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 subjected 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 4 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, 4, and 5 were decreased in control siRNA-treated cells (**Fig. 4C, right panel blue column**), while, in NF1 siRNA-treated cells, spots 3, 4, and 5 were increased and spots 1′, 1, and 2 were decreased. Notably, the expression ratio of spot 4 (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 COX-1, which was detected as a significantly upregulated protein in NF1-KD cells by iTRAQ analysis. To observe the expression pattern of COX-1, PC12 cells were transfected with control siRNA or NF1 (249) siRNA and stimulated with NGF. Proteins were harvested from the cells at each time point (n = 3) and subjected to immunoblotting using a COX-1-specific antibody. Compared with control siRNA-treated cells, COX-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 COX-1, and its mRNA expression was upregulated two-fold in NF1-KD cells compared with that of control cells after 48-h NGF stimulation, suggesting that GR activation causes the elevation of COX-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, 14). We prepared both cytoplasmic and nuclear protein fractions from NGF-stimulated NF1-KD or control cells and subjected 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-B 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 COX-1, supporting the involvement of dynein IC2-GR-COX-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 24 h. Then, after 48-h NGF treatment, dynein

expression in cells was analyzed by 2D-immunoblotting using anti-dynein IC antibody, confirming that expression of spots 4 and 5 (corresponding to dynein IC2-C) were reproducibly suppressed (**Fig. 5A**). We 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**). We 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. We confirmed that inhibition of dynein IC2-C decreased the intensities of spots 4 and 5 (corresponding to dynein IC2-C), and this inhibition was subsequently related to the increase in dynein IC2-B spots. In particular, spots 1′ and 1 (representing the phosphorylated form of dynein IC2-B) were increased with concomitant decrease of spot 3 (non-phosphorylated dynein IC2-B) and spot 2 compared with those of NF1-KD cells (**Fig. 5D**). These findings suggest that phosphorylation of dynein IC2-B 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-B 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-B 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 COX-1 upregulation (**Fig. 3B** and **4F**), we next analyzed the effect of treatment with a GR antagonist, mifepristone, on COX1 expression to investigate the interaction between GR and COX-1 in more detail. After 24-h NF1 or control siRNA transfection, PC12 cells were treated with 10 μM mifepristone for 30 min before treatment with NGF. After 48-h NGF treatment, cells were harvested for measurement of COX-1 expression by immunoblotting analysis. Upregulation of COX-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 COX-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 COX-1 expression in NF1-KD cells.

To validate this hypothesis, we analyzed the effect of dynein IC2 knockdown on the activation of GR and COX-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 subjected 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 COX-1 expression in NGF-stimulated PC12 cells. Upregulation of COX-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 COX-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-B function or the mutual interaction of both dynein IC2-B and -C may play an important role in the transportation of GR that affects the expression of COX-1.

Finally, we analyzed the effect of COX-1 knockdown on the cellular phenotypes of NF1-KD cells. COX-1 upregulation in NF1-KD cells was suppressed by the co-transfection of COX-1 siRNA (**Fig. 6F**), and, interestingly, the neurite retraction and spindle-like alteration observed in NF1-KD cells were recovered by treatment with COX-1 siRNA (**Fig. 6G**). We 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 COX-1 siRNA-treated cells (data not shown). These results suggest that the inhibition of COX-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-COX-1 signaling pathway is specifically activated in NF1-KD cells and related to the abnormal neuronal phenotype observed in the NF1 disease model.

In our previous study, molecules related to NGF-inducible neurite outgrowth in PC12 cells were analyzed using software called MANGO (8), which was reported as a novel, unique, and useful strategy in the global study of neural cells (8) (ASBMB Today p31 August 2009). We identified 39 upregulated-proteins and 33 downregulated-proteins in 48 h of NGF-treated PC12 cells in that study, and unique anti-apoptotic proteins were extracted as NGF induced proteins in the PC12 cells. Among those differentiated proteins, 21 (13 up-, 8 downregulated) proteins and 12 (7 up-, 5 downregulated) proteins were identified as up/down regulated in NF1-KD cells during NGF stimulation (0, 24, 48, and/or 72 h) in this proteomics study, respectively. Interestingly, among upregulated proteins induced by the NGF stimulation in the normal PC12 cells, the specific downregulated proteins in NF1-KD cells were found to be related to the neuronal differentiation and the stress responses. On the other hand, the upregulated proteins in NF1-KD cells were related to cell morphology, motility and cell survival. It was suggested that these specific proteins found commonly in the previous study and the present study regulate cellular differentiation, motility, and apoptosis. Here, in addition to MANGO, we performed a unique comprehensive assessment using integrated proteomics with iPEACH software, a sophisticated new strategy that combines the technology of proteomic techniques, such as 2D-DIGE and iTRAQ, and transcriptomic methods, such as DNA array, and tried to identify more specific signaling in NF1-KD cells. This study is the first to report the global molecular alteration in NF1 disease model PC12 cells, and is the largest study to comprehensively identify both proteins and mRNAs in neuronal disease model cells.

Neurofibromin, the gene product of NF1, has a region homologous to the Ras-GAP domain, a negative regular of Ras, whose precise cellular signals related to NF1 disease phenotypes have yet to be clarified. We previously reported NF1-KD PC12 cells as useful NF1 disease model cells (7) to elucidate

the function of clinical therapies and drug development for NF1 patients. In that study, we identified CRMP-2, which was also identified as a differentially expressed protein in this study (in the 2D-DIGE result; pH-4-7, spot no. 2389, **Supplemental Table S4**), as a functionally related protein to the neural differentiation in NF1 model cells, and demonstrated that several inhibitors for the CRMP-2 phosphorylation responsible kinases could be useful for improving NF1 disease phenotypes (7). Using this model system, we performed integrated proteomics, which enabled us to obtain global differential information on proteins and mRNAs. The advantage of this integrated proteomics approach is that a global alteration of molecules, linked with information concerning both protein and mRNA expression levels as well as post-translational modification, can be obtained using the same sets of sample sources, such as model cells compared with normal cells. In this study, using this strategy, we precisely analyzed the molecules with respect to expression levels, alternative splicing, and modification as they continuously changed during the process of neurite outgrowth mediated by NGF treatment, and observed the specific upregulation of dynein IC2-GR-COX-1 signaling in NF1-KD PC12 cells.

This signal consisted of dynein IC2 uniquely found in 2D-DIGE, GR detected by DNA array, and COX-1 identified by iTRAQ analysis; therefore, this finding could be the first successful example of novel identifications after the integration and extraction of all information obtained from those three methods. Based on *in silico* analysis of protein-protein interaction, we speculate that the dynein complex transports GR to the nucleus, where it acts as a transcriptional regulator of COX-1. Dynein is a motor protein that forms a cargo complex to transport various proteins and organelles to appropriate cellular positions (19, 20), and, especially in neuronal cells, the complex associates with kinesin to transport and import neurotransmitters (21). The dynein subunit is constituted of heavy and light chains, as well as intermediate chains, which associate with cargo proteins (22) and mediate their cellular transport

function (18). Three splicing isoforms of dynein IC2, dynein IC2-A, -B, and -C, have been reported (12), with dynein IC2-B and -C predominantly expressed in PC12 cells (23).

Our study clearly demonstrated that NF1 knockdown caused the upregulation of the alternative splicing of dynein IC2-C isoform even before and after NGF stimulation as shown in **Fig. 4C**. Decreased dynein IC2-C isoform and increased IC2-B isoform were observed in NGF stimulated PC12 cells (as suggested in the previous report (23)), however, in NF1-KD cells, dynein IC2-C isoform was increased and further upregulates its phosphorylation in response to NGF stimulation. Simultaneously, the phosphorylation of dynein IC2-B splice form was suppressed in the NF1-KD cells, suggesting that neurofibromin deficiency caused the upregulation of alternative splicing/phosphorylation of dynein IC2-C, and downregulation of that of IC2-B. We further confirmed that dynein IC2-C knockdown using siRNA significantly recovered the inhibition of neurite outgrowth in NF1-KD PC12 cells as well as the phosphorylation of dynein IC2-B. This abnormal regulation of splicing and phosphorylation of dynein IC2 found in NF1-KD cells, may be caused by the functionally associated proteins of neurofibromin and/or other factors altered in NF1-KD cells which are under our investigation.

The enzyme responsible for phosphorylation in this system has not been identified; however, polo-like kinase (24) and casein kinase (25, 26) have been reported to phosphorylate dynein IC *in vitro* and in *Xenopus melanophores* (26), and also activations of MAPK, CDK5, GSK3, and RhoK in NF1-KD PC12 cells were found in our previous study (7). Thus, these enzymes may be involved in the regulation of neurite outgrowth in NF1-KD cells.

GR upregulation in NF1-KD cells was detected by DNA array and extracted by the network analysis as an important factor related to the NF1-KD phenotypes. GR belongs to the nucleus receptor superfamily, members of which are normally activated in the nucleus after transportation from

cytoplasm, and functions as a regulatory factor in physiological and metabolic processes (13, 14). GR upregulation was observed during involution of hippocampus and related to depression (27); thus, GR may be a factor related to neuronal cell abnormalities. In our study, NF1 knockdown mediated the upregulation of GR protein nuclear translocation/accumulation. An interacting protein with GR in the nucleus, 14-3-3 eta, which is activated by hormonal stimulation (28), was also identified as an upregulated molecule in NF1-KD cells using both transcriptomics and proteomics. Binding of 14-3-3 eta inhibits the ubiquitination and degradation of GR protein (28); therefore, upregulation of 14-3-3 eta mediated by NF1 knockdown can be speculated to contribute to GR accumulation and activation as a transcription factor, in addition to the association of the dynein complex transporting GR into the nucleus.

COX-1, a prostaglandin synthase that synthesizes prostaglandin G/H from arachidonic acid (29, 30), was one of the most highly upregulated proteins identified among the downstream targets of GR (31) using our integrated proteomics approach. The COX family has two isoforms, COX-1 and COX-2, which are independently regulated in different manners. COX-2 expression is mediated by inflammatory response, while COX-1 is expressed constitutively (32). Upregulation of COX-2 expression has been reported in cancer cells including malignant neural cancer and malignant peripheral nerve sheath tumor, and in response to inflammation and cell proliferation, it upregulates prostaglandin synthases (33-35). Thus, many studies on COX-2 as a target of drug development have been conducted (36, 37). However, limited information concerning the function of COX-1, such as for the mucosal protection of the stomach and blood flow maintenance, has been available (38). Recently, COX-1, but not COX-2, was reported to show involvement in brain inflammation (39, 40), and, importantly, COX-1 expression is regulated in PC12 cells in response to NGF stimulation (41, 42), suggesting that COX-1 is associated

with neuronal differentiation. In our study, downregulation of COX-1 using COX-1 siRNA rescued the abnormal phenotypes observed in NF1-KD cells; thus, COX-1 may be a candidate clinical target for NF1-related disease pathogenesis. The precise mechanism of COX-1 expression and function in neuronal differentiation related to NF1 pathogenesis remains to be clarified in future investigations.

In this study, we found that COX-1 expression was regulated by GR, and dynein IC2-GR-COX-1 signaling was upregulated in NF1-KD cells. In NF1-KD cells, suppression of dynein IC2 inhibits nuclear translocation of GR and decreases COX-1 expression. However, which dynein isoform is associated with GR transport and what mechanisms of dynein phosphorylation are involved remain unclear. Since no change in COX-1 expression was induced by dynein IC2-C siRNA treatment, dynein IC2-B rather than dynein IC2-C may be associated with GR translocation. Interestingly, COX-1 siRNA effectively recovered the neurite retraction of NF1-KD cells. We speculate that prostaglandins upregulated by COX-1 may be associated with neurite outgrowth. Four prostaglandin E2 (PGE2) receptors, EP1, EP2, EP3, and EP4, have been identified (43), and EP2 and EP3 expression was upregulated at 48 h and 72 h, respectively, in our DNA array analysis. EP3 has been reported to couple with G protein receptors to activate RhoA (44, 45), which was also identified as upregulated in NF1-KD HeLa cells in our previous report (10). Several studies show that activation of RhoA mediates neurite retraction in PC12 cells (44, 45). Therefore, upregulation of COX-1 in NF1-KD cells is speculated to promote PGE2 synthesis and the activation of RhoA via EP3, followed by the inhibition of neurite outgrowth in PC12 cells. COX-1 knockdown may also block this signaling activation and improve the abnormal phenotype observed in NF1-KD cells.

In NF1 patients, a cognitive deficit has frequently been found as one of the typical NF1-related phenotypes and suggested that neurofibromin plays an essential role in neuronal cells. NF1 heterozygous

(Nf1+/-) mice indicated spatial learning disability by the Morris water maze test (3, 46) and other reports also showed retraction of neurite and growth corn in NF1 heterozygous (Nf1+/-) hippocampal neurons (47). Therefore abnormal neuronal assembly such as neurite retraction is related to learning disability in NF1 patients. In our study, we used PC12 cells as model cells of neuronal differentiation to evaluate the function of neurofibromin. We firstly demonstrated that neurite retraction observed in NF1-KD cells is recovered by co-transfected dynein IC2-C siRNA (**Fig 5B, C**) or COX-1 siRNA (**Fig. 6G, H**). These experimental evidences may explain the possible functional relevance of the abnormal regulation of dynein IC2-GR-COX1 signaling to the NF1 pathology. Further studies based on our findings will elucidate the mechanism of NF1-related neuronal pathogenesis, such as learning disability.

This study is the first demonstration using an integrated proteomics approach that the specific signaling of dynein IC2-GR-COX-1 extracted from NF1-disease model neuronal cells may be an important mechanism of NF1-related pathogenesis. Drugs inhibiting this signaling such as specific COX-1 inhibitors may be useful candidates for the treatment of NF1 patients with neuronal abnormalities.

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Figure legends

Fig. 1 Workflow of the integrated proteomics approach to identify the abnormal network in NF1-KD PC12 cells. A, Representative images of NGF-stimulated PC12 cells treated with NF1 siRNA. PC12 cells were transfected with NF1 siRNA (lower panel) or control siRNA (upper panel), treated with NGF, and observed at the indicated time points. **B,** Workflow of the integrated proteomics approach. 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 Rat 230 2.0 gene chip analysis (Affymetrix), respectively. After these analyses, an integrated chart from all data was generated using iPEACH to identify differentially

expressed genes and proteins. Further biological and functional interpretation of the differentially expressed genes and proteins was carried out by GO and network analyses followed by cell biological analyses.

Fig. 2 Cluster analysis and pathway-based characterization of differentially expressed genes and proteins from integrated proteomics. A, Venn diagrams of the number of total mRNAs/proteins identified by 2D-DIGE, iTRAQ, and DNA array. **B,** Venn diagrams of the number of up/downregulated proteins identified by iTRAQ. In total, 3,239 proteins were identified with a confidence limit of 95% by MALDI and ESI. The fold change of each protein expression ratio (NF1 siRNA/control siRNA) was calculated by average iTRAQ ratio in MALDI and ESI analyses at each time point. Proteins quantified with a fold change more than 20% (average iTRAQ ratio >1.20 or <0.83) were identified as differentially expressed proteins. For cluster analysis, continuously up/downregulated proteins were selected by Venn diagrams (yellow area, 62 upregulated proteins; 35 downregulated proteins). **C,** Hierarchical clustering of the 97 continuously up/downregulated proteins. Each vertical column represents a sample map, whereas each horizontal row represents an individual protein, with relative expression values displayed as an expression matrix (heat map) using a standardized log abundance scale ranging from –0.5 (blue) to +0.5 (red). Blue and red colors indicate decreased and increased expression, respectively: the brighter the color, the stronger the change.

Fig. 3 Molecular network analysis of upregulated genes and proteins in NF1-KD PC12 cells.

A, The list of genes/proteins upregulated by the NF1 siRNA treatment in PC12 cells (Supplemental Table S7) was imported into KeyMolnet. Using the "start points and end points" network search

algorithm, KeyMolnet illustrated a highly complex network of targets with the most statistically significant relationships. Orange nodes represent upregulated genes, pink nodes represent upregulated proteins, and red nodes represent upregulated genes and proteins. White nodes indicate additional nodes extracted automatically from the core contents of KeyMolnet to establish molecular connections. **B,** Extraction of the abnormal molecular network including dynein IC2, GR, and COX-1 from the molecular network shown in A. Color marks represent numerical data for each method as follows: color mark 1: 24 h mRNA, 2: 24 h iTRAQ, 3: 48 h iTRAQ, 4: 72 h iTRAQ, 5: 2D-DIGE. Color set point is described in right panel of (B). The cluster of upregulated proteins including dynein IC2, GR, and COX-1 is highlighted by a red circle. The molecular relationships are indicated by a solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dashed line with arrow (transcriptional activation), and dashed line with arrow and stop (transcriptional repression).

Fig. 4 Validation of dynein IC2, GR, and COX-1 from the results of integrated proteomics.

A and B, Cytoplasmic dynein 1 intermediate chain 2 (dynein IC2) in 2D-DIGE. Cellular proteins prepared from PC12 cells transfected with NF1 (249) siRNA and control siRNA were labeled with Cy3 (red) and Cy5 (green), respectively. **A,** Representative images of the dynein IC2 spot pattern in 2D-DIGE analysis. Dynein IC2 was identified as differentially expressed protein spots 1–5 between control and NF1 siRNA-treated PC12 cells. Arrows indicate the dynein IC2 spots (right panel). **B,** Time-course expression analysis of dynein IC2 by DeCyder 2D software (2-way ANOVA analysis). Suppression of NF1 expression by siRNA leads to alteration of the 2D-DIGE pattern in PC12 cells. The average normalized intensities of spots 1, 2, 3, 4, and 5 (n = 3) are shown in the graphs. Blue circle:

control siRNA, red circle: NF1 siRNA, x-axis: period of NGF stimulation, y-axis: normalized spot intensity. **C,** Identification and quantification of dynein IC2 protein spots by 2D-immunoblot analysis. Left panel shows the representative dynein IC2 protein spot pattern in 2D-immunoblotting with anti-dynein IC antibodies (lower: NF1 siRNA-treated cells, upper: control siRNA-treated cells, after 48-h NGF treatment). Protein spots corresponding to spots 1′, 1, 2, 3, 4, and 5. PC12 cells were transfected with NF1 (249) siRNA or control siRNA, stimulated with NGF, and harvested after 24 h, 48 h, or 72 h. Each cell lysate sample was subjected to 2D-immunoblot analysis (7 × 5 cm) using anti-dynein IC antibodies. Percentage ratios of the intensity of each spot to total spot intensity are indicated in each histogram. The data were obtained from three separate identical experiments; error bars represent S.E. **D and E,** Immunoblot images and quantification of COX-1 in PC12 cells transfected with NF1 siRNA or control siRNA. D, Representative image of COX-1 immunoblot analysis. The expression of COX-1 was analyzed by immunoblotting using anti-COX-1 antibody. E, The intensity of the COX-1 band was quantified with ImageQuant software and normalized to that of actin to calculate the ratio. Data were obtained from three separate identical experiments. Error bars represent S.E. **F,** Analysis of GR nuclear localization by immunoblotting using anti-GR antibody. PC12 cells were transfected with NF1 (249) siRNA or control siRNA, stimulated with NGF, and harvested after 48-h NGF treatment. The cytoplasmic and nuclear proteins enriched in the cells were subjected to immunoblot analysis using anti-GR antibody. Lamin B1 was used as a marker of the nuclear fraction. Representative images of three reproducible experiments are shown.

Fig. 5 siRNA knockdown of dynein IC2-C recovered the inhibition of neurite outgrowth in NF1-KD PC12 cells. PC12 cells were transfected for 24 h with control siRNA, NF1 (249) siRNA, both

NF1 (249) and dynein IC2-C (329) siRNAs, or both NF1 (249) and dynein IC2-C (331) siRNAs before treatment with NGF. After 48-h NGF treatment, dynein expression in the cells was analyzed by 2-D immunoblotting using anti-dynein IC antibody, and the neurite length of the cells was measured. **A,** Representative spot patterns of dynein IC2 in 2-D immunoblotting of PC12 cells treated with control siRNA [**a**], NF1 (249) siRNA [**b**], NF1 (249) siRNA and dynein IC2-C (329) siRNA [**c**], and NF1 (249) siRNA and dynein IC2-C (331) siRNA [**d**]. **B,** Differential interference contrast images of PC12 cells treated with control siRNA [**a**], NF1 (249) siRNA [**b**], NF1 (249) siRNA and dynein IC2-C (329) siRNA [**c**], and NF1 (249) siRNA and dynein IC2-C (331) siRNA [**d**]. **C,** Measurement of neurite length of PC12 cells treated with the siRNAs. The average of the total length of PC12 neurites are shown on the y-axis. Data are expressed as means and S.E. of three independent experiments. For each experiment, more than 50 cells were counted. **D,** Comparison of the intensity of each dynein IC2 spot after transfection with NF1 siRNA and dynein IC2-C (329) siRNA. The histogram shows the percentage ratio of the intensity of each spot to the total spot intensity of dynein IC2 in individual samples. The intensity of each sample was obtained by 2D-immunoblotting using anti-dynein IC antibody followed by Cy5-labeled secondary antibody. The data were obtained from the average values of three separate identical experiments. Error bars represent S.E. of each set of three experiments.

Fig. 6 Biological validation of the abnormal dynein IC2-GR-COX1 network in NF1-KD PC12 cells identified by integrated proteomics.

A and B, Upregulation of COX-1 is suppressed by GR antagonist mifepristone. After 24-h NF1 or control siRNA transfection, PC12 cells were treated with 10 μM mifepristone for 30 min before treatment with NGF. After 48-h NGF treatment, cells were harvested for measurements of COX1 by