Centaurin β1 Down-regulates Nucleotide-binding Oligomerization Domains 1- and 2-dependent NF-κB Activation*

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Jesus K. Yamamoto-Furusho, Nicolas Barnich, Ramnik Xavier, Tadakazu Hisamatsu, and Daniel K. Podolsky*

From the Gastrointestinal Unit, Department of Medicine, Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

Centaurin β1 (CENTB1), a GTPase-activating protein, is a member of the ADP-ribosylation factor family encoded by a gene located on the short arm of human chromosome 17. A yeast two-hybrid screen first suggested a direct interaction between CENTB1 and NOD2. Co-immunoprecipitation experiments confirmed direct interaction between CENTB1 and NOD2 and demonstrated similar interaction between CENTB1 and NOD1. We also demonstrate that endogenous CENTB1 interacts with endogenous NOD2 and NOD1 in SW480 and HT-29 intestinal epithelial cells. CENTB1 partially co-localized with NOD2 and NOD1 proteins in the cytoplasm of mammalian cells. CENTB1 expression in epithelial cells was highly induced by tumor necrosis factor α, interleukin 1β, and the NOD1 and NOD2 ligands (γ-D-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively). In addition, CENTB1 mRNA level is increased in the inflamed mucosa of patients with inflammatory bowel disease. Functionally, CENTB1 overexpression inhibited NOD1- and NOD2-dependent activation of NF-κB, whereas small inhibitory RNA against CENTB1 increased NF-κB activation following NOD1- or NOD2-mediated recognition of the bacterial components γ-D-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively. In contrast, CENTB1 had no effect on NF-κB activation induced by Toll-like receptors. In conclusion, CENTB1 selectively down-regulates NF-κB activation via NODs pathways, creating a “feedback” loop and suggesting a novel role of CENTB1 in innate immune responses to bacteria and inflammatory responses.

Nucleotide-binding oligomerization domain (NOD)3 containing proteins, including NOD1 and NOD2, have been implicated in intracellular recognition of bacterial components (1). The CARD15 (NOD2) gene is located on the long arm of human chromosome 16 and encodes a protein that contains two C-terminal caspase recruitment domains (CARDS), a central NOD (also known as a nucleotide-binding domain) and an N-terminal leucine-rich repeat region (2). NOD2 recognizes intracellular peptidoglycans from Gram-negative and Gram-positive bacteria through the detection of the minimal motif muramyl dipeptide (MDP) (3). Recognition of this specific dipeptide by NOD2 leads to nuclear factor κB (NF-κB) activation (4). This is thought to be relevant to disease pathogenesis given that NF-κB is a key intracellular signaling molecule in a variety of inflammatory pathways (5) and the finding of NOD2 mutations in patients with inflammatory diseases such as Crohn disease (CD) (6, 7) and Blau syndrome (BS) (8).

The NOD1 (CARD4) gene is located on the short arm of human chromosome 7. NOD1 is a cytosolic protein that is constitutively expressed by many cell types including intestinal epithelial cells (9) and has also been implicated in innate immune recognition. NOD1 contains CARD, NOD/nucleotide-binding domain, and leucine-rich repeat region domains. The CARD and NOD domains are required for NF-κB activation (10), whereas the leucine-rich repeat region domain recognizes the γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) derived from peptidoglycans of Gram-negative bacteria (11).

Because intestinal epithelial cells (IECs) are constantly exposed to bacteria and their components, IECs are considered to be not only a structural but also a functional barrier as the front line of host defense against microorganisms. Although IECs are hypo-responsive to normal flora in vivo, pathogenic bacteria or internalized bacterial components can initiate an inflammatory response (12–14). These data suggest that presentation of internalized bacterial components is a critical point in bacteria-epithelial interaction. We have previously shown that the NOD2 acts as a defensive factor against intracellular bacteria in IECs (20). In addition, membrane recruitment of NOD2 in IECs is essential for NF-κB activation in MDP recognition, which could be responsible for NOD2 anti-bacterial activity (21).

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‡ To whom correspondence should be addressed: Gastrointestinal Unit, MA General Hospital, 55 Fruit St., Boston, MA 02114. Tel: 617-726-7411; Fax: 617-724-2136; E-mail: dpodolsky@partners.org.

The abbreviations used are: NOD, nucleotide-binding oligomerization domain; CENTB1, Centaurin β1; MDP, muramyl dipeptide; NF-κB, nuclear factor κB; IEC, intestinal epithelial cell; siRNA, small inhibitory RNA; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; IL, interleukin; CARD, C-terminal caspase recruitment domain; CD, Crohn disease; BS, Blau syndrome; IBD, inflammatory bowel disease; RT, reverse transcription; PH, pleckstrin homology; FITC, fluorescein isothiocyanate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; LPS, lipopolysaccharide; TLR, Toll-like receptor; HA, hemagglutinin.
Interestingly, intraepithelial bacteria have been found in patients with inflammatory bowel disease (IBD) (15) and an increased prevalence of adherent-invasive *Escherichia coli* has been observed in ileal mucosa of CD patients (16). This is consistent with the hypothesis that recognition mechanisms of intracellular bacteria or their components likely play a key role in the pathophysiology of IBD. However, understanding of the mechanisms through which NOD proteins lead to effective responses remains substantially incomplete. Although some studies have suggested that NOD2 activation modulates TLR2 responses, which may be dysregulated when the NOD2 3202insC mutant associated with CD is present, others have not found comparable results (17–19).

A number of groups have endeavored to identify and characterize proteins that interact with NODs to better understand their functional effects and mechanisms of action. These efforts have previously suggested roles for GRIM-19 (22), TAK1 (23), Ipaf (24), and Erbin (25, 26) in the regulation of NOD2 activation. However, it is evident that these proteins are not sufficient to fully explain the regulation of NOD2-mediated NF-κB activation. To identify new molecules that could interact with these intracellular sensors, a yeast two-hybrid screen was undertaken using NOD2 protein as bait. Hybrid constructs comprising the NOD and leucine-rich repeat region domain led to the finding that Centaurin β1 protein (CENTB1) interacts with both the NOD1 and NOD2 proteins.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Cloning of NOD2-interacting Proteins—**Yeast two-hybrid screening was performed using MATCHMAKER GAL4 two-hybrid system 3 according to the manufacturer’s protocol (BD Biosciences Clontech, Palo Alto, CA). Briefly, pGBKTT7-NOD2 was transfected into the AH109 yeast strain. Expression of Myc-tagged NOD2 protein in yeast extract was confirmed by Western blot analysis using anti-Myc monoclonal antibody (Covance, Richmond, CA) and affinity-purified anti-NOD2 antiserum (19). Screening was performed using a plasmid library that had been purchased from Clontech (BD Biosciences Clontech) according to the manufacturer’s protocol (BD Biosciences Clontech) as described previously (20). Co-transformants were selected in S.D. medium lacking histidine, leucine, and tryptophan. Yeast α-galactosidase activity, expressed from the MEL1 gene response to GAL4 activation, was determined in plates containing X-gal (BD Biosciences Clontech).

**Cell Culture and Tissue—**COS-7, HEK293, THP-1, Jurkat, SW480, HT-29, Caco-2, T84, Colo205, and HCT116 cells were obtained from the American Type Culture Collection (Manassas, VA) and used for experiments after they had reached 90–100% confluence. SW480 and Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro Mediatech Ins., Herndon, VA) supplemented with 20% (v/v) heat-inactivated fetal calf serum (Atlanta Biologicals Inc., Norcross, GA). COS-7, HEK293, HT-29, Colo205, and HCT116 were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (Cellgro Mediatech Inc.) containing 10% heat-inactivated fetal calf serum. T-84 cells were cultured in Dulbecco’s modified Eagle’s medium with 1-glutamine (Cellgro Mediatech Inc.) supplemented with 10% (v/v) heat-inactivated fetal calf serum. Colonic mucosal biopsy samples from patients with ulcerative colitis and CD were immediately snap frozen in liquid nitrogen. Frozen biopsies were disrupted mechanically, and total RNA was isolated using TRizol reagent (Invitrogen) following the manufacturer’s protocols. cDNA from colon surgical specimens of IBD patients and normal controls were provided by Dr. Emiko Mizoguchi. All of the tissue samples were obtained under protocols approved by Partners IRB.

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Construction of Expression Plasmids—cDNA of CENTB1 was obtained by a PCR method using an Expand High Fidelity PCR system (Roche Applied Science) from the above mentioned cell lines. The PCR primers were: 5'-atgcacgccgagtctcgagtcaggg-3' (forward) and 5'-cagctgggtgaaggggtgcgattc-3' (reverse). The sequence of CENTB1 cDNA was confirmed using an ABI 3700 PRISM (PerkinElmer Life Sciences) automated sequencer. An expression plasmid-encoding Xpress-tagged CENTB1 mammalian expression vector (pcDNA4/HisMAX-Centaurin β1) was generated by RT-PCR from SW480 cells. An expression plasmid-encoding FLAG-tagged NOD2 (pCMV FLAG-NOD2) was previously generated (20). pCI CARD4/NOD1-HA expression vector was provided by Dr. John Bertin (Millennium Pharmaceuticals Inc). Two oligonucleotides, 19 residues in length (siRNA-1, gtaagaggtgtgacctg; and siRNA-2, aattcaccgcgggtgtaa), specific to the human CENTB1 were selected for synthesis of siRNA. pSUPER vector for siRNA was purchased from Oligoengine (Seattle, WA). The depletion of endogenous CENTB1 expression was confirmed by RT-PCR.

Crohn’s disease mutations plasids including FLAG-NOD2 L1007fs, FLAG–NOD2 R702W, and FLAG–NOD2 G908R have been previously generated (21). Blau syndrome mutants FLAG-NOD2 R334W and FLAG-NOD2 L496F were generated by PCR using the QuikChange site-directed mutagenesis kit (Strategene) according to the manufacturer’s protocols, and the mutations introduced were confirmed by sequencing. The CENTB1 deletion constructs were generated by PCR amplifying different regions of amino acids using specific primers (PH domain: 5'-atgcacggctcagttgcagctggag-3' (forward) and 5'-acgctccttctgctctgct-3' (reverse)); Arf-GAP domain + Ankyrin repeats: (5'-gcctgctgacctggctgctgacctggctg-3' (forward) and 5'-ccagctggctgctgacctggctgacctggctg-3' (reverse)); Ankyrin repeats (5'-aactccacggctgctgacctggctgctgacctggctg-3' (forward) and 5'-tacagctggctgctgacctggctgacctggctg-3' (reverse)). The PCR product was cloned into pcDNA4-Xpress vector to generate a fusion protein with an N-terminal Xpress tag.

Immunoprecipitation and Immunoblotting CENTB1 and NOD Proteins—Anti-CENTB1 sera were produced by co-immunizing rabbits with the synthesized polypeptide sequences: peptide 1, DPEKLSRRSHDLHTL (positions 726–740); and peptide 2, ARLGPPEPMMAECLE (positions 67–81) conjugated with glutaraldehyde. Anti-CENTB1 sera were affinity purified by using BioPure from ABR Affinity BioReagents.

The cells were grown in six-well plates. The medium was removed, and 300 μl of 1% Triton-X-lysis buffer (1% Triton X-100, 0.1 M NaCl, 10 mM Hepes, pH 5.6, 2 mM EDTA, 4 mM Na3VO4, and 40 mM NaF) supplemented with protease-inhibitor mixture, complete Mini (Roche Applied Science) was added. Supernatants of cell lysates were obtained by centrifuge.
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expression at 12,000 × g for 10 min. Protein concentration was determined using DC protein assay kit (Bio-Rad). Proteins were extracted from COS-7 cells (transiently co-transfected with pCMV FLAG-NOD2 and pCDNA4-HisMAX-CENTB1 plasmids). Two milligrams of total cell lysate protein were immunoprecipitated with 2 μg of anti-FLAG biotinylated M2 monoclonal or anti-Xpress monoclonal antibodies and 100 μl of Hiptrap protein A/G-Sepharose beads. After overnight incubation at 4 °C, the immunoprecipitated proteins were separated on 4 – 12% Tris-glycine gels (Invitrogen). The proteins were blotted onto polyvinylidene difluoride membranes and detected using anti-FLAG biotinylated M2 monoclonal antibody (Sigma-Aldrich) and anti-Xpress monoclonal antibody (Invitrogen).

**Co-localization of CENTB1 and NOD Proteins**—COS-7 cells were seeded on sterile Permanox coverslips, grown for 24 h, and then co-transfected with pCDNA4/HisMAX-CENTB1 and pCMV FLAG-NOD2. After 48 h, the cells were washed twice with ice-cold phosphate-buffered saline, fixed 20 min with cold methanol at −20 °C, and washed three times with ice-cold phosphate-buffered saline. The cells were saturated for 30 min with phosphate-buffered saline containing 5% horse serum and then incubated 2 h with primary antibody (mouse monoclonal anti-Xpress antibody and/or rabbit polyclonal anti-FLAG antibody from Sigma). Immunostaining was performed with Texas Red-conjugated anti-mouse IgG or with FITC-conjugated anti-rabbit IgG secondary antibodies (Vector Laboratories). The coverslips were mounted on Vectashield (Vector Laboratories) and examined at room temperature with a confocal laser scanning microscope (Radiance 2000 model; Bio-Rad) using multi-tracking (line switching) for two-color imaging (40×). Image acquisition was performed with LaserSharpScanning software (Bio-Rad).

**mRNA Expression of CENTB1**—Total RNA of intestinal epithelial cell lines was extracted by TRizol (Invitrogen) following the manufacturer’s instructions. For RT-PCR, 1 μg of total RNA was reverse transcribed using a SuperScript first strand synthesis system (Invitrogen). Real time RT-PCR was performed in an ABI Prism 7000 sequence detector using a SYBR synthesis system (Invitrogen). Real time RT-PCR was performed at 12,000 °C, the immunoprecipitated proteins were separated on 4 – 12% Tris-glycine gels (Invitrogen). The proteins were blotted onto polyvinylidene difluoride membranes and detected using anti-FLAG biotinylated M2 monoclonal antibody (Sigma-Aldrich) and anti-Xpress monoclonal antibody (Invitrogen).

**RESULTS**

**Identification of CENTB1 as a Novel NOD2-interacting Protein**—A yeast two-hybrid screen was performed to identify cellular proteins that interact with CARD15/NOD2. A NOD2 construct without the CARD15 domain was used as bait. We screened a human cDNA library expressing proteins fused to the active domain transcriptional activation domain. One of the positive clones was found to encode the human CENTB1. To confirm a specific interaction between NOD2 and CENTB1, we performed AH109 yeast survival assays in S.D./−Ade/−His/−Leu/−Trp/X-gal selective medium. Co-expression of NOD2 and CENTB1 in AH109 yeast showed a strong interaction between these two proteins (data not shown).

To confirm the interaction of NOD2 and CENTB1 in mammalian cells, COS7 and HEK293 cells were transiently transfected with FLAG-tagged NOD2 and Xpress-tagged CENTB1. After 48 h of transfection, NOD2 was immunoprecipitated with HM2559 anti-NOD2 serum (20), and CENTB1 was immunoprecipitated with an anti-Xpress monoclonal antibody. As shown in Fig. 1A, CENTB1 was detected in anti-HM2559 (20) immunoprecipitates from NOD2 co-transfectants, but not from cells co-transfected with a control plasmid. A reciprocal immunoprecipitation/blotting experiment using anti-Xpress monoclonal antibody also showed NOD2 co-precipitation with CENTB1, confirming the interaction of these two proteins in mammalian cells.

**Interaction between NOD1 and CENTB1 in Mammalian Cells**—To examine the extent of the interaction of CENTB1 with NOD proteins, the ability of NOD1, an other NOD family member, to co-immunoprecipitate with CENTB1 was investigated by Western blot (Fig. 1B). After 48 h of co-transfection of HEK293 cells with DNA constructs encoding HA-tagged NOD1 and Xpress-tagged CENTB1, immunoprecipitation experiments were performed using anti-NOD1 antisera HM3851 (9) and anti-Xpress monoclonal antibody to pull down NOD1 and CENTB1, respectively. CENTB1 was detected in anti-NOD1 immunoprecipitates from NOD1 co-transfectants. A reciprocal immunoprecipitation/Western blotting experiment using anti-Xpress monoclonal antibody also showed NOD1 co-precipitation with CENTB1, confirming the interaction of these two proteins.

**Expression of CENTB1 Messenger RNA in IBD Tissues and Immune Cell Lines**—CENTB1 mRNA level was assessed by real time PCR in immune cell lines as well as several human intestinal epithelial cell lines (SW480, HT-29, Caco-2, T84, Colo205, and HCT-116). THP-1 macrophages, Jurkat cells, granulocytes, and B lymphocytes showed expression of CENTB1 (Fig. 2A). In contrast, no basal CENTB1 mRNA was detectable in any of several intestinal epithelial cell lines, including SW480, HT-29, Caco-2, T84, Colo205, and HCT116 (data not shown).
Real time PCR showed that CENTB1 mRNA was present in surgical specimens from ulcerative colitis and CD patients, but not from normal controls (Fig. 2B).

### Induction of CENTB1 Expression in Intestinal Epithelial Cells by Inflammatory Stimuli

The finding that endogenous CENTB1 expression is increased in association with intestinal inflammation prompted an assessment of the effects of proinflammatory cytokines on CENTB1 expression. Two representative colonic cell lines (SW480 and HT-29) were stimulated with TNF-α (50 ng/ml) or IL-1β (15 ng/ml). CENTB1 mRNA was markedly increased after 1h of TNF-α stimulation (Fig. 3A). Comparable effects were observed following IL-1β stimulation. Similar results were also found in Jurkat and THP-1 cells when they were stimulated with these two proinflammatory cytokines (data not shown).

CENTB1 mRNA was also markedly induced in SW480 and HT-29 cells stimulated with the ligands for NOD1 and NOD2 MDP and iE-DAP, respectively (Fig. 3, B and C). Comparable increases in endogenous protein expression were also observed (Fig. 3, D and E). Indeed, CENTB1 expression was greater in epithelial cell lines stimulated with NODs ligands than when cells were stimulated with proinflammatory cytokines. In contrast, TLR2 and TLR4 prototype ligands lipoteichoic acid and lipopolysaccharide (LPS) had no effect on CENTB1 expression (data not shown).

### Interaction between Endogenous NODs and CENTB1 Proteins in Mammalian Cells

To assess the possible physiological significance of the NOD2/CENTB1 interaction, we investigated the interaction between endogenous NOD2 and CENTB1 using lysates from SW480 cells that express endogenous NOD2 (21) and stimulated them with MDP-LD (1 μg) to induce endogenous CENTB1 expression. For immunoprecipitation experiments, we used a newly produced rabbit antiserum against CENTB1. Anti-CENTB1 antibody specifically recognized CENTB1 in lysate of COS-7 cells transfected with the Xpress-tagged CENTB1 construct (data not shown). Endogenous NOD2 expression was observed in SW480 cells by using the rabbit antiserum against NOD2 HM2559 (20). Immunoprecipitation using anti-CENTB1 antiserum in SW480 cells stimulated with MDP-LD (1 μg) (Fig. 4A) confirmed the interaction between endogenous CENTB1 with NOD2 protein.

Similarly, interaction between endogenous NOD1 and CENTB1 proteins was assessed using HT-29 cells stimulated with the NOD1 ligand (iE-DAP) to induce CENTB1 expression. The HT-29 intestinal epithelial cell line has been previously

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**FIGURE 1.** Association between NOD proteins and CENTB1 in mammalian cells. A, co-immunoprecipitation of NOD2 and CENTB1. COS-7 cells were transfected with FLAG-tagged NOD2 and/or Xpress-tagged CENTB1. The cell lysates were immunoprecipitated (IP) with HM2559 anti-NOD2 antiserum (top left panel) or with anti-Xpress monoclonal antibody (top right panel). Western blots were performed with HM2563 anti-NOD2 antiserum or anti-Xpress monoclonal antibody. B, co-immunoprecipitation of NOD1 and CENTB1. COS-7 cells were transfected with HA-tagged NOD1 and/or Xpress-tagged CENTB1. The cell lysates were immunoprecipitated with HM3851 anti-NOD1 antiserum (bottom left panel) or with anti-Xpress antibody (bottom right panel). The membranes were blotted with HM3851 anti-NOD1 antiserum or anti-Xpress monoclonal antibody. Precipitates were fractionated through 4–12% Tris-glycine SDS-PAGE gel, transferred onto polyvinylidene difluoride membrane, and blotted with anti-NOD2, anti-NOD1 or anti-Xpress monoclonal antibodies. Total cell lysates were subjected to Western blot analysis using anti-Xpress antibody or anti-NODs antiserum to detect the expression of CENTB1, NOD1, or NOD2 in transfected COS-7 cells.

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**FIGURE 2.** CENTB1 expression in immune cells and IBD tissue. A, RT-PCR was performed to assess CENTB1 mRNA expression in immune cell lines. GAPDH (440 bp) was used as an internal control. The identity of each product was confirmed by sequencing. B, expression of CENTB1 mRNA in IBD tissues. CENTB1 mRNA was assessed in biopsies and surgical specimens of patients with CD and ulcerative colitis as well as normal controls using real time RT-PCR and compared with GAPDH mRNA as internal standard. The identity of each product was confirmed by sequencing. The data are means ± S.E. of three independent experiments. UC, ulcerative colitis.
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A, effect of TNFα on CENTB1 expression. HT-29 cells were seeded and grown in six-well plates for 24 h. The cells were stimulated with TNF-α (50 ng/ml) for different time periods, and the CENTB1 mRNA level was assessed by RT-PCR. B, effect of NOD2 ligand (MDP) stimulation on CENTB1 expression. SW480 cells were seeded in six-well plates and then transfected with 1 μg of MDP-LD/well for different time periods. Total RNA was isolated, and CENTB1 mRNA was determined by real-time RT-PCR, normalized to GAPDH mRNA. The values are the means ± S.D. from four experiments. C, effect of NOD1 ligand (iE-DAP) stimulation on CENTB1 expression. Real-time RT-PCR was performed using mRNA of SW480 cells stimulated with iE-DAP (100 ng/ml) for different time periods. The values were normalized to GAPDH. The sizes of PCR products were verified using 2% agarose gel electrophoresis, and all of the products were confirmed by sequencing. The values are the means ± S.D. from four experiments. D, effect of NOD2 ligand on CENTB1 protein expression. SW480 cells were stimulated with medium or MDP at 1 μg/ml for time intervals ranging from 1 to 24 h. Western blot was performed to determine CENTB1 protein expression in cell lysates using anti-CENTB1 antibody (top panel). Equal loading was confirmed using anti-β-actin antibody (bottom panel). E, effect of NOD1 ligand on CENTB1 protein expression. Western blot analysis was performed after stimulation of SW480 cells with iE-DAP for various lengths of time up to 24 h. Aliquots of cell lysates were immunoblotted with anti-CENTB1 antibody (top panel). Equal loading was confirmed by blotting with anti-β-actin antibody. All of the experiments were repeated at least three times.

Centaurin β1 and NODs Proteins Co-localize in the Cytosystem—
To determine whether NOD2 and CENTB1 co-localize as implied in the finding of a direct biochemical interaction, subcellular localization was evaluated by immunofluorescence confocal microscopy. COS-7 cells were transfected with Xpress-tagged CENTB1 and FLAG-tagged NOD2 expression plasmids. NOD2 protein was observed throughout the cytoplasm and also associated with the plasma membrane as previously reported (21). In COS-7 cells co-expressing FLAG-tagged NOD2 and Xpress-tagged CENTB1, CENTB1 co-localized with the cytoplasmic NOD2 but not with the NOD2 membrane-associated fraction (Fig. 5A). This partial co-localization of NOD2 with CENTB1 is consistent with the evidence that specific interactions occur between these two proteins in vitro. Using similar approaches CENTB1 was also found to co-localize with NOD1 in the intracytoplasmic compartment (Fig. 5B).

Cellular localization of endogenous CENTB1 and NOD2 was investigated in SW480 cells stimulated with MDP-LD. Co-localization between endogenous CENTB1 and NOD2 was found in the cytoplasm (Fig. 5C). Similar co-localization of endogenous CENTB1 and NOD1 was found in SW480 cells transfected with HA-NOD1 and stimulated with iE-DAP (Fig. 5D). These observations are consistent with interactions of endogenous NODs/CENTB1 proteins, suggesting physiological significance of these interactions.

Mapping of CENTB1 Domain Required for NODs Interaction—
To characterize the domain of CENTB1 responsible for interaction with the NODs, deletion constructs of CENTB1 protein were prepared that allowed discrimination between encompassed three regions: the PH domain, the Arf-GAP domain, and the Ankyrin repeats (Fig. 6A). Co-immunoprecipitation experiments were performed in HEK293 cells co-transfected with wild type NOD1 or NOD2 and the variant deletion constructs of CENTB1. Using this approach, the PH domain of CENTB1 was found to be able to co-immunoprecipitate full-length of NOD1 and NOD2 (Fig. 6, B and C), indicating that NODs/CENTB1 interaction occurs via the CENTB1 PH domain.

FIGURE 3. CENTB1 mRNA expression induced by TNFα and NOD ligands. A, effect of TNFα on CENTB1 expression. HT-29 cells were seeded and grown in six-well plates for 24 h. The cells were stimulated with TNF-α (50 ng/ml) for different time periods, and the CENTB1 mRNA level was assessed by RT-PCR. B, effect of NOD2 ligand (MDP) stimulation on CENTB1 expression. SW480 cells were seeded in six-well plates and then transfected with 1 μg of MDP-LD/well for different time periods. Total RNA was isolated, and CENTB1 mRNA was determined by real-time RT-PCR, normalized to GAPDH mRNA. The values are the means ± S.D. from four experiments. C, effect of NOD1 ligand (iE-DAP) stimulation on CENTB1 expression. Real-time RT-PCR was performed using mRNA of SW480 cells stimulated with iE-DAP (100 ng/ml) for different time periods. The values were normalized to GAPDH. The sizes of PCR products were verified using 2% agarose gel electrophoresis, and all of the products were confirmed by sequencing. The values are the means ± S.D. from four experiments. D, effect of NOD2 ligand on CENTB1 protein expression. SW480 cells were stimulated with medium or MDP at 1 μg/ml for time intervals ranging from 1 to 24 h. Western blot was performed to determine CENTB1 protein expression in cell lysates using anti-CENTB1 antibody (top panel). Equal loading was confirmed using anti-β-actin antibody (bottom panel). E, effect of NOD1 ligand on CENTB1 protein expression. Western blot analysis was performed after stimulation of SW480 cells with iE-DAP for various lengths of time up to 24 h. Aliquots of cell lysates were immunoblotted with anti-CENTB1 antibody (top panel). Equal loading was confirmed by blotting with anti-β-actin antibody. All of the experiments were repeated at least three times.
CENTB1 Down-regulates NOD-mediated NF-κB Activation

To assess the functional effect of NODs/CENTB1 interactions, constructs encoding CENTB1, NOD2, and different amounts of MDP-LD were transfected into HEK293 cells. Subsequent activation of NF-κB was determined using the NF-κB-driven luciferase reporter assay. CENTB1 was found to down-regulate NF-κB activation in a concentration-dependent manner (Fig. 7A). To confirm the role of CENTB1 in the down-regulation of NOD2, a siRNA approach was used to selectively inhibit the expression of CENTB1. HEK293 cells were transfected with different deletion constructs of CENTB1 and full-length NOD2. The cell lysates were immunoprecipitated (IP) and blotted with either anti-Xpress or anti-NOD2 antibodies. The lack of effect of a siRNA control (pSUPER vector) confirmed the specificity of these effects (Fig. 7B).

Similar studies were undertaken to evaluate the effect of CENTB1 on NOD1-mediated NF-κB activation. HEK-293 cells were co-transfected with different concentrations of CENTB1 and NOD1 DNA constructs and stimulated with iE-DAP. CENTB1 down-regulated NOD1-mediated NF-κB activation in a concentration-dependent manner resembling the effects of

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**Figure 5. Co-localization of NOD proteins and CENTB1.**
- **A**: Cellular localization of NOD2 and CENTB1 was assessed in COS-7 cells co-transfected with FLAG-tagged NOD2 and Xpress-tagged CENTB1. CENTB1 was detected using monoclonal anti-Xpress antibody, followed by Texas Red-conjugated anti-mouse IgG and NOD2 protein, was detected using polyclonal rabbit anti-NOD2 antibody followed by staining with FITC-conjugated anti-rabbit IgG.
- **B**: Cellular localization of NOD1 and CENTB1 in COS-7 cells co-transfected with HA-tagged NOD1 and Xpress-tagged CENTB1. CENTB1 was detected using monoclonal anti-Xpress and Texas Red-conjugated anti-mouse IgG for CENTB1, and NOD1 was detected using polyclonal rabbit anti-HA antibody and FITC-conjugated anti-rabbit IgG.
- **C**: Co-localization of endogenous CENTB1 and NOD2. SW480 cells were stimulated with 1 μg of MDP-LD to induce CENTB1 expression. CENTB1 was detected using anti-CENTB1 antibody as primary and Texas Red-conjugated anti-mouse IgG.
- **D**: Cellular localization of endogenous CENTB1 and transfected NOD1. SW480 cells were transfected with HA-tagged NOD1 construct and stimulated with iE-DAP (100 ng) to induce CENTB1 expression. Endogenous CENTB1 was detected as described previously. NOD1 was detected using anti-HA monoclonal antibody and stained with FITC-conjugated anti-mouse IgG.

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**Figure 6. Identification of domain of CENTB1 interacting with NODs.**
- **A**: Schematic representation and amino acid composition of the CENTB1 deletion mutants used in this study. **B**: PH domain of CENTB1 is necessary for interaction with NOD2. HEK293 cells were transfected with different deletion constructs of CENTB1 and full-length NOD2. The cell lysates were immunoprecipitated (IP) and blotted with either anti-Xpress or anti-NOD2 antibodies. **C**: PH domain of CENTB1 interacts with NOD1. The cell lysates were immunoprecipitated and blotted with anti-Xpress and anti-NOD1 antibodies from HEK293 cells transfected with different deletion constructs of CENTB1 and the full length of NOD1.
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CENTB1 on NOD2 mediated activation (Fig. 8A). Transfection of siRNA against CENTB1 significantly enhanced NF-κB activation in response to the NOD1 ligand (iE-DAP) in HEK293 expressing NOD1 and CENTB1 compared with those measured in HEK293 cells transfected with pSUPER control vector (Fig. 8B).

To determine whether the action of CENTB1 was specific to the NOD family or also encompassed other innate immune pathways, the effect of CENTB1 on the Toll-like receptor (TLR2 and TLR4) pathways was assessed. Activation of NF-κB was evaluated in HEK293 cells transiently transfected with different concentrations of CENTB1, DNA constructs encoding CENTB1 and TLR2 DNA constructs and stimulated with lipoteichoic acid. TLR2 pathway-mediated NF-κB activation was not affected by CENTB1 expression (Fig. 9A). A similar approach was undertaken to evaluate the effect of CENTB1 on TLR4-mediated NF-κB activation. HEK293 cells were transiently transfected with different concentrations of DNA constructs encoding CENTB1, TLR4, and MD-2 and then stimulated with LPS at 100 ng/ml for 4 h. Expressed CENTB1 exerted no effect on NF-κB activation via TLR4 (Fig. 9B). These findings were confirmed in colonic epithelial cells transfected with two different siRNA against CENTB1 and stimulation of endogenous TLR4 and TLR2 by the specific ligands LPS and lipoteichoic acid in SW480 and Caco-2 cells, respectively (data not shown). These findings confirm the specificity of CENTB1 in modulating NOD-mediated, but not TLR-mediated, NF-κB cell activation.

Analysis of the Interaction of CENTB1 with NOD2 Disease-associated Mutants—Specific mutations in the NOD2 gene have been associated with inflammatory diseases. To determine whether CD and Blau mutants exert an effect on the interaction and functional effect of CENTB1, we performed co-immunoprecipitation assays in HEK293 cells transfected with constructs encoding CENTB1 and disease-associated NOD2 mutants. CD and Blau mutations retained the ability to interact with CENTB1 (Fig. 10A). To assess the functional role of these mutations on CENTB1, NF-κB assays were performed in HEK293 cells transfected with wild type NOD2 or CD and Blau syndrome mutants. Interestingly, CENTB1 down-regulates NF-κB activation in Blau mutants, but the suppressive effect of NF-κB activation was lost when interacting with CD mutants (Fig. 10, B and C).

Up-regulation of NF-κB Activation by Endogenous NODs Pathways Using siRNA against CENTB1—To assess the functional effect of endogenous CENTB1 after stimulation with NODs ligands, we first confirmed that siRNA could specifically knock down endogenous CENTB1 induced by NOD ligands in TNF. Two siRNA against CENTB1 (siRNA-1 and siRNA-2) significantly knocked down the level of CENTB1 mRNA (around 75 and 60%, respectively) compared with cells transfected with control siRNA (pSUPER) (Fig. 11A). Following confirmation of effective knockdown, SW480 cells were stimulated with MDP and iE-DAP and transfected with siRNA against CENTB1 or siRNA control. Luciferase and Renilla reporters were transfected, and NF-κB assays were performed. SW480 cells transfected with siRNA-1 and siRNA-2 against CENTB1 showed significantly increased NF-κB activity compared with those measured in untransfected or transfected cells with siRNA control. (Fig. 11, B and C). These results confirm that endogenous CENTB1 down-regulates NF-κB activation after stimulation with NOD1 and NOD2 ligands in colonic epithelial cells.

DISCUSSION

In the present study, we describe a novel NOD2 interactor, CENTB1, found by yeast two-hybrid screening. Interaction between CENTB1 and NOD2 was confirmed in mammalian
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Centaurin β1 (CENTB1) is a protein thought to be involved in the regulation of innate immune responses to intracellular bacteria by down-regulating NF-κB activation in mammalian cells. The functional effects of CENTB1 contrast with those observed for two other NOD2 interactors, GRIM19 and Erbin. CENTB1 suppresses the activation of cytosolic NOD2, whereas Erbin may have an inhibitory effect on membrane-associated NOD2 fraction. These findings suggest that CENTB1 could play an important role in overall modulation of innate immune responses to intracellular bacteria by down-regulating NF-κB activation in mammalian cells. The functional effects of CENTB1 contrast with those observed for two other NOD2 interactors, GRIM19 and Erbin. CENTB1 is required for NF-κB activation following NOD2-mediated recognition of bacterial MDP and controls pathogen invasion of intestinal epithelial cells (22). TAK1 up-regulates NOD2-mediated NF-κB activation (23). Collectively, it appears that NOD-CENTB1 has a differential effect on the regulation of NF-κB activation. CENTB1 was still able to exert down-regulatory effects on B5-associated NOD2 mutants following ligand stimulation comparable with those observed with wild type NOD2. In contrast, CENTB1 had no effect on ligand-induced NF-κB activation by CD mutants. Conceptually, this could lead to a loss of feedback inhibition of NF-κB activation mediated by NOD2 in patients with CD. Paradoxically, the CD-associated mutant forms of NOD2 have previously been found to exhibit base line-reduced activation of NF-κB compared with wild type. However, the loss of the down-regulating effect mediated by CENTB1 might have pathophysiological significance by facilitating persistent basal activation.

CENTB1 was identified by sequencing clones obtained from a size-fractionated myeloid cell cDNA library (27, 28). The deduced protein contains 740 amino acids and shares 20% sequence identity with Centaurin α1, a protein thought to be involved in the activation of NF-κB pathway through intracellular recognition of the bacterial components iE-DAP and MDP-LD (4, 11).

To determine whether these associations have a functional role, we assessed NF-κB activation assays in mammalian cells co-transfected with NOD2 or NOD1 and CENTB1. These studies showed that CENTB1 down-regulates NF-κB activation via NOD1 and NOD2 pathways in a concentration-dependent manner. This functional interaction was confirmed by the finding that ligand-dependent NF-κB activation was enhanced when the expression of CENTB1 was inhibited using specific siRNA against CENTB1. In contrast, CENTB1 does not modify NF-κB activation via TLR-2 or -4, indicating a selective differential effect on NF-κB activation by these two families (NODs and TLRs) of innate immune pattern recognition receptors.

In addition to the demonstration of direct interactions between CENTB1 and the NODs proteins biochemically, characterization of the subcellular localization of NODs proteins and CENTB1 revealed intracellular co-localization, consistent with functional interaction between these proteins in situ. We previously reported that membrane localization of NOD2 is required for MDP-dependent activation of NF-κB (21). However, CENTB1 is located in the cytoplasm and interacts with NOD1 and NOD2 in this cellular compartment to modulate the NF-κB signaling. Recent studies demonstrated a direct interaction between NOD2 and Erbin that occurs near the basolateral membrane in differentiated Caco-2 cells, and Nod2-dependent activation of NF-κB is inhibited by Erbin overexpression (25, 26). In light of this observation, we hypothesize that CENTB1 suppresses the activation of cytosolic NOD2, whereas Erbin may have an inhibitory effect on membrane-associated NOD2 fraction. These findings suggest that CENTB1 could play an important role in overall modulation of innate immune responses to intracellular bacteria by down-regulating NF-κB activation in mammalian cells.
most highly expressed in neurons that has been associated with the formation of neuritic plaques in Alzheimer disease (29). CENTB1 (also named ACAP1), previously identified as a GTPase-activating protein, is a member of the ADP-ribosylation factor family. This protein is composed by a coiled-coil region at its N terminus, followed by an X-box domain, a PH domain, an ARF-GAP domain, and C-terminal ankyrin repeats (27). CENTB1 is encoded by a gene located on the short arm of human chromosome 17, and there has previously been little known about its function (28). To our knowledge this is the first protein interaction identified for CENTB1. A recent study showed that Centaurin 1 contributes to extracellular signal-regulated kinase activation induced by epidermal growth factor (30). Of note, a selected partner, Centaurin 4 protein appears to be involved in a variety of cellular signaling pathways including control of growth, survival, and movement and may act to directly assemble or disassemble molecular complexes (31). Its expression is significantly greater in high grade uveal melanomas with more significant risk of metastasis than in low grade tumors (32). Expression has also been found in breast cancer cell lines (33).

A previous report described high CENTB1 expression in spleen, thymus, and bone marrow; intermediate expression in lung and testis; and low expression in prostate and ovary. No expression was detected in heart, brain, placenta, liver, skeletal muscle, kidney, and pancreas (28). However, expression in the intestine and immune cells was apparently not assessed. In the present study, base-line endogenous expression of CENTB1 was not detected in several colonic epithelial cell lines but was present in several immune system cell lines such as THP-1,

![FIGURE 9. CENTB1 does not regulate TLR2- and TLR4-dependent NF-κB activation. A, effect of CENTB1 on TLR2-mediated activation. HEK293 cells were transfected with TLR2 (100 ng), CENTB1 at different concentrations (10 – 100 ng), NF-κB luciferase, and Renilla plasmids. The cells were stimulated with lipoteichoic acid (LTA, 10 μg/ml) and lysed 18h later. Luciferase activity was described previously. The results are the mean ± S.D. of fold induction of NF-κB activity. B, effect of CENTB1 on TLR4-mediated activation. HEK293 cells were transfected with TLR4 (100 ng), MD-2 (20 ng), CENTB1 (10 – 100 ng), NF-κB luciferase, and Renilla plasmids and stimulated with LPS (100 ng) for 4 h. The cells were lysed 18 h later. NT, non-transfected.](image)

![FIGURE 10. CENTB1 interaction with human disease-associated NOD2 mutants. A, immunoprecipitation (IP) shows direct binding of CENTB1 and the three most common mutants (L1007fs, R702W, and G908R) associated with Crohn’s disease or the two most common mutants (R334W and L496F) associated with Blau syndrome. HEK293 cells were co-transfected with Xpress-CENTB1 and FLAG-NOD2 wild type and different mutations. Immunoprecipitation showed interaction between CENTB1 with CD and BS mutants in HEK293 cells. B, CENTB1 does not down-regulate NF-κB activity of CD NOD2 mutants. HEK293 cells were seeded in 24-well plates and transfected with 1 ng of DNA encoding NOD2 BS mutants (R334W and L496F), NOD2 wild type (1 ng), NOD1 (10 ng), CENTB1 (100 ng), NF-κB luciferase, and Renilla reporters and stimulated with 1 μg of MDP for 18 h. NF-κB activity was determined by measurement of luciferase and normalization with Renilla. * , p<0.01. WB, Western blot.](image)
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In summary, CENTB1 acts as a component of the host innate immune response system by down-regulating NF-κB activation via the NOD2 and NOD1 pathways. CENTB1 expression is up-regulated by bacterial components (MDP-LD and iE-DAP) and proinflammatory molecules such as TNF-α and IL-1β and could play a moderating influence in the context of infectious and inflammatory challenges. These studies identify CENTB1 as a regulator of NOD2 signaling and demonstrate a novel role for CENTB1 in inflammatory responses.

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REFERENCES

1. Inohara, N., and Nunez, G. (2003) Nat. Rev. Immunol. 3, 371–382
2. Ogura, Y., Inohara, N., Benito, A., Chenn, F., Yamaoka, S., and Nunez, G. (2001) J. Biol. Chem. 276, 4812–4818
3. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nunez, G. (2003) J. Biol. Chem. 278, 5590–5592
4. Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D., and Sansonetti, P. J. (2003) J. Biol. Chem. 278, 8869–8873
5. Neurath, M. F., Fuss, I., Schurmann, G., Pettersson, S., Arnold, K., Muller-Loebeck, H., Strober, W., Herfarth, C., and Buschenfelde, K. H. (1998) Ann. N. Y. Acad. Sci. 859, 149–159
6. Ogura, Y., Bonen, D. K., Inohara, N., Nicole, D. L., Chen, F. F., Ramos, R., Briton, H., Moran, T., Karaliukas, R., Duerr, R. H., Ackbar, J. P., Brant, S. R., Rayess, T. M., Kirschen, B. S., Hanauer, S. B., Nunez, G., and Cho, H. J. (2001) Nature 411, 603–606
7. Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J. P., Belaiche, J., Almer, S., Tysk, C., O’Morain, C., A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J. F., Sahbatou, M., and Thomas, G. (2001) Nature 411, 599–603
8. Miceli-Richard, C., Lesage, S., Rybojad, M., Prieur, A. M., Manouvrier-Hanu, S., Hafner, R., Chamaillard, M., Zouali, H., Thomas, G., and Hugot, J. P. (2001) Nat. Genet. 29, 19–20
9. Hisamatsu, T., Suzuki, M., and Podolsky, D. K. (2003) J. Biol. Chem. 278, 32962–32968
10. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Nunez, G. (1999) J. Biol. Chem. 274, 14560–14567
11. Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., and Viala, J. (2003) Science 300, 1584–1587
12. Philpott, D. J., Yamaoka, S., Israel, A., and Sansonetti, P. J. (2000) J. Immunol. 165, 903–914
13. Savovic, S. D., Koutsiouris, A., and Hecht, G. (1997) Amer. J. Physiol. 278, C1160–C1167
14. Elewaut, D., DiDonato, J. A., Kim, J. M., Truong, F., Eckmann, L., and Kagnoff, M. F. (1999) J. Immunol. 163, 1457–1466
15. Swidsinski, A., Ladhoff, A., Perntehler, A., Swidsinski, S., Loening-Baucke, V., Orter, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M., and Lochs, H. (2002) Gastroenterology 122, 44–54
16. Darfeuille-Michaud, A., Boudou, J., Bulois, P., Neut, C., Glasser, A. L., Barnich, N., Bring, M. A., Swidsinski, A., Beaugerie, L., and Colombel, J. F. (2004) Gastroenterology 127, 412–421
17. Watanabe, T., Kitani, A., Murray, P. J., and Strober, W. (2004) Nat. Immunol. 5, 800–808
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18. Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R. A. (2005) *Science* **307**, 731–734
19. Maeda, S., Hsu, L. C., Liu, H., Bankston, L. A., Iimura, M., Kagnoff, M. F., Eckman, L., and Karin, M. (2005) *Science* **307**, 734–738
20. Hisamatsu, T., Suzuki, M., Reinecker, H. C., Nadeau, W. J., McCormick, B. A., and Podolsky, D. K. (2003) *Gastroenterology* **124**, 993–1000
21. Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R., and Podolsky, D. K. (2005) *J. Cell Biol.* **170**, 21–26
22. Barnich, N., Hisamatsu, T., Aguirre, J. E., Xavier, R., Reinecker, H. C., and Podolsky, D. K. (2005) *J. Biol. Chem.* **280**, 19021–19026
23. Chen, C. M., Gong, Y., Zhang, M., and Chen, J. J. (2004) *J. Biol. Chem.* **279**, 25876–25882
24. Damiano, J. S., Oliveira, V., Welsh, K., and Reed, J. C. (2004) *Biochem. J.* **381**, 213–219
25. McDonald, C., Chen, F. F., Ollendorff, V., Ogura, Y., Marcheto, S., LeCine, P., Borg, J. P., and Nunez, G. (2005) *J. Biol. Chem.* **280**, 40301–40309
26. Kufer, T. A., Kremmer, E., Banks, D. J., and Philpott, D. J. (2006) *Infect. Immun.* **74**, 3115–3124
27. Jackson, T. R., Brown, F. D., Nie, Z., Miura, K., Foroni, L., Sun, J., Hsu, V. W., Donaldson, J. G., and Randazzo, P. A. (2000) *J. Cell Biol.* **151**, 627–638
28. Nomura, N., Nagase, T., Miyajima, N., Sazuka, T., Tanaka, A., Sato, S., Seki, N., Kawarabayasi, Y., Ishikawa, K., and Tabata, S. (1994) *DNA Res.* **1**, 223–229
29. Reiser, G., and Bernstein, H. G. (2004) *Clin. Neuro. Neuropathol.* **15**, 147–148
30. Hayashi, H., Matsuura, O., Muramatsu, S., Tsuchiya, Y., Harada, T., Susuki, Y., Sugano, S., Matsuda, A., and Nishida, E. (2006) *J. Biol. Chem.* **281**, 1332–1337
31. Podolsky, D. K. (2002) *N. Engl. J. Med.* **347**, 417–429
32. Martin, R. K., and Jackson, T. R. (2005) *Biochem. Soc. Trans.* **33**, 1282–1284
33. Ehlers, J. P., Worley, L., Onken, M. D., and Harbour, J. W. (2005) *Clin. Cancer Res.* **11**, 3609–3613
34. Onodera, Y., Hashimoto, S., Hashimoto, A., Morishige, M., Mazaki, Y., Yamada, A., Ogawa, E., Adachi, M., Sakurai, T., Manabe, T., Wada, H., Matsuura, N., and Sabe, H. (2005) *EMBO J.* **24**, 963–973