

An essential role for p120-catenin in Src- and Rac1-mediated anchorage-independent cell growth

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p120-catenin regulates epithelial cadherin stability and has been suggested to function as a tumor suppressor. In this study, we used anchorage-independent growth (AIG), a classical *in vitro* tumorigenicity assay, to examine the role of p120 in a different context, namely oncogene-mediated tumorigenesis. Surprisingly, p120 ablation by short hairpin RNA completely blocked AIG induced by both Rac1 and Src. This role for p120 was traced to its activity in suppression of the RhoA–ROCK pathway, which appears to be essential for AIG. Remarkably, the AIG block associated

with p120 ablation was completely reversed by inhibition of the downstream RhoA effector ROCK. Harvey-Ras (H-Ras)-induced AIG was also dependent on suppression of the ROCK cascade but was p120 independent because its action on the pathway occurred downstream of p120. The data suggest that p120 modulates oncogenic signaling pathways important for AIG. Although H-Ras bypasses p120, a unifying theme for all three oncogenes is the requirement to suppress ROCK, which may act as a gatekeeper for the transition to anchorage independence.

Introduction

Normal epithelial architecture and function are maintained by integrins and cadherins, transmembrane receptors that mediate cell–ECM and cell–cell interactions, respectively. Importantly, signaling centers organized by these receptors modulate anchorage dependence and contact-dependent inhibition of cell growth, which are essential cellular functions often disrupted in cancer. Under normal conditions, cell proliferation is dependent on both growth factors (serum dependence) and adhesion to a solid substrate (anchorage dependence; Schwartz, 1997). When deprived of anchorage, normal epithelial cells undergo detachment-induced apoptosis, or anoikis, irrespective of the presence of growth factors (Gilmore, 2005). Anchorage dependence is thought to be mediated primarily by integrins (Ruoslahti and Reed, 1994; Meredith and Schwartz, 1997), and most oncogenes, including activated variants of Src, Rac1, and Harvey-Ras (H-Ras), are defined in part by their ability to permit or induce anchorage-independent growth (AIG; Wang, 2004). The transition to AIG, as measured *in vitro* by growth in inert 3D matrices (e.g., soft agar), is tightly correlated with the ability to form tumors *in vivo* (i.e., tumorigenicity; Freedman and Shin,

1974; Colburn et al., 1978) and is widely recognized as an *in vitro* hallmark of oncogenic transformation.

Cadherins constitute a family of cell–cell adhesion proteins important in development, morphogenesis, and cancer (Takeichi, 1995; Semb and Christofori, 1998; Yap, 1998; Vleminckx and Kemler, 1999; Tepass et al., 2000). Cadherins are thought to mediate contact inhibition of cell growth (Perrais et al., 2007) but are not obviously linked to anchorage dependence. Epithelial cadherin (E-cadherin) is the main cell–cell adhesion molecule in epithelial tissues and is regarded as a master organizer of the epithelial phenotype (Takeichi, 1995). Mutations or deletions in the gene itself are common in lobular breast carcinoma (Berx et al., 1995) and familial-linked gastric tumors (Oda et al., 1994), indicating a classical tumor suppressor role in some tissues. In most cancers, however, E-cadherin behaves more like a metastasis suppressor, and compromised cadherin expression or function in late-stage cancers is widely believed to mark the transition to metastasis. E-cadherin is regulated in part by cytoplasmic binding partners called catenins (α -catenin, β -catenin, and p120-catenin). α - and β -catenins are primarily associated with physical and/or functional linkage to the actin

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Abbreviations used in this paper: AIG, anchorage-independent growth; DA, dominant active; E-cadherin, epithelial cadherin; H-Ras, Harvey-Ras; HGF, hepatocyte growth factor; LIMK, LIM kinase; N-cadherin, neuronal cadherin; shRNA, short hairpin RNA.

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infrastructure (Gumbiner and McCrea, 1993; Kobiela and Fuchs, 2004; Yamada et al., 2005), whereas p120-catenin (hereafter p120) appears to modulate cadherin stability at the cell surface (Ireton et al., 2002; Davis et al., 2003; Xiao et al., 2003). The E-cadherin stabilizing effect of p120 is most consistent with an accessory role to E-cadherin as a tumor and/or metastasis suppressor.

p120 was originally identified as a prominent substrate for oncogenic Src and various receptor tyrosine kinases (Reynolds et al., 1989; Downing and Reynolds, 1991; Kanner et al., 1991). Although a theoretically attractive target for oncogenes, a direct role for p120 in the tumor-promoting activities of these proteins has yet to be demonstrated. In the absence of p120, E-cadherin (along with α - and β -catenins) is rapidly internalized and degraded, usually resulting in defective cell–cell adhesion (Davis et al., 2003; Xiao et al., 2003). In vitro studies show that cadherin stabilization by p120 is dependent on direct p120 binding to the cadherin cytoplasmic tail (Ireton et al., 2002) and may be regulated in part by physical or functional connections with ubiquitin ligases (Fujita et al., 2002) or elements of the endocytic machinery (Xiao et al., 2007).

p120 likely participates in the regulation of actin rearrangements that contribute to dynamic cadherin function via mechanisms involving Rho GTPases (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). p120 has been shown to inhibit RhoA directly via a guanine nucleotide dissociation inhibitor–like mechanism (Anastasiadis et al., 2000; Noren et al., 2000) and indirectly via recruitment of p190RhoGAP to cadherin complexes (Wildenberg et al., 2006). Though not mutually exclusive, the former mechanism is thought to involve cytoplasmic association of p120 and GDP-bound RhoA, whereas the latter suggests a role for p120 as a scaffold that coordinates the actions of other RhoA-modulating factors. Regardless of the exact mechanism, p120 appears to be essential in some cell types for inhibition of RhoA (Anastasiadis et al., 2000) and the functional coupling of Rac1 to the inhibition of RhoA (Wildenberg et al., 2006).

Although p120 is physically or functionally associated with a variety of oncogenes and tumor suppressors (e.g., Src, E-cadherin, and Rho GTPases), its role in cancer is not yet understood. Targeted p120 knockout in the salivary gland results in reduced E-cadherin levels and morphological changes resembling high-grade intraepithelial neoplasia, suggesting a tumor suppressor function (Davis and Reynolds, 2006). However, identification of p120 some time ago as a potentially transformation-specific Src substrate implies a central role in the mechanism of transformation by oncogenic Src (Reynolds et al., 1989). To date, however, there has been little evidence in support of the latter, and it remains unclear whether p120 has any role at all in this process.

In this study, we have identified an essential role for p120 in Rac1- and Src-induced, but not H-Ras–induced, AIG. Interestingly, all three oncogenes suppressed the RhoA–ROCK–LIM kinase (LIMK) pathway at one level or another, and this was required for AIG. Rac1 and Src appeared to act at the level of RhoA and, surprisingly, were fully dependent on p120 for this activity. However, H-Ras acted in a manner independent of

p120 and suppressed the pathway downstream of RhoA at the level of ROCK (or possibly LIMK). Thus, the ROCK pathway may be a critical target for all three of these otherwise dissimilar oncogenes, and suppression of the RhoA–ROCK–LIMK cascade is apparently an essential event in the oncogenic changes that lead to AIG. Oncogenic perturbation (suppression) of the ROCK pathway via p120-dependent or -independent mechanisms ultimately resulted in constitutive activation of the actin-severing protein cofilin, whose activity was necessary but not sufficient to permit the transition to anchorage independence and, presumably, tumorigenicity.

Results

Selective down-modulation of adherens junctions in p120-deficient MDCK cells

To study the effects of p120 ablation in cells, we generated p120 knockdown cell lines by clonal selection of MDCK cells expressing canine p120-specific short hairpin RNA (shRNA; hereafter MDCK-p120i). Multiple cell lines were characterized to control for clonal variation, and two representative lines were chosen for further experiments. Western blot analyses of MDCK-p120i-1 and MDCK-p120i-2 cells revealed a dramatic reduction of p120 protein levels compared with parental MDCK cells (Fig. 1 A). As expected, p120 loss led to a decrease in E-cadherin, neuronal cadherin (N-cadherin), β -catenin, and α -catenin protein levels (Fig. 1 A) as well as a decrease in E-cadherin junctional staining (Fig. 1 B), as has been described previously in other cell lines (Davis et al., 2003). However, although loss of p120 frequently disrupts intercellular adhesion (Ireton et al., 2002; Davis et al., 2003), p120-deficient MDCK cells formed compact colonies with normal appearing cell–cell junctions (Fig. 1 B). Interestingly, levels and localization of desmosomal (desmoglein) and tight junction (occludin and ZO-1) markers appeared to be unaffected by p120 loss (Fig. 1, A and B), which may account for the retention of cell–cell adhesion in the near absence of adherens junctions. Thus, p120 ablation in MDCK cells induces a selective loss of adherens junctions and, surprisingly, very little effect on gross colony morphology.

p120 is required for Rac1- and Src-induced AIG

To examine the effect of p120 loss in the context of several transforming oncogenes, we stably expressed dominant-active (DA) forms of Rac1(G12V), Src(Y527F), and H-Ras(G12V) in wild-type and p120-deficient MDCK cells (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200807096/DC1>). Interestingly, in standard 2D cell cultures (i.e., on plastic or glass), the morphologies induced by the three oncogenes differed markedly from one another, but p120 ablation had little or no additional effect on these phenotypes (Fig. 2, A–C, compare top panels with bottom panels). DA-Rac1 increased cortical actin staining and appeared to enhance epithelial morphology (Fig. 2 A). In contrast, DA-Src induced morphological changes and colony dispersal resembling classical epithelial to mesenchymal transition (Fig. 2 B), whereas DA-H-Ras led to decreased

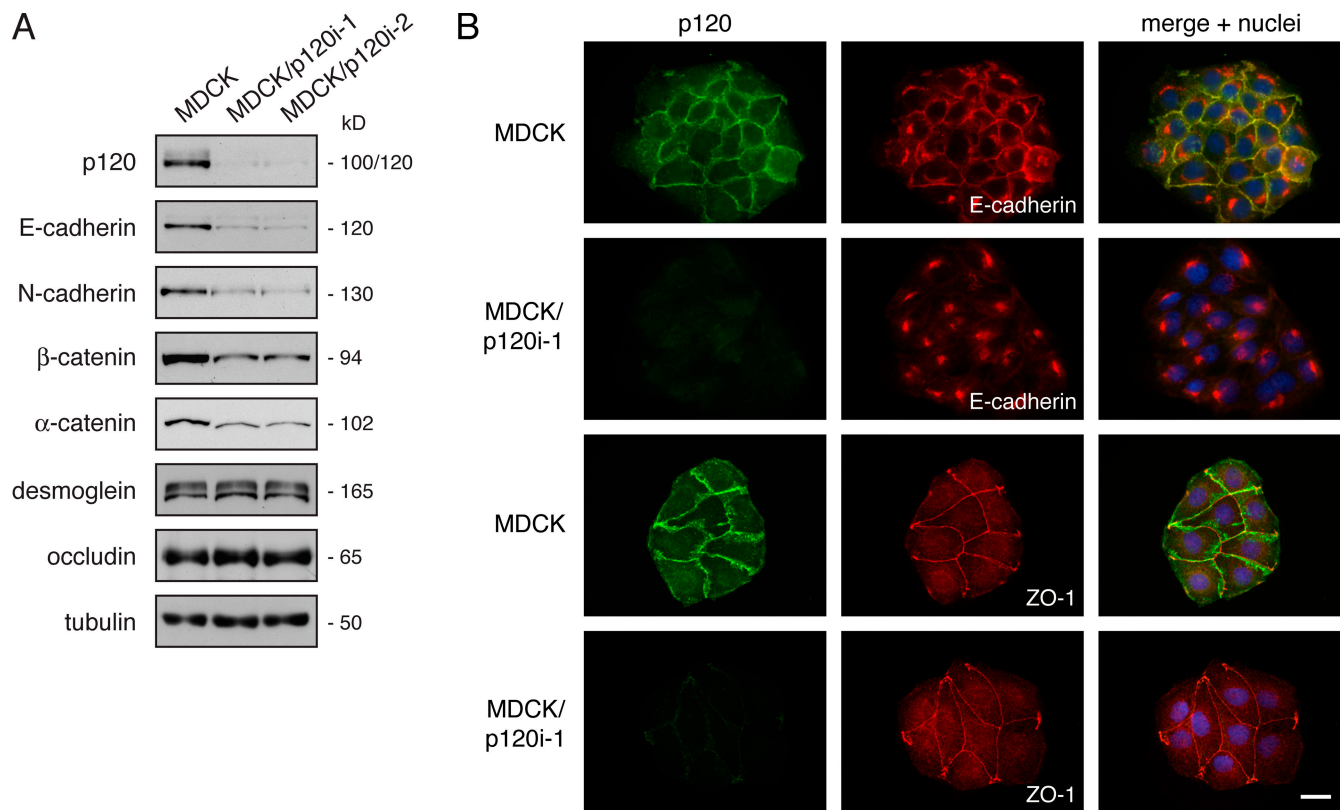


Figure 1. **shRNA knockdown of p120 in MDCK cells.** (A) Lysates from parental MDCK cells and two representative p120 knockdown monoclonal cell lines, MDCK-p120i-1 and MDCK-p120i-2 cells, were analyzed by Western blotting for levels of p120, E-cadherin, N-cadherin, β-catenin, α-catenin, desmoglein, and occludin. Tubulin levels were determined as a loading control. (B) MDCK and MDCK-p120i-1 cells plated on glass coverslips were stained for p120, E-cadherin, or ZO-1 as described in Materials and methods. Nuclei were visualized with Hoechst stain. Bar, 10 μm.

filamentous actin staining and a flattened morphology (Fig. 2 C). Thus, only DA-Src induced morphological changes that are typically associated with cell transformation in 2D cultures, and the effects of DA-Rac1 were distinctly different and essentially opposite from those normally associated with oncogenic transformation. These observations highlight the fact that despite an underlying commonality based on linkage to tumor formation, the morphological changes associated with the oncogene-driven phenomenon referred to as cell transformation can vary substantially from one oncogene to another and are often difficult to interpret.

Activated forms of Rac1 (Coniglio et al., 2001), Src (Wei et al., 2004), and H-Ras (Malaney and Daly, 2001) are known to promote AIG, a hallmark of transformation that tightly correlates with tumorigenicity in vivo (Freedman and Shin, 1974; Colburn et al., 1978). To examine the effect of p120 loss on AIG, the aforementioned DA-Rac1, DA-Src, and DA-H-Ras cell lines (with and without p120) were assayed for their ability to grow in soft agar. Soft agar consists of denatured collagen, which forms a 3D environment for cell growth but lacks the signaling activity of native collagens and therefore does not support proliferation of nontransformed cells. Wild-type and p120-ablated MDCK cells are nontumorigenic and were unable to proliferate in soft agar (Fig. 3 A, i, v, and ix), whereas expression of DA-Rac1, DA-Src, or DA-H-Ras in wild-type

MDCK cells induced rapid AIG and formation of large colonies (Fig. 3 A, ii, iii, and iv, respectively). Surprisingly, p120 ablation completely blocked DA-Rac1- and DA-Src-induced AIG (Fig. 3 A, vi, vii, x, and xi). In contrast, DA-H-Ras-induced AIG was unaffected by p120 loss (Fig. 3 A, viii and xii), indicating that the requirement for p120 applies to Rac1 and Src but not H-Ras (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200807096/DC1>).

To verify that the aforementioned effects were unique to cell growth in suspension (i.e., soft agar), we quantified proliferation rates of the wild-type and transformed cell lines grown on plastic under normal 2D culture conditions (Fig. 3 B). Proliferation was indeed moderately reduced (i.e., by 20–30%) after removal of p120, but the effect was similar in all of the cell lines. In contrast, the soft agar phenotypes were generally all or none, irrespective of their behavior in 2D cultures. For example, soft agar colonies from H-Ras-transformed cells were indistinguishable from those generated from the same cells lacking p120 (Fig. 3 A, compare iv with viii), whereas colony growth was virtually eliminated by p120 ablation in Rac1- or Src-transformed cells (Fig. 3 A, compare ii with vi and iii with vii). Thus, the effects of p120 ablation on cell growth are manifested selectively under conditions of AIG. Together, these observations suggest a role for p120 in signaling pathways relevant to tumorigenesis.

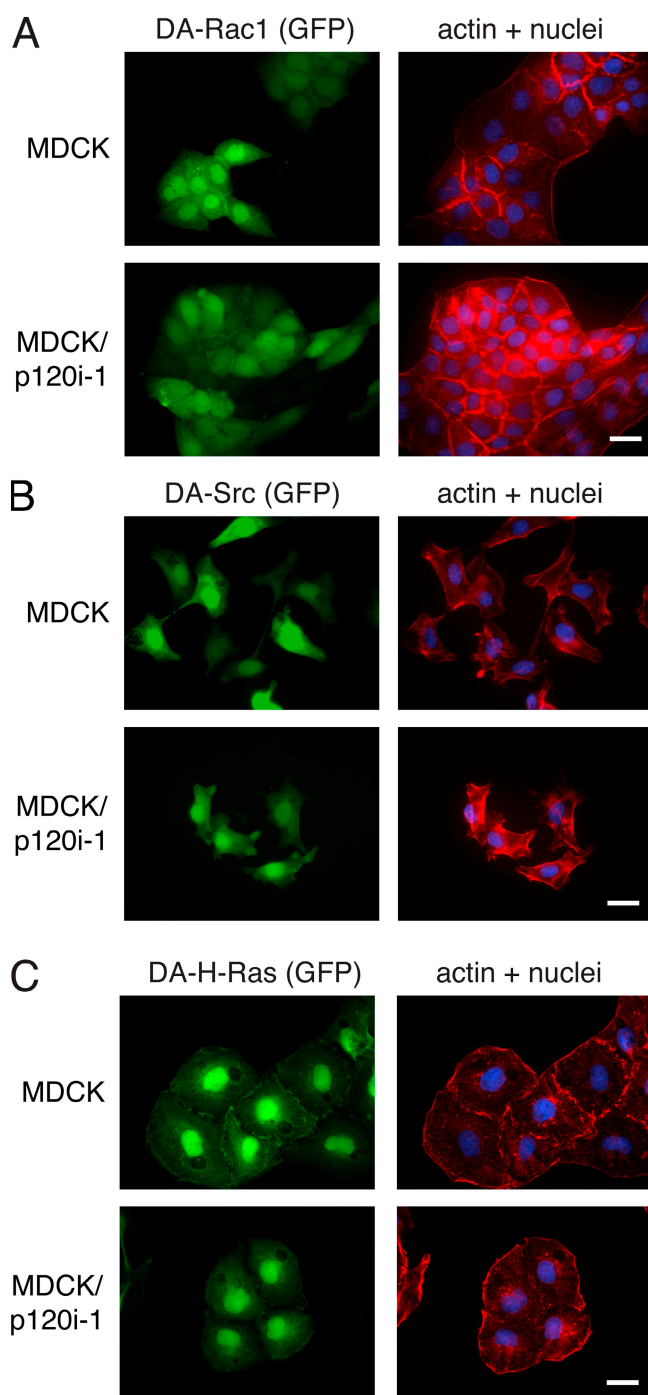


Figure 2. **p120 loss has no effect on Rac1-, Src-, or H-Ras-induced morphology in two dimensions.** (A–C) MDCK and MDCK-p120i-1 cells infected with DA-Rac1 (A), DA-Src (B), or DA-H-Ras (C) were plated on glass coverslips. Infected cells were visualized with GFP. Actin and nuclei were visualized with phalloidin and Hoechst staining, respectively. Bars, 10 μ m.

Rac1- and Src-induced AIG is dependent on p120's ability to suppress RhoA-ROCK

As p120 is known to negatively regulate RhoA, it is possible that elevated RhoA activity in p120-deficient cells might be relevant to the effects of p120 ablation on AIG. To test for RhoA activation, p120-deficient MDCK cells were stained for markers of RhoA activity (i.e., actin stress fibers and vinculin) and

examined biochemically for interaction with rhotekin. In serum-starved cells, we observed an approximately twofold increase in staining of actin stress fibers and vinculin in the absence of p120 (Fig. 4 A and Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200807096/DC1>), and rhotekin binding assays revealed an approximately twofold increase in RhoA activity, similar to that induced by hepatocyte growth factor (HGF; Fig. 4 B). Thus, p120 loss in MDCK cells increases RhoA activity and leads to RhoA-associated phenotypes.

To determine whether elevated RhoA signaling in p120-ablated MDCK cells is responsible for the block in DA-Rac1- and/or DA-Src-induced AIG, we treated cells with Y-27632, a potent and highly specific chemical inhibitor of the downstream RhoA effector ROCK. Treatment with Y-27632 markedly decreased actin stress fibers in standard 2D culture conditions (not depicted) and did not affect AIG of wild-type cells (Fig. 4 C). Interestingly, the strong suppression of AIG by p120 ablation in DA-Rac1- and DA-Src-transformed cells was completely reversed by ROCK inhibition (Fig. 4 C and Fig. S2 A). These findings strongly suggest that the failure of DA-Rac1 and DA-Src to drive AIG in the absence of p120 stems, at least in part, from constitutive elevation of RhoA activity and is further dependent on the activity of its downstream effector ROCK.

Inhibition of ROCK signaling correlates with AIG

The significant reduction in actin stress fibers upon ROCK inhibition is consistent with prior literature showing that RhoA-ROCK signaling is a principal mediator of stress fiber formation in MDCK cells (Ridley et al., 1995; Imamura et al., 1998; Nakano et al., 1999). Along with other substrates, ROCK activates LIMK, which phosphorylates the actin-severing protein cofilin on Ser-3 (for review see Amano et al., 2000). Cofilin's actin-severing activity is inactivated by phosphorylation at this site (Arber et al., 1998; Yang et al., 1998; Ohashi et al., 2000; Sumi et al., 2001), which can be monitored by Western blotting with phosphospecific antibodies. Fig. 4 D shows that indeed, in the absence of p120, basal cofilin phosphorylation was elevated (compare lane 1 with lane 2). In wild-type MDCK cells expressing DA-Rac1 or DA-Src (Fig. 4 D, lanes 3 and 5, respectively), cofilin phosphorylation was markedly decreased, whereas cofilin phosphorylation remained elevated in p120-deficient cells expressing DA-Rac1 or DA-Src (Fig. 4 D, lanes 4 and 6, respectively). In contrast, DA-H-Ras induced a marked decrease in cofilin phosphorylation independent of p120 (Fig. 4 D, lanes 7 and 8). Total cofilin levels were up-regulated in response to p120 ablation in wild-type and DA-Rac1- and DA-Src-expressing cells, possibly reflecting a feedback loop attempting to compensate for the unscheduled decrease in cofilin activity. Nonetheless, calculation of relative phosphocofilin/cofilin levels by quantitative densitometry revealed that even after correction for elevated cofilin, phosphocofilin levels were significantly elevated in p120-deficient wild-type, DA-Rac1, and DA-Src cell lines (Fig. 4 D, graph). Thus, the RhoA-ROCK-LIMK pathway in these cell lines is activated by p120 ablation. In sharp contrast, neither phosphorylation nor expression of cofilin was elevated by p120 ablation in cells transformed by H-Ras (Fig. 4 D,

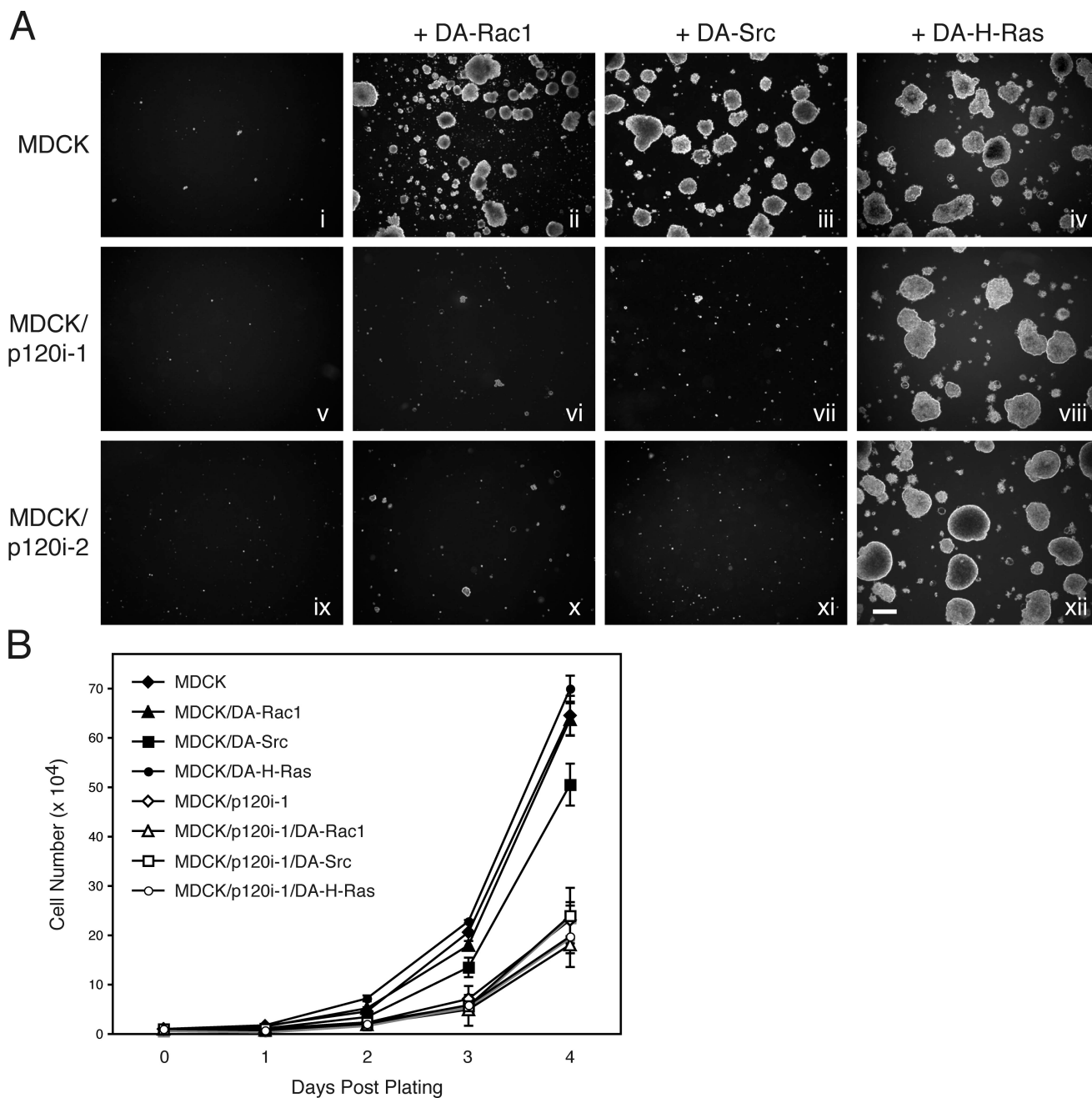


Figure 3. **p120 is required for Rac1- and Src-induced AIG.** (A) MDCK, MDCK-p120i-1, and MDCK-p120i-2 cells with and without DA-Rac1, DA-Src, or DA-H-Ras were plated in soft agar as described in Materials and methods. (B) MDCK (closed shapes) and MDCK-p120i-1 (open shapes) cells with and without DA-Rac1, DA-Src, or DA-H-Ras were seeded at 10^4 cells per well in 6-well dishes, and at the times indicated, cells were trypsinized and counted. Error bars represent standard deviation from three independent experiments. Bar, 0.5 mm.

lanes 7 and 8). These data reveal a strong correlation between inhibition of the ROCK pathway, activation of cofilin, and the ability of Rac1, Src, and H-Ras to induce AIG.

Inhibition of ROCK-LIMK pathway by H-Ras is independent of p120

The essential role for p120 with respect to Rac1- and Src-induced AIG appears to involve suppression of RhoA: for both oncoproteins, p120 ablation blocked AIG, and addition of a ROCK inhibitor reversed the block. Based on the cofilin phosphorylation data, H-Ras also appears to suppress the ROCK

pathway, suggesting a common underlying theme based on inhibition of the RhoA-ROCK cascade. However, the aforementioned observations do not account for how H-Ras might suppress ROCK independently of p120.

One possibility is that H-Ras suppresses the pathway at a point downstream of p120 and/or RhoA. Indeed, Ras has been shown previously in other cell types to inhibit ROCK by down-regulation of ROCKI and ROCKII proteins (Pawlak and Helfman, 2002b), up-regulation of RhoE (Hansen et al., 2000; Riento et al., 2003), or up-regulation of p21^(WAF1/Cip1) (Tanaka et al., 2002; Lee and Helfman, 2004). However, in our system, the

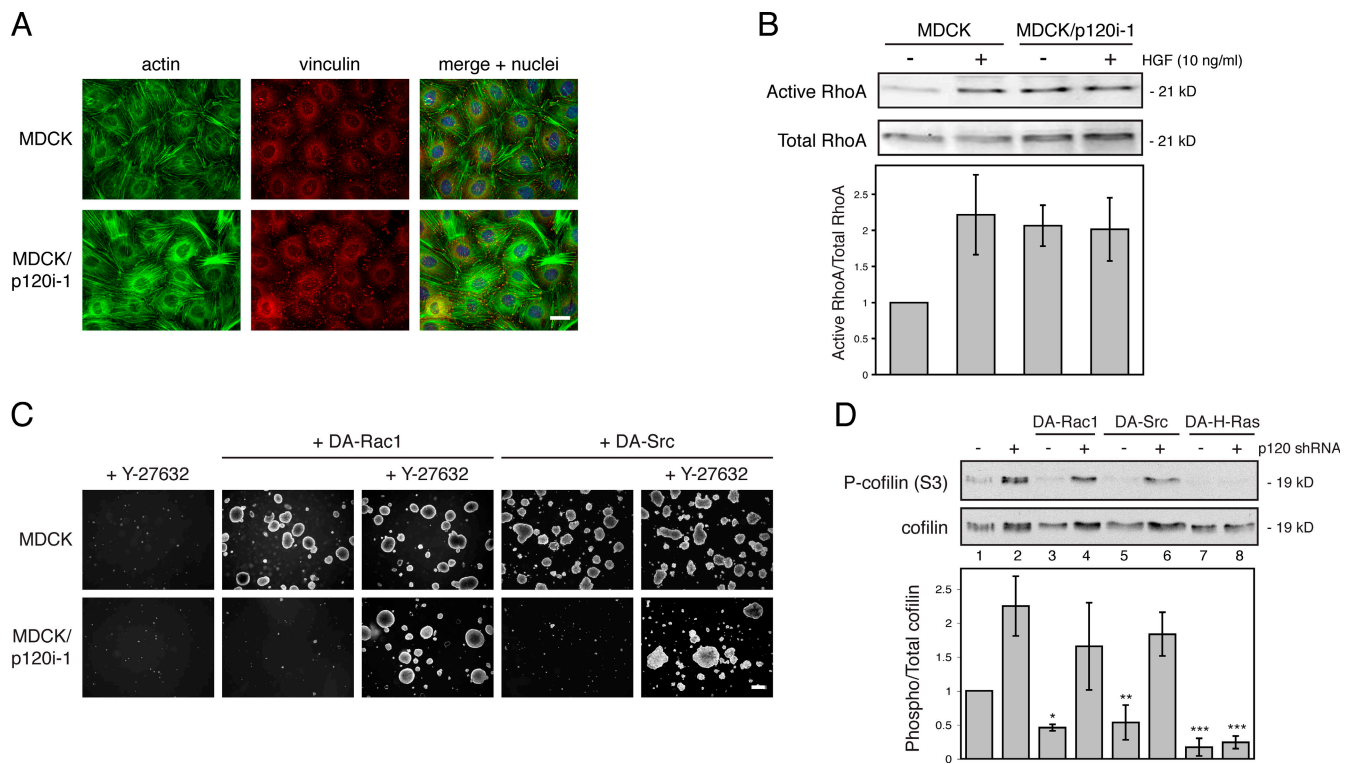


Figure 4. Inhibition of ROCK restores Rac1- and Src-induced AIG in the absence of p120. (A) MDCK and MDCK-p120i-1 cells plated on glass coverslips were serum starved (0.1% FBS) overnight and stained for actin and vinculin as described in Materials and methods. Nuclei were visualized with Hoechst stain. (B) MDCK and MDCK-p120i-1 cells were serum starved overnight and left untreated or treated with 10 ng/ml HGF for 30 min. Cell lysates were analyzed for RhoA GTPase activity by rhotekin-binding assay as described in Materials and methods, and the results from three separate experiments are depicted graphically. (C) MDCK and MDCK-p120i-1 cells with and without DA-Rac1 or DA-Src were plated in soft agar in the absence or presence of 10 μ M Y-27632 as described in Materials and methods. (D) Cell lysates from MDCK and MDCK-p120i-1 cells with and without DA-Rac1, DA-Src, or DA-H-Ras were analyzed by Western blotting for levels of Ser-3-phosphorylated cofilin and total cofilin. Phosphocofilin levels were normalized to total cofilin levels from three separate experiments and depicted graphically (*, $P < 0.003$; **, $P < 0.04$; ***, $P < 0.0002$). (B and D) Error bars represent standard deviation from three independent experiments. Bars: (A) 10 μ m; (C) 0.5 mm.

role of H-Ras with respect to these mechanisms was not entirely clear. Neither ROCK nor RhoE levels were significantly affected by H-Ras (Fig. S1, A and B), and although H-Ras induced expression of p21^(WAF1/Cip1), we were unable to detect an interaction between p21^(WAF1/Cip1) and ROCK1 or ROCK2 (Fig. S1 C) previously reported by others (Tanaka et al., 2002; Lee and Helfman, 2004). Therefore, it is unlikely that the mechanism involves direct down-regulation of ROCK or up-regulation of RhoE, but the suppression of ROCK via p21^(WAF1/Cip1) up-regulation remains a strong possibility.

Another possibility is that H-Ras inhibits the pathway at the level of LIMK. Fig. S4 shows that cofilin phosphorylation was efficiently suppressed by H-Ras and increased by exogenous LIMK1 expression as expected (Fig. S4 A, lanes 2 and 3, respectively; available at <http://www.jcb.org/cgi/content/full/jcb.200807096/DC1>). Moreover, exogenous LIMK1 overrode the effect of H-Ras on cofilin by maintaining it in a phosphorylated state (Fig. S4 A, lane 4), and these events correlated with the ability of exogenous LIMK to reverse H-Ras-induced AIG (Fig. 5 A). The precise effect of H-Ras on endogenous LIMK1 could not be directly determined because the canine LIMK1 protein was not detected by Western blotting with available LIMK antibodies (Fig. S4 A, lanes 1 and 2). However, exogenous coexpression of murine LIMK1 with

DA-H-Ras induced a marked band shift in LIMK1 protein migration as assessed by Western blotting (Fig. S4 A, lane 4), suggesting posttranslational modification. Interestingly, although total exogenous LIMK levels were clearly not reduced by H-Ras (Fig. S4 A, compare lane 3 with lane 4), staining by immunofluorescence of total LIMK with the same antibody was nearly eliminated (Fig. S4 B). Note that the overexpressed LIMK was detected only in cells where H-Ras staining was absent (Fig. S4 B, arrowheads). Although the exact reasons for these alterations are unknown, the data reflect clear changes in the status of LIMK when activated H-Ras is present. Thus, Rac1 and Src act upstream of p120 and are therefore p120 dependent, whereas H-Ras circumvents p120 by acting downstream, most likely at the level of ROCK. Overall, the data strongly suggest that all three oncogenes promote activation of cofilin via suppression of the RhoA-ROCK-LIMK pathway.

Activation of cofilin is necessary, but not sufficient, for oncogene-induced AIG

To further examine the relationship between inhibition of the ROCK pathway and oncogene-induced AIG, we coexpressed murine LIMK1 with DA-Rac1, DA-Src, or DA-H-Ras and again assayed for growth in soft agar (Fig. 5 A). Because LIMK lies

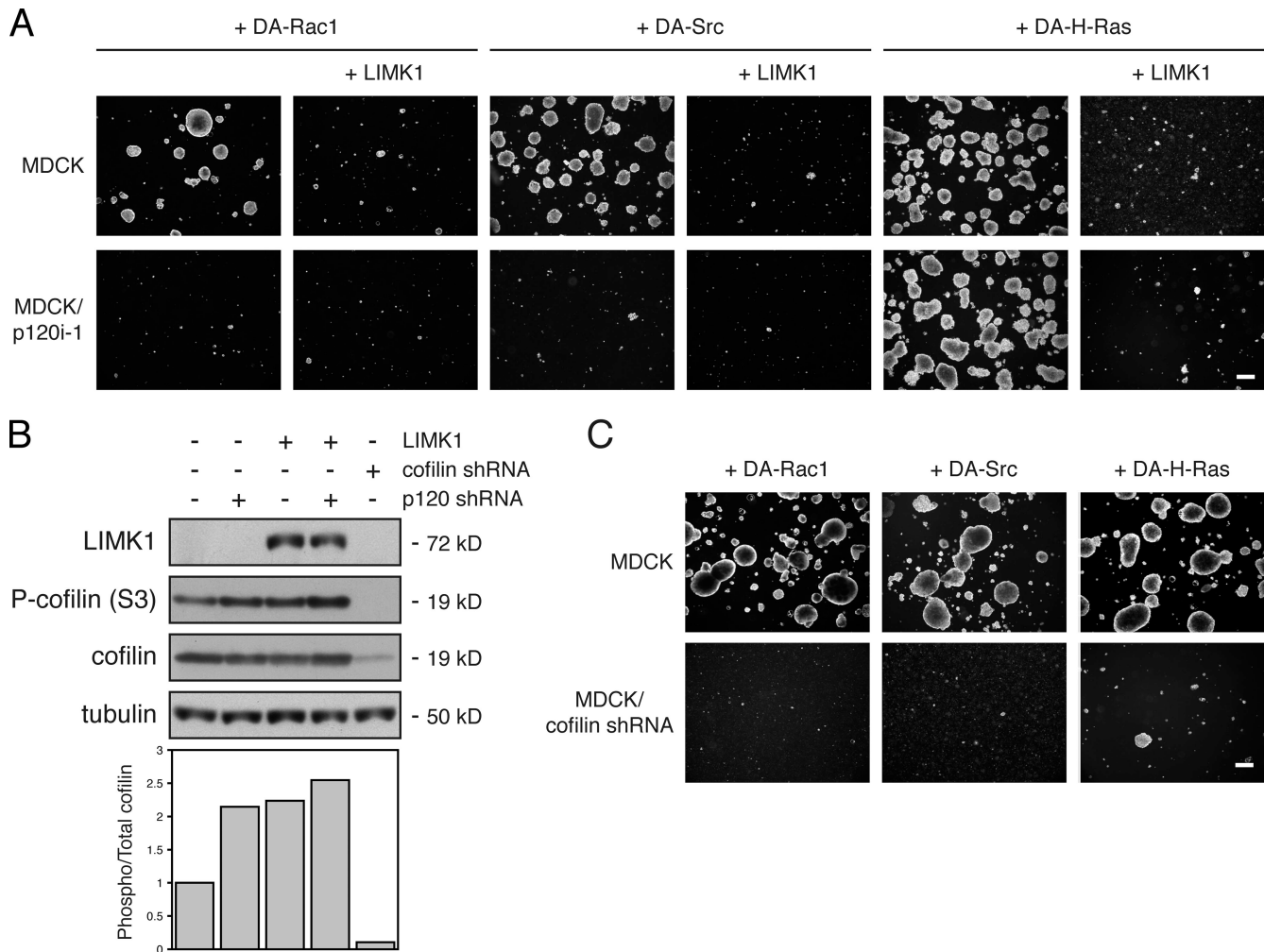


Figure 5. Direct inhibition of cofilin inhibits Rac1-, Src-, and H-Ras-induced AIG. (A) MDCK and MDCK-p120i-1 cells expressing DA-Rac1, DA-Src, or DA-H-Ras with and without murine LIMK1 were plated in soft agar as described in Materials and methods. (B) Lysates from MDCK and MDCK-p120i-1 cells with and without murine LIMK1 or cofilin shRNA were analyzed by Western blotting for levels of LIMK, Ser-3-phosphorylated cofilin, and total cofilin. Tubulin levels were determined as a loading control. Phosphocofilin levels were normalized to total cofilin levels and depicted graphically. (C) MDCK cells expressing DA-Rac1, DA-Src, or DA-H-Ras with and without cofilin shRNA were plated in soft agar as described in Materials and methods. Bars, 0.5 mm.

downstream of ROCK, forced expression of LIMK1 activates the pathway independent of upstream activities (as determined by Ser-3 phosphorylation of cofilin) and results in suppression of cofilin activity (Fig. 5 B). Importantly, for all three oncogenes, AIG was effectively blocked by either coexpression of LIMK (Fig. 5 A; quantified in Fig. S2 A) or knockdown of cofilin (Fig. 5, B and C; quantified in Fig. S2 A). Thus, as with DA-Rac1 and DA-Src, DA-H-Ras-induced AIG is strongly dependent on the activation of cofilin.

As in the case of p120 ablation (Fig. 3 B), forced expression of LIMK or knockdown of cofilin had measurable but relatively moderate effects on the proliferation of cells grown in 2D cultures (Fig. S2, B and C). Fig. S2 (B and C) shows the direct quantification of these effects in normal 2D cultures. LIMK had no effect on the proliferation of wild-type MDCK cells, and, surprisingly, proliferation rates were actually decreased by further addition of oncogenes (Fig. S2 B). Cofilin knockdown induced a moderate decrease in proliferation, which was not substantially altered by addition of oncogenes

(Fig. S2 C). Thus, although most of the aforementioned conditions were associated with reduced cell proliferation, the effects were unimpressive in comparison with the virtual absence of growth when the same cells were grown in soft agar over periods of time in excess of 4 wk. Therefore, it seems unlikely that the absence of AIG induced by exogenous LIMK or ablation of cofilin can be attributed to the effects on cell proliferation observed in 2D cultures.

To determine whether elevating cofilin activity is by itself sufficient to rescue the AIG block induced by p120 knockdown in DA-Rac1- or DA-Src-transformed cells, we introduced a constitutively active cofilin mutant (S3A; Fig. 6 A). However, in contrast to ROCK inhibition (Fig. 4 C), activated cofilin had no effect (Fig. 6 B). Thus, cofilin activity appears to be necessary but not sufficient to reverse the effects of p120 ablation on DA-Rac1- and DA-Src-induced AIG. Together, the data suggest that the LIMK/cofilin branch of the ROCK pathway is just one of several potentially important ROCK effectors that may participate in controlling AIG.

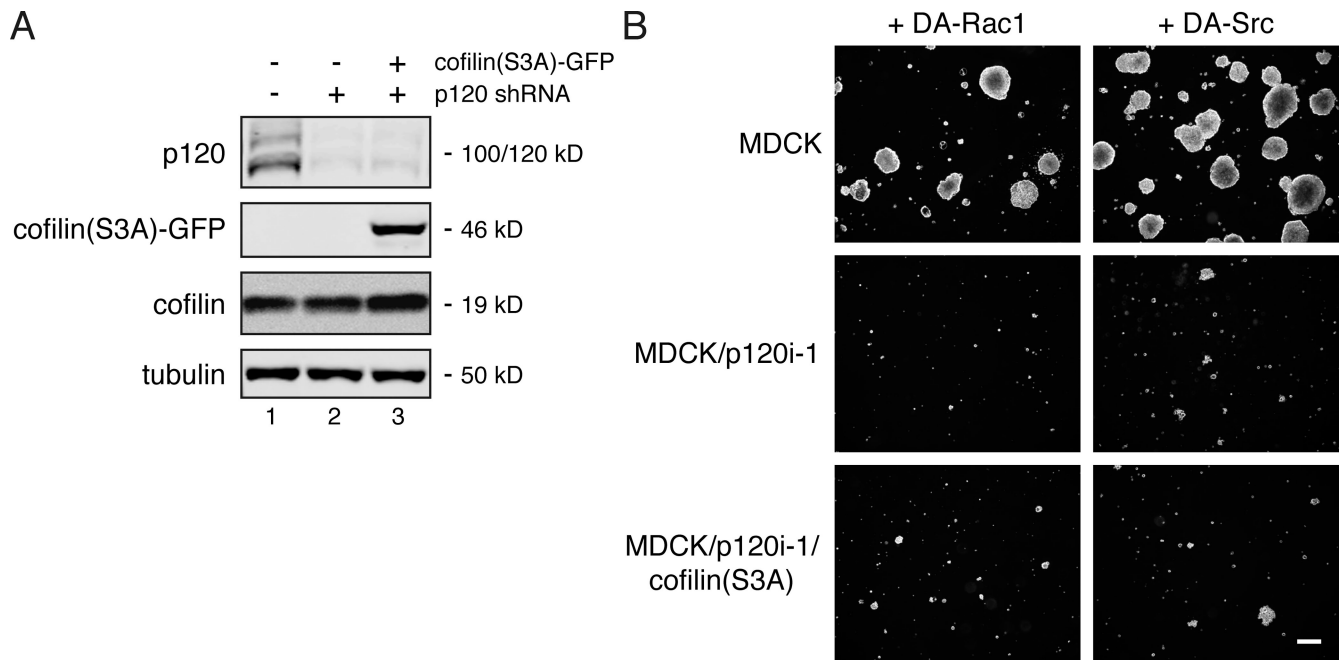


Figure 6. **DA cofilin is not sufficient to rescue the AIG block induced by p120 ablation in Rac1- and Src-transformed cells.** (A) Lysates from MDCK, MDCK-p120i-1, and MDCK-p120i-1 cells expressing DA cofilin(S3A)-GFP were analyzed by Western blotting for levels of p120, cofilin(S3A)-GFP, and total cofilin. Tubulin levels were determined as a loading control. (B) Cells in A stably expressing DA-Rac1 or DA-Src were plated in soft agar as described in Materials and methods. Bar, 0.5 mm.

p120-dependent and -independent AIG in human tumor cell lines

Although the wide variety of oncogenic alterations found in human tumor cell lines complicates analysis of relevant signaling pathways, we wondered whether the aforementioned phenomenon was restricted to MDCK cells or was more broadly applicable. Thus, we generated stable p120 knockdown cell lines from A431 (cervical carcinoma), A431D (a cadherin-negative variant of A431), MiaPaCa (pancreatic carcinoma), and DLD-1 (colon carcinoma) cells and assayed their behavior in soft agar. Parental A431 and A431D cells did not grow well in soft agar in the absence of exogenous oncogenes. However, the addition of DA-Rac1 induced strong AIG, which was effectively blocked by p120 ablation and subsequently restored by ROCK inhibition (Fig. S5 A, available at <http://www.jcb.org/cgi/content/full/jcb.200807096/DC1>). Thus, the behavior of A431 and A431D cells with respect to Rac1 and p120 was similar, if not identical, to that observed in MDCK cells.

In contrast, MiaPaCa and DLD-1 cells exhibited robust growth in soft agar without addition of exogenous oncogenes. Interestingly, p120 ablation severely compromised AIG of MiaPaCa cells, whereas DLD-1 cells were unaffected (Fig. S5 B). Thus, in the few human tumor cell lines analyzed to date, we found examples of both p120-dependent and -independent AIG phenotypes. Based on our observations in MDCK cells, we suggest that p120 dependence in tumor cell lines might be a function of whether relevant signaling pathways act up- or downstream of p120. Importantly, exogenous expression of LIMK1 suppressed the otherwise strong AIG in both MiaPaCa and DLD-1 cells (Fig. S5 B), as would be expected if cofilin activity constitutes an important contribution to AIG. Therefore, as in our oncogene-

transformed MDCK cell lines, this phenomenon may represent a common theme in various human tumors.

E-cadherin ablation suppresses Rac1- but not Src-induced AIG

Because p120 loss destabilizes cadherins, it is possible that the effects of p120 knockdown on oncogene-induced AIG could be linked to down-regulation of endogenous cadherins. To begin to address this question, we generated canine E-cadherin-specific shRNAs to knock down E-cadherin in MDCK cells. Expression of E-cadherin shRNA decreased E-cadherin protein levels to the same extent as observed after p120 knockdown, but without affecting p120 levels (Fig. 7 A, compare lane 2 with lane 3). However, in contrast to p120 knockdown data, the effects of E-cadherin knockdown in DA-Rac1- and DA-Src-transformed cells differed from one another. Surprisingly, E-cadherin knockdown had no effect on DA-Src-induced AIG but markedly reduced AIG in DA-Rac1-transformed cells (Fig. 7 B; quantified in Fig. 7 C). In addition, the suppression of AIG by E-cadherin knockdown in DA-Rac1-transformed cells was reversed by ROCK inhibition. Thus, in DA-Rac1-transformed cells, the effects of p120 and E-cadherin knockdown are similar, whereas in DA-Src-transformed cells, p120 ablation blocks AIG and E-cadherin ablation does not.

Discussion

Currently, anchorage independence is best understood in terms of constitutive oncogene-generated signals, which are thought to replace the normal integrin-dependent signaling required for anchorage-dependent cell growth and survival. Indeed, Rac1

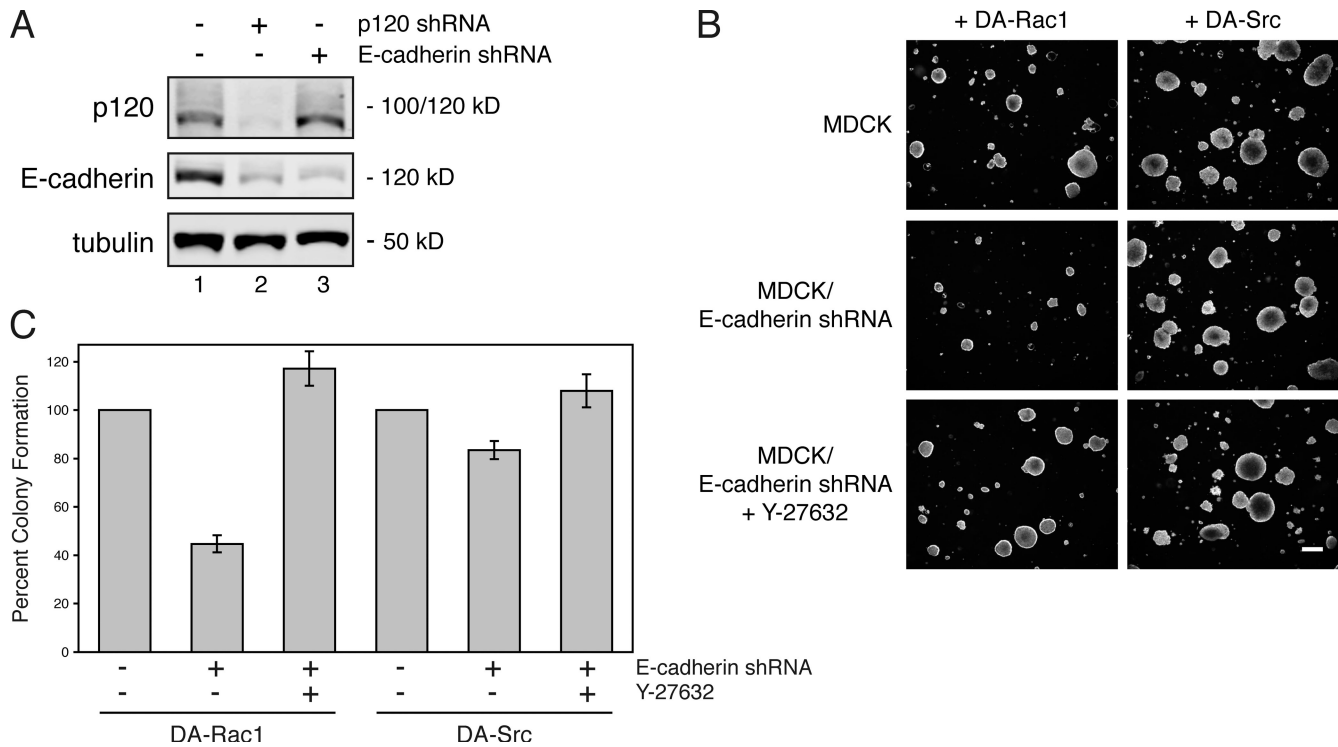


Figure 7. E-cadherin knockdown suppresses AIG induced by Rac1 but not Src. (A) Lysates from MDCK cells with and without p120 shRNA or E-cadherin shRNA were analyzed by Western blotting for levels of p120 and E-cadherin. Tubulin levels were determined as a loading control. (B and C) MDCK and MDCK/E-cadherin shRNA cells stably expressing DA-Rac1 or DA-Src were plated in soft agar in the presence or absence of the ROCK inhibitor Y-27632 (B) as described in Materials and methods, and the percentage of colony formation relative to wild-type MDCK cells was calculated for each oncogene (C). Error bars represent standard deviation from three independent experiments. Bar, 0.5 mm.

and Src are key effectors of normal integrin signaling cascades, and their activated counterparts are potent drivers of AIG. However, p120 has not been directly implicated in integrin signaling, and as a core component of the cadherin complex, a priori, one would not expect p120 to participate in signaling cascades that bypass anchorage dependence. Nonetheless, our data show that in MDCK cells, Rac1- and Src-mediated AIG is completely dependent on p120.

The experiments reveal for the first time a clear requirement for p120 in AIG induced by Src (and also by Rac1). Although p120 phosphorylation by Src was correlated with cell transformation nearly two decades ago (Reynolds et al., 1989), there has been little additional evidence that activated Src and p120 might be functionally interdependent. Indeed, Fig. 2 shows that when grown under normal 2D conditions on plastic, p120 knockdown did little to alter or reverse the Src-transformed morphology. However, when cultured in soft agar, the same cells were critically dependent on p120 for growth (Fig. 3 A). This result highlights the importance of context and is particularly interesting given the high correlation between AIG and tumorigenicity in vivo. Importantly, H-Ras-induced AIG was p120 independent, indicating that the requirement for p120 by Rac1 and Src is selective.

Several lines of evidence support these conclusions and suggest that the pathway delineated in Fig. 8 plays a central role in AIG and, by inference, tumorigenesis. The simplest interpretation of our data is that p120 is required for inhibition of RhoA by both Rac1 and Src. Clearly, p120 depletion elevates RhoA

activity and blocks AIG by a mechanism that requires activation of ROCK. Our working model is therefore similar in some respects to previously published observations in NIH3T3 fibroblasts describing obligatory roles for p120 and p190RhoGAP in Rac1-mediated inhibition of RhoA via the so-called “Bar-Sagi” pathway (Wildenberg et al., 2006). Indeed, both Rac1 (Nimnual et al., 2003) and Src (Fincham et al., 1999; Haskell et al., 2001) are known to inhibit RhoA via p190RhoGAP-dependent pathways, and transformation by Src depends in part on inhibition of RhoA via activation of p190RhoGAP (Fincham et al., 1999). However, we have not yet been able to validate a role for p190RhoGAP in MDCK cells. An efficient knockdown of the A isoform of p190RhoGAP (p190RhoGAP-A) had no discernible effect on either Rac1- or Src-mediated AIG (our unpublished data). p190RhoGAP-B is known to have partial functional redundancy to the A isoform, but we have not been able to effectively knock down this protein. Thus, p190RhoGAP-B remains a candidate, but other mechanisms are also possible, including direct p120-mediated inhibition of RhoA (Anastasiadis et al., 2000) or potentially direct inhibition of ROCK itself.

Although high RhoA–ROCK activity clearly suppresses AIG, the evidence does not fully exclude the possibility that p120’s role might be independent of Rac1 and Src signaling pathways, as is clearly the case with H-Ras. For example, Rac1 and Src might normally suppress RhoA–ROCK activity via p120-independent mechanisms that are then unable to compensate for elevated RhoA–ROCK activity caused separately by p120 ablation. It is worth noting, however, that the effects of

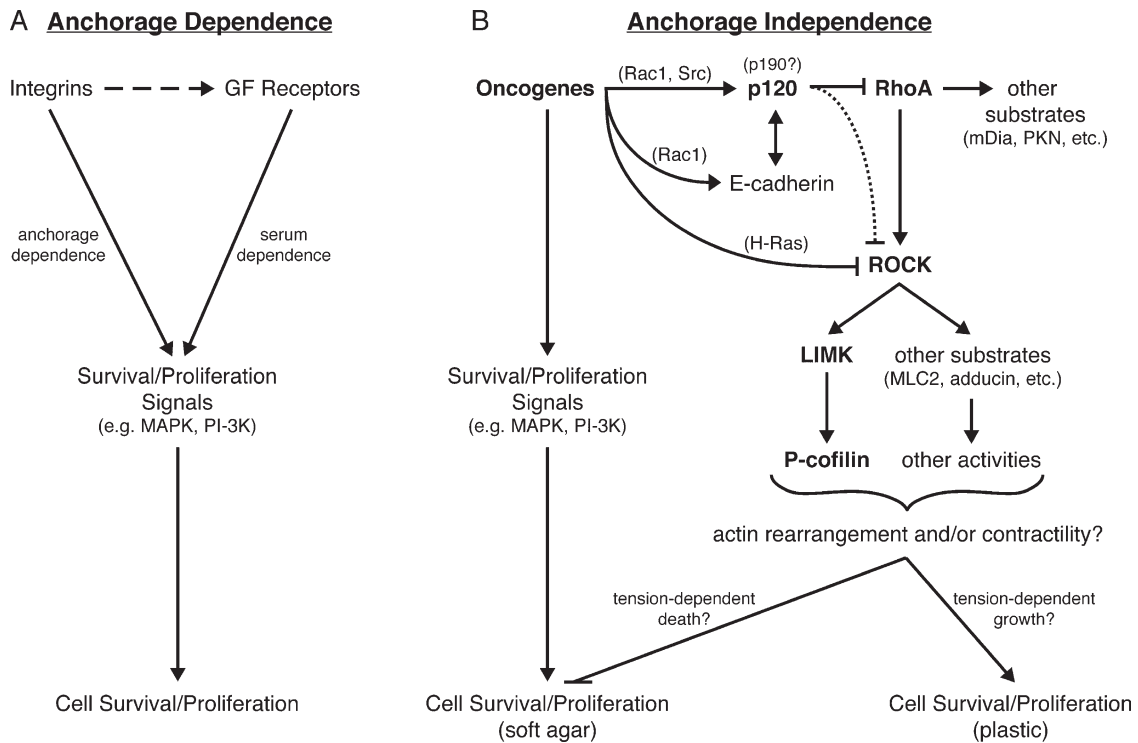


Figure 8. **p120-dependent and -independent control of AIG via inhibition of ROCK.** (A) Under normal conditions, cell proliferation is dependent on both growth factors (GFs; serum dependence) and adhesion to a solid substrate (anchorage dependence). Anchorage dependence is mediated primarily by integrins, whereas most oncogenes, including activated variants of Src, Rac1, and H-Ras, are defined in part by their ability to permit or induce AIG (B). Oncogenes are thought to circumvent anchorage dependence by constitutive activation of downstream survival and proliferation signals (e.g., MAPK and PI-3K) normally generated by integrins. Our model suggests that inhibition of ROCK is also essential. For Rac1 and Src, p120 is required and appears to function via suppression of RhoA–ROCK. H-Ras also suppresses ROCK but utilizes a different pathway that bypasses p120. The suppression of ROCK leads to the activation of cofilin, which is necessary but not sufficient for AIG, suggesting that other ROCK activities also play a role. The exact mechanisms underlying these events are not yet clear but likely involve rearrangement of the actin cytoskeleton and/or regulation of contractility. For normal cells, the effects of RhoA activity are context dependent. In soft agar, RhoA generates contractility and promotes anoikis, whereas on stiff matrices (e.g., plastic), RhoA participates in mechanical tension-based feedback loops that promote growth. According to our data, transformed cells circumvent RhoA-, actin-, and/or tension-dependent cell death via suppression of ROCK.

p120 ablation are not so potent as to be insurmountable, as indicated by the data on H-Ras. Regardless of the exact mechanism, the ultimate effects of both Rac1 and Src on the RhoA–ROCK pathway and on AIG are clearly dependent on p120.

Interestingly, although H-Ras did not require p120, its ability to induce AIG nonetheless depended on suppression of the ROCK–LIMK cascade and activation of cofilin (Fig. 5). Ras has been shown previously to inhibit ROCK by at least three different cell type–specific mechanisms, including down-regulation of ROCKI and ROCKII proteins (Pawlak and Helfman, 2002b), up-regulation of RhoE (Hansen et al., 2000; Riento et al., 2003), and up-regulation of p21^(WAF1/Cip1) (Tanaka et al., 2002; Lee and Helfman, 2004), all of which act downstream of p120. In our system, the mechanism may involve direct binding and suppression of ROCK activity by up-regulated cytoplasmic p21^(WAF1/Cip1), as has been reported previously in other Ras-transformed cells (Lee and Helfman, 2004). Consistent with this, we found that H-Ras up-regulated p21^(WAF1/Cip1) in MDCK cells (Fig. S1 C), induced marked changes in LIMK behavior (Fig. S4), and blocked cofilin phosphorylation (Fig. 4 D and Fig. S4 A). These data support a growing body of evidence suggesting that suppression of the RhoA–ROCK–LIMK cascade is a fundamentally important element of the transforming mechanisms for

several different oncogenes. For example, several MEK (MAPK/extracellular signal-regulated kinase kinase)-dependent and -independent mechanisms for inhibition of ROCK have been reported in cells transformed by Src (Pawlak and Helfman, 2002a), Ras (Hansen et al., 2000; Pawlak and Helfman, 2002b; Tanaka et al., 2002; Riento et al., 2003; Lee and Helfman, 2004), and Raf (Ehrenreiter et al., 2005). Interestingly, Ras is in fact dependent on RhoA activity for cell transformation in fibroblasts (Olson et al., 1998), but actin stress fibers are nonetheless absent because Ras simultaneously suppresses ROCK by one of the aforementioned mechanisms. Thus, it appears that oncogenic Ras goes to great lengths to silence the ROCK–LIMK cascade, and this in turn may be essential for cell growth in the absence of anchorage.

Regardless of the path to ROCK inhibition, the end result with respect to each oncogene’s effect on cofilin was unambiguous. Indeed, cofilin activity was markedly increased, and, importantly, blocking this activity downstream of ROCK (e.g., by LIMK overexpression or cofilin knockdown) also blocked AIG. Thus, upstream elements of the Rac1, Src, and H-Ras pathways differ, but all appear to converge downstream to suppress ROCK. The AIG block induced by either LIMK overexpression or cofilin knockdown shows clearly that cofilin activity

and/or events controlled by cofilin are necessary for AIG, irrespective of other essential growth and survival pathways activated by the oncogenes (e.g., MAPK, PI-3K, etc.). However, although ROCK inhibition by itself was sufficient to rescue the AIG block caused by p120 ablation (Fig. 4 C), cofilin activation by itself was clearly insufficient (Fig. 6 B). Thus, it appears that cofilin activity is necessary but not sufficient for AIG, and other ROCK substrates such as MLC2 or adducin are likely to make essential contributions. Together, the data suggest that ROCK is an important common effector for these otherwise dissimilar oncogenes, and one critical consequence is activation of cofilin.

Exactly how inhibition of ROCK signaling supports AIG is not yet clear. One possibility is that pathways normally suppressed by ROCK engage the cell cycle machinery via signaling events that are not well understood. For example, cofilin activation has been linked to up-regulation of the early response gene *c-Myc* (Kamaraju and Roberts, 2005; Honma et al., 2006), which modulates cell cycle progression via regulation of cyclin E, *cdk2*, and E2F-1. However, we did not find a consistent relationship between the status of cofilin and *c-Myc* in our transformed MDCK cells (unpublished data).

A more likely scenario is that ROCK signaling controls actin-dependent phenomena linked to anoikis. Indeed, several lines of evidence implicate actin-based mechanisms in sensing detachment and promoting tension-dependent cell death (Huang and Ingber, 1999; Ma et al., 2007; Gunning et al., 2008). The oncogenes described herein constitutively suppress RhoA–ROCK signaling, which may short-circuit surveillance mechanisms responsible for sensing tension, detecting cell detachment, and/or executing death programs. Thus, ROCK inhibition could directly promote AIG by mechanically disabling actin-dependent events responsible for detecting and ultimately eliminating unanchored cells. Several other factors that impact actin stability behave in ways that are consistent with these ideas. For example, high molecular weight tropomyosins, which are often down-regulated in cancer, induce anoikis by inhibiting cofilin and stabilizing actin filaments (Gunning et al., 2008). Similarly, anoikis mediated by p66^{Shc}, the long isoform of the Shc adapter protein, is dependent on its ability to activate RhoA and actomyosin contractility (Ma et al., 2007). Inhibition of RhoA or ROCK also suppresses anoikis of human embryonic stem cells (Watanabe et al., 2007), stem cell-derived neural precursors (Koyanagi et al., 2008), and neuronal cells plated in organotypic culture (Julien et al., 2008). Thus, suppression of ROCK may effectively circumvent actin-dependent mechanisms that link cell detachment to growth suppression and/or apoptosis.

These observations also provide an apparent explanation for the initially surprising finding that RhoA activity suppresses MDCK cell proliferation under anchorage-independent conditions. In cultured cells, RhoA activity is strongly associated with the opposite effect, namely promitogenic signaling and cell transformation (Frame and Brunton, 2002; Jaffe and Hall, 2002; Sahai and Marshall, 2002; Malliri and Collard, 2003). Attachment and growth on plastic is tightly coupled to RhoA activation (Huang and Ingber, 1999), and increased stiffness in premalignant breast tissue can promote tumor progression via

tension-induced activation of RhoA (Paszek et al., 2005). However, in detached cells, unopposed RhoA-dependent tension appears to be unfavorable for cell growth and in fact promotes tension-dependent cell death (Ma et al., 2007). Therefore, it is likely that the growth-promoting effects of RhoA, whether *in vitro* or *in vivo*, reflect anchorage-dependent tension and the associated positive feedback loops described previously in cells responding to tension (Wozniak et al., 2003; Zhao et al., 2007). In contrast, our observations coupled with the tight relationship between AIG and *in vivo* tumorigenicity highlight the importance of circumventing RhoA activity under conditions in which external tension is reduced, which is a potential consequence of the changes in tissue architecture during tumorigenesis. The importance of context is highlighted by the fact that p120 depletion in Src (or Rac1)-transformed MDCK cells can support growth on plastic but blocks growth completely in soft agar.

The opposite effects of p120 versus E-cadherin knockdown in Src-transformed cells (Fig. 7 B) highlight important features of p120 ablation that are not yet understood. Although E-cadherin levels in p120 knockdown cells are essentially equivalent to those in E-cadherin knockdown cells, p120 ablation in Src-transformed cells virtually eliminated AIG, whereas E-cadherin ablation had little or no effect. A potential explanation is that MDCK cells have other cadherins (e.g., N-cadherin and cadherin-6), most (if not all) of which are degraded along with E-cadherin in the absence of p120. In contrast, E-cadherin ablation actually increases levels of N-cadherin in MDCK cells (unpublished data), presumably because N-cadherin no longer has to compete with E-cadherin for limiting amounts of p120. Thus, it is possible that the presence of one or more cadherins at the cell surface is essential for AIG. In this scenario, p120-mediated stabilization of a cadherin might be required for a cadherin-dependent function. Alternatively, cadherin-mediated targeting of p120 to the membrane (or cadherin complex) may be required for a p120-dependent function. For example, Src mutants that cannot associate with membranes are unable to phosphorylate p120 and are transformation defective (Reynolds et al., 1989). However, we note that the scenario is now complicated by the fact that Src- and Rac1-transformed cells behave the same with respect to p120 ablation but different with respect to E-cadherin ablation. These interesting observations raise new questions that are beyond the scope of this study.

In summary, our work reveals a novel role for p120 in AIG and, by inference, tumorigenicity. Using growth in soft agar as an *in vitro* surrogate for tumorigenicity, we find that cell transformation by oncogenic forms of Rac1 and Src is functionally dependent on p120, which in turn appears to be essential for suppression of ROCK and induction of AIG. H-Ras-induced AIG is also dependent on the suppression of ROCK, but the mechanism involves alternate pathways that bypass p120. Therefore, ROCK may act as a gatekeeper for cofilin and other activities that appear to be essential for AIG. Although the obligatory participation of p120 in pathways associated with anchorage independence is surprising, Src substrates in general regulate the actin cytoskeleton (for review see Frame, 2004), which in turn coordinates a wide variety of activities necessary for cell proliferation and cell death. Although p120 phosphorylation by Src was linked to cell

transformation nearly two decades ago (Reynolds et al., 1989), there has been little additional evidence that activated Src and p120 might be functionally interdependent. The experiments herein demonstrate for the first time a clear requirement for p120 in both Src- and Rac1-mediated AIG.

Materials and methods

Cell lines, reagents, and constructs

All cells were grown in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. ROCK inhibitor Y-27632 was purchased from Enzo Biochem, Inc. HGF was purchased from EMD. DA forms of Rac1(G12V), Src(Y527F), and H-Ras(G12V) (gifts from A. Hall, Memorial Sloan-Kettering Cancer Center, New York, NY) and murine LIMK1 (a gift from J. Bamburg, Colorado State University, Fort Collins, CO) were cloned into pLZRS-IRES-GFP and pLZRS-IRES-Neo retroviral vectors as previously described (Ireton et al., 2002). pML-cofilin(S3A)-GFP was a gift from J. Bear (University of North Carolina Lineberger Comprehensive Cancer Center, Chapel Hill, NC). pRetroSuper retroviral vectors expressing shRNA directed against canine p120 and canine E-cadherin were generated as previously described (Davis et al., 2003). Production of pLZRS virus for protein expression and of pRetroSuper virus for shRNA expression was conducted in Phoenix 293 cells as previously described (Ireton et al., 2002).

Western blot analysis

Western blotting procedures were performed as previously described (Mariner et al., 2001). In brief, subconfluent cells were washed once with PBS, lysed with radio immunoprecipitation assay buffer, and clarified by centrifugation. Protein concentrations of lysates were determined by bicinchoninic acid assay (Thermo Fisher Scientific). The primary antibodies used were p120 (pp120; BD), β -catenin (Sigma-Aldrich), α -catenin (Sigma-Aldrich), E-cadherin (BD), N-cadherin (clone 13A9; a gift from P. Wheelock, University of Nebraska Medical Center, Lincoln, NE), occludin (Invitrogen), desmoglein (BD), tubulin (DM1A; Sigma-Aldrich), Ras (BD), Rac (clone 23A8; Millipore), Src (mAb327; EMD), ROCK1 (BD), ROCK2 (BD), cofilin (Cell Signaling Technology), phosphocofilin (Ser-3; Cell Signaling Technology), LIMK (Santa Cruz Biotechnology, Inc.), and p21^(WAF1/Cip1) (BD). The secondary antibodies used were HRP-conjugated donkey anti-mouse IgG and mouse anti-rabbit IgG (Jackson Immuno-Research Laboratories).

Immunofluorescence

Immunofluorescence procedures were performed as previously described (Davis et al., 2003). In brief, cells plated on glass coverslips were washed with PBS, fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 3% milk before staining with primary antibodies. p120 was detected with either monoclonal (pp120; BD) or polyclonal (F1; Reynolds et al., 1994) antibodies. Other antibodies used were E-cadherin, ZO-1 (Invitrogen), vinculin (Sigma-Aldrich), Ras, and LIMK. Secondary goat anti-mouse IgG1 Alexa Fluor 488, goat anti-mouse IgG1 Alexa Fluor 594, goat anti-mouse IgG2a Alexa Fluor 594, and goat anti-rabbit Alexa Fluor 488 antibodies were purchased from Invitrogen. Actin was detected by staining with phalloidin conjugated to Alexa Fluor 488 (Invitrogen). Nuclei were stained with Hoechst DNA stain (Sigma-Aldrich), and coverslips were mounted with Prolong Antifade (Invitrogen). Fluorescence images were taken at room temperature with a microscope (Axio-plan 2; Carl Zeiss, Inc.) using a 1.30 NA 40 \times oil or 1.4 NA 63 \times oil differential interference contrast objective. Digital images were captured with a camera (ORCA-ER; Hamamatsu Photonics) and processed with OpenLab (PerkinElmer) and MetaMorph (MDS Analytical Technologies) software. Photoshop (version CS2; Adobe) and Illustrator (version CS2; Adobe) were used to generate figures.

Growth in soft agar

5 \times 10³ cells were plated in 1 ml of 0.35% SeaPlaque agarose/DME/10% FBS on top of 1.5 ml of 0.7% SeaPlaque agarose/DME/10% FBS in 6-well dishes. Once solidified, the agarose was covered with 2.5 ml DME/10% FBS, which was changed twice weekly. Where indicated, 20 μ M Y-27632 was added to the top media for a final concentration of 10 μ M Y-27632. Digital images were captured 2–3 wk later at room temperature with a microscope (Axiovert 200M; Carl Zeiss, Inc.) using a 0.075 NA 2.5 \times objective with an ORCA-ER camera and processed with OpenLab and

MetaMorph software. Photoshop and Illustrator were used to generate figures. Growth in soft agar was quantified by determining the number of colonies >0.2 mm in diameter 2 wk after plating.

RhoA activity assays

The GST pull-down assay to measure RhoA activation was described previously (Ren et al., 1999). In brief, subconfluent cells were serum starved overnight before lysis in rhotekin lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, and 10 mM MgCl₂ plus protease inhibitors). Clarified lysates were incubated with the RhoA-binding domain of rhotekin fused to GST (Cytoskeleton, Inc.) to precipitate RhoA-GTP. GTP-bound (active) and total RhoA were detected by Western blotting with anti-RhoA antibody (26C4; Santa Cruz Biotechnology, Inc.). Densitometric analysis was performed using Odyssey software (LI-COR Biosciences). MetaMorph software was used for quantification of actin stress fibers and vinculin-positive focal contacts.

Detection of RhoE transcript by PCR

Total RNA was isolated from MDCK and MDCK/DA-H-Ras cells with the RNeasy Mini kit (QIAGEN) and reverse transcribed with the High Capacity cDNA Archive kit (Applied Biosystems). The resulting cDNA was used as a template for PCR with primers for canine RhoE (forward, 5'-AAAATGCTCTTGGTTGGCTGC-3'; reverse, 5'-TTTGTCTTCCGTAAGTCCGTG-3'), canine p120 (forward, 5'-AAATCTGGCTGTGGATGCTCGG-3'; reverse, 5'-GTG-GTCTCTCTCATTACAGTGTG-3'), and canine glyceraldehyde 3-phosphate dehydrogenase (forward, 5'-TCAAGGCTGAGAACGGGAAAC-3'; reverse, 5'-AACTGATACATTGGGGGTGGGGAC-3'). PCR reactions were terminated after the indicated number of cycles to detect relative transcript levels.

Online supplemental material

Fig. S1 shows that H-Ras does not inhibit ROCK activity by previously known mechanisms. Fig. S2 shows the effects of p120 shRNA, cofilin shRNA, and LIMK1 expression on colony formation and cell growth. Fig. S3 contains quantification of actin stress fibers and vinculin staining corresponding to the data in Fig. 4 A. Fig. S4 shows the effects of LIMK1 expression with and without DA-H-Ras on cofilin phosphorylation and LIMK1 staining. Fig. S5 depicts p120-dependent and -independent AIG in human tumor cell lines. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200807096/DC1>.

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