The Tumor Suppressor DiRas3 Forms a Complex with H-Ras and C-RAF Proteins and Regulates Localization, Dimerization, and Kinase Activity of C-RAF*†‡§

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Background: The tumor suppressor DiRas3 interferes with the Ras/MAPK mitogenic cascade.

Results: DiRas3 associates with H-Ras and C-RAF and regulates localization, dimerization, and kinase activity of C-RAF.

Conclusion: DiRas3 regulates the Ras/MAPK cascade at the level of Ras/RAF signal transmission.

Significance: Learning how DiRas3 affects the Ras/MAPK signaling is crucial for understanding the molecular mechanisms underlying DiRas3-mediated tumor suppression.

The maternally imprinted Ras-related tumor suppressor gene DiRas3 is lost or down-regulated in more than 60% of ovarian and breast cancers. The anti-tumorigenic effect of DiRas3 is achieved through several mechanisms, including inhibition of cell proliferation, motility, and invasion, as well as induction of apoptosis and autophagy. Re-expression of DiRas3 in cancer cells interferes with the signaling through Ras/MAPK and PI3K. Despite intensive research, the mode of interference of DiRas3 with the Ras/MAPK/mitogenic cascade is still a matter of speculation. In this study, we show that DiRas3 associates with the H-Ras oncogene and that activation of H-Ras enforces this interaction. Furthermore, while associated with DiRas3, H-Ras is able to bind to its effector protein C-RAF. The resulting multimeric complex consisting of DiRas3, C-RAF, and active H-Ras is more stable than the two protein complexes H-Ras-C-RAF or H-Ras-DiRas3, respectively. The consequence of this complex formation is a DiRas3-mediated recruitment and anchorage of C-RAF to components of the membrane skeleton, suppression of C-RAF/B-RAF heterodimerization, and inhibition of C-RAF kinase activity.

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The Ras/RAF/MEK/ERK mitogenic cascade is one of the essential signal transduction pathways in the cell involved in the control of different cellular processes, including proliferation, differentiation, transformation, survival, adherence, and motility. This pathway couples extracellular signaling via ligand-bound receptor tyrosine kinases, SOS (“son of sevenless”), and activated Ras to the cytoplasmic extracellular signal-regulated kinase (ERK), a multifunctional kinase, which in turn phosphorylates and activates several proteins in the cytosol and nucleus, including various transcription factors such as Elk-1, Ets, and Sp1 (1, 2). The interaction of the GTP-loaded Ras with RAF, the key regulator of the pathway, represents the initial and essential step in the activation of the mitogenic cascade.

The family of RAF protein kinases, which includes A-, B-, and C-RAF, shares three highly conserved regions: CR1, CR2, and CR3. The CR3 region represents the catalytic domain, whereas CR1 contains a Ras binding domain and a zinc binding domain that is also called the cysteine-rich domain. The serine/threonine-rich CR2 contains a conserved 14-3-3-binding motif. Cytosolic C-RAF exists as a multiprotein complex consisting of heat shock proteins, 14-3-3 proteins, and the kinase suppressor of Ras. Upon stimulation of cell surface receptors, C-RAF translocates to the plasma membrane, associates with Ras-GTP, and undergoes a series of activation events, including interaction with lipids and regulatory proteins, numerous phosphorylation and dephosphorylation events, as well as dimer formation (for review see Refs. 3–6). RAF dimerization proved to be one of the most decisive steps in the regulation of RAF activity, particularly in light of recent findings concerning the paradoxical behavior of some RAF inhibitors in cancer treatment (7–11).

The Ras subfamily of GTP-hydrolyzing oncoproteins is part of a large superfamily of more than 170 evolutionarily conserved proteins related to Ras. About 35 members constitute the Ras subfamily, whereby H-, N-, and K-Ras represent the best characterized members (12, 13). Ras proteins alternate between the GTP-bound (active “on” state) and GDP-bound (inactive “off” state) conformations. As the “switch-on” and “switch-off” reactions in the cycle of Ras are intrinsically very low, the regulatory input by guanine nucleotide exchange factors and GTase-activating proteins determines the lifetime of the two states (14–16). Binding of Ras effector proteins to GTP-Ras triggers distinct signaling cascades. RAF kinase was first discovered as a Ras effector followed by RalGDS and PI3K. The family of Ras effectors expanded over the past years and includes currently more than 10 different proteins (13). Ras proteins are synthesized as cytosolic precursors that undergo
post-translational processing (C-terminal prenylation and palmitoylation) to be able to associate with cellular membranes (12, 17, 18). Localization of Ras at the cytosolic leaflet of cellular membranes is believed to be required for their biological activity. Ras signaling events previously presumed to be restricted to the plasma membrane have now been observed on intracellular membranes, including endosomes, the endoplasmic reticulum, and the Golgi apparatus (19–24).

The small GTPase DiRas3 (also referred as NOEY2 and ARHI) belongs to the Ras family of proteins and shares 55–62% homology with Ras and Rap (25, 26). Intriguingly, in contrast to most Ras proteins and despite its high degree of GTP-bound state in resting cells, DiRas3 acts as a tumor suppressor, thus possessing entirely different functional properties compared to most Ras proteins and despite its high degree of GTP-bound homology with Ras and Rap (25, 26). Intriguingly, in contrast to ARHI) belongs to the Ras family of proteins and shares 55–62% homology with Ras and Rap (25, 26). Intriguingly, in contrast to ARHI. The DiRas3 gene encodes a 26-kDa protein that is monoallelically expressed and maternally imprinted (25). As a member of the Ras protein family, DiRas3 contains three typical motifs as follows: a GTP binding domain, a putative effector domain, and the membrane localization motif CAAAX (where AA is aliphatic amino acid and X is any amino acid) (15). However, there are also some unique characteristics, which distinguish DiRas3 from other members of the Ras protein family. It contains a 34-amino acid extension at the N terminus and differs from H-Ras in residues critical for GTPase activity and for putative effector function. The substitutions within the GTP binding domain of DiRas3 are consistent with the mutations of Ras responsible for its constitutive activation. Correspondingly, DiRas3 has been found predominantly in its GTP-bound state in cells (27). DiRas3 is lost or down-regulated in more than 60% of ovarian and breast cancers through several different mechanisms, including loss of heterozygosity, DNA hypermethylation, transcriptional regulation, and shortened mRNA half-life (26, 28). Loss of DiRas3 expression is associated with tumor progression and poor prognosis (29, 30). Re-expression of DiRas3 in cancer cells inhibits growth, decreases invasiveness, and induces apoptosis (25, 31). Signaling alterations caused by introduction of the DiRas3 gene into cancer cells lacking DiRas3 expression range between inhibition of the Ras/MAPK pathway, activation of JNK, inhibition of the STAT3 transcriptional activity, and down-regulation of cyclin D1 (25, 27, 32).

The studies reported on DiRas3 function so far suggest that the biological activities of DiRas3 GTPase could not only be explained by its effects on a single pathway. Despite considerable progress, the molecular mechanisms of the DiRas3 tumor suppressive activity are not sufficiently elucidated. In particular, the mode of DiRas3 interference with the Ras/MAPK signaling cascade is still a matter of speculation. In this study, we report that DiRas3 interacts with the H-Ras oncogene and that activation of H-Ras enforces its association with DiRas3, indicating that the tumor suppressive activity of DiRas3 is achieved, at least in part, at the level of Ras signaling. Furthermore, our study reveals that, although associated with DiRas3, H-Ras is able to bind to its effector C-RAF and that the multimeric complex consisting of DiRas3, C-RAF, and active H-Ras is more stable than the two-protein complexes H-Ras-C-RAF or H-Ras-DiRas3, respectively. The consequence of this complex formation is a DiRas3-coordinated translocation and anchorage of C-RAF to components of the membrane skeleton (MSK). 2 In addition, DiRas3 disrupts the H-Ras-induced heterodimerization of C-RAF with B-RAF and suppresses the kinase activity of C-RAF.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used: mouse anti-c-Myc (9E10), rabbit anti-C-RAF (RAF-1 and C-12), mouse anti-HA (12CA5), mouse anti-KDEL (10C3), mouse anti-pERK (E-4), rabbit anti-ERK2 (C-14), rabbit anti-B-RAF (C-19), and mouse anti-vimentin (V9) from Santa Cruz Biotechnology; mouse anti-H-Ras (catalog no. R02120) from BD Transduction Laboratories; rabbit anti-phospho-C-RAF-Ser-338 (catalog no. 56A6, was also used for detection of phosphoSer-446 in B-RAF) from Cell Signaling Technology; mouse anti-M2PK (catalog no. DF4) from Schebo Biotech; rabbit anti-EEA1 (catalog no. E3906) from Sigma; mouse anti-PARP-1 (catalog no. C-2-10) from Calbiochem; and mouse anti-penta-His™ (catalog no. 34660) from Qiagen. The anti-DiRas3 (6EC.2) antibody (kindly provided by R. Kroschewski) was raised in rabbit against partially purified full-length native His6-DiRas3. The horseradish peroxidase-labeled (for Western blot) and Cy2- or Cy3-conjugated (for indirect immunofluorescence microscopy) anti-mouse and anti-rabbit secondary antibodies were from Dianova.

**Plasmids**—The following plasmids were used: human C-RAF WT-Myc-His in pcDNA3, human HA-C-RAF WT, and HA-C-RAF-R89L in pcDNA3; human B-RAF WT-Myc-His in pcDNA3 and human B-RAF WT in pCMV; human DiRas3-Myc and ΔNDiRas3-Myc in pRK5; human H-Ras12V, H-Ras17N, H-Ras12V/35S, H-Ras12V/37G, H-Ras12V/40C, H-Ras12V/186S, and H-Ras12V/181/4SS in pcDNA3; and human K-Ras12V in pEXV3. The plasmids encoding mutants of H-Ras12V were kindly provided by I. Rubio (University of Jena).

**Immunoprecipitation**—The required cDNA plasmids were transfected into COS7 cells under starvation conditions using jetPEI transfection reagent (Polyplus Transfection). Cells were lysed 24 h after transfection with buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 30 mM sodium pyrophosphate, 100 μM Na3VO4, 1% Triton X-100, and standard proteinase inhibitors for 45 min at 4°C. The lysates were clarified by centrifugation at 27,000 × g for 15 min and incubated for 1 h at 4°C with the appropriate antibody. After addition of protein G-agarose, the incubation was continued for 2 h at 4°C. The agarose beads were washed three times with lysis buffer containing 0.1% Triton X-100. The immunoprecipitates were supplemented with Laemmli buffer, boiled for 5 min at 100°C, and applied to SDS-PAGE. After Western blotting, the isolated proteins were visualized by appropriate antibodies.

**In Vitro Kinase Assay**—The kinase assay was carried out directly with immunoprecipitated proteins in 25 mM Hepes (pH 7.6), 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM Na3VO4, and 500 μM ATP buffer (50-μl final volume). Recombinant MEK and ERK-2 were used as substrates. After incubation for 30 min at 30°C,
the kinase assay mixtures were supplemented with Laemml buffer, boiled for 5 min at 100 °C, and applied to SDS-PAGE. After Western blotting, the extent of ERK phosphorylation was determined by an anti-phospho-ERK antibody.

Subcellular Fractionation—Cell fractions were isolated using the ProteoExtract subcellular proteome extraction kit (Calbiochem). COS7 cells were grown on 10-cm Petri dishes and transfected with appropriate cDNA constructs under starvation conditions. The cells were fractionated into four subproteomic fractions (cytosolic and nuclear fractions, fractions of whole membranes, and cytoskeleton) according to the manufacturer’s protocol. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The selectivity of subcellular extraction was documented by immunoblotting against marker proteins (M2PK for cytosolic fraction, KDEL (endoplasmic reticulum retention signal) for membrane fraction, poly(ADP-ribose) polymerase for nuclear fraction, and vimentin for cytoskeletal fraction).

Indirect Immunofluorescence Microscopy—COS7 cells were grown on coverslips and transfected with required cDNA plasmids. The cell imaging was performed either with whole cells or following cytoplasm and/or membranes extraction by use of ProteoExtract subcellular proteome extraction kit (Calbiochem). For the whole cell imaging, cells were fixed with 4% paraformaldehyde in 1 × PBS (Morphisto) for 15 min and permeabilized with 0.1% v/v Triton X-100 in 1 × PBS for 5 min. For imaging of the cytoplasm/membrane-depleted cells, fixation (15 min) with 4% paraformaldehyde in 1 × PBS was performed immediately after extraction. In all cases, fixation was stopped by incubation with 50 mM NH4Cl in 1 × PBS for 15 min. Immunostaining of the proteins was performed with specific antibodies and fluorescently labeled secondary antibodies that were included in the staining procedure. The staining of the proteins was performed with specific antibodies and fluorescently labeled secondary antibodies that were included in the staining procedure. The staining of the proteins was performed with specific antibodies and fluorescently labeled secondary antibodies that were included in the staining procedure.

RESULTS

DiRas3 Binds Preferentially to the Active and Nonfarnesylated H-Ras—Several reports documented that the growth of the ovarian and breast cancer cell lines could be inhibited by exogenous expression of DiRas3 (25, 27, 32). The DiRas3-induced growth inhibition has been proved by use of various cell lines such as HeLa, Saos-1, NIH3T3, 293, and COS7 (25). Although much effort has been devoted to the study of this phenomenon, the mechanism by which DiRas3 inhibits cell proliferation is still not fully understood. Yu et al. (25) and Luo et al. (27) have reported that introduction of the DiRas3 gene into cancer cells triggers apoptosis, down-regulates cyclin D1, activates JNK, and impairs signaling through the Ras/MAPK pathway. As the Ras/RAF/MEK/ERK cascade is a central pathway coordinating cell proliferation, differentiation, migration, and apoptosis, it appears very likely that DiRas3 may interfere with signal transduction between the modules of this cascade.

The Ras/RAF interaction represents the initial and crucial step in the signal transduction of the Ras/RAF/MEK/ERK cascade. Regarding the mode of Ras coupling to RAF, the formation of Ras homodimers has been reported to be essential for C-RAF activation (33, 34). Also numerous other GTPases are regulated by dimerization (33, 35). Therefore, we hypothesized that DiRas3 may regulate the Ras/RAF/MEK/ERK cascade by formation of heterodimers with H-Ras. To test this issue, we performed coimmunoprecipitation of the constitutively active H-Ras12V or dominant-negative H-Ras17N with the Myctagged DiRas3. To this end, the proteins of interest were expressed in COS7 cells. The results of this assay revealed that DiRas3 indeed formed a stable complex with H-Ras in vivo (Fig. 1A). Moreover, DiRas3 bound preferentially to the H-Ras12V mutant suggesting that activation of H-Ras enforced its association with DiRas3. Interestingly, DiRas3 appeared to interact with both farnesylated (the lower band in the anti-H-Ras blot (36)) and nonfarnesylated (the upper band in the anti-H-Ras blot (36)) forms of the inactive H-Ras17N. On the contrary, in the case where the active H-Ras12V was assayed, DiRas3 bound exclusively to its nonfarnesylated form (Fig. 1A). As the unique N-terminal extension of DiRas3 has been shown to be important for its inhibitory effect on cell growth (27), we also tested the DiRas3 mutant lacking the 34 amino acid N-terminal extension (ΔNDiRas3) for its ability to interact with H-Ras. As shown in Fig. 1A, N-terminal deletion reduced the binding affinity of DiRas3 to H-Ras.

H-Ras Lipidation Sites Are Not Required for the DiRas3/H-Ras Interaction—As demonstrated above, the lipidation status of H-Ras plays a role in the interaction with DiRas3. To examine whether lipidation sites or lipidation of H-Ras in general was required for the interaction with DiRas3, we tested the binding of DiRas3 to the farnesyl-deficient mutant H-Ras12V/186S and palmitoyl-deficient mutant H-Ras12V/181/4SS (13, 15). As a control for the experimental conditions used in our coimmunoprecipitation assay, we made use of the interaction between the C-RAF and H-Ras. As reported previously, in contrast to B-RAF, which associates effectively with both farnesylated and nonfarnesylated H-Ras, C-RAF requires farnesylated H-Ras (37). As expected, the loss of the palmitoylation sites did not severely impair the association of H-Ras with C-RAF, but this binding was completely abolished, if the farnesylation site of H-Ras was mutated (Fig. 1B). These results are in line with published data (37). In contrast, loss of the palmitoylation or farnesylation sites in H-Ras did not affect its interaction with DiRas3 under the same experimental conditions (Fig. 1B). This finding is in agreement with the binding selectivity of DiRas3 toward the active nonfarnesylated H-Ras (Fig. 1A).

H-Ras Effector Domain Is Not Essential for the H-Ras/DiRas3 Interaction—Because the coimmunoprecipitation experiments did not provide information on whether the interaction between H-Ras and DiRas3 takes place directly or indirectly, the possibility that this association might be mediated by an effector protein of H-Ras could not be excluded. Therefore, we investigated the binding properties of DiRas3 toward the H-Ras mutants that were impaired in their binding to effector proteins. For that purpose, we made use of three different H-Ras effector domain mutants: H-Ras12V/355, H-Ras12V/37G, and H-Ras12V/40C, which are altered in activation of effector proteins RAF, RalGDS, and PI3K (38). Again, as a control for the
experimental settings of our coimmunoprecipitation assay, we used the interaction between C-RAF and the H-Ras mutants. All H-Ras effector domain mutants tested were indeed severely impaired in their binding to C-RAF (Fig. 1C). In contrast, the interaction between DiRas3 and H-Ras was not compromised by the mutations within the effector domain (Fig. 1C). These findings exclude the possibility that effector proteins mediate the association between DiRas3 and H-Ras. Furthermore, the data of this experiment indicate that the effector domain of H-Ras is not involved in the DiRas3/H-Ras interaction.

C-RAF Associates with the H-Ras/DiRas3 Complex—As the effector domain of H-Ras is not involved in the DiRas3/H-Ras interaction, H-Ras might still be able to bind to its effector proteins while associated with DiRas3. To explore this issue, we used C-RAF as the best characterized representative of the H-Ras effector proteins. To this end, we expressed Myc-DiRas3 and H-Ras12V together with C-RAF kinase in COS7 cells. The DiRas3-bound proteins were isolated by the anti-Myc immunoprecipitation and tested for the presence of H-Ras and/or C-RAF. Indeed, as shown in Fig. 2A, C-RAF WT coprecipitated with DiRas3 in the presence of H-Ras12V, suggesting that all three proteins form a multimeric complex, given that H-Ras is present in its active state. The association of C-RAF with the H-Ras/DiRas3 complex was disrupted by the R89L mutation within the Ras binding domain of C-RAF (Fig. 2A). This single amino acid exchange in C-RAF has been shown to disrupt H-Ras binding (39). Therefore, the data obtained with the C-RAF-R89L mutant suggest that the association of C-RAF with the H-Ras/DiRas3 complex occurs through the C-RAF/H-Ras interaction. This conclusion is also supported by previously published results demonstrating that DiRas3 does not bind to the Ras binding domain of C-RAF (27). Furthermore, data presented in Fig. 2A indicate that binding of C-RAF to the H-Ras/DiRas3 complex enforces the interaction between these two GTPases, as significantly more H-Ras coprecipitated with DiRas3, if C-RAF WT was coexpressed. In contrast, expression
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of the C-RAF-R89L mutant did not support H-Ras/DiRas3 interaction (Fig. 2A).

Binding of DiRas3 to H-Ras Supports the Interaction between H-Ras and Its Effector Protein C-RAF—The finding that C-RAF supports the H-Ras/DiRas3 interaction raises the question of whether it also works vice versa and whether DiRas3 might facilitate the binding of H-Ras to its effector C-RAF. To answer this question, we expressed H-Ras12V and HA-tagged C-RAF together with or without DiRas3 in COS7 cells. The C-RAF-bound proteins were isolated by anti-HA immunoprecipitation and tested for the presence of H-Ras. The C-RAF-R89L mutant was used as a negative control. The results of this experiment revealed that the amount of the coprecipitated H-Ras was two times higher if DiRas3 was coexpressed, suggesting that the binding of DiRas3 to H-Ras indeed supports the interaction between H-Ras and C-RAF (Fig. 2B). Of note, as shown in Fig. 2B, in the absence of DiRas3 coexpression, the signal of the coprecipitated H-Ras appeared as a doublet of two bands with equal intensity, indicating that both farnesylated and nonfarnesylated H-Ras12V were bound to C-RAF WT. In contrast, upon coexpression with DiRas3, the upper band of the doublet, which represents the nonfarnesylated H-Ras, clearly dominated. This observation suggests that DiRas3 stabilizes selectively the complex between C-RAF and the nonfarnesylated H-Ras. These data are in line with the finding that DiRas3 binds preferentially to the nonfarnesylated H-Ras12V (Fig. 1A). Collectively, the results of the experiments presented in Fig. 2 lead to the conclusion that the multimeric complex consisting of DiRas3, C-RAF and active H-Ras is more stable than the two protein complexes consisting of H-Ras-C-RAF or H-Ras-DiRas3, respectively.

DiRas3 Induces Accumulation of C-RAF within the Cytoskeletal Fraction—As the coimmunoprecipitation experiments revealed that C-RAF associates with the H-Ras-DiRas3 complex (see also Fig. 2A), we asked whether this association may affect the subcellular distribution of C-RAF. To address this question, we expressed different combinations of C-RAF, DiRas3, H-Ras12V, and H-Ras17N in COS7 cells as indicated in Fig. 3A. Next, we analyzed the subcellular distribution of these proteins by use of the ProteoExtract subcellular proteome extraction kit as described under “Experimental Procedures.” If DiRas3 was expressed alone, relatively small portions of this GTPase were found in the cytoplasmic and the membrane fractions. In contrast, more than 50% of DiRas3 localized to the cytoskeletal fraction (F4) of COS7 cells (data not shown). Similar subcellular distribution has been observed for ΔNDiRas3 (data not shown), suggesting that DiRas3 is associated either directly or indirectly with the cytoskeletal components of the cell. With regard to the subcellular distribution of C-RAF, in accordance with the published data (40), coexpression of C-RAF together with active H-Ras induced translocation of C-RAF from the cytosolic fraction to the membrane fraction, whereas nonactive H-Ras did not alter the subcellular localization of C-RAF (Fig. 3, A, panels 1–3, and B). When C-RAF was coexpressed with DiRas3, a considerable portion of C-RAF was translocated to the cytoskeletal fraction (Fig. 3, A, panel 4, and B). Of note, the DiRas3–induced accumulation of C-RAF within the cytoskeletal fraction was increased upon coexpression with the active H-Ras and almost abolished upon coexpression with the nonactive H-Ras mutant (Fig. 3, A, panels 5 and 6, and B). These results suggest that binding of C-RAF to H-Ras is a prerequisite for the DiRas3-mediated cytoskeletal localization of C-RAF. This conclusion is supported by the observation that the DiRas3-induced cytoskeletal localization of C-RAF was strongly reduced by the R89L mutation in C-RAF, which impairs the high affinity binding of C-RAF to H-Ras (Fig. 3, C and D) (39).

H-Ras12V and DiRas3 Colocalize at the Plasma Membrane-decorated Cortical Cytoskeleton—To investigate the topology of the interaction between DiRas3 and H-Ras, we next performed colocalization experiments examining transiently expressed epitope-tagged proteins by confocal microscopy. For that purpose, the proteins were expressed in COS7 cells, and the subcellular localization of DiRas3 and constitutively active H-Ras12V was analyzed following sequential cytoplasm and membrane extractions. We show here that after cytoplasm extraction, the localization pattern of H-Ras12V appears as a network. In addition, localization of H-Ras12V was detected at the juxtanuclear structures under these conditions (Fig. 5A). After membrane extractions, the staining of H-Ras12V disappeared (data not shown). As it has been previously shown that H-Ras localizes to the Golgi (19–20), we concluded that the juxtanuclear localization of H-Ras12V shown in Fig. 5A could be assigned to the association of H-Ras12V with the Golgi. The netlike localization of H-Ras12V resembles the MSK web, the portion of the cytoskeleton that is closely associated with the cytoplasmic surface of the plasma membrane (41). We propose that the observed netlike pattern of H-Ras12V localization is formed by the collapse of the plasma membrane caused by cytoplasm extraction, resulting in decoration of the MSK by the plasma membrane–located and -associated proteins. Indeed, a similar localization pattern has been observed by Cole et al. (42) and Lallemant et al. (43) for the membrane–associated protein merlin. Under conditions where permeabilization was carried out after fixation, an enrichment of the merlin protein at the plasma membrane was found. When fixation and permeabilization were performed simultaneously, the localization pattern of the merlin protein appeared as a network that resembled the cortical actin web.

Regarding DiRas3, our results revealed that after cytoplasm extraction, this GTPase localized to the EEA1 (early endosome antigen 1 protein)-positive endosomes and to the netlike structures (Fig. 4). The netlike pattern of DiRas3 localization suggests that, similar to H-Ras, DiRas3 is associated with the plasma membrane, which decorates MSK upon cytoplasm extraction. However, in contrast to H-Ras, the netlike pattern of the DiRas3 localization persisted after membrane extractions (Fig. 4, A and B), whereas endosomal localization disappeared. These data suggest that DiRas3 located at the plasma membrane is connected to the cortical components of the cytoskeleton. If DiRas3 was coexpressed together with H-Ras12V, both proteins colocalized at the plasma membrane–decorated MSK but not at the Golgi (Fig. 5B). The fact that DiRas3 does not associate with the Golgi is explained by the absence of the palmitoylation sites in DiRas3, which are necessary for plasma membrane targeting of Ras proteins through the Golgi pathway (21, 44).
Because the association of H-Ras with the plasma membrane and Golgi requires palmitoylation and farnesylation of H-Ras, respectively (22), mutation of the corresponding lipidation sites in H-Ras should alter its subcellular localization. Indeed, the palmitoylation-deficient mutant H-Ras12V/181/4SS revealed dramatic redistribution of the staining after cytoplasm extraction (compare Fig. 5, A with C). The H-Ras12V/181/4SS mutant did not localize to the Golgi, and only a very small fraction of the protein accumulated at the plasma membrane-decorated MSK. Most of the H-Ras12V/181/4SS was found in small vesicles distributed throughout the cell, probably representing the collapsed endoplasmic reticulum (Fig. 5C). As expected, the farnesylation-deficient mutant H-Ras12V/186S did not associate with the plasma membrane, and the endomembranes of the cell and the staining of the H-Ras12V/186S disappeared almost completely after cytoplasm extraction (compare Fig. 5, A with E). In contrast, when coexpressed with DiRas3, both lipidation mutants of H-Ras12V revealed accumulation at the plasma membrane-decorated MSK where they colocalize with DiRas3 (see Fig. 5, D and F). These findings are in line with our coimmunoprecipitation results suggesting that the lipidation-deficient mutants of H-Ras were targeted to the plasma membrane by the recruitment through DiRas3.

**DiRas3 Recruits C-RAF to Cytoskeletal Components in an H-Ras-dependent Manner**—Our results of cell fractionation reveal an accumulation of C-RAF within the cytoskeletal fraction upon coexpression with DiRas3 and H-Ras12V. To further investigate these findings, we analyzed the subcellular distribution of C-RAF-R89L compared with C-RAF WT. D, to demonstrate the differences more clearly, the samples from C were separated again by electrophoresis using an alternative loading scheme. IB, immunoblot.
the membrane-associated C-RAF WT staining was found associated with the EEA1-positive endosomes, in the case that C-RAF WT was expressed alone (Fig. 6C). Exactly the same subcellular localization was observed for C-RAF-R89L mutant (data not shown). Coexpression of C-RAF WT or C-RAF-R89L with DiRas3 did not change the pattern of C-RAF staining (Fig. 6D, the data for C-RAF-R89L are not shown), suggesting that DiRas3 alone does not alter the subcellular localization of C-RAF. In contrast, when the constitutively active H-Ras12V mutant was coexpressed with C-RAF WT, a dramatic redistribution of C-RAF WT from the EEA1-positive endosomes to the plasma membrane-decorated MSK was observed following cytoplasm extraction (Fig. 6E). This observation, together with the fact that the C-RAF-R89L mutant was not redistributed by coexpression with H-Ras12V (data not shown), suggests that the observed relocation of C-RAF and accumulation at the plasma membrane are driven by binding of C-RAF to activated H-Ras. Importantly, upon coexpression with both DiRas3 and H-Ras12V, C-RAF WT showed a complete colocalization with DiRas3 at the plasma membrane-decorated MSK after cytoplasm extraction (Fig. 6F, left panel). This netlike C-RAF staining and colocalization with DiRas3 persisted even after removal of membranes (Fig. 6F, right panel), revealing an association of both proteins with components of the cytoskeleton. In contrast, the fluorescence signal of C-RAF almost completely disappeared following membrane extraction, if coexpressed with H-Ras12V alone (data not shown). The findings presented in Fig. 6 support our data obtained by immunoprecipitation and fractionation assays (see Figs. 1–3) and indicate that the DiRas3-mediated recruitment of C-RAF to the cytoskeletal components occurs in an H-Ras-dependent manner.

**DiRas3-mediated Regulation of Ras/RAF Signaling Is Not Limited to H-Ras and C-RAF Only**—We next addressed the question whether the DiRas3-mediated regulatory mechanism presented above is limited to H-Ras and C-RAF or whether it is also valid for other members of the Ras protein subfamily and for other RAF isoforms. Regarding Ras proteins, we tested the validity of the proposed mechanism for K-Ras, as this GTPase differs from H-Ras and N-Ras in its membrane targeting motif lacking the palmitoylation site but containing a polybasic sequence (12, 23). The results of coimmunoprecipitation revealed that similar to H-Ras12V (Fig. 2A), K-Ras12V supports the association between C-RAF and DiRas3, as much more C-RAF coprecipitated with DiRas3 in the presence of K-Ras12V (supplemental Fig. S1A). Consistently, we observed accumulation of C-RAF within the cytoskeletal fraction upon coexpression with DiRas3 and K-Ras12V (supplemental Fig. S1B).

Regarding the RAF protein family, we examined the validity of the proposed mechanism for B-RAF isoform, whose regulation differs significantly from that of C-RAF (4, 5). The results presented in supplemental Fig. S2A revealed that similar to C-RAF, B-RAF associates with DiRas3 as well and that this interaction strongly depends on the presence of activated H-Ras. In accordance with this finding, B-RAF accumulated to a high degree within the cytoskeletal fraction, if coexpressed with H-Ras12V and DiRas3 (supplemental Fig. S2B). Taken together, these data suggest that the DiRas3-mediated regulation of Ras/RAF signaling is not limited to H-Ras and C-RAF but is valid for other members of the Ras and RAF protein families.

**DiRas3 Impairs the Dimer Formation of RAF Proteins and Suppresses the Kinase Activity of C-RAF**—As the catalytic activity of RAF proteins is required for the signal transmission from Ras to MEK/ERK, we asked whether the complex formation between Ras, RAF, and DiRas3 may affect the activity of RAF proteins. To address this question, we expressed C-RAF-Myc-His or B-RAF-Myc-His constructs either alone or in combination with H-Ras12V/Di-
Ras3 (triple transfection). RAF proteins were isolated by anti-His immunoprecipitation and subjected to an in vitro kinase assay using recombinant MEK and ERK as substrates. Surprisingly, the results of this experiment revealed that DiRas3 strongly suppressed the H-Ras12V-induced kinase activity of C-RAF but not that of B-RAF (Fig. 7, A and B). Similar results were obtained for the K-Ras12V-mediated activation of RAF (see supplemental Fig. S3). Importantly, the activating phosphorylation of C-RAF at Ser-338 (in B-RAF Ser-446) within the regulatory N-region was not impaired by DiRas3 (Fig. 7, A and B), thus excluding the possibility that the DiRas3-mediated inhibition of C-RAF activity may occur through the interference with the phosphorylation within the N-region. However, an unexpected effect of DiRas3 expression on the electrophoretic mobility of B-RAF has been observed. As shown in Fig. 7C, the intensity of the shifted B-RAF bands was significantly reduced upon coexpression with DiRas3. This finding suggests that DiRas3 impairs Ras-induced phosphorylation of B-RAF on one or several sites different from Ser-446.

These data raised the following question. What is the molecular mechanism of the DiRas3-mediated suppression of C-RAF kinase activity? RAF dimerization proved to be one of the most decisive steps in the regulation of RAF activity, especially with respect to C-RAF activation. It has been previously shown that C-RAF is activated by B-RAF through the mechanism involving heterodimerization (45). Therefore, we investigated whether DiRas3 may affect the dimerization of RAF proteins. To monitor the dimer formation between C-RAF and B-RAF, either C-RAF-Myc-His was expressed together with untagged B-RAF (see Fig. 7D) or B-RAF-Myc-His was expressed together with
HA-tagged C-RAF (see Fig. 7E) in COS7 cells. As Weber et al. (46) reported that dimer formation of RAF proteins is induced by active Ras and this study revealed that DiRas3 forms a complex with H-Ras, we additionally cotransfected the cells with H-Ras12V and DiRas3. The RAF dimers were isolated by anti-His immunoprecipitation. As shown in Fig. 7, D–F, C-RAF dimerized effectively with B-RAF in the presence of active H-Ras12V mutant. In contrast, the Ras-induced dimerization of RAF was strongly impaired (up to ∼50% reduction, see Fig. 7F) upon coexpression with DiRas3. These data suggest that DiRas3 inhibits the kinase activity of C-RAF through suppression of heterodimer formation between C-RAF and B-RAF.
Almost 10 years ago, Luo et al. (27) reported that the small GTPase DiRas3 interferes with the Ras/MAPK signaling. However, the underlying molecular mechanism has not been further investigated. Data presented in this study reveal that the tumor suppressor protein DiRas3 associates with H-Ras and that activation of H-Ras enforces this interaction (Fig. 1), indicating that the tumor suppressive activity of DiRas3 may be achieved, at least in part, at the level of Ras effector proteins. Interestingly, our experiments document that DiRas3 interacts with the nonprocessed form of activated H-Ras, indicating the association of DiRas3 with nascent H-Ras before the post-translational lipidation process of H-Ras began (see Fig. 1).

Prenylation of the wild type H-Ras is required for its efficient activation by the guanine nucleotide exchange factor SOS, whereas the constitutive active H-Ras12V mutant is independent of SOS-mediated activation and therefore is also active in its nonprocessed state (17). Moreover, B-RAF has been shown to associate effectively with both farnesylated and nonfarnesylated H-Ras, and it has been proposed that activation of B-RAF may take place both at the plasma membrane and in the cytosol (37). Considering these facts, it appears plausible that to ensure its tumor suppressive impact at all stages of H-Ras signaling, DiRas3 interacts with the nonprocessed and active H-Ras. In addition, capturing the nonprocessed H-Ras by DiRas3 before the attachment of the farnesyl isoprenoid took place prevents the further palmitoylation of H-Ras and targeting to the plasma membrane microdomains, where the activity of H-Ras is normally required to promote processes such as cell survival and proliferation. Instead, DiRas3 may target H-Ras to the signaling

**DISCUSSION**

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**FIGURE 7. DiRas3 disrupts the C-RAF/B-RAF heterodimer formation and suppresses the kinase activity of C-RAF.** Myc-His-tagged C-RAF (A) or B-RAF (B) was expressed either alone or together with H-Ras12V and DiRas3 in COS7 cells. The RAF kinase was immunoprecipitated (IP) by anti-His antibody and catalytic activity was analyzed in the in vitro kinase assay using purified MEK and ERK as substrates. Phosphorylation status of C-RAF at Ser-338 and B-RAF at Ser-446 was analyzed by use of an appropriate phosphospecific antibody. C-RAF was expressed in COS7 cells together with H-Ras12V and DiRas3 as indicated. 24 h after transfection, cells were lysed, and the proteins were separated on the 8% polyacrylamide gel. To examine the impact of DiRas3 on dimer formation between B- and C-RAF, either C-RAF-Myc-His, B-RAF and DiRas3-Myc (D) or B-RAF-Myc-His, HA-C-RAF, and DiRas3-Myc (E) were transfected into COS7 cells as indicated. Dimerization of RAF proteins was induced by H-Ras12V. RAF dimers were isolated from cell lysates by anti-His immunoprecipitation. F, data from four independent experiments (representative blots are shown in D and E) were quantified by optical densitometry. The quantification results are expressed in terms of %dimer formation, where 100% represents the amount of immunoprecipitated RAF dimer complexes from the cells lacking DiRas3 cotransfection. IB, immunoblot.
Regulation of Ras/RAF Signaling by DiRas3

platforms involved in induction of autophagy and apoptosis. There is indeed increasing appreciation that Ras, and other oncogenes, paradoxically induce both pro- and anti-apoptotic signaling and that the balance of positive and negative signals may differ according to kinetics, stoichiometry, availability of different binding partners, and activation of other similar or countervailing forces (for review see Ref. 47). Our results support the view that DiRas3 may relocate H-Ras to the signaling platforms involved in control of the anti-tumorigenic processes. In fact, we observed that DiRas3 was able to recruit the lipidation-deficient mutants of H-Ras to the plasma membrane (Fig. 5, C–F). DiRas3 itself is post-translationally processed, and prenylation of the CAAX box is essential for membrane association of DiRas3 (27). However, the mechanism of trafficking DiRas3 to the plasma membrane is still unknown. Considering the fact that there is no palmitoylation site in DiRas3, the translocation of this GTPase to the plasma membrane cannot occur through the Golgi pathway. Our results revealing that DiRas3 does not colocalize with the Golgi-associated H-Ras (Fig. 5B) support this view. It is conceivable that the N-terminal domain of DiRas3, which is unique for this member of the Ras proteins, may contribute to the plasma membrane localization of DiRas3 and may target this GTPase to specific microdomains. The presence of the conserved myristoylation site (see Fig. 2 in Ref. 12) within the N-terminal extension of DiRas3 and the fact that deletion of this DiRas3-specific extension nearly abolishes its inhibitory effect on cell growth (27) corroborate this assumption. In addition, in our study we found that the N-terminal extension of DiRas3 supports its association with H-Ras (Fig. 1A). Concerning the possible binding of DiRas3 to the fully lipidated form of H-Ras, this interaction cannot be excluded so far. The fact that the processed H-Ras12V was not coprecipitated with DiRas3 could be due to the insolubility of the DiRas3 fraction, which may be associated with the lipidated H-Ras at the plasma membrane. This assumption is supported by our finding that most of the plasma membrane-located DiRas3 is associated with the insoluble cytoskeletal fraction even after membrane extractions (Figs. 3 and 4).

In addition to the discussed role of DiRas3 in the targeting of H-Ras to particular plasma membrane microdomains, DiRas3 interferes with the effector proteins of H-Ras. In this study, we focused our attention on the RAF kinase, as this effector protein connects H-Ras to the MAPK pathway via direct activation of MEK. Of particular importance are experiments indicating that the effector domain of H-Ras is not involved in the DiRas3/H-Ras interaction (Fig. 1C) and that H-Ras is able to bind to C-RAF while it is associated with DiRas3 (Fig. 2). Moreover, our data document that the complex consisting of DiRas3, H-Ras, and C-RAF is more stable than the complexes between H-Ras and C-RAF or H-Ras and DiRas3 (Fig. 2). Presently, it cannot be excluded that other proteins also support the formation of the multimeric DiRas3/H-Ras–C-RAF complex. The formation of large Ras-signaling complexes, which may work as platforms for transducing the Ras signal to effector molecules, has been assumed by several groups. Based on the results of the single molecule imaging analysis of Ras activation in living cells, Murakoshi et al. (48) proposed a model in which activated Ras molecules may be bound by Ras-specific scaffolding proteins, which might initiate the cooperative formation of transient signaling complexes, including the effector molecules like C-RAF, and deactivating proteins for Ras. This group also reported that the activated Ras molecules (perhaps temporarily) become immobile in the plasma membrane. They concluded that immobilization may be induced by the formation of such a large signaling complex on the plasma membrane, which becomes connected to the actin-based membrane skeleton mesh (48). This view is supported by our results presented in this study. Our data reveal that DiRas3 colocalizes with H-Ras at the plasma membrane-decorated MSK (Fig. 5B). However, although the H-Ras staining almost disappears, DiRas3 localization at the MSK network structures persists after membrane extraction (Fig. 4, A and B). This observation suggests that DiRas3, but not H-Ras, is either directly or indirectly connected to the components of the cytoskeleton. Moreover, our data reveal that DiRas3 induces massive recruitment of C-RAF to the MSK mesh in an H-Ras-dependent manner (Figs. 3 and 6). The recruitment of C-RAF to the cytoskeletal elements has been first reported by Stokoe et al. (40). These authors found that C-RAF, which has been induced to translocate to the plasma membrane by activated Ras, cannot be solubilized with a buffer containing 1% of the detergent Nonidet P-40. They concluded that once recruited to the plasma membrane, C-RAF becomes tightly associated with the cytoskeletal elements underlying the plasma membrane but not with the lipid bilayer (40). Our data are in agreement with this model and suggest that the tumor suppressor DiRas3 supports the anchorage of C-RAF to the MSK.

Furthermore, the data presented here revealed that in addition to subcellular localization, DiRas3 regulates the kinase activity of C-RAF. Although DiRas3 associates in a Ras-dependent manner with both C-RAF (Fig. 2) and B-RAF (supplemental Fig. S2), the results of the in vitro kinase assay clearly show that DiRas3 suppresses specifically the catalytic activity of C-RAF but not that of B-RAF (Fig. 7 and supplemental Fig. S3). How could this C-RAF-specific inhibition be explained? The answer to this question is delivered by the results of RAF dimerization assay. We show here that DiRas3 disrupts the Ras-induced heterodimerization between C-RAF and B-RAF (Fig. 7, D–F). Previously, Garnett et al. (45) reported that B-RAF activates C-RAF through the mechanism involving heterodimerization. They have shown that B-RAF can activate C-RAF downstream of Ras but that C-RAF does not activate B-RAF. In light of these data, we propose that DiRas3 inhibits specifically the kinase activity of C-RAF through suppression of heterodimer formation between C-RAF and B-RAF. We also found that expression of DiRas3 affects the phosphorylation status of B-RAF (Fig. 7C). At present, we cannot definitely state whether the impaired B-RAF phosphorylation is a result of the disrupted C-RAF/B-RAF dimerization or, vice versa, whether the disrupted C-RAF/B-RAF dimerization is a result of impaired B-RAF phosphorylation. However, we propose that there is interplay between the RAF heterodimerization and the B-RAF phosphorylation status. This suggestion is supported by the studies of Rushworth et al. (49) and Ritt et al. (50), who reported that RAF dimerization is coupled to the ERK-induced phosphorylation of B-RAF. In addition, Heidorn et al. (8) pre-
viously showed that B-RAF undergoes a mobility shift in cells treated with the dimerization-inducing RAF inhibitors. Moreover, they suggested that the B-RAF bound to C-RAF is hyperphosphorylated through MEK-ERK-dependent and MEK-ERK-independent mechanisms but that this phosphorylation is not required for B-RAF binding to C-RAF (8). Considering all these findings, the possibility that DiRas3 reduces hyperphosphorylation of B-RAF by suppressing B-RAF/C-RAF dimerization appears more likely.

Taken together, our considerations raise the following question. What is the physiological output of the DiRas3/Ras/RAF interaction? The tumor suppressor DiRas3 has been shown to play a role in the control of cell proliferation, apoptosis, and cell motility (31, 51), the cellular processes that are also regulated by C-RAF (52–54). One outcome could be that DiRas3 can modify MEK/ERK activity specifically through C-RAF to provide fine-tuning of signaling intensity or duration. Alternatively, DiRas3 may utilize the C-RAF pathway to signal to other C-RAF effectors. One common linker that connects C-RAF to the regulation of the quite different processes, such as apoptosis and cell motility, is Rok-α, the kinase involved in the Rho-GTPase-induced rearrangement of the cytoskeleton in migrating cells (55). Furthermore, through its effect on the cytoskeleton, Rok-α regulates clustering and internalization of the death domain-containing receptor Fas, which has a central role in the regulation of the programmed cell death (56). Recently, it has been shown that, in the open state, the C-RAF regulatory domain of active H-Ras, may bind directly to the regulatory domain of the Rho signaling. DiRas3, which is recruited to C-RAF by its enzymatic activity directly and independently of C-RAF kinase activity (52, 57). Thus, restraining Rok-α is a common molecular basis of the essential function of C-RAF in apoptosis and cell migration. Binding of DiRas3 to the H-Ras-C-RAF complex may interfere with the inhibition of Rok-α by C-RAF, which may result, depending on physiological context, in altered cell motility and/or induction of apoptosis. Indeed, Badgwell et al. (51) reported that DiRas3 suppresses ovarian cancer cell migration partly through inhibition of the FAK/RhoA pathway. However, the underlying molecular mechanism is still unknown. Our study may provide the missing link between DiRas3 and the Rho signaling. DiRas3, which is recruited to C-RAF by active H-Ras, may bind directly to the regulatory domain of C-RAF and prevent its interaction with the kinase domain of Rok-α. A DiRas3-mediated recruitment of other yet unknown protein, which would compete with Rok-α for the binding to C-RAF, is also conceivable. The anchorage of DiRas3 to the components of the MSK may deliver this protein to the specific sites of action and avoid undesirable effects.

In conclusion, our data establish a new insight into regulation of Ras/RAF signaling. The finding that DiRas3 forms a complex with the activated Ras and its effector RAF has important biochemical implication. Tumor suppressor functions of DiRas3 are not limited to its antiproliferative activity but also rely on its combined effects on apoptosis, autophagy, and cell migration (27, 31, 58). Our data suggest Ras/RAF signaling as a common target for the tumor suppressive activity of DiRas3 in all these cellular processes. However, several questions remain to be answered, and future studies will be necessary to elucidate in more detail the physiological output of the DiRas3-mediated regulation of Ras/RAF signal transduction.

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