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The Tumor Suppressor Hamartin Enhances Dbl Protein Transforming Activity through Interaction with Ezrin*

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The Rho guanine nucleotide exchange factor (GEF) Dbl binds to the N-terminal region of ezrin, a member of the ERM (ezrin, radixin, moesin) proteins known to function as linkers between the plasma membrane and the actin cytoskeleton. Here we have characterized the interaction between ezrin and Dbl. We show that binding of Dbl with ezrin involves positively charged amino acids within the region of the pleckstrin homology (PH) domain comprised between β1 and β2 sheets. In addition, we show that Dbl forms a complex with the tuberous sclerosis-1 (*TSC-1*) gene product hamartin and with ezrin. We demonstrate that hamartin and ezrin are both required for activation of Dbl. In fact, the knock-down of ezrin and hamartin, as well as the expression of a mutant hamartin, unable to bind ezrin, inhibit Dbl transforming and exchange activity. These results suggest that Dbl is regulated by hamartin through association with ezrin.

ERMs (ezrin, radixin, moesin)[2] are ubiquitously expressed proteins that serve as membrane-cytoskeleton linkers and control diverse actin-based cellular functions including cell adhesion, cell motility, and protein localization, and participate in signaling pathways (1). ERM proteins share a conserved structure containing two domains: the C-terminal domain, that binds to actin, and the N-terminal FERM domain, which binds to protein targets at the plasma membrane and with several signaling proteins (2–14). In the inactive conformation the C terminus binds to the N terminus, thus preventing both interaction with membrane targets and actin. Activation of ERM proteins causes the release of this intramolecular binding and subsequent association of ERMs with the cytoskeleton and membrane proteins.

Two distinct signals have been proposed as effectors of ERM protein activation: the phosphorylation, by Rho-dependent kinase ROCK, of a conserved C-terminal threonine residue, localized in a consensus C-terminal region of ERM proteins (15–20), and the binding of phosphatidylinositol-4,5-biphosphate (21–23), produced by the activity of phopshatidylinositol 4-phosphate 5-kinase (24, 25), which in turn is stimulated by activated ROCK (26). Thus Rho, upon activation by guanine nucleotide exchange factors, may act as an upstream activator of ERMs.

On the other hand, *in vitro* and *in vivo* studies have indicated that ERM proteins can act as upstream regulators of Rho GTPases by binding to the Rho GDP dissociation inhibitor (RhoGDI). This association is thought to displace RhoGDI from Rho GTPases, allowing them to be activated by their specific guanine nucleotide exchange factors (2). In this regard, a functional dependence of Rho GEFs on ezrin has been shown (27) and the association of ERM proteins with Rho GEF Dbl has been demonstrated (28–30). Moreover, association of ezrin with a novel GEF that activates the small GTPase RhoG has been reported (8). Therefore, ERM proteins may act as upstream activators of Rho GTPases not only through their association with Rho GDI but also through their interaction with Rho GEFs.

Link between ERM proteins and the GTP-binding protein Rho has also been reported by Lamb *et al.* (31), who provided evidence that activation of Rho by ERM proteins requires the interaction of the TSC-1 gene product hamartin with ERM proteins. In their model ERM proteins are first activated by lysophosphatidic acid (LPA) and serum, allowing hamartin to associate with the ERM N-terminal domain, causing the subsequent activation of Rho through the N-terminal domain of hamartin by an unknown mechanism. Activation of Rho, in response to LPA, is thought to involve stimulation of the α-subunit of the heterotrimeric G_{12}/G_{13} proteins that act on a family of highly related Rho-specific GEFs, including p115-RhoGEF, PDZ-RhoGEF, and LARG (32–34). Moreover, we have shown that activated $G\alpha_{13}$ induces activation of the GEF Dbl stimulating its association with ezrin (14). The two mechanisms by which Rho acts both upstream and downstream of ERM proteins are compatible with a system that creates a positive feedback loop which promotes activation of Rho by ERM association with hamartin and/or by inhibition of Rho GDI.

In this study, we further characterized the interaction of the Rho GEF Dbl with ezrin. We show here that interaction of ezrin with a specific region of Dbl PH domain is necessary for Dbl-induced cell transformation and activation of Cdc42 and Rac

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[2] The abbreviations used are: ERM, ezrin, radixin, moesin; FERM, four.one protein, ERM; ROCK, Rho-dependent kinase; GEF, guanine nucleotide exchange factor; GDI, GDP dissociation inhibitor; LARG, leukemia-associated Rho GEF; LPA, lysophosphatidic acid; PDZ, post synaptic density protein, discs large protein, zonula occludens; TSC, Tuberous Sclerosis Complex; PH, pleckstrin homology; DH, Dbl homology; MEF, mouse embryonic fibroblasts; PAK-CRIB, p21-activated kinase-Cdc42/Rac interactive binding.

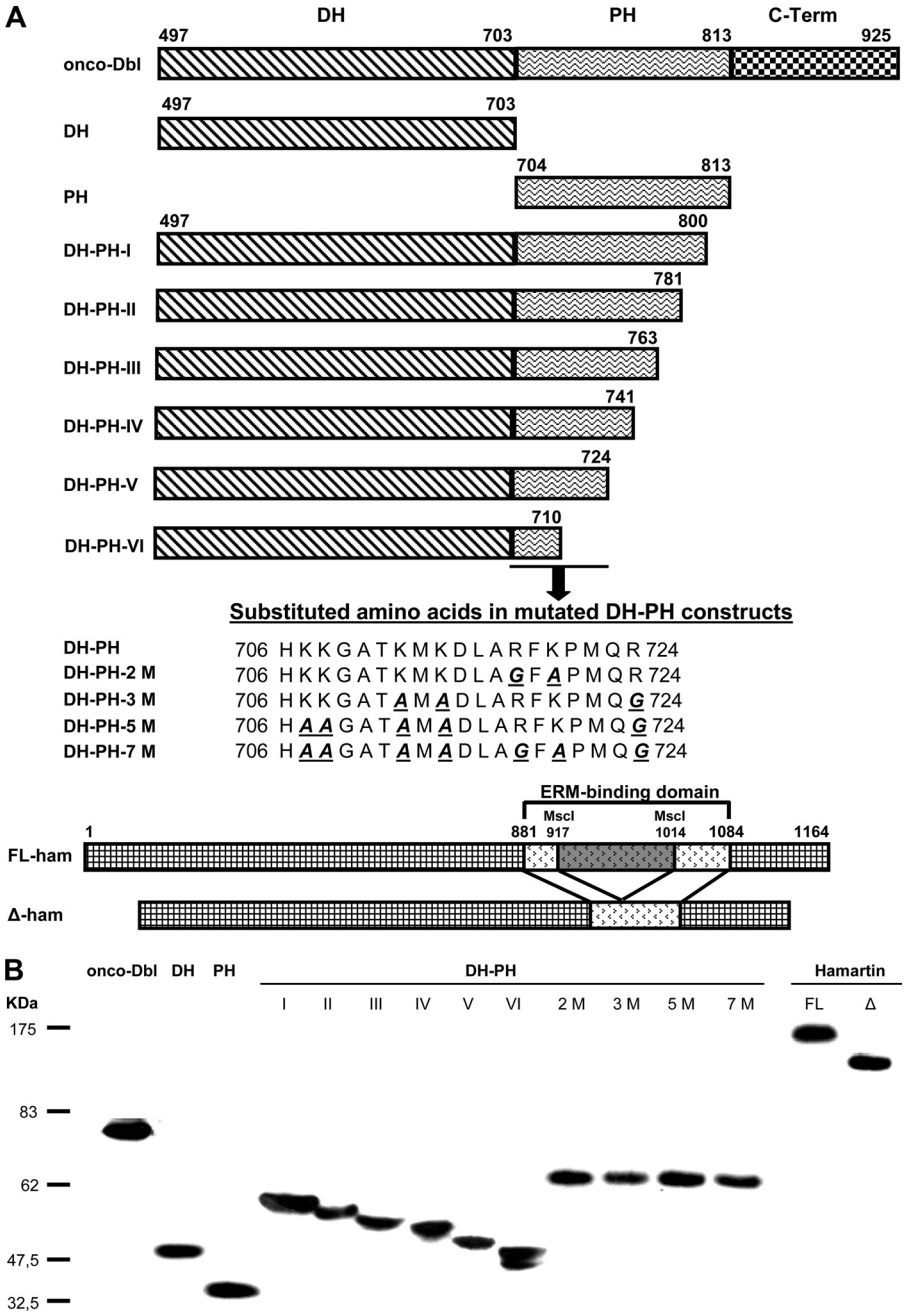


FIGURE 1. **Schematic representation and expression of Dbl and hamartin mutants.** *A*, Dbl oncogene lacks the N-terminal fragment of proto-Dbl; PH represents the isolated pleckstrin homology domain; *DH* represents the isolated Dbl homology domain; the DH-PH deletion mutants DH-PH-I to DH-PH-VI were derived from the DH-PH fragment by progressive truncations. The amino acid substitutions in the DH-PH mutants, DH-PH-2 M to DH-PH-7 M, were introduced between positions 707 and 724 in the PH domain of the DH-PH fragment. FL-ham represents the full-length hamartin. Hamartin deletion mutant, Δ-ham, lacks 98 amino acids within the C-terminal ERM-binding domain. *B*, expression of various Dbl and hamartin constructs. Transient transfectants of Dbl and hamartin were generated in COS7 cells. Cell lysates were subjected to Western blotting. Dbl products were visualized with anti-GST antibody, while FL-ham and Δ-ham were detected using anti-hamartin and anti-Xpress antibody, respectively.

GTPases. We also show that hamartin binds to Dbl, stimulating ezrin-Dbl interaction and Dbl activity. Finally, we show that knock-out of both ezrin and hamartin inhibit Dbl activity. Our results indicate that ezrin and hamartin work in concert to activate the Rho GEF Dbl.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—pCEFL-GST-onco-Dbl, pCEFL-GST-PH, and pCEFL-GST-DH constructs were previously described (35, 36). GDI cDNA, provided by Dr Y. Zheng, and full-length hamartin cDNA (FL-ham), provided by Dr D. J. Kwiatkowski, were subcloned into pCEFL-GST vector. Plasmid expressing onco-Vav (pAX142) was provided by Dr C. J. Der (37). DH-PH-2 M, DH-PH-3 M, DH-PH-5 M, and DH-PH-7 M were obtained by mutagenesis of the Dbl DH-PH fragment: substitution of Lys^{707} to Ala, Lys^{708} to Ala, Lys^{712} to Ala, Lys^{714} to Ala, Arg^{718} to Gly, Lys^{720} to Ala, and Arg^{724} to Gly were introduced by QuikChange Site-directed Mutagenesis kit

(Stratagene-La Jolla, CA). The mutant cDNAs were subcloned into pCEFL-GST vector and sequenced by a Beckman-Coulter Sequenator (Brea, CA). The cDNAs encoding the truncated DH-PH fragments (amino acids 497–800, 497–781, 497–763, 497–741, 497–724, and 497–710) were obtained by PCR amplification, subcloned into pCEFL-GST vector and sequenced by a Beckman-Coulter Sequenator. The deleted cDNA of hamartin (Δ-ham), lacking 98 amino acids within the C-terminal ERM-binding region, was generated utilizing the two unique MscI sites at position 2981 bp and 3270 bp of hamartin cDNA. Following digestion of hamartin cDNA with MscI restriction enzyme, the excised fragment was removed and the N-terminal and C-terminal cDNA fragments obtained were religated at the MscI site. The resulting deleted hamartin cDNA was subcloned into pEF1B vector (Invitrogen-Carlsbad).

Cell Cultures and Transfections—COS7 cells, wild type MEF (MEF-WT), and MEF knock-out for the ezrin gene (MEF-KO) provided by Dr. A. I. McClatchey (38) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were grown to 70% confluency in 100-mm tissue culture dishes and transiently transfected with 4 μg of the indicated plasmids using Lipofectamine PLUS as described by the manufacturer (Invitrogen-Carlsbad).

Mass cultures of stable transfected cell lines were generated by transfecting NIH-3T3 fibroblasts with 0.01 to 1.5 μg of each plasmid DNA by the calcium phosphate coprecipitation method and culturing them in DMEM supplemented with 5% calf serum. 14–21 days after transfection, foci of transformed cells were scored.

Small Interfering RNA—The expression arrest mouse retroviral shRNAmir-ezrin (sh-ezrin), the shRNAmir-hamartin (sh-ham) construct, and the shRNA non-silencing (sh-ns) control plasmid were purchased from Open Biosystems (Huntsville, AL). NIH-3T3 cells were stably cotransfected with 300 ng of onco-Dbl together with 1 μg of sh-ezrin plasmid or cotransfected with 100 ng of onco-Dbl or onco-Vav together with 500 ng of sh-ham plasmid, and selected in DMEM supplemented with 7% calf serum, 378 μg/ml of G418 (Invitrogen-Carlsbad), and 1 μg/ml of puromycin (Sigma) for 14–21 days. Cells were starved in DMEM containing 0.5% serum 18 h before kinase and pull-down assays.

Lentivirus Production—The pLVX-DsRed-Monomer-C1 vector (Clontech-Mountain View) was utilized to obtain a lentiviral construct expressing Dbl conjugated with a red fluorescent protein. Recombinant lentiviruses were produced in HEK-293T cells using the Lenti-X Lentiviral Expression kit (Clontech-Mountain View) as described by the manufacturer. MEF cells were infected with lentiviruses expressing the Dbl product fused to the red fluorescent protein (Red-Dbl) or the red fluorescent protein alone (Red) in the presence of 10 μg/ml of polybrene.

In Vivo GTPase Activation Assay—The GST-PAK-CRIB domain fusion protein (residues 56–141) containing the Cdc42 and Rac binding region of human PAK1, was expressed and purified as described previously (39). NIH-3T3 cells were stably transfected with onco-Dbl alone or together with control sh-ns or sh-ezrin. COS7 cells were transiently cotransfected with onco-Dbl together with FL-ham, Δ-ham, or the empty vector, as control. Analysis of Cdc42 and Rac activation was performed as previously described (14).

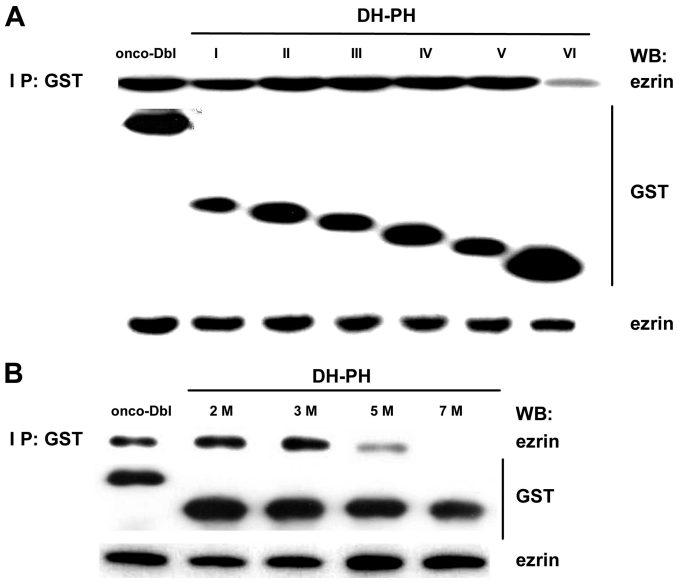


FIGURE 2. **Mapping of the binding site for ezrin on Dbl PH domain.** COS7 cells were transiently transfected with (*A*) onco-Dbl, DH-PH-I, DH-PH-II, DH-PH-III, DH-PH-IV, DH-PH-V, or DH-PH-VI and with (*B*) onco-Dbl, DH-PH-2M, DH-PH-3M, DH-PH-5M, or DH-PH-7M. Cells lysates were subjected to anti-GST immunoprecipitation followed by anti-ezrin Western blot. The amount of GST constructs and endogenous ezrin was determined using anti-GST and anti-ezrin antibodies, respectively. The results shown are representative of three independent experiments.

Kinase Activation Assay—COS7 cells were transiently cotransfected with onco-Dbl and FL-ham, Δ-ham, or the empty vector as control. MEF-WT and MEF-KO were transiently transfected with onco-Dbl, onco-Vav, or with the empty vector as control. NIH-3T3 cells were stably transfected with onco-Dbl alone or together with sh-ezrin or sh-ns. The levels of activated p38MAPK and JNK were assessed as previously described (14).

In Vivo Binding Assay—COS7 cells were transiently transfected with PH, DH, onco-Dbl, DH-PH-I, DH-PH-II, DH-PH-III, DH-PH-IV, DH-PH-V, DH-PH-VI, DH-PH-2M, DH PH-3M, DH-PH-5M, or DH-PH-7M. Alternatively, COS7 cells were transiently transfected with onco-Dbl alone or cotransfected with PH, DH-PH-7M, GDI, FL-ham or Δ-ham. MEF-WT and MEF-KO were transiently transfected with onco-Dbl, onco-Vav, or the empty vector, as control. NIH-3T3 cells were stably cotransfected with onco-Dbl together with sh-ham or sh-ns. Cell lysates were obtained and processed as previously described (14). Immunoprecipitation was performed by incubating cell lysates with polyclonal anti-GST antibody (Molecular Probes) to detect GST-Dbl fusion proteins, monoclonal anti-ezrin antibody (Santa Cruz Biotechnology), polyclonal anti-hamartin antibody (Santa Cruz Biotechnology) for detection of endogenous and FL-ham, or with monoclonal antibody against Xpress epitope (Invitrogen), for detection of Δ-ham. Immunoprecipitates were processed for immunoblotting with polyclonal anti-Dbl antibody (Santa Cruz Biotechnology), monoclonal anti-ezrin antibody, monoclonal anti-hamartin antibody (Sigma), or monoclonal anti-HA antibody (Covance), to detect onco-Vav, as previously described (14).

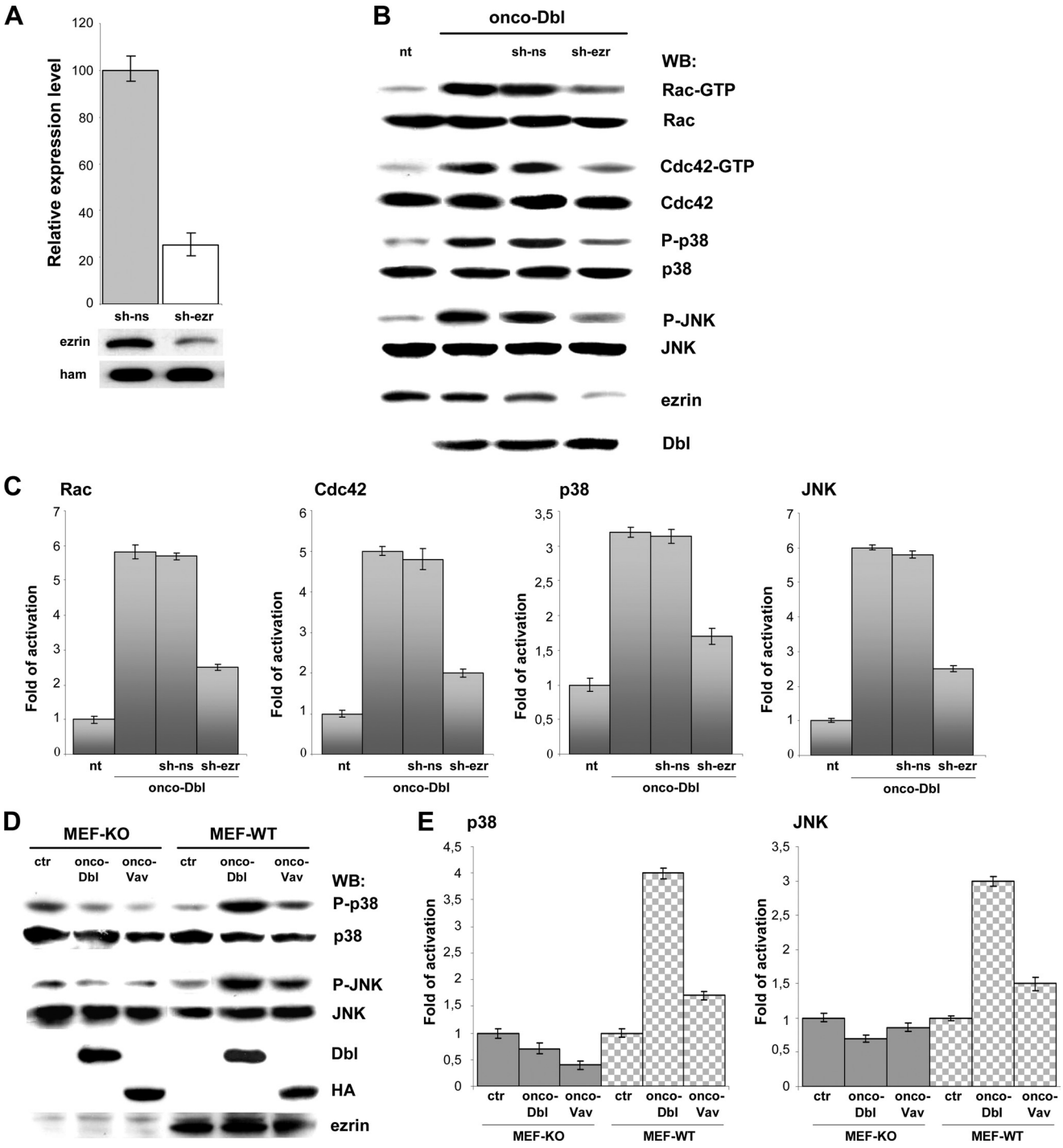


FIGURE 3. **Knockdown of ezrin expression inhibits activation of Rho GTPases, JNK, and p38 in Dbl-expressing cells.** *A*, quantitative Western blot evaluation of ezrin expression level after silencing. Stable transfectants of sh-ezr or sh-ns were generated in NIH 3T3 cells by puromycin selection. Cell lysates of the puromycin-resistant clones were subjected to anti-ezrin Western blotting. The blot was re-probed with anti-hamartin antibody as loading control. A 75% reduction in total ezrin level was achieved in comparison with the non-silenced control, considered as 100%. Data are mean ± S.D., $n = 3$. *B*, untransfected NIH-3T3 cells (nt), and NIH-3T3 cells stably transfected with onco-Dbl alone or together with shRNA-ns (sh-ns) or shRNA-ezrin (sh-ezr) were lysed. Whole cell lysates were subjected to GST-PAK pull-down assay and anti-Rac or anti-Cdc42 Western blot analysis. Alternatively, cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho-p38 and anti-phospho-JNK antibodies. The amount of Rac, Cdc42, p38, JNK, ezrin, and onco-Dbl in total cell lysates was determined by Western blotting with the respective antibodies. The results shown are representative of three independent experiments. *C*, amount of activated Rac and Cdc42 and the phosphorylation of p38 and JNK were quantified by densitometry and normalized to the content of each total protein in cell extracts. The optical density of the scanned film was measured with Quantity One v. 2–3 Image software (Versa Doc, Bio-Rad). Results represent the mean values ± S.D. from three different experiments. *D*, MEF-WT and MEF-KO were transiently transfected with onco-Dbl, onco-Vav, or with the empty vector, as control (*ctr*). Whole cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho-p38 and anti-phospho-JNK antibodies. The amount of p38, JNK, onco-Dbl, and endogenous ezrin in cell lysates was determined by Western blotting with the respective antibodies, while the amount of onco-Vav was determined with anti-HA antibody. The results shown are representative of three independent experiments. *E*, phosphorylation of p38 and JNK was quantified by densitometry and normalized to the content of each total protein in cell extracts. The optical density of the scanned film was measured with Quantity One v. 2–3 Image software (Versa Doc, Bio-Rad). Results represent the mean values ± S.D. from three different experiments.

Immunofluorescence—MEF-WT and MEF-KO were infected with Red-Dbl or the Red recombinant lentiviruses, plated onto glass coverslips and fixed in 4% paraformaldehyde in PBS for 20 min at 25 °C, followed by treatment with 0.1 M glycine in PBS for 20 min at 25 °C, and with 0.1% Triton X-100 in PBS for additional 5 min at 25 °C to allow permeabilization. Cells were then incubated for 1 h at 25 °C with anti-ezrin monoclonal antiboby (Santa Cruz Biotechnology), followed by Alexa Fluor 350-conjugated goat anti-mouse IgG (Molecular Probes) for 30 min at 25 °C, and for 1 h at 25 °C with anti-hamartin polyclonal antibodies (Santa Cruz Biotechnology), followed by FITC-conjugated goat anti-rabbit IgG (Cappel, Organon Teknica Corp.) for 30 min at 25 °C. Coverslips were mounted with Mowiol (Calbiochem) for observation. Fluorescence signals were analyzed by recording and merging stained images using an ApoTome system (Zeiss-Ober Kochen, Germany), connected with an Axio Observer inverted microscope (Zeiss), and Axiovision software (Zeiss).

RESULTS

Identification of the Ezrin Binding Site on Dbl PH Domain— We have previously demonstrated that ezrin binds through its N-terminal region to Dbl pleckstrin homology (PH) domain (30). To map the binding site of ezrin on Dbl PH domain we fused to pCEFL-GST expression vector six different fragments of the DH-PH domain carrying progressive truncations (Fig. 1*A*). COS7 cells were transiently transfected with each construct. Cell lysates were subjected to Western blot with anti-GST antibody to verify the proper expression of each mutant protein (Fig. 1*B*). To assess the ability of each truncated Dbl products to bind ezrin COS7 cells were transfected with each of these constructs or with onco-Dbl and the coimmunoprecipitation pattern of Dbl proteins with ezrin was determined by anti-GST immunoprecipitation and anti-ezrin Western blot. All the Dbl products truncated up to residue 724 formed a stable complex with ezrin (Fig. 2*A*) indicating that the binding site for ezrin is located upstream of residue 724. In fact, binding of Dbl with ezrin was strongly reduced when the construct DH-PH VI, truncated up to residue 710, was used. Therefore the stretch of amino acids between residues 704 and 724 represents the binding site for ezrin. It has been reported that different ERM substrates contain positively charged amino acids in their binding sites (1, 40). To assess whether positively charged amino acids are involved in Dbl binding to ezrin, we generated four DH-PH fragments containing 2, 3, 5, and 7 substitutions of positively charged amino acids with neutral ones (Fig. 1*A*). Each fragment was subcloned into pCEFL-GST vector and transiently transfected in COS7 cells. Expression of the mutant proteins was verified by anti-GST Western blot (Fig. 1*B*). COS7 cells were transfected with each DH-PH mutant constructs or with onco-Dbl, and cell lysates were subjected to an *in vivo* binding assay by anti-GST immunoprecipitation and anti-ezrin Western blot. While up to three mutations did not affect binding, five mutations strongly reduced it and the presence of seven mutations completely abolished the interaction of Dbl with ezrin (Fig. 2*B*). Therefore, the positively charged amino acids within residues 707 and 724 are necessary for Dbl-PH interaction with ezrin.

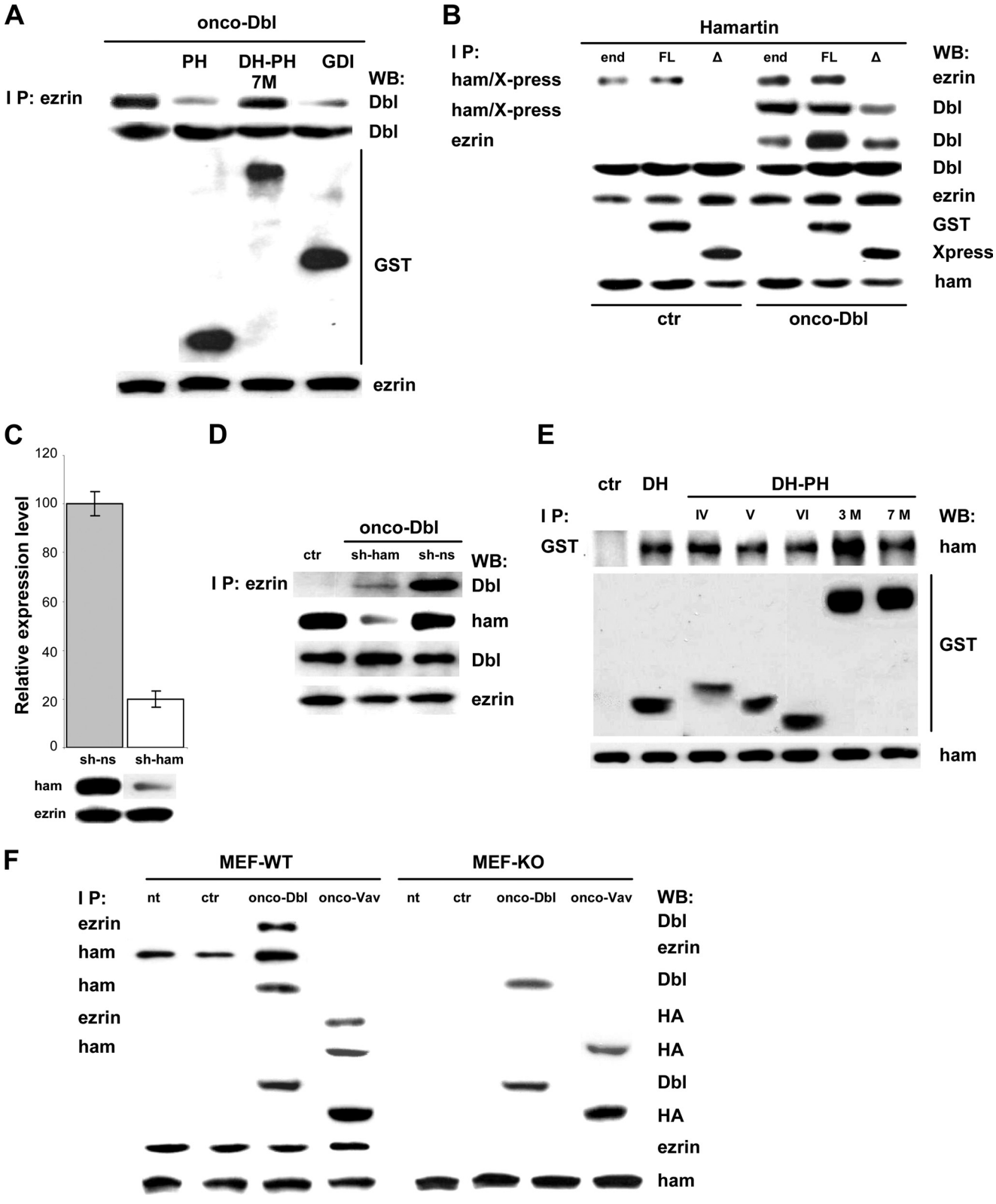
TABLE 1
Knock-down of ezrin inhibits Dbl-transforming activity
MEF-WT and MEF-KO were transiently transfected with onco-Dbl or with the empty vector, as control. The number of focus forming units (F.f.u.) was evaluated 15–21 days after transfection. The results shown represent an average of five independent experiments.

MEF-WT	
DNA	F.f.u.
Control	<0.001
Onco-Dbl	2×10^2
MEF-KO	
DNA	F.f.u.
Control	<0.001
Onco-Dbl	<0.001

Knockdown of Ezrin Expression Inhibits the Activation of Rho GTPases and Their Effector Kinases in Dbl Oncogene-expressing Cells—Ezrin function is required for Rho- and ROCK-mediated fibroblast transformation by Dbl (27). We further evaluated the effects of ezrin on Dbl activity by using silencing RNA approach to determine whether Dbl-mediated activation of Cdc42 and Rac is dependent on a functional ezrin. NIH-3T3 cells were stably cotransfected with onco-Dbl and a retroviral construct expressing an antisense RNA targeted against ezrin (sh-ezrin) or with a retroviral construct expressing a non-silencing control RNA (sh-ns). Stable mass cultures were generated by double selection with G418 and puromycin. We achieved a knockdown of ezrin of ~75% (Fig. 3*A*). Transfected cells were harvested 18 h after incubation in serum-free medium and the active Cdc42 and Rac were collected on GST-PAK-CRIB domain. As shown in Fig. 3*B*, knockdown of ezrin reduced the ability of Dbl to activate both Rac and Cdc42 by ~60% (Fig. 3*C*), indicating that activation of these GTPases by Dbl is dependent on ezrin. We then evaluated whether Dbl-induced serine-threonine kinase activation is dependent on ezrin expression. NIH-3T3 cells cotransfected with onco-Dbl and sh-ezrin or sh-ns were lysed after 18 h incubation in serum-free medium. p38 and JNK activation was then determined by Western blot with phosphospecific antibodies. As shown in Fig. 3, *B* and *C*, the activation of both p38 and JNK in cells depleted of ezrin was reduced by ~50 and ~60%, respectively. No significant inhibition of Rac, Cdc42, p38, and JNK activation was observed when cells were cotransfected with onco-Dbl and sh-ns constructs. These results were confirmed by performing similar experiments on MEF-WT and MEF-KO transiently transfected with onco-Dbl and harvested 18 h after incubation in serum-free medium. Cell lysates were subjected to SDS-PAGE and immunoblotting. As shown in Fig. 3, *D* and *E*, Dbl expression in MEF-WT induced ~4.0- and ~3.0-fold of activation of p38 and JNK, respectively, in comparison with control cells. No activation of these kinases can be detected in Dbl expressing MEF-KO. The oncogenic form of another GEF, Vav (41), was used as control in these experiments. Onco-Vav expression in MEF-WT induced ~1.7- and ~1.5-fold of activation of p38 and JNK, respectively, while no activation could be observed in MEF-KO, indicating that ezrin affects Vav activity as well. We determined the effects of the lack of ezrin on Dbl transforming activity. MEF-WT and MEF-KO were transfected with onco-Dbl and foci of transformed cells were scored 15–21 days later. As shown in Table 1, lack of ezrin expression caused the inabil-

ity of Dbl to transform cells and induce foci. Overall these results indicate that expression of ezrin is critical for Dbl-induced transformation and exchange activity on Rac and Cdc42.

GDI and Hamartin Differently Affect the Interaction of Dbl with Ezrin—We evaluated whether the interaction of Dbl with ezrin is affected by the expression of proteins that bind ERMs, thereby regulating Rho family member activation. We examined two ezrin-binding proteins, RhoGDI, the GDP dissociation inhibitor of Rho family proteins (2), and the tumor suppressor hamartin (42), known to interact with ezrin and to regulate Rho GTPases and thus cytoskeleton structure and cell adhesion (31). As controls, we used the construct expressing



the Dbl PH domain (Fig. 1*A*), that binds ezrin, or the construct expressing the DH-PH-7M that, as shown above, does not interact with ezrin. As expected, overexpression of the Dbl PH domain inhibited the binding, while overexpression of DH-PH-7M did not. Overexpression of GDI strongly reduced the formation of a stable complex between Dbl and ezrin, as revealed by the anti-Dbl Western blot of the anti-ezrin immunoprecipitates (Fig. 4*A*). It has been reported that the ERM protein radixin interacts with Rho GDI displacing Dbl from radixin (28). While a direct interaction of Dbl with radixin *in vivo* has not been demonstrated, we have shown that Dbl protein can effectively bind *in vivo* with ezrin (30). Therefore, ezrin, like radixin, may sequester Rho GDI for release of GDP-bound form of the Rho family members to be converted to GTP-bound form by a Rho GEF-like Dbl. On the other hand, when FL-ham (*FL*) (Fig. 1, *A* and *B*) was coexpressed in COS7 cells with onco-Dbl, we observed a stimulation of the association of ezrin to hamartin (Fig. 4*B*). Similarly, expression of onco-Dbl alone stimulated the interaction of ezrin with endogenous hamartin (end). Moreover, a larger amount of Dbl bound to ezrin could be immunoprecipitated in the presence of overexpression of exogenous hamartin (Fig. 4*B*). It is possible that the overexpression of hamartin induces an increased amount of open/activated ezrin able to bind Dbl. When a deletion mutant of hamartin, Δ-ham (Δ), in which a region spanning 98 amino acids within the ERM binding domain had been deleted (Fig. 1, *A* and *B*), was coexpressed with onco-Dbl in COS7 cells, no stimulation of the association of ezrin to Dbl could be detected, indicating that the activity of hamartin is dependent on its association with ezrin (Fig. 4*B*). We then stably cotransfected NIH-3T3 with onco-Dbl and a retroviral construct expressing an antisense RNA targeted against hamartin (sh-ham) or with the non silencing control RNA (sh-ns). Stable mass cultures were generated by double selection with G418 and puromycin. We achieved a knockdown of hamartin of ~80% (Fig. 4*C*). As shown in Fig. 4*D*, expression of sh-ham inhibited binding of onco-Dbl with ezrin confirming the role of hamartin in Dbl/ezrin interaction. Therefore, differently from the results obtained with GDI, hamartin appears to stimulate ezrin-Dbl binding supporting the hypothesis that hamartin is required for Rho GTPase activation. The results of these experiments also indicate that Dbl can directly interact with hamartin. In fact, Dbl could be coimmunoprecipitated with both endogenous hamartin and exogenous FL-ham (Fig. 4*B*), and, to a minor extent, with Δ-ham. These observations suggest that the site of interaction of hamartin with Dbl is different from the one necessary for binding of hamartin to ezrin, in agreement with what has been proposed, *i.e.* that hamartin may interact with a GEF through its conserved N-terminal region (31). To identify the binding site of hamartin on Dbl we used the DH-PH mutants carrying progressive truncations or substitutions of positively charged amino acids with neutral ones (Fig. 1*A*). As shown in Fig. 4*E*, truncations of up to residue 710 as well as mutations of up to 7 amino acids did not affect binding of Dbl with hamartin. These results indicate that the site of interaction of Dbl with hamartin does not overlap with the one involved in the interaction of Dbl with ezrin. This was confirmed by the observation that hamartin binds to the DH domain of Dbl (Fig. 4*E*). Finally, to determine whether ezrin mediates the association between Dbl and hamartin, MEF-WT, and MEF-KO were transfected with onco-Dbl. Onco-Vav was used as control. As shown in Fig. 4*F*, hamartin formed a stable complex with Dbl, regardless of the cell type used indicating that interaction between Dbl and hamartin occurs independently of ezrin expression. We observed that Vav associates with ezrin, as shown by the anti-ezrin immunoprecipitation and anti-HA Western blot of lysates from MEF-WT transfected with onco-Vav. No binding was observed in MEF-KO (Fig. 4*F*). Moreover, binding was observed between hamartin and onco-Vav in both MEF-WT and MEF KO (Fig. 4*F*). These results suggest that hamartin may interact with various GEF and that this interaction does not require ezrin expression.

Transforming Activity of Dbl Oncogene Is Enhanced by Hamartin—The results obtained suggest that hamartin may affect Dbl functions. Therefore, we evaluated the effect of hamartin expression on Dbl transforming activity. We cotransfected onco-Dbl with FL-ham, Δ-ham, or the empty vectors, as controls. Foci of Dbl-transformed cells were scored 15–21 days after transfection. As shown in Table 2, expression of FL-ham results in stimulation of Dbl transforming activity. The number

FIGURE 4. **Overexpression of GDI and hamartin induces opposite effects on Dbl GEF activity.** *A*, COS7 cells were transiently transfected with onco-Dbl or cotransfected with onco-Dbl and PH, DH-PH-7M, or GDI. Cells lysates were subjected to anti-ezrin immunoprecipitation followed by anti-onco-Dbl Western blot. Protein expression level was determined by Western blot analysis with specific anti-Dbl, anti-GST, and anti-ezrin antibodies using total cell lysates. The results shown are representative of three independent experiments. *B*, COS7 cells were transiently cotransfected with the empty vector (*ctr*) or onco-Dbl, together with FL-ham (*FL*) or Δ-ham (Δ). The effects of the endogenous hamartin (*end*) on Dbl activity was evaluated by transfecting COS7 cells with onco-Dbl alone. Cells lysates were subjected to anti-hamartin, to visualize FL-ham, to anti-Xpress, to visualize Δ-ham, or to anti-ezrin immunoprecipitation followed by anti-ezrin or anti-Dbl Western blot. Onco-Dbl, endogenous ezrin, FL-ham, Δ-ham, and endogenous hamartin expression level was determined by Western blot analysis with anti-Dbl, anti-ezrin, anti-GST, anti-Xpress, and anti-hamartin specific antibodies. The results shown are representative of three independent experiments. *C*, quantitative Western blot evaluation of hamartin expression level after silencing. Stable transfectants of sh-ham or sh-ns were generated in NIH 3T3 cells by puromycin selection. Cell lysates of the puromycin-resistant clones were subjected to anti-hamartin Western blotting. The blot was re-probed with anti-ezrin antibody as loading control. A 80% reduction in total hamartin level was achieved in comparison with the non-silenced control, considered as 100%. Data are means ± S.D., $n = 3$. *D*, NIH-3T3 cells were stably transfected with the empty vector (*ctr*) or cotransfected with onco-Dbl together with shRNA-hamartin (*sh-ham*) or with shRNA-ns (*sh-ns*). Cell lysates were subjected to anti-ezrin immunoprecipitation followed by anti-Dbl Western blot. Protein expression level of onco-Dbl, endogenous hamartin and endogenous ezrin was determined by Western blot analysis with specific antibodies. *E*, binding of hamartin with Dbl constructs. COS7 cells were transiently transfected with the empty vector (*ctr*), DH, DH-PH-IV, DH-PH-V, DH-PH-VI, DH-PH-3M, or DH-PH-7M. Cells lysates were subjected to anti-GST immunoprecipitation followed by anti-hamartin Western blot. The amount of GST-constructs and endogenous hamartin was determined using anti-GST and anti-hamartin antibodies, respectively. The results shown are representative of three independent experiments. *F*, *in vivo* interaction of hamartin with Dbl. MEF-WT and MEF-KO were left untreated (*nt*) or transiently transfected with the empty vector (*ctr*), onco-Dbl, or onco-Vav. Cells lysates were subjected to anti-ezrin or anti-hamartin immunoprecipitation followed by anti-Dbl, anti-ezrin, or anti-HA Western blot. Protein expression level of onco-Dbl, onco-Vav, endogenous ezrin, and endogenous hamartin was determined by Western blot analysis with anti-Dbl, anti-HA, anti-ezrin, and anti-hamartin specific antibodies. The results shown are representative of three independent experiments.

TABLE 2

Hamartin enhances Dbl-transforming activity

NIH-3T3 cells were stably cotransfected with onco-Dbl and FL-ham, Δ-ham, or with the empty vectors as controls. The number of focus forming units (F.f.u./μg DNA) was evaluated 15–21 days after transfection. The results shown represent an average of five independent experiments.

DNA	F.f.u./μg DNA
Control (GST)	$<1 \times 10^0$
Onco-Dbl + GST	2.3×10^4
Onco-Dbl + FL-ham	4.0×10^4
Control (pEF1B)	$<1 \times 10^0$
Onco-Dbl + pEF1B	5.0×10^3
Onco-Dbl + Δ-ham	2.2×10^3

TABLE 3

Knockdown of hamartin strongly inhibits Dbl-transforming activity

NIH-3T3 cells were stably cotransfected with onco-Dbl or onco-Vav and sh-ns or sh-ham plasmids. Cells were transfected with the empty vector or with the shRNA plasmids alone, as controls. The number of focus forming units (F.f.u./μg DNA) was evaluated 15–21 days after transfection. The results shown represent an average of three independent experiments.

DNA	F.f.u./μg DNA
Control (GST)	$<1 \times 10^0$
Control (sh-ns)	$<1 \times 10^0$
Control (sh-ham)	$<1 \times 10^0$
Onco-Dbl + sh-ns	6.0×10^3
Onco-Dbl + sh-ham	1.7×10^3
Onco-Vav + sh-ns	4.0×10^3
Onco-Vav + sh-ham	1.9×10^3

of foci of transformed cells detectable in NIH-3T3 was increased by 74%. On the other hand, coexpression of Δ-ham induced up to 56% inhibition of Dbl-induced foci (Table 2). To further establish the role of hamartin in Dbl transformation, we cotransfected onco-Dbl with the retroviral constructs sh-ham and sh-ns. Onco-Vav was used as control. As shown in Table 3, silencing of hamartin with sh-ham induced up to 72% inhibition of Dbl-induced foci. Moreover, onco-Vav transforming activity was inhibited by 52% when sh-ham was coexpressed. These results indicate that hamartin is essential for Dbl-induced transformation and suggest that hamartin may act as a regulator for the activity of other GEFs.

The role of hamartin on Dbl activity was further evaluated by analyzing whether hamartin affects Dbl GEF activity on Rac and Cdc42 and Dbl-induced activation of serine-threonine kinases. COS7 cells, cotransfected with onco-Dbl and FL-ham or Δ-ham, were harvested 18 h after incubation in serum-free medium and lysed. Active Rac and Cdc42 were collected on GST-PAK-CRIB domain. Expression of FL-ham (FL) increased the ability of Dbl to activate both Rac and Cdc42 by ~2.0-fold. Expression of Δ-ham, (Δ), on the contrary, caused a reduction of Dbl-induced Rac and Cdc42 activation of 70 and 65%, respectively (Fig. 5, *A* and *B*). p38 and JNK activation was then determined by Western blot with phosphospecific antibodies. Coexpression of FL-ham increased p38 and JNK activation by 2.2- and 1.6-fold respectively, compared with activation in cells expressing Dbl alone. Expression of Δ-ham, on the other hand, reduced activation of both p38 and JNK by ~40% (Fig. 5, *A* and *B*). Similar results were obtained with stably transfected NIH-3T3 cells (data not shown). These results indicate that hamartin stimulates Dbl-induced activation of JNK and p38 MAPK signaling pathways.

Knock-out of Ezrin Expression Prevents the Translocation of Dbl and Hamartin to the Plasma Membrane—We have previously demonstrated that Dbl interacts with ezrin, with a consequent relocalization of both proteins to the plasma membrane (30). Moreover, hamartin colocalizes with ezrin at the cell periphery (31). Here, we analyzed hamartin and Dbl subcellular localization in the absence of ezrin expression. MEF-WT and MEF-KO were infected with a lentivirus expressing onco-Dbl fused to the fluorescence Red epitope (Red-Dbl) or with the control lentivirus (Red) and plated onto glass coverslips. Cells were double labeled with anti-ezrin antibody and Alexa Fluor 350-labeled secondary antibody, for ezrin detection, and with anti-hamartin antibody and FITC-labeled secondary antibody, for hamartin detection. Protein intracellular localization was evaluated by an Apotome system, connected with an inverted microscope. In MEF-WT Dbl oncogene appeared colocalized with both ezrin and hamartin at the cell surface (Fig. 6). A typical Dbl-transformed phenotype, characterized by an enlarged and polygonal cell shape, with production of ruffles and lamellipodia can be observed. On the contrary, in cells infected with the control lentivirus, hamartin and ezrin can be visualized as diffuse signal in the cytoplasm. When MEF-KO were used no translocation to the cell periphery of hamartin or Dbl could be observed. Both proteins maintain a diffuse cytosolic localization, and cells show an elongated shape similar to the morphology of cells infected with control lentivirus. Therefore, these results indicate that ezrin is indispensable for Dbl and hamartin colocalization at the cell periphery. We conclude that interaction of both hamartin and ezrin with Dbl is required for relocalization of the three proteins to the cell periphery and for Dbl GEF activity, as indicated by the acquisition of the transformed phenotype of Dbl-expressing MEF-WT.

DISCUSSION

In this study we provide evidence that the tumor suppressor hamartin can directly interact with Dbl oncogene. This interaction occurs in intact cells between onco-Dbl and either the full-length hamartin or its mutant form lacking the ERM-binding domain. To our knowledge this is the first report of a direct interaction between a GEF and hamartin.

We have previously shown that PH domain mutants, unable to associate with PIPs or with actin cytoskeleton, retained the ability to associate with ezrin. Those data implied that different amino acids in the PH domain have distinct specificity for defined substrates and that the PH region between β1 and β2 sheets was not responsible for Dbl binding to ezrin (30). In the present study we have extended our previous investigation and determined that the binding site for ezrin on the Dbl PH domain involves all 7 positively charged amino acids within the region of the PH domain comprised between β1 and β2 sheets. These results are consistent with data on other ERM substrates, which in most cases possess ezrin binding sites constituted by three or more positively charged amino acids (1). Our present observations indicate that the interactions of Dbl with PIPs, actin cytoskeleton and ezrin occur at overlapping binding sites. Further studies will be necessary to determine whether these interactions are concomitant or successive, cooperative or competitive.

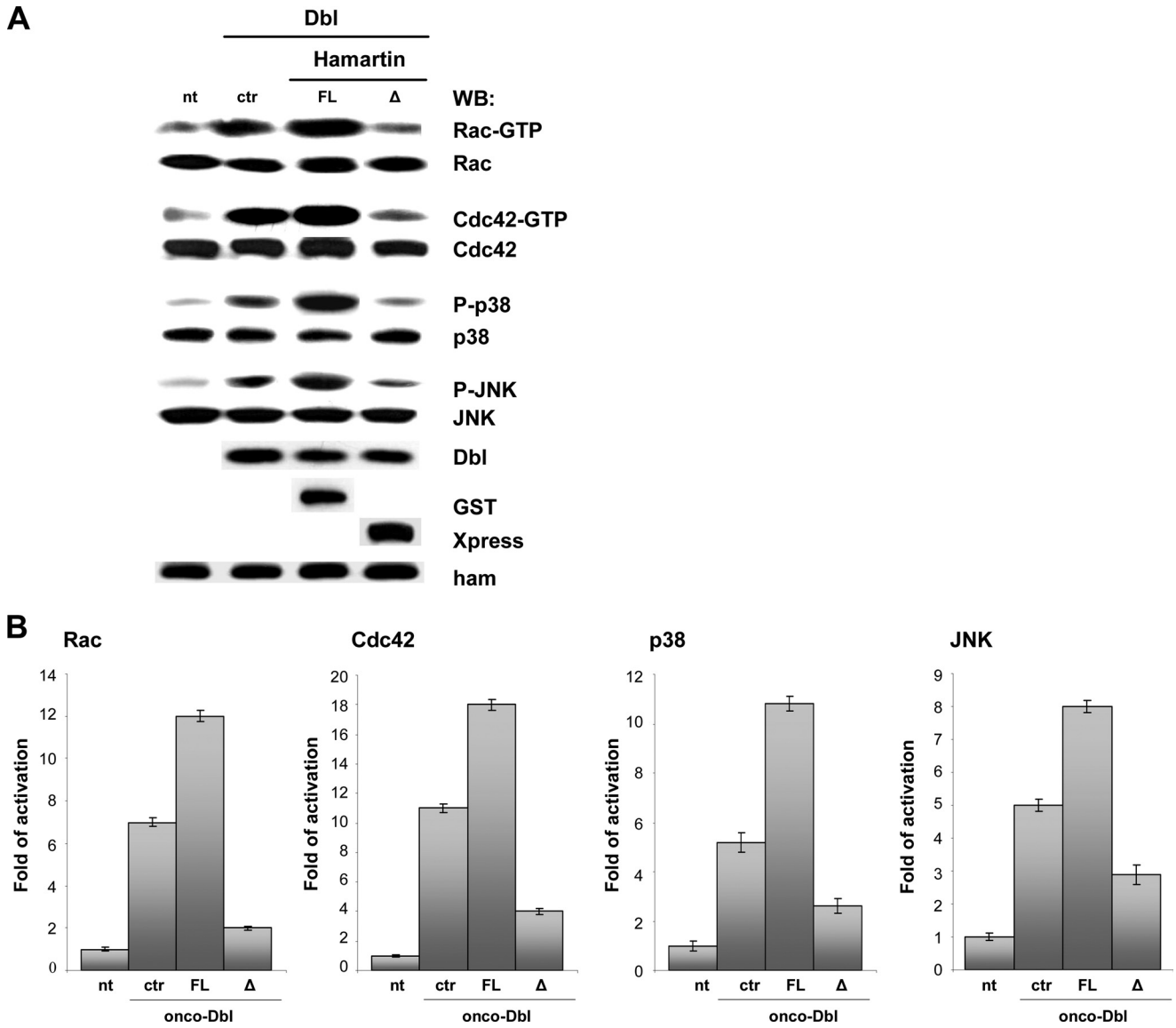


FIGURE 5. **Hamartin enhances activation of JNK and p38 in Dbl-expressing cells.** *A*, COS7 cells were used untransfected (*nt*) or transiently cotransfected with onco-Dbl and the empty vector (*ctr*), FL-ham (*FL*), or Δ-ham (Δ). Whole cell lysates were subjected to GST-PAK pull-down assay and anti-Rac or anti-Cdc42 Western blot analysis. Alternatively, cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane and probed with anti-phospho-p38 and anti-phospho-JNK antibodies. Expression level of Rac, Cdc42, p38, JNK, onco-Dbl, FL-ham, Δ-ham, and endogenous hamartin was determined by Western blot analysis with specific antibodies using total cell lysates. The results shown are representative of three independent experiments. *B*, amount of activated Rac and Cdc42 and phosphorylated of p38 and JNK were quantified by densitometry and normalized to the content of each total protein in cell extracts. The optical density of the scanned film was measured with Quantity One v. 2–3 Image software (Versa Doc, Bio-Rad). Results represent the mean values ± S.D. from three different experiments.

By using both an antisense RNA targeted against ezrin and MEF knock-out for the ezrin gene we established that ezrin is not required for hamartin interaction with Dbl but necessary for hamartin-mediated activation of Dbl exchange and transforming activity. Therefore, upon interaction of hamartin with Dbl ezrin may promote GTPase activation by inhibiting RhoGDI. In some experiments we used Vav oncogene (41) as control and found that the activity of this GEF is also affected by the presence of a functional ezrin. In fact, knock-out of ezrin abolishes onco-Vav-induced activation of JNK and p38. These results are not surprising since it was previously shown that ezrin function is necessary for cell transformation induced by other GEFs such as Net (27) and PLEKHG6, a novel GEF that activates the small GTPase RhoG (8). Therefore, ERM proteins may act as upstream activators of Rho GTPases not only through their association with Rho GDI but also through their interaction with Rho GEFs.

We explored the functional implications of Dbl/hamartin interaction. The results obtained indicate that hamartin overexpression stimulates Dbl activity and interaction with ezrin. It was previously reported that hamartin binds to ezrin regulating cell adhesion and inducing activation of Rho. Lamb *et al.* (31) suggested that hamartin might interact through its conserved N-terminal region with Rho-specific exchange factors. Here we show that in fact hamartin interacts with Dbl DH domain inducing Dbl-mediated activation of downstream kinases (Fig. 7). This was proven by the fact that inhibition of Dbl activities occurs when a deletion mutant of hamartin, unable to bind

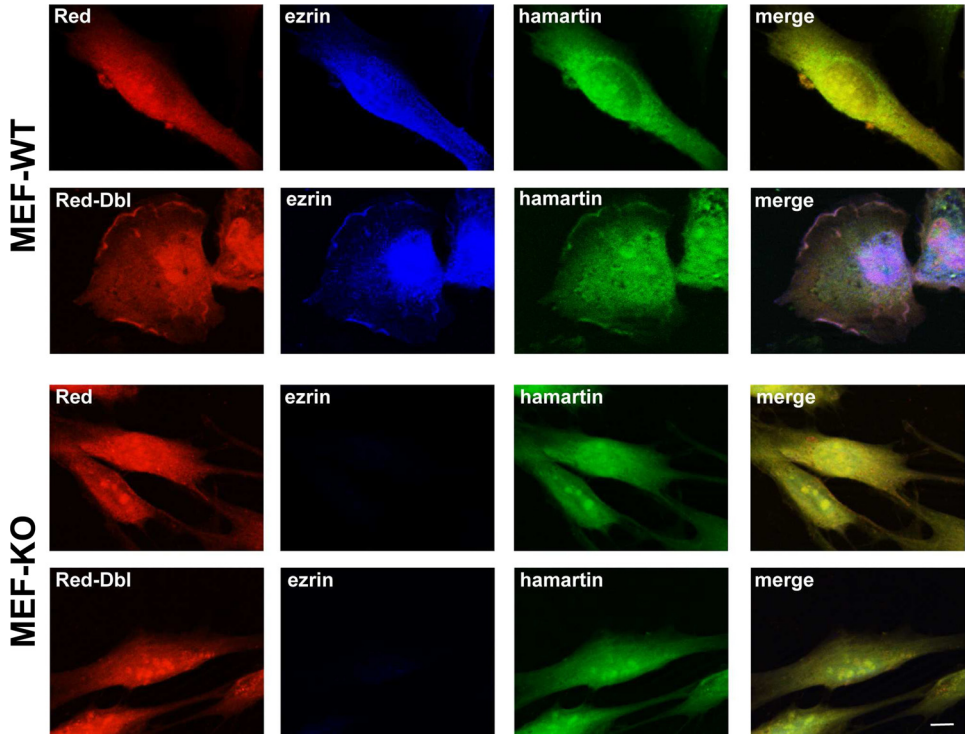


FIGURE 6. **Knock-out of ezrin expression prevents the translocation of Dbl and hamartin to the plasma membrane.** MEF-WT and MEF-KO were infected with the lentivirus expressing onco-Dbl fused to the fluorescence Red protein (*Red-Dbl*) or with the control lentivirus (*Red*). Cells were double immunolabeled for ezrin (*blue*) and hamartin (*green*), as described under "Experimental Procedures." MEF-WT infected with Red-Dbl show the typical Dbl phenotype and localization (*red*) along the plasma membrane. Colocalization of Dbl with ezrin and hamartin is shown (*merge*). Staining of Red-Dbl, hamartin, and ezrin appears diffuse in the cytoplasm of the elongated MEF-KO cells, and no localization along the plasma membrane is evident. Cells infected with Red appear elongated, and the red fluorescence is diffuse in the cytoplasm of both MEF-WT and MEF-KO, with no plasma membrane localization of either ezrin or hamartin (bar, 10 μm).

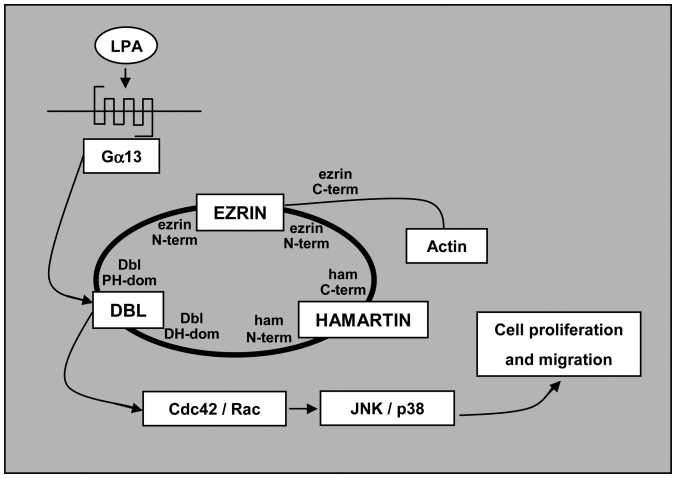


FIGURE 7. **Schematic model of Dbl activation by ezrin and hamartin.** Activation of Gα13 by stimulation of Gα13-associated G-protein-coupled receptors by the agonist LPA (48) induces Dbl activation (14). Hamartin associates with its N-terminal domain to Dbl DH domain and with its C-terminal domain to ezrin N-terminal domain (31). Ezrin associates with Dbl PH domain through its N-terminal domain (30) and with actin through its C-terminal domain, inducing Dbl activity on Rho GTPases by inhibition of Rho GDI. Activation of Cdc42 and Rac by Dbl induces cell proliferation and migration.

ezrin, was overexpressed or when hamartin was silenced by sh-RNA in Dbl-expressing cells. We also show that hamartin associates with onco-Vav and that its expression is necessary for efficient Vav-induced cells transformation. Even though the effects of hamartin on Vav activity were not as strong as the ones observed on Dbl, these data imply that ezrin and hamartin may regulate several exchange factors for Rho GTPases.

It has been reported that Rho activity is affected by ezrin and activated by hamartin (27, 31). We show here that association of hamartin with onco-Dbl also causes activation of Cdc42 and Rac and their downstream substrates, JNK and p38, and that this association is dependent on a functional ezrin. In fact, lack of ezrin inhibits both Dbl transforming activity and downstream signaling pathway. Small GTP-binding proteins Rac-1 and Cdc42 activate JNK and p38 (43–45) and activation of JNK directly correlates with Dbl transforming activity (36). On the other hand, we have shown that activation of p38 in Dbl-expressing cells correlates with inhibition of cell proliferation and induction of cell migration with localized activation of Rac (46). A potential balance of MAPK activities exists in which inhibition of p38 leads to an increase in cell proliferation while preventing cell migration and the activation of p38 leads to a decrease in cell proliferation while inducing cell migration (47). Activation of Rac by onco-Dbl causes formation of membrane ruffles and cell migration (46) and Dbl transformed cells are characterized by abundant lamellipodia (30). Such morphology was evident in MEFs infected with a Dbl recombinant lentivirus, but disappeared when MEF knock-outs for the ezrin gene were used. Therefore, the ability of Dbl to activate both Cdc42 and Rac is reflected by the concomitant activation of JNK and p38, cell proliferation, and cell migration.

These data not only establish that ezrin interaction with Dbl is dependent on hamartin containing a functional ezrin binding site but also that regulation of Rho GTPases by hamartin may be affected when a constitutively active GEF is involved. The association of hamartin with onco-Dbl would stimulate Dbl activity

with the consequent induction of cell transformation. Therefore, disruption of the normal balance of ezrin, hamartin, and GEF activities would contribute to cell transformation.

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