M-Ras/Shoc2 signaling modulates E-cadherin turnover and cell–cell adhesion during collective cell migration

Pradeep Kota1,2, Elizabeth M. Terrell2, Daniel A. Ritt3, Christine Insinna4, Christopher J. Westlake5, and Deborah K. Morrison6,1

1Laboratory of Cell and Developmental Signaling, National Cancer Institute-Frederick, Frederick, MD 21702

Collective cell migration is required for normal embryonic development and contributes to various biological processes, including wound healing and cancer cell invasion. The M-Ras GTPase and its effector, the Shoc2 scaffold, are proteins mutated in the developmental RASopathy Noonan syndrome, and, here, we report that activated M-Ras recruits Shoc2 to cell surface junctions where M-Ras/Shoc2 signaling contributes to the dynamic regulation of cell–cell junction turnover required for collective cell migration. MCF10A cells expressing the dominant-inhibitory M-Ras52V variant or those lacking Shoc2 exhibited reduced junction turnover and were unable to migrate effectively as a group. Through further depletion/reconstitution studies, we found that M-Ras/Shoc2 signaling contributes to junction turnover by modulating the E-cadherin/p120-catenin interaction and, in turn, the junctional expression of E-cadherin. The regulatory effect of the M-Ras/Shoc2 complex was mediated at least in part through the phosphoregulation of p120-catenin and required downstream ERK cascade activation. Strikingly, cells rescued with the Noonan-associated, myristoylated Shoc2 mutant (Myr-Shoc2) displayed a gain-of-function (GOF) phenotype, with the cells exhibiting increased junction turnover and reduced E-cadherin/p120-catenin binding and migrating as a faster but less cohesive group. Consistent with these results, Noonan-associated C-Raf mutants that bypass the need for M-Ras/Shoc2 signaling exhibited a similar GOF phenotype. M-Ras/Shoc2 signaling contributes to the dynamic control of coordinated collective cell movement.

Significance

Noonan syndrome belongs to a group of related developmental disorders termed the RASopathies, and both the M-Ras GTPase and its effector Shoc2 are signaling proteins mutated in specific subtypes of this disorder. Previously, the M-Ras/Shoc2 complex has been shown to act as a positive regulator of growth factor–mediated ERK cascade activation, and, here, we demonstrate a function for M-Ras/Shoc2/ERK cascade signaling in the dynamic control of cell-cell adhesion that is required for collective cell migration. Given the essential role of collective cell migration in human embryogenesis and organ development, our elucidation of the M-Ras/Shoc2 function in this highly regulated process may provide insight regarding the cellular basis of defects associated with Noonan syndrome and other RASopathies.
migration. Here, we report that activated M-Ras recruits Shoc2 to cell–cell adherens junctions where M-Ras/Shoc2/ERK cascade signaling functions to modulate E-cadherin turnover and cell–cell adhesion during the coordinated movement of cells. Notably, in depletion/reconstitution studies, we found that cells expressing the Noonan-associated Myr-Shoc2 mutant or either of two Noonan-associated C-Raf mutants (S257L- and P261S-C-Raf) display a less cohesive migratory behavior, which correlates with the reduced junctional expression of E-cadherin. Finally, expression of the Noonan-associated Myr-Shoc2 or C-Raf mutants also induced defects in coordinated convergent/extension cell movements during zebrafish gastrulation, further supporting a regulatory role for the M-Ras/Shoc2/ERK cascade signaling axis in cell migratory events.

Results

Activated M-Ras Recruits Shoc2 to Cell–Cell Junctions. As Shoc2 has been shown to bind M-Ras in a GTP-dependent manner, we initiated experiments to further investigate the function of Shoc2 as an effector of M-Ras. For these studies, we first examined the interaction of Shoc2 with active M-RasQ71L in live 293FT cells using the proximity-based, bioluminescence resonance energy transfer (BRET) assay (23). In this system, a BRET signal is generated when a protein tagged with an energy donor interacts with, and can transfer energy to, a protein tagged with an energy acceptor. In our studies, Shoc2 served as the energy donor tagged at the C terminus with the Rluc8 enzyme whereas activated versions of M-Ras and the canonical Ras proteins functioned as the energy acceptors tagged at the N terminus with the Venus fluorophore. In saturation curve analyses, a strong BRET signal was observed between Shoc2 and activated M-RasQ71L with a BRETso of 1.200 milliBRET units (mBU) and a BRET50 of 0.103 (Fig. 1 A, Left). A strikingly lower BRET signal was observed when Shoc2 was coexpressed with analogous, activated versions of the canonical Ras proteins (H-RasQ61L, K-RasQ61L, or N-RasQ61L). In contrast, when C-Raf was used as the energy donor, the strongest and highest affinity interaction was observed with the activated canonical Ras members (Fig. 1 A, Right). These findings demonstrate the preferential binding between active M-Ras and Shoc2 in live cells.

As a model system for further investigation of collective cell migration, we chose to use MCF10A cells, a nontransformed breast epithelial cell line with well-characterized migratory properties (24).

Fig. 1. Activated M-Ras recruits Shoc2 to cell–cell junctions. (A) Shown are BRET saturation curves examining the interaction of Shoc2-Rluc8 or C-Raf-Rluc8 and Venus-M-RasQ71L, K-RasQ61L, N-RasQ61L, or H-RasQ61L. BRET50 values, indicative of binding affinity, are also listed. (B) Endogenous Shoc2 complexes were immunoprecipitated (IP) from MCF10A cells stably expressing HA-Tagged M-RasQ71L, HA-K-RasQ61L, or the indicated M-RasG71T variants, and the complexes examined for HA-Ras binding and Shoc2 levels. (C) MCF10A cells stably expressing HA-M-RasQ71L, HA-K-RasQ61L, or vector alone were fractionated into cytosol and membrane-rich fractions, following which the fractions were probed for Shoc2, HA-Ras, E-cadherin (E-Cad), RhogDI (cytosolic), and the transferrin receptor (TRFrc, membrane). (D) Live cell imaging of cells expressing GFP-WT-Shoc2 and mCherry, GFP, and mCherry-M-RasQ71L, or GFP-WT-Shoc2 and mCherry-M-RasQ71L, showing that M-RasQ71L can recruit Shoc2 to cell–cell junctions. (E) Immunofluorescent staining of endogenous Shoc2 and M-Ras proteins at cell-cell junctions in serum-stimulated MCF10A cells. Magenta lines indicate free cell edges, and white arrows indicate cell–cell junctions.
Moreover, in wound-healing assays, depletion of Shoc2 had no significant effect on the ability of cells at the leading edge to form lamellipodia or become polarized toward the wounded edge (as determined by the orientation of the microtubule organizing center, MTOC) (SI Appendix, Fig. S2 C and D), indicating that the lack of collective cell movement was not due to defects in these processes.

Another important aspect of collective migration is the ability of the monolayer to regulate its cell–cell cohesive bonds (26). Cell–cell adhesive contacts are dynamic structures, being continually formed, disassembled, and reformed during many physiological processes, including collective cell migration (27). Therefore, to examine the role of M-Ras/Shoc2 signaling in the dynamic regulation of intercellular adhesion, cell scatter assays were performed. In this assay, cells plated in low density clusters were serum-starved, and then growth media was added to induce the severing of adhesive bonds and the migration of cells away from the cluster. As depicted in Fig. 2D, MCF10A cells expressing WT and active M-RasQ71L were able to dissociate and scatter in response to growth media addition whereas cells expressing dominant-inhibitory M-RasS27N failed to scatter. When we next examined the effect of Shoc2 depletion on the scattering of MRasQ71L-expressing cells, we found that, while cells transfected with control siRNA showed efficient scattering, those transfected with Shoc2 siRNA were unable to scatter (Fig. 2D and SI Appendix, Fig. S2E), indicating that M-Ras/Shoc2 signaling may contribute to the dynamic regulation of cell–cell cohesion.

**Analysis of Shoc2 Mutants: Protein Interactions, Localization, and Migratory Properties.** To further define Shoc2 as an M-Ras effector in cell migration, we utilized various Shoc2 mutants identified in previous studies (9). C260Y-Shoc2 is fully competent of M-Ras binding whereas D175N- and E457K-Shoc2 were defective in their ability to bind to-glycine substitution at amino acid position 2, generating a nonmyristoylated Shoc2 proteins must be competent to bind active M-Ras could restore EGF-induced ERK activation (9), the Shoc2 proteins competent to bind M-Ras could restore EGF-induced ERK activation in Shoc2-depleted cells (SI Appendix, Fig. S3C).

The Shoc2 scaffold has also been reported to mediate the assembly of a larger complex that contains Scribble (6). To determine whether the failure of D175N- and E457K-Shoc2 to interact with active M-RasQ71L might correlate with a defect in Scribble binding, the Shoc2 immunoprecipitates were probed for the presence of Scribble. As shown in Fig. 3, endogenous Scribble interacted with the D175N mutants at levels comparable with the WT protein whereas the E457K mutants failed to bind Scribble even when localized to the plasma membrane. It should be noted that C260Y is a mutation analogous to a LOF mutation identified in C. elegans genetic screens (19); however, in agreement with previous studies (9), we found that C260Y-Shoc2 is fully competent to bind active M-RasQ71L as well as Scribble (Fig. 3). Taken together, these findings reveal that Shoc2 proteins containing the D175N mutation are defective in M-Ras binding whereas the E457K mutant fails to bind both M-Ras and Scribble, with the caveat that forced membrane localization of this mutant (Myr-E457K) can restore M-Ras binding, but not the interaction with Scribble (SI Appendix, Fig. S3B).

The M-Ras binding properties of the Shoc2 mutants were further confirmed in live cell studies using the BRET assay and confocal imaging. As shown in Fig. 3B, both WT-Shoc2 and the C260Y mutant exhibited a strong interaction with M-RasQ71L by BRET analysis whereas a significant reduction in the BRET signal was observed when the D175N- and E457K-Shoc2 mutants were analyzed. Consistent with the colocalization results, forced membrane localization restored binding of Myr-E457K-Shoc2 to M-RasQ71L; however, the Myr-D175N mutant remained defective (Fig. 3B). In confocal imaging studies using MCF10A cells coexpressing GFP-tagged versions of the Shoc2 mutants with mCherry-M-RasQ71L, we found that the D175N- and E457K-Shoc2 mutants defective in M-Ras binding localized primarily in the cytosol whereas WT-, C260Y-, and Myr-Shoc2 colocalized with mCherry-M-RasQ71L at the cell–cell junctions (Fig. 3C and SI Appendix, Fig. S3D). Thus, our biochemical and live cell studies demonstrate that nonmyristoylated Shoc2 proteins must be competent to bind active M-Ras to be recruited to the cell surface junctions.
Next, to investigate the migratory properties of the Shoc2 mutants, wound healing assays were performed under conditions where growth media were used to stimulate the activation and GTP-loading of endogenous M-Ras. As shown in Fig. 3D, cells lacking Shoc2 exhibited a significantly reduced ability to fill the wound; however, the migration defect observed in these cells could be rescued by reexpression of WT-Shoc2, as well as C260Y- or Myr-Shoc2, but not by mutants defective in M-Ras binding (D175N-, Myr-D175N-, or E457K-Shoc2). Notably, reexpression of membrane-localized Myr-E457K Shoc2, which has restored M-Ras binding, could largely rescue the wound-healing defect of cells lacking Shoc2 (Fig. 3D), demonstrating that both the membrane recruitment of Shoc2 as well as its interaction with M-Ras are needed to drive collective cell movement.

When the Shoc2 mutants were evaluated in cell scatter assays, we found that Shoc2-depleted cells reexpressing the Shoc2 proteins competent to bind M-Ras (WT-, Myr-, and Myr-E457K Shoc2) were all able to detach from their neighbors and scatter whereas those reexpressing mutants defective in M-Ras binding (D175N-, E457K-, and Myr-D175N-Shoc2) failed to do so (Fig. 3E). It should
be noted that similar effects on wound healing and cell scattering were observed when the Shoc2 mutant analysis was performed using an shRNA to deplete Shoc2 ([SI Appendix, Fig. S4]). Moreover, consistent with the role of the Shoc2/M-Ras complex in promoting ERK cascade signaling, we found that treatment of MCF10A cells with the MEK inhibitor U0126 inhibited the migration of wounded MCF10A monolayers to a similar extent as did Shoc2 depletion (∼35% of control cells) (Fig. 3D) whereas treatment with the PI3K inhibitor LY294002 had a less severe effect. MEK inhibitor treatment was also found to prevent cell scattering (Fig. 3E), suggesting that the regulatory effects of the M-Ras/Shoc2 scaffold on cell migration require ERK cascade signaling.

**Computational Analysis of Shoc2 Function in Collective Cell Migration.** To further delineate the function of Shoc2 in coordinated cell movement, the wound healing and cell scatter assays were analyzed in more detail. As shown in Fig. 4, an initial comparison of Shoc2-depleted cells or those rescued with WT- or Myr-Shoc2 revealed that cells rescued with Myr-Shoc2 were found to fill the wounded area faster and scattered...
further from their neighbors than did cells rescued with WT-Shoc2, indicating a gain-of-function (GOF) phenotype. Based on these findings, time-lapse movies of the migrating cells were analyzed by particle image velocimetry (PIV) to generate velocity fields, which were then used to quantify the speed and directionality of cell movement (Fig. 4 A–F, SI Appendix, Fig. S5 A and B, and Movies S1–S4). PIV analysis of the wounded monolayers revealed that the Shoc2-depleted cells moved at significantly slower speeds (Fig. 4C), showed more variation in their directionality of movement (Fig. 4D), and exhibited reduced lengths of coordinated movement (Fig. 4E) than did control cells or those rescued with WT-Shoc2. In contrast, cells rescued with Myr-Shoc2 demonstrated a clear GOF phenotype, with the Myr-Shoc2 cells migrating at significantly faster speeds than did control cells or those expressing WT-Shoc2, albeit the movement of the Myr-Shoc2 cells was less cohesive, with individual cells occasionally detaching from the leading edge (Fig. 4 B and C). Biorthogonal decomposition (BOD) analysis further revealed that cells lacking Shoc2 migrated in a less collective manner as their velocity field modes converged more slowly during cell movement (energy recovery in the first two modes for Shoc2-depleted cells was ~40% versus 70% for control shNeg cells) (Fig. 4F). Importantly, the defects observed in the Shoc2-depleted cells could be rescued by reexpression of WT-Shoc2 (Fig. 4B–F). It should also be noted that, while all of the parameters of collective cell migration were impacted in cells lacking Shoc2, no significant effect on cell matrix adhesion was observed (SI Appendix, Fig. S5C). Therefore, these findings, together with the results from the random migration assays (Fig. 2C), support the model that Shoc2-depleted cells fail to scatter or migrate effectively as a group due to an altered ability to regulate their intercellular cohesive bonds.

M-Ras/Shoc2 Signaling Regulates Cell–Cell Adhesion by Modulating the E-Cadherin/p120-Catenin Interaction. To more directly assess the role of M-Ras/Shoc2 signaling in regulating intercellular adhesion, we first examined whether the presence or absence of Shoc2 had any effect on the junctional turnover rate of a known component of adherens junctions, α-catenin. For these studies, MCF10A cells stably expressing a GFP-tagged α-catenin were depleted, or not, of Shoc2, following which the dynamics of junction turnover was monitored in fluorescence recovery after photobleaching (FRAP) assays. Loss of Shoc2 was found to have no effect on the junctional expression of GFP-α-catenin before photobleaching; however, recovery of GFP-α-catenin into the junctions after bleaching was significantly slower (Fig. 5A and B), consistent with reduced junction turnover and an increase in adhesion strength. The recovery rate of GFP-α-catenin into the bleached junctions could be returned to control times by the reexpression of WT-Shoc2 but not the D175N mutant that is competent to bind Scribble but defective in M-Ras binding (Fig. 5A and B). These findings indicate that the effect on junction turnover is a direct consequence of Shoc2 depletion and reiterate the importance of the membrane recruitment of Shoc2 via the interaction with M-Ras. Interestingly, we also found that cells rescued with Myr-Shoc2 exhibited an accelerated recovery of GFP-α-catenin into the bleached junctions at early time points (0 to 50 s), an observation consistent with their increased migratory behavior and indicative of enhanced junction turnover. Because the cell surface expression of E-cadherin is an important determinant for cell–cell adhesiveness (28), we next examined the effect of Shoc2 depletion on E-cadherin localization in actively scattering MCF10A cells. Before scatter induction, both control and Shoc2-depleted cells were densely packed with strong E-cadherin staining at the cell–cell junctions (Fig. 5C). Nine hours after scatter induction, junction breakdown and dissipation of E-cadherin staining at the cell surface were observed in control shNeg cells whereas intact junctions with strong E-cadherin staining were still detected in Shoc2-depleted cells (Fig. 5C), indicating reduced E-cadherin turnover. Consistent with a model in which alterations in E-cadherin turnover contribute to the migratory defects observed in Shoc2-depleted cells, we found that treating MCF10A cells with the dynamin inhibitor dynasore to block E-cadherin endocytosis/turnover severely reduced cell migration in wound-healing assays (SI Appendix, Fig. S6D). Moreover, the wound-healing defect observed in Shoc2-depleted cells could be partially rescued when EGTA treatment was used to chelate Ca2+ and disrupt the trans-junctional Ca2+-mediated cadherin–cadherin interactions (SI Appendix, Fig. S6B).

To begin to address the mechanism by which Shoc2 regulates E-cadherin turnover, MCF10A cells were evaluated for the colocalization of p120-catenin and E-cadherin, given that binding of p120-catenin is known to control the cell-surface expression of E-cadherin by blocking its endocytosis and degradation (29). In subconfluent MCF10A monolayers that had been depleted, or not, of Shoc2, both p120-catenin and E-cadherin were found to colocalize at cell–cell junctions (Fig. 5D). Strikingly, E-cadherin and p120-catenin staining at cell–cell junctions was more intense and continuous in cells depleted of Shoc2 whereas the junctions in control cells exhibited a serrated discontinuous staining pattern. Moreover, we found that the appearance of the junctions in Shoc2-depleted cells could be reverted by reexpression of WT-Shoc2, but not the D175N-Shoc2 mutant that fails to bind M-Ras but is still competent to bind Scribble (Fig. 5D), confirming a requirement for the Shoc2/M-Ras interaction but not Scribble binding in the dynamic control of cell–cell junctions.

To further monitor the cell surface expression of E-cadherin, proteins localized at the cell surface were biotinylated and then isolated using avidin beads. In these pull-down assays, the EGFR receptor was detected at equivalent levels in either control or Shoc2-depleted cells; however, E-cadherin levels were increased in cells lacking Shoc2 (Fig. 5E), confirming the reduced junction turnover of E-cadherin in the absence of Shoc2. Consistent with these results, an increased interaction between p120 catenin and E-cadherin was also observed in Shoc2-depleted cells (Fig. 5F).

Although the mechanisms that control p120-catenin and E-cadherin binding are not fully understood, changes in the phosphorylation state of p120-catenin have been reported to modulate the p120-catenin/E-cadherin interaction (30, 31). The phosphorylation of p120-catenin is highly complex, with over 50 sites of phosphorylation identified, including tyrosine as well as serine and threonine residues. Despite this complexity, some studies have found that enhanced tyrosine phosphorylation of p120-catenin correlates with an increased binding affinity for E-cadherin (32–34) whereas phosphorylation at T310 has been reported to be a requirement for disrupting p120-catenin/cadherin binding during the collective migration of primary rat astrocytes (35). Strikingly, when the phosphorylation state of p120-catenin was examined, we found that, in MCF10A cells depleted of Shoc2, the phosphorylation of p120-catenin at the T310 site was significantly reduced whereas the tyrosine phosphorylation state of p120-catenin was increased (Fig. 5F), changes consistent with a more stable p120-catenin/E-cadherin interaction. In addition, treatment of MCF10A cells with the MEK inhibitor U0126 was found to have similar effects on p120-catenin phosphorylation and binding to E-cadherin as did Shoc2 depletion (Fig. 5G), suggesting the need for localized ERK cascade signaling induced by the active M-Ras/Shoc2 complex.

Because the T310 site of p120-catenin is found within the sequence context of T9GTP and given that ERK is a proline-directed kinase, we next examined whether p120-catenin might be a target of active ERK. Utilizing purified proteins and γ[32P]ATP, we found that active ERK could phosphorylate p120-catenin in vitro whereas little phosphorylation was observed in the absence of active ERK or in the presence of active GSK3, another proline-directed kinase (Fig. 5H). When the 32P-labeled p120-catenin was examined by trypsin digestion and HPLC analysis, only one peptide showed significant phosphorylation, and analysis of that peptide by Edman degradation, phosphoamino acid analysis, and sequencing revealed...
that the site phosphorylated was T310 (Fig. 5f). Taken together, these findings suggest that M-Ras/Shoc2 signaling contributes to the regulation of E-cadherin turnover at cell–cell junctions by modulating the interaction with p120-catenin in a manner that correlates with ERK cascade activation and p120-catenin phosphoregulatory events.
Noonan-Associated C-Raf Mutants Exhibit a Similar GOF Activity as Does the Noonan-Associated Myr-Shoc2 Mutant. To confirm that the effects of the M-Ras/Shoc2 complex on cell migration and intercellular adhesion require ERK cascade signaling, we utilized two C-Raf mutants most frequently detected in Noonan patients, S257L- and P261S-C-Raf. Importantly, both of these mutations result in the constitutive dephosphorylation of the C-Raf S259/14-3-3 binding site (Fig. 6A)—the known target of the M-Ras/Shoc2/PP1c ternary complex (9). Therefore, if the effects of the M-Ras/Shoc2 complex on cell–cell adhesion are mediated by ERK cascade signaling, then expression of these C-Raf mutants would be predicted to bypass the need for the M-Ras/Shoc2/PP1c complex and restore migration in Shoc2-depleted cells. As shown in Fig. 6B and C, expression of either S257L- or P261S-C-Raf, but not WT-C-Raf, could restore wound healing and cell scattering in cells lacking Shoc2. However, as is observed for cells rescued with the Noonan-associated Myr-Shoc2, cells expressing the Noonan-associated C-Raf mutants exhibited a GOF phenotype. More specifically, these cells were found to migrate in a less cohesive manner, with cells detaching from the leading edge in wound healing assays (Fig. 6B). Moreover, cells rescued with any of the Noonan-associated mutants, Myr-Shoc2, S257L-C-Raf, or P261S-C-Raf, displayed reduced binding between E-cadherin and p120 catenin, as well as reduced staining of E-cadherin at cell–cell junctions (Fig. 6D and E).

To further assess the GOF activity of the Noonan-associated mutants, the effect of these proteins on collective cell movements in
zebrafish embryos was evaluated. Previous studies have shown that E-cadherin turnover, as well as ERK signaling, contributes to the dynamic regulation of cell movement during zebrafish gastrulation and epiboly (36, 37), and expression of Noonan-associated PTP11/Shp2 and N-Ras mutants has been reported to alter the coordinated convergent-extension cell movements required for these processes, resulting in oblong embryos with an abnormal axis ratio (38, 39). As shown in Fig. 6F, expression of Myr-Shoc2, S257L-C-Raf, or P261S-C-Raf mutant. The GOF activity of the Noonan-associated mutants was provided by Dom Esposito, Frederick National Laboratory, Frederick, MD.

## Discussion

Shoc2 was first identified as an effector of M-Ras through its function as a positive regulator of growth factor-mediated ERK cascade activation. In this study, we have elucidated a distinct activity of the M-Ras/Shoc2 complex in the dynamic regulation of adherens junction turnover that is required for efficient collective cell migration. Moreover, we found that M-Ras/Shoc2/ERK cascade signaling plays a role in regulating the p120-catenin/E-cadherin interaction, which in turn controls the cell surface expression and junctional turnover of E-cadherin, a critical component of cell–cell junctions that controls intercellular adhesiveness. Through cell fractionation and live cell imaging studies, we found that E-cadherin turnover, as well as ERK signaling, contributes to the dynamic regulation of cell movement during zebrafish gastrulation and epiboly. Expression of Noonan-associated PTP11/Shp2 and N-Ras mutants has been reported to alter the coordinated convergent-extension cell movements required for these processes, resulting in oblong embryos with an abnormal axis ratio. As shown in Fig. 6F, expression of Myr-Shoc2, S257L-C-Raf, or P261S-C-Raf mutants were further confirmed in zebrafish assays demonstrating the requirement of E-cadherin/p120-catenin in concert with studies demonstrating the requirement of E-cadherin turnover and reduced binding to the cell surface, is critical for the regulation of junction turnover and coordinated cell movement. Moreover, our analysis of Shoc2 mutants, we found that the interaction of Shoc2 with M-Ras, as well as the localization of the active M-Ras/Shoc2 complex to the cell surface, is critical for the regulation of junction turnover and coordinated cell movement. Although we did not conduct a comprehensive analysis of p120-catenin phosphorylation, alterations in the phosphorylation state of p120-catenin were detected in Shoc2-depleted cells that would be predicted to stabilize the interaction with E-cadherin. In particular, we found that p120-catenin phosphotyrosine levels were increased whereas phosphorylation of a site previously reported to inhibit cadherin binding, T310 (35), was reduced. Moreover, our results indicate that the effects of the M-Ras/Shoc2 complex on p120-catenin phosphorylation, as well as cell migration and junction turnover, require ERK cascade signaling as MEK inhibitor treatment caused similar effects on these processes as did loss of Shoc2. In addition, active ERK could directly phosphorylate p120-catenin on the T310 site, suggesting a model whereby Shoc2 binds active M-Ras at the cell–cell junctions to form the M-Ras/Shoc2/PP1c ternary complex that facilitates the localized activation of Raf/MEK/ERK at junctions, allowing active ERK to phosphorylate p120-catenin and perhaps other substrates, which contribute to the dynamic turnover of cell–cell junctions required for the coordinated movement of cells. In support of this model, two Noonan-associated C-Raf mutants, S257L- and P261S-C-Raf, that bypass the need for the M-Ras/Shoc2/PP1c-mediated dephosphorylation of the C-Raf S259/14-3-3 binding site could rescue the migratory defects observed in Shoc2-depleted cells. However, it is important to note that cells rescued with the Noonan-associated C-Raf mutants exhibited a similar GOF phenotype as those rescued with the Noonan-associated Myr-Shoc2 mutant. The GOF activity of the Myr-Shoc2 mutant was first observed in the wound-healing assays where the Myr-Shoc2–expressing cells were found to migrate at significantly faster speeds than did WT-Shoc2–expressing cells. Notably, the Myr-Shoc2–expressing cells moved in a less cohesive manner, with cells occasionally breaking away from the leading edge, and they exhibited increased cell–cell junction turnover and reduced binding to E-cadherin and p120-catenin. Likewise, the junctional expression of E-cadherin and the E-cadherin/p120-catenin interaction was reduced in cells rescued with the Noonan-associated C-Raf mutants, and these cells were also found to dissociate more readily from the leading edge in wound-healing assays. The GOF activities of the Noonan-associated Myr-Shoc2, S257L-C-Raf, and P261S-C-Raf mutants were further confirmed in zebrafish assays monitoring cell movement during gastrulation/epiboly. Consistent with studies demonstrating the requirement of E-cadherin turnover and ERK signaling in the regulation of coordinated convergent/extension cell movements during zebrafish gastrulation/epiboly (36, 37), this assay has been used to evaluate other Noonan-associated mutant proteins, such as PTPN11/Shp2 and N-Ras (38). Using this assay, we found that expression of Myr-Shoc2, S257L-C-Raf, and P261S-C-Raf, but not WT-Shoc2 or WT-C-Raf, induced defects in the embryo axis ratio, which are indicative of aberrant cell migration. Moreover, we found that these defects could be reversed by treatment with the MEK inhibitor PD0325901, further confirming the participation of the ERK cascade in mediating the M-Ras/Shoc2 effects on coordinated cell movement. Taken together, our findings have revealed a previously unappreciated function for the M-Ras/Shoc2 complex in the regulation of cell–cell adhesion and collective cell migration, with potentially important implications for normal human development, as well as tumor metastasis.

## Methods

### DNA Constructs, siRNAs, and shRNA Vectors

Shoc2 cDNA was a kind gift of Jean Paul Borg, INSERM, Marseille, France, and M-Ras expression plasmids were provided by Dom Esposito, Frederick National Laboratory, Frederick, MD.
α-Catenin-EGFP and mCherry-NLS were purchased from Addgene. Shoc2 and M-Ras mutants were generated using the QuikChange site-directed mutagenesis kit from Stratagene. Constructs were tagged at the C terminus with either EGFP or Rluc8 or were constructed to contain a Pyo-epitope tag. M-Ras proteins were tagged at the N terminus with either mCherry, Venus, or the HA-epitope tag. All genes of interest were cloned into the pCDH-CMV or pCDH-PGK lentiviral vectors for close to endogenous level expression in MCF10A cells. Oligos used for Shoc2 deletion were as follows: sense, 5'-GGCAGGGAAGCUGAUUATT-3'; antisense, 5'-UAUAAUGCAGUCCGACGATC-3'. All-stars Neg control and custom Shoc2 siRNA were purchased from Qiagen and transfected into MCF10A cells using lipofectamine RNAiMAX (ThermoFisher). Stable Shoc2 deletion was achieved by infecting MCF10A cells with lentiviruses encoding human mir-30-based shRNA duplexes (6).

Cell Culture, Generation of Lentiviruses, and Lentiviral Infection. MCF10A cells were purchased from ATCC and cultured in DMEM/F12 supplemented with 5% horse serum, 10% Shglycerol, 20 ng/mL EGF, 100 ng/mL cholera toxin, 10 μg/mL insulin, and 1% penicillin/streptomycin as described previously (40). HEK-293T cells were cultured in DMEM supplemented with 10% PBS and 1% penicillin/streptomycin. Lentiviruses were generated by transfection of the pCDH-constructs with packaging plasmids PMD2.G and psPAX2 (1:2 ratio) into 293T cells, followed by collection of virus-containing supernatants at 48 and 72 h posttransfection. Supernatants were pooled, cleared of debris, and stored at −80 °C until use. MCF10A cells were infected with lentivirus supernatants containing 8 μg/mL polybrene (Sigma) for 24 h, following which growth media supplemented with the appropriate antibiotic selection was added (puromycin, 1 μg/mL; hygromycin, 40 μg/mL; blastidicin, 10 μg/mL). Lentiviral culture supernatants were also used for the transient expression of proteins in MCF10A cells, with cells being harvested at 48 h postinfection.

Cell Lysis and Immunoprecipitation Assays. Cells were washed twice with cold PBS and lysed for 15 min at 4 °C in 1% Nonidet P-40 buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 alternative, 0.15 U/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium vanadate, 20 μM leupeptin). Lysates were cleared of insoluble material by centrifugation at 16,000 × g for 30 min at 4 °C. Protein content was determined by bichinonic acid (BCA) analysis. Lysates containing equivalent amounts of protein were incubated with the appropriate antibody and protein G Sepharose beads for 2 to 3 h at 4 °C on a rocking platform. Complexes were washed extensively with 1% Nonidet P-40 buffer and then examined by immunoblot analysis together with equalized lysates.

Wound-Healing and Cell-Scattering Assays. For wound-healing assays, MCF10A cells stably expressing the indicated proteins were plated in 12-well dishes and grown to confluency. Monolayers were then wounded with a 20-gauge pipet tip to create a 400- to 600-μm scratch, washed with PBS, and incubated in growth media. Migration of the cells into the denuded area was monitored by phase contrast time-lapse microscopy, with images acquired every 5 min for 24 h. For scatter assays, cells were plated at low density (1.5 × 104) in 12-well dishes and incubated for 16 to 24 h. Cells were then starved for 24 h with serum-free media functions, following which cell scattering was induced by the addition of growth medium. Phase-contrast images were acquired at 10-min intervals for 12 to 18 h.

Additional experimental procedures are in SI Appendix, Supplementary Methods and Materials.

ACKNOWLEDGMENTS. We thank Suzanne Specht and Dr. Jimmy Staufer for excellent technical assistance and Dr. Russell Smith for help with confocal imaging. This project was supported by federal funds from the National Cancer Institute, National Institutes of Health, under Project Z01 BC 010329.