Typhoon □□00 variable mode imager (GE Healthcare) at 100 □m resolution using excitation/emission wavelengths of □88 nm/520 nm for Cy2, 532 nm/580 nm for Cy3, and 633 nm/670 nm for Cy5, respectively. The intensity was ad□usted to ensure that the maximum volume of each image was within 60,000□□0,00 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 □h, and then washed with destain solution (5□ 1 M sodium acetate, pH □0, in 20□ ACN) three times for 60 min. Gels were washed twice with deionized water for 5 min and scanned with a Typhoon□□00 variable mode imager (GE Healthcare). For S□PRO Ruby gel staining, gels were fixed in 10□ methanol containing 7.5□ acetic acid for 30 min, stained with S□PRO Ruby protein gel stain (Invitrogen) overnight, and washed with 10□ methanol containing 7.5□ acetic acid for 1 h and scanned with a Typhoon□□00 imager (GE Healthcare). Fluorescent images stained with Pro-Q Diamond and SyproRuby were overlaid using Multi Gauge software (Fu□ifilm).

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

To identify proteins detected in 2D-DIGE, 150 □g 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 □□00Imager (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 □l of 0.1□ (v/v) TFA in 2□ (v/v) ACN. These samples were desalted with a □ipTip C18 pipette tip (Millipore) and sub□ected to MALDI-MS/MS analysis using the □700 Proteomics analyzer (Applied □iosystems/A□ SCIE□) or LC-ESI-MS/MS analysis using the API QSTAR Pulsar i time-of-flight mass spectrometer (Applied □iosystems/A□ SCIE□) coupled with the UltiMate NanoLC system (Thermo Scientific Dionex).

iTRAQ sample preparation, fractionation, and desalting

Protein samples (100 □g) were precipitated using a 2-D Clean-Up kit (GE Healthcare), and the precipitates were dissolved in 10 □l 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, 11□ for 2□-h control, 115 for □8-h control, 116 for 72-h control, 117 for 0-h NF1 siRNA, 118 for 2□-h NF1 siRNA, 11□for □8-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 □ (10 mM potassium phosphate, pH 3.0, and 500 mM KCl in 20□ ACN) as follows□0□2 min, 0□7□ □□at 10 min, to 1□□ □□at 1□min, to 32□ □□at 1□ min, to 70□ □□and at 2□ min, to 100□ □. 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 □ipTip □-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 (K□A Tech Corp.) equipped with a device spotting eluted fractions onto a MALDI plate. Sample was in□ected onto a C18 column (0.5-mm inner diameter × 1-mm length, K□A 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□K□A Tech Corp.) at a flow rate of 300 nl/min with a 113-min gradient of solvent □ (70□ ACN and 0.1□ TFA) as follows□0□ 22□ □ from 0 to 10 min, to 3□□ □ at 53 min, to □5□ □ at 83 min, and to 100□ □ at □3 min. Column effluent was mixed with matrix (3 mg/ml alpha-cyano-□-hydroxycinnamic acid in 50□ ACN and 0.1□ TFA) at a flow rate of 1.□ □l/min. Fractions were spotted at 30-s intervals onto a stainless steel MALDI target plate (38□wells/plate□Applied □iosystems). Mass spectra of the peptides were acquired on an A□ SCIE□ TOF/TOF 5800 system (A□ SCIE□) using TOF/TOF Series Explorer software (□ersion □0.0). Mass spectra from m/z 800 to □,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 k□.

LC-ESI-MS/MS analysis

Samples were analyzed by nano-LC-ESI-MS/MS using the LC Packings Ultimate instrument fitted with a 20-□l sample loop. Samples were loaded onto a 5-mm RP C18 precolumn (Thermo Scientific Dionex) at 25 □l/min and washed for 10 min before switching the precolumn in line with the separation column, which was a 75-□m internal diameter × 150-mm length PepMap RP column (Thermo Scientific Dionex) packed with 3-□m C18 beads with 100-□ pores. The flow rate used for separation on the RP column was 200 nl/min with a 120-min gradient of solvent □ (85□ ACN and 0.1□ formic acid) as follows□0□3□ □ from 0□5 min, to 15□ □ at 10 min, to □0□ □ at □5 min, to 100□ □ at 100 min. Sample analysis was performed using a QSTAR Elite mass spectrometer (Applied □iosystems/A□ SCIE□), and Analyst QS 2.0 software (Applied □iosystems/A□ SCIE□) 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 2.1.0□(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 □□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 (□) of ProteinPilot □ersion □1 (A□ SCIE□), and the UniProt Rat proteome database (release2012□02, 37,10□ 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 □5□. 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 □ersion □.1 (A□ SCIE□). The data analysis parameters were set as follows□Sample type□iTRAQ (peptide labeled)□□ias Correction□Auto□□ackground Correction□□es.

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□M□, Keyword, GO□□iological□Process, GO□Molecular□Function,

GO□Cellular□Component, SwissProt□□CC□) and original data information (Post-Translational Modification, Cleavage sites, Theoretical□pI, Observed□M□, 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 pH□-7 gels). First, iPEACH integrates genes and proteins identified in the different experiments using Entrez ID. Then, iPEACH calls R/□ioconductor 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, 2□h, □8 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□$z = \Sigma\{ |log_2(x_0)\square\square |log_2 (x_{2\square})\square\square |log_2 (x_{\square 8})\square\square |log_2 (x_{72})\square\square$

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 CO□-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′-2□□CAAGGAGTGTCTGATCAACTT-3′ (for NF1 2□□siRNA) and 5′-611GGTTACAGGAGTTGACTGTTT-3′ (for NF1 611 siRNA), the rat dynein IC2-C sequences 5′-32□-GATCTAGACGAGGACCTATTT-3′ (for dynein IC2-C 32□siRNA) and 5′-331-TCTAGACGAGGACCTATTATT-3′ (for dynein IC2-C 331 siRNA), and the rat CO□-1 sequences 5′-1023-CCATCGAGATTATCATCGATT-3′ (for CO□-1 1023 siRNA) and 5′-2618-CAGGTGGACTCATCTACGATT-3′ (for CO□-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 P□DF membrane by electroblotting, and sub□ected to immunoblotting with the indicated antibody. Membranes were probed with different primary antibodies followed by horseradish peroxidase-con□ugated 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 □□00 imager (GE Healthcare). The following primary antibodies were used□NF1, dynein IC, TCPε1

(Santa Cruz □iotechnology), CO□-1, GR, Lamin □1 (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 □orkstation 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 □g) were mixed with rehydration solution (8 M urea, 2□ (w/v) CHAPS, 1.0□ (v/v) Destreak Reagent, 0.□□ (v/v) IPG buffer (pH □-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 □ for 15 min, ramped to 1,000 □ in 15 min, ramped to 3,000 □ in 15 min, ramped to 6,000 □ in 15 min, and held at 6,000 □ for 15 min. Strips were equilibrated in solution (□5□ (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 P□DF membrane by electroblotting and sub□ected to immunoblotting with the indicated antibody. After immunoblotting, the intensities were measured using ProGenesis □orkstation 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 (□ako) 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 □□ paraformaldehyde in P□S for 15 min at room temperature and then permeabilized with 0.1□ (w/v) Triton □-100 in P□S for 15 min. After washing with P□S, cells were incubated in primary antibodies diluted in P□S containing 0.2□ (w/v) □SA, followed by a secondary antibody con□gated with a fluorescent dye for 1 h at room temperature and observed with a fluorescence microscope (Olympus I□81 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 (2□□) siRNA and stimulated with NGF, and morphological changes were observed at the indicated time points (0 h, NGF-□2□h, □8 h, and 72 h□ NGF□). □e 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 sub□ected to integrated proteomics analyses as shown in **Fig. 1B.** PC12 cells were transfected with control siRNA or NF1 (2□□) siRNA and stimulated with NGF, and protein and mRNA samples were prepared from the cells at the indicated time points (0 h, NGF-□2□h, □8 h, and 72 h□NGF□). The protein and mRNA samples were sub□ected 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 (2□□) siRNA and stimulated with NGF for 0 h, 2□h, □8 h, or 72 h. Proteins were extracted from the cells at each time point (n □ 3), desalted, and sub□ected to 2D-DIGE. To obtain as much protein information as possible, each sample was separated into two fractions and sub□ected to IEF using both strip ranges of pH 3□11 and pH □□7. An average of

□,157□160 spots in pH 3□11 gels and an average of □,007□176 spots in pH □□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, 1□5 spots (pH 3□11) and 187 spots (pH □□7) were found to be significant differentially expressed, and 123 non-redundant proteins (pH 3□11□128, pH □□7□ 12□ 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 □□ fractions with cation exchange column chromatography, and analyzed by nanoLC-MALD-TOF/TOF MS and nanoESI-Qq-TOF MS. Total spectral data (MALDI□□□1533, ESI□ 286025) were analyzed with ProteinPilot □1 software using the UniProt Rat proteome database. In LC-MALDI-TOF/TOF analysis, 3,02□ proteins were identified from □6,766 peptide sequences□ in LC-ESI-MS/MS analysis, 3,582 proteins were identified from 68,7□0 peptide sequences with the criterion of unused protein score □ 1.3 (□5□ 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 sub□ected to GeneChip Rat Genome 230 2.0 Array analyses. After obtaining normalized signal data, 10,868 genes (21,3□□probes with present call among 31,0□□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, 3,23□ 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,□21lines 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, 3,1□3 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 2□□□8h, from □8□72 h, or from 2□□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 □7 proteins and mRNA sequences were sub□ected to hierarchical clustering analysis using uncentered correlation (**Fig. 2C**). In the heat map, we observed that a cluster of 2□ h mRNA and 2□-72 h protein expression patterns were closely related, suggesting that protein expression was affected by mRNA expression at 2□ 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 (2□ h □ 1.2 and □8 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 (2□ h □ 0.83 and □8 h □ 0.67). GO analysis identified "Calcium signaling" (P □ 1.380×10^{-5}), "Transcriptional regulation by GR" (P □ □.□63$10^{-5}$), "Granzyme signaling pathway" (P □ 8.112×10^{-5}), "MMP (matrix metalloproteinase) signaling pathway" (P □ $1.118\times10^{-□}$), and "Intermediate filament signaling pathway" (P □ 1.78□$\times10^{-□}$) as upregulated in NF1-KD cells (**Table 1A**), and "Serotonin signaling pathway" (P □ 6.580×10^{-6}), "CaSR (calcium-sensing receptor) signaling pathway" (P □ 3.507×10^{-5}), "AMPAR (alpha-amino-3-hydroxy-5-methyl-□-isoxazolepropionic acid receptor) signaling pathway" (P □ 5.266×10^{-5}), "Calcium signaling pathway" (P □ 6.266×10^{-5}), and "GA□A (gamma-aminobutyric acid) signaling pathway" (P □ □.□8□$\times10^{-5}$) 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. □e performed the "start points and end points" network search algorithm using the start points of mRNA at 2□h □1.2 and at □8h □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. □e focused on a highly clustered area consisting of upregulated proteins in this network (**Fig. 3A, red circle**), and particularly a specific network consisting of CO□-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. □e named this signaling pathway "dynein IC2-GR-CO□-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 □0 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 CO□-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, □, 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 □ 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 □ 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-□, 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-□ or IC2-C.

To confirm each spot identity, we next designed specific dynein IC2-C siRNAs (termed 32□ and 331) able to distinguish IC2-C from IC2-□. Knockdown of IC2-C by treatment with both 32□ and 331 siRNAs significantly diminished both spots □ 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-2□. The pIs of these dynein IC2 spots were shifted in 2D-PAGE, suggesting that dynein IC2 was post-translationally modified. □e 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 □ were responsive to the Pro-Q Diamond staining (yellow), indicating that these spots were phosphorylated. The phosphorylation of spots 2 and □ was demonstrated on Ser 87/81/81 (dynein IC2-A/-□/-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

them by 2D-immunoblotting using a specific dynein IC antibody. Cell lysates were prepared identically to the method used for 2D-DIGE analyses with NF1 siRNA-treated or control siRNA-treated PC12 cells and sub□cted to 2D-immunoblotting analysis. Six spots were identified by the anti-dynein IC antibody (**Fig. 4C, left upper and lower panel**), and the ratios of the intensity of each spot relative to total spot intensity were calculated (**Fig. 4C, right panel**). Similar results to those of 2D-DIGE, except for an additional spot 1′, were obtained. Spots 1′, 1, 2, and 3 were downregulated, and spots □ and 5 were upregulated in NF1-KD cells compared with control cells. In response to NGF stimulation, phospho-staining-positive spots 1′, 1, and 2 were increased, and phospho-staining-negative spots 3, □, and 5 were decreased in control siRNA-treated cells (**Fig. 4C, right panel blue column**), while, in NF1 siRNA-treated cells, spots 3, □, and 5 were increased and spots 1′, 1, and 2 were decreased. Notably, the expression ratio of spot □ (phosphorylated IC-2C) was increased in NF1-KD PC12 cells. These results suggest that suppression of NF1 causes dynamic changes of the regulation pattern of dynein IC2 alternative splicing and phosphorylation in NGF-treated PC12 cells, which may affect the abnormal neuronal differentiation related to the NF1 disease phenotype.

COX-1 and GR protein upregulation in NF1-KD cells

Next, we analyzed the functional regulation of CO□-1, which was detected as a significantly upregulated protein in NF1-KD cells by iTRAQ analysis. To observe the expression pattern of CO□-1, PC12 cells were transfected with control siRNA or NF1 (2□□) siRNA and stimulated with NGF. Proteins were harvested from the cells at each time point (n □ 3) and sub□cted to immunoblotting using a CO□-1-specific antibody. Compared with control siRNA-treated cells, CO□-1 expression was upregulated up to two-fold in NF1-KD cells, and after 72 h of NGF stimulation, it reached three times that of non-stimulated NF1-KD cells (**Fig. 4D, E,** n □ 3). These results are consistent with the result of

quantitative analysis by iTRAQ. In the network extracted by KeyMolnet, GR was assumed as an expression regulator of CO□-1, and its mRNA expression was upregulated two-fold in NF1-KD cells compared with that of control cells after □8h NGF stimulation, suggesting that GR activation causes the elevation of CO□-1 expression.

Thus, we next analyzed the GR activation pattern in NF1-KD cells. Usually, GR exists in cytoplasm in an inactive state, and upon activation, it is transported to the nucleus (13, 1□). □e prepared both cytoplasmic and nuclear protein fractions from NGF-stimulated NF1-KD or control cells and sub□ected these fractions to immunoblot analysis using an anti-GR antibody. No difference in GR expression between NF1-KD cells and control cells was observed in the cytoplasmic fraction, whereas in the nuclear fraction, GR expression was significantly higher in NF1-KD cells compared with control cells (**Fig. 4F**), suggesting that elevated nuclear translocation of GR caused the increase of transcriptional activity.

These results demonstrate that suppression of NF1 causes the downregulation of dynein IC2-□ expression and its phosphorylation, upregulation of alternative splicing and phosphorylation of dynein IC2-C, and also upregulates the nuclear translocation and activation of GR followed by upregulation of CO□-1, supporting the involvement of dynein IC2-GR-CO□-1 network activation in NF1-KD cells.

Regulation of dynein IC2 splicing and phosphorylation by NF1 is related to NGF-induced neurite outgrowth in PC12 cells.

This study demonstrated that the upregulation of dynein IC2-C expression and phosphorylation is a significant event in NF1-KD cells. To investigate the function of dynein IC2-C in NF1-KD cells, the effects of the dynein IC2-C siRNA knockdown on cellular phenotype were analyzed. PC12 cells were co-transfected with NF1 and dynein IC2-C siRNAs for 2□h. Then, after □8-h NGF treatment, dynein

expression in cells was analyzed by 2D-immunoblotting using anti-dynein IC antibody, confirming that expression of spots □and 5 (corresponding to dynein IC2-C) were reproducibly suppressed (**Fig. 5A**). □e observed PC12 phenotypes using time-lapse microscopy and found that knockdown of cytoplasmic dynein IC2-C effectively recovered the inhibition of neurite outgrowth observed in NF1-KD PC12 cells (**Fig. 5B**). □e measured the total length of neurites in siRNA-treated cells and found that neurite extension in co-transfected cells significantly recovered to the same level as that of control siRNA-treated cells (**Fig. 5C**). The number of neurites did not differ between NF1 siRNA-treated cells and both NF1 and dynein IC2-C siRNA-treated cells (data not shown). These results strongly suggest that dynein IC2-C is an important effector for the inhibition of neurite outgrowth in NF1-KD PC12 cells.

To study the alternative splicing and phosphorylation patterns of dynein IC2 after the knockdown both of NF1 and dynein IC2-C, we analyzed changes in the intensity of 6 dynein IC2 spots with 2D-immunoblotting. □e confirmed that inhibition of dynein IC2-C decreased the intensities of spots □ and 5 (corresponding to dynein IC2-C), and this inhibition was subsequently related to the increase in dynein IC2-□ spots. In particular, spots 1′ and 1 (representing the phosphorylated form of dynein IC2-□) were increased with concomitant decrease of spot 3 (non-phosphorylated dynein IC2-□) and spot 2 compared with those of NF1-KD cells (**Fig. 5D**). These findings suggest that phosphorylation of dynein IC2-□ is promoted by suppression of dynein IC2-C in NF1-KD cells and is an important process underlying neurite extension in PC12 cells. This observation was also validated by the use of pan-dynein IC siRNA, which inhibits both dynein IC2-□ and -C. Since neurite retraction by NF1 siRNA was not recovered by pan-dynein siRNA (**Supplemental Fig. S4A**), increased expression and phosphorylation of dynein IC2-□ may be important for the recovery of neurite outgrowth.

Validation of the dynein IC2-GR-COX-1 network

Having demonstrated the potential for GR upregulation in NF1-KD cells to directly affect CO□-1 upregulation (**Fig. 3B** and **4F**), we next analyzed the effect of treatment with a GR antagonist, mifepristone, on CO□1 expression to investigate the interaction between GR and CO□-1 in more detail. After 2□-h NF1 or control siRNA transfection, PC12 cells were treated with 10 □M mifepristone for 30 min before treatment with NGF. After □8-h NGF treatment, cells were harvested for measurement of CO□-1 expression by immunoblotting analysis. Upregulation of CO□-1 by NF1 siRNA was significantly decreased by mifepristone treatment to the basal level of control siRNA-treated cells (**Fig. 6 A, B**), suggesting that the expression of CO□-1 is regulated by GR activity in NF1-KD cells. As shown in **Fig. 4F**, in NF1-KD cells, GR was transported to the nucleus from cytoplasm, and this transportation could be supported by other binding proteins such as chaperones and motor proteins to form a cargo complex (15-17). Notably, dynein intermediate chain is known to play an important role in this transportation as a cargo protein (18). Thus, we speculated that dynein IC2 may be associated with this transportation of GR to induce CO□-1 expression in NF1-KD cells.

To validate this hypothesis, we analyzed the effect of dynein IC2 knockdown on the activation of GR and CO□-1 expression in NF1-KD cells. First, to examine whether dynein IC2 is involved in the increase of GR nuclear translocation triggered by NF1 knockdown, PC12 cells were co-transfected with dynein IC2 siRNA and NF1 siRNA and fractionated into cytoplasmic and nuclear proteins, which were sub□ected to immunoblot analysis using anti-GR antibody. Nuclear translocation of GR by NF1 knockdown was greatly decreased by co-transfection of both NF1 and dynein IC2 siRNAs (**Fig. 6C**).

Next, we analyzed the effects of double-knockdown of NF1 and dynein IC2 on CO□-1 expression in NGF-stimulated PC12 cells. Upregulation of CO□-1 by NF1 knockdown was significantly decreased by co-transfection with NF1 and dynein IC2 siRNAs to the level of control siRNA-treated cells (**Fig. 6**

D, E), while no effects on CO□-1 expression in cells co-transfected with NF1 siRNA and dynein IC2-C siRNA were observed (**Supplemental Fig. S4C**). From these experimental results, we speculate that dynein IC2-□ function or the mutual interaction of both dynein IC2-□ and -C may play an important role in the transportation of GR that affects the expression of CO□-1.

Finally, we analyzed the effect of CO□-1 knockdown on the cellular phenotypes of NF1-KD cells. CO□-1 upregulation in NF1-KD cells was suppressed by the co-transfection of CO□-1 siRNA (**Fig. 6F**), and, interestingly, the neurite retraction and spindle-like alteration observed in NF1-KD cells were recovered by treatment with CO□-1 siRNA (**Fig. 6G**). □e calculated the total length of neurites in the double-knockdown PC12 cells and observed significant extension up to 1.8-times longer than those of NF1-KD cells (**Fig. 6H,** n □ 3). The number of neurites demonstrated no difference between NF1 siRNA-treated cells and NF1 siRNA- and CO□-1 siRNA-treated cells (data not shown). These results suggest that the inhibition of CO□-1 upregulation in NF1-KD cells recovers the cellular phenotypes that are abnormally altered in NF1-KD PC12 cells.

Discussion

In this study, we clarified global alterations of the molecular network in NF1-disease model PC12 cells, which demonstrate abnormal phenotypes caused by NF1 knockdown. Using an integrated proteomics approach followed by network analysis *in silico*, we extracted key molecules that were validated after sequential cellular functional analysis using siRNA and found that a novel dynein IC2-GR-CO□-1 signaling pathway is specifically activated in NF1-KD cells and related to the abnormal neuronal phenotype observed in the NF1 disease model.