

Arp2/3- and Cofilin-coordinated Actin Dynamics Is Required for Insulin-mediated GLUT4 Translocation to the Surface of Muscle Cells

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GLUT4 vesicles are actively recruited to the muscle cell surface upon insulin stimulation. Key to this process is Rac-dependent reorganization of filamentous actin beneath the plasma membrane, but the underlying molecular mechanisms have yet to be elucidated. Using L6 rat skeletal myoblasts stably expressing myc-tagged GLUT4, we found that Arp2/3, acting downstream of Rac GTPase, is responsible for the cortical actin polymerization evoked by insulin. siRNA-mediated silencing of either Arp3 or p34 subunits of the Arp2/3 complex abrogated actin remodeling and impaired GLUT4 translocation. Insulin also led to dephosphorylation of the actin-severing protein cofilin on Ser-3, mediated by the phosphatase slingshot. Cofilin dephosphorylation was prevented by strategies depolymerizing remodeled actin (latrunculin B or p34 silencing), suggesting that accumulation of polymerized actin drives severing to enact a dynamic actin cycling. Cofilin knockdown via siRNA caused overwhelming actin polymerization that subsequently inhibited GLUT4 translocation. This inhibition was relieved by reexpressing *Xenopus* wild-type cofilin-GFP but not the S3E-cofilin-GFP mutant that emulates permanent phosphorylation. Transferrin recycling was not affected by depleting Arp2/3 or cofilin. These results suggest that cofilin dephosphorylation is required for GLUT4 translocation. We propose that Arp2/3 and cofilin coordinate a dynamic cycle of actin branching and severing at the cell cortex, essential for insulin-mediated GLUT4 translocation in muscle cells.

INTRODUCTION

A major function of insulin is to regulate glucose uptake by muscle and fat tissues. This is achieved through a rapid and dynamic gain in glucose transporter-4 (GLUT4) at the cell surface (Huang and Czech, 2007; Larance *et al.*, 2008; Zaid *et al.*, 2008). Notably, this process becomes defective in insulin resistance states and type 2 diabetes (Klip *et al.*, 1990; Zierath *et al.*, 1996; Garvey *et al.*, 1998; Mora and Pessin, 2002). To date, defects in insulin signaling and GLUT4 traffic per se have been invoked to underlie such defects (Krook *et al.*, 2004; Patel *et al.*, 2006).

Skeletal muscle is the primary site of insulin-dependent glucose disposal in vivo, and muscle cells in culture are useful to scrutinize principles of this response. L6 myoblasts, like further differentiated myotubes and skeletal muscle, mount robust responses of insulin signaling via insulin receptors, the insulin receptor substrate-1 (IRS-1), phosphati-

dylinositol 3-kinase (PI3K), and Akt (Ruderman *et al.*, 1990; Tsakiridis *et al.*, 1995; Thong *et al.*, 2007). In muscle cells, signaling bifurcates downstream of PI3K into two independent arms characterized by phosphorylation of Akt (Wang *et al.*, 1999) and GTP activation of the small GTPase Rac leading to actin remodeling (Khayat *et al.*, 2000; JeBailey *et al.*, 2004). The two pathways are independent of one another because neither Akt dominant-negative mutants (Wang *et al.*, 1999) nor the Akt inhibitor Akti (A. Koshkina and A. Klip, unpublished data) prevent insulin-induced Rac activation or its consequent actin remodeling, and disruption of Rac via small inhibitory RNA (siRNA) fails to reduce Akt phosphorylation by insulin (JeBailey *et al.*, 2007). Both signaling arms are required to elicit proper insulin-mediated GLUT4 translocation as perturbation of either one significantly reduces the GLUT4 response to insulin in muscle cells (Wang *et al.*, 1999; JeBailey *et al.*, 2007; Ishikura and Klip, 2008; Zaid *et al.*, 2008).

Although much emphasis has been placed on the effectors downstream of Akt such as Akt substrate of 160 (AS160) and Rab GTPases (Sano *et al.*, 2003; Miinea *et al.*, 2005; Gonzalez and McGraw, 2006; Ishikura *et al.*, 2007; Sano *et al.*, 2007; Thong *et al.*, 2007; Ishikura and Klip, 2008), the function of Rac remains less explored. Rac belongs to the small Rho GTPase family whose activity is regulated by GTP loading (Bernards and Settleman, 2004; Rossman *et al.*, 2005). Insulin promotes GTP loading of Rac within the first 1–5 min of stimulation (JeBailey *et al.*, 2004; Ishikura *et al.*, 2008). Once activated, Rac induces the reorganization of cortical actin

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filaments (Khayat *et al.*, 2000; JeBailey *et al.*, 2007). This actin remodeling is a critical component in insulin-stimulated GLUT4 translocation because overexpression of a dominant negative Rac mutant (Khayat *et al.*, 2000) or siRNA-mediated Rac knockdown (JeBailey *et al.*, 2007) not only prevent actin remodeling but also markedly diminish the insulin-mediated recruitment of GLUT4 to the surface. A similar reduction in insulin response is observed upon preventing actin remodeling with inhibitors of actin polymerization such as latrunculin B (LB) and cytochalasin D (Tsakiridis *et al.*, 1994; Khayat *et al.*, 2000) or by precluding actin depolymerization with jasplakinolide (Tong *et al.*, 2001). Altogether, these findings reveal the importance of peripheral actin reorganization in insulin-dependent GLUT4 translocation in muscle cells. However, the precise regulation of this dynamic actin change and the elements acting downstream of Rac are undefined. Moreover, a model that incorporates both actin polymerization and depolymerization, as required for dynamic remodeling, has not been proposed.

Here we test the hypothesis that insulin produces a dynamic regulation of actin remodeling involving cycles of branching and depolymerization. Using a well-established muscle cell model of L6GLUT4myc myoblasts displaying insulin-induced GLUT4myc exocytosis, we identify the Arp2/3 complex as a downstream effector of Rac that governs cortical actin polymerization and also identify cofilin as a regulator promoting actin filament depolymerization. Cofilin dephosphorylation requires prior buildup of polymerized actin driven by Arp2/3. Therefore, an integrated mode for active actin cycling is proposed that enables insulin-mediated GLUT4 translocation/insertion into the muscle cell membrane.

MATERIALS AND METHODS

Reagents, siRNA, and Constructs

Polyclonal anti-*myc* antibody (A-14) and anti-p34-ARC were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phosphorylated cofilin-1, anti-Akt, anti-phosphorylated Akt (Ser473), and anti-phosphorylated LIMK1 antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-Arp3 was from BD Biosciences (San Jose, CA). Polyclonal anti-slingshot-1 (anti-SSH1) was from ECM Biosciences (Versailles, KY). Affinity-purified antibodies against P-AC and cofilin-1 were as described (Meberg *et al.*, 1998; Shaw *et al.*, 2004). Monoclonal anti-ACTN1 (mouse IgM isotype, clone BM-75.2), anti- β -actin, polyclonal anti-LIMK1, LB, and DMSO (high-pressure liquid chromatography grade) were from Sigma-Aldrich (St. Louis, MO). Indocarbocyanine (Cy3) or horseradish peroxidase (HRP)-conjugated goat-anti-mouse, goat-anti-rabbit, and rabbit-anti-goat IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Rhodamine-bound phalloidin, Alexa 488-conjugated goat anti-mouse and anti-rabbit IgG antibodies were from Invitrogen (Carlsbad, CA). Human insulin was purchased from Eli Lilly (Indianapolis, IN). siRNAs targeted against p34 (AAGGAAC-TCAGGCACACGGA), Arp3 (GAAAGCGTGGATGACCTATT; Korobova and Svitkina, 2008), cofilin-1 (AAGGTGTCAATGACATGAAA), LIMK1 (AAGGAATGTGCCGCTGGCAGATT), slingshot-1 (AAGAATGAGCGTC-TCATTA), and nonrelated control (AATAAGGCTATGAAGAGATAC) were purchased from Qiagen (Chatsworth, CA) and are here named, respectively, sip34, siArp3, siCofilin, siLIMK, and siSSH1. Green fluorescent protein (GFP)-tagged *Dictyostelium discoideum* Arp3 was kindly provided by Dr. Sergio Grinstein (University of Toronto, Toronto, ON, Canada). GFP-tagged wild-type (WT) and S3E *Xenopus* cofilin were generated in the laboratory of Dr. J. Bamberg (Colorado State University; Ashworth *et al.*, 2003).

Cell Culture and Transfection

Rat L6 muscle cells expressing GLUT4 with an exofacial *myc* epitope (L6 GLUT4myc) were cultured as described previously (Ueyama *et al.*, 1999). GLUT4 transfected into these cells segregates away from the recycling pathway and responds to insulin to a similar extent in myoblasts and myotubes (Ueyama *et al.*, 1999; Niu *et al.*, 2003). Myoblasts, however, offer the advantage of being easier to transfect and to scrutinize by confocal fluorescence microscopy. Transfection of siRNAs used the calcium phosphate-based CellPect Transfection Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). siRNA-calcium phosphate precipitates, 200 nM, were removed 12–16 h after addition,

and cells were maintained for 72 h until experimentation. Transfection of cDNA used Lipofectamine 2000 (Invitrogen). The cDNA-Lipofectamine mixture was removed after 4–6-h incubation, and cells were allowed to recover for 24 h before experimentation.

Cell-Surface GLUT4myc detection by Immunofluorescence Microscopy

Immunofluorescent detection of surface GLUT4myc in adhered L6 myoblasts was performed as previously described (Torok *et al.*, 2004). After 3-h serum starvation, cells were stimulated with and without 100 nM insulin for 10 min at 37°C. Cells were quickly washed twice with cold PBS, fixed with 3% (vol/vol) paraformaldehyde, and blocked with 5% (vol/vol) milk. Surface GLUT4myc was stained by incubating anti-*myc* primary antibody followed by Cy2- or Cy3-coupled secondary antibody. For costaining of surface GLUT4myc and intracellular filamentous (F)-actin, surface GLUT4myc was labeled first before membrane permeabilization for subsequent F-actin staining. Fluorescent images were obtained with a Zeiss LSM 510 laser-scanning confocal microscope (Thornwood, NY). Whole cells were scanned along the z-axis, and a single composite image (collapsed *xy* projection) of the optical cuts per cell was generated using LSM5 Image software. Pixel intensity of single cells (>30 cells per condition) was quantified by ImageJ software (<http://rsb.info.nih.gov/ij/>). Surface GLUT4myc was also detected by immunofluorescence in intact rounded-up myoblasts prepared as described previously (Randhawa *et al.*, 2008; Talior-Volodarsky *et al.*, 2008).

Detection and Quantification of Actin Remodeling/Polymerized Dorsal Actin

After treatment with or without insulin for 10 min at 37°C, adhered or rounded-up L6 myoblasts were fixed, permeabilized with 0.1% Triton X-100 in PBS for 3 min, blocked with 5% milk, and stained with rhodamine-phalloidin for F-actin. Cells were imaged by Zeiss LSM 510 laser scanning confocal microscope. Acquisition parameters were adjusted to minimize signal saturation. To quantify actin remodeling, the F-actin pixel intensity from compiled dorsal optical slices of basal and insulin-stimulated cells were analyzed with ImageJ software (>30 cells per condition). The optical slices quantified began from the outermost confocal fluorescent signal detected and continued toward the interior of the cell but skipped the last 2–3 optical slices dominated by actin stress fibers. By eliminating from the quantification the focal planes enriched in parallel arrays of stress fibers, which are still devoid of crisscrossed arrays of branched actin (vastly present in the top half of the cells toward the dorsal surface), we quantify cortical remodeling. The method may potentially underestimate this response, but not magnify it.

Cell Lysates and Immunoblotting

After treatments, cells were washed quickly with cold phosphate-buffered saline (PBS) and lysed with 1% Triton X-100 in PBS. Lysates were passed through a 29-gauge syringe needle five times, and supernatants were collected by a 10-min spin at 12,000 \times g. Equal protein samples were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA), and immunoblotted with respective antibodies. Primary antibodies were detected with the appropriate HRP-conjugated species-specific IgG antibodies. Detection was completed with Western Lightning Chemiluminescence Reagent Plus and HyBlot CL autoradiography film from Denville Scientific (Denville, NJ).

¹²⁵I-Transferrin Recycling

The recycling of transferrin (Tf) was measured essentially as previously described (Yan *et al.*, 2005). After siRNA treatment and 3-h serum starvation, myoblasts were labeled with ¹²⁵I-Tf (1 μ g/ml) for 30 min at 37°C. Cells were placed on ice, washed once with cold medium (α -MEM, 1% BSA, 20 mM HEPES), once with cold acid solution (0.15 M NaCl, 0.1 M glycine, pH 3.0), and again with cold medium. Cells were then chased with medium containing 200 μ g/ml holo-Tf with/without insulin at 37°C, and the medium was collected at the indicated time points. After another wash with cold medium, cells were scraped and lysed in 1 M NaOH. Radioactivity in cell lysates (internalized Tf) and medium collected (externalized Tf) was measured in a gamma counter. Data for each time point were measured in triplicate. Tf recycling was expressed as the ratio of externalized Tf to the internalized Tf. Data were corrected for nonspecific binding of ¹²⁵I-Tf by the addition of 200 μ g/ml holo-Tf during the initial labeling.

Statistical Analysis

Statistical analyses were carried out using Prism 4.0 software (GraphPad Software, San Diego, CA). Groups were compared using one-way ANOVA analysis. $p < 0.05$ was considered statistically significant.

RESULTS

Arp2/3 Is Required for Insulin-mediated Actin Remodeling

We have previously reported that insulin causes marked actin polymerization at the cortical periphery of L6 myoblasts and myotubes that has a branched appearance when visualized by fluorescence (Khayat *et al.*, 2000; Patel *et al.*, 2003) and electron (Randhawa *et al.*, 2008) microscopy and that manifests even in suspended myoblasts lacking stress fibers (JeBailey *et al.*, 2004; Randhawa *et al.*, 2008). The small GTPase Rac is a major regulator of such cortical actin remodeling (Khayat *et al.*, 2000; JeBailey *et al.*, 2004; JeBailey *et al.*, 2007).

Known effectors of the Rho-family of small G proteins that modify actin filament organization in general include the branching complex Arp2/3 (Goley and Welch, 2006), capping/severing proteins such as gelsolin, and the severing protein cofilin (Ono, 2007). Here we hypothesize that Arp2/3 acts directly downstream of Rac to initiate the insulin-dependent actin polymerization process. Arp2/3 is a seven-subunit complex consisting of Arp2, Arp3, p16, p20, p21, p34, and p40 (Goley and Welch, 2006). By transiently expressing the Arp3-GFP subunit into L6-GLUT4myc myoblasts, we sought to examine changes in the localization of Arp2/3 after insulin stimulation. In the unstimulated state, myoblasts displayed normal cellular stress fibers and Arp3-GFP was mainly cytosolic (Figure 1A). When challenged with insulin, an actin meshwork formed at the periphery of the cells, and Arp3-GFP redistributed to the zone of remodeled actin, suggesting its involvement at this region (Figure 1A).

To functionally establish the role of Arp2/3 in insulin-responsive actin remodeling, we interfered with the complex by silencing expression of its Arp3 subunit via siRNA-mediated knockdown. Significant down-regulation of Arp3 (by 77%) was achieved in siArp3-treated cells compared with cells treated with a nonrelated siRNA (siNR) sequence (Figure 1B). In Arp3 knockdown cells, the aspect and density of basal-state stress fibers remained unchanged. However, the insulin-induced actin remodeling was lost (Figure 1C). Quantitative analysis of remodeled actin revealed a 70% decrease in dorsal actin remodeling upon Arp3 down-regulation compared with control siNR-treated cells (Figure 1D). This deleterious effect was further validated by inhibiting the function of Arp2/3 via down-regulation of another Arp2/3 subunit, p34. As with siArp3, siRNA-mediated knockdown of p34 (sip34) by 60% also prevented the formation of remodeled actin structures at the cell periphery upon insulin stimulation (Figure 2, A and B).

To illustrate that Arp2/3 functions downstream of Rac to generate the dorsal actin meshwork, we took advantage of a constitutively active Rac-GFP mutant (CA-Rac-GFP) that remodels actin without insulin stimulation when transfected into myoblasts. In the control of siNR-transfected cells expressing CA-Rac-GFP, distinctive actin remodeling was observed, compared with neighboring nontransfected cells. However, CA-Rac-GFP failed to elicit actin remodeling after Arp3 knockdown (Figure S1A). This result suggests that Arp2/3 is a major effector downstream of Rac governing actin remodeling in muscle cells.

Depletion of Arp2/3 Reduces Insulin-mediated GLUT4 Gain on the Cell Surface

Because inhibition of Arp2/3 function averted actin remodeling, we explored its effect on insulin-responsive GLUT4 translocation. After siRNA-mediated disruption of Arp2/3 function, costaining of surface GLUT4myc and intracellular F-actin was

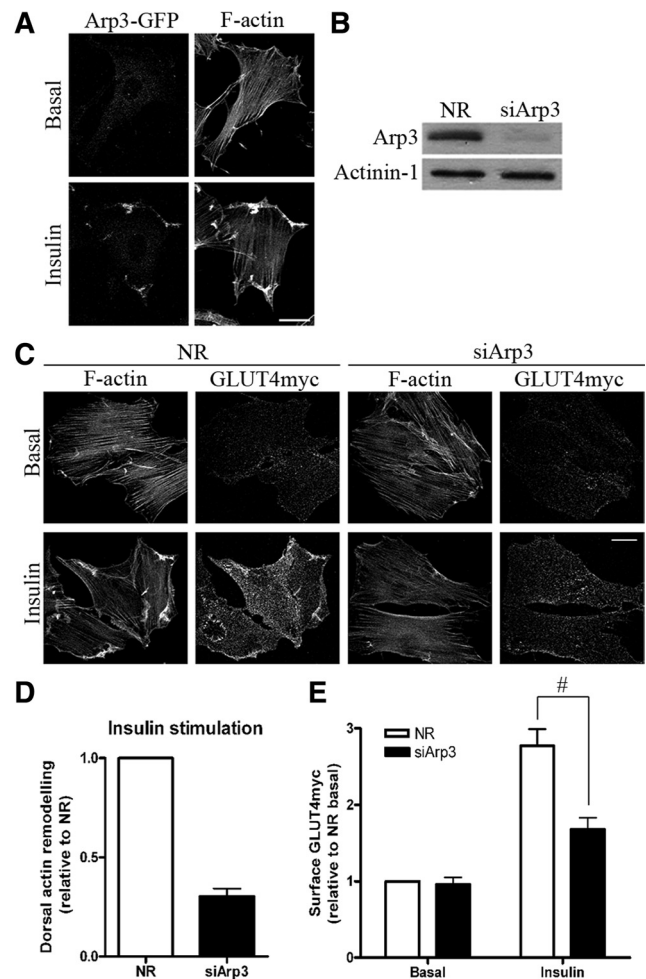


Figure 1. Down-regulation of Arp3 prevents insulin-induced actin remodeling and reduces GLUT4 translocation. (A) L6GLUT4myc myoblasts were transiently transfected with Arp3-GFP. After 3-h serum starvation, cells were stimulated with 100 nM insulin for 10 min. Subsequent staining of F-actin by rhodamine-phalloidin was performed to observe the changes in the localization of Arp3-GFP with respect to the remodeled actin. Representative images of four independent experiments are shown. Bars, 20 μ m. (B) Myoblasts were transfected with 200 nM of nonrelated (NR) siRNA control or Arp3 siRNA for 72 h. Total cell lysates were prepared, and 10 μ g protein was loaded and immunoblotted for Arp3 and actinin-1 (as loading control). Representative blots of five independent experiments are shown. (C) Myoblasts transfected with NR or Arp3 siRNA were treated with/without insulin for 10 min followed by staining surface GLUT4myc in nonpermeabilized cells and then permeabilized to label actin filaments with rhodamine-phalloidin. Dorsal actin remodeling was calculated from the pixel quantification in fluorescence optical cuts of the dorsal surface of adhered myoblasts (see *Materials and Methods*). Representative images of three independent experiments are shown. Bars, 20 μ m. (D) Quantification of changes in insulin-stimulated dorsal actin remodeling relative to NR control (mean \pm SE). (E) Quantification of fold increases in surface GLUT4myc relative to NR basal in NR and Arp3 knockdown conditions (mean \pm SE, #*p* < 0.05).

applied to identify cells lacking the insulin-induced actin rearrangement and their corresponding GLUT4myc levels on the plasma membrane. Surface GLUT4myc showed the typical speckled distribution (Patel *et al.*, 2003; Thong *et al.*, 2007) that may suggest insertion into hot spots. These may represent specific plasma membrane domains or may correspond to

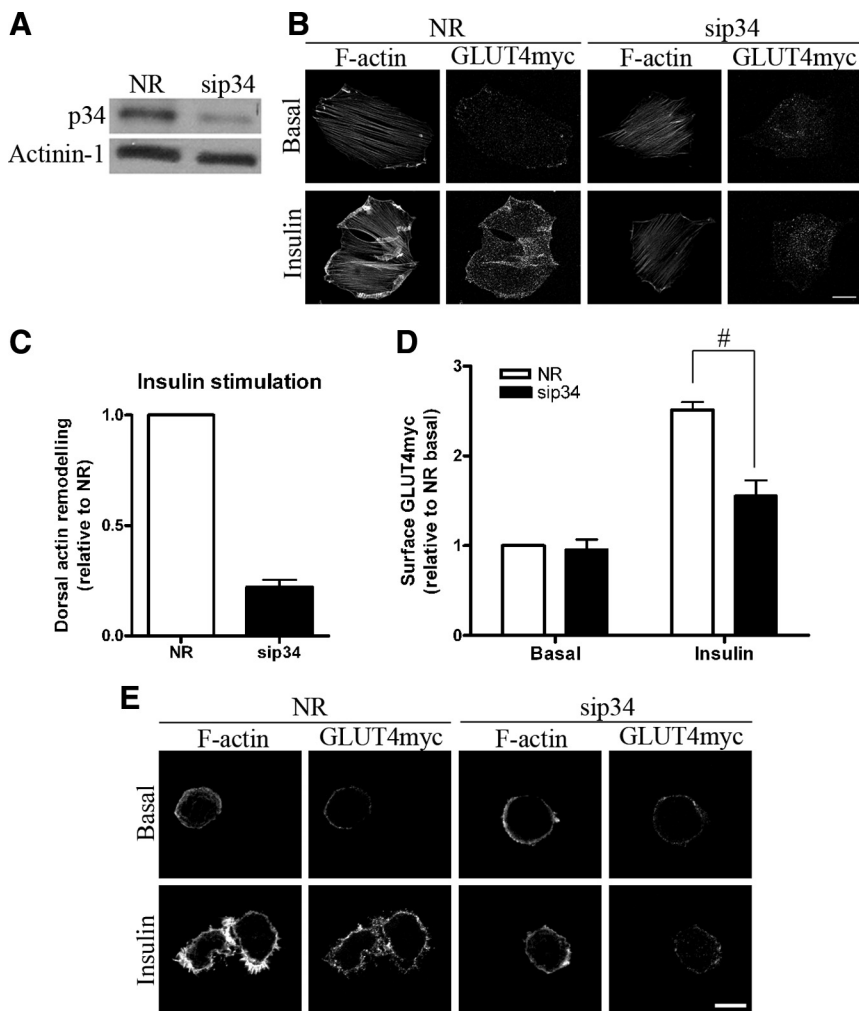


Figure 2. Down-regulation of p34 inhibits the formation of remodeled actin and decreases GLUT4 translocation. (A) L6GLUT4myc myoblasts were transfected with 200 nM of non-related (NR) siRNA control or p34 siRNA for 72 h. Total cell lysates were prepared, and 10 μ g protein was loaded and immunoblotted for p34 and actinin-1 (as loading control). Representative blots of five independent experiments are shown. (B) Myoblasts transfected with NR or p34 siRNA were treated with/without insulin for 10 min followed by costaining of surface GLUT4myc in the nonpermeabilized state and then permeabilized to label actin filaments with rhodamine-phalloidin. Representative images of five independent experiments are shown. Bars, 20 μ m. (C) Quantification of changes in insulin-stimulated dorsal actin remodeling relative to NR control (mean \pm SE). (D) Quantification of fold increases in surface GLUT4myc relative to NR basal in NR and p34 knockdown conditions (mean \pm SE, #*p* < 0.05). (E) p34 knockdown abolishes cortical actin remodeling in rounded-up myoblasts and prevents gain in surface GLUT4myc in response to insulin. Bars, 10 μ m.

ruffled areas supported by the remodeled actin, consistent with the predominant localization of GLUT4myc in ruffles determined by immunogold labeling and scanning electron microscopy (Tong *et al.*, 2001). Down-regulation of Arp3 did not alter the surface level of GLUT4myc in the basal state, compared with control cells treated with siNR (Figure 1, C and E). In contrast, siRNA to Arp3 not only prevented insulin-mediated peripheral actin remodeling but also significantly decreased the amount of GLUT4myc at the plasma membrane (Figure 1C). Quantification of surface GLUT4myc indicated a 44% inhibition in GLUT4 translocation after the down-regulation of Arp3 (Figure 1E). Furthermore, a strong reduction (60%) was achieved by knocking down the p34 subunit of Arp2/3 (Figure 2, B and D). Neither Arp3 nor p34 knockdown altered the overall insulin-stimulated Akt phosphorylation (Figure S1B), suggesting that the Akt signaling arm of insulin action remained intact.

To demonstrate the specificity of Arp2/3 in GLUT4 translocation, we rescued Arp2/3 expression by transfecting *D. discoideum* Arp3-GFP into siArp3-treated cells. Under this setting, insulin-stimulated actin remodeling was restored (Figure S2, A and B), and the deleterious effect of Arp3 down-regulation on insulin-mediated GLUT4 translocation was alleviated (Figure S2C). More importantly, expression of *Dictyostelium* Arp3-GFP alone did not change the basal actin filament morphology or surface GLUT4 in unstimu-

lated cells, indicating that Arp2/3 only exerts its functional action upon insulin stimulation.

The fact that depletion of Arp2/3 components only altered the insulin-dependent component of surface GLUT4 but did not change the basal levels of the transporters at the cell membrane suggests that the constitutive recycling of GLUT4 does not require Arp2/3 input. To establish if the effect of Arp2/3 interference is selective to GLUT4 traffic, we examined the effect of depletion of p34 of the Arp2/3 complex on Tf recycling, which depends on endosome recycling. As shown later in Figure 6, Tf recycling in either basal or insulin stimulated state was similar in cells treated with siRNA to p34 as in siNR-treated cells, implying that the inhibition of Arp2/3 did not affect the traffic of Tf.

The above results indicate that Arp2/3 is required for cortical actin remodeling and GLUT4 translocation including full exposure at the cell surface. To ascertain that the effect of Arp2/3 knockdown is independent of possible changes in actin stress fibers, we analyzed suspended myoblasts devoid of stress fibers. In this rounded-up configuration, myoblasts show actin filaments exclusively at the cell periphery (JeBailey *et al.*, 2004; Randhawa *et al.*, 2008; Talior-Volodarsky *et al.*, 2008). Myoblasts treated with siRNA to p34 of the Arp2/3 complex or with siNR were suspended, stimulated with insulin, and analyzed for actin remodeling and surface GLUT4 levels. Although siNR-treated cells

showed the habitual cortical actin arborizations and gain in surface GLUT4 in response to insulin, sip34 treatment markedly abolished both responses to insulin (Figure 2E). These results suggest that it is the cortical actin structures regulated by Arp2/3 that are required for GLUT4 translocation in response to the hormone.

Insulin Causes Cofilin Dephosphorylation That Depends on Slingshot

Actin dynamics is regulated by concerted actin polymerization and depolymerization. In fact, a spatial/temporal burst in actin polymerization and branching, as caused by Arp2/3, requires available sources of actin monomer, which are typically provided by the continuous depolymerization of actin filaments (Pollard, 2007; Insall and Machesky, 2009). Hence, we sought to identify molecules in the actin-depolymerization pathway that may respond to insulin, which would contribute to the balance of actin dynamics. ADF/cofilin are highly related, actin-severing, and monomer-sequestering proteins whose activity is mainly regulated by the phosphorylation status of Ser3 (Agnew *et al.*, 1995; Moriyama *et al.*, 1996). Phosphorylation and dephosphorylation of ADF/cofilin leads to its inactivation and activation, respectively. Insulin elicited ADF/cofilin dephosphorylation in cells that do not represent metabolically determining tissues and do not express GLUT4 (HT4 neuronal and 293T cells; Meberg *et al.*, 1998; Nishita *et al.*, 2004), but the function of cofilin in these cells and the metabolic consequences of its activation were not investigated. We therefore examined whether insulin stimulation in muscle cells alters the ADF/cofilin phosphorylation status and its possible contribution to GLUT4 traffic. We utilized a rabbit antibody that recognizes both ADF and cofilin-1 and their phosphorylated forms to an equal extent and cofilin-2 to a somewhat lesser extent (Shaw *et al.*, 2004). By two-dimensional (2D) gel electrophoresis of L6 cell lysates, we calculated the molar ratio of cofilin to ADF in L6 myoblasts to be 7:1. The major cofilin isoform present is cofilin-1 with a faint immunoreactive spot probably representing cofilin-2 focusing at the more acidic position identified for mouse cofilin-2 (Ono *et al.*, 1994). This 2D immunoblotting approach also revealed that ~9% of the cellular content of cofilin is phosphorylated (inactive) in unstimulated cells, and insulin caused a fourfold decrease in the phosphorylation of this protein (Figure 3). By SDS-PAGE and immunoblotting with an antibody selective to cofilin (1 and 2) phosphorylated on p-Ser3, phosphorylation of cofilin in unstimulated cells was also ascertained (Figure 4A). On insulin stimulation, a reduction in the level of phosphorylated cofilin (P-cofilin) was observed without changes in total cofilin, indicating a shift to its active state (Figure 4A). This was evident as early as 3 min and was most significant 10 min after insulin treatment (Figure 4B). Inhibiting PI3K with wortmannin prevented cofilin dephosphorylation in response to insulin (not shown), paralleling the response in 293T cells (Nishita *et al.*, 2004).

The balance of cofilin phosphorylation/dephosphorylation is primarily achieved by the action of kinases LIM kinase (LIMK) and testicular kinase and of the phosphatases slingshot (SSH), chronophin (Van Troys *et al.*, 2008), and to some degree PP1/PP2A (Meberg *et al.*, 1998). Because net dephosphorylation of cofilin was observed after insulin stimulation, we examined first the participation of the phosphatases in this response. Treatment of myoblasts with SSH1 siRNA reduced the expression of SSH1 by 73% (Figure 4C). Down-regulation of this phosphatase notably prevented the dephosphorylation of cofilin evoked by insulin compared with the degree observed in NR siRNA controls (Figure 4C).

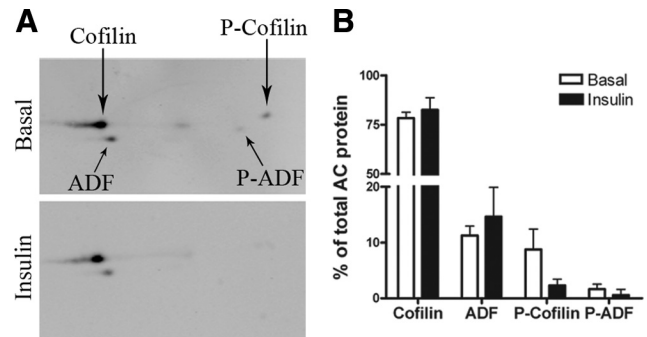


Figure 3. 2D Western blots of ADF/cofilin from which the relative percent of cofilin, ADF, phosphorylated-cofilin, and phosphorylated ADF can be calculated. Basal and insulin-stimulated L6GLUT4myc myoblasts were lysed and subjected to 2D gel electrophoresis and assessment of ratios of ADF, cofilin, and their phosphorylated versions, essentially as described (Shaw *et al.*, 2004). (A) Representative gel. Faint spot midway between cofilin and phospho-cofilin probably represents the cofilin-2 isoform. (B) Quantification of three experiments.

This revealed that insulin signals primarily via SSH1 to dephosphorylate cofilin, which would lead to cofilin activation. Regarding the kinases mediating cofilin phosphorylation, LIMK is attractive because it is typically activated by phosphorylation via Rac-dependent, p21-activated-kinase (Edwards *et al.*, 1999). Although LIMK1 knockdown did not increase the steady-state phosphorylation of cofilin in the basal state, LIMK knockdown potentiated cofilin dephosphorylation in response to insulin (Figure S3). This observation suggests that, in response to insulin, LIMK1 may partially restore phosphorylation of cofilin, which is however more dominantly dephosphorylated by SSH1.

Arp2/3-mediated Actin Remodeling Signals to Insulin-stimulated Cofilin Dephosphorylation

The net effect of cofilin dephosphorylation induced by insulin suggests the activity of SSH1 outweighed that of LIMK1. One of the possible explanations could be a surge in the phosphatase activity of SSH1 after insulin stimulation. Indeed, adding F-actin to purified SSH1 markedly augments its phosphatase activity (Nagata-Ohashi *et al.*, 2004; Soosairajah *et al.*, 2005; Kurita *et al.*, 2008). Analogously, the remodeled actin produced in muscle cells by insulin could serve as the stimulus that boosts the activity of SSH1 in vivo to cause a shift toward cofilin dephosphorylation because SSH1 accumulated at the remodeled actin after insulin stimulation (Figure S4). Knowing that the inhibition of Arp2/3 prevented actin remodeling, we examined this hypothesis by examining changes in P-cofilin levels upon p34 knockdown. In control cells treated with siNR, cofilin dephosphorylation occurred normally as evident from the decrease in P-cofilin level after 5 and 10 min of insulin stimulation (Figure 4D). In contrast, and notably, this insulin-dependent decrease in the level of P-cofilin was lacking in p34 knockdown cells (Figure 4D). Similar results were obtained when the actin filament-disrupting agent LB was used to stop the formation of remodeled actin by insulin. Under LB treatment, the dephosphorylation of cofilin by insulin was also prevented, so that the levels of P-cofilin at 5 and 10 min of stimulation were comparable to that of unstimulated cells (Figure 4E). In neither case was the ability of insulin to signal to Akt affected. These findings support the idea that the remodeled actin functions as a stimulus that augments the

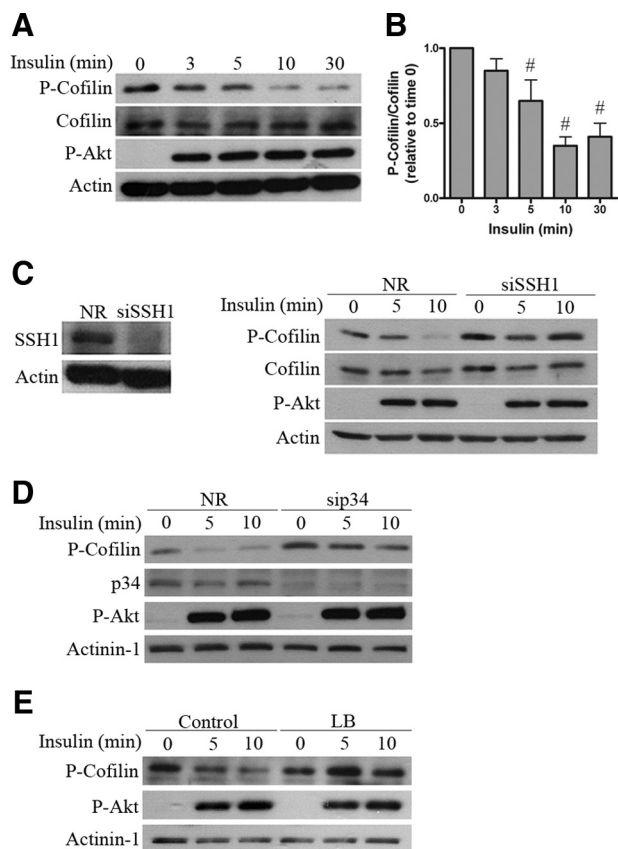


Figure 4. Insulin stimulation in L6GLUT4myc muscle cells causes dephosphorylation of cofilin that is dependent on SSH1. L6GLUT4myc myoblasts were serum-starved for 3 h before insulin stimulation (100 nM) for the indicated time periods. (A) Total lysates immunoblotted for P-cofilin, cofilin, P-Akt, and actin (as loading control). Representative blots of four experiments are shown. (B) Quantification of insulin-dependent cofilin dephosphorylation expressed as P-Cofilin/Cofilin ratio relative to time 0 ($n = 4$, mean \pm SE, $\#p < 0.05$). (C) Lysates from myoblasts treated with SSH1 siRNA (siSSH1) were prepared to determine the knockdown effect on SSH1 and its contribution toward insulin-induced cofilin dephosphorylation and Akt phosphorylation by immunoblotting for P-cofilin and p-Akt (Ser-473). Myoblasts were (D) transfected with p34 siRNA or (E) subjected to 250 nM LB treatment for 30 min, and the effect of insulin on P-Cofilin was assessed. Representative blots of more than three independent experiments are shown.

activity of SSH1 to generate the net dephosphorylation of cofilin after insulin stimulation in muscle cells.

Cofilin Knockdown Promotes F-Actin Accumulation and Decreases Insulin-mediated GLUT4 Translocation

Given that insulin activates cofilin via its dephosphorylation, it became intriguing to explore the functional implication that cofilin has on the dynamics of insulin-responsive actin remodeling. Immunofluorescence detection of endogenous cofilin in myoblasts revealed its redistribution from the cytosol to the peripheral zone where actin remodels upon insulin stimulation (Figure 5A). The localization of the phosphorylated form was determined using an antibody to phosphorylated ADF/cofilin that is compatible with immunofluorescence approaches. Costaining of total cofilin and phosphorylated ADF/cofilin (P-AC) revealed that insulin stimulation decreased the ratio of P-AC/cofilin at the cell periphery/remodeled actin (by 31%, $p < 0.05$), indicating

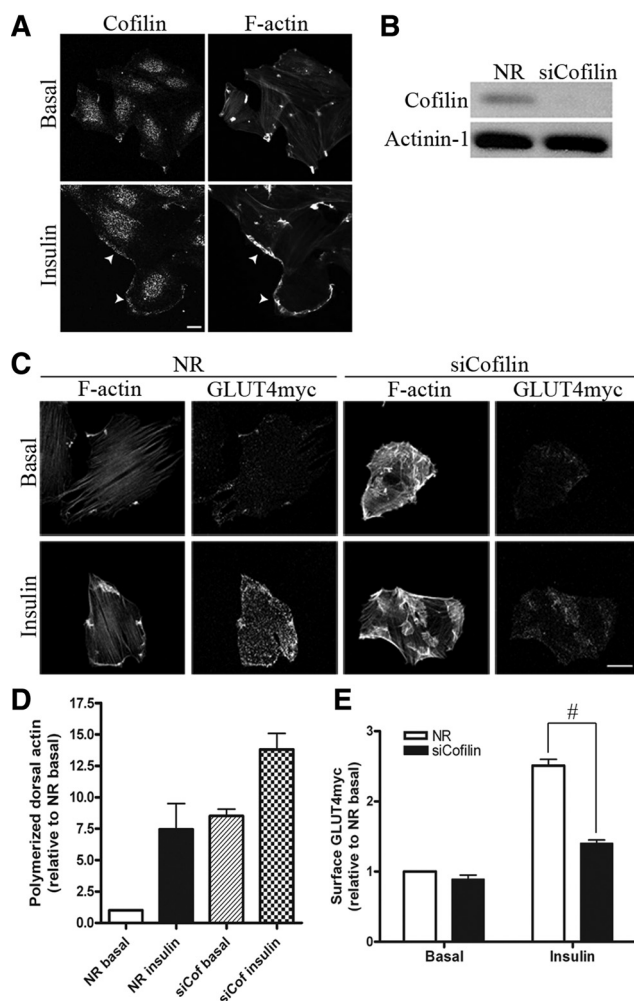


Figure 5. Cofilin localizes with insulin-induced remodeled actin and down-regulation of cofilin increases F-actin aggregates and reduces insulin-induced GLUT4 translocation. (A) L6GLUT4myc myoblasts were serum-starved for 3 h and stimulated with 100 nM insulin for 10 min, followed by labeling with rhodamine-phalloidin for F-actin and cofilin-specific antibody for endogenous cofilin. Representative images of three independent experiments are shown. Bars, 10 μ m. (B) Total lysates from myoblasts transfected with NR or cofilin siRNA were prepared and immunoblotted for cofilin and actinin-1 (loading control). Representative blots of five independent experiments are shown. (C) Myoblasts with siNR or siCofilin were treated with/without insulin followed by costaining of surface GLUT4myc and F-actin. Representative images of four independent experiments are shown. Bars, 20 μ m. (D) Quantification of dorsal polymerized actin relative to NR basal after cofilin knockdown (mean \pm SE). (E) Quantification of fold increases in surface GLUT4myc relative to NR basal in NR and siCofilin conditions (mean \pm SE, $\#p < 0.05$).

that cofilin undergoes dephosphorylation in response to the hormone at this region of the cell (Figure S5, A and B), consistent with the localization of SSH1.

To explore if cofilin dephosphorylation is required for insulin action on actin dynamics and GLUT4 translocation, siRNA against cofilin-1 was applied to achieve an 82% knockdown compared with the siNR control (Figure 5B). The siRNA sequence used to target cofilin-1 is unlikely to target cofilin-2, given a four-base difference (rat sequences). Under these conditions, the signal of phospho-cofilin was proportionately reduced (results not shown), confirming the

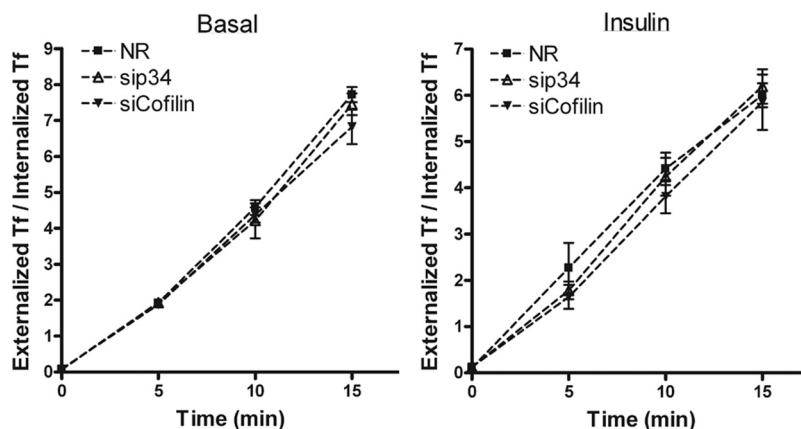


Figure 6. Knockdown of either p34 or cofilin does not alter Tf recycling. L6GLUT4myc myoblasts were treated with NR, p34, or cofilin siRNA. After 3-h serum starvation, ^{125}I -Tf recycling was determined as described in *Materials and Methods*, in basal (left) and insulin-stimulated (right) conditions. Tf recycling is displayed as the ratio of externalized Tf to internalized Tf (mean \pm SD).

2D immunoblot data showing that cofilin 1 is the predominant isoform expressed and phosphorylated in L6 myoblasts. As expected from existing literatures (Nishita *et al.*, 2005; Sidani *et al.*, 2007), down-regulation of cofilin caused a massive increase in the level of random F-actin aggregates in the basal state, and this morphological change was scored via polymerized dorsal actin assay (Figure 5, C and D). When challenged with insulin, these cells displayed a further elevation in polymerized actin (Figure 5D), suggesting that potentially there were still some available actin monomers to respond to the actin polymerization cues evoked by insulin. However, this remodeled actin was morphologically more diverse and was not confined to the cell periphery compared with that in NR control cells stimulated by insulin (Figure 5C).

Although insulin-dependent actin polymerization continued upon down-regulation of cofilin, the visually excessive/abnormal remodeling suggests that the dynamics of F-actin at the zone of remodeling may have been impeded. We therefore explored whether under these conditions the insulin-dependent GLUT4 translocation was affected. As shown in Figure 5C and E, cofilin knockdown caused a major reduction (66%) in insulin-dependent gain in surface GLUT4, yet Tf recycling, in either absence or presence of insulin, was not significantly disrupted (Figure 6). This observation allowed us to test whether cofilin reexpression restores normal GLUT4 translocation and if such restoration would be dependent on the phosphorylation status of cofilin. Transfecting *Xenopus* cofilin-WT-GFP into myoblasts with down-regulated cofilin expression restored the normal F-actin pattern in the basal state by eliminating the excessive F-actin accumulation (Figure 7, A and B). Moreover, such reexpression also alleviated the defect in insulin-dependent GLUT4 translocation (Figure 7C). Strikingly, neither the recovery of actin dynamics nor that of GLUT4 translocation was achieved when the inactive cofilin-S3E-GFP mutant was transfected into myoblasts with down-regulated cofilin. This mutant is unable to sever actin filaments. Overall, these results argue that the insulin-induced increase in the severing function of cofilin is critical for the dynamics of actin remodeling, which in turn allows proper insulin-stimulated GLUT4 translocation to proceed.

DISCUSSION

Because muscle is the major insulin-regulated storage of blood glucose, it is imperative to define the signaling events that promote the gain of GLUT4 at the surface to increase glucose uptake into this tissue. L6GLUT4myc myoblasts

have been a bona fide cell culture model to study GLUT4 traffic in a muscle cell context (Ueyama *et al.*, 1999; Wang *et al.*, 1999; Khayat *et al.*, 2000; Tong *et al.*, 2001; JeBailey *et al.*, 2004, 2007; Thong *et al.*, 2007; Ishikura and Klip, 2008). In these cells, we found that in parallel to the well-established Akt-AS160 pathway (Thong *et al.*, 2007), insulin induces a rapid activation of the small GTPase Rac that is PI3K-dependent and leads to cortical actin remodeling (Khayat *et al.*, 2000; Tong *et al.*, 2001; JeBailey *et al.*, 2004, 2007). However, as with any cell culture system, lessons learned from L6 muscle cells should eventually be tested in the mature tissue. In this regard, insulin-dependent reorganization of non-sarcomeric actin and Rac activation were recently confirmed in rodent skeletal muscles (Brozinick *et al.*, 2004; Ueda *et al.*, 2010; Hansen *et al.*, 2010). Rac activation and actin remodeling fail in several models of insulin resistance, without detectable defects in upstream IRS-1 phosphorylation or PI3K activation (Tong *et al.*, 2001; McCarthy *et al.*, 2006; JeBailey *et al.*, 2007). Here we identify Arp2/3 as the effector downstream of Rac that promotes formation of remodeled actin, whereas the activity of cofilin maintains the active turnover of actin in the remodeling zone. Disruption of either protein yielded abnormal actin remodeling and subsequent inhibition in insulin-mediated GLUT4 translocation (Figures 1C, 2B, and 5C).

Insulin Induces Concerted Actin Branching and Severing

F-actin contributes to maintaining structural integrity, promoting cellular migration, and aiding in vesicle traffic. To be functional, these cellular processes depend on the dynamic nature of actin regulation. Cytoplasmic actin exists as monomers and oligomerized F-actin. Uncapped F-actin undergoes constant polymerization at the barbed ends, whereas depolymerization occurs at the point ends to regenerate a steady pool of monomeric actin for further rounds of polymerization (Pollard, 2007). Such dynamic turnover is tightly controlled by actin-modifying proteins including actin-nucleating and -severing proteins (Pollard, 2007). Arp2/3 initiates branching at 70° on existing actin filaments and is firmly established in the Rac-dependent formation of lamellipodia in migrating cells (Ridley *et al.*, 1992; Miki *et al.*, 2000). Here we report that Arp2/3 contributes to the Rac-mediated, cortical actin remodeling after insulin stimulation. Down-regulation of Arp2/3 through siRNA against its Arp3 or p34 subunits abrogated insulin-induced actin remodeling, suggesting that Arp2/3-initiated actin polymerization is responsible for the genesis of dorsally remodeled actin in muscle cells (Figures 1C and 2B). Overexpressing Arp3-GFP

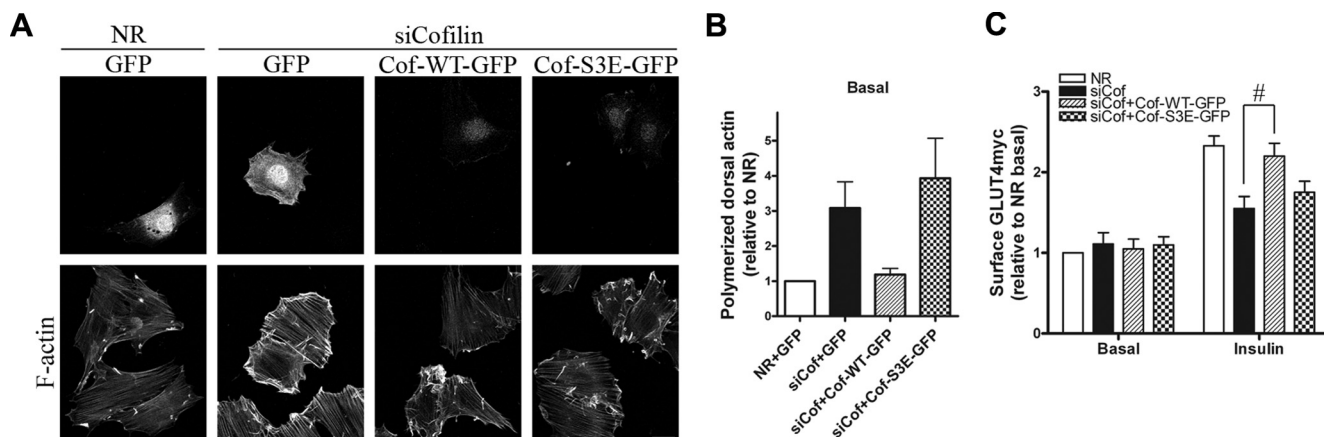


Figure 7. Expression of *Xenopus* cofilin-WT-GFP, but not cofilin-S3E-GFP mutant, restores normal F-actin morphology and GLUT4 translocation. (A) L6GLUT4myc myoblasts transfected with NR or cofilin siRNA were further transfected with cDNA to either GFP as control, cofilin-WT-GFP, or cofilin-S3E-GFP. After serum starvation, F-actin was labeled with rhodamine-phalloidin in the basal state to detect changes in actin morphology. Representative images of six independent experiments are shown. Bars, 20 μ m. (B) Quantification of F-actin aggregates relative to NR+GFP basal (mean \pm SE). (C) Surface GLUT4myc levels in siCofilin-treated myoblasts cotransfected with cofilin-WT or S3E mutant expression in siCofilin myoblasts were measured by fluorescent detection of single cells and are presented as fold increases in surface GLUT4myc relative to NR basal are also shown (mean \pm SE, $n = 6$, # $p < 0.05$).

did not promote actin polymerization in the basal state (Figure 1A), suggesting that Arp2/3 activation is tightly controlled by insulin stimulation. Conceivably, this occurs in response to insulin-activated Rac and its downstream effectors. Indeed, constitutively active Rac caused Arp2/3-mediated actin branching (Figure S1A).

In both L6 muscle cells (this study) and 3T3-L1 adipocytes (Jiang *et al.*, 2002) insulin caused Arp2/3 colocalization with the peripheral remodeled actin, and cortical actin remodeling is required for GLUT4 translocation (Kanzaki and Pessin, 2001; Tong *et al.*, 2001; Torok *et al.*, 2004). However, this phenomenon is differentially regulated in both cell types, because only in the muscle cells is the insulin-dependent actin remodeling downstream of PI3K (Patel *et al.*, 2003; JeBailey *et al.*, 2004). Moreover, the Rho-family G proteins engaged by each cell type also differ, with Rac determining actin remodeling in the muscle cells and TC10 (related to Cdc42) in adipocytes (Kanzaki *et al.*, 2002; JeBailey *et al.*, 2004). Arp2/3 is regulated by the nucleation-promoting factors N-WASP, WAVE, and cortactin (Machesky *et al.*, 1999; Suetsugu *et al.*, 1999; Weed *et al.*, 2000), and a dominant-negative mutant of N-WASP prevented the TC10-mediated insulin-induced actin reorganization in adipocytes (Jiang *et al.*, 2002). In contrast, WAVE, rather than N-WASP, is the nucleating factor downstream of Rac that activates Arp2/3 and its consequent actin-filament branching (Suetsugu *et al.*, 1999). Thus, WAVE2 may be the Arp2/3 activator in insulin-stimulated muscle cells, a possibility worthy of future exploration.

Insulin also activated the actin-severing protein cofilin by inducing its dephosphorylation on Ser3 (Figure 3A). This may be required to generate a dynamic, rapid turnover of the branching network and to provide a continuous supply of monomeric actin.

Because cofilin phosphorylation and dephosphorylation are governed by LIMK1 and SSH1, respectively (Yang *et al.*, 1998; Niwa *et al.*, 2002), we propose that insulin must activate the phosphatase and that this enzyme has a predominant effect over the kinase to produce the net dephosphorylation (Soosairajah *et al.*, 2005). In muscle cells, cofilin is already phosphorylated in the absence of stimuli (Figures 3 and 4), ostensibly due to a low, tonic activity of LIMK1 and

a relatively inactive SSH1. On insulin stimulation, cofilin undergoes SSH1-dependent dephosphorylation (Figure 4C). The surge in SSH1 activity may arise in response to the formation of the cortical network of branched, polymerized actin, because F-actin binding is required for significant phosphatase activity of SSH1 (Nagata-Ohashi *et al.*, 2004; Soosairajah *et al.*, 2005; Kurita *et al.*, 2008). This model is supported by our observations that LB and p34 knockdown, strategies that prevent insulin-induced actin polymerization, avert cofilin dephosphorylation (Figure 4, D and E). Consistent with this scenario, SSH1 concentrated along the peripherally remodeled actin upon insulin stimulation (Figure S4). Moreover, there is precedent for F-actin tilting the balance from LIMK1 to SSH1 dominance over cofilin (Jovceva *et al.*, 2007). A dynamic interplay between LIMK1 and SSH1 likely enables spatial temporal control over cofilin activity especially at the zone of insulin-induced actin remodeling.

Arp2/3 Activation and Cofilin Dephosphorylation Are Required for Insulin-dependent GLUT4 Translocation

A key observation of this study is that silencing two components of the Arp2/3 complex prevents GLUT4 translocation in response to insulin. This result is reminiscent of the well-documented abrogation of GLUT4 translocation by LB in fat cells (Wang *et al.*, 1998; Kanzaki and Pessin, 2001) and muscle cells (Khayat *et al.*, 2000; Torok *et al.*, 2004) in culture and in primary adipocytes (Omata *et al.*, 2000) and muscle tissue (Brozinick *et al.*, 2004). However, because LB inhibits actin remodeling by sequestering actin monomers, it impedes actin polymerization not only at the cell cortex but also at the level of stress fiber (Spector *et al.*, 1983). Instead, disruption of Arp2/3 allowed us to assign the selective participation of branched actin remodeling in GLUT4 translocation, irrespective of stress fibers or other actin filament-containing structures. The inhibition of GLUT4 translocation by Arp2/3 silencing is here ascribed to inhibition of actin branching, but may also be due to the consequent loss of insulin-dependent cofilin dephosphorylation, consistent with an Arp2/3- and cofilin-controlled actin polymerization-depolymerization cycle.

When actin severing was impaired by siRNA-mediated knockdown of cofilin, actin polymerization was extensive

and widely prevalent across the cell (Figure 5C), and this abnormality paralleled a reduction in insulin-dependent GLUT4 translocation (Figure 5E). This finding is reminiscent of the effect of jasplakinolide, an actin-stabilizing agent that causes excessive actin aggregates resembling those produced in cells with cofilin knockdown (Bubb *et al.*, 1994). Likewise, jasplakinolide also interferes with GLUT4 vesicle traffic to the surface of muscle and adipose cells (Kanzaki and Pessin, 2001; Tong *et al.*, 2001; Torok *et al.*, 2004). All these considerations point to the need for a dynamic, concerted actin polymerization and severing induced by insulin and required for GLUT4 translocation. Perhaps the strongest support of this model is the fact that the defect in GLUT4 traffic caused by cofilin knockdown was rescued by expression of active cofilin-WT-GFP (which is amenable to phosphorylation and dephosphorylation) but could not be restored by the expression of inactive cofilin-S3E-GFP (Figure 7C). These results buttress the participation of insulin-induced, cofilin-mediated actin depolymerization to facilitate GLUT4 translocation. Further, although other interpretations may be possible, the results are consistent with cofilin dephosphorylation being required for insulin-dependent vesicle traffic.

Possible Mechanisms Whereby a Dynamic Actin Network Supports GLUT4 Traffic

The remodeled actin filaments may act as a tether for GLUT4 vesicles close to the plasma membrane, so that their subsequent docking/fusion can occur more readily (Khayat *et al.*, 2000; Tong *et al.*, 2001; Randhawa *et al.*, 2008). Indeed, in insulin-stimulated muscle cells, GLUT4 vesicles accumulate within the cortical actin mesh visualized by electron microscopy (Tong *et al.*, 2001; Randhawa *et al.*, 2008). GLUT4 itself may tether to actin filaments via α -actinin-4 (Foster *et al.*, 2006; Talior-Volodarsky *et al.*, 2008), and indeed upon α -actinin-4 silencing, GLUT4 vesicles do not accumulate at the muscle cell periphery, and there is no insulin-dependent gain in surface GLUT4 (Talior-Volodarsky *et al.*, 2008). Similarly, when actin filament remodeling is inhibited by expressing dominant negative Rac or by LB, the enrichment of GLUT4 beneath the plasma membrane is lost, leading to the collapse of GLUT4 vesicles back to perinuclear regions (Randhawa *et al.*, 2008). It is also plausible that the remodeled actin clusters insulin-signaling molecules close to GLUT4 vesicles near the plasma membrane (Patel *et al.*, 2003). Although preventing actin remodeling by Arp2/3 knockdown did not reduce the overall level of P-Akt (Figure S1B), failure to accumulate phosphorylated Akt near the membrane may be a factor in the loss of insulin-induced GLUT4 translocation (Peyrollier *et al.*, 2000; Eyster *et al.*, 2005; Ng *et al.*, 2008; Gonzalez and McGraw, 2009).

On the other hand, actin polymerization is abundant in cells with down-regulated cofilin, yet the resulting polymerized actin filaments seem morphologically distinct and disseminated across the cell (Figure 5C). We speculate that, in this case, the impaired gain in surface GLUT4 is due to vesicle retention in a static mesh that no longer undergoes severing. By analogy, in neuronal cells, thick patches of submembrane F-actin present at rest act as a barrier to inhibit basal exocytosis of neurotransmitters (Trifaro *et al.*, 2008). In cells with down-regulated cofilin, the imbalance in dynamic cycles of actin depolymerization and branching may lead to uncoordinated growth of "static" actin filaments that could similarly turn into a barrier for GLUT4 vesicles, preventing them from gaining access to interaction with membrane SNAREs required for fusion (Randhawa *et al.*, 2008).

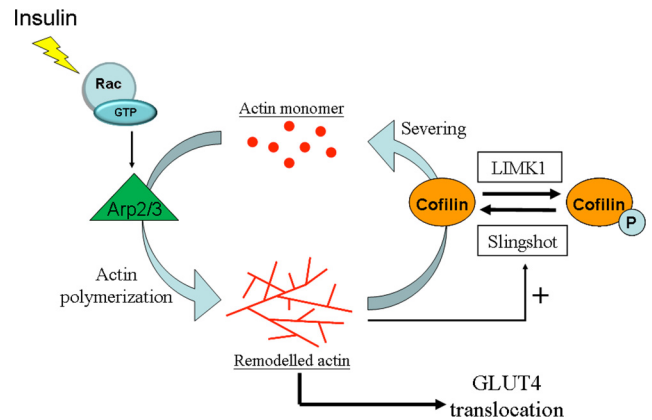


Figure 8. Proposed mechanism of insulin-regulated actin dynamics in muscle cells. Insulin stimulation in muscle cells promotes dynamic actin remodeling. The formation of the remodeled actin is achieved by the polymerization activity of Arp2/3 acting downstream of active Rac. The accumulation of polymerized F-actin poses a stimulatory factor in the phosphatase activity of SSH, which leads to net dephosphorylation and activation of cofilin. Hereon, the actin-severing function of cofilin maintains the flexibility of remodeled actin and enables regeneration of free monomeric actin for further polymerization. This active cycling of actin mediated by insulin keeps proper actin dynamics at the cortical zone in order to facilitate GLUT4 insertion onto the cellular surface.

We propose a model (Figure 8) whereby insulin stimulation leads to Arp2/3 activation downstream of Rac that initiates dorsal actin polymerization. The increase in polymerized actin stimulates the phosphatase activity of SSH1, leading to cofilin dephosphorylation and consequential increase in its severing action. Active actin depolymerization by cofilin can then free up actin monomers for Arp2/3-dependent polymerization and a dynamic actin turnover of insulin-stimulated actin remodeling is achieved. This dynamic status must be sustained to allow GLUT4 vesicle positioning and proper interaction with elements of the vesicle fusion machinery. This framework will allow testing the fidelity of its individual steps during insulin resistance and may reveal so far unsuspected steps that may be altered in this condition.

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