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# Myosin Light Chain Kinase and Acto-Myosin Contractility Modulate Activation of the ERK Cascade Downstream of Oncogenic Ras

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**Abstract** The actin cytoskeleton is recognized as an important component of both adhesion- and growth factor-dependent signaling, but its role in oncogene-dependent signaling has received much less attention. In this study, we investigated the role played by the acto-myosin cytoskeleton and its main regulators, i.e., myosin light chain kinase and Rho kinase, in oncogenic Ki-Ras-induced signaling. We found that activation of the ERK cascade by Ras is dependent on acto-myosin contractility, under the regulation of myosin light chain kinase but not Rho kinase. Inhibition of myosin II or myosin light chain kinase caused a complete loss of ERK phosphorylation in a time- and dose-dependent manner, but proved dispensable for activation of the PI3K pathway. We also provide evidence that the target of myosin light chain kinase lays at the level of Raf activation. Since myosin light chain kinase is a target of ERK, these results suggest a previously uncharacterized signaling pathway involving Ras-mediated alterations of the actin cytoskeleton, which might play a critical role in ERK activation by the Ras oncogene and contribute to aberrant signaling and enhanced cell motility. In addition, restoration of stress fibers following ectopic expression of tropomyosin 2 resulted in reduced levels of ERK phosphorylation. Finally, these studies suggest that myosin light chain kinase but not Rho kinase plays an essential role in the generation of ERK signaling in transformed cells and indicate distinct cellular roles for Rho-kinase and myosin light chain kinase-dependent functions involving the regulation of acto-myosin contractility. J. Cell. Biochem. 95: 1069–1080, 2005. 

**Key words:** oncogenic Ras; ERK; actin cytoskeleton; myosin II; MLCK; Rho kinase

Actin filaments are crucial for cell migration and the maintenance of cellular morphology. In addition, a role for actin in cell signaling is emerging. Signals generated by growth factor stimulation and cell adhesion are required for cell proliferation, and the actin cytoskeleton has been implicated in the integration of these signals by forming a platform that regulates signaling events [Assoian and Schwartz, 2001]. Most studies on the regulation of cell signaling by the actin cytoskeleton have focused on growth factor signaling, using cells in monolayer versus suspension culture, but the importance of cytoskeleton organization in oncogenic signaling has received much less attention. Alterations in the actin-based cytokeleton are an established part of the neoplastic phenotype, and it is becoming clear that such alteration is not a byproduct of cellular transformation but contributes to malignant transformation [Pawlak and Helfman, 2001; Rao, 2002]. These results raised the hypothesis that the actin cytoskeleton may play a direct role in the regulation of oncogenic signaling.

The actin cytoskeleton is a dynamic system, in which the association of actin filaments with the molecular motor myosin II drives the generation of intracellular contractile forces required for the formation of stress fibers and focal adhesions [Burridge et al., 1997; Helfman et al., 1999]. The activity of myosin II is

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controlled by phosphorylation of a serine residue on its regulatory light chain (MLC) by the enzyme myosin light chain kinase (MLCK) [Sellers, 1991; Tan et al., 1992]. Phosphorylation of MLC by MLCK activates myosin II. This action is opposed by dephosphorylation of the MLC, which is mediated by a specific myosin light chain phosphatase. The Rho kinase catalyzes the inhibitory phosphorylation of myosin light chain phosphatase [Kawano et al., 1999], and the resulting increase in steady-state MLC phosphorylation by MLCK promotes both myosin filament assembly and actin-activated myosin ATPase activity, thus generating actomyosin contractility [Chrzanowska-Wodnicka and Burridge, 1996; Fukata et al., 2001]. MLC has also been identified as a direct substrate of Rho kinase [Amano et al., 1996; Totsukawa et al., 2000].

The ERK (extracellular-signal regulated kinase) cascade impacts on cell proliferation and survival, and recent studies have demonstrated a close link between organization of the actin cytoskeleton and activity of the ERK pathway. Growth factor-mediated activation of ERKs is adhesion-dependent and relies upon an intact cytoskeleton [Renshaw et al., 1997; reviewed in Howe et al., 2002]. Recent studies have also shown that stress fiber formation is required for sustained activation of ERKs in NIH3T3 fibroblasts, therefore allowing cyclin D1 expression and cell cycle progression [Roovers et al., 1999; Bottazzi et al., 2001; Roovers and Assoian, 2003]. In addition, translocation of ERKs from the cytoplasm to the nucleus is also regulated by adhesion and cytoskeletal integrity in fibroblasts and endothelial cells [Aplin and Juliano, 2001a,b; Smith et al., 2004].

The adhesion-dependent activation of ERKs is by-passed in ras-transformed cells, leading to constitutive activation of this pathway. Mechanistically, activity of oncogenic Ras might promote adhesion-independent signaling by causing the ERK cascade to bypass the requirement for cytoskeletal integrity. Alternatively, because oncogenic Ras can profoundly affect microfilament organization [Reuveni et al., 2000; Sahai et al., 2001; Pawlak and Helfman, 2002], it is also possible that Ras might remodel the actin cytoskeleton into structures required for efficient signaling to ERK. To examine the contribution of myosin II and the actin cytoskeleton to the signaling activity of the Ras oncogene, we have measured the basal level of ERK activation in NRK (normal rat kidney) fibroblasts transformed by the Ki-ras oncogene (NRK/ras cells), and cultured in the presence of various cytoskeleton-perturbing agents. We have found that myosin II and MLCK inhibitors are potent and specific inhibitors of ERK activation, thus demonstrating the importance of myosin II in oncogenic Ras signaling. Furthermore, restoration of stress fibers following ectopic expression of tropomyosin 2 in NRK/ras cells resulted in reduced levels of ERK phosphorylation, suggesting that alterations in the actin cytoskeleton contribute to oncogene-mediated signaling events. In addition, these studies suggest distinct cellular roles for Rho kinase- and MLCK-dependent functions involving the regulation of myosin II.

## MATERIALS AND METHODS

### Cell Culture and Pharmacological Reagents

NRK and NRK/ras cells (NRK ATCC CRL 1570 and 1569, respectively) were maintained in DMEM containing 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified air–5% $CO_2$ atmosphere. Cells were allowed to proliferate in 60 mm tissue culture dishes (Corning, Corning, NY) until they reached 80% confluence, and then exposed to different cytoskeleton-modifying drugs. For some experiments, NRK/ras cells were detached with 0.05% trypsin-EDTA and collected in DMEM containing 2% bovine serum albumine (BSA). Cells were then incubated non-adherently at 37°C for 2 h in a rotator to allow kinases to become quiescent. Cytoskeleton-modifying drugs were eventually included at this step. At the end of treatments, cells were rinsed rapidly with ice-cold phosphate-buffered saline and solubilized for western-blot analysis. The pharmacological compounds Blebbistatin, ML-7, ML-9, and Y27632 were purchased from Calbiochem (La Jolla, CA); 2,3-butanedione monoxime (BDM) was from Sigma (St. Louis, MO).

### Preparation of Ligand-Coated Dishes and Adhesion-Induced Signaling

Tissue culture dishes were incubated with 10 μg/ml fibronectin (Sigma) or 100 μg/ml poly-L-lysine (Sigma) in PBS overnight at room temperature, followed by blocking with 2% BSA in DMEM for 1 h at 37°C. Dishes were rinsed twice with PBS prior to use. Cells were then replated

onto these ligand-coated dishes and allowed to proliferate for various times.

### Retrovirus Production and Infection

Vectors for the inducible expression of active forms of Raf1 (pBP3ΔRaf1:ER[YY]) and MEK1 (pBP3ΔMEK1:ER) were kindly provided by M. McMahon (ICSF, CA). The plasmid pDCR-Ha-Ras (G12V, T35S) was a generous gift of M.A. White (University of Texas, Southwestern Medical Center, Dallas, TX). Ecotropic retrovirus stocks were obtained by Lipofectamine-mediated transient transfection of the retroviral vector DNAs into Phoenix packaging cells. Target NRK cells were infected in the presence of 4 μg/ml polybrene then cultured in medium containing 4 μg/ml puromycin (NRK/ΔRaf1:ER and NRK/MEK1:ER) or 500 μg/ml G418 (NRK/RasV12S35) to select for virus-infected cells. Following selection, cells were pooled, expanded, and tested for the expression of RasV12S35, ΔRaf1:ER, and ΔMEK1:ER proteins by Western blotting, using antibodies to the HA tag (12CA5 mAb; Cold Spring Harbor Laboratory Antibody Facility) and ER protein (HC-20 pAb; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. For the induction of Raf-1:ER or MEK1:ER, cells were grown in medium containing 4-HT (1 μg/ml) or ethanol (as a control) for up to 72 h. 4-HT (Sigma) was stored at −20°C as a 1 mM stock in ethanol and diluted directly into the cell culture medium.

### Cell Extracts and Immunoblotting

Cells were washed with ice-cold PBS containing 1 mM sodium orthovanadate before direct extraction in 2% SDS Laemmli sample buffer. Lysates were clarified by centrifugation (16,000*g*, 15 min at 4°C) and protein concentrations were measured by bicinchoninic acid protein assay (Biorad, Hercules, CA). Equal amounts of proteins were resolved by SDS–PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The following primary antibodies were used: mAb anti-β-actin (Sigma), polyclonal anti-ERK1 (Santa Cruz Biotechnology), mAb anti-phospho-ERK1/2 (Cell Signaling Technology), polyclonal anti-AKT (Cell Signaling Technology, Beverly, MA), polyclonal anti-phospho-AKT (Cell Signaling Technology), mAb anti-MEK (Transduction Laboratory, San Diego, CA), polyclonal anti-phospho-MEK1/2 (Cell Signaling Technology), mAb anti-tropomyosin (TM311; Sigma), polyclonal anti-phospho-Thr18/Ser19-MLC (Cell Signaling Technology), mAb anti-MLC (MY-21; Sigma). The phospho-ERK1/2 antibody is directed against tyrosine 202 and threonine 204 of ERK, the phospho-AKT antibody against serine 473, and the phospho-MEK1/2 antibody against serine 217 and serine 221. These sites have been shown to correlate with the activated forms of ERK, AKT, and MEK, respectively [Yan and Templeton, 1994; Alessi et al., 1996; Xu and Goldfarb, 2001]. After incubation with appropriate HRP-conjugated secondary antibodies, the immunoreactive bands were detected by chemiluminescence (NEN) according to the manufacturer's instructions. Signals were quantitated by densitometry using an image analyzer (AlphaImager 2200 v5.5 software).

### Immunofluorescence

Cells grown on glass coverslips were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, then blocked with 1% BSA, at room temperature. Incubations with primary antibodies against vinculin (Sigma; 1:400) were conducted at room temperature for 1 h, followed by incubation with Cy3-conjugated secondary antibodies (Molecular Probes, Eugene, OR; 1:500) for 45 min. To stain actin, fixed cells were incubated with Oregon Green-conjugated phalloidin Molecular Probes). Cells were finally stained with 4′6-diamidino-2-phenylindole (DAPI) and coverslips were mounted using Prolong Antifade (Molecular Probes). Samples were examined and pictures acquired on a Zeiss Axiophot microscope equipped with a Photometrics SenSys cooled CCD camera using Image 2.0.5 software (Oncor). All photographs were taken at the same magnification.

### Statistical Analysis

All the experiments were performed at least three times. For statistical analysis of data, Student's *t*-test was used. Values are expressed as mean ± standard error of the mean. Data were considered statistically significant at a $P$ value of $<0.01$.

## RESULTS

### Oncogenic Ras Requires Acto-Myosin Contractility to Activate ERK1/2

Studies performed with untransformed cells have shown that assembly of the acto-myosin

cytoskeleton modulates activation of the ERK cascade, in response to various stimuli [Yanazume et al., 2002; Roovers and Assoian, 2003]. On the other hand, the ERK signaling cascade induced by the Ras oncogene targets the actin cytoskeleton and induces a remodeling of structures such as stress fibers and focal adhesions [Reuveni et al., 2000; Sahai et al., 2001; Pawlak and Helfman, 2002]. These results raised the hypothesis that the cytoskeletal rearrangements induced by oncogenic Ras may play a direct role in the regulation of ERK signaling. To test whether the actin-based cytoskeleton is relevant for Ras-induced ERK activation, we blocked myosin II in Ki-ras-transformed NRK cells (NRK/ras cells), using 2,3-butanedione monoxime (BDM) as a broad-spectrum inhibitor of myosin II function [Ostap, 2002; Yarrow et al., 2003]. ERK activity was monitored with phospho-specific antibodies, since phosphorylation of ERKs on both threonine and tyrosine residues has been shown to be a good indicator of the kinase activity of these proteins [Egea et al., 2000]. The constitutive activation of ERKs observed in control NRK/ras cells was dramatically reduced by 25 mM BDM (Fig. 1A), revealing that oncogenic Ras signaling activity requires myosin II-induced actin contractility to activate ERKs. Since recent findings have questioned the mechanism by which BDM functions [Duran et al., 2003; Titus, 2003; Yarrow et al., 2003], we tested blebbistatin, a recently described highly specific myosin II inhibitor [Straight et al., 2003; Limouze et al., 2004]. Treatment of NRK/ras cells with blebbistatin indeed led to reduced ERK phosphorylation (Fig. 1B), thus confirming the implication of myosin II activity in oncogenic Ras signaling.

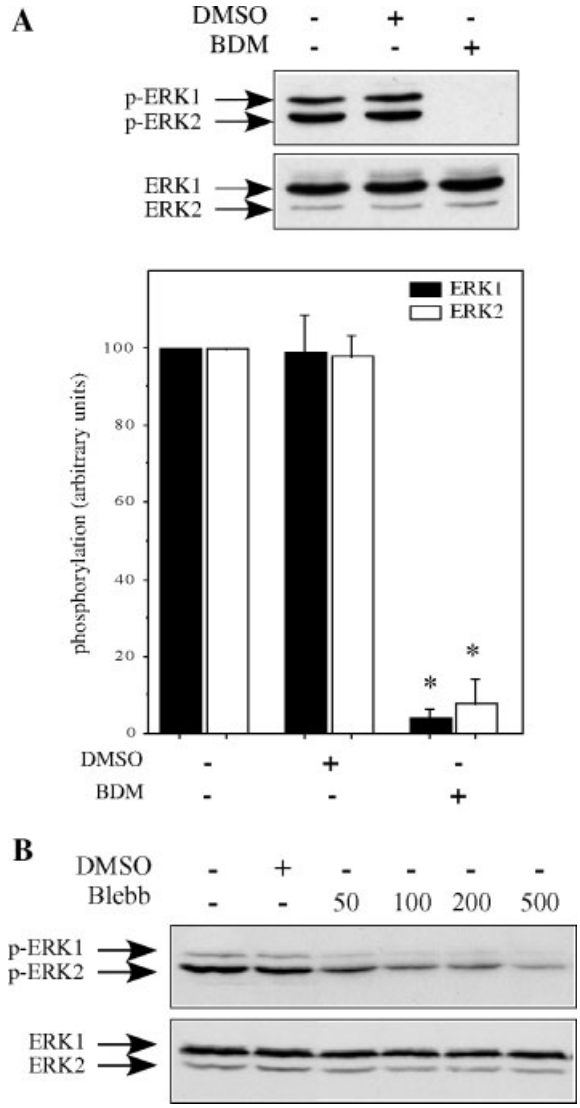


**Fig. 1.** Inhibition of myosin II ATPase activity prevents phosphorylation of ERK by Ki-Ras. **A**: NRK/ras cells were treated (+) or not (−) with 25 mM BDM or vehicle only (DMSO) for 30 min. After treatment, cells were lysed, and protein extracts were analyzed by Western-blotting with an anti-phospho-ERK antibody (**upper panel**). The membrane was then stripped and reprobed with an anti-ERK antibody (**lower panel**) as a control for the protein content per lane. Graph shows the average ERK phosphorylation from five independent experiments. *, *P* value of <0.001, as determined by Student's *t*-test. Arrows labeled p-ERK1 and p-ERK2 or ERK1 and ERK2 indicate the positions of phosphorylated and total ERK1 and ERK2 proteins, respectively. **B**: NRK/ras cells were treated (+) or not (−) with Blebbistatin (50, 100, 200, or 500 μM) or vehicle only (DMSO) for 30 min. After treatment, protein extracts were analyzed by Western blotting for ERK phosphorylation as described above.

## Functional Inhibitors of MLCK Prevent Activation of the ERK Cascade by Oncogenic Ras

The activity of myosin II is mainly regulated by phosphorylation of its regulatory light chain (MLC), under the control of two major kinases, MLCK and Rho-kinase [Amano et al., 1996; Totsukawa et al., 2000]. To test whether MLCK- and Rho-kinase-dependent signaling play a role in Ki-ras-induced ERK activation, we assayed ERK phosphorylation in cells that had been pretreated with ML-7, one of the most specific small molecule inhibitors which blocks MLCK function [Saitoh et al., 1987; Chrzanowska-Wodnicka and Burridge, 1996], or Y-27632, a well-established inhibitor of Rho-kinase [Uehata et al., 1997].

NRK/ras cells were treated with ML-7 or ML-9 (an other MLCK inhibitor), or the Rho kinase

inhibitor Y27632, for 30 min. Protein extracts were analyzed with an anti-phospho ERK antibody, and results are shown in Figure 2. Both ML-7 and ML-9 blocked the phosphorylation of ERKs, while inhibition of Rho kinase by Y27632 had no effect on ERK phosphorylation (Fig. 2), even at concentrations as high as 50 μM. As a control of the effectiveness of ML-7, ML-9, and Y27632 at the concentrations used, they were shown to induce characteristic morphological changes in parental NRK cells (data not shown). Thus, Rho-kinase does not play a role in myosin II activation during Ki-ras signaling and this activation is largely regulated by MLCK alone.

The effects of MLCK inhibitors on Ki-ras-induced ERK activation were also investigated over time. ERK phosphorylation was inhibited as soon as 5 min after the addition of ML-7 and remained undetectable for almost 2 h (Fig. 2B). The level of ERK phosphorylation eventually returned to the control level, typically after 3–4 h of incubation (not shown). The ML-7 effects were reversible, since ERK phosphorylation quickly returned to control levels when the drug was washed out (Fig. 2B), ruling out the possibility that inhibition of ERK phosphorylation upon ML-7 treatment could result from toxicity followed by cell death. Collectively, these data suggest that activation of myosin II via MLCK, but not Rho kinase, plays a critical role in Ki-Ras-induced ERK activation.

Having shown the implication of both myosin II and MLCK in oncogenic Ras signaling, we then analyzed the state of MLC phosphorylation. We found that MLC is phosphorylated in NRK/ras cells, and that treatment with ML7 does indeed reduce the level of phosphorylation of MLC, whereas Y27632 has no significant effect (Fig. 2C). As expected, blebbistatin did not affect MLC phosphorylation, since this compound inhibits the myosin II ATPase activity without affecting MLCK [Straight et al., 2003].

To determine whether our findings apply to cells not transformed by Ras, the effects of

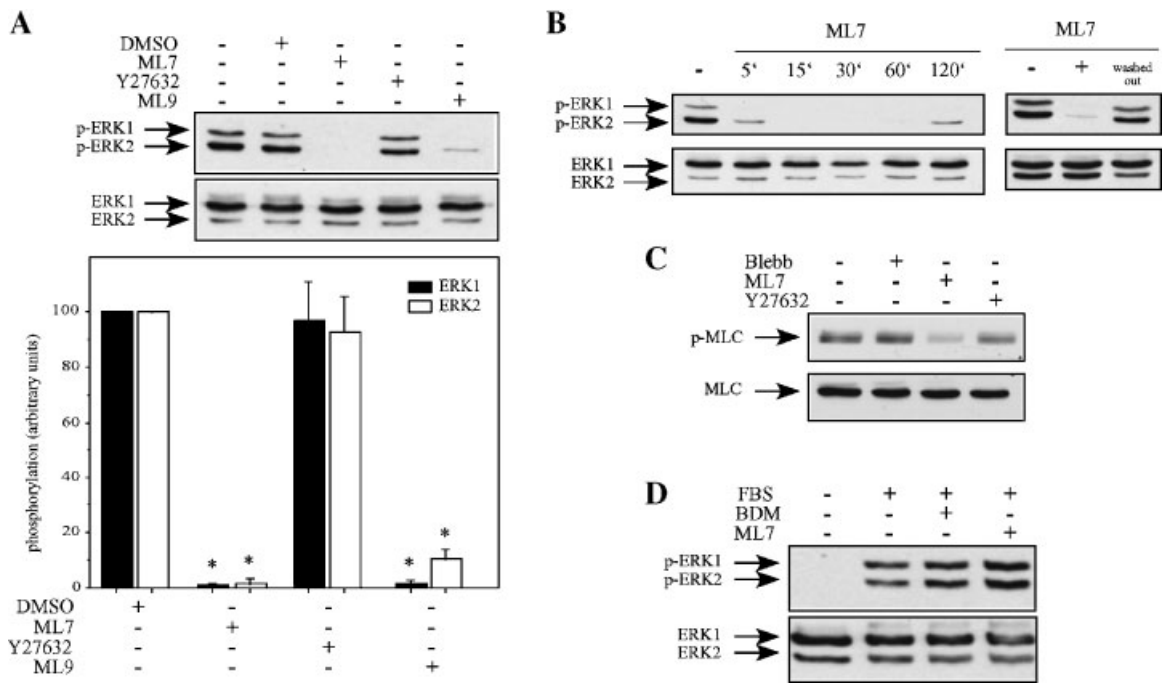


**Fig. 2.** MLCK inhibitors block Ki-Ras-induced ERK phosphorylation. **A**: NRK/ras cells were treated (+) or not (−) with ML-7 (25 μM), ML-9 (50μM), Y27632 (20 μM) or vehicle only (DMSO) for 30 min. After treatment, cells were lysed, and ERK phosphorylation was analyzed as described in the legend to Figure 1. *, *P* value of <0.001, as determined by Student's *t*-test. **B**: NRK/ras cells were not treated (−) or treated with 25 μM ML-7 for various times (**left panel**). After 60 min of incubation in the presence of ML-7, NRK/ras cells were washed with media and incubated for another hour (right panel). After treatment, cells were lysed, and ERK phosphorylation was analyzed as described in the legend to Figure 1. **C**: NRK/ras cells were treated (+) or not (−) with blebbistatin (200 μM), ML-7 (25 μM), or Y27632 (20 μM) for 30 min. Protein extracts were analyzed by Western blotting with an anti-phospho-Thr18/Ser19-MLC antibody (**upper panel**). The membrane was then stripped and reprobed with an anti-MLC antibody (**lower panel**) as a control for the protein content per lane. D: Adherent NRK cells were serum-starved overnight in DMEM/2% BSA, pre-incubated for 15 min with BDM (20 mM) or ML-7 (25 μM), and finally stimulated by 10% FBS for 30 min. Protein extracts were analyzed by Western blotting for ERK phosphorylation as described in the legend to Figure 1.

acto-myosin inhibitors were analyzed in untransformed parental NRK cells. Adherent serum-starved NRK fibroblasts were pre-incubated with BDM or ML-7, and then stimulated by FBS. We found that acto-myosin contractility was not required for serum-induced ERK phosphorylation in normal adherent cells, since none of the inhibitors tested could impair the timing or the intensity of ERK activation (Fig. 2D and data not shown). Longer pre-incubation periods or increased dosages did not change the outcome (not shown). This result shows that the MLCK-dependent pathway we identified is specific to cells transformed by Ras.

### MLCK Modulates ERK Activity Downstream of Ras

Another effector pathway that becomes activated upon Ras induction is the PI3-kinase/AKT pathway. To test whether all of oncogenic Ras signaling activities were under the control of myosin II and MLCK, we monitored the activity of the PI3-kinase/AKT pathway upon incubation of NRK/ras cells with MLCK inhibitors, by measuring the phosphorylation level of AKT. We found that treatment of NRK/ras cells with ML-7 (Fig. 3A) or BDM (data not shown) did not significantly modify the constitutive level of AKT phosphorylation, suggesting that the inhibitory effects of BDM and ML-7 on ERK activity are not mediated through the modulation of Ras itself.

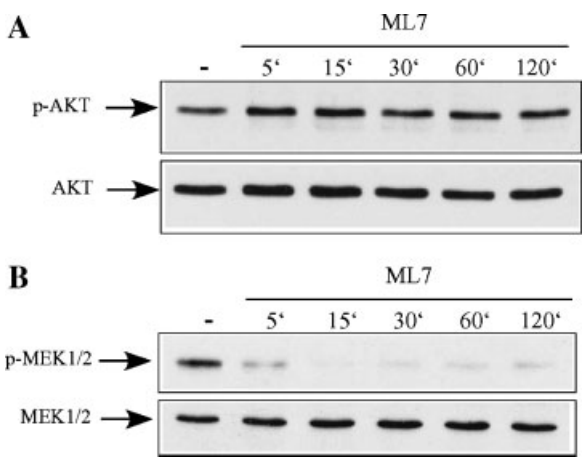


**Fig. 3.** MLCK modulates ERK activity downstream of Ki-ras but does not affect AKT. NRK/ras cells were not treated (−) or treated with 25 μM ML-7 for the time indicated. After treatment, cells were lysed, and protein extracts were analyzed by Western-blotting using an anti-phospho-AKT antibody (**A**), or an anti-phospho-MEK1/2 antibody (**B**). Membranes were then stripped and reprobed with an anti-AKT antibody (A) or an anti-MEK1/2 antibody (B) as a control for the protein content per lane.

ERKs are activated by phosphorylation on threonine and tyrosine residues by MEK1 and MEK2. MEK itself is activated by phosphorylation on specific serine residues and this phosphorylation is generally attributed to Raf kinases. To get more insight into the mechanism by which myosin II and MLCK regulate ERK activation, we analyzed the effect of myosin inhibitors on MEK activation using an anti-phospho-MEK1/2 antibody that recognizes phosphorylated Ser217 and Ser221 residues. The phosphorylation of these residues correlates with its functional activation [Egea et al., 2000]. Ki-ras-induced MEK phosphorylation was inhibited by ML-7 (Fig. 3B) and BDM (data not shown) treatments, following a pattern similar to that found for the inhibition of ERK phosphorylation. This result suggests that the effects of myosin inhibitors on ERK activation were due to an inhibitory effect on MEK activity. More specifically, the lack of MEK phosphorylation in the BDM- and ML-7-treated cultures indicates that myosin modulates MEK activity through the regulation of an upstream MEK kinase. Taken together, these results indicate that MLCK specifically regulates the ERK pathway downstream of Ras and upstream of MEK.

### MLCK Modulates the ERK Cascade at the Level of Raf Activation

Because commercially available phospho-specific Raf antibodies did not work in our hands, constitutively active components of the ERK cascade were used to further assess the myosin-regulated step leading to ERK activation in NRK/ras cells. Stable cell lines were established by retroviral infection, using RasV12S35, a Ras effector loop mutant activating specifically the Ras/ERK pathway [White et al., 1995], or inducible active forms of Raf (ΔRaf1:ER) and MEK (ΔMEK1:ER) [McMahon, 2001]. The ability of the different inhibitors to prevent ERK phosphorylation was tested for each cell line and the results are presented in Figure 4. Similar to NRK/ras cells, NRK/RasV12S35 cells were found sensitive to BDM and ML-7. Both inhibitors were able to inhibit ERK phosphorylation in a time- and dose-dependent manner, whereas treatment with the Rho kinase inhibitor had no effect (Fig. 4A and data not shown). These results further demonstrate that Rho kinase is not involved in oncogenic Ras signaling to ERK, and strengthen

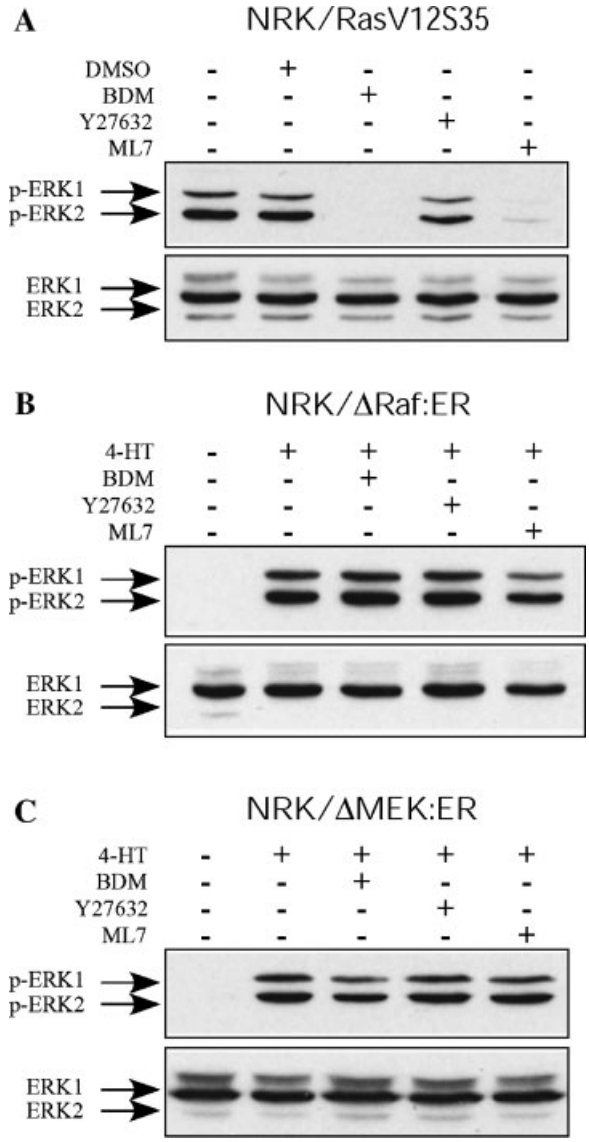


**Fig. 4.** Activation of the ERK cascade by estrogen inducible ΔRaf1:ER and ΔMEK1:ER is not inhibited by MLCK or Rho kinase inhibitors. NRK cells infected with RasV12S35 (**A**), an inducible active mutant of Raf (ΔRaf1:ER) (**B**) or an inducible active mutant of MEK1 (ΔMEK1:ER) (**C**) were treated (+) or not (−) with BDM (20 mM), Y27632 (20 μM), or ML-7 (25 μM) for 30 min. Prior to drug treatment, NRK/ΔRaf1:ER and NRK/ΔMEK1:ER cells were incubated (+) or not (−) with 4-HT (1 μg/ml) for 4 h, to induce ERK activation. After treatment, protein extracts were analyzed by Western blotting for ERK phosphorylation as described in the legend to Figure 1.

the idea that Rho kinase and MLCK play distinct roles despite common substrates such as MLC.

By contrast, ERK was found to be phosphorylated in a myosin-independent manner in NRK/ΔRaf1:ER cells as well as NRK/ΔMEK1:ER cells (Fig. 4B,C). The same results were obtained when increasing the dosages up to 100 mM (BDM) or 100 μM (ML-7 and Y27632), or when prolonging the incubation time period up to 2 h (data not shown). The ability of active Raf (i.e., ΔRaf1:ER), but not active Ras (i.e., RasV12S35), to induce ERK phosphorylation in a myosin-independent manner suggest that the target of MLCK inhibitors lies upstream or at the level of Raf activation.

### Inhibition of MLCK Prevents Activation of the ERK Cascade Independently of the Cell Adhesive State

Normal cells deprived of attachment to the extracellular matrix are no longer able to signal to the ERK cascade [Renshaw et al., 1997]. To test whether MLCK could impact on ERK signaling by regulating signals generated by adhesion, NRK/ras cells were kept in suspension for several hours then treated by the different compounds. Consistent with their anchorage-independent phenotype, keeping NRK/ras cells in suspension did not per se decrease the efficiency of Ras signaling to ERK (Fig. 5A). Under these conditions, inhibition of myosin by either BDM or ML-7 dramatically decreased the level of ERK phosphorylation (Fig. 5A), suggesting that the effects of these compounds were not mediated through changes in integrin engagement. To further confirm this result, the different compounds were tested on NRK/ras cells grown on plates coated with either fibronectin, to induce integrin engagement, or poly-L-lysine, a non-specific adhesive polymer. None of these conditions impaired the ability of BDM or ML-7 to prevent ERK phosphorylation, while Y27632 was still unable to do so (Fig. 5B). These results show that the ability of myosin inhibitors to modulate the ERK cascade is independent of the adhesion state of the cells and integrin engagement.

### Restoration of Microfilament Organization Decreases the Efficiency of Oncogenic Ras Signaling to ERK

Collectively, our results suggest that the cytoskeletal rearrangements induced by oncogenic Ras in NRK fibroblasts somehow contributes to activation of the ERK signaling pathway. Consequently, we reasoned that restoration of an organized cytoskeleton in NRK/ras cells would impact on the level of activation of the ERK pathway. Tropomyosins are

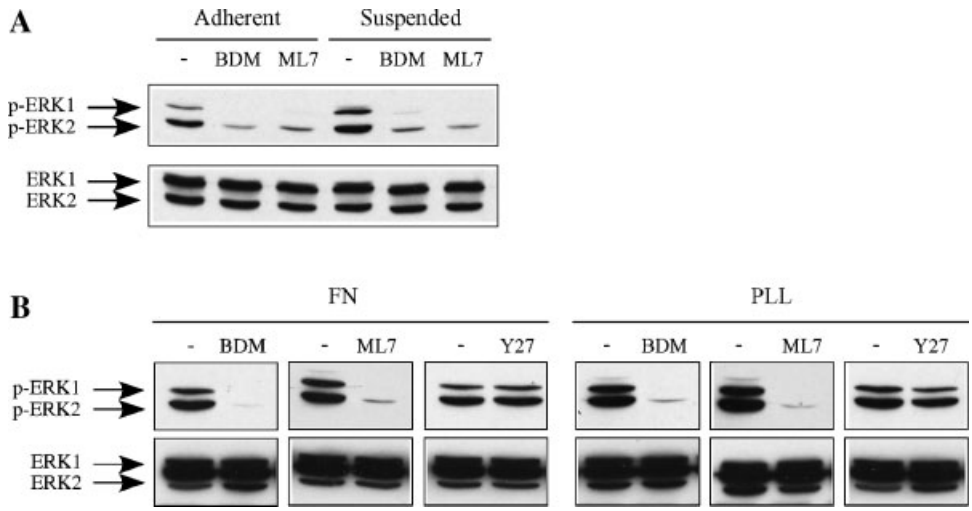


**Fig. 5.** MLCK inhibitors prevent activation of the ERK cascade independently of cell adhesion. **A**: NRK/ras cells grown as adherent cells or kept in suspension for 2 h were treated or not with BDM (25 mM) or ML-7 (25 μM) for 10 min. After treatment, cells were lysed, and ERK phosphorylation was analyzed as described in the legend to Figure 1. **B**: NRK/ras cells grown on fibronectin-coated plates (FN) or poly-L lysine-coated plates (PLL) for 24 h were treated or not with BDM (25 mM), ML-7 (25 μM), or Y27632 (20 μM), for 10 min. After treatment, cells were lysed, and ERK phosphorylation was analyzed as described in the legend to Figure 1.

actin-associated proteins involved in stabilization of actin filaments, and are often down-regulated in oncogenically transformed cells and patient tumor samples [Pawlak et al., 2004, and references therein]. We have previously shown that forced expression of Tropomyosin-2 (TM-2) in NRK/ras cells restores microfilament organization concomitantly with the growth properties of these cells [Gimona et al., 1996]. Therefore, we asked whether the TM-2-induced restoration in cytoskeleton architecture was associated with changes in ERK signaling. Cells expressing low levels of TM-2 (NRK/ras/TM-2*l*) were indistinguishable from parental NRK/ras cells and exhibited a disrupted actin cytoskeleton, associated with high levels of ERK1 and ERK2 phosphorylation (Fig. 6). By contrast, cells expressing higher levels of TM-2 (NRK/ras/TM-2*h*), i.e., the level observed in untransformed NRK cells, showed restored actin filament assembly correlated with a significant decrease in ERK phosphorylation (Fig. 6). These results demonstrate that stable expression of TM-2 in NRK/ras cells leads to decreased activation of the ERK pathway, and further suggest that organization of the actin cytoskeleton strongly impacts on ERK signaling.

## DISCUSSION

Constitutive activation of the ERK cascade is the hallmark of a variety of tumor cell types, and has been implicated in tumor cell migration, regulation of apoptosis, and angiogenesis, events that are essential for the successful completion of metastasis [Hoshino et al., 1999; Reddy et al., 2003]. Alterations in the actin-based cytoskeleton is another common feature of transformed cells [Pawlak and Helfman, 2001; Rao, 2002], which prompted us to address whether the actin cytoskeleton plays a role in oncogene-mediated signaling events. Here, we demonstrate that activation of the ERK cascade by oncogenic Ras is dependent on actomyosin contractility, under the regulation of MLCK but not Rho kinase. Since MLCK is a target of ERK [Klemke et al., 1997], these results suggest a novel signaling pathway involving Ras-mediated alterations of the actin cytoskeleton, which might play a critical role in ERK activation by the Ras oncogene and contribute to aberrant signaling and enhanced cell motility.

Our data indicate an essential role for myosin II and MLCK in activation of the Ras/ERK cascade in NRK (normal rat kidney) cells transformed with Ki-Ras or RasV12S35. ERK activation in NRK cells transformed with Ki-Ras was inhibited following addition of BDM, blebbistatin ML-7, and ML-9, but not the Rho kinase inhibitor Y27632. In addition, activation of the Ras/ERK pathway in cells expressing either estrogen inducible ΔRaf-1ER or ΔMEK1-ER was not inhibited by agents that inhibit

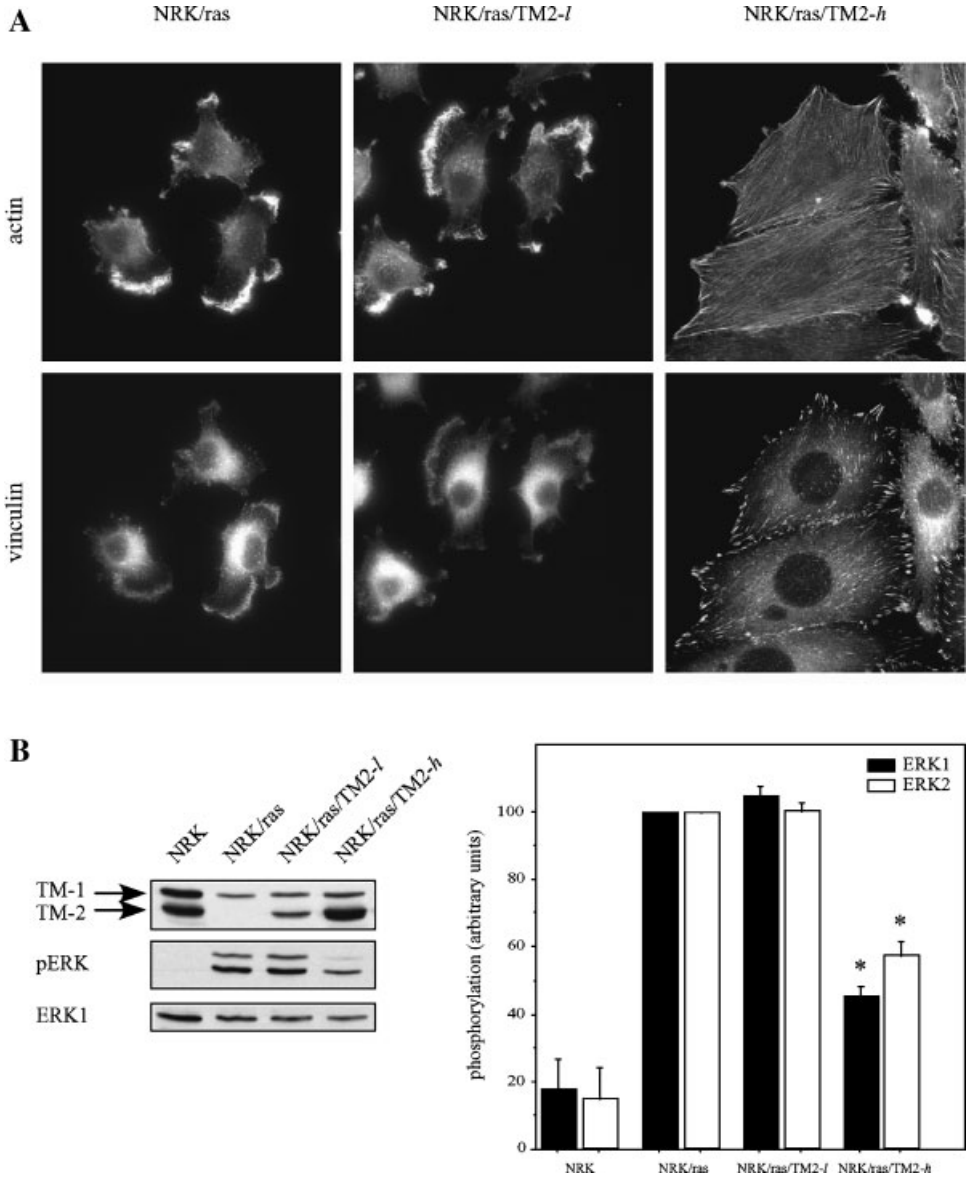


**Fig. 6.** Restoration of microfilament assembly by TM-2 is associated with decreases in the efficiency of oncogenic signaling to ERK. **A**: NRK/ras, NRK/ras/TM2-*l*, and NRK/ras/TM2-*h* cells grown on glass coverslips were stained with oregon green-conjugated phalloidin and anti-vinculin mAb, followed by Cy3-conjugated anti-mouse antibody. Note the reappearance of actin bundles and vinculin-containing focal adhesions in NRK/ras/TM2-*h* cells. **B**: Exponentially growing cells were harvested and protein extracts analyzed by Western blotting for expression of Tropomyosins (TM-1 and TM-2 isoforms) and phospho-ERK, as described in the legend to Figure 1. Graph on the right shows the average ERK phosphorylation from three independent experiments. *, *P* value of $<0.001$, as determined by Student's *t*-test.

myosin, MLCK or Rho kinase. Thus, our data demonstrates that actomyosin contractility is required for activation of the Ras/ERK pathway in cells expressing Ki-Ras or RasV12S35, but not following activation of ΔRaf-1ER and ΔMEK1-ER. Furthermore, the ability of active ΔRaf-1ER and ΔMEK1-ER, but not active Ras (Ki-Ras or RasV12S35), to induce ERK phosphorylation in a myosin-independent manner suggest that the target of myosin and MLCK inhibitors lies upstream or at the level of Raf activation. Thus, in NRK/ras cells, it is likely that the point of convergence between Ras signaling and myosin II occurs at or above Raf activation, because ERK signaling is myosin-independent in NRK/ΔRaf1:ER cells.

It is of note that the activation of the ERK cascade by oncogenic Ras was found to depend

on actomyosin contractility, under the regulation of MLCK but not Rho kinase. Rho kinase, like MLCK, is a major serine/threonine kinase that phosphorylates MLC, leading to activation of myosin ATPase activity [Amano et al., 1996; Leung et al., 1996]. Previous studies performed on non-transformed cells indicated Rho kinase is necessary for sustained ERK activation and inhibition of Rho kinase suppresses ERK activation [Yanazume et al., 2002; Roovers and Assoian, 2003], thus suggesting a function for Rho kinase in ERK activation. Using Y27632, we demonstrated a negligible effect of Rho kinase inhibition on ERK phosphorylation suggesting that contrary to what was observed in normal cells, Rho kinase plays no major role in the signaling events downstream of oncogenic Ras. This result could also be explained by our previous finding that NRK/ras cells have impaired expression of both Rho kinase I and Rho kinase II [Pawlak and Helfman, 2002]. However, we also showed that NRK/RasV12S35 cells have an intact level of expression of both kinases [Pawlak and Helfman, 2002], yet the Y27632 compound still does not have any effect on the level of ERK phosphorylation (Fig. 4). Several studies have demonstrated a spatial regulation of MLCK and Rho kinase activity. During the migration of T cells, initiated by LFA-1 binding to ICAM-1, MLCK operates at the leading edge while Rho kinase is found in highest concentration in the body and trailing edge of the migrating cell. The distribution of these two kinases suggests that there are two compartments with distinct functions operating in the migrating T cell [Smith et al., 2004]. Separation of these two kinases has also been observed in murine 3T3 cells [Totsukawa et al., 2000] and human fibroblasts [Katoh et al., 2001]. In these studies, Rho kinase activity was associated with stress fibers in the center of cells, whereas MLCK was associated with peripheral cortical microfilaments, which corresponds to the predominant actin structures in NRK/ras cells (see Fig. 6).

Here, we demonstrate that activation of the ERK cascade by oncogenic Ras is dependent on actomyosin contractility, under the regulation of MLCK but not Rho-kinase. The only recognized substrate of MLCK is MLC, which is phosphorylated on Thr18 and Ser19, thereby activating myosin ATPase activity [reviewed in Bresnick, 1999]. The inhibitor ML-7 used in this study targets the kinase domain and blocks the catalytic activity of MLCK [Saitoh et al., 1987; Tanaka et al., 1995]. The fact that this agent prevents ERK phosphorylation in response to oncogenic Ras signaling provides evidence that it is the kinase activity of MLCK that is paramount. Since MLCK is a target of ERK [Klemke et al., 1997], these results suggest a novel signaling pathway involving Ras-mediated alterations of the actin cytoskeleton, which might play a critical role in ERK activation by the Ras oncogene. Two mechanisms have been implicated in Ras activation of ERK. One involves activation of G-protein coupled receptors via the Shc/Grb2/SOS pathway. The other involves a β-arrestin-dependent scaffold [Pierce et al., 2001]. Interestingly, MLCK is implicated in pseudopod formation, and a β-arrestin scaffold is associated with prolonged ERK activation during pseudopodia formation [Brahmbhatt and Klemke, 2003; Ge et al., 2003]. The observation that inhibition of actomyosin contractility and myosin light chain kinase blocks ERK phosphorylation suggests that constitutive activation of the ERK pathway is through a β-arrestin scaffold. Our results further suggest that the cytoskeletal rearrangements induced by oncogenic Ras in NRK fibroblasts are functionally required for activation of ERK signaling. Interestingly we found that stable expression of TM-2 in NRK/ras cells leads to decreased activation of the ERK pathway. The expression of HMW TMs is associated with a more well-spread morphology, i.e., less lamellipodia, and might compromise ERK activation by limiting pseudopod growth. A β-arrestin scaffold is associated with prolonged ERK activation in pseudopodia [Ge et al., 2003]. Thus it is possible that myosin and MLCK function in a β-arrestin-dependent scaffold required for ERK activation. Experiments are in progress to examine whether the actomyosin-dependent activation of ERK in Ras-transformed cells is dependent on a β-arrestin scaffold.

In conclusion, we have shown that inhibition of actomyosin contractility inhibits ERK activation in Ras-transformed NRK cells. Inhibition of the ERK pathway following modulation of myosin II activity is likely meditated through alterations in Raf functions. These studies also demonstrate that MLCK but not Rho kinase plays an essential role in the generation of ERK signaling and indicate distinct cellular roles for Rho kinase and MLCK-dependent functions involving the regulation of myosin II.

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## REFERENCES

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 15: 6541–6551.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase. J Biol Chem 271:20246–20249.

Aplin AE, Juliano RL. 2001a. Regulation of nucleocytoplasmic trafficking by cell adhesion receptors and the cytoskeleton. J Cell Biol 155:187–191.

Aplin AE, Stewart SA, Assoian RK, Juliano RL. 2001b. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J Cell Biol 153:273–282.

Assoian RK, Schwartz MA. 2001. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. Curr Opin Genet Dev 11:48–53.

Bottazzi ME, Buzzai M, Zhu X, Desdouets C, Brechot C, Assoian RK. 2001. Distinct effects of mitogens and the actin cytoskeleton on CREB and pocket protein phosphorylation control the extent and timing of cyclin A promoter activity. Mol Cell Biol 22:7607–7616.

Brahmbhatt AA, Klemke RL. 2003. ERK and RhoA differentially regulate pseudopodia growth and retraction during chemotaxis. J Biol Chem 278:13016–13025.

Bresnick AR. 1999. Molecular mechanisms of nonmuscle myosin-II regulation. Curr Opin Cell Biol 11:26–33.

Burridge K, Chrzanowska-Wodnicka M, Zhong C. 1997. Focal adhesion assembly. Trends Cell Biol 7:342–347.

Chrzanowska-Wodnicka M, Burridge K. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 133:1403–1415.

Duran JM, Valderrama F, Castel S, Magdalena J, Tomas M, Hosoya H, Renau-Piqueras J, Malhotra V, Egea G. 2003. Myosin motors and not actin comets are mediators of the actin-based Golgi-to-endoplasmic reticulum protein transport. Mol Biol Cell 14:445–459.

Egea J, Espinet C, Soler RM, Peiro S, Rocamora N, Comella JX. 2000. Nerve growth factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca(2+) and calmodulin. Mol Cell Biol 20:1931–1946.

Fukata Y, Amano M, Kaibuchi K. 2001. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends Pharmacol Sci 22:32–39.

Ge L, Ly Y, Hollenberg M, DeFea K. 2003. A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. J Biol Chem 278:34418–34426.

Gimona M, Kazzaz J, Helfman DM. 1996. Forced expression of tropomyosin 2 or 3 in v-Ki-ras-transformed fibroblasts results in distinct phenotypic effects. Proc Natl Acad Sci USA 93:9618–9623.

Helfman DM, Levy ET, Berthier C, Shtutman M, Riveline D, Grosheva I, Lachish-Zalait A, Elbaum M, Bershadsky AD. 1999. Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. Mol Biol Cell 10:3097–3112.

Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M. 1999. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 18:813–822.

Howe AK, Aplin AE, Juliano RL. 2002. Anchorage-dependent ERK signaling—Mechanisms and consequences. Curr Opin Genet Dev 12:30–35.

Katoh K, Kano Y, Amano M, Kaibuchi K, Fujiwara K. 2001. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. Am J Physiol Cell Physiol 280:1669–1679.

Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, Matsumura F, Inagaki M, Kaibuchi K. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. J Cell Biol 147:1023–1038.

Klemke R, Cai S, Giannini AL, Gallagher PJ, deLanerolle P, Cheresh DA. 1997. Regulation of cell motility by mitogen-activated protein kinase. J Cell Biol 137:481–492.

Leung T, Chen X, Manser E, Lim L. 1996. The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 16:5313–5327.

Limouze J, Straight AF, Mitchison T, Sellers JR. 2004. Specificity of blebbistatin, an inhibitor of myosin II. J Muscle Res Cell Motil 25:337–341.

McMahon M. 2001. Steroid receptor fusion proteins for conditional activation of Raf-MEK-ERK signaling pathway. Methods Enzymol 332:401–417.

Ostap EM. 2002. 2,3-Butanedione monoxime (BDM) as a myosin inhibitor. J Muscle Res Cell Motil 23:305–308.

Pawlak G, Helfman DM. 2001. Cytoskeletal changes in cell transformation and tumorigenesis. Curr Opin Genet Dev 11:41–47.

Pawlak G, Helfman DM. 2002. Post-transcriptional down-regulation of ROCKI/Rho-kinase through a MEK-dependent pathway leads to cytoskeleton disruption in ras-transformed fibroblasts. Mol Biol Cell 13:336–347.

Pawlak G, McGarvey TW, Nguyen TB, Tomaszewski JE, Puthiyaveettil R, Malkowicz SB, Helfman DM. 2004. Alterations in tropomyosin isoform expression in human transitional cell carcinoma of the urinary bladder. Int J Cancer 110:368–373.

Pierce KL, Luttrell LM, Lefkowitz RJ. 2001. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 20:1532–1539.

Rao J. 2002. Targeting actin remodeling profiles for the detection and management of urothelial cancers—A perspective for bladder cancer research. Front Biosci 7:e1–e8.

Reddy KB, Nabha SM, Atanaskova N. 2003. Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev 22:395–403.

Renshaw MW, Ren XD, Schwartz MA. 1997. Growth factor activation of MAP kinase requires cell adhesion. EMBO J 16:5592–5599.

Reuveni H, Geiger T, Geiger B, Levitzki A. 2000. Reversal of the ras-induced transformed phenotype by HR12, a novel ras farnesylation inhibitor, is mediated by the Mek/Erk pathway. J Cell Biol 151:1179–1192.

Roovers K, Assoian RK. 2003. Effects of rho kinase and actin stress fibers on sustained extracellular signal-regulated kinase activity and activation of G(1) phase cyclin-dependent kinases. Mol Cell Biol 23:4283–4294.

Roovers K, Davey G, Zhu X, Bottazzi ME, Assoian RK. 1999. Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. Mol Biol Cell 10:3197–3204.

Sahai E, Olson MF, Marshall CJ. 2001. Cross-talk between ras and rho signalling pathways in transformation favours proliferation and increased motility. EMBO J 20:755–766.

Saitoh M, Ishikawa T, Matsushima S, Naka M, Hidaka H. 1987. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. J Biol Chem 262: 7796–7801.

Sellers JR. 1991. Regulation of cytoplasmic and smooth muscle myosin. Curr Opin Cell Biol 3:98–104.

Smith ER, Smedberg JL, Rula ME, Xu XX. 2004. Regulation of Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endoderm differentiation of embryonic carcinoma and stem cells. J Cell Biol 164:689–699.

Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, Sellers JR, Mitchison TJ. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science 299:1743–1747.

Tan JL, Ravid S, Spudich JA. 1992. Control of nonmuscle myosins by phosphorylation. Annu Rev Biochem 81:721–759.

Tanaka M, Ikebe R, Matsuura M, Ikebe M. 1995. Pseudo-substrate sequence may not be critical for autoinhibition of smooth muscle myosin light chain kinase. EMBO J 14:2839–2846.

Titus MA. 2003. Caveat experimentor—Is your myosin really inhibited? Nat Cell Biol 5:95.

Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F. 2000. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J Cell Biol 150:797–806.

Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 389:990–994.

White MA, Nicolette C, Minden A, Polverino A, Van Aelst L, Karin M, Wigler MH. 1995. Multiple Ras functions can contribute to mammalian cell transformation. Cell 80: 533–541.

Xu H, Goldfarb M. 2001. Multiple effector domains within SNT1 coordinate ERK activation and neuronal differentiation of PC12 cells. J Biol Chem 276:13049–13056.

Yan M, Templeton DJ. 1994. Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. J Biol Chem 269:19067–19073.

Yanazume T, Hasegawa K, Wada H, Morimoto T, Abe M, Kawamura T, Sasayama S. 2002. Rho/ROCK pathway contributes to the activation of extracellular signal-regulated kinase/GATA-4 during myocardial cell hypertrophy. J Biol Chem 277:8618–8625.

Yarrow JC, Lechler T, Li R, Mitchison TJ. 2003. Rapid de-localization of actin leading edge components with BDM treatment. BMC Cell Biol 4:5–14.