The Role of Transcriptional Corepressor Nif3l1 in Early Stage of Neural Differentiation via Cooperation with Trip15/CSN2*

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Mouse Nif3l1 gene is highly conserved from bacteria to human. Even though this gene is expressed throughout embryonic development, its biological function is still obscure. Here, we show that Nif3l1 participates in retinoic acid-primed neural differentiation of P19 embryonic carcinoma cells through cooperation with Trip15/CSN2, a transcriptional corepressor/component of COP9 signalosome. We isolated Nif3l1 cDNA from P19 cell cDNA library by a yeast two-hybrid screening using Trip15/CSN2 as a bait. This interaction was confirmed by a pull-down assay and an epitope-tagged communoprecipitation. Although Nif3l1 was mainly detected in the cytoplasm, the translocation of Nif3l1 into the nuclei was observed in retinoic acid-primed neural differentiation of P19 cells and enhanced by the enforced expression of Trip15/CSN2. Furthermore, enforced expression of sense Nif3l1 RNA, but not antisense RNA, enhanced the neural differentiation of P19 cells accompanying the intense down-regulation of Oct-3/4 mRNA expression and the rapid induction of Mash-1 mRNA expression. Luciferase reporter assay showed that Nif3l1 could act as a transcriptional repressor and synergized the transcriptional repression by Trip15/CSN2. These results indicate that Nif3l1 implices in neural differentiation through the cooperation with Trip15/CSN2.

The differentiation of mammalian neurons during development is a highly complex process involving regulation and coordination of gene expression at multiple steps. To understand the basic mechanisms underlying this complex pathway, identification of genes that are differentially expressed during neural differentiation is an important approach. P19 embryonal carcinoma (EC)3 cells derived from a mouse embryo have been used extensively as a model system for in vitro neural differentiation (1, 2). Exposure of aggregated P19 cells with retinoic acid (RA) results in the differentiation of cells with many fundamental phenotypes of mammalian nervous system (3). During the early stages of neuronal differentiation of P19 cells, the mammalian homologues of several Drosophila gene products such as Motch, Mash-1, Wnt-1, and transduction-like enhancers of split are expressed (4–7). Moreover, a number of proteins including retinoic acid receptors, retinoid X receptors, epidermal growth factor receptor, and transcription factors such as Oct-3/3, Brn-2, and Bcm-1 have been identified (8–10). Perhaps a limited number of RA- and aggregation-responsive genes trigger the neuronal differentiation pathway of P19 cells.

Recently we found that a transcriptional corepressor, a component of COP9 signalosome, Trip15/CSN2, was highly expressed at the early stage of neural differentiation of RA-treated P19 cells. The deduced amino acid sequence of rat Trip15/CSN2 gene is completely identical with those of mouse and human homologues (DDBJ/EMBL/GenBank™ accession no. AB081072; Ref. 11). Enforced expression of sense rat Trip15/CSN2 RNA was sufficient to convert P19 cells into neurons, but not glial cells in the absence of RA, only after the aggregation treatment, accompanying the down-regulation of Oct-3/4 transcript, which maintains the undifferentiated state of P19 cells. Thus, the induction of Trip15/CSN2 prior to down-regulation of Oct-3/4 gene expression is required for the commitment of P19 cells to neuronal lineage.

Trip15/CSN2 was originally identified as a thyroid hormone receptor (TR)-interacting protein and acts as a transcriptional corepressor (12). Trip15/CSN2 interacts with a subset of nuclear hormone receptors such as DAX-1, ecdysone receptor, chicken ovalbumin promoter transcription factor 1 (COUP-TF1), its Drosophila homologue Seven-up, Fushi-tarazu-F1 (Ftz-F1), and TRs, but not with retinoic acid receptors and retinoid X receptors (13, 14). Trip15/CSN2 is conserved in a wide range of organisms and was also identified as a component of a 26 S proteasome lid-like complex termed COP9 signalosome (CSN; Refs. 15 and 16). The CSN complex was originally identified as a repressor of light-controlled development in Arabidopsis thaliana (17). In animals, the CSN complex is localized in the nucleus and possesses a kinase activity that specifically phosphorylates transcriptional regulators such as p105, IκBα, c-Jun, and p53 (18–20). Although the mutual interaction between Trip15-nuclear receptor complex and CSN complex is still unknown, these facts indicate that the CSN complex participates in various signal transduction pathways. The proteasome-COP9 complex-initiation factor 3 domain in the C-terminal region of Trip15/CSN2 stabilizes protein-protein interaction within the CSN complex (21–24). The N-terminal region of Trip15/CSN2 has been reported to be sufficient for the effector functions of Trip15/CSN2. In fact, Trip15/CSN2
associated with its binding partners, such as TR and DAX-1, through its N-terminal region (12–14).

Considering the importance of Trip15/CSN2 in neuronal differentiation, we tried to find out novel molecules that bind to Trip15/CSN2 and possibly act as new downstream targets or regulators of Trip15/CSN2. We used the N-terminal region of rat Trip15/CSN2 as a bait for the yeast two-hybrid screening of RA-treated P19 cell cDNA library. Five of 17 positive cDNA clones have been turned out to be mouse Nif3l1 (Ngg1-interacting factor 3-like) 1 gene. Nif3l1 cDNA was isolated through a suppression subtractive hybridization between brain and P19 embryonic stem cells. The expression of Nif3l1 gene encodes a cytoplasmic protein consisted of 376 amino acids and is highly conserved from bacteria to mammals. Expression of Nif3l1 transcript is detected through mouse embryonic development (25); however, the real biological function of Nif3l1 is still unknown.

Yeast Nif3 was originally identified in yeast two-hybrid screening as a NGG1-interacting protein (26). Ngg1 was isolated based on its requirement for the full inhibition of transcriptional activity by GAL4 protein in glucose media (27). Independently, ADA3/NGG1 was isolated based on the ability to suppress the toxic effects of overexpression of the viral activator VP16 in yeast (28). Therefore, ADA3/NGG1 was involved in transcriptional activation and repression (26, 29, 30). Alternative deficiency in activation (ADA) proteins have been found to be required for transcriptional activation by a number of yeast activators (28, 31, 32). In yeast, ADA3/NGG1 is found as multisubunit complexes containing three to four additional ADA proteins and different TAFs and Spt (30, 31, 33–36). In mammalian cells, the majority of ADA3/NGG1 protein also seems to be complexed with Spt and TAF or TAF-like factors, making up several types of complexes (37–41). These complexes are thought to be functional homologs of the yeast ADA complexes (37, 39, 41). Recently, genetic studies in yeast have demonstrated a crucial role of ADA complex in the transcription activation function of mammalian nuclear hormone receptors (42–45). Nonetheless, there is no report concerning about the function of Nif3l1 in transcriptional regulation.

In this study, we show that the cytoplasmic Nif3l1 protein could be translated into the nuclei by the association with Trip15/CSN2 and that it synergized the transcriptional repression activity of Trip15/CSN2. In addition, Nif3l1 implicates in neural differentiation of P19 cells, perhaps through the down-regulation of Oct-3/4 transcript, which suppresses neurogenic genes including Mash-1 to maintain the undifferentiated state of P19 cells. Considering these results and the expression of Nif3l1 in early developing brain, it seems likely that both Nif3l1 and Trip15/CSN2 play an important role in neural differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Experimental Animals—**Mouse P19 embryonal carcinoma cells were obtained from American Type Culture Collection (Bethesda, MD). Cells were maintained in α-minimal essential medium (Invitrogen) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO2 in air. To induce neural differentiation, 1 × 106 P19 cells aggregated in 10-cm bacteriological grade dishes were cultivated in 10 ml of α-minimal essential medium containing 10% FCS and 5 × 10−7 M all-trans-retinoic acid (RA) (Sigma) for 4 days. Cell aggregates were then suspended with mild pipetting and transferred to tissue culture dishes. The cells were cultivated in RA-free α-minimal essential medium containing 10% FCS for additional 3 days to induce β-tubulin type III-positive neurons and for 5 days of the culture media fibroblasts were used for the transfected P19 cells. Genes including Mash-1 to maintain the undifferentiated state of P19 cells. Considering these results and the expression of Nif3l1 in early developing brain, it seems likely that both Nif3l1 and Trip15/CSN2 play an important role in neural differentiation.

**Immunoprecipitation Assay—**Flag-tagged Nif3l1 expression vector was constructed by the insertion of blunt-ended Neo-I-Sau96I ORF fragment of pGEM-Saf II (+) into Nif3l1 into the blunt-ended EcoRI-SalI site of pFLAG-CMV2 (Sigma). Full-length rat Trip15/CSN2 cDNA clone was then amplified from pGEM-7zf (+) (Promega) to EcoRI and SalI. HA-tagged Trip15/CSN2 expression vector was constructed by the insertion of blunt-ended XhoI-BglII ORF fragment of pACT2-Trip15/CSN2 into the blunt-ended BamHI-EcoRV site of pCDNA (Invitrogen). For immunoprecipitation studies, COS-7 cells were transfected with pDNA-HA-Trip15/CSN2 and pFLAG-CMV2-Nif3l1 by lipofection with DOTMA and DMEM (CPS Biochemicals). Forty-eight hours after the transfection, the cells were lysed in 0.5 ml of TM buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was incubated with 2 μg of Flag M2-agarose affinity gel (Sigma) at 4 °C overnight. The affinity gels were washed three times with 1 ml TM buffer and suspended in 15 μl of the reaction mix containing 5% mercaptoethanol. The affinity gels were heated at 95 °C for 5 min and subjected to 12% SDS-PAGE. The proteins were transferred to a Clear Blot Membrane (Atto, Tokyo, Japan). The membrane were blocked in TBSST (20 mM Tris-HCl, pH 7.4-buffered saline, 0.1% Tween 20) for 1 h at room temperature. Blots were incubated with antibodies against Flag or HA-tag for 1 h at room temperature. After incubation, the blots were washed three times with TBSST and incubated with the secondary antibodies for 1 h at room temperature. After incubation, the blots were washed three times with TBSST and incubated with the secondary antibodies for 1 h at room temperature. Finally, the blots were washed three times with TBSST and incubated with 2 μg/ml of a donor fluorophore solution for 1 h. Chemiluminescence was then visualized by x-ray film.
Nif3l1 Participates in Neural Differentiation

0.02% Tween 20 containing 1% nonfat dry milk for 1 h at room temperature and incubated overnight at 4°C with anti-HA antibody (1:1000 dilution, Sigma), anti-Flag antibody (1:400 dilution, Sigma), or anti-rat Trip15/CSN2 antibody (1:1000 dilution; Ref. 11). After washing three times with TBST, the membrane was incubated with anti-mouse IgG conjugated with horseradish peroxidase (1:5000 dilution, Sigma). Signals were visualized with the ECL system (Amersham Biosciences) according to the protocol from the manufacturer.

Northern Blot Analysis—Total RNAs were extracted from P19 cells and various mouse tissues by the acidic guanidinium thiocyanate-phenol-chloroform method (47). Aliquots of 20 μg total RNA were electrophoresed on 1% agarose, 6% formaldehyde gel, transferred to Hybond-N+ nylon membrane (Amersham Biosciences), and hybridized with 32P-labeled 0.6-kb EcoRI-DraI fragment of pGEM5zf(+)-Nif3l1, 2.1-kb BamHI-EcoRI fragment of pGEM-rTrip15/CSN2 (11), and PCR-amplified mouse Oct-3/4 cDNA (5’-CAA GCG GCC AAG CAG CAG-3’, 5’-GTT CTA GCT CCT TCT GCA GGG C-3’) (48), Mash-1 cDNA (5’-GCC TGG CTC GAN GAG TAG T-3’, 5’-CAC AGA TCC CAC GCC GGC CAG-3’), Mash-1 3’UTR fragment (5’-CAC TGG ACA GGA AAG CAC GAG-3’, 5’-CAC CCC CAC ACC TCC GCT CAG-5’), Trp-2 cDNA (5’-GGC-3’, 5’-GAT CCC TCG TCG GAG GAG TAG-3’), Mash-1 cDNA (5’-GAT CCC TCG TCG GAG GAG TAG-3’, 5’-GAC CTC GAG TGG AGA AAC TGC TG-3’), and acidic ribosomal phosphoprotein (PO) cDNA (5’-GAC CTC GAG TGG AGA AAC TGC TG-3’, 5’-GAC CTC GAG TGG AGA AAC TGC TG-3’). The samples were then hybridized with 32P-labeled probes and washed according to the manufacturer’s instructions.

Construction of Tetracycline (Tet)-controlled Nif3l1 Expression System—Tet-controlled Nif3l1 expression in P19 cells was performed using the Tet-Off™ gene expression system (Clontech). At the first step, P19 cells were transfected with a pTet-Off vector using DOTAP and cultivated in the presence of 400 μg/ml G418 (Wako, Tokyo, Japan) for selection.

b) Tet-controlled Nif3l1 expression in P19 cells was performed using the Tet-Off™ gene expression system (Clontech). At the first step, P19 cells were transfected with a pTet-Off vector using DOTAP and cultivated in the presence of 400 μg/ml G418 (Wako, Tokyo, Japan) for selection.

Western Blot Analysis—For Western blot analysis, cells were collected, lysed in the SDS sample buffer without bromphenol blue and 2-mercaptoethanol, and sonicated for 4 s. The resulting lysates were cleared by centrifugation at 15,000 rpm for 10 min. After protein concentration was determined by BCA kit (Pierce), 5% 2-mercaptoethanol (final concentration) was added. Aliquots of 20 μg of cell lysate were loaded onto an SDS-PAGE gel and blotted onto a Hybond-N+ membrane (Amersham Biosciences), and hybridized with 32P-labeled probes.

Immunocytochemistry—P19 cells were cultivated in a Lab-Tek II Chamber slide (Nalgene Nunc International, Naperville, IL) and fixed with 4% paraformaldehyde. The cell samples were incubated in Ca2+/Mg2+-free phosphate-buffered saline (PBS(−)) containing 10% normal rabbit serum for 30 min at 37°C. The samples were then incubated for 2 h at 37°C with antibodies against β-tubulin III (1:1000 dilution, Sigma) or GFAP (1:400 dilution, Sigma) in the same solution described above. After washing with PBS(−), the samples were incubated with a biotin-conjugated rabbit anti-mouse IgG+IgA+IgM (Nichirei, Tokyo, Japan) as a secondary antibody and followed by the incubation with a peroxidase-conjugated streptavidin (Nichirei). Visualization of the signal was carried out using 3,3’diaminobenzidine.

Nif3l1 Participates in Neural Differentiation

Fig. 1. Characterization of binding region of Trip15/CSN2 to Nif3l1. A, yeast two-hybrid analysis of the Trip15/CSN2 domain required for binding to Nif3l1. Various fragments of Trip15/CSN2 cDNA were ligated in frame with the GAL4DBD in pAS2–1 and used as baits. Nif3l1 cDNA corresponding to amino acid residues 243–376 was ligated with the GAL4 activation domain in pACT-2. These Nif3l1 and Trip15/CSN2 expression constructs were simultaneously introduced into yeast strain Y153 cells. The cells were streaked on both selection (Leu-, Trp−, His−, and 3-aminotriazole) and non-selection (Leu+, Trp+, His+, and 3-aminotriazole) media. The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52). The PO gene, also called 36B4 (51), was used as an internal control gene, because its expression level has been shown to be invariant among mouse tissues (52).
heated at 95 °C for 5 min and subjected to 12% SDS-PAGE. The proteins were transferred to a Clear Blot Membrane (Atto). The membrane was blocked in TBST containing 1% nonfat dry milk for 1 h at room temperature and incubated overnight at 4 °C with anti-β-tubulin type III antibody (1:1000 dilution, Sigma) or anti-GAP antibody (1:400 dilution, Sigma). After washing three times with TBST, the membrane was incubated with anti-mouse IgG conjugated with horseradish peroxidase (1:15,000 dilution, Sigma). Signals were visualized with the ECL system (Amersham Biosciences) according to the protocol from the manufacturer. Densitometric analysis was performed using Image 1.62 ppc program as described above.

Luciferase Reporter Assay—A luciferase reporter construct was generated by the insertion of the thymidine kinase (TK) basal promoter derived from pMC1neoPolyA (Stratagene) into XhoI-BamHI site of pTRE2-Luc (Clontech) containing luciferase gene. In addition, bluntended fragment containing five copies of GAL4 binding sequence was inserted into blunt-ended XhoI sites of pTRE2-Luc. The plasmid was designated as pGAL4-TK-Luc. The GAL4DBD expression vector was constructed by the insertion of the GAL4DBD sequence derived from pMC1neoPolyA into the BamHI-EcoRI fragment containing luciferase gene. The GAL4DBD-fused Nif3l1 and Trip15/CSN2 expression vector was constructed by insertion of the entire coding region of Nif3l1 and Trip15/CSN2 in frame into the downstream of GAL4DBD-coding sequence in pcDNA3GAL4DBD and designated as pGAL4DBD-Nif3l1 and pGAL4DBD-Trip15/CSN2, respectively. Trip15/CSN2 expression vector was constructed by insertion of the BamHI-EcoRI fragment containing entire ORF of pGEM7zf+ (21) into pcDNA3 and designated as pcDNA3-Trip15/CSN2 (11). For each transfection, 2 × 10^5 P19 cells/35-mm diameter dish was transfected with 1 μg of pGAL4-TK-Luc, 0.5 μg of β-gal expression vector pCDNA3/Myc/His/loc2 (Invitrogen), 0.5 μg of GAL4DBD-fused Nif3l1 or Trip15/CSN2 expression vector or empty expression vector (pcDNA3) which indicated, 0.5 μg of Trip15/CSN2 expression vector was also cotransfected. At 48 h after transfection, cells were lysed with the lysis buffer of luciferase assay kit (Promega), and luciferase activities were determined according to the instructions from the manufacturer using a Luminous CT9000D (Dia- Iatron, Tokyo, Japan). Reporter gene activities were normalized using β-gal activity as an internal control.

Cytological Analysis—Enhanced green fluorescent protein (EGFP)-fused Nif3l1 and Trip15/CSN2 expression vectors were constructed by the insertions of entire cording region of Nif3l1 and Trip15/CSN2 into the downstream of EGFP-coding sequence in frame in pEGFP-C1 expression vector (Clontech) and designated as pEGFP-Nif3l1 and pEGFP-Trip15/CSN2, respectively. COS-7 cells (5 × 10^5 cells/6-cm dish) were transfected with these vectors in various combinations using DOTAP. The cell lysates were analyzed by 12% SDS-PAGE. The retained proteins were coprecipitated with anti-Flag antibody, and subjected to Western blot with anti-HA antibody. The signals were visualized with ECL system (Amersham Biosciences) according to the protocol from the manufacturer. Densitometric analysis was performed using Image 1.62 ppc program as described above.

Identification of Trip15/CSN2-binding Proteins—The N-terminal region of Trip15/CSN2 has been shown to be sufficient for its effective function and for association with its binding partners including TR and DAX-1 via its N-terminal region (12–15). To analyze the role of Trip15/CSN2 in the process of neuronal differentiation, cDNA clones encoding novel Trip15/CSN2-binding proteins were searched for by using a yeast two-hybrid screening system. A yeast strain Y153 (His , Trp , Leu ) was cotransfected with the bait vector, which expresses a fusion protein composed of GAL4DBD and the N-terminal region (amino acids 1–275) of rat Trip15/CSN2, and a RA-treated P19 cell cDNA library, which direct the synthesis of fusion proteins composed of cDNA-encoded proteins and the GAL4 transcriptional activation domain. Y153 strain contains the His3 and LacZ genes linked to the GAL4 promoter. By the transfection of 3 × 10^4 Y153 cells, 52 His colonies were developed and 17 of 52 colonies were β-gal-positive. Sequence analysis of these clones showed that 5 of 17 clones encode the C-terminal region (amino acids 243–376) of mouse Nif31 (Ngg1-interacting factor 3 like-1), which possesses a high homology to yeast Ngg1-interacting factor 3 homolog (25). Based on this finding, we focused on the functional analysis of Nif31 in neural differentiation. Full-length Nif31 cDNA was amplified by RT-PCR using total RNA from mouse P19 cells and Nif31-specific primers (25).

Interaction of Nif31 with Trip15/CSN2 in Vitro and in Vivo—We found that Nif31 possesses two putative leucine zipper (Leu Zip) motifs (210–224 and 264–278) mediating a protein-protein interaction (Fig. 1B) (52). To determine the binding region of Trip15/CSN2 to the C-terminal region (243– 376) of Nif31, various regions of Trip15/CSN2 were inserted into pAS2–1 and obtained the bait constructs that express fusion proteins of GAL4DBD and Trip15/CSN2 fragments. These constructs, Full (amino acids 1–443), N1 (1–127), N2 (1–275), M (128–275), C1 (128–443), and C2 (276–443), are shown in Fig. 1A. These bait constructs were transfected into yeast strain Y153 cells, which allow selection for two different markers: His and β-gal. Cotransformation with the vector for GAL4 activation domain-fused C-terminal region of Nif31

![Image](image-url) # FIG. 2. Interaction between Nif31 and Trip15/CSN2 in vivo and in vitro. A, binding of Trip15/CSN2 with Nif31 analyzed by GST pull-down assay. Bacterially expressed GST-Trip15/CSN2 or GST were preloaded to glutathione-Sepharose beads. The beads were incubated with [35S]methionine-labeled Nif31 protein. The retained proteins were analyzed by 12% SDS-PAGE. B, interaction between Trip15/CSN2 and Nif31 in COS-7 cells. COS-7 cells were transfected with pFlag-CMV2-Nif31, pcDNA3-HA-Trip15/CSN2, or both, and the cell lysates were prepared after 48 h were subjected to immunoprecipitation with a monoclonal anti-Flag antibody. The immunoprecipitates were analyzed by Western blot with anti-HA antibody (upper panel). Expression of Flag-Nif31 and HA-Trip15 was confirmed in the cells transfected with the corresponding vector alone by Western blot (middle and lower panels, respectively). C, interaction of Nif31 with endogenous Trip15/CSN2. P19 cells were transfected with pFlag-CMV2 or pFlag-CMV2-Nif31 vector and then treated with RA for 12 h to induce the Trip15/CSN2 gene expression. The cell lysates were prepared, immunoprecipitated with anti-Flag antibody, and subjected to Western blot with anti-Trip15/CSN2 antibody (upper panel; Ref. 11) and anti-Flag antibody (lower panel). The cell lysate from parental P19 cells treated with RA for 12 h was also analyzed to confirm the induction level of Trip15/CSN2.

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Nif3l1 Participates in Neural Differentiation

The transient expression detected two proteins of 43 and 53 kDa that correspond to Flag-tagged Nif3l1 and HA-tagged Trip15/CSN2, respectively, judging from the estimated molecular mass of these constructs (Fig. 2B, middle and lower panels, respectively). Immunoprecipitation of the extracts prepared from the cells transfected with both vectors with anti-Flag antibody revealed coprecipitation of Trip15/CSN2 (Fig. 2B, upper panel). Coprecipitation was not observed in the cells transfected with either one of expression vectors. No effect on the expression level of either Trip15/CSN2 or Nif3l1 was observed in the cotransfected cells as determined by Western blot analysis. To examine further the interaction between Nif3l1 and endogenous Trip15/CSN2, P19 cells were transfected with pFlag-CMV2-Nif3l1 vector and then treated with RA to induce the Trip15/CSN2 gene expression. After 12 h when the maximal induction of Trip15/CSN2 protein was observed (11), the cell lysate was prepared, immunoprecipitated with anti-Flag antibody and subjected to Western blot with polyclonal anti-Trip15/CSN2 antibody (11). As shown in Fig. 2C, endogenous Trip15/CSN2 was communoprecipitated with Nif3l1. These results indicate that the interaction between Nif3l1 and Trip15/CSN2 occurs in vivo.

Expression Pattern of the Nif3l1 Gene during Neural Differentiation—To examine the expression level of Nif3l1 mRNA during the neural differentiation of P19 cells, total RNAs were extracted from the aggregated P19 cells treated with $5 \times 10^{-7}$ M RA for various times and analyzed by Northern blotting with the 0.6-kb EcoRI-DraI fragment of Nif3l1 cDNA as a probe. As shown in Fig. 3A, the Nif3l1 gene was mainly expressed as a 1.85-kb mRNA and the levels of two additional transcripts of 2.4 and 3.4 kb were very low. The expression pattern of the Nif3l1 gene in P19 cells resembles those of the spermatogonia-derived GC-1 spg cells and the mouse teratocarcinoma F9 cells (25). Three species of mRNAs might be generated by alternative splicing, because the Nif3l1 gene has been shown to be a single-copy gene (25). During neural differentiation of RA-treated P19 cells, the level of Nif3l1 gene ex-

FIG. 3. Expression levels of Nif3l1 mRNA in RA-treated P19 cells and in various mouse tissues. A, expression patterns of Nif3l1 and Trip15/CSN2 mRNAs during RA-primed P19 cell neural differentiation. Total RNAs were extracted from the P19 cells treated with $5 \times 10^{-7}$ M RA for various times and analyzed by Northern blot using a 0.6-kb EcoRI-DraI fragment of mouse Nif3l1 cDNA (upper panel) and a 2.1-kb EcoRI fragment of rat Trip15/CSN2 cDNA (middle panel) as probes. Expression levels of Nif3l1 mRNA in various adult mouse tissues (B), embryonic mouse brain (C), and postnatal mouse brain (D) were similarly analyzed. Pictures of ethidium bromide-stained 28 S ribosomal RNA are included for comparison of total amount of RNA employed.

(243–376) showed that the N2 region selectively binds to the C-terminal region of Nif3l1 (Fig. 1A). The full-length Trip15/CSN2 bound to Nif3l1 weakly. The result suggests that the N2 region of Trip15/CSN2 containing a putative Leu Zip, nuclear localization signal (NLS), and corepressor motif ([I/L]XX[V/I]; Ref. 53), which is required for binding to a nuclear hormone receptor DAX-1 (14), is necessary and sufficient for interaction with the C-terminal region of Nif3l1 containing a putative Leu Zip (Fig. 1C). It seems likely that these putative motifs mediate the interaction between Trip15/CSN2 and Nif3l1 and the weak interaction of full-length Trip15/CSN2 with Nif3l1 is related to the transcriptional co-repressor activity of Trip15/CSN2 (12–14).

To further confirm the interaction between the full-length Nif3l1 and Trip15/CSN2 in vitro, GST pull-down assay was performed. GST-fused Trip15/CSN2 protein was bound to glutathione-Sepharose beads, and then $^{35}$S-labeled Nif3l1 synthesized by in vitro translation was added to the beads. After washing with the binding buffer, $^{35}$S-labeled Nif3l1 retained in the beads was eluted with the SDS sample buffer and analyzed by 12% SDS-PAGE. As shown in Fig. 2A, the 42-kDa Nif3l1 protein specifically bound to GST-Trip15/CSN2 fusion protein.

To demonstrate that the interaction between these two proteins also occurs in vivo, C57 cells were transfected with expression vectors for pcDNA3-HA-Trip15/CSN2, pFlag-CMV2-Nif3l1, or both vectors to express HA-tagged Trip15/CSN2 and Flag-tagged Nif3l1 proteins. After 48 h, the cell lysates were prepared and subjected to Western blot analysis.

FIG. 4. Induction of sense and antisense Nif3l1 RNAs by Tet removal. Expression levels of Nif3l1 mRNA in R13 cells in the presence and absence of Tet (A) and in R13NifA cells after removal of Tet (B). The levels were analyzed by Northern blotting using a 0.6-kb EcoRI-DraI fragment of Nif3l1 cDNA as a probe. C, reduction of endogenous Nif3l1 mRNA expression in R13NifA cells after removal of Tet. Expression levels of endogenous Nif3l1 mRNA were analyzed by RT-PCR. The primers annealed to mouse Nif3l1 cDNA (25) are as follows: 5'-primer, 5'-CAG CGG CCT GGA GTG GGA AGC AG-3'; 3'-primer, 5'-CTC CTC CAG TAC CTG CTC CGA G-3'. Expression levels of PO mRNA were also analyzed by RT-PCR as an internal control using the following primers, 5'-primer, 5'-CAG CTC GGT AGA AAC TGC TG-3'; 3'-primer, 5'-GTG TAC TCA GTC TCC ACA GA-3' (50).

Expression levels of Nif3l1 mRNA in RA-treated P19 cells and in various mouse tissues. A, expression patterns of Nif3l1 and Trip15/CSN2 mRNAs during RA-primed P19 cell neural differentiation. Total RNAs were extracted from the P19 cells treated with $5 \times 10^{-7}$ M RA for various times and analyzed by Northern blot using a 0.6-kb EcoRI-DraI fragment of mouse Nif3l1 cDNA (upper panel) and a 2.1-kb EcoRI fragment of rat Trip15/CSN2 cDNA (middle panel) as probes. Expression levels of Nif3l1 mRNA in various adult mouse tissues (B), embryonic mouse brain (C), and postnatal mouse brain (D) were similarly analyzed. Pictures of ethidium bromide-stained 28 S ribosomal RNA are included for comparison of total amount of RNA employed.
Nif3l1 Participates in Neural Differentiation

Fig. 5. Effects of enforced expression of sense and antisense mouse Nif3l1 RNAs on RA-primed P19 cell neural differentiation. A, effects of Tet-controlled expression of sense and antisense Nif3l1 RNAs on differentiation to β-tubulin III-positive neurons. R13 (a–c), R13NifS (d–f), and R13NifA (g–i) cells were treated without RA (a, d, and g) and with RA (b, c, e, f, h, and i) in the presence (b, c, e, f, h, and i) of Tet for 4 days. Immunoocytochemical analysis was performed after 3 days of replating. Scale bar presents 100 μm. B, quantification of effects of sense and antisense Nif3l1 RNAs on differentiation to β-tubulin III-positive neurons. β-Tubulin III-positive neurons were counted at least 5 fields/slide under a microscope (original magnification, ×100) and estimated the Tet(-)/Tet(+) ratios. Each value is the average ± S.E. of triplicate chamber slides. *p < 0.001; **p < 0.001 compared with the control R13 cells. C, effects of Tet-controlled expression of sense and antisense Nif3l1 RNAs on differentiation to GFAP-positive glial cells. R13 (j and k), R13NifS (l and m), and R13NifA (n and o) cells were treated with RA in the presence (j, l, and n) and absence (k, m, and o) of Tet for 4 days. Immunoocytochemical analysis was carried out at 7 days after replating. Scale bar presents 500 μm. D, quantification of effects of sense and antisense Nif3l1 RNAs on differentiation to GFAP-positive glial cells. GFAP-positive areas were measured at least 6 fields/slide using a Image Gauge software (Fujifilm) and estimated the Tet(-)/Tet(+) ratios. Each value is the average ± S.E. of triplicate chamber slides. *p < 0.001; **p < 0.001 compared with the control R13 cells.

expression was not changed. On the other hand, the expression of Trip15/CSN2 mRNA was induced shortly after the addition of RA reaching a maximal level at 3 h after the treatment. The level then decreased and became barely detectable after 12 h. However, Trip15/CSN2 seemed to be accumulated during this period, and its ability to translocate Nif3l1 into the nuclei was augmented along with the progression of neural differentiation as shown below (see Fig. 10B). The result suggested that Nif3l1 interacts with Trip15/CSN2 for only the limited period during differentiation.

B The second up-regulation was observed at postnatal day (P5), until E14.5 (Fig. 3C). The levels decreased steeply after E16.5. Nif3l1 was already detectable in the brain at E10.5 and continued augmented along with the progression of neural differentiation period, and its ability to translocate Nif3l1 into the nuclei was clear at present. These results support the idea that the Nif3l1 gene plays an important role in neural differentiation and development, and in maintenance of neural function.

Implication of Nif3l1 in Neural Differentiation—To demonstrate the implication of Nif3l1 in neural differentiation, we established P19 derivative cell lines in which the exogenous expressions of sense and antisense mouse Nif3l1 RNAs could be initiated by the withdrawal of Tet. Briefly, pTRE2-sense Nif3l1 and pTRE2-antisense Nif3l1 vectors were transfected into the pTet-Off vector-introduced P19 (termed as R13) cells as described under “Experimental Procedures.” The resulting stable transfectants introduced with sense and antisense Nif3l1 vectors were designated as R13NifS and R13NifA, respectively. To examine whether the expression of exogenous sense and antisense Nif3l1 RNA could be induced by the removal of Tet, we analyzed the expression levels of Nif3l1 mRNA in R13, R13NifS, and R13NifA cells in the presence and absence of Tet (Fig. 4). The endogenous expression level of Nif3l1 mRNA in R13 cells was not changed in the Tet-free medium (Fig. 4A). In R13NifS cells, the expression of Nif3l1 1.85-kb mRNA analyzed by Northern blot was substantially induced by the withdrawal of Tet and after 12 h, the level increased to 6.5-fold higher than that expressed in the presence of Tet (0 h) (Fig. 4B). In contrast, the expression level of endogenous Nif3l1 mRNA in R13NifA cells detected by RT-PCR was decreased after the removal of Tet and lowered to 20% of the original level after 36–48 h (Fig. 4C). The expression...
Implication of Nif3l1 Gene in Early Commitment of P19 Cells to Neural Lineage—The enforced expression of sense Nif3l1 RNA in R13NifS cells by the removal of Tet enhanced differentiation into β-tubulin III-positive neurons in the presence of RA. These results were consistent with the data obtained from immunocytochemical analysis as shown in Fig. 5. Taken together, it seems likely that the Nif3l1 gene plays a crucial role in the RA-primed neuronal differentiation signal pathway.

Implication of Nif3l1 Gene in Early Commitment of P19 Cells to Neural Lineage—The enforced expression of sense Nif3l1 RNA in R13NifS cells by the removal of Tet enhanced differentiation into β-tubulin III-positive neurons in the presence of RA.
RA (Fig. 5A). To investigate which periods of sense Nif3l1 RNA expression are effective for RA-primed neuronal differentiation, we removed Tet for various time periods and the extents of cell differentiation and the levels of β-tubulin III were analyzed by immunocytochemistry and Western blot, respectively. We confirmed that sense Nif3l1 RNA expression was induced just after 30 min of the Tet removal (Fig. 7B). Immunocytochemical analysis revealed that neuronal differentiation of R13Nifs cells was significantly stimulated by Tet removal, only for 3 h (Fig. 7, B and E). Longer removal of 12 h stimulated the differentiation further, but only to a small extent, and the extent was even slightly decreased by removal for 24 h (Fig. 7, C, D, and E). Western blot analysis also showed that the expression of β-tubulin III was induced to near maximal level during 3 h of Tet removal (Fig. 7, F and G). These results indicate that Nif3l1 acts on the very early stage of RA-primed neuronal differentiation.

Effect of Nif3l1 on Oct-3/4 and Neurogenic Gene Expressions—Transcription factors regulate expression of specific genes to control cellular phenotype. Oct-3/4 is a transcription factor and acts to maintain the undifferentiated state of P19 cells as well as embryonic stem cells. Its mRNA expression is dramatically diminished within 24 h of the RA treatment (3). Thus, the down-regulation of Oct-3/4 is required for neural differentiation. In addition, Nif3l1 and Trip15/CSN2 are known as transcriptional regulator and transcriptional corepressor, respectively (12–14, 25, 26). On the basis of these facts, whether Nif3l1 participates in neural differentiation through the down-regulation of Oct-3/4 gene expression was analyzed in aggregated R13Nifs cells after the removal of Tet in the presence and absence of RA by Northern blot (Fig. 8A). When the cells were treated with RA in the absence of Tet, the level of Oct-3/4 mRNA began to decrease after 36 h and lowered to an almost undetectable level after 60 h (Fig. 8A). The reduction of Oct-3/4 mRNA level after 60 h, however, was not observed in the presence of Tet, suggesting that Nif3l1 is involved in the repression of the Oct-3/4 gene and the effect became visible after 60 h. This residual effect of Nif3l1 was also seen in the absence of RA, and the level was reduced to 75% of the original level at 96 h after removal of Tet (Fig. 8A).

We further analyzed the expression level of neurogenic Mash-1 gene during neural differentiation, because it plays a key regulatory role in the downstream of Oct-3/4 pathway, which leads to RA-primed neuronal differentiation of P19 cells (6). As shown in Fig. 8B, the expression of Mash-1 mRNAs was hardly detected in R13Nifs cells not treated with RA. By the addition of RA, expression of Mash-1 mRNA became detectable after 60 h when the down-regulation of Oct-3/4 mRNA was enhanced. This induction of Mash-1 gene expression seemed to be influenced by exogenously expressed Nif3l1, although the effects was not so evident. Under these conditions, the level of PO mRNA, analyzed as an internal control, was not significantly altered, although some bias was observed (Fig. 8C).

Nif3l1 harbors an autonomous silencing function. A nuclear localization of GAL4DBD and GAL4DBD-fused Nif3l1 proteins. P19 cells were transfected with either pGAL4DBD (upper panel) or pGAL4DBD-Nif3l1 (middle panel) and stained with anti-GAL4 antibody after 48 h (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclei were stained with hematoxylin (lower panel). Scale bar presents 20 μm. B, both luciferase reporter plasmid pGAL4-TK-Luc (1 μg) and β-gal expression plasmid pCDNA3.1/Myc-His/lacZ (0.5 μg) were transfected into P19 cells together with 0.5 μg of pGAL4DBD, pGAL4DBD-Nif3l1, or pGAL4DBD-Trip15/CSN2 in combination with 0.5 μg of pCDNA3-Trip15/CSN2 or empty pcDNA3. At 48 h after transfection, the cells were lysed and assayed for luciferase activity. Luciferase activities were normalized using β-gal activity as an internal control, and the activity expressed by cotransfection with pGAL4DBD as a control was taken as 1. Each value is shown as average ± S.E. of triplicate culture dishes. *p < 0.002; **p < 0.001; ***p < 0.001 compared with the pGAL4DBD control.

Nif3l1 Harbors an Autonomous Silencing Function and Cooperates with Trip15/CSN2—Trip15/CSN2 interacts with a subset of nuclear hormone receptors such as DAX-1, edysone receptor, COUP-TF1, Ftz-F1, and TRs, but not with retinoic acid receptors and retinoid X receptors (12–14). Trip15/CSN2 interacts with TRs and harbors an autonomous silencing function. This interaction is inhibited by increasing amounts of thyroid hormone (13). Thus, Trip15/CSN2 represents a member of novel class of corepressors specific for a selected member of the nuclear hormone receptor family (13, 14). In addition, Nif3l1 interacts with transcriptional activator/repressor protein ADA3/NGG1 (26, 29, 30). Therefore, if Nif3l1 is involved in a transcriptional silencing, we expected that Nif3l1 should harbor an autonomous silencing function and cooperate with Trip15/CSN2 in a transcriptional silencing.
Nif3l1 Participates in Neural Differentiation

To localize Nif3l1 and Trip15/CSN2 effectively in nuclei, expression vectors containing the full-length Nif3l1 and Trip15/CSN2 cDNAs fused to the GAL4DBD coding sequence downstream of the CMV promoter were constructed, because GAL4DBD possesses PKTKRSP sequence as a NLS. GAL4DBD itself and GAL4DBD-fused Nif3l1 transiently expressed in P19 cells were actually localized in nuclei (Fig. 9A). The effect of Nif3l1 and Trip15/CSN2 on the suppression of TK promoter activity in P19 cells was studied using these vectors and a luciferase reporter construct, pGAL4-TK-Luc which contains GAL4 binding sequence upstream of the TK basal promoter. Trip15/CSN2 expressed in P19 cells was actually localized in nuclei (Fig. 9A). When Nif3l1 was coexpressed with Trip15/CSN2 by cotransfection of COS-7 cells with pEGFP-Nif3l1 alone (c) or together with pDNA3-Trip15/CSN2 in the ratio of 1:9 (d), COS-7 cells were also transfected with pEGFP-Trip15/CSN2 alone (c) or together with pDNA3-Nif3l1 in the ratio of 1:9 (d). Transfection with pEF/Myc/ER/GFP (Invitrogen) was performed as a marker of ER (e). Nuclei were stained with Hoechst 33258 (f). At 48 h after transfection, the cells were observed under a fluorescent optics (Axioplan2; Carl Zeiss). Scale bar presents 40 µm. Quantitative analysis of the effect of Trip15/CSN2 on intracellular localization of EGFP-fused Nif3l1. The cells exhibiting distinct fluorescence in the cytoplasm and nuclei as shown in a and b, respectively, were counted. Each value is the average ± S.E. of triplicate culture dishes. *, p < 0.004 compared with the cells transfected with pEGFP-Nif3l1 alone. b, quantitative analysis of the effect of coexpression of Nif3l1 on intracellular localization of EGFP-fused Trip15/CSN2. Each value is the average ± S.E. of triplicate culture dishes. b, translocation of Nif3l1 during neural differentiation. The pEGFP-Nif3l1-transfected P19 cells were cultivated in the absence of RA (a–c) or in the presence of RA (d–f) for 12 h. Intracellular localization of EGFP-Nif3l1 (a and d) and the nuclei stained with Hoechst 33258 (b and e) were observed under a confocal microscope (Radiance2100; Bio-Rad). c and f present merges of a and b, and d and e, respectively. Scale bar presents 40 µm. g, time-dependent translocation of EGFP-fused Nif3l1 during RA-primed P19 cell neural differentiation. Values are presented as the average ± S.E. of four independent dishes. *, p < 0.03 compared with RA (−) control.

Cellular Localization of Nif3l1—To investigate the subcellular localization of Nif3l1, we constructed a EGFP-fused Nif3l1 expression vector by the insertion of full-length Nif3l1 cDNA into pEGFP-C1 downstream of EGFP coding sequence in frame. The EGFP-fused Nif3l1 expression vector was introduced into COS-7 cells, and subcellular distribution of the fused protein was observed after 48 h. As shown in Fig. 10A (a), EGFP-Nif3l1 fusion protein was localized predominantly in the cytoplasm, although fluorescence was homogenously detected both in the nuclei and cytoplasm when only EGFP protein was expressed (data not shown). The localization of EGFP-Nif3l1 was different from that of ER retention signal-tagged GFP, which was concentrated around the nuclei (Fig. 10A (e)). On the other hand, EGFP-fused Trip15/CSN2 was localized in the nuclei predominantly (Fig. 10A (c)) and showed an image similar to the nuclei that are stained with Hoechst 33258, which intercalate to DNA (Fig. 10A (c) and f). For the expression of transcriptional silencing activity, however, the translocation of Nif3l1 from the cytoplasm into the nuclei is absolutely required. As described above, Nif3l1 bound to Trip15/CSN2 in vivo and in vitro, and cooperated with Trip15/CSN2 for the suppression of the TK promoter activity, suggesting that Trip15/CSN2 participates in the translocation of Nif3l1 from the cytoplasm to the nuclei. To confirm this assumption, the effect of Trip15/CSN2 on the intracellular localization of Nif3l1 was examined by cotransfection of COS-7 cells with pEGFP-Nif3l1 and pDNA3-Trip15/CSN2 and the cells exhibiting distinct fluorescence in the nucleus or cytoplasm were counted. The fraction of EGFP-fused Nif3l1 localized predominantly in the nuclei was increased depending on the increase in the amount of the Trip15/CSN2 vector transfected, and in the ratio of 1:9, the fraction of cells exhibiting EGFP-fused Nif3l1 in the nuclei (Fig. 10A (b)) was ~4-fold higher than that observed in the absence of Trip15/CSN2 (Fig. 10A (g)). In contrast, the inter-
cellular localization of EGFP-fused Trip15/CSN2 was not affected by the coexpression of Nif3l1 (Fig. 10A (d and h)).

Because the Trip15/CSN2 gene was highly expressed in an early stage of neural differentiation of RA-treated P19 cells (Fig. 3A), we assumed that the translocation of Nif3l1 from the cytoplasm into the nuclei might be enhanced at this early stage of neuronal differentiation. To confirm this assumption, P19 cells were transfected with pEGFP-Nif3l1 and cultivated with and without RA for various times and the intracellular localization of EGFP-fused Nif3l1 was examined. The localization in the nuclei was confirmed by staining the cells with Hoechst 33258. Although in the absence of RA, EGFP-fused Nif3l1 was mainly detected in the cytoplasm (Fig. 10B (a–c)), the fraction of cells exhibiting EGFP-fused Nif3l1 predominantly in the nuclei was augmented by the treatment with RA in a time-dependent manner and, after 24 h, increased to ~3.5-fold of that observed in the absence of RA (Fig. 10B (d–g)). The merge of two images obtained by the EGFP fluorescence and by staining with Hoechst 33258 showed that Nif3l1 localizes as nuclear patches (Fig. 10B (f)).

**DISCUSSION**

The data presented here provide the first insight into the molecular mechanism underlying the involvement of Nif3l1 in neural differentiation through the association with Trip15/CSN2.

Using a yeast two-hybrid method, we found that the N-terminal region (amino acids 1–275) of Trip15/CSN2 bound to the C-terminal region (amino acids 243–376) of Nif3l1 (Fig. 1). The interaction was confirmed by a pull-down assay and an epitope-tagged coimmunoprecipitation (Fig. 2, A and B). In addition, the interaction between Nif3l1 and endogenous Trip15/CSN2 in RA-primed P19 cells was also confirmed (Fig. 2C). Trip15/CSN2 possesses a putative NLS and Leu Zip sequence and is functioning in the nucleus (54), whereas its C-terminal region (amino acids 243–376) of Trip15/CSN2 bound to Nif3l1. The molecular mechanism underlying the involvement of Nif3l1 could be that Nif3l1 binds to Trip15/CSN2 in the cytoplasm and is functioning in the nucleus (54), whereas its C-terminal region (amino acids 243–376) of Trip15/CSN2 bound to Nif3l1. It appeared that Nif3l1-Trip15/CSN2 complex could be implicated in the commitment of multipotent P19 cells to neural lineage via the Oct-3/4 down-regulation. In the developing brain, a common cortical progenitor cell gives rise first to a variety of layer-specific neurons and then switches to producing astrocytes and ultimately oligodendrocytes (59, 60). As in the case of neurogenesis in vivo, neurons appear earlier than glial cells during RA-primed P19 cell neural differentiation (61). Therefore, the enhancement of both neurogenesis and gliogenesis by Nif3l1 might be caused by the increase in the fraction of cells committed to differentiate to neural lineage, although the precise estimation of the fraction of neural lineage was difficult because of the occurrence of the cells that underwent proliferation and apoptosis during P19 cell neural differentiation (62).

The mouse Nif3l1 gene has been isolated by a suppression subtractive hybridization between the spermatogonia-derived cell line GC-1 spg and spermatocyte-derived cell line GC-4 spc (25). Yeast Nif3 and NGG1 form a complex for effective inhibition of the transcriptional activation by GAL4 (27). The Nif3l1 gene was expressed throughout embryonic development and in the various adult mouse tissues ubiquitously accompanying the putative alternative splicing (Fig. 3). The widespread expression of Nif3l1 gene suggests that the gene acts as a common repressor and is unlikely to be restricted to specify neural identities by itself. The expression of Nif3l1 gene was also observed in the undifferentiated P19 cells, and the expression level was not changed significantly during RA-primed neural differentiation. On the other hand, binding partner Trip15/CSN2 gene expression was markedly induced by the treatment of RA (Fig. 3A; Ref. 11). These observations suggest that Nif3l1-Trip15/CSN2 complex is specifically required for the early stage of neural differentiation and various regulatory factors distinct from Trip15/CSN2 cooperate with Nif3l1 in the process of the important cellular events such as spermatogenesis and maintenance of cell type-specific functions.

P19 cells possess many properties similar to embryonic stem cells isolated from mice and humans (63). Therefore, it may be possible that the expression system of Nif3l1-Trip15/CSN2 complex can be utilized for the production of large amount of neurons from human embryonic stem cells in combination with the expression of key neurogenic genes.

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