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RhoA GTPase Is Dispensable for Actomyosin Regulation but Is Essential for Mitosis in Primary Mouse Embryonic Fibroblasts*[S]

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RhoA, the founding member of mammalian Rho GTPase family, is thought to be essential for actomyosin regulation. To date, the physiologic function of RhoA in mammalian cell regulation has yet to be determined genetically. Here we have created *RhoA* conditional knock-out mice. Mouse embryonic fibroblasts deleted of *RhoA* showed no significant change in actin stress fiber or focal adhesion complex formation in response to serum or LPA, nor any detectable change in Rho-kinase signaling activity. Concomitant knock-out or knockdown of RhoB and RhoC in the $RhoA^{-/-}$ cells resulted in a loss of actin stress fiber and focal adhesion similar to that of C3 toxin treatment. Proliferation of $RhoA^{-/-}$ cells was impaired due to a complete cell cycle block during mitosis, an effect that is associated with defective cytokinesis and chromosome segregation and can be readily rescued by exogenous expression of RhoA. Furthermore, *RhoA* deletion did not affect the transcriptional activity of Stat3, NFκB, or serum response factor, nor the expression of the cell division kinase inhibitor $p21^{Cip}1$ or $p27^{Kip1}$. These genetic results demonstrate that in primary mouse embryonic fibroblasts, RhoA is uniquely required for cell mitosis but is redundant with related RhoB and RhoC GTPases in actomyosin regulation.

RhoA is a founding member of the mammalian Rho GTPase family and has been implicated in a variety of roles in cell regulation, mostly by the dominant negative mutant expression approach or applying C3 bacterial toxin that covalently modifies the effector domain of RhoA and other related Rho GTPases (1–3). Among the widely accepted cell functions, RhoA is considered essential for actin stress fiber formation and actomyosin contractility, focal adhesion complex and adherens junction complex formation, gene transcription, cell cycle progression, and survival (1, 4, 5).

Vertebrates have two closely related family members, RhoB and RhoC, that share significant sequence homology with RhoA (6). RhoB is localized primarily on endosome membranes, whereas RhoC, like RhoA, is cytosolic (7, 8). Additionally, RhoB and RhoC appear to show different, and sometimes opposite, cell functions (8, 9). Neither RhoB nor RhoC knock-out mice display detectable developmental defects, and no clear phenotypes were reported from primary cells derived from these gene-targeted animals (10–12).

In the present studies, we present genetic data to unambiguously demonstrate an essential role of RhoA in regulating cell cytokinesis and a dispensable, redundant role with RhoB and RhoC in regulating the actomyosin and adhesion machineries in primary mouse embryonic fibroblasts (MEFs).[2]

EXPERIMENTAL PROCEDURES

Mice—Mice harboring conditional *RhoA* alleles, in which exon 3 is flanked by loxP sites ($RhoA^{f/f}$), were generated as depicted in supplemental Fig. S1.

Mouse Embryonic Fibroblast Isolation and Cell Culture—Primary MEFs were isolated from mouse embryos at embryonic day 12.5 as described previously (13). See supplemental Experimental Procedures for additional details.

RESULTS AND DISCUSSION

RhoA Is Dispensable for Actomyosin Signaling—Our current knowledge of the cellular functions of RhoA is based primarily on numerous studies performed in fibroblast or epithelial cells by nonspecific mutant overexpression or toxin treatment (13–18). To investigate the physiologic role of RhoA in cell regulation, we generated a conditional knock-out mouse model in which exon 3 of the *RhoA* gene, which encodes the guanine nucleotide binding sequences, was sandwiched between loxP sequences to allow Cre recombinase-mediated gene targeting (supplemental Fig. S1). Primary MEFs were derived from homozygous embryos, and deletion of *RhoA* gene was achieved by infection of the cells with adenovirus transiently expressing Cre (Fig. 1*A*). Deletion of RhoA in MEFs did not affect the expression or activity of the Rho family members Rac1 or Cdc42 but resulted in a compensatory increase in the expression and activity of the more closely related RhoB and RhoC proteins (Fig. 1*B*). RhoA-null MEFs were morphologically normal but appeared to occupy a larger spreading area than control cells (Fig. 1*C*). Surprisingly, deletion of *RhoA* did not significantly reduce actin stress fibers (Fig. 1*C*) or focal adhesion com-

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[2] The abbreviations used are: MEF, mouse embryonic fibroblast; MLC, myosin light chain; 7-AAD, 7-aminoactinomycin D; FAK, focal adhesion kinase; ROCK, Rho-associated coiled-coil protein kinase; Pax, paxillin; p, phospho.

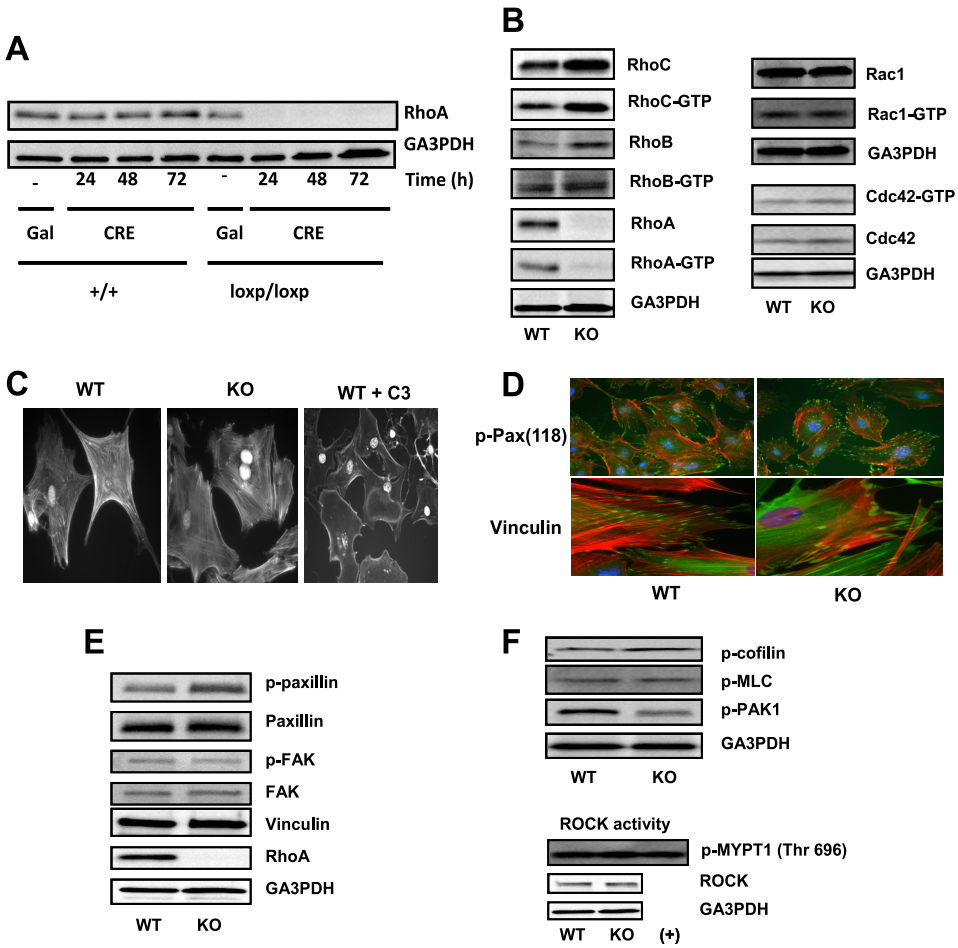

FIGURE 1. **RhoA is dispensable for actin stress fiber and focal adhesion formation in MEFs.** *A*, deletion of *RhoA* in loxp/loxp MEF cells using Cre-expressing adenovirus. Cells were infected with adeno-Cre or β-Gal virus. Cells were infected twice, and the levels of RhoA protein were evaluated by Western blotting. *GA3PDH*, glyceraldehyde 3-phosphate dehydrogenase. *B*, *RhoA* deletion affected expression and activity of RhoC and RhoB but not Cdc42 or Rac1 GTPases. GST-PAK1 pZ1-binding domain and GST-rhotekin Rho-binding domain were used for pulldown experiments to evaluate the respective GTPase activities 48 h after virus infection. *C*, actin stress formation was detected by rhodamine-phalloidin staining of the cells. Control and *RhoA* KO cells were compared with C3 toxin-treated WT cells (2 μg/ml for 4 h) in the F-actin network. *D*, focal adhesion complex proteins were examined by immunofluorescence in control and RhoA-deficient MEF cells. Cells were plated and fixed, and focal adhesion proteins were revealed using specific antibodies for p-Pax(118) (40×) or vinculin (63×) (in *green*). Cells were co-stained with rhodamine-phalloidin for F-actin (in *red*). *E*, Western blots for focal adhesion proteins in control and RhoA-deficient cells. *F*, effects of *RhoA* KO in MEF cells on ROCK and ROCK-mediated actomyosin activity. Cells were plated for 48–72 h before being processed for Western blotting with p-MLC and p-cofilin specific antibodies. For measuring ROCKII activity, ROCKII was immunoprecipitated with an anti-ROCKII antibody followed by an *in vitro* kinase activity assay using MYPT-1 polypeptide as a substrate.

plex formation (Fig. 1, *D* and *E*) under serum or lysophosphatidic acid stimulation. In fact, immunostaining of vinculin revealed that the focal adhesion complex in the RhoA-null cells appeared to be enhanced, rather than reduced, as compared with that in WT cells. However, RhoA-deficient cells did exhibit increased cell spreading area with a multinucleus phenotype (Fig. 1*C* and supplemental Fig. S2). Additionally, PDGF-stimulated lamellipodia or bradykinin-stimulated filopodia formation was not affected by *RhoA* deletion (data not shown). Consistent with the lack of effects on actin stress fibers, RhoA-null cells did not show a significant change in Rho-kinase activity (Fig. 1*F*). Furthermore, the phosphorylation status of two signaling components of the previously implicated actomyosin machinery regulated by RhoA, myosin light chain (MLC) and cofilin (5, 14), was not affected by *RhoA* knock-out, but phospho-Pak1 levels appeared reduced (Fig. 1*F*). When cells were treated with C3 toxin, stress fiber formation was eliminated in both WT and RhoA-null cells, but the RhoA-null cells appeared to remain responsive to the FBS stimulation in producing lamellipodia-like structures (supplemental Fig. S2). These results demonstrate unambiguously that RhoA is dispensable for previously attributed functions of actin stress fiber and focal adhesion formation and is not required for Rho-kinase and downstream actomyosin signaling. The results also raise the possibility that previously defined cell functions of RhoA may be attributable to other related Rho GTPases or that multiple RhoA-related GTPases share redundancy.

RhoA Is Required for MEF Cell Proliferation during Mitosis—In addition to actomyosin signaling, RhoA has been implicated in the regulation of essential gene transcription activities involved in cell growth (19, 20). Reporter assays were carried out to determine whether *RhoA* deletion alters the transcriptional activity of NFκB, SRF, and STAT3, all of which have been proposed to be regulated by RhoA-mediated activation (21–24). We have found that RhoA-null cells activate these transcription factors similarly to WT cells following serum stimu-

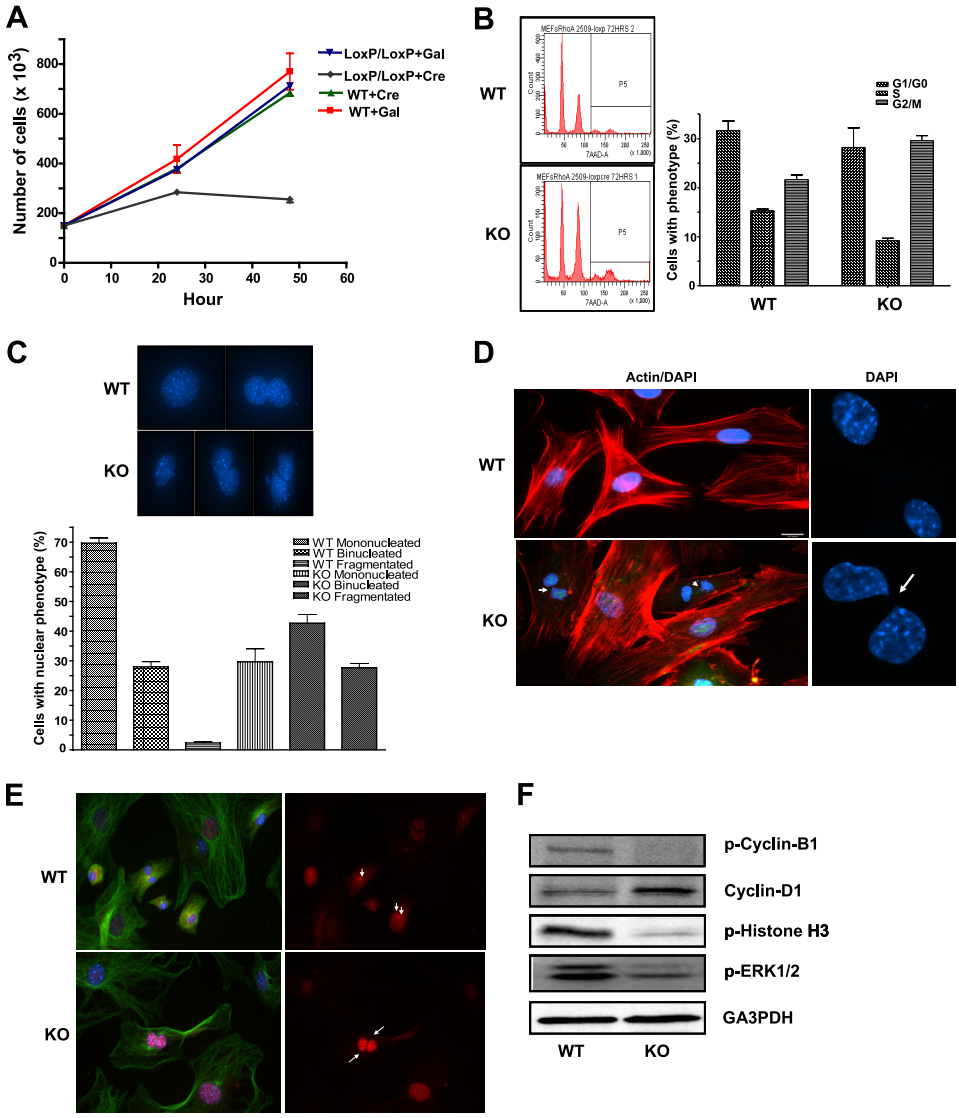

FIGURE 2. **RhoA-deficient cells are impaired in proliferation due to a mitotic defect during cytokinesis.** *A*, proliferation assays of control cells (Loxp-Gal, WT-Gal, and WT-Cre) and RhoA-deficient cells (Loxp-Cre) were carried out at a density of 1 × 105 cells/well. Cells were counted every 24 h. Cell viability was estimated by using trypan blue staining. Assays were performed in triplicate. *Error bars* represent S.D. *B*, representative cell cycle profile of RhoA-deficient cells 48 h in culture. Cells were plated at a density of 1 × 105 cells/well, fixed, and analyzed by flow cytometry following propidium iodide/7-AAD staining. *Error bars* represent S.D. *C*, nuclear morphology in RhoA-deficient cells. Control and RhoA-deficient cells were plated for 24–96 h. Cells were stained with DAPI. The percentages of cells with different nuclear phenotypes at 72 h of culture were quantified. *Error bars* represent S.D. *D*, evidence of DNA bridges between nuclei in RhoA-deficient cells. Cells were stained with DAPI to reveal nuclear morphology and were co-stained with rhodamine-phalloidin for F-actin. *Arrowheads* indicate DNA-bridges. *E*, α/β- and γ-tubulin immunostaining in RhoA-deficient cells. Cells were plated for 48 h, fixed, and incubated with antibodies for α/β-tubulin (*green*) and γ-tubulin (*red*). γ-Tubulin staining was used to determine centrosome duplication. Nuclei staining was revealed with DAPI. *F*, evaluation of cell cycle markers in RhoA-deficient cells. Plated cells were extracted at 72 h of culture, and Western blots were conducted for p-histone H3, cyclin D1, cyclin B1, cyclin E, cyclin A, and p-ERK2 as markers for cell cycle progression. *GA3PDH*, glyceraldehyde 3-phosphate dehydrogenase.

lation (supplemental Fig. S3), indicating that RhoA is also dispensable for the transcription activities of these important cell proliferation proteins. In the case of SRF activation, it was previously shown that RhoA-mediated Rho-kinase activity and actin bundling regulate the nuclear translocation of the SRF co-factor, MAL (25, 26). In light of our finding that RhoA is not required for actin stress fiber formation and SRF transcription regulation, we conclude that such a RhoA/Rho-kinase/F-actin/MAL/SRF pathway is not functionally essential for the transcription regulation.

RhoA has been found to be a central player in cytokinesis by regulating cortical contractility and cleavage furrow formation and is considered essential for G_1/S phase transition during proliferation (27, 28). We next examined whether RhoA-null cells showed any defects in proliferation under normal culture conditions. Control cells proliferated exponentially in a 4-day proliferation assay, whereas RhoA-null cells stopped proliferating after 24 h in culture (Fig. 2*A*). To better understand this effect on proliferation, we conducted cell cycle analysis following staining with 7-AAD. The resulting data revealed an accumulation of RhoA-deficient cells in G_2/M (4N DNA content) as compared with controls (Fig. 2*B*). Additionally, MPM-2 immunostaining, which shows mitotic cells (29), demonstrated a strong reduction of the frequency of RhoA-null cells in mitosis at 48 h in culture as compared with controls (supplemental Fig. S4). Combined, these results indicate that loss of RhoA results

in a cell cycle arrest. This conclusion is further supported by the finding that RhoA-null cells displayed a significant decrease in BrdU incorporation as compared with control (supplemental Fig. S5). The impaired proliferation in these cells was not associated with an induction of apoptosis, as determined by TUNEL and DNA laddering assays, or an induction of senescence, as determined by β-Gal staining and the expression of p16 (supplemental Fig. S6). Additionally, no changes in the expression of the cell cycle inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, were observed (supplemental Fig. S6).

The nuclei of RhoA-null cells showed two predominant patterns, binucleated and micronucleated (Fig. 2*C*). These phenotypes were further observed by lamin B staining for the nuclear envelope (supplemental Fig. S7). The frequency of these phenotypes in the knock-out cells plateaued at 72 h in culture and was significantly different from that of control cells (Fig. 2*C*). 7-AAD staining also revealed increased DNA content in RhoA-null populations, consistent with an aneuploid state (Fig. 2*B*). This raises the possibility that at least a subset of cells with 4N DNA content is in tetraploid G_1, rather than diploid G_2. Several studies have documented arrest of cells in tetraploid G_1 after cytokinesis failure (29–32). Interestingly, the levels of phospho-cyclin B1 and phospho-histone H3, markers for G_2/M phase, as well as phospho-ERK, were drastically lower in RhoA-null cells than in controls, and the level of the G_1/S marker, cyclin D1, was higher (Fig. 2*F*). This further supports the conclusion that RhoA-null cells were arrested in a tetraploid G_1 state. Although no RhoA-null cells were observed in mitosis, consistent with an interphase arrest, no significant changes in the interphase microtubule pattern, as detected with an antibody against α-β tubulin, were observed, however minor abnormalities were visible in the KO cells (supplemental Fig. S8). Furthermore, although supernumerary centrosomes can cause cytokinetic failure, *RhoA* deletion did not impact centrosome duplication in these cells, as demonstrated by γ-tubulin staining (Fig. 2*E*).

The presence of high levels of micronucleation and binucleation suggests that RhoA-null cells were defective for chromosome segregation and cytokinesis, respectively. The possibility of a segregation defect is further supported by the finding of chromosome bridges in interphase RhoA-null cells, which presumably returned to interphase following incomplete segregation during mitosis (Fig. 2*D*). Previous work has demonstrated that chromosome bridges can induce failure of cytokinesis and thereby result in tetraploidization (30, 32).

Cytokinesis of mammalian cells is initiated by assembly of the contractile actomyosin ring at the equatorial cell cortex during anaphase(33). Using an RNAi-based approach, it has been shown that RhoA is essential for cytokinesis in *Caenorhabditis elegans* embryos (34). RhoA appears to be required for the assembly and ingression of the actomyosin filament ring (35). RhoA activation during initiation of furrowing depends on the Rho guanine nucleotide exchange factor protein Ect2. Ect2 is phosphorylated during mitosis by Cdk1 and the Polo-like kinase Plk1, and in turn, activates RhoA at the equatorial region and the cleavage furrow (36–38). Activated RhoA recruits two effector kinases, citron kinase and Rho-kinase, along with other mediators such as diaphanous-related protein mDia1, to assemble the contractile ring and mediate cleavage furrow ingression and subsequent abscission (39–44). Because deletion of RhoA strongly inhibited mitosis in our experiments, we were unable to directly observe the formation of furrowing or abscission, recruitment of ECT-2, anillin, or mDia1, or activation of Aurora B in the knock-out cells (data not shown).

Our results show that genetic deletion of *RhoA* causes binucleated and fragmented nuclei in the absence of cytokinesis. In HeLa cells, siRNA-mediated depletion of citron kinase also resulted in the formation of multinucleated cells (36). However, a mild defect in cytokinesis was observed in citron kinase knock-out mice, suggesting its redundancy with other Rho effectors responsible for filament contraction (45). Similarly, depleting mDia2 in NIH3T3 cells also increased the number of binucleated cells (46). In the same context, Rosario *et al.* (47) recently demonstrated that $Plk4^{+/-}$ MEF, the Polo family kinase, which is required for late mitotic progression, showed a high incidence of multinucleation, supernumerary centrosomes, and a near tetraploid karyotype. In addition to the binucleation phenotype associated with a cleavage failure, RhoA-null MEFs displayed micronucleation. This further suggests that RhoA is involved in chromosome segregation. In the future, it will be interesting to dissect this novel function for RhoA during mitosis.

RhoA, RhoB, and RhoC Are Redundantly Involved in Actomyosin Regulation in MEFs—To more rigorously test the possibility that RhoA is essential for cell proliferation, an add-back rescue experiment was performed. $RhoA^{flox/flox}$ cells were infected with retrovirus expressing RhoA-GFP (Fig. 3*A*). Subsequently, endogenous RhoA was deleted by using an adeno-Cre virus. Proliferation assays demonstrate that expression of exogenous wild-type RhoA is sufficient to rescue and support normal proliferation in the endogenous *RhoA* gene deleted cells (Fig. 3*B*). Thus, RhoA is required for normal cell cycle progression. Consistently, reintroduction of wild-type RhoA significantly reduced the percentage of cells containing binucleated or micronucleated nuclei as compared with controls (data not shown). Also consistent with a dispensable role in actomyosin regulation, exogenous expression of RhoA-GFP in MEF cells in the absence of endogenous RhoA did not affect the actin network or the expression and distribution of focal adhesion proteins (data not shown).

Initial characterization of RhoA-deficient cells showed an increase in both RhoC protein and its activity (Fig. 1*A*). $RhoC^{-/-}$ MEFs behaved similarly to wild-type cells in actin reorganization, focal adhesion complex formation and proliferation (Fig. 3*D* and supplemental Fig. S10), indicating that RhoC plays a redundant role in these cell functions. To determine the contribution of RhoC in RhoA-deficient cells, $RhoA^{flox/flox}$ mice that were heterozygous for *RhoC* knock-out were crossed to generate RhoA/RhoC-deficient MEF cells. In the absence of RhoA and RhoC, RhoB protein level and activity were increased (Fig. 3*C*). Surprisingly, RhoA/RhoC-deficient cells displayed completely normal actin filaments and focal adhesions (Fig. 3*D*). Further, there were no abnormalities in the actomyosin signaling of p-MLC and p-cofilin in $RhoA^{-/-}/RhoC^{-/-}$ cells as compared with WT cells or cells deficient for only RhoA (supplemental Fig. S9). These data indicate that RhoC itself is not

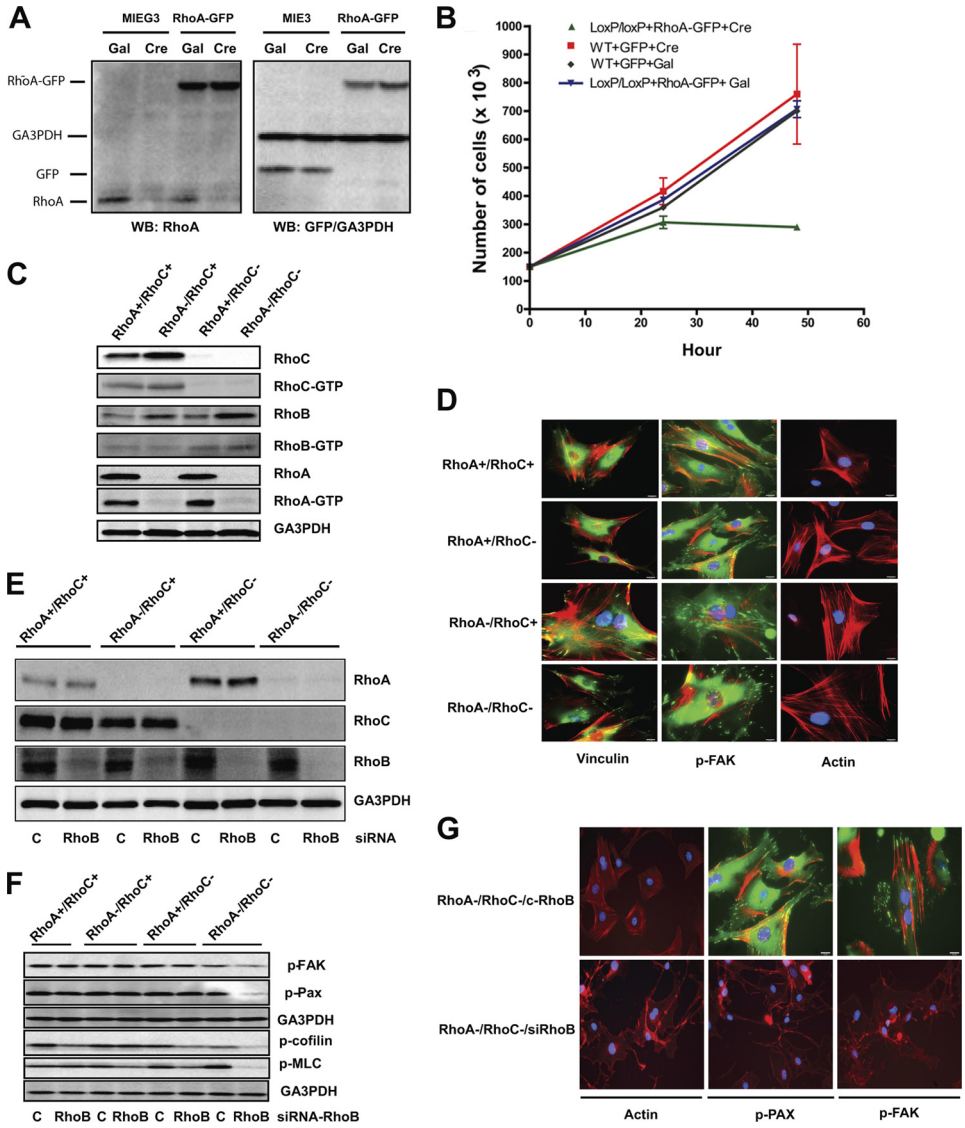

FIGURE 3. **RhoB and RhoC serve a redundant role with RhoA in regulating actomyosin activity.** *A*, *RhoA*$^{flox/flox}$ and WT cells were infected with MIEG3-GFP (control vector) or MIEG3-RhoA-GFP retrovirus. The transduced cells were sorted and plated for 24 h, and adeno-Cre or β-Gal adenovirus infection was carried out. Western blots (*WB*) for endogenous RhoA or transduced RhoA/GFP were performed. *GA3PDH*, glyceraldehyde 3-phosphate dehydrogenase. *B*, a cell proliferation assay was performed in WT, *RhoA* deleted, or RhoA reconstituted cells. Cells grown in triplicate plates were quantified at the indicated times. *Error bars* represent S.D. *C*, RhoB and RhoC activities in relation to that of *RhoA* deletion. WT and *RhoC* KO cells were plated, and 24 h later, endogenous RhoA was deleted by adeno-Cre treatment. Cells were extracted for RhoA, RhoB, and RhoC activity measurements by GST-rhotekin pulldown. *D*, RhoA/RhoC-deficient cells do not show adhesion defects. WT and *RhoC* KO cells were stained for vinculin, p-FAK (*green*), and F-actin (*red*). Nuclei were stained with DAPI (*blue*). *E*, effect of RhoB suppression in RhoA/RhoC-deficient cells on F-actin and focal adhesion. WT and *RhoC* KOs were plated, and 24 h later, they were incubated with an on-TARGETplusR control non-targeting pool or an on-TARGETplus SMARTpool mouse RhoB. Endogenous *RhoA* were deleted by adenovirus treatment. Cells were processed for Western blotting to determine the levels of RhoA, RhoB, and RhoC proteins. *F*, effect of RhoB knockdown on focal adhesion proteins and actomyosin components in RhoA/RhoC-deficient cells. Cells were treated with rhoB-specific siRNA, and the levels of p-MLC, p-cofilin, p-FAK, and p-Pax were determined by Western blotting. *G*, effect of RhoB knockdown on F-actin structure and focal adhesion complex in RhoA/RhoC-deficient cells. Cells were stained for p-Pax, p-FAK (*green*), and F-actin (*red*).

sufficient for the redundant actomyosin regulatory role with RhoA.

Population doubling experiments showed that RhoA/RhoC-deficient cells exhibited a partial increase in the rate of proliferation as compared with RhoA-deficient cells, an effect associated with a compensatory elevation of RhoB activity in the cells (supplemental Fig. S10). To evaluate the contribution of RhoB to the cellular phenotypes of these *RhoA*/*RhoC* double knock-out cells, siRNA specifically targeting RhoB was used to suppress the expression of the RhoB protein (Fig. 3*E*). The lack of RhoB expression in *RhoA*/*RhoC* double knock-out cells not only significantly reduced the levels of p-cofilin and p-MLC, but also the phosphorylation of paxillin and FAK (Fig. 3*F*). In fact, cultured RhoA/RhoB/RhoC-deficient cells showed a complete deficiency in actin stress fiber formation and displayed signs of detachment (Fig. 3*G*). These phenotypes are similar to those observed in studies utilizing C3 toxin (Fig. 1*C*), which inhibits all three Rho subfamily members. Our study shows that RhoB, together with RhoC, functions as a redundant part of the regulatory signals in RhoA-mediated pathways and that these three subfamily members of Rho GTPase family collectively, rather than individually, control cell actomyosin machinery.

Conclusions—In the present studies, we have presented genetic data to unambiguously demonstrate an essential role of RhoA in regulating cell mitosis, but surprisingly, a dispensable, redundant role in regulating cell actomyosin machineries in MEFs. In particular, RhoA is not essential for actin stress fiber and focal adhesion formation in MEFs. Rather, these functions are redundant with closely related RhoC and RhoB GTPases. RhoA is essential for cell proliferation at the cytokinesis stage of cell cycle, and neither RhoB nor RhoC is required for this important function. RhoA is dispensable for regulation of NFκB, Stat3, and SRF transcriptional activities, which were previously implicated in RhoA-mediated cell growth regulation. RhoA is also dispensable for cell survival and for tumor suppressor $p21^{Cip1}$ and $p27^{Kip1}$ regulation that was previously suggested to be involved in RhoA-mediated signaling during S phase transition (48–50). Finally, our data indicate that RhoA is dispensable for signaling events of Rho-kinase, p-MLC/cofilin, JNK, ERK, and cyclin D1, which were implicated as RhoA-regulated events in numerous previous studies (14, 28). In the context of a recent observation that RhoA appears to be dispensable for skin development in mice (51), our studies raise the possibility that the general principles of RhoA function defined by *in vitro* biochemical methods in clonal cell lines may not necessarily apply to primary cells and that the physiological function and signaling pathways regulated by RhoA are tissue cell type-specific.

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