^{+/+}$; C and D) or $p190^{rhogap-/-}$ ($p190^{-/-}$; E and F) fibroblasts expressing YFP (C and E) or Arg-YFP (D and F) were plated on FN-coated coverslips and fixed and stained for F-actin and paxillin. Images were obtained with a 20 $\times$ objective lens. Merged image (merge) shows YFP (blue), F-actin (red), and paxillin (green). Bar, 10 μ m. (G and H) Quantitation of peripheral FA and stress fiber density in $p190^{+/+}$ + YFP or Arg-YFP and $p190^{-/-}$ + YFP or Arg-YFP cells. Mean $\pm$ SE. Twenty cells were analyzed for each genotype and both quantifications. ANOVA between data for all genotypes, $p < 0.0001$. *post hoc Student-Newman-Keuls, $p < 0.05$.

wt cells at 30 min (Figure 4A). Interestingly, a kinase-inactive Arg point mutant (ArgKI-YFP) expressed in $arg^{-/-}$ cells does not restore adhesion-dependent Rho inhibition, but instead exhibits a slight increase in Rho activity as observed in $arg^{-/-}$ + YFP cells. These observations indicate that Arg kinase activity is necessary for adhesion-dependent Rho inhibition (Figure 4A). Control experiments confirm that total Rho levels are similar at each time point in each cell type (Figure 4B).

Arg phosphorylates and activates the Rho inhibitor p190RhoGAP after integrin-mediated adhesion (Hernandez *et al.*, 2004b; Bradley *et al.*, 2006). We monitored FA and stress fiber structure in $p190^{rhogap-/-}$ ($p190^{-/-}$) and lit-

termate control $p190^{rhogap+/+}$ ($p190^{+/+}$) fibroblasts expressing YFP or Arg-YFP (Figure 4, C–F). Under these conditions, Arg-YFP was expressed at levels 2- to 2.5-fold over endogenous Arg levels (Supplementary Figure S2). Arg-YFP expression causes a similar reduction of FAs in both $p190^{+/+}$ and $p190^{-/-}$ cells (Figure 4, D, F, and G). Interestingly, Arg-YFP inhibits stress fibers in $p190^{+/+}$ cells, but not in $p190^{-/-}$ cells (Figure 4, D, F, and H). ArgKI-YFP expression in $p190^{+/+}$ cells has no effect on stress fibers, despite its slight ability to reduce FA staining (data not shown). Together, these data indicate that Arg signaling through p190RhoGAP is required to inhibit stress fibers.

Figure 5. Arg inhibits contractility via p190RhoGAP. (A and B) wt (A) or *arg*^{-/-} (B) cells were plated on FN-coated coverslips, fixed, and stained with antibodies to phosphoSer19 of myosin light chain (pMLC) and pan-myosin II (MyoII) heavy chain, and Alexa350-phalloidin to visualize F-actin. Top row images were obtained with a 40× objective lens and bottom row images were obtained with a 100× objective lens and are magnified images of the top row's boxed regions. Merged image (merge) shows F-actin (red) and pMLC (green). Bar, 10 μ m. (C) Contractility of wt + YFP cells in collagen/FN gels with DMSO (control) or blebbistatin. The percent decrease in gel diameter is shown for each treatment. Mean $\pm$ SE; n = 4 experiments; ANOVA between data for both treatments, $p = 0.0199$. *post hoc Student-Newman-Keuls, $p < 0.05$. (D) Contractility of wt + YFP and *arg*^{-/-} + YFP or Arg-YFP cells in collagen/FN gels. The percent decrease in gel diameter is shown for each genotype. Mean $\pm$ SE; wt + YFP, n = 9 experiments; *arg*^{-/-} + YFP, n = 5 experiments; and *arg*^{-/-} + Arg-YFP, n = 5 experiments; ANOVA between data for all genotypic classes, $p < 0.0001$. *post hoc Student-Newman-Keuls, $p < 0.05$. (E) Contractility of *arg*^{-/-} + YFP, Arg-YFP, Arg Δ C-YFP, or ArgKI-YFP cells in collagen/FN gels. The percent decrease in gel diameter is shown for each genotype. Mean $\pm$ SE; n = 5 experiments for each genotype. ANOVA between data for all genotypic classes, $p < 0.0001$. *post hoc Student-Newman-Keuls, $p < 0.05$. (F) Differential effects of Arg-YFP expression on contractility of *p190*^{+/+} or *p190*^{-/-} cells. Mean $\pm$ SE; n = 5 experiments for each genotype. ANOVA between data for all genotypic classes, $p = 0.0003$. *post hoc Student-Newman-Keuls, $p < 0.05$.

Arg Acts through p190RhoGAP to Regulate Cell Contractility

The observation that stress fiber bundles are increased in *arg*^{-/-} cells led us to test whether these cells have increased contractility. Activation of Rho activity results in increased regulatory myosin light chain (MLC) phosphorylation on Ser19, leading to increased actomyosin contractility (Chrza-

nowska-Wodnicka and Burridge, 1996). MLC phosphorylation and myosin activation relocalizes myosin heavy chain to actin filaments, thereby forming contractile actomyosin stress fibers (Chrzanowska-Wodnicka and Burridge, 1996).

wt cells show some staining for phosphorylated MLC (pMLC) within the cell body (Figure 5A). In contrast, *arg*^{-/-}

cells exhibit more intense and concentrated pMLC staining at the cell periphery, a portion of which overlaps with the increased F-actin stress fiber network (Figure 5B). Similarly, myosin II heavy chain (Myo II) staining is weaker in wt cells, whereas Myo II staining in *arg*^{-/-} cells overlaps with pMLC staining, with strong localization along stress fibers (Figure 5, A and B). Together, the pMLC, Myo II, and phalloidin staining indicates that *arg*^{-/-} cells have more prominent actomyosin complex staining than wt cells.

Fibroblasts suspended in a collagen:FN gel exert actomyosin-derived, contractile forces resulting in gel shrinkage (Murphy and Daniel, 1987; Arora and McCulloch, 1994). This gel contraction can be inhibited by treatment with blebbistatin (Straight *et al.*, 2003), an inhibitor of myosin II (Figure 5C). When suspended in gels, *arg*^{-/-} + YFP fibroblasts cause a $52 \pm 2\%$ shrinkage of the gel, whereas wt + YFP cells contract the gel by only $33 \pm 3\%$ (Figure 5D). Importantly, collagen gels become more difficult to contract as they shrink. Calibration experiments reveal that about threefold more wt cells than *arg*^{-/-} cells are required to achieve similar gel contractility (data not shown). Thus, *arg*^{-/-} cells have an approximately threefold increased contractility per cell relative to wt cells in this assay.

We measured the contractility of *arg*^{-/-} cells re-expressing Arg/Arg mutant-YFP fusions to determine the mechanism by which Arg attenuates cell contractility. Although *arg*^{-/-} + YFP cells contract the gels by $52 \pm 2\%$, *arg*^{-/-} + Arg-YFP-expressing cells only contract the gel by $4.6 \pm 1.7\%$, demonstrating that Arg-YFP expression can relax cell contractility (Figure 5D). Arg Δ C-YFP-expressing *arg*^{-/-} cells also do not appreciably contract the gels ($0.8 \pm 1.1\%$; Figure 5E). This apparently increased potency of Arg Δ C-YFP relative to Arg-YFP may result in part from its greater expression level (4–5-fold) relative to Arg-YFP (2–3-fold) over normal endogenous Arg levels (Supplementary Figure S2). *arg*^{-/-} cells expressing ArgKI-YFP contract the gels by $38 \pm 5\%$, indicating that the C-terminus has only modest effects on contractility relative to Arg-YFP (Figure 5E). These experiments indicate that Arg kinase activity is important to inhibit contractility, but that the Arg C-terminal half is largely dispensable for this function. Thus, the Arg functional domains required for Arg to inhibit cell contractility mirror those required to inhibit stress fiber formation (Table 1 and Figures 3 and 5).

We also investigated whether p190RhoGAP is required for the effects of Arg on cell contractility. Arg-YFP expression in *p190*^{+/+} cells resulted in a decrease in contractility ($13 \pm 2\%$), relative to *p190*^{+/+} cells expressing YFP alone ($34 \pm 2\%$; Figure 5F). Arg-YFP did not lead to a significant decrease in contractility of *p190*^{-/-} cells ($18 \pm 2\%$ shrinkage) relative to YFP-expressing *p190*^{-/-} cells ($23 \pm 1\%$ shrinkage; Figure 5F). Control experiments indicated that Arg-YFP is expressed at similar levels in *p190*^{+/+} and *p190*^{-/-} cells (Supplementary Figure S2). Together, these data indicate that Arg acts through p190RhoGAP to attenuate F-actin stress fiber formation and cell contractility.

FA Dynamics and Anchoring to the Substrate are Altered in *arg*^{-/-} fibroblasts

We examined how increased contractility influences the fate of FAs by monitoring the localization of a paxillin-GFP fusion in *arg*^{-/-} relative to wt fibroblasts. wt cells have numerous paxillin-GFP-positive puncta, and most of these puncta turn over rapidly during a 30-min observation period (Supplementary Video SV6). Although *arg*^{-/-} fibroblasts have larger paxillin-GFP-positive adhesions, adhe-

sions at both the leading and lagging edges slide relative to the substrate, moving as a complex without disassembling (Supplementary Video SV7). We documented the FA movements using “rainbow analysis” (Smilenov *et al.*, 1999), in which fluorescence images of FAs taken at successive time points in a time-lapse series are pseudocolored different colors. In overlay images, FAs that turn over rapidly appear as a single color, stationary long-lived FAs appear as a uniform white color, and FAs that move relative to the substrate appear as a “rainbow,” with adjacent spots of color aligned next to one another. FAs turn over rapidly in wt cells, appearing often as a dimmer single color, indicating their appearance and disappearance within the observation period (see enlargements 1 and 2, Figure 6A). In contrast, most of the larger brighter, clustered FAs in *arg*^{-/-} cells have a rainbow appearance (Figure 6B). These FAs usually move concertedly in large clusters that align with prominent stress fibers. They also rarely turn over during the observation period (see enlargements 1 and 2, Figure 6B).

The large paxillin-positive FAs in *arg*^{-/-} cells (Figure 2B) could result from more rapid FA formation or a decrease in FA turnover. FA formation and disassembly rate constants for wt and *arg*^{-/-} cells were determined from the rate of change in paxillin-GFP fluorescence intensity, as described previously (Webb *et al.*, 2002). FAs assemble slightly faster in *arg*^{-/-} cells compared with wt cells, although the difference is not significant (Figure 6D). Most FAs at the rear of the *arg*^{-/-} cells do not disassemble normally. Instead, they elongate, fuse with other FA complexes, and slide along the substrate as cells move. For this reason, we were only able to measure the rate constants for FA disassembly at the more stable and leading edges, where disassembly rates were 6.1-fold slower than in wt cells (Figure 6E). We also noted several examples where *arg*^{-/-} cells lurch forward and leave spots of FA material behind. This analysis indicates that the loss of Arg function inhibits FA turnover.

We noted that re-expression of ArgKI-YFP in *arg*^{-/-} cells leads to reduced staining for paxillin and vinculin in FAs (Figure 3, C and D, and data not shown). We monitored paxillin-dsRed fluorescence in these cells to examine FA formation and turnover (Supplementary Video SV8). FAs in *arg*^{-/-} + ArgKI-YFP cells appeared uniformly white in rainbow analysis, suggesting that they were more stable than in *arg*^{-/-} cells (Figure 6C). Some FAs slide at the trailing edge of *arg*^{-/-} + ArgKI-YFP cells, but the degree of sliding is much less than *arg*^{-/-} cells (Figure 6C; Supplementary Video SV8). Measurement indicated that FAs form 2.3-fold slower and disassemble 5.9-fold slower than in wt cells (Figure 6, D and E). This observation indicates that ArgKI-YFP inhibits FA formation and cannot complement the FA turnover defects in *arg*^{-/-} cells.

Both Arg Δ C and ArgKI Inhibit Cell Migration

Having established that Arg Δ C can inhibit cell contractility, whereas ArgKI alters FA formation and disassembly, we examined how these mutants affect cell migration. *arg*^{-/-} + Arg Δ C-YFP fibroblasts migrate even slower than Arg-YFP-expressing cells, with an average velocity 50% slower than YFP-expressing wt fibroblasts (Figure 1G). Significantly, the ability of Arg-YFP and Arg Δ C-YFP to inhibit migration is proportional to their ability to inhibit cell contractility. Interestingly, although *arg*^{-/-} + ArgKI-YFP cells are more contractile than wt cells, they migrate with a 29.5% slower average velocity than YFP-expressing wt cells (Figure 1G). This reduced migration speed correlates with an inability of these cells to turn over substrate attachments (Figure 6, C and E). Together, our data suggest that Arg reduces cell

A wt cell expressing paxillin-GFP **B *arg*^{-/-} cell expressing paxillin-GFP** **C *arg*^{-/-} cell expressing ArgKI-YFP and paxillin-DsRed**

Figure 6. FA dynamics are altered in *arg*^{-/-} cells. (A–C) Fluorescence images of a time-lapse sequence of paxillin-GFP (A and B) or paxillin-DsRed (C) dynamics in a wt (A; see Supplementary Video SV6), *arg*^{-/-} (B; Supplementary Video SV7), or *arg*^{-/-} + ArgKI-YFP (C; Supplementary Video SV8) fibroblast. Images obtained at 1, 14, and 27 min (left panels) were colored red, green, and blue, respectively, and overlaid to form the merge image. Stationary FAs appear as a single color, whereas FAs that move have a rainbow appearance. Boxed areas in the central merge image are enlarged 400% for the merge and 1-, 14-, and 27-min time-point panels on the right to show FA dynamics in different parts of the fibroblasts. Arrows mark the trailing edge(s) of the cell. The FA in enlargement (1) of the wt cell (A) assembles between the 1- and 14-min time points and disassembles between the 14- and 27-min time points. The FA in enlargement (2) of the wt cell assembles between the 1- and 14-min time points, but does not disassemble. The FAs in *arg*^{-/-} cells (B) slide, but do not significantly decrease in intensity during filming period. FAs form slowly in *arg*^{-/-} + ArgKI-YFP cells (C) during the 27-min time period, and once formed, do not change intensity significantly over the time period. The FAs in *arg*^{-/-} + ArgKI-YFP cells do not slide significantly (white appearance). Note that FAs in wt cells are dimmer than in either *arg*^{-/-} or *arg*^{-/-} + ArgKI-YFP cells. Bar, 10 μ m. (D and E) Rate constants for FA formation (D) and disassembly (E) for wt, *arg*^{-/-}, and *arg*^{-/-} + ArgKI-YFP cells are given. Mean $\pm$ SE. Assembly and disassembly rate constants were determined respectively from 21 FAs from five and four wt cells, from 20 FAs each from four *arg*^{-/-} cells, and from 19 FAs from four *arg*^{-/-} + ArgKI-YFP cells. For D and E, ANOVA between different cell types, * p = 0.0001. *post hoc Student-Newman-Keuls, p < 0.05.

migration both by attenuating cell contractility and by regulating focal adhesion dynamics and stability.

DISCUSSION

Genetic studies indicate that Abl family kinases translate signals from cell surface receptors into changes in cell shape and movement, but it is unclear which cellular processes are controlled by these kinases. We report here that integrin signaling through Arg regulates fibroblast migration on FN by inhibiting cellular actomyosin contractility and regulating focal adhesion turnover. We show that Arg kinase activity acts through p190RhoGAP to in-

hibit stress fibers, resulting in reduced actomyosin contractility. We also show that the loss of Arg function slows FA turnover, leading to larger focal adhesions. However, the increased contractility in *arg*^{-/-} cells can tear FAs from the substrate, resulting in an aberrant snap-like mode of migration in these cells. These data indicate that Arg regulates integrin-mediated cell migration by attenuating cell contractility and regulating its coupling to adhesion sites.

Arg is a New, Important Regulator of FAs in Migrating Cells

Our findings show that Arg plays an important role in governing the size, distribution, and dynamics of FAs

within migrating cells. Using *arg*^{-/-} cells reconstituted with various Arg mutants, we separated the different Arg functions in FA regulation. Our results indicate that Arg kinase activity is required for normal FA turnover and stable anchoring of FAs to the substrate. The Arg C-terminal half inhibits FA formation. The absence of *both* functions leads to the inhibition of FA disassembly, a modest increase in FA formation, and sliding of FAs at the trailing edge in *arg*^{-/-} cells. Arg therefore serves as a new central regulator of FAs in a migrating cell; it inhibits FA formation and increases turnover in dynamic protrusive areas of migrating cells.

***arg*^{-/-} Cells Exhibit an Imbalance of Adhesive Strength and Contractile Forces**

Despite having large FAs that do not turn over efficiently, *arg*^{-/-} cells migrate faster than wt cells. This observation indicates that the increased contractile forces in these cells are sufficient to overcome the larger FAs in these cells, especially in the trailing edge. Indeed, FAs slide in groups relative to the substrate in Arg-deficient cells. We also find that *arg*^{-/-} cells migrate aberrantly in large steps that result from the sudden detachment at the cell's trailing edge.

Genetic studies have revealed critical roles for Abl family kinases in regulating cell migration and tissue morphogenesis in developing organisms. Arg is required for integrin-dependent neurite branching *in vitro* and dendrite branch stabilization in the developing mouse brain (Moresco *et al.*, 2005). Proper mouse neural tube morphogenesis requires Abl and Arg (Koleske *et al.*, 1998). *Drosophila* contain a single Abl family kinase, Abl, which is equally related to Abl and Arg phylogenetically. *Drosophila* Abl localizes to cell-cell contact points (Bennett and Hoffmann, 1992), and loss of its function leads to defects in epithelial cell morphogenesis (Grevengeot *et al.*, 2001, 2003) and axon guidance (Wills *et al.*, 1999a,b, 2002; Bashaw *et al.*, 2000; Liebl *et al.*, 2000). Our results suggest that these phenotypes may result in part from a failure to properly regulate actomyosin contractility. We anticipate that it will be possible to suppress some Arg-dependent phenotypes via genetic or chemical inhibition of actomyosin contractility.

Do Abl and Arg Play Different Roles in the Regulation of Cell Migration?

Chemical or dominant-negative inhibition of Abl or Arg can lead to increased cell migration (Frasca *et al.*, 2001; Kain and Klemke, 2001). *abl*^{-/-}*arg*^{-/-} fibroblasts migrate faster than wt fibroblasts in wound healing assays, and this migration can be slowed by re-expression of Abl (Kain and Klemke, 2001). The slowing of cell migration by Abl correlates with phosphorylation of the Crk adaptor protein, which blocks Crk association with the Crk-associated substrate p130Cas (Kain and Klemke, 2001). The Crk-CAS complex is believed to promote cell migration by activating the Rac1 GTPase, and Kain and Klemke (2001) propose that Abl slows cell migration by disruption of a Crk-CAS complex. However, Crk phosphorylation is normal in single mutant *abl*^{-/-} fibroblasts, which do not exhibit altered migration properties (Kain and Klemke, 2001). It is possible that Arg plays a redundant role with Abl in Crk phosphorylation and subsequent Crk-CAS coupling in fibroblasts during adhesion to FN. It is not clear if Abl and/or Arg kinase signaling through Crk-CAS affects focal adhesion dynamics or actomyosin contractility to alter cell migration rate.

Our experiments describe important novel functions for Arg in the regulation of actomyosin contractility and focal adhesion dynamics. We demonstrate that Arg acts

through its substrate p190RhoGAP to inhibit Rho-induced stress fiber formation and actomyosin contractility. We also find that FAs are larger and do not turn over efficiently in *arg*^{-/-} cells. Interestingly, the larger FAs in *arg*^{-/-} can be partially reduced by expression of either wt Arg or a kinase-inactive Arg mutant. This observation suggests that Arg may act via a kinase-independent mechanism to control FA dynamics.

The effects of Abl family kinases on overall cell migration depend on the migration stimulus. Modest elevation of Abl expression can promote cell migration in response to platelet-derived growth factor (Plattner *et al.*, 2003), but Arg does not share this property (Plattner *et al.*, 2004). These data point to clear differences in the regulation of cell migration by Abl and Arg. However, the downstream effectors that mediate Abl's promigratory effects are not yet clear.

Arg May Coordinate Cell Edge Protrusion with Actomyosin Contractility in Migrating Cells

Actin polymerization promotes cell edge protrusion, whereas actomyosin contractility generates traction forces that translocate the cell body. Analysis of neuronal growth cone and epithelial cell migration on adhesive surfaces indicates that protrusion and contractility are coordinately regulated and sensitive to the density of adhesive cues (Lin and Forscher, 1995; Suter *et al.*, 1998; Gup-ton and Waterman-Storer, 2006).

The regulatory pathways that coordinate cell edge protrusion and actomyosin contractility during migration are not well understood. We have previously shown that Arg promotes F-actin-rich protrusions in fibroblasts as they adhere and spread on FN (Miller *et al.*, 2004). We demonstrate here a novel role for Arg as an important attenuator of actomyosin contractility. Importantly, we have shown that Arg-deficient cells exhibit reduced cell edge protrusion during adhesion and spreading (Miller *et al.*, 2004), but they migrate faster than wt cells due to increased actomyosin contractility. Conversely, modest elevation of Arg activity promotes protrusions, reduces contractility, and inhibits overall cell migration.

We propose that Arg coordinates cell protrusion and contractility during migration on adhesive surfaces (Supplementary Figure 3). According to this model, localization of Arg to the leading edge might simultaneously promote actin polymerization and locally inhibit Rho-induced contractility at the periphery via p190RhoGAP (Pertz *et al.*, 2006). Integrin signaling through Arg generates numerous cell protrusions (Miller *et al.*, 2004), allowing the cell to sample the local adhesive environment. Moreover, Arg localization to the leading edge would reduce Arg levels in the trailing edge, resulting in increased Rho-induced cell contractility required for tail retraction (Pertz *et al.*, 2006). According to this model, Arg must somehow be released from complexes at the leading edge to allow formation of focal adhesions and stress fibers that propel the cell forward. The dual roles for Arg in cell protrusion and relaxation of contractility may allow cells greater sensitivity to subtle differences in adhesive cues. Arg-induced protrusion may help cells survey the adhesive landscape, whereas Arg signaling through p190RhoGAP could attenuate contractility during this surveying process.

ACKNOWLEDGMENTS

We thank Xianyun Ye for expert technical assistance, Pam Arora for advice on collagen gel contraction assays, and Jeff Settleman (Massachusetts General Hospital) for *p190*^{+/+} and *p190*^{-/-} cells. We thank Scott Boyle, Paul Forscher, Rick Horwitz, Michael Koelle, and Clare Waterman-Storer for logistical sup-

port, helpful discussions, and critical comments on the manuscript. This work was supported by Public Health Service Grants NS39754 and MH77306. A.L.M. was supported by a predoctoral National Research Service Award (NS45477). J.G.P. and O.C.R. are National Science Foundation Fellows. W.D.B. is a Scottish Rite Schizophrenia Research Fellow. A.J.K. is a Scholar Leukemia and Lymphoma Society of America and an Established Investigator of the American Heart Association.

REFERENCES

- Affolter, M., and Weijer, C. J. (2005). Signaling to cytoskeletal dynamics during chemotaxis. *Dev. Cell* 9, 19–34.
- Arora, P. D., and McCulloch, C. A. (1994). Dependence of collagen remodeling on alpha-smooth muscle actin expression by fibroblasts. *J. Cell. Physiol.* 159, 161–175.
- Bashaw, G. J., Kidd, T., Murray, D., Pawson, T., and Goodman, C. S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell* 101, 703–715.
- Bennett, R. L., and Hoffmann, F. M. (1992). Increased levels of the *Drosophila* Abelson tyrosine kinase in nerves and muscles: subcellular localization and mutant phenotypes imply a role in cell-cell interactions. *Development* 116, 953–966.
- Bradley, W. D., Hernandez, S. E., Settleman, J., and Koleske, A. J. (2006). Integrin signaling through Arg activates p190RhoGAP by promoting its binding to p120RasGAP and recruitment to the membrane. *Mol. Biol. Cell* 17, 4827–4836.
- Chrzanowska-Wodnicka, M., and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* 133, 1403–1415.
- de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M. A., and Waterman-Storer, C. M. (2005). Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J. Cell Biol.* 171, 153–164.
- Frasca, F., Vigneri, P., Vella, V., Vigneri, R., and Wang, J. Y. (2001). Tyrosine kinase inhibitor ST1571 enhances thyroid cancer cell motile response to hepatocyte growth factor. *Oncogene* 20, 3845–3856.
- Grevengoed, E. E., Fox, D. T., Gates, J., and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J. Cell Biol.* 163, 1267–1279.
- Grevengoed, E. E., Loureiro, J. J., Jesse, T. L., and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in *Drosophila*. *J. Cell Biol.* 155, 1185–1198.
- Gupton, S. L., and Waterman-Storer, C. M. (2006). Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell* 125, 1361–1374.
- Hernandez, S. E., Krishnaswami, M., Miller, A. L., and Koleske, A. J. (2004a). How do Abl family kinases regulate cell shape and movement? *Trends Cell Biol.* 14, 36–44.
- Hernandez, S. E., Settleman, J., and Koleske, A. J. (2004b). Adhesion-dependent regulation of p190RhoGAP in the developing brain by the Abl-related gene tyrosine kinase. *Curr. Biol.* 14, 691–696.
- Jay, P. Y., Pham, P. A., Wong, S. A., and Elson, E. L. (1995). A mechanical function of myosin II in cell motility. *J. Cell Sci.* 108(Pt 1), 387–393.
- Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995). The molecular architecture of focal adhesions. *Annu. Rev. Cell Dev. Biol.* 11, 379–416.
- Kaibuchi, K. (1999). Regulation of cytoskeleton and cell adhesion by Rho targets. *Prog. Mol. Subcell. Biol.* 22, 23–38.
- Kain, K. H., and Klemke, R. L. (2001). Inhibition of cell migration by Abl family tyrosine kinases through uncoupling of Crk-CAS complexes. *J. Biol. Chem.* 276, 16185–16192.
- Koleske, A. J. (2006). Regulation of Cytoskeletal Dynamics and Cell Morphogenesis by Abl Family Kinases, Georgetown, TX: Landes Bioscience.
- Koleske, A. J., Gifford, A. M., Scott, M. L., Nee, M., Bronson, R. T., Miczek, K. A., and Baltimore, D. (1998). Essential roles for the Abl and Arg tyrosine kinases in neurulation. *Neuron* 21, 1259–1272.
- Kruh, G. D., Perego, R., Miki, T., and Aaronson, S. A. (1990). The complete coding sequence of arg defines the Abelson subfamily of cytoplasmic tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 87, 5802–5806.
- Lauffenburger, D. A., and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* 84, 359–369.
- Liebl, E. C., Forsthoefel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M., and Seeger, M. A. (2000). Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. *Neuron* 26, 107–118.
- Lin, C. H., and Forscher, P. (1995). Growth cone advance is inversely proportional to retrograde F-actin flow. *Neuron* 14, 763–771.
- Miller, A. L., Wang, Y., Mooseker, M. S., and Koleske, A. J. (2004). The Abl-related gene (Arg) requires its F-actin-microtubule cross-linking activity to regulate lamellipodial dynamics during fibroblast adhesion. *J. Cell Biol.* 165, 407–419.
- Mitchison, T. J., and Cramer, L. P. (1996). Actin-based cell motility and cell locomotion. *Cell* 84, 371–379.
- Moresco, E. M., Donaldson, S., Williamson, A., and Koleske, A. J. (2005). Integrin-mediated dendrite branch maintenance requires Abelson (Abl) family kinases. *J. Neurosci.* 25, 6105–6118.
- Murphy, K. H., and Daniel, J. C. (1987). Human periodontal ligament in vitro: cell culture passage effect on collagen gel contraction. *J. Periodont. Res.* 22, 342–347.
- Nobes, C. D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53–62.
- Pendergast, A. M. (2002). The Abl family kinases: mechanisms of regulation and signaling. *Adv. Cancer Res.* 85, 51–100.
- Pertz, O., Hodgson, L., Klemke, R. L., and Hahn, K. M. (2006). Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* 440, 1069–1072.
- Plattner, R., Irvin, B. J., Guo, S., Blackburn, K., Kazlauskas, A., Abraham, R. T., York, J. D., and Pendergast, A. M. (2003). A new link between the c-Abl tyrosine kinase and phosphoinositide signalling through PLC-gamma1. *Nat. Cell Biol.* 5, 309–319.
- Plattner, R., Koleske, A. J., Kazlauskas, A., and Pendergast, A. M. (2004). Bidirectional signaling links the Abelson kinases to the platelet-derived growth factor receptor. *Mol. Cell Biol.* 24, 2573–2583.
- Pollard, T. D., and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 112, 453–465.
- Ponti, A., Machacek, M., Gupton, S. L., Waterman-Storer, C. M., and Danuser, G. (2004). Two distinct actin networks drive the protrusion of migrating cells. *Science* 305, 1782–1786.
- Ridley, A. J. (2001). Rho GTPases and cell migration. *J. Cell Sci.* 114, 2713–2722.
- Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70, 389–399.
- Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* 302, 1704–1709.
- Rottner, K., Hall, A., and Small, J. V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* 9, 640–648.
- Smilenov, L. B., Mikhailov, A., Pelham, R. J., Marcantonio, E. E., and Gundersen, G. G. (1999). Focal adhesion motility revealed in stationary fibroblasts. *Science* 286, 1172–1174.
- Straight, A. F., Cheung, A., Limouze, J., Chen, I., Westwood, N. J., Sellers, J. R., and Mitchison, T. J. (2003). Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. *Science* 299, 1743–1747.
- Suter, D. M., Errante, L. D., Belotserkovsky, V., and Forscher, P. (1998). The Ig superfamily cell adhesion molecule, apCAM, mediates growth cone steering by substrate–cytoskeletal coupling. *J. Cell Biol.* 141, 227–240.
- Wang, B., Mysliwiec, T., Feller, S. M., Knudsen, B., Hanafusa, H., and Kruh, G. D. (1996). Proline-rich sequences mediate the interaction of the Arg protein tyrosine kinase with Crk. *Oncogene* 13, 1379–1385.
- Wang, Y., Miller, A. L., Mooseker, M. S., and Koleske, A. J. (2001). The Abl-related gene (Arg) nonreceptor tyrosine kinase uses two F-actin-binding domains to bundle F-actin. *Proc. Natl. Acad. Sci. USA* 98, 14865–14870.
- Webb, D. J., Parsons, J. T., and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells—over and over and over again. *Nat. Cell Biol.* 4, E97–E100.
- Wills, Z., Bateman, J., Korey, C. A., Comer, A., and Van Vactor, D. (1999a). The tyrosine kinase Abl and its substrate enabled collaborate with the receptor phosphatase Dlar to control motor axon guidance. *Neuron* 22, 301–312.

Wills, Z., Emerson, M., Rusch, J., Bikoff, J., Baum, B., Perrimon, N., and Van Vactor, D. (2002). A *Drosophila* homolog of cyclase-associated proteins collaborates with the Abl tyrosine kinase to control midline axon pathfinding. *Neuron* 36, 611–622.

Wills, Z., Marr, L., Zinn, K., Goodman, C. S., and Van Vactor, D. (1999b). Profilin and the Abl tyrosine kinase are required for motor axon outgrowth in the *Drosophila* embryo. *Neuron* 22, 291–299.

Woodring, P. J., Hunter, T., and Wang, J. Y. (2003). Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. *J. Cell Sci.* 116, 2613–2626.

Woodring, P. J., Litwack, E. D., O'Leary, D. D., Lucero, G. R., Wang, J. Y., and Hunter, T. (2002). Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. *J. Cell Biol.* 156, 879–892.

Woodring, P. J. *et al.* (2004). c-Abl phosphorylates Dok1 to promote filopodia during cell spreading. *J. Cell Biol.* 165, 493–503.