Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominantly inherited disorder with an estimated prevalence of 1 in 3,000 people (1). The hallmarks of NF1 include development of benign tumors of the peripheral nervous system and increased risk of malignancies. The phenotype of NF1 is highly variable, with several organ systems affected including the skin, bones, irises, and central and peripheral nervous systems. The effects on the nervous system are manifested in multiple neurofibroma, gliomas, and learning disabilities.

The NF1 gene is located on chromosome 17q11.2 and encodes a large protein of 2,818 amino acids, neurofibromin (2). Because the great majority of NF1 gene mutations frequently found in NF1 patients disturb the expression of intact neurofibromin, functional disruption of neurofibromin is potentially relevant to the expression of some or all of the abnormalities that occur in NF1 patients (3). A region centered around 360 amino acid residues encoded by the NF1 gene shows significant homology to the known catalytic domains of mammalian Ras GTPase-activating protein (p120 GAP). This region is also similar to yeast IRA1/2 proteins, which have been shown to interact with Ras and mediate hydrolysis of Ras-bound GTP to GDP, resulting in inactivation of Ras protein function. The GAP-related domain of the NF1 gene product also stimulates Ras GTPase and consequently inactivates Ras protein (4-6). In our previous report, we demonstrated a novel role of neurofibromin in neuronal differentiation in conjunction with regulation of Ras activity via its GAP-related domain using nerve growth factor (NGF)-stimulated PC12 cells as a model for neuronal cells (6). We also found that a novel neurofibromin interacting protein CRMP-2, identified with the screening of binding proteins of neurofibromin C-terminal domain by iTRAQ, upregulates the phosphorylation patterns in the NF1 siRNA treated PC12 cells compared with those of control cells through two-dimensional (2D) fluorescence difference gel

electrophoresis (DIGE) analysis coupled with Pro-Q Diamond staining, and demonstrated that the functional association of neurofibromin and CRMP-2 is essential for neuronal cell differentiation (7). In these studies, neurofibromin expression was suppressed using small interfering RNA (siRNA) directed against NF1, and the inhibition of neurofibromin functions caused neurite retraction via the regulation of Ras-MAPK-CDK5-GSK3/ROCK activation in differentiated PC12 cells stimulated by NGF (7). These results indicated that the neurofibromin-deficient PC12 cell is a useful model for analyzing NF1-related molecular pathology in detail.

Our previous research has examined specific genes and proteins related to phenotypic changes in neural tumors using integrated proteomic techniques such as 2D-DIGE combined with Pro-Q Diamond staining to identify phosphorylated proteins, isobaric tagging for relative and absolute quantitation (iTRAQ) (7, 8), which provides information on peptide/protein quantitative expression levels from different sources in a single experiment by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), as well as DNA array technology, utilizing the original data mining tool, MANGO (Molecular Annotation by Gene Ontology) (8). Processing the voluminous data arising from each analysis highlighted the need for a mining system for data integration. Therefore, we created the "iPEACH" (Integrated Protein Expression Analysis Chart, PCT/JP2011/58366) application to integrate information from several analysis types into a useful data file that provides comprehensive proteomic data including post-translational modification, transcriptomic data, and functional annotations from several databases, with a tool for quickly organizing, enriching, and sorting these data to reveal candidate molecules. Using iPEACH, sample data obtained from our transcriptomic and proteomic (iTRAQ and 2D-DIGE) analyses of NF1 disease model cells were integrated and stored in a database.

In this study, we constructed an iPEACH database for neurofibromin-deficient PC12 cells

compared with normal cells after NGF stimulation, and used Gene Ontology (GO) and knowledge-based network analyses targeting upregulated signals in the NF1 model cells, which lost their normal differentiation activity, to extract a novel candidate signal network for NF1-related phenotype, termed "dynein IC2-GR-COX1" signaling. Statistical analysis of the expression or modification of these proteins in NF1-knockdown (NF1-KD) cells and subsequent biological validation by sequential analyses using siRNAs and the glucocorticoid receptor (GR) antagonist led to the successful identification of protein targets of the network most likely to be involved in the abnormal PC12 differentiation caused by neurofibromin deficiency. In addition, this network involved the alternative splicing and specific phosphorylation of cytoplasmic dynein 1 intermediate chain 2 isoform B (dynein IC2-B) and isoform C (dynein IC2-C) via NGF stimulation in NF1-KD PC12 cells.

Here, we demonstrate that the function of neurofibromin for neurite outgrowth in PC12 cells may involve the regulation of cyclooxygenase-1 (COX-1) via glucocorticoid receptor (GR) and dynein IC2 splicing and phosphorylation. We also discuss the implications of a functional association between neurofibromin and COX-1 for neuronal regulation in relation to NF1 pathogenesis. This report is the first to identify a signal network related to NF1 phenotype in a neurofibromin-deficient neural model using our new integrated proteomics strategy.

Experimental procedures

Cell culture, NGF treatment, transfection, and preparation of cell lysate

PC12 cells were cultured under 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Invitrogen)

supplemented with 10% horse serum and 5% fetal bovine serum. Transfection of siRNA was performed with the Neon transfection system (Invitrogen) according to the manufacturer's protocol (1100 V, 20 ms, two times). PC12 cells were transfected with siRNA and then seeded onto collagen-coated culture dishes (Iwaki). At 24 h after transfection, PC12 cells were stimulated with 50 ng/ml 2.5S NGF (Wako) until harvest. Cells were washed twice in PBS and solubilized with lysis buffer containing 8 M urea, 2% (w/v) CHAPS, 1 mM DTT, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1 μM okadaic acid, and 1% (v/v) protease inhibitor mixture (Sigma). Lysates were passed through a 25-gauge syringe 20 times and centrifuged at 20,000 × *g* for 15 min at 4 °C, and the protein concentrations of the supernatants were determined using Protein Assay Dye Reagent Concentrate (Bio-Rad). Cellular phenotypic changes were monitored on a time-lapse microscope equipped with a CO_2 incubation chamber system (IX81, Olympus) using MetaMorph software ver. 7.5.5.0 (Molecular Devices).

RNA isolation and microarray analysis

Total RNA was extracted and purified with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated RNA was assessed for quantity and quality using a NanoDrop ND1000 spectrophotometer (Thermo Scientific) and a 2100 Bioanalyzer (Agilent). Double-stranded complementary DNA (cDNA) and labeled complementary RNA (cRNA) were synthesized from the total RNA using the 3′ IVT Express Kit and hybridized to Affymetrix Rat 230 2.0 gene chips (Affymetrix). The chips were scanned with a Gene Chip Scanner 3000. The probe-level raw data and CEL files were obtained using GeneChip Operating Software. The raw expression data from CEL files was normalized by MAS5 algorism and analyzed by Subio Platform software (ver. 1.12, Subio, Japan).

2D-DIGE and image analysis

PC12 cell lysates were desalted using a 2D Clean-up kit (GE Healthcare) and dissolved in lysis buffer

containing 8 M urea, 2% (w/v) CHAPS, and 30 mM Tris-HCl (pH 8.5). Each sample (50 μg) was minimally labeled with 400 pmol of either Cy3 or Cy5 for comparison on the same gel. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was prepared and labeled with Cy2 (400 pmol/50 μg) as an internal standard for all gels. The labeling mixture was incubated on ice in the dark for 30 min, and the reaction was terminated by addition of 10 nmol lysine. The differentially Cy3- and Cy5-labeled samples were mixed with Cy2-labeled internal standard and incubated with equal volumes of 2× sample buffer containing 8 M urea, 2% (w/v) CHAPS, 2.4% (v/v) Destreak Reagent (GE Healthcare), and 1% (v/v) IPG buffer (pH 3–11 or pH 4–7, GE Healthcare) for 10 min on ice. The mixed samples were supplemented with 1× sample buffer containing 8 M urea, 2% (w/v) CHAPS, 1.2% (v/v) Destreak Reagent, and 0.5% (v/v) IPG buffer to reach a final volume of 450 μl and loaded into strip holders for first-dimension isoelectric focusing (IEF). IPG strips (pH 3–11 NL or pH 4–7, 24 cm, GE Healthcare) were rehydrated with the Cy-labeled samples in the dark at room temperature overnight. IEF was performed using a Multiphor II apparatus (GE Healthcare) under the following conditions: held at 100 V for 2 h, held at 500 V for 2 h, ramped to 1,000 V in 5 h, ramped to 8,000 V in 3 h, and held at 8,000 V for 9 h for pH 3–11 NL strips; held at 100 V for 2 h, held at 500 V for 4 h, ramped to 1,000 V in 5 h, ramped to 8,000 V in 3 h, and held at 8,000 V for 9.5 h for pH 4–7 strips. Strips were equilibrated in solution containing 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue, and 1 mM DTT for 20 min and then in the same buffer additionally containing 1 mM iodoacetamide for 20 min. The equilibrated strips were then transferred onto 10% gels (24 × 20 cm) for second-dimension electrophoresis using the Ettan DALTsix system (GE Healthcare). SDS-PAGE was run at 10 mA/gel for 1 h and then 12 mA/gel at 30°C until the bromophenol dye front reached the gel end. Images of protein spots on the gels were scanned with a

Typhoon 9400 variable mode imager (GE Healthcare) at 100 μm resolution using excitation/emission wavelengths of 488 nm/520 nm for Cy2, 532 nm/580 nm for Cy3, and 633 nm/670 nm for Cy5, respectively. The intensity was adjusted to ensure that the maximum volume of each image was within 60,000–90,000 pixels. Image analysis was performed using Decyder2D software version 5.2 (GE Healthcare) according to the manufacturer's instructions. The differential in-gel analysis module was used for spot detection, spot volume quantification, and volume ratio normalization of different samples in the same gel. Then, the biological variation analysis module was used to match protein spots across different gels and to identify spots demonstrating significant differences. Differentially expressed spots were determined statistically using two-way analysis of variance (condition 1: $P < 0.05$ for the treatment of NF1 siRNA and condition 2: $P < 0.05$ for the period of NGF treatment).

Staining of 2D gels with Pro-Q Diamond phosphoprotein gel stain

Two-dimensional gels were fixed in 50% methanol containing 10% acetic acid once for 1 h and again overnight. Gels were washed three times in water for 15 min, stained with Pro-Q Diamond phosphoprotein gel stain (Invitrogen) in the dark for 4 h, and then washed with destain solution (5% 1 M sodium acetate, pH 4.0, in 20% ACN) three times for 60 min. Gels were washed twice with deionized water for 5 min and scanned with a Typhoon9400 variable mode imager (GE Healthcare). For SYPRO Ruby gel staining, gels were fixed in 10% methanol containing 7.5% acetic acid for 30 min, stained with SYPRO Ruby protein gel stain (Invitrogen) overnight, and washed with 10% methanol containing 7.5% acetic acid for 1 h and scanned with a Typhoon9400 imager (GE Healthcare). Fluorescent images stained with Pro-Q Diamond and SyproRuby were overlaid using Multi Gauge software (Fujifilm).

In-gel digestion of proteins identified in 2D-DIGE

To identify proteins detected in 2D-DIGE, 150 μg of proteins was separated onto 2D gels. The gels were

fixed with 10% methanol and 7.5% acetic acid and then stained with Deep Purple total protein stain (GE Healthcare) according to the manufacturer's instructions. After scanning the gel image with a Typhoon 9400 imager (GE Healthcare), protein spots of interests were selected with DeCyder software and picked by an Ettan spot picker (GE Healthcare). The gel pieces were washed three times with 50 mM ammonium bicarbonate in 50% (v/v) ACN, dehydrated in 100% (v/v) ACN, and vacuum-dried. Sequencing grade modified trypsin (Promega) was added to the gel pieces at a concentration of 50 ng/mL in 10% ACN including 50 mM ammonium bicarbonate, and the mixture was incubated at 37 °C overnight. The trypsinized peptides were sequentially extracted from the gels with 0.1% (v/v) TFA in 30% (v/v) ACN, 0.1% (v/v) TFA in 50% (v/v) ACN, and 0.1% (v/v) TFA in 80% (v/v) ACN, for 5 min each. The extracted peptides were vacuum-dried and dissolved in 20 μl of 0.1% (v/v) TFA in 2% (v/v) ACN. These samples were desalted with a ZipTip C18 pipette tip (Millipore) and subjected to MALDI-MS/MS analysis using the 4700 Proteomics analyzer (Applied Biosystems/AB SCIEX) or LC-ESI-MS/MS analysis using the API QSTAR Pulsar i time-of-flight mass spectrometer (Applied Biosystems/AB SCIEX) coupled with the UltiMate NanoLC system (Thermo Scientific Dionex).

iTRAQ sample preparation, fractionation, and desalting

Protein samples (100 μg) were precipitated using a 2-D Clean-Up kit (GE Healthcare), and the precipitates were dissolved in 10 μl of 6 M urea. iTRAQ sample labeling was performed according to the manufacturer's protocol with minimum modification (8). The digests were incubated with eight different iTRAQ reagents (113–121) for 2 h as follows: iTRAQ 113 for NGF 0 h and control siRNA, 114 for 24-h control, 115 for 48-h control, 116 for 72-h control, 117 for 0-h NF1 siRNA, 118 for 24-h NF1 siRNA, 119 for 48-h NF1 siRNA, and 121 for 72-h NF1 siRNA. The labeled samples were then mixed together and fractionated to remove excess, unbound iTRAQ reagent and to simplify the peptide mixture

using a GE Healthcare AKTA system. The mixed sample was diluted in loading buffer (20% ACN and 10 mM potassium phosphate, pH 3.0) and loaded onto a Mono S column (GE Healthcare) equilibrated with loading buffer. Peptides were eluted with a gradient of solvent B (10 mM potassium phosphate, pH 3.0, and 500 mM KCl in 20% ACN) as follows: 0–2 min, 0–7% B; at 10 min, to 14% B; at 14 min, to 32% B; at 19 min, to 70% B; and at 24 min, to 100% B. Forty-four fractions that included the iTRAQ-labeled peptides were used for analysis. The fractions were dried in a vacuum centrifuge and rehydrated with solution containing 2% ACN and 0.1% TFA. The samples were desalted with ZipTip μ-C18 pipette tips (Millipore). The desalted peptides were divided into two fractions to analyze the same samples by LC-MALDI-MS/MS and LC-ESI-MS/MS analyses

LC-MALDI-MS/MS analysis

Samples were separated by C18 nano-LC using a DiNa MaP system (KYA Tech Corp.) equipped with a device spotting eluted fractions onto a MALDI plate. Sample was injected onto a C18 column (0.5-mm inner diameter × 1-mm length, KYA Tech Corp.) equilibrated with solvent A (2% ACN and 0.1% TFA) and resolved on a C18 nanocolumn (0.15-mm inner diameter × 50-mm length; KYA Tech Corp.) at a flow rate of 300 nl/min with a 113-min gradient of solvent B (70% ACN and 0.1% TFA) as follows: 0–22% B from 0 to 10 min, to 39% B at 53 min, to 45% B at 83 min, and to 100% B at 93 min. Column effluent was mixed with matrix (3 mg/ml alpha-cyano-4-hydroxycinnamic acid in 50% ACN and 0.1% TFA) at a flow rate of 1.4 μl/min. Fractions were spotted at 30-s intervals onto a stainless steel MALDI target plate (384 wells/plate; Applied Biosystems). Mass spectra of the peptides were acquired on an AB SCIEX TOF/TOF 5800 system (AB SCIEX) using TOF/TOF Series Explorer software (Version 4.0.0). Mass spectra from m/z 800 to 4,000 were acquired for each fraction with 1,500 laser shots. To analyze the less abundant peptides, all peaks with a signal-to-noise ratio threshold from 25 to 50, from 50 to 100,

and more than 100 in each MS spectrum were sequentially selected for MS/MS analysis. Fragmentation of the labeled peptides was induced by the use of atmosphere as a collision gas with a pressure of 1×10^{-6} Torr and a collision energy of 1 kV.

LC-ESI-MS/MS analysis

Samples were analyzed by nano-LC-ESI-MS/MS using the LC Packings Ultimate instrument fitted with a 20-µl sample loop. Samples were loaded onto a 5-mm RP C18 precolumn (Thermo Scientific Dionex) at 25 µl/min and washed for 10 min before switching the precolumn in line with the separation column, which was a 75-µm internal diameter × 150-mm length PepMap RP column (Thermo Scientific Dionex) packed with 3-µm C18 beads with 100-Å pores. The flow rate used for separation on the RP column was 200 nl/min with a 120-min gradient of solvent B (85% ACN and 0.1% formic acid) as follows: 0–3% B from 0–5 min, to 15% B at 10 min, to 40% B at 95 min, to 100% B at 100 min. Sample analysis was performed using a QSTAR Elite mass spectrometer (Applied Biosystems/AB SCIEX), and Analyst QS 2.0 software (Applied Biosystems/AB SCIEX) was used for data acquisition with the scan cycles set to perform a 1-s MS scan followed by three MS/MS scans of the three most abundant peaks for 2 s each. Data acquisition was performed with an exclusion of 60 s for previous target ions. The labeled peptides were fragmented under CID conditions designed to yield iTRAQ reporter ions.

Protein identification in 2D-DIGE and iTRAQ

Data from MALDI and ESI analysis of proteins identified in 2D-DIGE were analyzed using MASCOT software application 2.1.04 (Matrix Sciences). The UniProt database (release-2010_03) was used for the search. The following search parameters were used: taxonomy, rat; cleavage enzyme, trypsin; variable modifications, carbamidomethyl (C), oxidation (M); max missed cleavage, 1. Peptide tolerance of 0.3 Da, fragment mass tolerance of 0.3 Da, and peptide charge of +1 for MALDI or +2, +3, and +4 for ESI

were considered significant. Confident identification required a statistically significant ($P < 0.05$) protein score based on combined MS and MS/MS spectra. Data from MALDI or ESI analysis with the iTRAQ method and from ESI analysis with 2D-DIGE were analyzed using the Paragon^{TM} algorithm (9) of ProteinPilot Version 4.1 (AB SCIEX), and the UniProt Rat proteome database (release2012_02, 37,104 entries) was searched. Identified proteins were grouped by the Paragon algorithm to minimize redundancy. The six user-defined options included (i) cysteine alkylation, methyl methane thiosulfate in iTRAQ or iodoacetic acid in 2D-DIGE; (ii) digestion, trypsin digestion; (iii) special factors, none in iTRAQ or phosphorylation emphasis in 2D-DIGE; (iv) species, none; (v) identification focus, biological modifications, amino acid substitutions; and (vi) search effort, thorough identification search. The ProteinPilot cutoff score used was 1.3, which corresponds to a confidence limit of 95%. The protein identification confidence for the dataset employing the iTRAQ method was further evaluated by false discovery rate in the concomitant search of the UniProt Rat proteome database for the reverse sequences.

Protein quantification for iTRAQ analysis

Quantitation of proteins was carried out based on the relative intensities of iTRAQ reporter ions released during MS/MS fragmentation of peptides according to the Paragon^{TM} algorithm of ProteinPilot Version 4.1 (AB SCIEX). The data analysis parameters were set as follows: Sample type: iTRAQ (peptide labeled); Bias Correction: Auto; Background Correction: Yes.

Data processing of integrated proteomics and network analysis

All the data obtained by transcriptomic and proteomic analysis were integrated into one chart by iPEACH. The iPEACH tool has following function such as, mutual conversions of probe ID, UniProt ID, and Entrez ID, calculations and outputs of iPEACH indices from DNA microarray, iTRAQ, and 2D-DIGE data, and annotations of UniProt descriptions (Organism, Amino Acid_Seqence_Length, Theoretical_MW, Keyword, GO_Biological_Process, GO_Molecular_Function,

GO_Cellular_Component, SwissProt:[CC]) and original data information (Post-Translational Modification, Cleavage sites, Theoretical_pI, Observed_MW, Observed_pI) obtained from iTRAQ, and 2D-DIGE analysis. Input multi types of files for iPEACH are CEL files (DNA microarray), peptide summary and protein summary files from ProteinPilot (iTRAQ experiments using ESI and MALDI), and xml output files from Decyder 2D software (2D-DIGE experiment using pH3-11 and pH4-7 gels). First, iPEACH integrates genes and proteins identified in the different experiments using Entrez ID. Then, iPEACH calls R/Bioconductor with affy package to normalize the gene expression data with MAS5 algorithm. iTRAQ and 2D-DIGE data are already normalized by data processing softwares. And then fold changes (control vs treated samples at each time point: 0 h, 24 h, 48 h, 72 h) are calculated within each experiment. Finally, iPEACH integrates the calculated fold changes and outputs iPEACH_sum (sum of fold changes at same time points), iPEACH_log2 (sum of logarithmical fold changes at same time points), and iPEACH_Index (sum of absolute value of logarithmical fold changes at same time points) followed by the sorting of iPEACH_index descending order (**Supplemental Table S1**). iPEACH indices (z) were calculated as follows: $z = \Sigma\{ |log_2(x_0)| + |log_2 (x_{24})| + |log_2 (x_{48})| + |log_2 (x_{72})|\}$ where x = ratio of gene or protein expression in the NF1 siRNA treatment to control siRNA treatment groups. Each suffix indicates stimulation time. UniProt release 2012_02 was used for the annotation in this study. The iPEACH column legends are listed in the supplemental information. The extraction of differentially expressed genes/proteins and cluster analysis were performed based on the protein ratio (NF1 siRNA/control treatment) using the Subio platform. Network analysis was performed using KeyMolnet (Institute of Medicinal Molecular Design). The lists of differentially expressed genes/proteins were imported into KeyMolnet, and the "start points and end points" network search algorithm was used to generate the network of all types of molecular interactions between these

genes/proteins. The network included direct and indirect activation, transcriptional activation, and complex formation.

siRNA

The target sequences for rat NF1, dynein IC2-C, dynein IC2, and COX-1 siRNA were designed as follows: a 21-oligonucleotide siRNA duplex was designed as recommended elsewhere (10) and synthesized by Nippon EGT to target the rat NF1 sequences 5′-249CAAGGAGTGTCTGATCAACTT-3′ (for NF1 249 siRNA) and 5′-611GGTTACAGGAGTTGACTGTTT-3′ (for NF1 611 siRNA), the rat dynein IC2-C sequences 5′-329-GATCTAGACGAGGACCTATTT-3′ (for dynein IC2-C 329 siRNA) and 5′-331-TCTAGACGAGGACCTATTATT-3′ (for dynein IC2-C 331 siRNA), and the rat COX-1 sequences 5′-1023-CCATCGAGATTATCATCGATT-3′ (for COX-1 1023 siRNA) and 5′-2618-CAGGTGGACTCATCTACGATT-3′ (for COX-1 2618 siRNA). A 25-oligonucleotide siRNA duplex was synthesized by Nippon EGT to target the rat dynein IC2 sequence 5′-CCCTTTGCTTTGGATTGGTGTCATT-3′ (11). Silencer Negative Control siRNA 1 (Ambion) was used as a control.

Immunoblotting

Cell lysate samples were electrophoresed on SDS-polyacrylamide gels, transferred onto a PVDF membrane by electroblotting, and subjected to immunoblotting with the indicated antibody. Membranes were probed with different primary antibodies followed by horseradish peroxidase-conjugated mouse or rabbit secondary antibodies or ECL-Plex rabbit IgG-Cy2 and ECL-Plex mouse IgG-Cy5 (GE Healthcare). The blot images were developed with ECL Prime reagent and exposed to Hyperfilm ECL (GE Healthcare). Fluorescent images visualized with CyDye patterns were processed with a Typhoon 9400 imager (GE Healthcare). The following primary antibodies were used: NF1, dynein IC, TCPε1

(Santa Cruz Biotechnology), COX-1, GR, Lamin B1 (Abcam), and beta-actin (Sigma). The intensities were measured using an ImageQuant system (GE Healthcare) with background subtraction and normalization by total spot volume. For 2D-immunoblotting, the intensities were measured using ProGenesis Workstation version 2005 (PerkinElmer Life Sciences). The intensity of each spot was recorded as digital data and processed with Microsoft Office Excel. Experimental values are expressed as mean ± S.E.

Two-dimensional immunoblotting using Auto-2D

PC12 cell lysates were desalted using a 2D clean-up kit (GE Healthcare) and dissolved in lysis buffer (8 M urea, 2% (w/v) CHAPS). Samples (5 μg) were mixed with rehydration solution (8 M urea, 2% (w/v) CHAPS, 1.0% (v/v) Destreak Reagent, 0.4% (v/v) IPG buffer (pH 4-7)) and loaded into strip holders for first-dimension IEF. IEF was performed using Auto-2D (Sharp Manufacturing Systems) with the following conditions: held at 200 V for 15 min, ramped to 1,000 V in 15 min, ramped to 3,000 V in 15 min, ramped to 6,000 V in 15 min, and held at 6,000 V for 15 min. Strips were equilibrated in solution (45% (v/v) NuPAGE (Invitrogen), 50 mM DTT) for 5 min and transferred to 8% gels (5 × 7.5 cm) for second-dimension electrophoresis. After electrophoresis, gels were transferred onto a PVDF membrane by electroblotting and subjected to immunoblotting with the indicated antibody. After immunoblotting, the intensities were measured using ProGenesis Workstation version 2005 (PerkinElmer Life Sciences).

Neurite outgrowth analysis

To quantify the neurite outgrowth of PC12 cells, cells transfected with siRNAs were cultured on collagen-coated culture dishes (Iwaki) and stimulated with 50 ng/ml 2.5S NGF (Wako) at 72 h. Total neurite length of NGF-stimulated PC12 cells was measured using MetaMorph software (Molecular Devices). The total number of tip ends was manually counted to represent the number of neurites from

individual cells. For each measurement, at least 50 cells per dish were analyzed from randomly selected fields. Each experiment was repeated three times.

Immunocytochemistry

PC12 cells grown on a 6-well plate were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and then permeabilized with 0.1% (w/v) Triton X-100 in PBS for 15 min. After washing with PBS, cells were incubated in primary antibodies diluted in PBS containing 0.2% (w/v) BSA, followed by a secondary antibody conjugated with a fluorescent dye for 1 h at room temperature and observed with a fluorescence microscope (Olympus IX81 at 20× magnification).

Preparation of nuclear and cytoplasmic protein extracts

PC12 cells were collected, and the nuclear and cytoplasmic proteins were obtained using the 2-D Sample Prep for Nuclear Proteins kit (Thermo Fisher scientific) according to the manufacturer's instructions.

Results

Integrated proteomics using iPEACH to identify specific molecules related to the phenotypic change caused by NF1 knockdown in PC12 cells.

According to our previous study (7), we prepared NF1-KD PC12 cells using NF1 siRNA. PC12 cells were transfected with control siRNA or NF1 (249) siRNA and stimulated with NGF, and morphological changes were observed at the indicated time points (0 h, NGF-; 24 h, 48 h, and 72 h; NGF+). We confirmed the suppression of the NF1 gene product, neurofibromin, by immunoblot analysis and immunocytochemistry in PC12 cells (**Fig. 1A, Supplemental Fig. S1A-C**). PC12 cells that express neurofibromin normally extend neurites upon NGF treatment, while NF1-KD PC12 cells consistently adopted spindle bipolar shapes and inhibited neurite outgrowth (**Fig. 1A, Supplemental Fig. S1C**). Cells were then subjected to integrated proteomics analyses as shown in **Fig. 1B.** PC12 cells were transfected with control siRNA or NF1 (249) siRNA and stimulated with NGF, and protein and mRNA samples were prepared from the cells at the indicated time points (0 h, NGF-; 24 h, 48 h, and 72 h; NGF+). The protein and mRNA samples were subjected to 2D-DIGE and iTRAQ-8plex methods and DNA microarray analysis using Rat 230 2.0 gene chips (Affymetrix). All the results were then integrated using iPEACH (**Fig. 1B**).

Results of 2D-DIGE

PC12 cells were transfected with control siRNA or NF1 (249) siRNA and stimulated with NGF for 0 h, 24 h, 48 h, or 72 h. Proteins were extracted from the cells at each time point (n = 3), desalted, and subjected to 2D-DIGE. To obtain as much protein information as possible, each sample was separated into two fractions and subjected to IEF using both strip ranges of pH 3–11 and pH 4–7. An average of

4,157±160 spots in pH 3–11 gels and an average of 4,007±176 spots in pH 4–7 gels were detected. Differentially expressed spots were statistically determined using two-way analysis of variance (condition 1: $P < 0.05$ for the treatment of NF1 siRNA and condition 2: $P < 0.05$ for the period of NGF treatment) with DeCyder 2D software. As a result, 145 spots (pH 3–11) and 187 spots (pH 4–7) were found to be significant differentially expressed, and 123 non-redundant proteins (pH 3–11: 128, pH 4–7: 124 spots) were identified by MALDI-TOF/TOF or LC-ESI-Qq-TOF MS. The proteins identified with 2D-DIGE are shown in **Supplemental Table S4** and integrated into the iPEACH Table (**Supplemental Table S1**).

Results of iTRAQ (8-Plex) analysis

iTRAQ analysis was performed with the same samples used for 2D-DIGE analysis. For 8-plex iTRAQ labeling, each protein sample obtained from the three pooled independent experiments at four time points of NGF stimulation after NF1 or control siRNA treatment was digested with trypsin after reduced alkylation and labeled with iTRAQ reagents. The labeled samples were mixed together, fractionated into 44 fractions with cation exchange column chromatography, and analyzed by nanoLC-MALD-TOF/TOF MS and nanoESI-Qq-TOF MS. Total spectral data (MALDI:141533, ESI: 286025) were analyzed with ProteinPilot 4.1 software using the UniProt Rat proteome database. In LC-MALDI-TOF/TOF analysis, 3,024 proteins were identified from 46,766 peptide sequences; in LC-ESI-MS/MS analysis, 3,582 proteins were identified from 68,790 peptide sequences with the criterion of unused protein score = 1.3 (95% confidence) (**Fig. 1B, Supplemental Fig. S2B**). The iTRAQ data is shown in **Supplemental Table S2** and **S3** and integrated into the iPEACH Table (**Supplemental Table S1**).

DNA array results

Total RNAs extracted from cells prepared in the same manner described for proteome analyses at each time points were subjected to GeneChip Rat Genome 230 2.0 Array analyses. After obtaining normalized signal data, 10,868 genes (21,349 probes with present call among 31,099 total probes) were used for differential expression analysis.

Integration of all data obtained from proteomics and transcriptomics

Data obtained from four time-course samples with or without NF1 knockdown using two differential proteome analyses and a transcriptomic analysis (123 proteins from 2D-DIGE, 3,239 proteins from 8-plex iTRAQ, and 10,868 genes from DNA array) were combined by iPEACH (**Fig. 1B, Supplemental Table S1**). The iPEACH list contains all molecular information, including molecular ID (Entrez gene ID, UniProt), GO, post-translational modifications, expression ratios, and analysis methods in an integrated chart (international patent PCT/JP2011/58366). The integrated total data from the original iPEACH in this study consisted of 16,421 lines of comprehensive molecular information. After obtaining this chart, the integrated data were reprocessed for GO analysis or knowledge-based network analysis by KeyMolnet, and the specific signal networks mediated by upregulated molecules in NF1-KD samples were extracted.

Data mining

To identify the specific molecules differentially up- or downregulated in NF1-KD cells, all data listed in iPEACH were rearranged based on the ratio of molecular expression (NF1-KD/control), and groups of up- or downregulated molecules were extracted. In total, 3,193 molecules were identified by both proteomics (2D-DIGE or iTRAQ) and transcriptomics (**Fig. 2A**). Upon NGF stimulation, proteins that were identified by iTRAQ as continuously (from 24–48 h, from 48–72 h, or from 24–72 h)

upregulated (62 proteins) or downregulated (35 proteins) in NF1-KD cells compared with control cells were selected as the differentially expressed core proteins (**Fig. 2B**). The expression patterns of these 97 proteins and mRNA sequences were subjected to hierarchical clustering analysis using uncentered correlation (**Fig. 2C**). In the heat map, we observed that a cluster of 24 h mRNA and 24-72 h protein expression patterns were closely related, suggesting that protein expression was affected by mRNA expression at 24 h.

GO analysis

GO analysis was performed by an analysis with "pathway based on molecule" (based on the GO criterion) in KeyMolnet. Upregulated molecules comprised 62 continuously upregulated proteins identified in iTRAQ, 32 upregulated proteins identified in 2D-DIGE, 185 genes selected based on fold-change analysis of DNA array data (24 h > 1.2 and 48 h > 1.5). Downregulated molecules comprised 35 proteins continuously downregulated in iTRAQ, 20 proteins downregulated in 2D-DIGE, and 186 genes selected based on fold-change analysis of DNA arrays (24 h < 0.83 and 48 h < 0.67). GO analysis identified "Calcium signaling" ($P = 1.380\times10^{-5}$), "Transcriptional regulation by GR" ($P = 4.963\times10^{-5}$), "Granzyme signaling pathway" ($P = 8.112\times10^{-5}$), "MMP (matrix metalloproteinase) signaling pathway" ($P = 1.118\times10^{-4}$), and "Intermediate filament signaling pathway" ($P = 1.789\times10^{-4}$) as upregulated in NF1-KD cells (**Table 1A**), and "Serotonin signaling pathway" ($P = 6.580\times10^{-6}$), "CaSR (calcium-sensing receptor) signaling pathway" ($P = 3.507\times10^{-5}$), "AMPAR (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) signaling pathway" ($P = 5.266\times10^{-5}$), "Calcium signaling pathway" ($P = 6.266\times10^{-5}$), and "GABA (gamma-aminobutyric acid) signaling pathway" ($P = 9.489\times10^{-5}$) as downregulated in NF1-KD cells (**Table 1B**). In addition, cell

communication, epidermis development, and apoptotic process were noted as significant biological events in the upregulated group, while those of the downregulated group were localization, transport, and response to wounding (data not shown). These gene ontologies identified in NF1-KD cells may relate to the tumorigenesis and abnormal neuronal differentiation in NF1 pathology.

Network analysis

To extract abnormal signaling patterns in NF1-KD PC12 cells, we conducted a precise network analysis focused on upregulated proteins. We performed the "start points and end points" network search algorithm using the start points of mRNA at 24 h > 1.2 and at 48 h > 1.5 and end points of upregulated proteins identified by iTRAQ and 2D-DIGE. **Figure 3A** shows the upregulated total network in NF1-KD cells illustrated by KeyMolnet. We focused on a highly clustered area consisting of upregulated proteins in this network (**Fig. 3A, red circle**), and particularly a specific network consisting of COX-1, which was continuously upregulated at all time points in iTRAQ analysis, dynein IC2, which was highly differentially expressed by 2D-DIGE (listed as a top molecule in the iPEACH index), and GR, which was detected as an upregulated gene in DNA array and a significant key molecule in the GO-based pathway analysis. We named this signaling pathway "dynein IC2-GR-COX-1" (**Fig. 3B**).

This *in silico* network analysis by KeyMolnet indicated that dynein is associated with GR via cargo proteins such as heat shock protein 90 and FK506-binding protein, and that the dynein complex can transport GR into the nucleus, where this GR signal may function as a transcriptional factor to upregulate COX-1 expression. This network was strongly suggested to be upregulated and related to the abnormal neuronal differentiation of NF1-KD cells.

Biological validation of a unique pathway of dynein IC2 regulation, COX-1 expression, and GR nuclear translocation in NF1-KD PC12 cells

Cytoplasmic dynein 1 intermediate chain 2 (dynein IC2) was the most significantly altered protein in 2D-DIGE analysis. Dynein IC2 was identified in 5 spots, and each spot was differentially expressed upon NF1 knockdown (**Fig. 4A**). Each spot was defined as spots 1, 2, 3, 4, and 5 from the lower pI on the 2D-DIGE, and the alteration of sequential expression levels in each spot is shown in **Fig. 4B**. Interestingly, spots 1–3 were continuously downregulated, while spots 4 and 5 were upregulated in NF1-KD cells. After NGF stimulation in control cells, the temporal expression of spots 1 and 2 increased, and that of spots 3 and 5 decreased. Spot 4 was also decreased in the control condition but increased in NF1-KD cells. Rat dynein IC2 is known to have two alternative splicing sites, and three isoforms were identified as IC2-A, IC2-B, and IC2-C in rat hippocampal neurons (12). The combined information of the observed molecular weight and pI in 2D-DIGE and the MS/MS analysis of spots 1–5 (**Supplemental Table S5, Supplemental Fig. S3B**) identified the detected spots as IC2-B or IC2-C.

To confirm each spot identity, we next designed specific dynein IC2-C siRNAs (termed 329 and 331) able to distinguish IC2-C from IC2-B. Knockdown of IC2-C by treatment with both 329 and 331 siRNAs significantly diminished both spots 4 and 5, which were thus concluded to be IC2-C (**Supplemental Fig. S3D, E**). These results further indicated that spots 1, 2, and 3 are IC-2B. The pIs of these dynein IC2 spots were shifted in 2D-PAGE, suggesting that dynein IC2 was post-translationally modified. We analyzed their phosphorylation patterns, which may reflect dynein IC2-related cellular signaling, using a phospho-specific protein stain, Pro-Q Diamond (**Supplemental Fig. S3A**). Dynein IC2 spots 1, 2, and 4 were responsive to the Pro-Q Diamond staining (yellow), indicating that these spots were phosphorylated. The phosphorylation of spots 2 and 4 was demonstrated on Ser 87/81/81 (dynein IC2-A/-B/-C) by MS/MS analyses (**Supplemental Table 5, Supplemental Fig. S3A and C**).

To observe the sequential alternation of dynein IC2 isoform expression patterns, we next analyzed