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Divergence of Rho residue 43 impacts GEF activity

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RhoA, RhoB and RhoC GTPases are over 85% identical at the amino acid level, with RhoA and RhoC differing at only one residue (43) across the initial two-thirds of their sequences. A putative regulatory distinction between the molecules is their capacity to be uniquely activated by guanine nucleotide exchange factors (GEFs). We hypothesize that variation of amino acid residue 43 between RhoA/B (valine) and RhoC (isoleucine) impacts GEF activity. Direct participation of residue 43 in GEF-catalyzed exchange was confirmed by the observation that mutation of this position to a threonine reduced GEF-catalyzed nucleotide exchange activity in vitro (Vav2, XPLN, GEFT, Dbl and Dbs) and greatly depressed RhoA and RhoC GTP-loading profiles in cell lysates. Using a residue swap approach, substitution of RhoA Val 43 with an Ile was found to significantly promote basal nucleotide exchange activity and enhance GTP-loading in cells. Substitution of Val 43 with an Ile in RhoB negatively affected nucleotide exchange in vitro. Substitution of RhoC Ile 43 with a Val increased GEF-catalyzed exchange in vitro. In addition, RhoC-I43V was more efficacious at driving ovarian cancer cell invasion through matrigrel than wild-type RhoC, RhoC-I43T, wild-type RhoA, RhoA-V43I or RhoA-V43T GTPases. These findings suggest that a divergence between RhoA/B and RhoC at residue 43 impacts basal and GEF-stimulated nucleotide exchange activity.

Introduction

Members of the Rho family of small intracellular GTPases are protein mediators of a multitude of cellular events, including proliferation, migration and transformation.[1-4] Rho GTPases become activated in cells by binding a GTP nucleotide and, as molecular switches, turn themselves "off" through their intrinsic GTPase activity. Upon activation, Rho GTPases favor conformations which permit binding of intracellular effectors molecules, including kinases and lipases, which in turn coordinate cellular events.[5]

Although RhoA, RhoB and RhoC are perhaps the most closely related of the 22 human Rho GTPases, sharing over 85% sequence identity, they possess clear differences in cellular activity.[6] One major function of RhoA signaling is to orchestrate actin-based contractility and adhesion turnover at the rear of migrating cells.[7,8] RhoB, on the other hand, has been linked to regulation of endosomal trafficking and apoptosis.[9,10] RhoC has been positively linked with migration and metastatic potential of cancer cells.[11-13] Peptide analysis indicates that the greatest amino acid sequence divergence of the three isoforms lies within the carboxyl terminus, a region largely responsible for intracellular localization.[14,15] In agreement with assigned function, RhoB associates with late endosomes and lysosomes, whereas RhoA and RhoC are either plasma membrane bound or cytoplasmic.[15–17]

Amino acid switch regions one and two, which are responsible for binding effector molecules, are nearly identical between the three isoforms.[6] Not surprisingly, RhoA/B/C have all been reported to bind many of the same effector molecules, such as Rhotekin and Citron.[6,18,19] More recent work, however, has revealed distinctions in effector engagements that directly contribute to isoform-specific function, especially in the pro-motility aspects of RhoC. Vega et al. reported that RhoC uniquely works through FMNL3 to restrict lamellopodia broadening in promotion of polarized migration, whereas RhoA engages ROCK1 and ROCK2 at the rear of cells in order to maintain a migratory axis.[20] Kitzing et al. discovered that RhoC specifically engages FMNL2 during an amoeboid migratory response, while Bravo-Codero et al. found that spatiotemporal restricted RhoC signaling dictates the level of cofilin phosphorylation, thereby fostering the freeing of actin-barbed ends required for invadopodia formation during cellular migration.[21,22]

In addition to effector-based signaling differences, selective activation of RhoA/B/C GTPases by guanine nucleotide exchange factors (GEFs) is also likely to produce isoform-specific signaling.[23,24] Two possible mechanisms for selective GEF activation of Rho GTPases are the regulated spatiotemporal intersection of a Rho GTPase and a given GEF, such as through molecular scaffolding, and innate or directed differences in binding affinity between a GEF and the Rho isoforms. Toward an understanding of the latter, Snyder et al. found that Lys 758 of the exchange factor Dbs, once positioned through electrostatic interaction with Glu 54 and Asp 45 of RhoA, participates in van der Waals contacts with Val 43 of RhoA.[25] Subsequent studies with LARG, PDZ-RhoGEF, and p63RhoGEF have all found Val 43 of RhoA hydrophobically engages either a Lys or Arg residue of the

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participating GEF, thereby supporting the conclusion that an R/KL motif at the N-terminal position of the α5 helix of the DH domain is a conserved feature for the vast majority of GEFs which display activity against RhoA.[26-28]

RhoA and RhoC are identical across the initial two-thirds of their amino acid sequences except at position 43, which is an isoleucine in RhoC. As the aforementioned Val 43 of RhoA functions as a "hydrophobic knob" that participates directly in Rho GEF binding, the presence of a bulkier isoleucine in RhoC could sterically disrupt binding of some Rho GEFs.[27] In support, we and others previously isolated a novel GEF, called XPLN, that catalyzed RhoA and RhoB nucleotide exchange in vitro, but exhibited little activity against RhoC.[29] In a residue swapping experiment, XPLN was found to catalyze RhoC-I43V to a much greater extent than wild-type RhoC, while its activity against RhoB-V43I was dramatically lowered compared with RhoB. Here, we advance this initial work by examining the impact of residue 43 identity on both basal and GEF-catalyzed GTPase activity in vitro and in cells. Our findings support the hypothesis that a divergence between RhoA/B and RhoC at residue 43 impacts both basal and GEF-stimulated nucleotide exchange activity.

Results

GST-RhoA/B/C GTPases were mutated at residue 43 to a non-conserved threonine in order to potentially handicap this position's participation in GEF binding. The threonine mutation did not detectably disrupt protein folding, as all three mutant isoforms exhibited statistically equivalent EDTA-stimulated nucleotide loading as equimolar amounts of their wild-type GTPase counterparts (**Table 1**). Although GST-RhoA-43T and GST-RhoC-43T basal nucleotide exchange rates were moderately lower than wild-type GST-RhoA or GST-RhoC, respectively, they were still consistent with EDTA-stimulated nucleotide-loading results, indicating that properly-folded mutant GTPases possess approximately similar core nucleotide rates of exchange as their wild-type GTPase counterpart (**Table 1**).

Wild-type Myc-RhoA produced a round contractile phenotype when transiently expressed in NIH 3T3 fibroblasts (**Fig. 1A**). On

Table 1. Percent wild-type activity of Rho 43T mutants

GST-Protein	% EDTA Loading Capacity	% Basal Exchange Rate
RhoA-43T	92 ± 31	86 ± 11*
RhoB-43T	90 ± 11	105 ± 19
RhoC-43T	86 ± 13	73 ± 11*

Evaluation of Rho 43T fusion protein integrity and basal nucleotide exchange activity. Affinity-purified GST-Rho proteins were incubated in the presence of N-methylanthraniloyl (mant)-GDP and the chelating agent EDTA in order to chemically stimulate nucleotide binding. The extent of nucleotide uptake by the GTPases was measured by a fluorescence shift that was normalized to that of wild-type protein counterparts (provided as % wild type; performed in quadruplicate wells; mean ± st.dev). Disruption of protein folding due to the 43T mutation, which would result in loss of nucleotide binding capacity, was minimal. GST-Rho proteins were alternatively incubated with mant-GDP without EDTA and basal nucleotide uptake examined (provided as % wild-type activity; mean ± st.dev of three separate experiments, n = 8–12 for each experiment). Basal nucleotide exchange rates were consistent with nucleotide-loading results, indicating that properly-folded mutant GTPases possess similar core nucleotide exchange as their wild-type counterparts. *p ≤ 0.03 compared with wild-type enzyme, two-tailed unpaired t-test.

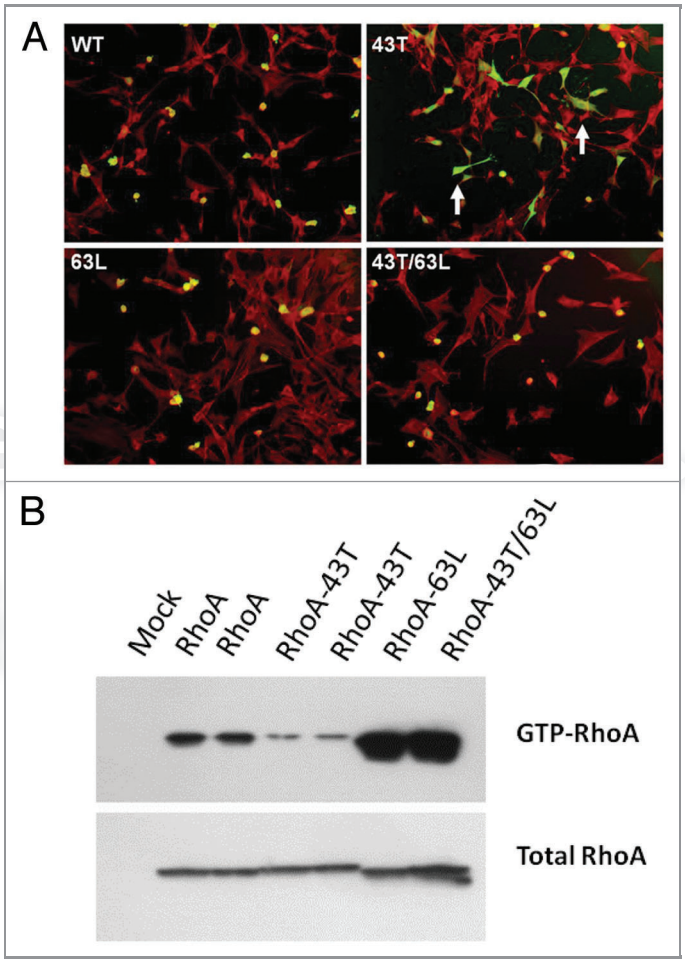

Figure 1. Effect of V43T mutation on RhoA activity in NIH 3T3 fibroblasts. (A) Wild-type Myc-RhoA or Myc-RhoA-43T protein was transiently expressed and the ability to stimulate a round contractile phenotype in serum-deprived NIH3T3 fibroblasts determined through immunofluorescence. Mutation of RhoA residue 43 to a threonine hampered the ability of the GTPase to promote a contractile cell phenotype (white arrows, non-contractile cells). At the same time, expression of either constitutively active Myc-RhoA-63L or Myc-RhoA-43T-63L stimulated similar cell contractility, suggesting the V43T mutation does not detectably impair the ability of Myc-RhoA to bind GTP or effectors involved in the cell contractility response. Green, Myc-RhoA expressing cells; red, phalloidin-bound filamentous actin; yellow, merged. (B) GST-RBD effector pulldown assays were performed for transiently expressed Myc-RhoA, Myc-RhoA-43T, Myc-RhoA-63L and Myc-RhoA-43T-63L, as indicated. Myc-RhoA-43T displayed reduced GTP-loading compared with wild-type GTPase, whereas constitutively active Myc-Rho-43T-63L presented a similar GTP binding profile as Myc-RhoA-63L (comparing levels of GTP-bound to total RhoA). Together, these data indicate that the Myc-RhoA-43T mutation negatively impacts the GTPase at the level of its activation.

the other hand, Myc-RhoA-43T produced an attenuated phenotype in which a number of expressing fibroblasts appeared similar to control cells. Moreover, transiently-expressed Myc-RhoA-43T displayed reduced GTP-loading compared with wild-type Myc-RhoA (**Fig. 1B**), indicating the observed impairment in RhoA signaling may occur at the level of GTPase activation. To examine this possibility further, the V43T mutation was expressed against a constitutively active background (Myc-RhoA-43T-63L). Transiently transfected Myc-RhoA-43T-63L cells all exhibited a round contractile phenotype, and Myc-RhoA-43T-63L exhibited high GTP loading, as would be expected from constitutively-active protein with defective GTP-hydrolyzing capability (**Fig. 1A and B**). Taken together, these data support the conclusion that the V43T mutation does not appreciably impair RhoA folding, but does negatively impact its activation.

To directly examine the impact of the V43T mutation on GEF-catalyzed exchange, purified GST-Rho proteins were incubated in the presence of GEF and a labeled (mant) guanine nucleotide, which exhibits a fluorescence shift when bound into the hydrophobic core of a Rho GTPase.[32] The V43T mutation significantly reduced the activity of Vav2, XPLN, GEFT, Dbs and Dbl against GST-RhoA and GST-RhoB (**Fig. 2**). Presence of the I43T mutation in GST-RhoC significantly reduced GEFT stimulated-exchange, but only weakly impacted activity of Vav2, Dbl and Dbs. XPLN had little to no activity against either GST-RhoC or GST-RhoC-43T. These data support previous work that residue 43 directly contributes to GEF-catalyzed exchange and suggests that the identity of this residue can directly influence GEF activity.[25-28]

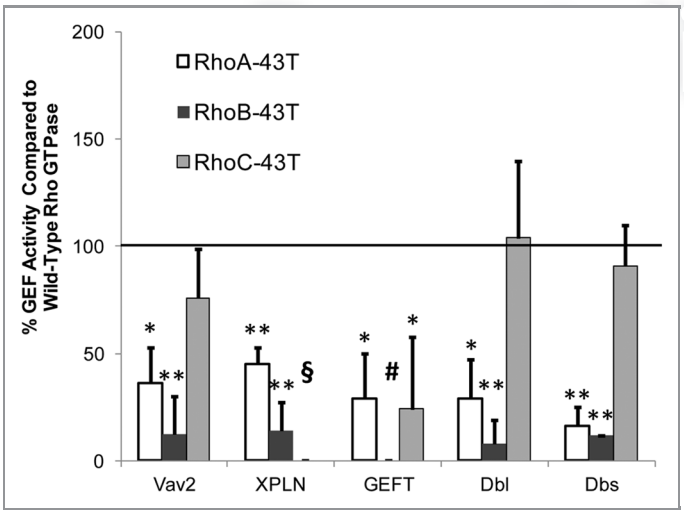

Figure 2. Effect of Rho residue 43 threonine mutation on GEF-stimulated exchange activity. Affinity-purified GST-Rho proteins (3 μM) were equilibrated for 30 min in the presence of mant-GDP in exchange buffer before the indicated GEF protein or control buffer was added and steady-state velocities of nucleotide uptake obtained. Data are presented with standard deviation of mean produced from at least two independent experiments performed in duplicate or triplicate for each condition. Mutation of RhoA or RhoB residue 43 to a threonine negatively impacted GEF-catalyzed exchange for all enzymes examined. The equivalent substitution for RhoC greatly reduced GEFT stimulated-exchange, but only weakly impacted activity of Vav2, Dbl and Dbs. *$p \leq 0.04$, **$p \leq 0.01$ compared with wild-type enzyme, two-tailed unpaired t-test. §, No to little XPLN activity detected against GST-RhoC (1.1-fold) or GST-RhoC-43T (1.4-fold). #, GEFT had no detectable exchange activity against GST-RhoB-43T.

In a residue swap approach, GST-RhoA/B/C GTPases were mutated to either an isoleucine (RhoA and RhoB) or a valine (RhoC) at position 43. GST-RhoA-43I and GST-RhoB-43I exhibited statistically similar EDTA-stimulated nucleotide loading as equimolar amounts of their wild-type GTPase counterparts (**Table 2**), indicating that the mutations did not detectably impact protein folding. GST-RhoC-43V exhibited a higher, but not statistically significant, EDTA-stimulated nucleotide loading profile than wild-type GST-RhoC, suggesting that the presence of the valine at position 43 may facilitate protein folding and/or stabilize wild-type GST-RhoC to a small degree.

Consistent with our previous results, the V43I mutation significantly accelerated basal nucleotide exchange activity of GST-RhoA (**Table 2**).[29] Although GEF-stimulated nucleotide exchange rates were higher for GST-RhoA-43I than GST-RhoA, the fold-stimulation of GEF-catalyzed exchange was, with the exception of GEFT, significantly lower for GST-RhoA-43I due to its elevated basal nucleotide exchange activity (**Fig. 3A and Table 2**). The positive impact of the V43I mutation on RhoA nucleotide exchange was visible in effector pulldown assays of Myc-RhoA and Myc-RhoA-43I transiently expressed in NIH 3T3 fibroblasts (**Fig. 3B**).

The V43I mutation reduced GST-RhoB basal nucleotide exchange activity (**Table 2**). Further, GST-RhoB-V43I was a poorer substrate for XPLN, Dbl, Dbs and to a lesser extent GEFT, whereas Vav2 was relatively insensitive to the GST-RhoB V43I mutation (**Figs. 4 and 5**). Although the I43V mutation reduced GST-RhoC basal nucleotide exchange activity (**Table 2**), it also produced a GTPase that was a significantly better substrate for XPLN, GEFT and Dbl (**Fig. 4**). Transiently-expressed wild-type Myc- or GFP-tagged RhoC and RhoC-43V, however, exhibited no significant difference in their GTP-loading profile, while Myc-RhoC-43T displayed, as expected, reduced GTP-loading compared with wild-type Myc-RhoC (**Fig. 6A and B**).

Table 2. Percent wild-type activity of Rho 43I/V mutants

GST-Protein	% EDTA Loading Capacity	% Basal Exchange Rate
RhoA-43I	115 ± 26	290 ± 107*
RhoB-43I	101 ± 3	69 ± 8*
RhoC-43V	143 ± 56	62 ± 18*

Evaluation of Rho 43I/V fusion protein integrity and basal nucleotide exchange activity. Mutating RhoA or RhoB residue 43 to an isoleucine had little impact on folding/GTP-loading potential, but strongly increased GST-RhoA-43I basal exchange activity (values normalized and collected as described in **Table1**). Mutating RhoC residue 43 to a valine produced slightly higher, but not statistically significant, loading capability for the GTPase. GST-RhoB-43I and GST-RhoC-43V basal exchange rates were significantly less than wild-type GST-RhoB or GST-RhoC, respectively. These data suggest that swapping hydrophobic residues between RhoA and RhoC impacts their rates of basal nucleotide exchange. *$p \leq 0.02$ compared with wild-type enzyme, two-tailed unpaired t-test.

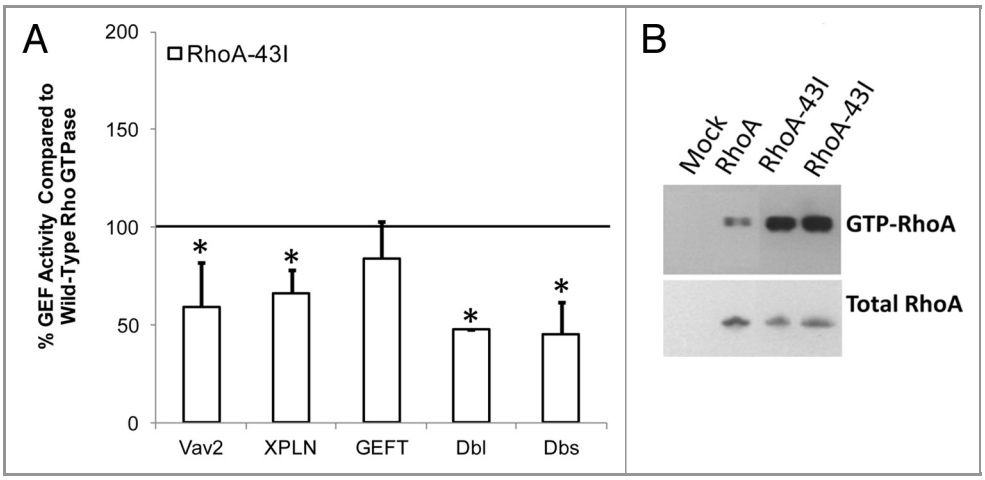

Figure 3. Impact of RhoA-V43I mutation on in vitro GEF activity and GTP-loading in fibroblast. (A) GST-RhoA or GST-RhoA-V43I basal and GEF-catalyzed nucleotide exchange were measured (data are presented with standard deviation of mean produced from at least two independent experiments performed in duplicate or triplicate for each condition). Although GEF-stimulated nucleotide exchange rates were higher for GST-RhoA-43I than GST-RhoA, the fold-stimulation of GEF-catalyzed exchange was, with the exception of GEFT, significantly lower for GST-RhoA-43I due to its elevated basal nucleotide exchange activity (**Table 2**). *$p \leq 0.02$ compared with wild-type enzyme, two-tailed unpaired t-test (B) GST-RBD effector pulldown assays were performed for transiently expressed Myc-RhoA or Myc-RhoA-43I. Consistent with the more robust exchange activity observed for GST-RhoB-43I in vitro, Myc-RhoA-43I protein displayed greater GTP-loading than wild-type RhoA GTPase when expressed in fibroblasts.

As GST-RhoC-43V was a better GEF substrate in vitro, the effect of transient expression of GFP-RhoC and its mutants on ovarian cancer invasion of matrigrel-coated chambers was assessed. In support of the conclusion that divergence at position 43 influences Rho GTPase activity, GFP-RhoC-43V was significantly more effective at driving ovarian cancer cell invasion than either wild-type GFP-RhoC or GFP-RhoC-43T (**Fig. 7A**), even though its transient expression levels were found to be consistently lower in these experiments (**Fig. 7B**). Neither wild-type GFP-RhoA, GFP-RhoA-43T or GFP-RhoA-43I were capable of stimulating ovarian cancer cell invasion when transiently expressed (data not shown).

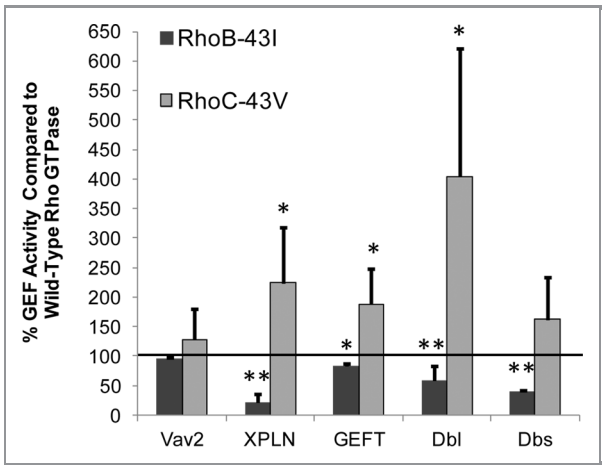

Figure 4. GEF-stimulated exchange activity against GST-RhoB-43I and GST-RhoC-43V. Data are presented with standard deviation of mean produced from at least two independent experiments performed in duplicate or triplicate for each condition. Mutation of RhoB residue 43 to an isoleucine strongly and negatively impacted XPLN-, Dbl- and Dbs-catalyzed exchange, modestly reduced GEFT-catalyzed exchange and had little impact on Vav2-stimulated nucleotide exchange. Mutation of RhoC residue 43 to a valine produced a GTPase that was a better substrate for XPLN and Dbl and to a lesser extent GEFT and Dbs. Vav2 was not sensitive to the I43V mutation of GST-RhoC. *$p \leq 0.05$, **$p \leq 0.01$ compared with wild-type enzyme, two-tailed unpaired t-test.

Discussion

In this work, we found a divergence between RhoA/B and RhoC at residue 43 impacts basal and GEF-stimulated nucleotide exchange activity. The inability of a small polar threonine to provide similar functionality as a hydrophobic valine at amino acid position 43 in RhoA and RhoB provides additional evidence that the van der Waals interaction between Val 43 and a basic Lys or Arg of the participatory GEF is indispensible for proper exchange activity.[25-28] At the same time, Dbs and Dbl and to a lesser extent Vav2 had similar activity against GST-RhoC and GST-RhoC-43T, suggesting that molecular engagement between GEFs and RhoC varies in residue 43 participation. Such differential effects of the threonine substitution on three GTPase isoforms supports the conclusion that a GEF which stimulates RhoA exchange should not necessarily be expected to possess activity against RhoC.

In addition to differences in GEF-catalyzed exchange, RhoC has also been reported to possess a higher intrinsic rate of nucleotide exchange than RhoA.[33] In support, we found that GST-RhoA-43I possesses faster basal nucleotide exchange activity than wild-type enzyme, while the reverse was observed for GST-RhoC-43V (**Table 2**). As such, evolutionary mutation of RhoC to an isoleucine at position 43 may provide the GTPase with faster core nucleotide exchange necessary for isoform-specific function, whereas RhoA remained a more GEF-inducible GTPase. In

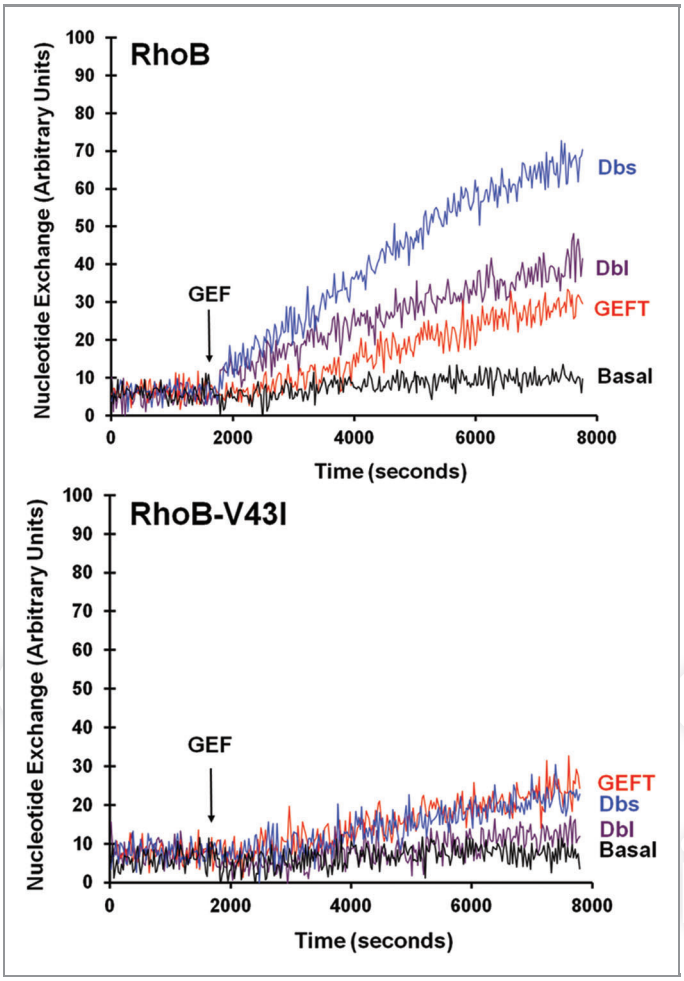

Figure 5. GEF-catalyzed exchange activity against GST-RhoB and GST-RhoB-43I. In this representative experiment, GST-RhoB or GST-RhoB-43I protein were equilibrated in the presence of mant-GDP in exchange buffer before addition of GEF or control buffer (basal) at the indicated time (arrow). Presence of the isoleucine mutation negatively impacted Dbs- and Dbl-catalyzed exchange, but exhibited only a modest impact on GEFT-stimulated exchange activity.

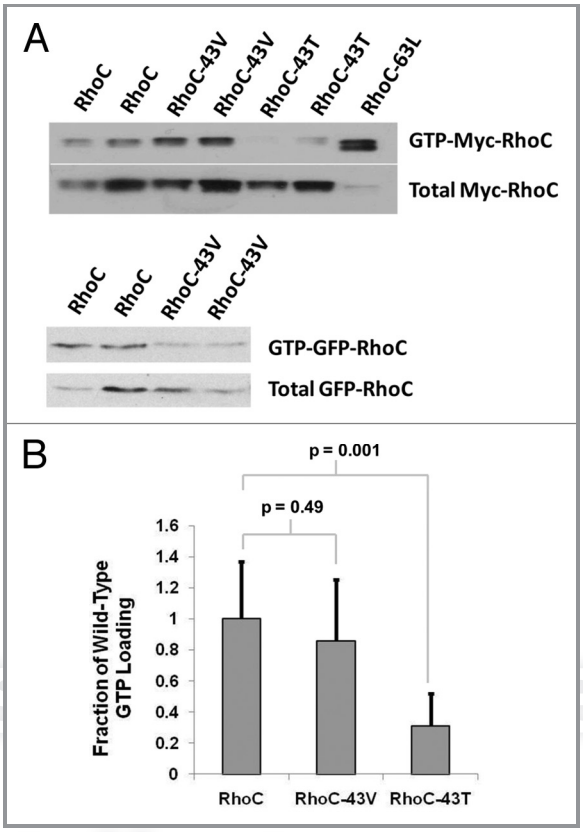

Figure 6. Profile of RhoC residue 43 mutations on GTP loading profile in ovarian cancer cells. (A) Myc-RhoC or GFP-RhoC fusion proteins were transiently expressed in OVCA 429 ovarian cancer cells and their GTP loading profiles analyzed by effector pulldown (RBD) assays. In these representative experiments, RhoC-43V displayed variable but statistically similar GTP-loading profile as wild-type RhoC, whereas RhoC-43T consistently exhibited reduced GTP-loading. Myc-RhoC-63L was included as a constitutively active positive control for the Myc-RhoC pulldowns. (B) Densitometry of four unique experiments (duplicate) involving Myc- or GFP-tagged RhoC fusion proteins support the conclusion that a similar GTP-loading profile exists between wild-type RhoC and RhoC-43V, whereas RhoC-43T has significantly reduced GTP-loading (two-tailed unpaired t-test).

accordance, switching of nucleotide-binding phenotype through residue swapping was not limited to basal exchange, as GST-RhoA-43I (or GST-RhoB-43I) was a poorer substrate for a number of the RhoGEFs examined, while GST-RhoC-43V was a better substrate for some of these same GEFs, including XPLN (**Figs. 3 and 4**).

Somewhat unexpectedly, the V43I mutation of GST-RhoB yielded a slower, not faster, basal nucleotide exchange rate compared with wild-type enzyme (**Table 2**). However, while RhoA and RhoC differ at only residue 43 across the initial two-thirds of their amino acid sequences, RhoB additionally diverges at five other positions across the same stretch (Val 10, Glu 29, Val 86, Val 100 and Ala 116); a dissimilarity that could potentially change the overall structure of RhoB such that the V43I mutation differently affects core nucleotide exchange as compared with RhoA. Regardless, theV43I mutation was still sufficient to change the substrate profile of GST-RhoB for XPLN, GEFT, Dbs and Dbl, further strengthening the conclusion that residue 43 is a key contributor to GEF-catalyzed exchange of all three Rho isoforms (**Figs. 4 and 5**).

Vav2, which is considered to be a GEF with a broad range of substrates, was relatively insensitive to the swapping of residue 43 identities in RhoB and RhoC.[24] In addition, GEFT, which has been reported to be both a Rac and RhoA-specific GEF, was found to be somewhat refractory to the presence of a valine or isoleucine in all three isoforms.[34,35] At the same time, the other GEFs (XPLN, Dbl and Dbs) were sensitive to the residue switch; they were less accommodating of the bulkier isoleucine in either GST-RhoA-43I or GST-RhoB-43I and demonstrated higher activity against GST-RhoC-43V. The breakdown in GEF exchange profiles were not without exception, however, as GST-RhoA-43I was a poorer substrate for Vav2. Nonetheless, these data clearly indicate that Rho GEFs exhibit variable sensitivity to valine vs. isoleucine at position 43. As such, our work demonstrates that

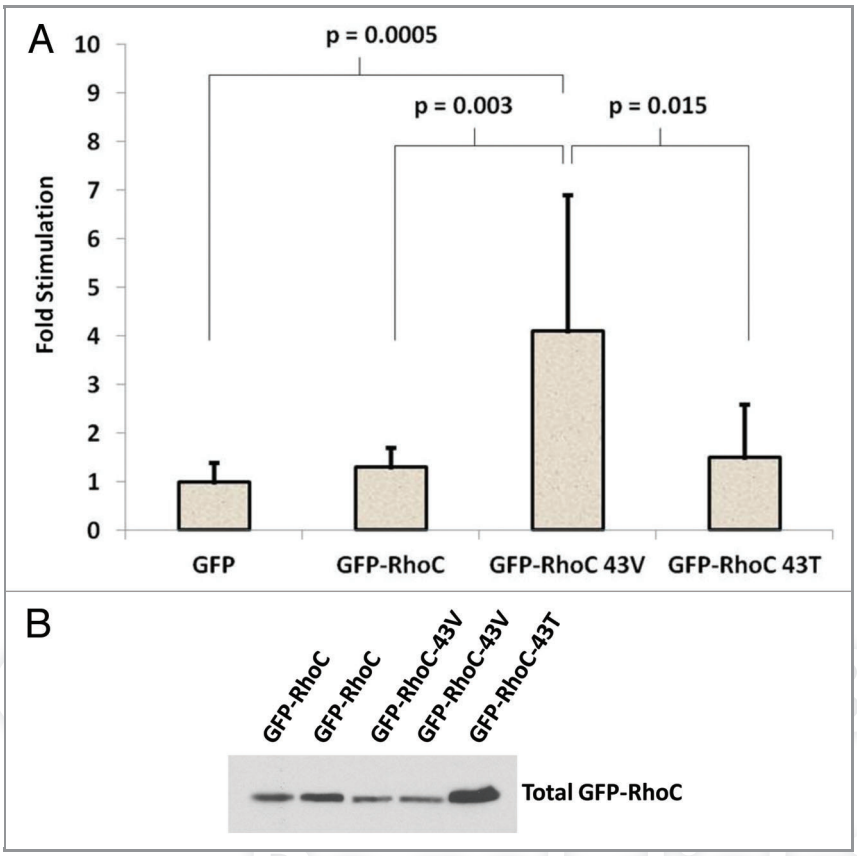

Figure 7. Impact of RhoC on ovarian cancer invasion. (A) Although RhoC-43V displays similar GTP-loading as RhoC, in vitro exchange data using purified protein indicates that RhoC-43V is better GEF substrate and therefore may possess altered signaling activity when expressed in cells. GFP or GFP-RhoC fusion proteins were transiently expressed in ovarian cancer cells and then examined for their ability to drive matrigel invasion. GFP-RhoC-43V significantly promoted ovarian cancer invasion in three different experiments done with triplicate filters using two different ovarian cancer cell lines (OVCA 429 n = 2 and OVCA 433 n = 1). Reported p values were obtained from Mann-Whitney U analysis of the number of counted cells produced from each filter. In data not shown, transient expression GFP-RhoA, GFP-RhoA-43I, and GFP-RhoA-43T had no impact on ovarian cancer cell invasion. (B) Although comparable transfection efficiency was confirmed by fluorescence analysis of seeded cells, expression levels of GFP-RhoC-43V were reproducibly lower and GFP-RhoC-43T consistently higher than wild-type GFP-RhoC in these experiments.

divergence at this position is sufficient to offer a viable mechanism through which GEFs can selectively activate RhoA or RhoC.

The finding that wild-type Myc-RhoC and Myc-RhoC-43V displayed similar GTP-loading in effector pulldown assays may be due to reduction in basal nucleotide exchange activity of Myc-RhoC-43V (**Fig. 6 and Table 2**). Alternatively, the I43V mutation may change the availability of the GTPase for an exchange event, such as through RhoGDI binding and sequestration. Although GTP-loading profiles were similar, exogenous expression of Myc-RhoC-43V was more effective than Myc-RhoC in facilitating ovarian cancer invasion, thus demonstrating that modification of residue 43 can directly affect enzyme signaling/function. While our data suggest that the increase in Myc-RhoC-43V activity in ovarian cancer cells is the result of increased GEF-exchange against the GTPase, the lack of an increase in GTP-loading profile suggests this is an incomplete interpretation. There is a dearth of work exploring whether the residue 43 divergence impacts effector binding, however, Kitzing et al. have recently reported that the I43V mutation weakened binding of the effector FMNL2 to RhoC.[22] As FMNL2 signaling is expected to facilitate invasion, it is worth noting that FMNL2 still bound RhoC-I43V. Further, it is unclear how much FMNL2 might contribute to OVCA cellular invasion because RhoC/FMNL2 signaling was positively linked to amoeboid-based, but not mesenchymal-based, invasion.[22] Nevertheless, the FMLN2 work raises the possibility that the I43V mutation potentially change RhoC signaling in addition to modifying GEF-catalyzed activation such that stimulation of OVCA cellular invasion is advanced.

Altogether, the work presented here offers insight on the contribution of a key molecular divergence between three closely related Rho isoforms. Specifically, the mutation of RhoC to a bulkier isoleucine residue at position 43 is postulated to have enhanced basal nucleotide exchange while variably reducing GEF-stimulated nucleotide exchange. Future work will continue to explore the potential impact of this residue's identity on RhoA and RhoC membrane association and effector binding.

Materials and Methods

Materials. Bovine serum albumin, c-Myc monoclonal antibody (clone 9E10), and buffer reagents were acquired from Sigma. Monoclonal antibody against GFP (clones 7.1 and 13.1) was purchased from Roche. Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore. NIH 3T3 fibroblast cells were maintained in growth medium (DMEM) supplemented with 10% bovine calf serum (Biowhittaker). OVCA 429 and OVCA 433 ovarian cancer cell lines were maintained in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Biowhittaker).

Expression constructs. Creation of human RhoA, RhoB, RhoC, XPLN and RhoA 63L pGEX4T and pCMV-Myc expression plasmids was previously described.[29] pEGFP-RhoA and pEGFP-RhoC were created by subcloning RhoA or RhoC cDNA into pEGFP-C1 (Clontech) using BglII and SalI restriction sites. Rho mutations (RhoA-V43I, RhoA-V43T, RhoA-V43T/Q63L, RhoB-V43I, RhoB-V43T, RhoC-I43V, and RhoC-I43T) were created through PCR mutagenesis using the Quickchange mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing, and cDNAs subcloned into either pGEX4T-1 (Amersham Biosciences), pCMV-Myc (Clontech), or pEGFP-C1 plasmids. pProEX-HT-Dbl (DH/PH) was a gift of Dr Kent Rossman

(University of North Carolina at Chapel Hill). pProEX-HT-GEFT was a gift of Dr Krister Wennerberg (Institute for Molecular Medicine Finland). RhoC-63L cDNA was a gift of Dr Natalia Mitin (University of North Carolina at Chapel Hill) and subcloned into pCMV-Myc using EcoRI and XhoI restriction sites.

Fusion proteins. Human GST-Rho and GST-XPLN (full length) fusion proteins were purified from BL21 *E. coli* cells (Stratagene) using glutathione-Sepharose 4B (Amersham Biosciences). Proteins were eluted with free and reduced glutathione in TBSM (50 mM Tris, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) and stored in 30% glycerol. 6xHis-Dbl DH/PH (murine) and full-length 6xHis-GEFT (murine) were purified from BL21 *E. coli* cells using Ni NTA-sepharose (Qiagen) with a gradient imidazole elution. Free imidazole was cleared with a PD10 desalting column (Amersham Biosciences) prior to protein storage in TBS containing 30% glycerol. Purified human 6xHis-Vav2 DH/PH/CRD (192–573) was a gift of Dr M. Booden (University of North Carolina at Chapel Hill). Murine 6xHis DH/PH Dbs (628-967) was a gift of Dr. K. Rossman (University of North Carolina at Chapel Hill).

Transfections. NIH 3T3 fibroblast or OVCA ovarian cancer cells were transfected in the presence of serum with the indicated expression vectors according to the manufacturer's protocol using LipofectAMINE and PLUS reagents (Invitrogen).

RhoA and RhoC GTP profile assays. The amount of GTP-bound RhoA or RhoC protein was examined using a technique similar to the method described by Ren and colleagues.[30] Briefly, transfected cells were lysed in 300 µL of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate and protease inhibitors. Five hundred to 750 µg of lysates were cleared at 16,000 x g for 5 min, and the supernatant rotated for 30 min with 30 µg GST-RBD [GST fusion protein containing the Rho-binding domain (RBD), amino acids 7–89 of Rhotekin] bound to glutathione-sepharose beads. Samples were washed in 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100 and protease inhibitors. GST-RBD pulldowns and lysates were then western blotted with anti-c-myc antibodies. To quantify GST-RBD pulldowns, western blots of lysates and corresponding GST-RBD pulldowns from multiple unique experiments done in duplicate were scanned and densitometry performed using Photoshop imaging software.

In vitro guanine nucleotide exchange factor assays. Fluorescence spectroscopic analysis (excitation = 360 nm, emission = 460 nm) of N-methylanthraniloyl (mant)-GTP or mant-GDP (Biomol) incorporation into GST-Rho proteins was performed using a Varian fluorescence microplate (96-well) reader at 25°C similar to as described previously.[29] Briefly, 3 µM of GST-Rho GTPase was prepared and allowed to equilibrate in exchange buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 µg/ml bovine serum albumin, 1% glycerol) containing 750 nM mant-GTP for 30 min at 25°C to ensure equivalent and consistent baseline nucleotide incorporation activity across wells. Varying amounts (100–500 nM) of DH/PH (Dbl, Dbs), DH/PH/CRD (Vav2), or full-length (XPLN, GEFT) protein or buffer control were subsequently added and relative mant-nucleotide fluorescence immediately monitored. Each condition was performed in duplicate or triplicate for every experiment. Steady-state velocity of basal or GEF-catalyzed nucleotide exchange was determined as previously described.[31] Briefly, baseline or GEF-induced rates of nucleotide exchange were calculated by dividing the change in emission at 460 nm by change in time and found to be linear with correlations at or above 0.9. Calculated steady-state GEF-catalyzed rates were averaged between samples and normalized to wild-type Rho protein basal exchange activity in order to compare results across different experiments.

EDTA-loading of GST-Rho proteins. GST-RhoA and GST-RhoB fusion proteins immobilized on sepharose were incubate for 45 min at 32°C in loading buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1 mg/mL BSA, 2 mM DTT, 10 mM EDTA) with either 5 µM GDP or mant-GDP. Loading was stabilized by adding 40 mM $MgCl_2$. Proteins were then incubated on ice for 15 min, washed with TBS with 5 mM $MgCl_2$ and eluted with 250 mM free glutathione. The extent of loading was measured as the fluorescence intensity of mant-GDP loaded samples normalized to GST-Rho protein concentration and corrected for values obtained from GDP control. Alternatively, GST-RhoC proteins, which were otherwise unstable during the aforementioned loading process, were eluted from sepharose and immediately incubated in loading buffer containing 5 µM mant-GDP. The fluorescence intensity of each sample was then measured after a 5 min equilibration, normalized to GST-RhoC protein concentration, and corrected for fluorescence intensity obtained from samples containing loading buffer with mant-GDP alone.

Immunofluorescence. NIH 3T3 fibroblasts were grown overnight on ethanol-washed glass coverslips in the presence of serum prior to transfection. Transfected cells were allowed to express exogenous protein overnight before a 10 min fixation in 3.7% formaldehyde in PBS. Washed and fixed cells were permeabilized for 5 min in 0.5% Triton X-100 in PBS prior to staining. Filamentous actin was labeled with Texas Red conjugated phalloidin (Molecular Probes). Exogenous RhoA expressing cells were visualized using anti-c-myc monoclonal antibody and an Alexa Fluor 488 goat anti-mouse secondary antibody (Jackson ImmunoResearch). Images were obtained on an Olympus spinning disc confocal microscope using a CoolSNAP E2 CCD camera (Photometrics) and Metamorph Image software (Universal Imaging Corp.).

Matrigel invasion assays. OVCA cells were transfected with equivalent amount of pEGFP or the indicated pEGFP-Rho expression plasmid. Following an over-night expression, cells were collected with trypsin, neutralized with serum, and washed twice with large volumes of serum-free minimum essential media. Transfection efficiency was ascertained for each condition by visually counting both the total number of cells and the number of green fluorescing cells (Zeiss Axiophot microscope) in random fields of a hemocytometer. The percentage (12–15%) of cells scored as expressing was consistent between expression conditions for every experiment. For each condition, approximately 100,000 OVCA cells were seeded into hydrated 8 µm BioCoat Matrigel invasion chambers (BD Biosciences). Serum-containing growth media was administered to outer wells. After 24 h incubation under standard cell culture conditions, individual chambers were

fixed, stained and scored for cell invasion according to manufacturer's instructions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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