THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 2, Issue of January 10, pp. 1108–1114, 2003

Printed in U.S.A.

Erbin Suppresses the MAP Kinase Pathway*

Received for publication, May 31, 2002, and in revised form, October 7, 2002
Published, JBC Papers in Press, October 11, 2002, DOI 10.1074/jbc.M205413200

Yang Z. Huang‡, Mengwei Zang§, Wen C. Xiong¶, Zhijun Luo§, and Lin Mei‡‖

From the ‡Departments of Neurobiology, Pathology, and Physical Medicine and Rehabilitation, Civitan International Research Center, University of Alabama at Birmingham, Birmingham, Alabama 35294-0021, the §Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, and the ¶Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0007

We present evidence here that Erbin is a negative regulator of the Ras-Raf-Erk signaling pathway. Expression of Erbin decreases transcription of the AChR ε-subunit gene, an event that is mediated by Erk activation. Although it interacts with the ErbB2 C terminus through the PDZ domain, Erbin has no effect on ErbB2 tyrosine phosphorylation or binding to the adaptor proteins Shc and Grb2. In contrast, expression of Erbin greatly impairs activation of Erk, but not Akt, by ligands that activate receptor tyrosine kinases. Moreover, Erbin inhibits the Erk activation by active Ras, while it fails to do so in the presence of active Raf-1. Erbin associates with active Ras, but not inactive Ras nor Raf. Consistently, Erbin interferes with the interaction between Ras and Raf both *in vivo* and *in vitro*. Finally, overexpression of Erbin leads to inhibition of NGF-induced neuronal differentiation of PC12 cells, whereas down-regulation of endogenous Erbin by specific siRNA exhibits an opposite effect. Collectively, our study has identified Erbin as a novel suppressor of the Ras signaling by disrupting the Ras-Raf interaction.

Extracellular signal-regulated kinases (Erk)[1] are a subfamily of mitogen-activated protein kinases (MAPK) that play important roles in a great array of cell programs including proliferation, differentiation, and apoptosis (1, 2). As exemplified by binding to growth factors such as EGF, receptor tyrosine kinases are activated and undergo autophosphorylation on tyrosine residues (3, 4). Phosphorylated tyrosine residues recruit adaptor proteins to the plasma membrane by directly interacting with modules including Src homology 2 (SH2) or phosphotyrosine binding domain (PTB). Grb2, one of such adaptors, brings guanyl nucleotide exchange factor (SOS) to the plasma membrane in proximity with Ras and expedites exchange of GDP for GTP on Ras (5). Activated Ras (GTP-bound) then directly binds to Raf and allows the latter to be activated (1, 6). Active Raf triggers sequential activation of MEK, a MAPK kinase, and Erk, leading to phosphorylation of various regulatory proteins including nuclear transcription factors such as Elk-1 and Myc as well as many cytoplasmic proteins (7, 8).

In the past, extensive efforts have been made to identify factors that participate in regulation of the Ras-Raf-Erk pathway. Several modulators have been identified that positively influence the pathway at different levels. For example, the MEK partner 1 (MP1) was isolated as a binding protein that interacts with both MEK1 and Erk1 to enhance the efficiency of Erk phosphorylation by MEK (9). A second protein is the kinase suppressor of Ras (KSR) that is believed to act as a scaffold for Raf-1, MEK, and Erk (10). The Connector enhancer of KSR (CNK) directly binds to Raf and is involved in activation of the Raf/MEK/Erk pathway (11). Sur-8 is an interesting protein that contains multiple leucine-rich repeats (LRRs) (12) and binds to both Ras and Raf-1. Although Ras can directly associates with Raf upon activation, the presence of Sur-8 increases the interaction between Ras and Raf and the activation of downstream signaling events (10). These non-enzymatic factors are important regulators for normal cell proliferation and differentiation.

In addition, there are negative regulators of the Ras pathway in cells. Sprouty, a Ras suppressor in *Drosophila* and its mammalian homologue Spred (Sprouty-related EVH1 domain-containing protein) appear to serve as negative feedback regulators of growth factor-mediated Erk pathway (13, 14). The Ras effector RIN1 has been shown to inhibit Ras-induced activation of Raf by competitively binding to active Ras (15). Additionally, the Raf kinase inhibitory protein (RKIP), initially isolated as a phosphatidylethanolamine-binding protein, binds directly to the kinase domains of both Raf and MEK and inhibit MEK phosphorylation (16). These negative regulators are important to ensure that all programs are adequately executed through autonomous turn-on and -off mechanisms. In addition, they may counterbalance overamplified proliferative signals that are caused by Ras mutation frequently occurring in human cancers. Such an inhibitory mechanism is key to maintaining normal cell growth rate or function.

Erbin is a protein that was identified as a binding partner for ErbB2, delta-catenin, and ARVCF (17–22). The 180-kDa protein contains two identifiable domains: LRR and PDZ (17, 19). Because of the essential role of ErbB2 in neuregulin (NRG)-induced synthesis of acetylcholine receptors (AChR) (23–26), we investigated the effect of Erbin on AChR subunit expression. Unexpectedly, Erbin was found to inhibit AChR subunit transcription, an event that requires Erk activation (23–26), suggesting that it may play a role in regulating Erk activation. This study presents evidence that Ras-mediated Erk activation is indeed negatively regulated by Erbin. We have explored the possible mechanism by which Erbin inhibits Erk activation. Our results identify Erbin as a novel suppressor of the Ras signaling.

* This work was supported by National Institutes of Health Grant NS40480. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. E-mail: lmei@nrc.uab.edu.

[1] The abbreviations used are: Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; RIPA, radioimmune precipitation assay buffer; GFP, green fluorescent protein; LRR, leucine-rich repeat; GST, glutathione *S*-transferase; AChR, acetylcholine receptor; NGF, nerve growth factor; NRG, neuregulin.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The human Erbin N-terminal domain (amino acids 1–391) consisting of 16 LRRs was generated by PCR amplification using sense primer containing *Bam*HI and antisense primer containing *Xho*I. The resulting 1.2 kb-fragment was digested with *Bam*HI and *Xho*I, and subcloned in the *Bam*HI-*Sal*I sites of yeast vector pGBT9 downstream of the Gal4 DNA binding domain (Clontech). Myc-Erbin LRR, Myc-ErbinΔ965–1371 and Myc-ErbinΔPDZ were generated by introducing a stop codon after the LRR domain following amino acids 965 or 1279 in pRK5-Myc-Erbin (19, 27). The N-terminal deletion mutant (pRK5-Erbin965) was described previously (19). A fragment encoding the full-length Akt cDNA generated by PCR using sense primer containing *Eco*RI and antisense primer containing *Xho*I. The resulting 1.6-kb fragment was digested with *Eco*RI and *Xho*I and subcloned into *Eco*RI-*Xho*I sites of the mammalian expression vector pCS2+MT (for the Myc tag at the N terminus). Wild type-ErbB2 and constitutively active form of ErbB2 (NeuT) (generously provided by Dr. M. C. Hung, University of Texas M. D. Anderson Cancer Center) were subcloned downstream of the FLAG tag and an artificial signal peptide in pCMV. pCMV-FLAG-Erk1 was generously provided by Dr. Mike Weber (University of Virginia). FLAG-Ras, FLAG-RasV12, FLAG-RasN17, and GST-Raf-BXB were described as previously (28).

Cell Culture and Transfection—HEK 293 cells and COS-1 cells were cultured as described previously (29). The C2C12 cells were maintained as undifferentiated myoblasts in Dulbecco's modified Eagle's medium with high glucose supplemented with 20% fetal bovine serum, and 0.5% chicken embryo extract. Fusing of myoblasts into myotubes was induced by culturing myoblasts for 48 h in differentiation medium DM (Dulbecco's modified Eagle's medium plus 4% horse serum). Mouse lung epithelial Mv1Lu cells and breast cancer MCF-7 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Rat pheochromocytoma-derived PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% horse serum. HEK293, COS-1, and C2C12 cells were transfected with the standard calcium phosphate technique (29). PC12 cells and Mv1Lu cells were transfected with SuperFect (Qiagen). Two days after transfection, cells were washed with phosphate-buffered saline and lysed in the modified RIPA buffer containing 20 mM sodium phosphate, pH 7.4, 50 mM sodium fluoride, 40 mM sodium pyrophosphate, 1% Triton X-100, 2 mM sodium vanadate, 10 mM *p*-nitrophenyl phosphate, and protease inhibitors (25). Lysed cells were incubated on ice for 20 min and centrifuged at 13,000 × *g* for 10 min at 4 °C. The clear supernatant was designated as cell lysates.

Immunoprecipitation and Immunoblotting—Cell lysates (~400 μg of protein) were incubated without or with indicated antibodies 1 h at 4 °C and subsequently with protein A- or protein G-agarose beads overnight at 4 °C on a rotating platform. After centrifugation, beads were washed five times with the modified RIPA buffer. Bound proteins were eluted with the SDS sample buffer, resolved by SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher and Schuell). Nitrocellulose membranes were incubated at room temperature for 1 h in the blocking buffer containing Tris-buffered saline with 0.1% Tween (TBS-T) containing 5% milk or 5% bovine serum albumin followed by incubation with indicated antibodies in the blocking buffer. After washing three times for 5 min each with TBS-T, membrane was incubated with horseradish peroxidase-conjugated donkey anti-mouse, or anti-rabbit IgG (Amersham Biosciences), or anti-rat IgG (Santa Cruz Biotechnology) followed by washing. Immunoreactive bands were visualized with enhanced chemiluminescence substrate (Pierce). In some experiments, the nitrocellulose filter was incubated in a buffer containing 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS at 50 °C for 30 min, and washed with 0.1% Tween 20 in 50 mM TBS at room temperature for 1 h, and reblotted with different antibodies. The following antibodies were used: FLAG (M2, Sigma), Myc (9E10, Santa Cruz Biotechnology), phospho-MAPK (Promega), phospho-Akt (Ser-473, New England Biolab), H-Ras (238, Santa Cruz Biotechnology), and Erbin (19).

Luciferase Assay—C2C12 myoblasts were co-transfected with or without Myc-Erbin, plus the ε-subunit promoter-luciferase transgene that contains 416 nucleotides of the 5′-untranslated region of the ε-subunit gene (25) and a control plasmid pRL-SV40 (Promega). 24 h after transfection, myoblasts were incubated in DM to induce myotube formation. Myotube formation was complete 48 h after switch to DM. The C2C12 myotubes were stimulated with NRG at a final concentration of 10 nM at 37 °C for 24 h. Mv1Lu cells were transiently transfected with the promoter reporter construct p3TP-Lux, which contains three AP-1 sites and the plasminogen activator inhibitor-1 (PAI-1) promoter (30), and firefly luciferase gene. pRL-SV40 (Promega) that express *Renilla* luciferase under the control of SV40 promoter was cotransfected as a control to monitor the transfection efficiency. 48 h after transfection, cells were lysed and activities of the two different luciferases were assayed with respective substrates with a dual luciferase assay kit (Promega).

Differentiation of PC12 Cells—PC12 cells were cotransfected pEGFP-C1 with empty vector pRK5-Myc, Myc-Erbin or its mutants, or Erbin-siRNA duplex. 48 h after transfection, PC12 cells were stimulated by 100 ng/ml or 20 ng/ml NGF for 2 days. Cells were examined by fluorescence microscopy. Cells with processes 1.5 times longer than the diameter of the cell body were considered to be differentiated.

Inhibition of Erbin Expression by RNA Interference (RNAi)—The target region of siRNA was 540 nucleotides downstream of the start codon (31), which contained ~50% G/C content. The nucleotide sequence was 5′-UAG ACU GAC CCA GCU GGA A dTdT-3′ (nucleotides 866–884) (27). We searched the NCBI sequence bank against this segment of DNA using the BLAST program, which revealed no match, suggesting of the specificity of target recognition by siRNA. The 21-nucleotide RNAs were chemically synthesized by Dharmacon Research Inc. Synthetic oligonucleotides were deprotected and gel-purified. To demonstrate the silencing effect of endogenous Erbin expression by siRNA, cells in a 60-mm culture dish were co-transfected with empty vector pEGFP-C1 and with siRNA duplex using SuperFect. Briefly, 2 μg of pEGFP-C1 and 30 μl of 20 μM Erbin-siRNA duplex were mixed with 300 μl of Opti-MEM (Invitrogen). After incubating 10 min at room temperature, add Opti-MEM to obtain a final volume of 1 ml. Cells were incubated with the mixture for 2–3 h at 37 °C and 5% CO_2 before the addition of 5 ml of growth medium. 72 h after transfection, cells were resuspended in phosphate-buffered saline buffer. GFP-positive cells were collected by fluorescence-activated cell sorting (FACS) analysis with the CellQuest software. Cells were lysed in modified RIPA buffer, and lysates were subjected to immunoblotting for expression of Erbin. In parallel experiments, GFP-positive PC12 cells were scored for differentiation.

Protein Assay—Protein was assayed with Coomassie Protein Assay Reagent (Pierce) using bovine serum albumin as a standard (32).

RESULTS AND DISCUSSION

Erbin Inhibits Erk Activation—Previous studies from our laboratory and others have demonstrated that NRG-induced AChR expression requires ErbB2 tyrosine phosphorylation and activation of the Ras-Raf-Erk signaling pathway (23–26,33). We speculated that Erbin, interacting with ErbB2, may play a role in regulating NRG signaling. To test this hypothesis, we examined the effect of Erbin on the promoter activity of ε416-Luc, a transgene reporter that contains 416 nucleotides of the 5′-untranslated region of the ε-subunit gene (25). Expression of this transgene is up-regulated by NRG or active forms of Ras and Raf and requires Erk activation (25, 33). Unexpectedly, we found that Erbin inhibited expression of the ε416-Luc transgene in control as well as NRG-stimulated muscle cells (Fig. 1*A*), suggesting that Erbin may regulate the Erk activation.

To test this hypothesis, we characterized effects of Erbin on Erk1 activation in COS-1 cells. FLAG-Erk1 was activated in response to NRG in cells cotransfected with ErbB4 (Fig. 1*B*) (29). Coexpression of Erbin caused a decrease in phospho-Erk (Fig. 1*B*) and as well as Erk kinase activity (Fig. 1*C*), indicating that Erbin negatively regulates the Ras-Raf-MEK-Erk pathway. The inhibitory effect of Erbin featured the following: 1) It was dose-dependent (Fig. 1*D*). 2) Erbin did not seem to delay the peak Erk activation that usually occurred within 5 min of stimulation (Fig. 1*E*, Ref. 25). 3) The inhibition was not growth factor-specific. The expression of Erbin inhibited EGF- and NGF-induced Erk activation (Fig. 1*F* and data not shown); and 4) it was Erk activation-specific, since expression of Erbin had no apparent effect on NRG activation of Akt (Fig. 1*G*). Thus, the results demonstrate that Erbin specifically inhibits Erk activation with no effect on the PI 3-kinase pathway.

To identify the domain that inhibits the Erk activation, we examined the effect of a series of Erbin mutants (Fig. 2*A*). The results revealed that the inhibition of Erk did not require PDZ

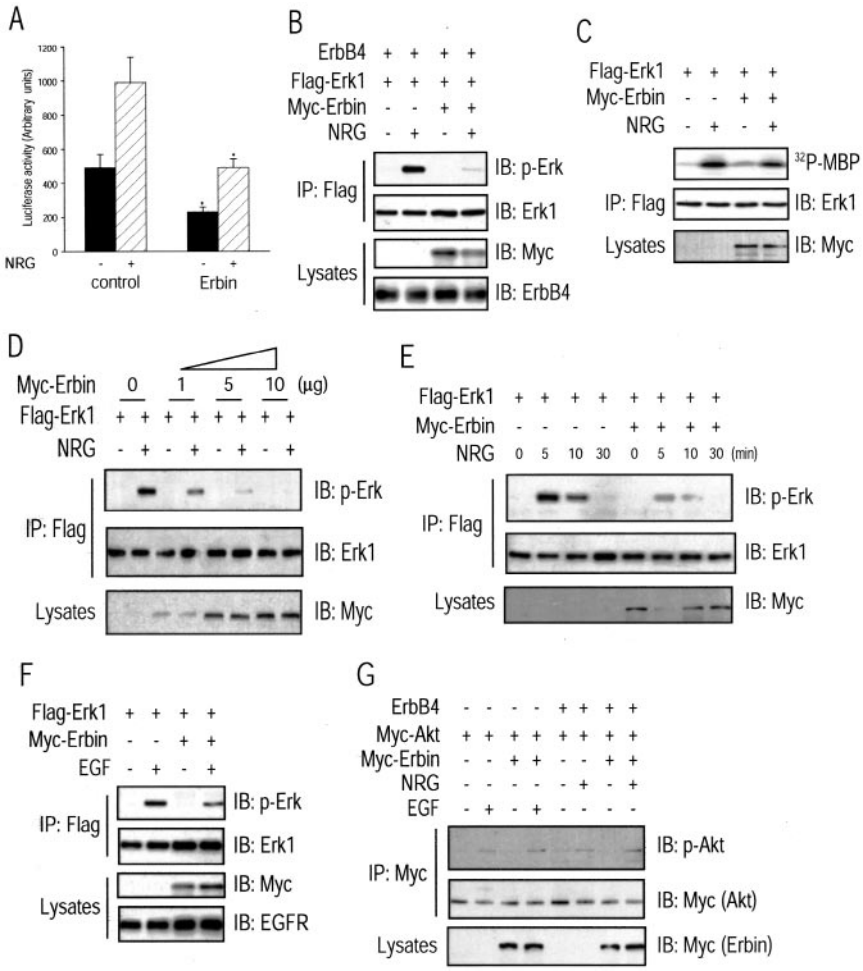

FIG. 1. **Inhibition of NRG-induced transcription of AChR ϵ-subunit gene and Erk activation by Erbin.** *A*, inhibition of the NRG-induced expression of an AChR ϵ-subunit reporter gene by Erbin. C2C12 cells were cotransfected with empty vector pRK5-Myc or Myc-Erbin and ϵ416-luc, which contains the ϵ-subunit 5′-flanking region driving the expression of firefly luciferase. A *Renilla* luciferase plasmid pRL-SV40 under the constitutive control of HSV promoter was cotransfected as a control to monitor the transfection efficiency and sample handling. After differentiation, transfectants were treated with or without 10 nM NRG for 24 h. Firefly luciferase activity was normalized to *Renilla* luciferase activity. *B*, Erbin inhibits Erk activation by NRG. COS-1 cells in 100-mm culture dishes were transfected with FLAG-Erk1 (1 μg) either alone or with Erbin (10 μg) and ErbB4 (5 μg). Transfected cells were serum-starved for 6 h and then stimulated with 10 nM NRG for 10 min. From the cell lysates, FLAG-Erk1 was immunoprecipitated (IP) using a mouse anti-FLAG antibody (M2) coupled with Sepharose beads. Resulting immunoprecipitates were then subjected to SDS-PAGE and immunoblotting with anti-phospho-Erk and anti-FLAG antibodies. Cell lysates were also subjected to immunoblotting (IB) with anti-Myc, and anti-ErbB4 antibodies to detect expression of Erbin and ErbB4. *C*, Erk kinase activity. Cells were transfected as in *B*. Immunopurified FLAG-Erk1 was assayed using myelin basic protein (*MBP*) as substrate in the presence of [γ-^{32}P]ATP *in vitro* as described previously (25). *D*, dose-dependent inhibition of Erk activation by Erbin. COS-1 cells were co-transfected with FLAG-Erk1 and ErbB4 and increasing concentrations of Myc-Erbin and then challenged with 10 nM NRG for 10 min. Erk activation was assayed as in *B*. Equal amount of FLAG-Erk1 was shown in a reblot of the same membrane in a representative experiment. *E*, Erbin had no effect on the Erk activation time course. *F*, inhibition of EGF-mediated Erk activation by Erbin. COS-1 cells were transfected with Flag-Erk1 (1 μg) with empty vector or Erbin (10 μg). Cells were stimulated with 100 ng/ml EGF for 10 min. Phospho-Erk was visualized with anti-phospho-MAPK antibody as described in *B*. Equal amounts of FLAG-Erk1 and EGFR were shown in a reblot. *G*, no effect of Erbin on Akt/protein kinase B kinase activation. COS-1 cells co-transfected with ErbB4 (5 μg), Myc-Akt (1 μg) without or with Myc-Erbin (10 μg). 48 h after transfection, cells were stimulated with 100 ng/ml EGF or 10 nM NRG for 10 min. Myc-Akt was immunoprecipitated with anti-Myc antibody, and subjected to SDS-PAGE and immunoblotting for phospho-Akt with a rabbit anti-phospho-Akt antibody.

domain (Fig. 2*B*), which is essential for interaction with ErbB2 (19, 27) or the region between the LRR domain and the PDZ domain (Fig. 2*B*). In contrast, deletion of the LRR domain disabled Erbin to inhibit Erk activation (Fig. 2*C*). Furthermore, we demonstrated that the LRR domain was sufficient to mediate the inhibitory effect (Fig. 2*B*). These results are in agreement with the differentiation assay (see Fig. 5) and point to an important role of Erbin in regulating the Ras-Raf-MEK-Erk pathway.

Erbin Inhibits Raf Activation—Next we attempted to dissect the position for Erbin action by walking upstream of Erk. Since Erbin directly interacts with the cytoplasmic domain of ErbB2, it is possible that Erbin interferes with the tyrosine kinase activation and/or subsequently binding to adaptor proteins. To test these hypotheses, we employed NeuT, an active form of ErbB2 (34). When expressed in COS-1 cells, NeuT was tyrosine-phosphorylated (Fig. 3*A*), resulting in an increase in the promoter activity of p3TP-Lux (Fig. 3*B*). When NeuT was immunoprecipitated, association of Shc and Grb2 could be easily detected (Fig. 3*A*). Coexpression of Erbin had no effect on either tyrosine phosphorylation of NeuT or its association with Shc and Grb2 (Fig. 3*A*). However, the NeuT-induced promoter activity of 3TP was greatly inhibited by Erbin (Fig. 3*B*), suggesting that the site of Erbin action is downstream of the adaptor proteins. Thus, we examined whether Erbin inhibits Erk activation by Raf or Ras. Since expression of the active Raf can bypass the requirement of upstream components for the MEK/Erk activation (7), Erbin would attenuate Erk activation by

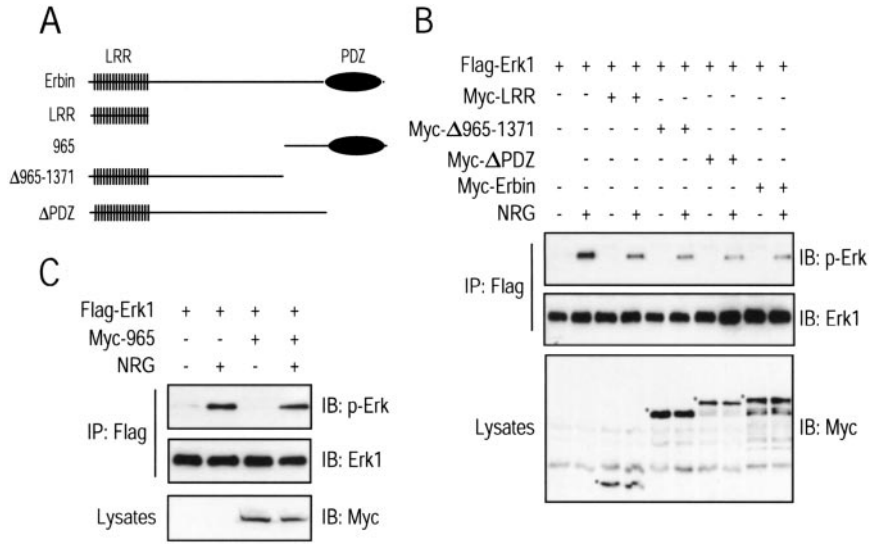

FIG. 2. **The LRR domain was required and sufficient to inhibit Erk activation.** *A*, schematic diagrams of Erbin expression constructs. *B*, LRR was sufficient to inhibit Erk activation. *C*, dependence of the inhibitory effect on the LRR domain. COS-1 cells were transfected with FLAG-Erk1 alone, or cotransfected with Myc-tagged LRR, Δ965–1371, ΔPDZ, Erbin in *A* or 965 in *B*. Transfected cells were stimulated NRG and assayed for Erk1 activation as described in Fig. 1. Lysates were immunoblotted with anti-Myc antibody to demonstrate the expression of Erbin and its mutants.

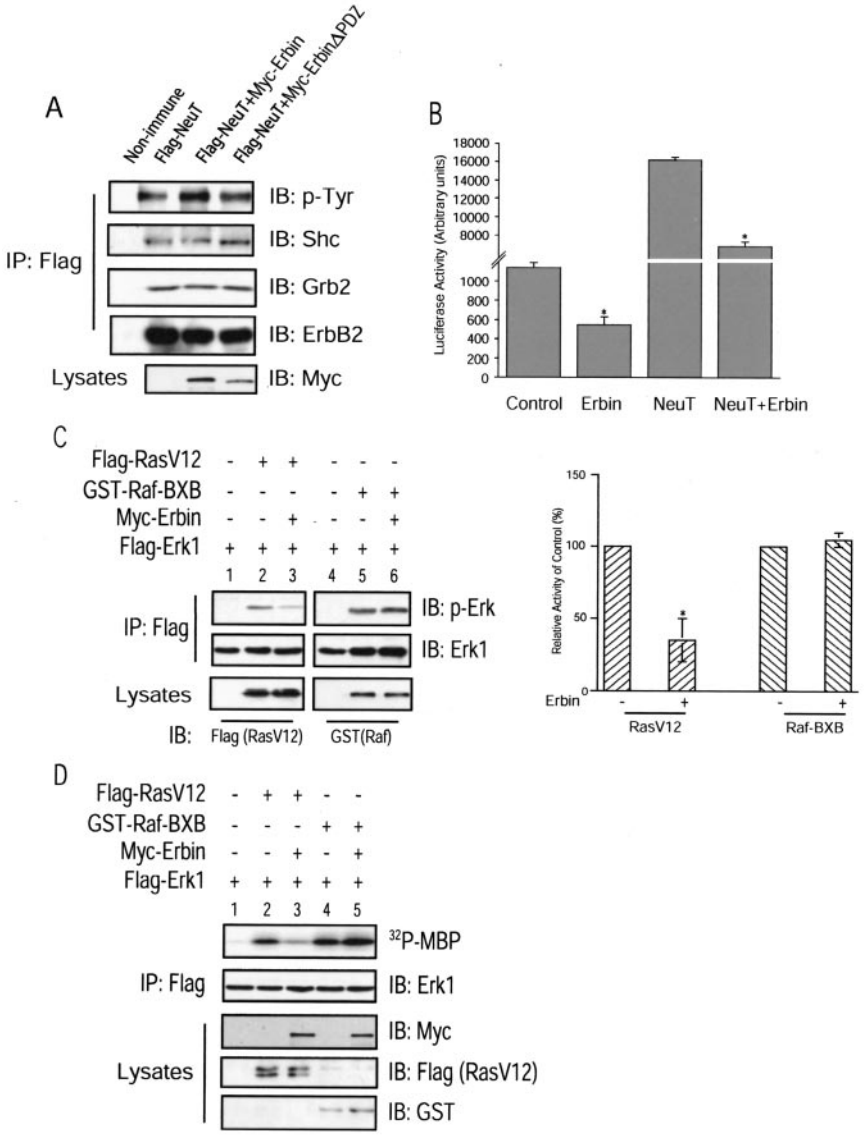

FIG. 3. **Erbin acts above Raf.** *A*, Erbin did not alter NeuT tyrosine phosphorylation or binding to Shc or Grb2. COS-1 cells transfected with FLAG-NeuT with or without Myc-Erbin or Myc-ErbinΔPDZ. NeuT was immunoprecipitated with anti-FLAG antibody. Resulting immunocomplexes were probed with antibodies against phosphotyrosine, Shc, Grb2, or ErbB2. Expression of Erbin and its mutant was detected by immunoblotting with anti-Myc antibody. *Non-immune*, no antibody in immunoprecipitation. *B*, Erbin inhibited NeuT-mediated gene expression. p3TP-Lux was transfected alone or cotransfected with FLAG-NeuT without or with Myc-Erbin in Mv1Lu cells. Cells were lysed, and luciferase activity measured and normalized to cotransfected pRL-SV40. Data are shown as means ± S.D. ($n = 3$). *Asterisk*, $p < 0.01$, Student's t test. *C*, inhibition of active Ras-, but not active Raf-mediated Erk activation. COS-1 cells were cotransfected with FLAG-Erk1, constitutively active Ras (V12) or Raf (BXB) without or with Myc-Erbin. Erk1 activation was assayed as described in the legend to Fig. 1. Results of densitometric analysis is shown on the *right*. Data are shown as means ± S.D. ($n = 3$). *Asterisk*, $p < 0.01$, Student's t test. *D*, Erk kinase activity. Cells were transfected as in *C*. Immunopurified FLAG-Erk1 was assayed using myelin basic protein (*MBP*) as substrate in the presence of [γ-^{32}P]ATP *in vitro* as described previously.

active Raf, if Erbin acts downstream of Raf. The results in Fig. 3, *C* and *D* showed that coexpression of Erbin with active Raf did not have an effect on Erk activation. In contrast, Erk activation by active Ras was evidently inhibited (*lanes 2* and *3*, Fig. 3, *C* and *D*). These results suggest that Erbin acts between Raf and Ras.

Erbin Disrupts the Ras-Raf Interaction—In considering the mechanism of Erbin-induced inhibition of the Erk pathway, it

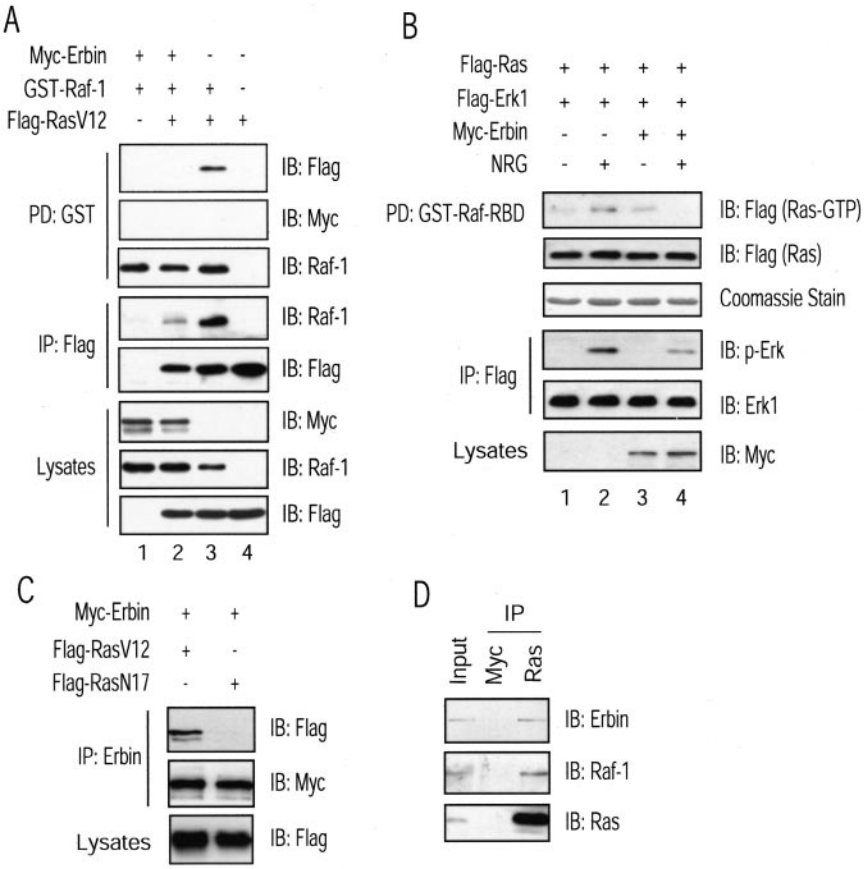

FIG. 4. **Erbin blocks the interaction of Ras and Raf.** *A*, Erbin inhibited RasV12 binding to Raf. Cells were transfected with Flag-RasV12, GST-Raf1, or Myc-Erbin. GST-Raf1 was pull-downed from cell lysates with GSH-agarose beads. Bound proteins were resolved by SDS-PAGE and then subjected to immunoblotting with antibodies against FLAG, Myc, or Raf1. In reciprocal experiments, FLAG-Ras was immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-Raf1 or anti-FLAG antibodies. Lysates were also blotted with anti-FLAG, anti-Myc, and anti-Raf1 antibodies to show the expression of indicated proteins. *B*, Erbin inhibited NRG-activated Ras binding to Raf. FLAG-Ras (wild type) and FLAG-Erk1 were cotransfected without or with Myc-Erbin. After stimulation with NRG, cells were lysed and cell lysates incubated with 5 μg of GST-Raf-RBD to purify activated GTP-bound Ras. Bound proteins were resolved on SDS-PAGE and subjected to immunoblotting with anti-FLAG antibody. Erk1 activation was assayed as described in the legend to Fig. 1. Lysates were also immunoblotted with anti-Myc or anti-FLAG antibodies to demonstrate expression of Erbin and Ras, respectively. *C*, Erbin interaction with active GTP-bound Ras. Cells were co-transfected with Myc-Erbin with FLAG-RasV12 or FLAG-RasN17. Myc-Erbin was immunoprecipitated from cell lysates with anti-Erbin antibodies. Resulting immunoprecipitates were subjected to SDS-PAGE and then immunoblotting with anti-FLAG or anti-Myc antibodies. Cell lysates were also blotted with anti-FLAG antibody to show expression of Ras constructs. *D*, interaction between endogenous Erbin and Ras. MCF-7 cells (500 μg of protein) were incubated with anti-Ras antibody, and anti-Myc antibody as a negative control. Resulting immunocomplexes were subjected to immunoblotting with antibodies against Erbin, Raf, or Ras. Input, 5% of proteins for immunoprecipitation.

is reasonable to postulate that Erbin inhibits the interaction between active Ras and Raf by competitive binding to either of them. To test this, active Ras (FLAG-RasV12) was coexpressed with GST-Raf1 into HEK293 cell. As shown in Fig. 4*A*, FLAG-RasV12 was found to copurify with GST-Raf1. In a reciprocal experiment, Raf1 was also detected in the immunoprecipitates of active Ras. Remarkably, coexpression of Erbin decreased the interaction between active Ras and Raf. In an alternative experiment, we employed the Ras pull-down assay developed by Rooij and Bos, which takes advantage of high affinity of GTP-Ras for the Ras-binding domain (RBD) of Raf-1 as compared with GDP-Ras (35). The RBD (amino acids 50–150) was expressed as a GST fusion protein and used to pull down GTP-Ras that had been activated in cells cotransfected with or without Erbin. Basal Ras binding to RBD was usually very low in control or Erbin-expressing cells. The effect of Erbin, if any, on Ras-RBD binding was inconsistence. However, in cells transfected with FLAG-Ras, GST-Raf-RBD could pull-down activated Ras (Fig. 4*B, lane 2*). Expression of Erbin inhibited the interaction of Raf with growth factor-activated Ras, which was accompanied by a decrease in phospho-Erk (Fig. 4*B, lane 4*). These results suggest that Erbin inhibits the Erk pathway by disrupting the interaction between active Ras and Raf.

Because our data did not support Erbin interaction with Raf1 (Fig. 4*A*), we attempted to determine whether Erbin associated with Ras. In this experiment recombinant Erbin was immunoprecipitated from HEK293 cells cotransfected with a constitutively active mutant of Ras (V12) or a dominant negative mutant of Ras (N17). The resulting immunocomplex was probed with anti-FLAG antibody. As shown in Fig. 4*C*, only the active Ras (V12) was co-immunoprecipitated with Erbin, suggesting that transfected Erbin and active Ras may interact in cells. We employed the yeast two-hybrid system to determine whether Erbin and active Ras interact directly. Yeast Y190 cells were cotransformed with pACT2-RasV12 and pGBT9-LRR, which contains the LRR domain of Erbin. Cotransformation of Ras and Erbin did not result in a significant change in β-galactosidase activity as compared with the negative control, while the interaction between Ras and Raf was evidently detected (36).[2] This suggests that the two proteins may not in-

[2] Y. Huang and L. Mei, unpublished observations.

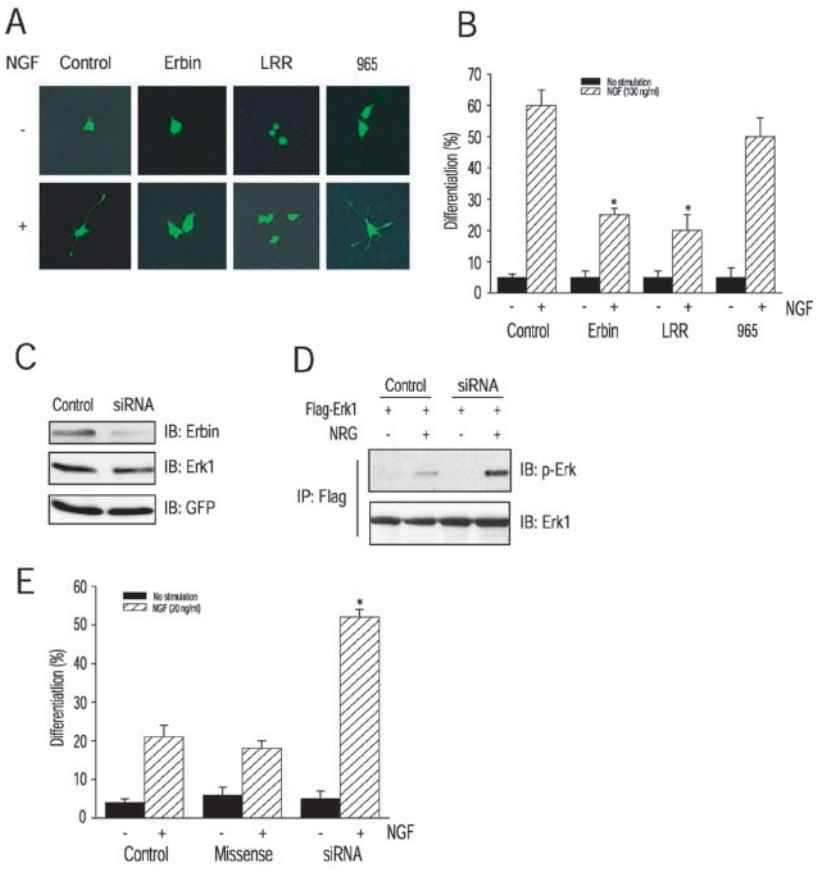

FIG. 5. **Erbin inhibition of PC12 cell differentiation.** *A*, PC12 cells were co-transfected with pEGFP-C1 and empty vector pRK5-Myc or wild-type Erbin, LRR domain, or C-terminal domain of Erbin. Transfected cells were stimulated without or with NGF (100 ng/ml) for 48 h. Representative fluorescent images of cells were shown. *B*, quantitative analysis of data in *A* (means ± S.D., $n = 3$). *Asterisk*, $p < 0.01$, Student's t test. *C*, inhibition of endogenous Erbin expression by RNAi. Cells were cotransfected with pEGFP-C1 expression vector either with RNAi against Erbin (10 μM), or with missense scramble RNA (10 μM) as a control. Two days after transfection, GFP-positive cells were sorted and lysed in RIPA buffer. Cell lysates were subjected to SDS-PAGE and Western blotting with anti-Erbin, Erk1, and GFP antibodies. *D*, increase in NRG-activated Erk activity in Erbin-siRNA expressing cells. COS-1 cells were co-transfected with FLAG-Erk1 and Erbin-siRNA, which were then challenged with NRG for 10 min. Erk activation was assayed as in the legend to Fig. 1. Equal amounts of FLAG-Erk1 was shown in a reblot of the same membrane in a representative experiment. *E*, increase in differentiated PC12 cells by Erbin-siRNA. PC12 cells cultured on 12-well plates were cotransfected with pEGFP-C1 either alone or with Erbin-siRNA (10 μM), or with missense RNA (10 μM) as a control. Transfected cells were stimulated without or with NGF (20 ng/ml) for 48 h. Data shown were means ± S.D. ($n = 3$). *Asterisk*, $p < 0.01$, Student's t test.

teract directly. To demonstrate the possible interaction of endogenous proteins, MCF-7 cell lysates were incubated with monoclonal antibody against Ras and the resulting immunocomplex was blotted for Erbin, Raf1, and Ras. Immunoprecipitation brought down Raf1 (Fig. 4*D*). Of note was the presence of Erbin in the complex, suggesting that endogenous Erbin and Ras may interact or be in the same complex.

Inhibition of NGF-induced PC12 Cell Differentiation by Erbin—To further study the physiological importance of the Ras inhibition by Erbin, we examined the effect of its overexpression on neuronal differentiation of rat pheochromocytoma (PC12) cells. By chronic incubation with NGF, these cells differentiated and developed sympathetic neuron-like phenotypes (37). The NGF-treated cells stopped to divide and in turn developed long, sometimes branched processes. The Ras-Raf-Erk pathway plays an essential role in NGF-induced differentiation of PC12 cells (38–40). The inhibition of Erk activation by Erbin suggests that it may alter NGF-induced differentiation of PC12 cells. Expression of enhanced green fluorescent protein (EGFP) and the pRK5-Myc empty vector (control) had no apparent effect on differentiation whereas Erbin-transfected PC12 cells exhibited altered morphology (Fig. 5*A*). The neurites became shorter and were less branched. Quantitative analysis of Erbin's effect was shown in Fig. 5*B*. Under the control condition, cells bearing neurites 1.5 times longer than the cell body accounted for 60 ± 5% of the total cell population, whereas the number of differentiated cells are significantly reduced by Erbin (Fig. 5*B*). Erbin contains three domains: an N-terminal region that contains 16 LRRs, a C-terminal PDZ domain, and a middle region that show no homology to known proteins (17–22). Ecptopic expression of the LRR domain showed similar effect on differentiation, suggesting that the inhibitory activity was contained in this domain. In contrast, cells transfected with Δ1–965 encoding C-terminal domain of Erbin appeared to have normal differentiation.

To confirm the inhibitory effect of Erbin on PC12 cell differentiation, we examined the effect of suppressed Erbin expression in PC12 cells. To this end, we employed the RNAi technique, which diminishes expression of a specific gene in cells (41). Recently, RNAi has been shown to specifically suppress the expression of endogenous and heterologous genes in mammalian cell lines (31, 42). Thus, 21 nucleotide small interfering RNA (siRNA) duplexes directed against Erbin (nucleotides 866–884, Ref. 27) were synthesized and transfected in PC12 cells to suppress expression of endogenous Erbin. Two days after transfection, cells expressing co-transfected EGFP were sorted out and analyzed for Erbin expression by Western blot. As shown in Fig. 5*C*, expression of Erbin in Erbin-siRNA-transfected cells was significantly decreased in comparison with missense RNAi-transfected cells (control). The suppressing effect by Erbin-siRNA appeared to be specific since it had no effect on expression of endogenous Erk1. Moreover, expression of cotransfected EGFP was unaffected (Fig. 5*C*). In agreement of results with Erbin expression experiments, inhibition of Erbin expression by Erbin-siRNA caused an increase in phospho-Erk in response to NRG stimulation (Fig. 5*D*). Having demonstrated that Erbin-siRNA inhibits expression of endogenous Erbin and increases NRG-induced activation of Erk, we studied the effect of Erbin-siRNA on PC12 cell differentiation. To capture the maximal effect of Erbin-siRNA, a submaximal effective concentration of NGF was used, as at this concentration, NGF caused differentiation of only 25 ± 2% PC12 cells (Fig. 5*E, control*). Remarkably, suppressing Erbin expression by Erbin-siRNA enhanced NGF-mediated cell differentiation (52 ± 2%), while missense RNA had no significant effect (18 ±

2%). These results demonstrate that Erbin inhibits NGF-induced differentiation of PC12 cells.

The current study showing that Erbin inhibits Erk activation together with previous findings that it associates with ErbB2, p0071, δ-catenin, and ARCVS (17–22) raises a possibility that Erbin may participate in targeting signaling complex to specific subcellular compartment. The mechanism of Erbin inhibition remains unclear. Erbin may compete with Raf in binding to active Ras. However, we have no evidence at present that Erbin interacts directly with Ras. Alternatively, Erbin may compete with Sur-8 for binding to Ras. Sur-8 is an LRR-containing protein that potentiates Ras signaling presumably by enhancing the interaction between Ras and Raf-1 (10, 12). Sur-8 contains 18 LRRs, two more than Erbin and the LRRs of Sur-8 and Erbin share only 30% amino acid identity (17, 19). The structure differences may account for distinct roles of these proteins in regulating Ras signaling. Erbin binding to Ras may dissociate the Sur-8/Ras/Raf ternary complex, resulting in down-regulation of the Erk pathway. Active mutations of the Ras gene render it to be the most frequent oncogene found in human cancers and even more, many other oncogenes exploit Ras and its downstream cohorts to execute their functions. Further study on how Erbin inhibits Erk activation may not only contribute to a better understanding of cell signaling, but also identify targets in development of cancer diagnosis and therapy.

Erbin belongs to the LAP (LRR and PDZ) protein family of PDZ domain-containing proteins (17–22). In addition to Erbin, the family members include LET-413 in *Caenorhabditis elegans* (27, 43); Scribble, a *Drosophila* protein essential for epithelial integrity (44, 45); and Densin-180 (46) and Lano in mammals (47). Genetic studies in non-vertebrates have demonstrated that LAP proteins play a role in cell polarity and cell morphology of epithelial cells (43–45). Our results raised the possibility that these previously thought scaffold proteins may participate in regulation of cell signaling.

In summary, we provide evidence that Erbin is a negatively regulator of the Ras signaling pathway. Since Erbin associates with activated, but not inactive Ras, our results suggest a turn-off mechanism through which Ras signaling to Raf is inhibited. Ras, upon activation by extracellular signals, forms a complex with Erbin and thus becomes unable to activate Raf.

Acknowledgments—We thank Dr. M. Weber, M. C. Hung, and M. Slikowski for reagents.

REFERENCES

1. Robinson, M. J., and Cobb, M. H. (1997) *Curr. Opin. Cell Biol.* **9,** 180–186
2. Bar-Sagi, D., and Hall, A. (2000) *Cell* **103,** 227–238
3. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61,** 203–212
4. Schlessinger, J. (2000) *Cell* **103,** 211–225
5. Pawson, T., and Scott, J. D. (1997) *Science* **278,** 2075–2080
6. Katz, M. E., and McCormick, F. (1997) *Curr. Opin. Genet. Dev.* **7,** 75–79
7. Kerkhoff, E., and Rapp, U. R. (1998) *Oncogene* **17,** 1457–1462
8. Kerkhoff, E., Houben, R., Loffler, S., Troppmair, J., Lee, J. E., and Rapp, U. R. (1998) *Oncogene* **16,** 211–216
9. Schaeffer, H. J., Catling, A. D., Eblen, S. T., Collier, L. S., Krauss, A., and Weber, M. J. (1998) *Science* **281,** 1668–1671
10. Li, W., Han, M., and Guan, K. L. (2000) *Genes Dev.* **14,** 895–900
11. Therrien, M., Wong, A. M., and Rubin, G. M. (1998) *Cell* **95,** 343–353
12. Sieburth, D. S., Sun, Q., and Han, M. (1998) *Cell* **94,** 119–130
13. Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M., Komiya, S., Baron, R., and Yoshimura, A. (2001) *Nature* **412,** 647–651
14. Sasaki, A., Taketomi, T., Wakioka, T., Kato, R., and Yoshimura, A. (2001) *J. Biol. Chem.* **276,** 36804–36808
15. Wang, Y., Waldron, R. T., Dhaka, A., Patel, A., Riley, M. M., Rozengurt, E., and Colicelli, J. (2002) *Mol. Cell. Biol.* **22,** 916–926
16. Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K. D., Rose, D. W., Mischak, H., Sedivy, J. M., and Kolch, W. (1999) *Nature* **401,** 173–177
17. Borg, J.-P., Marchetto, S., Le Bivic, A., Ollendorff, V., Haulin-Bastard, F., Saito, H., Fournier, E., Adelaide, J., Margolis, B., and Birnbaum, D. (2000) *Nat. Cell Biol.* **2,** 407–413
18. Favre, B., Fontao, L., Koster, J., Shafaatian, R., Jaunin, F., Saurat, J. H., Sonnenberg, A., and Borradori, L. (2001) *J. Biol. Chem.* **276,** 32427–32436
19. Huang, Y. Z., Wang, Q., Xiong, W. C., and Mei, L. (2001) *J. Biol. Chem.* **276,** 19318–19326
20. Jaulin-Bastard, F., Saito, H., Le Bivic, A., Ollendorff, V., Marchetto, S., Birnbaum, D., and Borg, J.-P. (2001) *J. Biol. Chem.* **276,** 15256–15263
21. Jaulin-Bastard, F., Arsanto, J. P., Le Bivic, A., Navarro, C., Vely, F., Saito, H., Marchetto, S., Hatzfeld, M., Santoni, M. J., Birnbaum, D., and Borg, J. P. (2002) *J. Biol. Chem.* **277,** 2869–2875
22. Laura, R. P., Witt, A. S., Held, H. A., Gerstner, R., Deshayes, K., Koehler, M. F., Kosik, K. S., Sidhu, S. S., and Lasky, L. A. (2002) *J. Biol. Chem.* **277,** 12906–12914
23. Altiok, N., Altiok, S., and Changeux, J. P. (1997) *EMBO J.* **16,** 717–725
24. Jo, S. A., Zhu, X., Marchionni, M. A., and Burden, S. J. (1995) *Nature* **373,** 158–161
25. Si, J., Luo, Z., and Mei, L. (1996) *J. Biol. Chem.* **271,** 19752–19759
26. Tansey, M. G., Chu, G. C., and Merlie, J. P. (1996) *J. Cell Biol.* **134,** 465–476
27. Borg, J. P., Marchetto, S., Le Bivic, A., Ollendorff, V., Jaulin-Bastard, F., Saito, H., Fournier, E., Adelaide, J., Margolis, B., and Birnbaum, D. (2000) *Nat. Cell Biol.* **2,** 407–414
28. Zang, M., Hayne, C., and Luo, Z. (2002) *J. Biol. Chem.* **277,** 4395–4405
29. Huang, Y. Z., Won, S., Ali, D. W., Wang, Q., Tanowitz, M., Du, Q. S., Pelkey, K. A., Yang, D. J., Xiong, W. C., Salter, M. W., and Mei, L. (2000) *Neuron* **26,** 443–455
30. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996) *Nature* **383,** 832–836
31. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* **411,** 494–498
32. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
33. Fu, A. K., Fu, W. Y., Cheung, J., Tsim, K. W., Ip, F. C., Wang, J. H., and Ip, N. Y. (2001) *Nat. Neurosci.* **4,** 374–381
34. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) *Cell* **45,** 649–657
35. de Rooij, J., and Bos, J. L. (1997) *Oncogene* **14,** 623–625
36. Luo, Z. J., Zhang, X. F., Rapp, U., and Avruch, J. (1995) *J. Biol. Chem.* **270,** 23681–23687
37. Greene, L. A., and Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 2424–2428
38. Gamett, D. C., and Cerione, R. A. (1994) *FEBS Lett.* **351,** 335–339
39. Gamett, D. C., Greene, T., Wagreich, A. R., Kim, H. H., Koland, J. G., and Cerione, R. A. (1995) *J. Biol. Chem.* **270,** 19022–19027
40. Marshall, C. J. (1995) *Cell* **80,** 179–185
41. Hammond, S. M., Caudy, A. A., and Hannon, G. J. (2001) *Nat. Rev. Genet.* **2,** 110–119
42. Paddison, P. J., Caudy, A. A., and Hannon, G. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1443–1448
43. Legouis, R., Gansmuller, A., Sookhareea, S., Bosher, J. M., Baillie, D. L., and Labouesse, M. (2000) *Nat. Cell Biol.* **2,** 415–422
44. Bilder, D., and Perrimon, N. (2000) *Nature* **403,** 676–680
45. Bilder, D., Li, M., and Perrimon, N. (2000) *Science* **289,** 113–116
46. Apperson, M. L., Moon, I. S., and Kennedy, M. B. (1996) *J. Neurosci.* **16,** 6839–6852
47. Saito, H., Santoni, M. J., Arsanto, J. P., Jaulin-Bastard, F., Le Bivic, A., Marchetto, S., Audebert, S., Isnardon, D., Adelaide, J., Birnbaum, D., and Borg, J. P. (2001) *J. Biol. Chem.* **276,** 32051–32055