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Rac1-dependent phosphorylation and focal adhesion recruitment of myosin IIA regulates migration and mechanosensing

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Summary

Background—Cell migration requires coordinated formation of focal adhesions (FAs) and assembly and contraction of the actin cytoskeleton. Non-muscle myosin II (MII) is a critical mediator of contractility and FA dynamics in cell migration. Signaling downstream of the small GTPase Rac1 also regulates FA and actin dynamics, but its role in regulation of MII during migration is less clear.

Results—We found that Rac1 promotes association of MIIA with FA. Live-cell imaging showed that while most MIIA at the leading edge assembled into dorsal contractile arcs, a substantial subset assembled in or was captured within maturing FA, and this behavior was promoted by active Rac1. Protein kinase C (PKC) activation was necessary and sufficient for integrin- and Rac1-dependent phosphorylation of MIIA heavy chain (HC) on serine1916 (S1916) and recruitment to FA. S1916 phosphorylation of MIIA HC and localization in FA was enhanced during cell spreading and ECM stiffness mechanosensing, suggesting up-regulation of this pathway during physiological Rac1 activation. Phospho-mimic and non-phosphorylatable MIIA HC mutants demonstrated that S1916 phosphorylation was necessary and sufficient for the capture and assembly of MIIA mini-filaments in FA. S1916 phosphorylation was also sufficient to promote the rapid assembly of FAs to enhance cell migration and for the modulation of traction force, spreading, and migration by ECM stiffness.

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Experimental Procedures

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Conclusions—Our study reveals for the first time that Rac1 and integrin activation regulates MIIA HC phosphorylation through a PKC -dependent mechanism which promotes MIIA association with FAs, and acts as a critical modulator of cell migration and mechanosensing.

Introduction

Cell migration is mediated by coupling forces generated in the actin cytoskeleton to integrin focal adhesions (FAs) that adhere to the extracellular matrix (ECM). Non-muscle Myosin II (MII) is the main generator of forces that drive motility [1]. In migrating cells, the two most widely expressed isoforms, MIIA and MIIB, exhibit distinct localizations and functions [2, 3]. MIIB assembles and localizes in the cell center and rear where it promotes front-back polarity and forms a retracting tail [3, 4]. MIIA assembles in the leading edge [5, 6], where it promotes contractile actin arcs [7, 8]. Despite their distinct localizations, both isoforms regulate FA maturation and dynamics. MIIB promotes long-lived FA in the cell center and rear, while MIIA promotes maturation and turnover of leading edge FA [6]. However, MIIs have not been localized to FAs, but are thought act on FAs "at a distance" by transmitting tension through the actin cytoskeleton [9]. Furthermore, how MII is controlled by upstream regulators to specifically modulate FA dynamics during cell migration is not known.

Rac1 is a small GTPase of the Rho family that mediates cell motility, mechanosensing and invasion [10]. Rac1 activation is promoted by integrin engagement [11, 12], and Rac1 downstream effectors including the p21-activated kinases (PAKs) regulate leading edge cytoskeletal and FA dynamics [10]. Rac1 promotes actin polymerization to drive protrusion of the leading edge and retrograde flow [13]. In conjunction with lamellipodial protrusion, Rac1 also promotes formation of small FAs near the leading edge [14]. Although leading edge actin retrograde flow and FA assembly and turnover dynamics are known to be modulated by MII activity [15], it is not known how Rac1 regulates the coordination of F-actin dynamics and adhesion, or whether it is through MII.

The regulation of MII is well-studied, however Rac1's role in this regulation is less clear. Phosphorylation of the MII light chains (MLC) on serine19 (S19) promotes activation of MII ATPase and filament assembly [16, 17]. It is controversial as to whether Rac1 promotes or inhibits cellular contractility via this mechanism in a PAK-dependent manner [18–20]. Phosphorylation of the MII heavy chain (HC) at several sites in the rod and non-helical tailpiece inhibits MII filament assembly, and thus may negatively regulate contractility [21]. Rac1 can promote phosphorylation and redistribution of MII HC via PAK [22, 23], however the sites targeted for phosphorylation in MII are not known. Other direct mediators of phosphorylation on MII HC sites include protein kinase C (PKC), TRPM7, and casein kinase II (CKII) [21]. Rac1 can regulate some PKCs indirectly [24], and PKCs are additionally activated by integrin engagement and regulate FA formation [25], and thus are good candidates for mediating the coordination of MII and adhesion in cell migration.

Here, we examined the role of Rac1 in regulation of MII in FA dynamics and cell migration. We find that Rac1 activity promotes capture and assembly of MIIA mini-filaments in maturing FA in an integrin-dependent manner. We show that this is mediated by PKC-dependent phosphorylation of serine 1916 on MIIA HC. We demonstrate that this

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phosphorylation is necessary and sufficient for MIIA recruitment to FA, facilitating rapid FA assembly, enhancing cell migration and modulating ECM stiffness mechanosensing.

Results

Active Rac1 promotes MIIA association with focal adhesions

We sought to determine the role of Rac1 activity in MII organization and its role in FA dynamics in the leading edge of migrating cells. We utilized human osteosarcoma U2OS cells plated on fibronectin-coated coverslips and transfected with blue fluorescent protein (BFP)-tagged constitutively active (Rac1V12) or dominant negative (Rac1N17) Rac1 mutants to manipulate Rac1 activity. We immuno-localized MII (either A or B isoforms) and paxillin as a marker of FAs [26], and used fluorescent phalloidin to visualize F-actin and imaged cells by epi-fluorescence microscopy (Epi). In mock-transfected controls, MIIA was localized along stress fibers (SFs) in the cell center and to actin arcs in the lamella, but was absent from lamellipodia, FAs, and radial SFs (Figure 1A), as reported [8, 9, 27]. A similar localization was seen in cells expressing Rac1N17, although lamellipodia were absent (Figure 1A). Expression of Rac1V12 induced a discoid or crescent-shaped morphology with MIIA localized to arcs and SFs in the cell center, similar to controls. However, Rac1V12 caused the additional accumulation of MIIA towards the distal ends of radial SFs that emanated from FA (Figure 1A). Similar analyses showed that MIIB predominantly localized to SFs in the cell center and rear and was absent from lamellipodia, radial SFs, and FAs, independent of the state of Rac1 activity (Figure S1). Thus, Rac1 activation promotes association of MIIA with SF termini in the lamella.

To determine if Rac1 activation caused MIIA to specifically localize within FA or to radial SFs [8, 27], we utilized TIRF microscopy and cell fractionation. Comparison of TIRF and Epi images of immuno-stained cells showed that in mock-transfected controls or cells expressing Rac1 N17, MIIA was associated with SFs on the ventral cell surface in the cell center and rear and SFs and arcs on the dorsal cortex [8], but was rarely in FA and lamellipodia (Figure 1B,S2). In cells expressing Rac1V12, MIIA localized to dorsal arcs and dorsal and ventral SFs, similar to controls, but was additionally prominently co-localized with paxillin in FA (Figure 1B,S2). Quantification showed that in controls or cells expressing Rac1N17, 46% of FAs contained some MIIA staining; expression of Rac1V12 increased this to 65% (Figure 1C). Similarly, in controls or cells expressing Rac1N17, the fraction of FA area that was co-occupied by MIIA and paxillin was relatively low (17%), but was significantly increased in cells expressing Rac1V12 (25%) (Figure 1D). To confirm our localization data, we separated the cell body fraction from the substrate-adhered FA fraction, with or without additional Rac1V12 expression, and performed western blot analysis. This showed that constitutive activation of Rac1 increased MIIA in the FA fraction 1.6 fold compared to control (Figure 1E). Thus, active Rac1 specifically enhances MIIA association with FAs.

Rac1 activation induces capture and assembly of MIIA mini-filaments in maturing FAs

We next sought to determine how Rac1 activity affects the association of MIIA with FA in live cells. We performed time-lapse TIRF microscopy of cells expressing MIIA HC fused

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via its n-terminus (head domain) to GFP (GFP-MIIA), a fusion protein that complements MIIA null in mice [28]. This was co-expressed with mApple paxillin, with or without the additional expression of Rac1 mutants. In peripheral regions of the cell where MIIA density was low, GFP-MIIA was always present as dual diffraction-limited puncta whose peaks were separated by 300 +/– 16 nm (Figure 2A). Immuno-staining of cells expressing GFP-MIIA with antibodies that recognize an epitope in the C-terminus of MIIA revealed that the rod was localized between the dual punctae of GFP-labelled heads (Figure 2A). Thus, dual punctae of GFP-MIIA represent individual bipolar MIIA mini-filaments in which two bouquets of actin-binding heads are separated by a 300nm bare zone of bundled MII rods [29–31].

Time-lapse movies (at 10s intervals) showed that in control cells, GFP-MIIA mini-filaments exhibited two distinct dynamic behaviors. In the predominant behavior, as the leading edge underwent protrusion/retraction dynamics, randomly oriented individual mini-filaments appeared in the lamellipodium, underwent rapid retrograde flow for a few microns, and then oriented parallel to the leading edge concurrent with slowing of retrograde flow, without association with mApple paxillin-labeled FA (Figure 2C, Movies S1,S2). The assembly, retrograde flow, and parallel orientation of many GFP-MIIA mini-filaments contributed to formation of concave arcs that spanned between FA [7, 27]. In the second, less prevalent behavior, GFP-MIIA mini-fiIaments appeared perpendicular to the leading edge in maturing FA near the base of the lamellipodium (Figure 2B, Movie S2). Quantification showed that mini-filaments within FA were predominantly oriented parallel to the FA, while mini-filaments outside of FA were not (Figure 2E). Thus, most GFP-MIIA mini-filaments assemble in the lamellipodium to promote formation of contractile arcs, while a subset assembles in maturing FA, where they align along the FA long axis.

In cells expressing Rac1V12, GFP-MIIA also exhibited these two behaviors, but mini-filament assembly within FAs was much more prevalent than in controls (Figure 2D, Movies S2,S3). In addition, mini-filaments undergoing retrograde flow in the lamellipodium were often captured by FA, where they either remained stationary or moved rearward in tandem with FA growth. Quantification demonstrated that like controls, in cells expressing Rac1V12 mini-filaments in FAs were oriented along the FA axis (Figure 2E). In contrast, in cells expressing DN Rac1, GFP-MIIA did not associate with FA (Figure 1A,B; Movie S4). Thus, Rac1 activation promotes the assembly and capture of MIIA mini-filaments in maturing FA, however mini-filament orientation is driven by FA association rather than Rac1 activation *per se*.

Rac1 activation promotes phosphorylation of MIIA HC on S1916 in FAs in an integrin-dependent manner

To determine how adhesion and Rac1 activation regulate MIIA association with FA, we analyzed their effects on MII phosphorylation. Integrin engagement to fibronectin or collagen extracellular matrices (ECMs) induces Rac1 activation [11, 12], while fibronectin engagement promotes phosphorylation of MIIA HC on serine 1916 (pS1916 MIIA HC) [32]. We performed western blot analysis with pS1916 MIIA HC phospho-specific antibodies of lysates from cells with Rac1 activity manipulated that were plated on either

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fibronectin or collagen. This showed that on either ECM, Rac1V12 increased pS1916 MIIA HC over two-fold compared to controls. However expression of Rac1N17 did not reduce pS1916 MIIA below control levels, suggesting that Rac1-independent mechanisms promote pS1916 (Figures 3B,C). Plating cells on poly-l-lysine to inhibit integrin engagement showed that Rac1V12 expression did not increase pS1916 MIIA HC over Rac1N17 expression or controls, in spite of the fact that Rac1V12 activated downstream signaling, as evidenced by an increase in phosphorylation of one of its downstream targets, Pak1 (Figure 3A). Thus, Rac1 mediates pS1916 MIIA HC in an integrin-dependent, Pak1-independent manner.

To determine the relationship between Rac1-induced MIIA recruitment to FAs and pS1916 phosphorylation, cells were immuno-stained for pS1916 MIIA HC and paxillin followed by TIRF imaging. This showed that in controls, pS1916 MIIA HC localized to peripheral SFs and a small fraction of FA. Rac1V12 markedly increased co-localization of pS1916 MIIA HC with FA, while expression of Rac1N17 reduced pS1916 MIIA HC staining throughout the cell (Figure 3E). Because Rac1 activity and MIIA HC phosphorylation are thought to inhibit MII filament assembly *in vitro* and in cells [33, 34], we examined the effects of Rac1 activation on the triton solubility of MIIA. Although RacV12 promoted pS1916 MIIA HC (Figure 3B,C), it induced no major change in total MIIA solubility compared to controls or cells expressing Rac1N17. However, the fraction of MIIA that was phosphorylated became concentrated in the soluble fraction (Figure 3D). This agrees with the previous studies, and further suggests that Rac1-mediated phosphorylation affects only a small fraction of total MIIA, and that the FA-associated fraction of pS1916 MIIA is locally protected against cytoskeletal dissociation. Thus, Rac1 activation promotes pS1916 MIIA HC in an integrin-dependent, Pak1-independent manner to promote specific association of pS1916 MIIA mini-filaments with FAs.

Indicators of Rac1 activation during cell spreading and ECM stiffness mechanosensing correlate with pS1916 MIIA HC at FAs

To determine if the Rac1-dependent pathway of MIIA phospho-regulation occurs in response to physiological stimulation of Rac1 activity, we examined MIIA HC phosphorylation and localization during cell spreading [11] and ECM stiffness mechanosensing [35]. Cells were lysed in suspension or during spreading at 15, 30, 45, 60 and 90min after plating on fibronectin and analyzed by western blotting. This showed that, compared to suspended cells, the level of pS1916 MIIA HC increased threefold and plateaued 45min after plating (Figure 4 A, B). Probing for phosphorylation of downstream targets of Rac1 (Pak1 and Lim kinase) and integrin signaling (FAK) showed that indicators of Rac1 activity increased and plateaued with kinetics similar to those of pS1916 IIA HC, while FAK activity peaked at 60min and declined by 90min (Figure 4A,B). TIRF images of cells expressing GFP-MIIA-HC and mApple paxillin at specific times after plating on fibronectin showed that GFP-MIIA-HC recruitment and localization to FA mirrored the kinetics of pS1916 MIIA HC seen by western blot (Figure 4C). Thus, activation of downstream targets of Rac1during cell adhesion and spreading correlate with pS1916 MIIA HC and localization in FA.

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Recent studies have shown that cellular mechanosensation in response to increased stiffnesses induces Rac activation [36]. To determine if Rac1 activation induced by ECM stiffness mechanosensing promotes S1916 MIIA phosphorylation and FA localization, we plated cells on fibronectin-coupled polyacrylamide gels of defined stiffness (0.7kPa, 8.6kPa, 55kPa; Figure 4D,E). Western blot showed that pS1916 MIIA HC was higher in cells adhered to intermediate stiffness ECM compared to either lower (0.7kPa) or higher (55kPa) stiffness ECMs (Figure 4E). Confocal imaging of cells expressing GFP-MIIA-HC and mApple paxillin on compliant ECMs showed that GFP-MIIA FA recruitment was highest in cells adhered to imtermediate stiffness ECMs (Figure 4D). Time-lapse phase-contrast imaging of cells plated on different stiffness ECMs showed that cells migrated the fastest on intermediate stiffness (Figure 4F). These results show that, similar to downstream indicators of Rac1 activation, pS1916 MIIA HC and localization to FA is induced by cell spreading and ECM stiffness mechanosensing, suggesting that these stimuli induce Rac1-mediated phospho-regulation of MIIA-HC.

PKC activity is necessary and sufficient for Rac1-dependent pS1916 MIIA HC at FA

Rac1 and PKCs are activated by integrin engagement [10, 11, 25], and PKCs can phosphorylate S1916 of MIIA HC [21]. We used pharmacological manipulation of PKC activity to determine the involvement of PKC in the Rac1- and integrin-mediated regulation of MIIA HC. Treatment of mock-transfected controls or cells expressing Rac1 V12 with either the pan-PKC inhibitor Go6976 or the PKC βII-specific inhibitor CGP53353 blocked pS1916 MIIA HC and localization of MIIA to FA in cells expressing Rac1 V12, but did not block Pak1 activation induced by Rac1 V12 or baseline pS1916 MIIA in control or RacN17 transfected cells (Figure 5A,B). In contrast, compared to cells expressing Rac1 N17 alone, treatment of cells expressing Rac1 N17 with phorbol-12-myristate-13-acetate (PMA) to activate PKCs increased pS1916 MIIA HC and induced localization of MIIA to FA (Figure 5A,B). Thus, PKC activity is necessary and sufficient for Rac1-dependent induction of pS1916 MIIA HC and recruitment to FA, and specifically implicates PKCβII in this process.

pS1916 MIIA HC is necessary and sufficient for enhancing its assembly at FAs

Rac1 and PKC have multiple targets in addition to MIIA HC [37]. To determine the specific role of S1916 phosphorylation in MIIA function, we generated non-phosphorylatable (GFP-S1916A MIIA HC) and phospho-mimic (GFP-S1916D MIIA HC) mutants of GFP-MIIA-HC (wild type, WT). Experiments were performed in cells depleted of MIIA siRNA (~90% knockdown after 72hrs, no effect on MIIB, Figure 6A). MIIA HC knockdown produced long cell tails, randomly directed lamellipodia and loss of mature FA (Supplemental Figure 3), similar to effects of blebbistatin [38]. Expression of WT GFP-MIIA-HC in cells depleted of MIIA rescued these effects (Figure 3).

We then examined the effect of phospho-mutants on MIIA localization and dynamics. GFP-tagged MIIA HC mutants were co-expressed with m-Apple paxillin in MIIA-knockdown cells at ~80% of the level of endogenous MIIA (Figure 6A). TIRF imaging and quantification showed that the fraction of FA area that was occupied by MIIA and the fraction of FA containing MIIAs was significantly higher in cells expressing GFP-S1916D MIIA HC than in cells expressing GFP MIIA HC or GFP-S1916A MIIA HC (Figure

6B,G,H, Movie S5). Furthermore, co-expression of Rac1 V12 with GFP-S1916A MIIA HC did not enhance its localization to FAs compared to expression of GFP-S1916A MIIA HC alone, and GFP-S1916D MIIA HC localized to the FAs even in the presence of Rac1N17 (Figure 6B). These results suggest that S1916 phosphorylation is necessary and sufficient for MIIA association with FA.

We next determined how pS1916 MIIA HC associates with FA in live cells by TIRF microscopy of cells depleted of endogenous MIIA and co-expressing GFP-tagged MIIA HC mutants and mApple-paxillin. This showed that like WT GFP-MIIA HC, both phospho-mutants formed bipolar mini-filaments as evidenced by dual diffraction-limited punctae. For all three GFP-MIIA-HC variants, most mini-filaments appeared and assembled into arcs, and did not co-localize with FAs (Figures 6C,D, Movie S5). However, in the presence or absence of Rac1N17, a substantial subset of GFP-S1916D MIIA HC mini-filaments were captured and oriented or underwent *de novo* assembly at maturing FA (Figure 6D,F, Movies S5,6), similar to the behavior of GFP-MIIA in cells expressing Rac1V12 (Figure 2D). This behavior was rare in cells expressing GFP-S1916A MIIA HC, even in the presence of Rac1V12 (Figure 6D; Movies S5,6). Together, these results suggest that S1916 phosphorylation is necessary and sufficient for MIIA assembly and capture at FA.

pS1916 MIIA HC promotes FA assembly, cell migration, and modulates ECM stiffness mechanosensing

We next sought to determine the effects of pS1916 MIIA on FA dynamics and cell behavior. We analyzed FA morphometry and dynamics in TIRF movies of cells depleted of endogenous MIIA and co-expressing GFP-tagged MIIA-HC mutants and mApple-paxillin. This showed that depletion of MIIA reduced FA size, while GFP-S1916D MIIA HC or GFP-S1916A MIIA HC had no effect FA size compared to WT-GFP-MIIA-HC (Figure 7A). However, GFP-S1916D MIIA HC increased FA number and density compared to WT GFP-MIIA-HC, while GFP-S1916A-MIIA-HC had no effect (Figures 7B, C). Movies (Movie S7) or time projections of mApple-paxillin-labeled FAs (Figure 7D) suggested that GFP-S1916D MIIA HC promoted FA turnover compared to WT GFP-MIIA HC, while GFP-S1916A MIIA HC appeared to reduce FA dynamics. Image autocorrelation analysis of FA in TIRF movies as a general measure of FA stability [39] supported this notion, demonstrating that GFP-S1916D MIIA HC reduced the half-time of image correlation decay relative to that of WT or GFP-S1916A MIIA HC (Figure 7E). Analysis of individual FA assembly/disassembly rates showed that compared to WT GFP-MIIA HC, GFP-S1916D MIIA HC increased FA assembly rate but did not affect FA disassembly rate, while GFP-S1916A MIIA HC did not affect FA assembly rate, but slowed FA disassembly (Figures 7F,G). Time-lapse phase-contrast imaging of cells migrating on fibronectin-coated coverslips showed that GFP-S1916D MIIA HC increased while GFP-S1916A MIIA HC decreased cell migration velocity compared to WTGFP-IIA HC (Figure 7H), similar to effects of these mutants on FA dynamics. Together, these results suggest that MIIA phosphorylation and/or recruitment to FAs promotes dynamic FA turnover and enhances cell migration.

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Finally, we determined the role of S1916 MIIA HC in ECM stiffness mechanosensing. We assayed the ability of cells depleted of endogenous MIIA HC and re-expressing GFP-MIIA HC mutants to modulate traction force, spread area, and migration speed in response to different stiffnesses of fibronectin-coupled polyacrylamide substrates. High-resolution traction force microscopy (TFM) on cells depleted of endogenous MIIA HC and co-expressing GFP-tagged MIIA HC mutants and mApple-paxillin was used to assess total traction force per cell and spread cell area (Figure 7I,J). This showed that in cells expressing WT-GFP-MIIA HC or GFP-S1916A MIIA HC that traction force and cell area ~doubled in response to ~doubling ECM stiffness from 4.1 to 8.6 kPa, and force increased ~6 fold and cells spread significantly further when stiffness was further raised ~6 fold from 8.6 to 55kPa. In contrast, cells expressing GFP-S1916D MIIA HC failed to increase traction force or spread area in response to shifting substrate stiffness from 4.1 to 8.6 kPa, but were still responsive to the shift from 8.6 kPa to 55kPa, increasing both total traction force and cell area. Analysis of cell velocity showed that cells expressing WT GFP MIIA-HC migrated faster on 8.6 kPa compared to on stiffer 55kPa ECMs (Figure 7K), similar to the behavior of untransfected cells (Figure 4F). In contrast, although cells expressing either GFP-S1916A MIIA HC or GFP-S1916D MIIA HC exhibited faster and slower migration than wild type GFP-MIIA HC, respectively, neither mutant showed differences in migration speed between soft and stiff ECMs. Thus, phosphoregulation of S1916 on MIIA HC is a critical modulator of cellular traction stress, migration speed, and cellular response to ECM stiffness.

Discussion

Our study reveals that Rac1 and integrin signaling induce PKC-mediated S1916 MIIA HC phosphorylation, and shows for the first time that this signaling pathway promotes MIIA specific association with FAs, and acts as a critical regulator of cellular traction stress, migration, and mechanosensation. We found that active Rac1 enhanced the association of MIIA, but not MIIB with FAs. PKC activity was necessary and sufficient for integrin- and Rac1-dependent phosphorylation of MIIA HC on S1916 and recruitment into FA, and may be mediated by the calcium-activated PKCβII. We demonstrated that Rac1-dependent S1916 phosphorylation and FA localization of MIIA HC occurs during cell spreading and ECM stiffness mechanosensing. Experiments with phospho-mimic and non-phosphorylatable mutants of MIIA HC demonstrated that S1916 phosphorylation was necessary and sufficient for enhancing the capture and assembly of MIIA mini-filaments at FA, and that phospho-regulation at this site promotes FA turnover to enhance cell migration and modulates the range ECM stiffnesses to which cells respond by changing their traction force, spreading, and migration speed.

The discovery of specific recruitment of MIIA to FA is surprising, as MII has not been considered as a *bona fide* FA protein. In spite of the fact that it has been identified in FA proteomes [40–42], it is generally thought that MII regulates FA maturation and turnover by promoting actin bundling or transmitting force through the actin network to FAs from a distance [4, 9]. Furthermore, recent evidence suggests that high traction forces at FA are generated by acto-myosin arcs, not by radial SFs that are attached to FAs [43]. Our data supports this notion, as we found that S1916 MIIA HC phospho-mimic that promotes MIIA recruitment to radial SF and FA decreased traction force. However, whether individual FA

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containing this mutant had altered force or if this was due to effects on global MII assembly/dissasembly was not determined.

So what is MIIA doing in FA? We suggest that myosin IIA could be directly promoting assembly and maturation at the single FA level by locally driving integrin clustering and/or activation by applying localized force to the ECM bound integrin-linked actin filaments [44, 45]. Alternatively, MIIA mini-filaments within FA could mediate recently observed traction force fluctuations in single FAs that are required for ECM stiffness mechanosensing and durotaxis [46]. In support of this, both traction force fluctuations and MIIA recruitment only occur in a subset of FA, and force fluctuations are not synchronized between neighboring FA in the same cell [46], implying that fluctuations are produced by very local contraction. Whether Rac1 activation or MIIA HC phosphorylation regulates force fluctuations in single FAs or is required for durotaxis remains to be determined.

Our results showing that phosphorylation of MIIA HC promotes assembly of mini-filaments specifically within FAs contradicts the notion that phosphorylation of MIIA HC promotes mini-filament disassembly both *in vitro* and in cells [33, 47]. How phosphorylation at S1916 targets MIIA to FA and how this location protects phosphorylated mini-filaments from disassembly we do not know. However, the calcium-activated PKCβII, as well as Rac1, have been identified as members of the FA proteome [41]. Thus, it is possible that local phosphorylation of MIIA within FAs and/or binding of pS1916 by an FA protein modulates the effect of phosphorylation on mini-filament assembly dynamics at the single FA level.

Our findings support previous studies that integrins [32], Rac1 [23], and PKC [33] are critical physiological regulators of MII HC phosphorylation. However, these regulators have not previously been linked in a pathway for site-specific phosphorylation of the IIA isoform or its association with FAs. Previous reports showed that fibronectin and collagen both induce Rac1 activation [11, 12] while fibronectin, but not collagen, induces PKCα activation [25, 48]. However, our findings suggest a novel pathway in which Rac1 and collagen or fibronectin-mediated activation of PKCβII promotes MIIA phosphorylation. Furthermore, while previous studies implicate Pak in mediating MII HC phosphorylation at unknown sites [23, 49], our results suggest Rac1-mediated phosphorylation of MIIA HC S1916 is independent of Pak. It is possible that this novel pathway mediates the up-regulation of S1916 MIIA HC phosphorylation in epithelial-to-mesenchymal transition [50], mast cell degranulation [51] and tumor cell migration [32], all of which have been linked to Rac1 activity [52–54].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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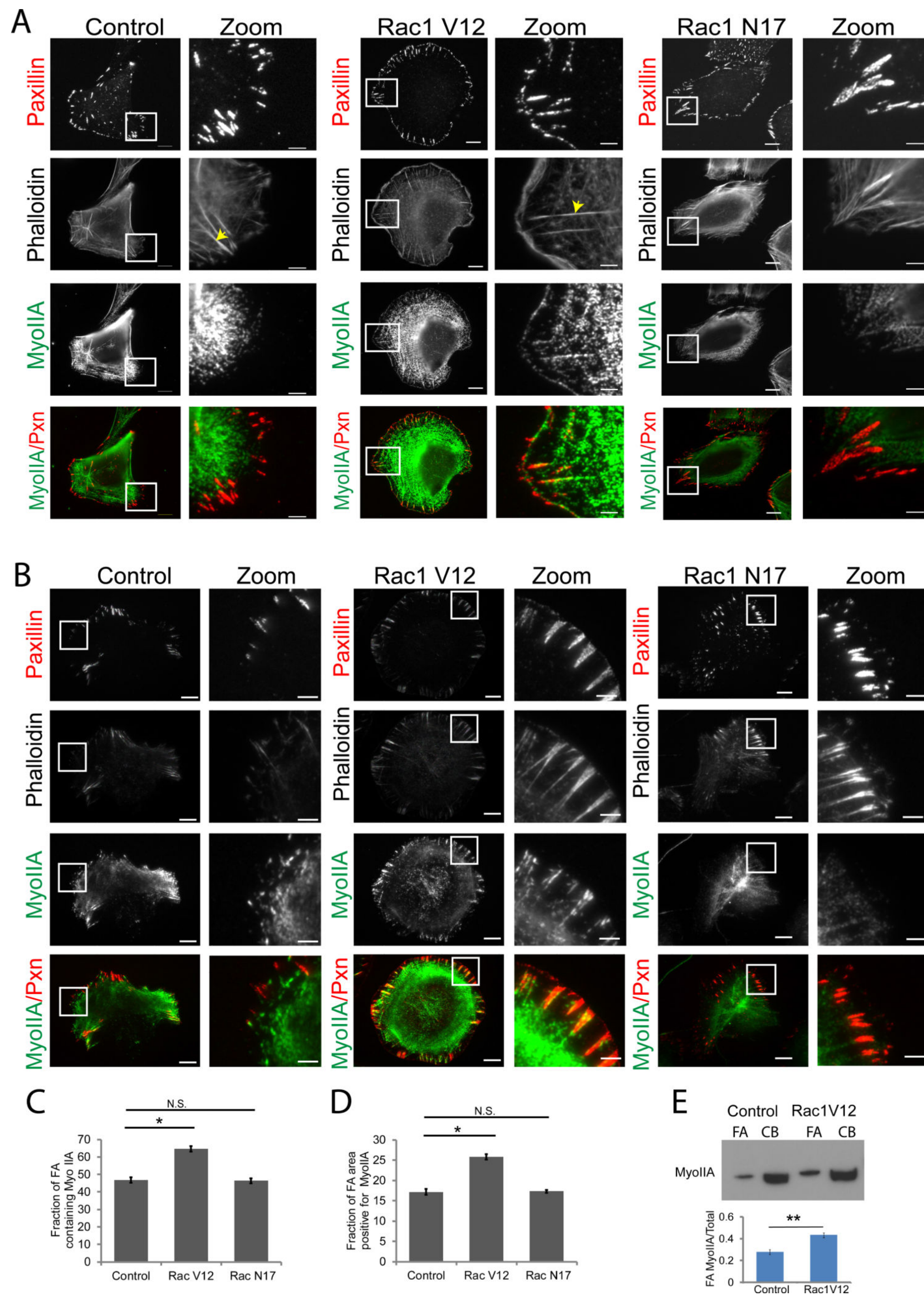

Figure 1.

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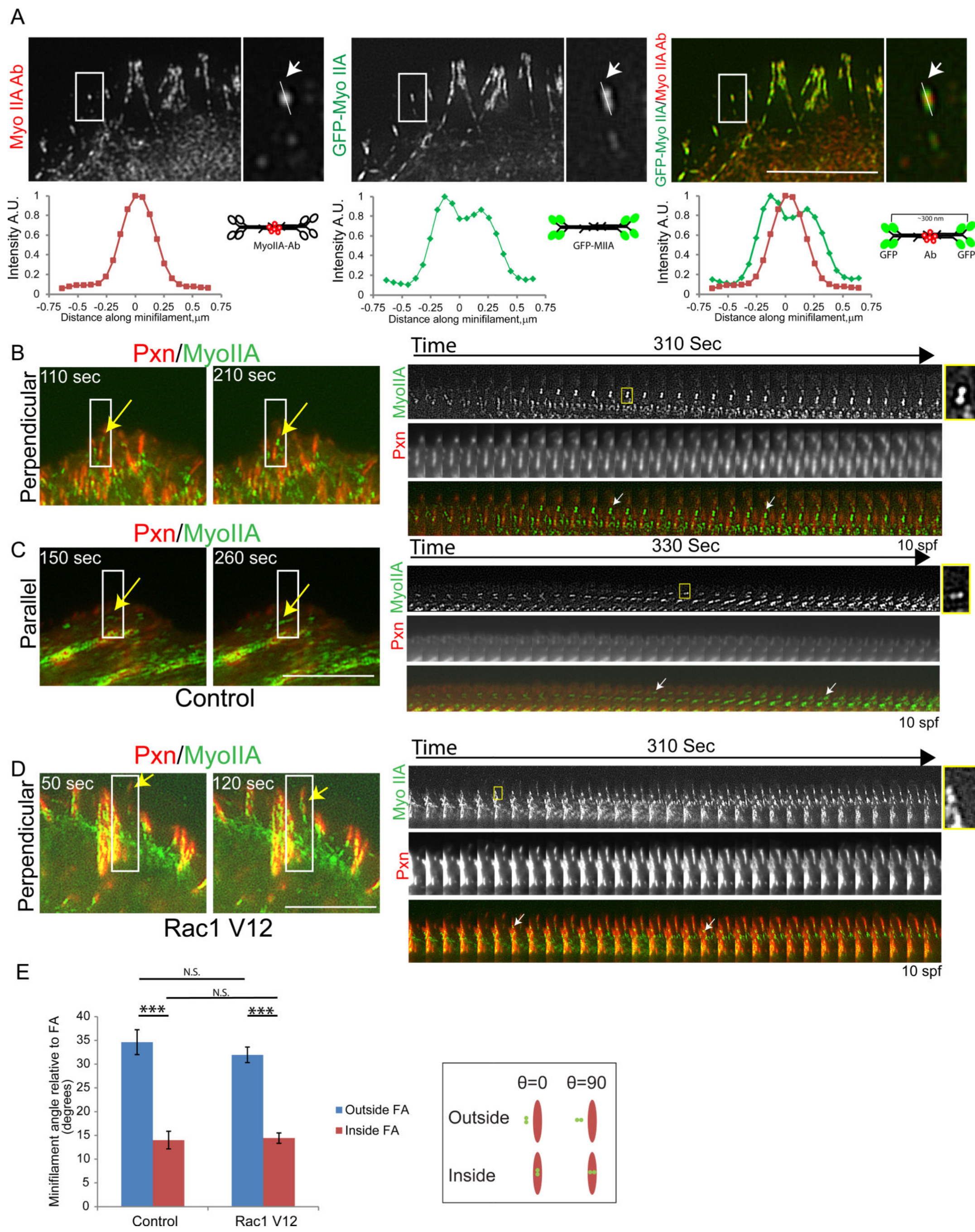

Figure 2.

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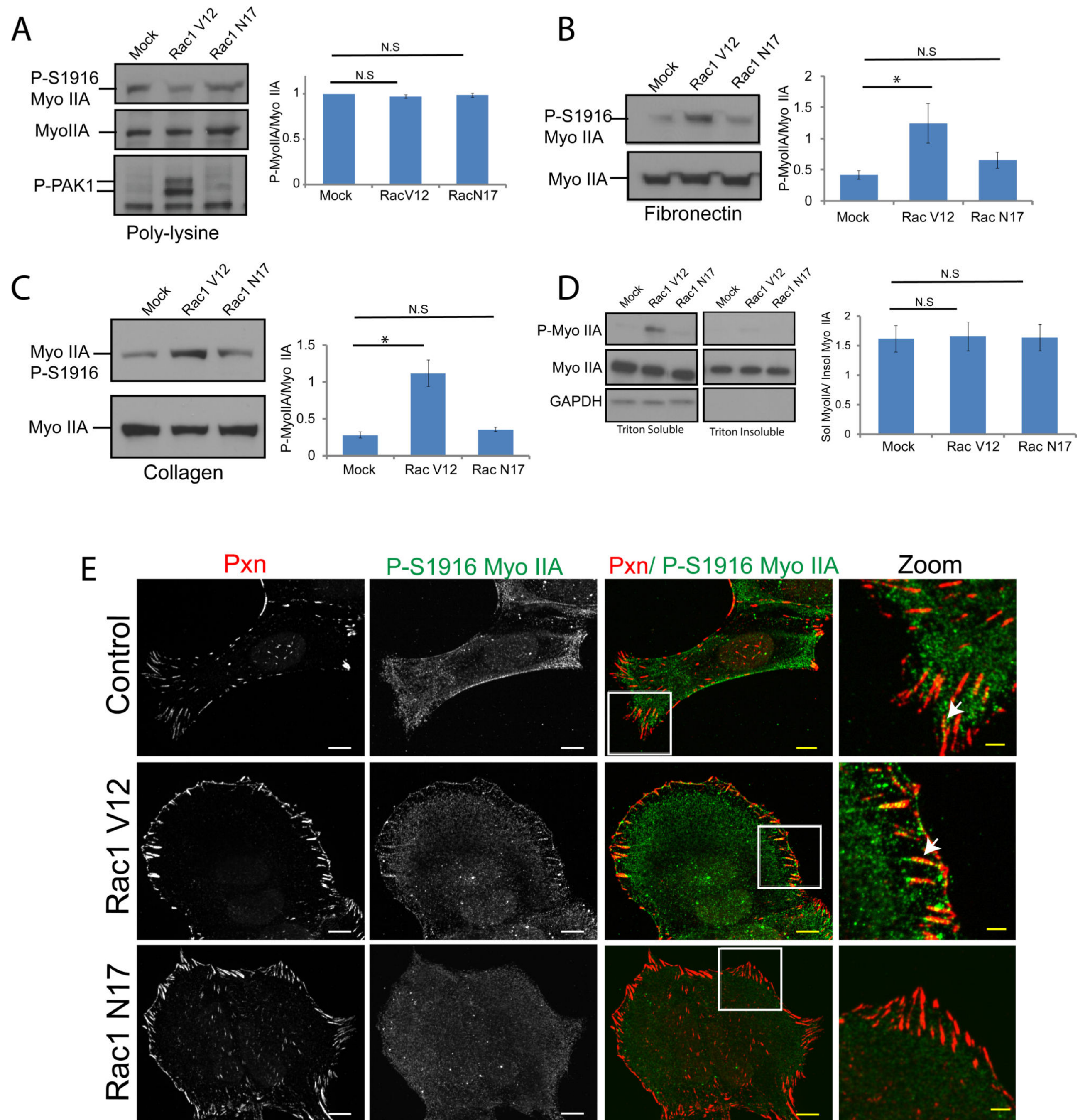

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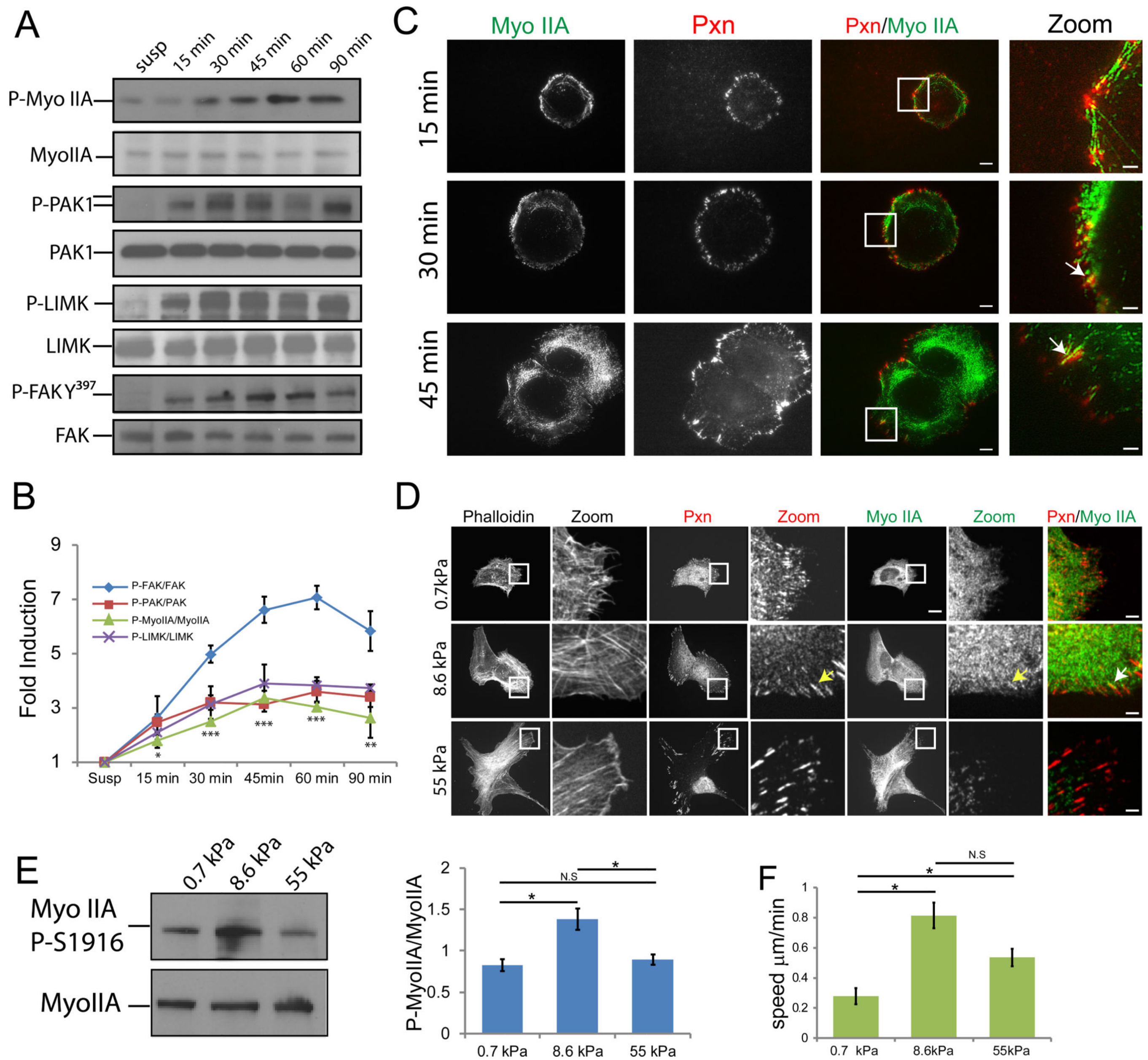

Figure 4.

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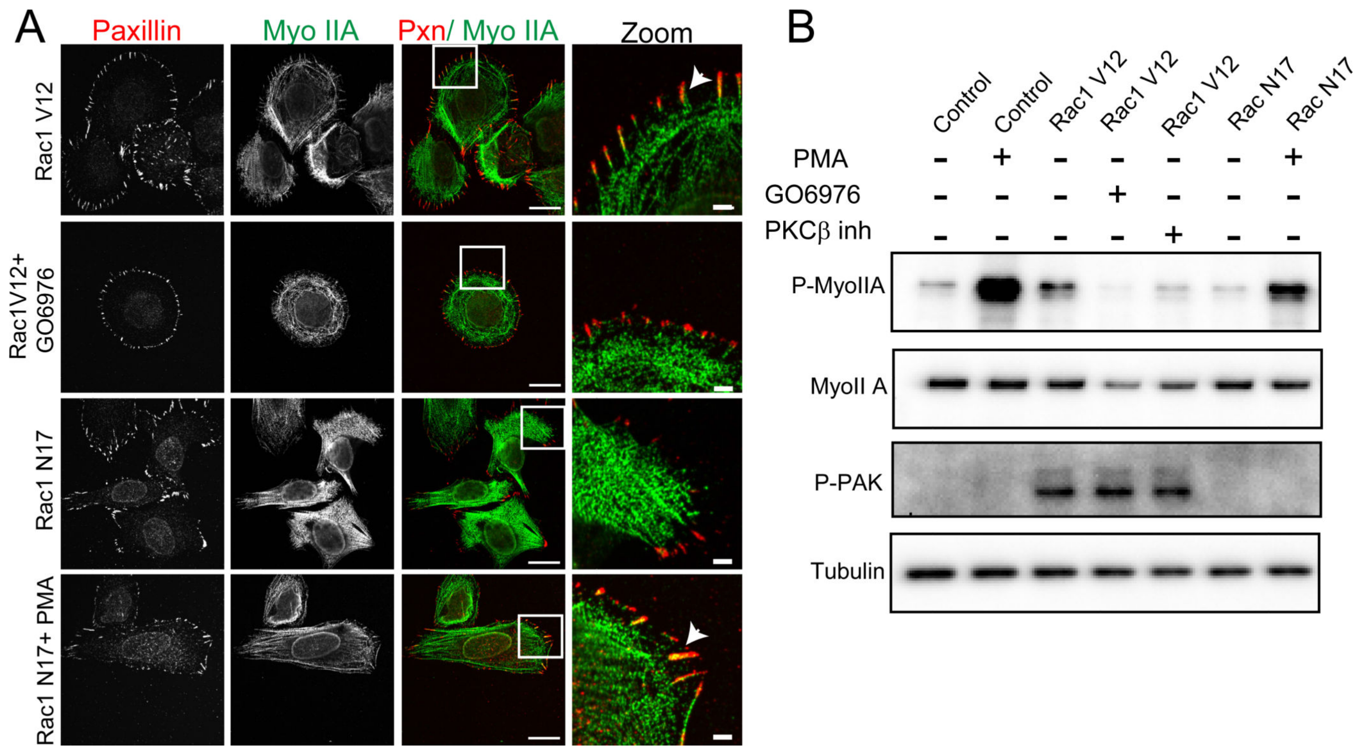

Figure 5.

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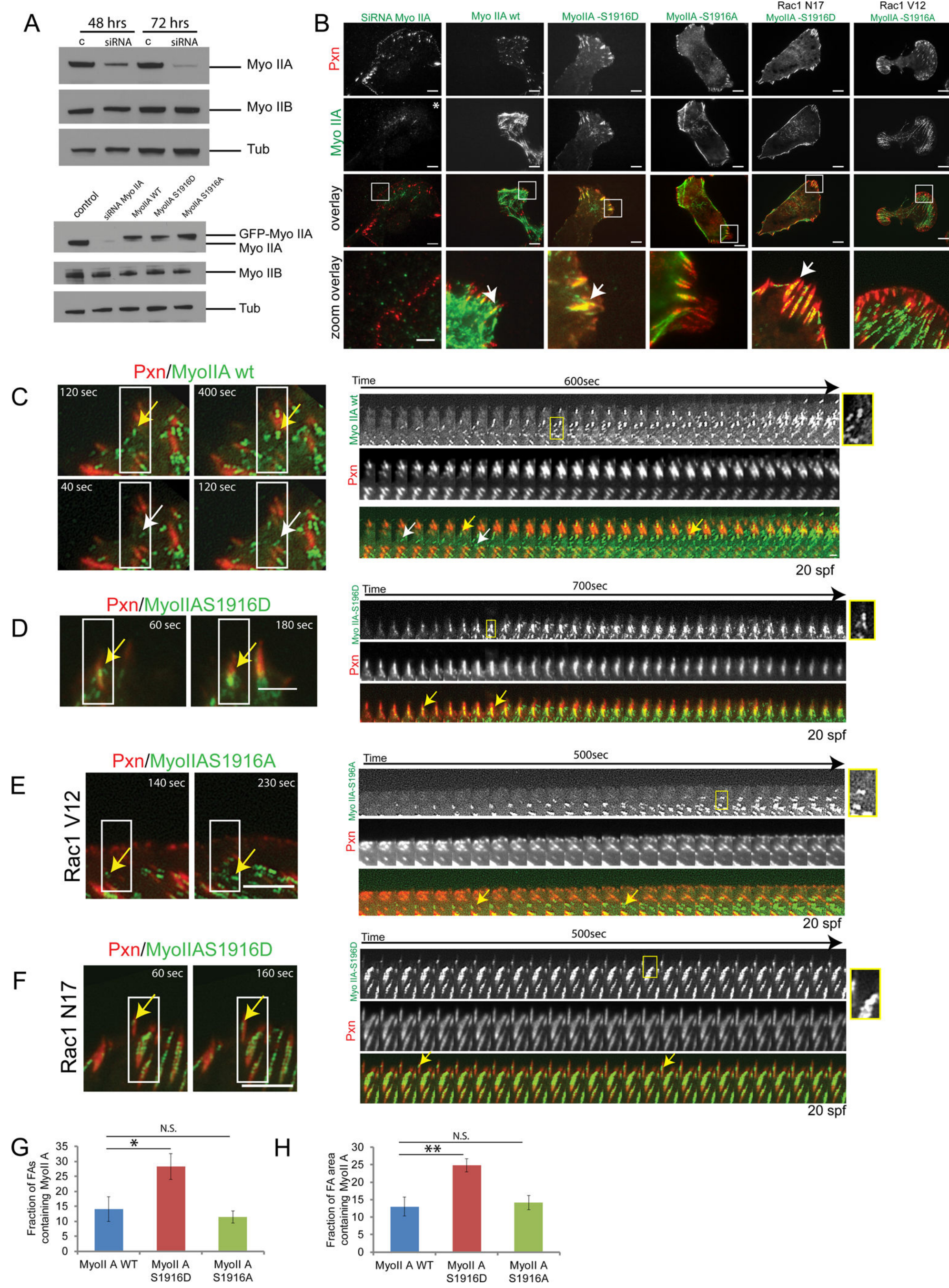

Figure 6.

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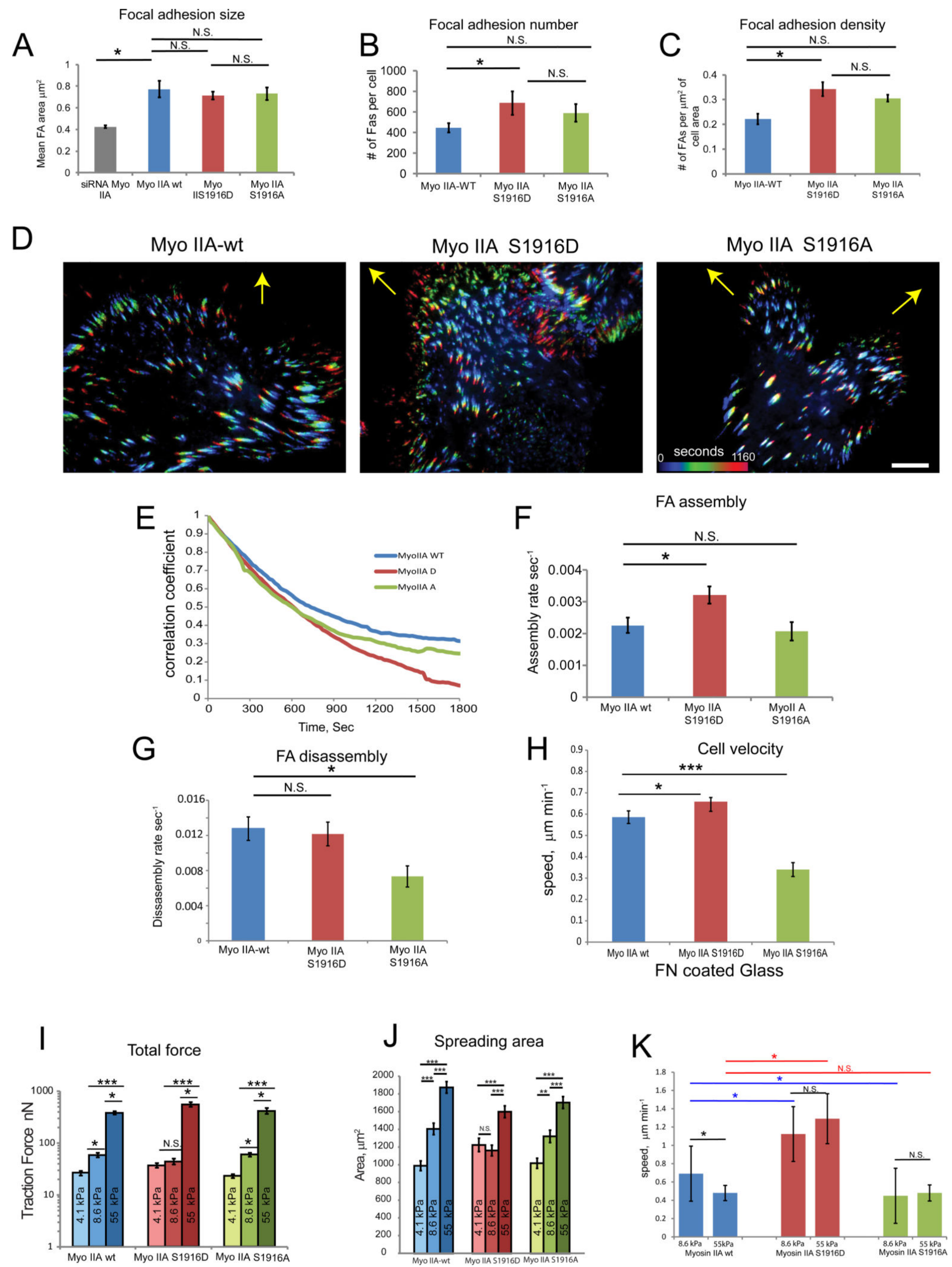

Figure 7.