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) (AS□M□ Today p31 August 200□). □e identified 3□ upregulated-proteins and 33 downregulated-proteins in □8 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, 2□, □8, 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. □e 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-□-7, spot no. 238□ **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-CO□-1 signaling in NF1-KD PC12 cells.

This signal consisted of dynein IC2 uniquely found in 2D-DIGE, GR detected by DNA array, and CO□-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. □ased 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 CO□-1. Dynein is a motor protein that forms a cargo complex to transport various proteins and organelles to appropriate cellular positions (1□, 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, -□, and -C, have been reported (12), with dynein IC2-□ 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-□ 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-□ 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-□. □e 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-□. 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 (2□) 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, 1□). 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, 1□-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. □inding of 1□-3-3 eta inhibits the ubiquitination and degradation of GR protein (28)□therefore, upregulation of 1□-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.

CO□-1, a prostaglandin synthase that synthesizes prostaglandin G/H from arachidonic acid (2□, 30), was one of the most highly upregulated proteins identified among the downstream targets of GR (31) using our integrated proteomics approach. The CO□ family has two isoforms, CO□-1 and CO□-2, which are independently regulated in different manners. CO□-2 expression is mediated by inflammatory response, while CO□-1 is expressed constitutively (32). Upregulation of CO□-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 CO□-2 as a target of drug development have been conducted (36, 37). However, limited information concerning the function of CO□-1, such as for the mucosal protection of the stomach and blood flow maintenance, has been available (38). Recently, CO□-1, but not CO□-2, was reported to show involvement in brain inflammation (3□, □0), and, importantly, CO□-1 expression is regulated in PC12 cells in response to NGF stimulation (□1, □2), suggesting that CO□-1 is associated

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

In this study, we found that CO□-1 expression was regulated by GR, and dynein IC2-GR-CO□-1 signaling was upregulated in NF1-KD cells. In NF1-KD cells, suppression of dynein IC2 inhibits nuclear translocation of GR and decreases CO□-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 CO□-1 expression was induced by dynein IC2-C siRNA treatment, dynein IC2-□ rather than dynein IC2-C may be associated with GR translocation. Interestingly, CO□-1 siRNA effectively recovered the neurite retraction of NF1-KD cells. □e speculate that prostaglandins upregulated by CO□-1 may be associated with neurite outgrowth. Four prostaglandin E2 (PGE2) receptors, EP1, EP2, EP3, and EP□, have been identified (□3), and EP2 and EP3 expression was upregulated at □8 h and 72 h, respectively, in our DNA array analysis. EP3 has been reported to couple with G protein receptors to activate RhoA (□□, □5), 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 (□□, □5). Therefore, upregulation of CO□-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. CO□-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, □6) and other reports also showed retraction of neurite and growth corn in NF1 heterozygous (Nf1□/-) hippocampal neurons (□7). 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. □e firstly demonstrated that neurite retraction observed in NF1-KD cells is recovered by co-transfected dynein IC2-C siRNA (**Fig 5B, C**) or CO□-1 siRNA (**Fig. 6G, H**). These experimental evidences may explain the possible functional relevance of the abnormal regulation of dynein IC2-GR-CO□1 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-CO□-1 extracted from NF1-disease model neuronal cells may be an important mechanism of NF1-related pathogenesis. Drugs inhibiting this signaling such as specific CO□-1 inhibitors may be useful candidates for the treatment of NF1 patients with neuronal abnormalities.

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References

1. Stephens, K., Riccardi, V. M., Rising, M., Ng, S., Green, P., Collins, F. S., Rediker, K. S., Powers, J. A., Parker, C., and Donis-Keller, H. (1987) Linkage studies with chromosome 17 DNA markers in 45 neurofibromatosis 1 families. *Genomics* 1, 353-357.

2. Cawthon, R. M., Weiss, R., Xu, G. F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., and et al. (1990) A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* 62, 193-201.

3. Costa, R. M., Federov, N. B., Kogan, J. H., Murphy, G. G., Stern, J., Ohno, M., Kucherlapati, R., Jacks, T., and Silva, A. J. (2002) Mechanism for the learning deficits in a mouse model of neurofibromatosis type 1. *Nature* 415, 526-530.

4. Guo, H. F., Tong, J., Hannan, F., Luo, L., and Zhong, Y. (2000) A neurofibromatosis-1-regulated pathway is required for learning in Drosophila. *Nature* 403, 895-898.

5. Gregory, P. E., Gutmann, D. H., Mitchell, A., Park, S., Boguski, M., Jacks, T., Wood, D. L., Jove, R., and Collins, F. S. (1993) Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. *Somat. Cell Mol. Genet.* 19, 265-274.

6. Yunoue, S., Tokuo, H., Fukunaga, K., Feng, L., Ozawa, T., Nishi, T., Kikuchi, A., Hattori, S., Kuratsu, J., Saya, H., and Araki, N. (2003) Neurofibromatosis type I tumor suppressor neurofibromin regulates neuronal differentiation via its GTPase-activating protein function toward Ras. *J. Biol. Chem.* 278, 26958-26969.

7. Patrakitkomjorn, S., Kobayashi, D., Morikawa, T., Wilson, M. M., Tsubota, N., Irie, A., Ozawa, T., Aoki, M., Arimura, N., Kaibuchi, K., Saya, H., and Araki, N. (2008) Neurofibromatosis type 1 (NF1) tumor suppressor, neurofibromin, regulates the neuronal differentiation of PC12 cells via its associating protein, CRMP-2. *J. Biol. Chem.* 283, 9399-9413.

8. Kobayashi, D., Kumagai, J., Morikawa, T., Wilson-Morifuji, M., Wilson, A., Irie, A., and Araki, N. (2009) An integrated approach of differential mass spectrometry and gene ontology analysis identified novel proteins regulating neuronal differentiation and survival. *Mol. Cell. Proteomics* 8, 2350-2367.

9. Shilov, I. V., Seymour, S. L., Patel, A. A., Loboda, A., Tang, W. H., Keating, S. P., Hunter, C. L., Nuwaysir, L. M., and Schaeffer, D. A. (2007) The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol. Cell. Proteomics* 6, 1638-1655.

10. Ozawa, T., Araki, N., Yunoue, S., Tokuo, H., Feng, L., Patrakitkomjorn, S., Hara, T., Ichikawa, Y., Matsumoto, K., Fujii, K., and Saya, H. (2005) The neurofibromatosis type 1 gene product neurofibromin enhances cell motility by regulating actin filament dynamics via the Rho-ROCK-LIMK2-cofilin pathway. *J. Biol. Chem.* 280, 39524-39533.

11. Ha, J., Lo, K. W., Myers, K. R., Carr, T. M., Humsi, M. K., Rasoul, B. A., Segal, R. A., and Pfister, K. K. (2008) A neuron-specific cytoplasmic dynein isoform preferentially transports TrkB signaling endosomes. *J. Cell Biol.* 181, 1027-1039.

12. Pfister, K. K., Salata, M. W., Dillman, J. F., 3rd, Vaughan, K. T., Vallee, R. B., Torre, E., and Lye, R. J. (1996) Differential expression and phosphorylation of the 74-kDa intermediate chains of cytoplasmic dynein in cultured neurons and glia. *J. Biol. Chem.* 271, 1687-1694.

13. Picard, D., and Yamamoto, K. R. (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.* 6, 3333-3340.

14. Madan, A. P., and DeFranco, D. B. (1993) Bidirectional transport of glucocorticoid receptors across the nuclear envelope. *Proc. Natl. Acad. Sci. U. S. A.* 90, 3588-3592.

15. Galigniana, M. D., Radanyi, C., Renoir, J. M., Housley, P. R., and Pratt, W. B. (2001) Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J. Biol. Chem.* 276, 14884-14889.

16. Harrell, J. M., Murphy, P. J., Morishima, Y., Chen, H., Mansfield, J. F., Galigniana, M. D., and Pratt, W. B. (2004) Evidence for glucocorticoid receptor transport on microtubules by dynein. *J. Biol. Chem.* 279, 54647-54654.

17. Wochnik, G. M., Ruegg, J., Abel, G. A., Schmidt, U., Holsboer, F., and Rein, T. (2005) FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. *J. Biol. Chem.* 280, 4609-4616.

18. Vaughan, P. S., Leszyk, J. D., and Vaughan, K. T. (2001) Cytoplasmic dynein

intermediate chain phosphorylation regulates binding to dynactin. *J. Biol. Chem.* 276, 26171-26179.

19. Schnapp, B. J., and Reese, T. S. (1989) Dynein is the motor for retrograde axonal transport of organelles. *Proc. Natl. Acad. Sci. U. S. A.* 86, 1548-1552.

20. Schroer, T. A., Steuer, E. R., and Sheetz, M. P. (1989) Cytoplasmic dynein is a minus end-directed motor for membranous organelles. *Cell* 56, 937-946.

21. Hirokawa, N., Niwa, S., and Tanaka, Y. (2010) Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* 68, 610-638.

22. Pfister, K. K., Shah, P. R., Hummerich, H., Russ, A., Cotton, J., Annuar, A. A., King, S. M., and Fisher, E. M. (2006) Genetic analysis of the cytoplasmic dynein subunit families. *PLoS Genet* 2, e1.

23. Salata, M. W., Dillman, J. F., 3rd, Lye, R. J., and Pfister, K. K. (2001) Growth factor regulation of cytoplasmic dynein intermediate chain subunit expression preceding neurite extension. *J. Neurosci. Res.* 65, 408-416.

24. Bader, J. R., Kasuboski, J. M., Winding, M., Vaughan, P. S., Hinchcliffe, E. H., and Vaughan, K. T. (2011) Polo-like kinase1 is required for recruitment of dynein to kinetochores during mitosis. *J. Biol. Chem.* 286, 20769-20777.

25. Karki, S., Tokito, M. K., and Holzbaur, E. L. (1997) Casein kinase II binds to and phosphorylates cytoplasmic dynein. *J. Biol. Chem.* 272, 5887-5891.

26. Ikeda, K., Zhapparova, O., Brodsky, I., Semenova, I., Tirnauer, J. S., Zaliapin, I., and Rodionov, V. (2011) CK1 activates minus-end-directed transport of membrane organelles along microtubules. *Mol. Biol. Cell* 22, 1321-1329.

27. Anacker, C., Zunszain, P. A., Cattaneo, A., Carvalho, L. A., Garabedian, M. J., Thuret, S., Price, J., and Pariante, C. M. (2011) Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. *Mol. Psychiatry* 16, 738-750.

28. Kim, Y. S., Jang, S. W., Sung, H. J., Lee, H. J., Kim, I. S., Na, D. S., and Ko, J. (2005) Role of 14-3-3 eta as a positive regulator of the glucocorticoid receptor transcriptional activation. *Endocrinology* 146, 3133-3140.

29. Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S., and Lanzo, C. A. (1999) Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J. Biol. Chem.* 274, 22903-22906.

30. Smith, W. L., Garavito, R. M., and DeWitt, D. L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.* 271, 33157-33160.

31. Sun, H., Sheveleva, E., and Chen, Q. M. (2008) Corticosteroids induce cyclooxygenase 1 expression in cardiomyocytes: role of glucocorticoid receptor and Sp3 transcription factor. *Mol. Endocrinol.* 22, 2076-2084.

32. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69, 145-182.

33. Dang, I., and De Vries, G. H. (2011) Aberrant cAMP metabolism in NF1 malignant peripheral nerve sheath tumor cells. *Neurochem. Res.* 36, 1697-1705.

34. Park, S. W., Kim, H. S., Choi, M. S., Jeong, W. J., Heo, D. S., Kim, K. H., and Sung, M. W. (2011) The effects of the stromal cell-derived cyclooxygenase-2 metabolite prostaglandin E2 on the proliferation of colon cancer cells. *J. Pharmacol. Exp. Ther.* 336, 516-523.

35. Rundhaug, J. E., Pavone, A., Kim, E., and Fischer, S. M. (2007) The effect of cyclooxygenase-2 overexpression on skin carcinogenesis is context dependent. *Mol. Carcinog.* 46, 981-992.

36. Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C., and Seibert, K. (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. U. S. A.* 91, 3228-3232.

37. Muraki, C., Ohga, N., Hida, Y., Nishihara, H., Kato, Y., Tsuchiya, K., Matsuda, K., Totsuka, Y., Shindoh, M., and Hida, K. (2012) Cyclooxygenase-2 inhibition causes antiangiogenic effects on tumor endothelial and vascular progenitor cells. *Int. J. Cancer* 130, 59-70.

38. Peskar, B. M. (2001) Role of cyclooxygenase isoforms in gastric mucosal defence. *J. Physiol. Paris* 95, 3-9.

39. Anrather, J., Gallo, E. F., Kawano, T., Orio, M., Abe, T., Gooden, C., Zhou, P., and Iadecola, C. (2011) Purinergic signaling induces cyclooxygenase-1-dependent prostanoid synthesis in microglia: roles in the outcome of excitotoxic brain injury. *PLoS One* 6, e25916.

40. Choi, S. H., Aid, S., and Bosetti, F. (2009) The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research. *Trends Pharmacol. Sci.* 30, 174-181.

41. Kaplan, M. D., Olschowka, J. A., and O'Banion, M. K. (1997) Cyclooxygenase-1 behaves as a delayed response gene in PC12 cells differentiated by nerve growth factor. *J. Biol. Chem.* 272, 18534-18537.

42. Yung, H. S., Chow, K. B., Lai, K. H., and Wise, H. (2009) Gi-coupled prostanoid receptors are the likely targets for COX-1-generated prostanoids in rat pheochromocytoma

(PC12) cells. *Prostaglandins Leukot. Essent. Fatty Acids* 81, 65-71.

43. Sugimoto, Y., and Narumiya, S. (2007) Prostaglandin E receptors. *J. Biol. Chem.* 282, 11613-11617.

44. Tamiji, J., and Crawford, D. A. (2010) Prostaglandin E(2) and misoprostol induce neurite retraction in Neuro-2a cells. *Biochem. Biophys. Res. Commun.* 398, 450-456.

45. Katoh, H., Negishi, M., and Ichikawa, A. (1996) Prostaglandin E receptor EP3 subtype induces neurite retraction via small GTPase Rho. *J. Biol. Chem.* 271, 29780-29784.

46. Silva, A. J., Frankland, P. W., Marowitz, Z., Friedman, E., Laszlo, G. S., Cioffi, D., Jacks, T., and Bourtchuladze, R. (1997) A mouse model for the learning and memory deficits associated with neurofibromatosis type I. *Nat. Genet.* 15, 281-284.

47. Brown, J. A., Gianino, S. M., and Gutmann, D. H. (2010) Defective cAMP generation underlies the sensitivity of CNS neurons to neurofibromatosis-1 heterozygosity. *J. Neurosci.* 30, 5579-5589.

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,** □orkflow of the integrated proteomics approach. 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, □8h, and 72 h□NGF□). The protein and mRNA samples were sub□ected 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, □enn diagrams of the number of total mRNAs/proteins identified by 2D-DIGE, iTRAQ, and DNA array. **B,** □enn diagrams of the number of up/downregulated proteins identified by iTRAQ. In total, 3,23□proteins were identified with a confidence limit of □5□ 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 □enn diagrams (yellow area, 62 upregulated proteins□35 downregulated proteins). **C,** Hierarchical clustering of the □7 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). □lue 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. □ hite 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 CO□-1 from the molecular network shown in A. Color marks represent numerical data for each method as follows□color mark 1□2□h mRNA, 2□2□h iTRAQ, 3□□8 h iTRAQ, □□72 h iTRAQ, 5□2D-DIGE. Color set point is described in right panel of (□). The cluster of upregulated proteins including dynein IC2, GR, and CO□-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 (2□□) 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 ANO□A 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, □, and 5 (n □ 3) are shown in the graphs. □lue 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 □8h NGF treatment). Protein spots corresponding to spots 1′, 1, 2, 3, □, and 5. PC12 cells were transfected with NF1 (2□□) siRNA or control siRNA, stimulated with NGF, and harvested after 2□h, □8 h, or 72 h. Each cell lysate sample was sub□ected 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 CO□-1 in PC12 cells transfected with NF1 siRNA or control siRNA. D, Representative image of CO□-1 immunoblot analysis. The expression of CO□-1 was analyzed by immunoblotting using anti-CO□-1 antibody. E, The intensity of the CO□-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 (2□□) siRNA or control siRNA, stimulated with NGF, and harvested after □8-h NGF treatment. The cytoplasmic and nuclear proteins enriched in the cells were sub□ected to immunoblot analysis using anti-GR antibody. Lamin □1 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 2□h with control siRNA, NF1 (2□□) siRNA, both

NF1 (2□□) and dynein IC2-C (32□) siRNAs, or both NF1 (2□□) and dynein IC2-C (331) siRNAs before treatment with NGF. After □8-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 (2□□) siRNA □**b**□, NF1 (2□□) siRNA and dynein IC2-C (32□) siRNA □**c**□, and NF1 (2□□) siRNA and dynein IC2-C (331) siRNA □**d**□. **B,** Differential interference contrast images of PC12 cells treated with control siRNA □**a**□, NF1 (2□□) siRNA □**b**□, NF1 (2□□) siRNA and dynein IC2-C (32□) siRNA □**c**□, and NF1 (2□□) 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 (32□) 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 CO□-1 is suppressed by GR antagonist mifepristone. 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 measurements of CO□1 by

immunoblot analysis. **A,** representative immunoblot image of CO□1. Actin was used as the internal loading control. **B,** Quantification of CO□1 expression. The normalized intensities of CO□1 obtained from three separate identical experiments are shown in the histogram. Error bars represent S.E. of each sets of three experiments. **C,** Nuclear translocation of GR was decreased by the knockdown of dynein IC2. PC12 cells were transfected for 2□h with control siRNA, NF1 (2□□) siRNA, both NF1 (2□□) and dynein IC2 siRNAs before treatment with NGF. After □8-h NGF treatment, cells were harvested, and the cytoplasmic and nuclear proteins were extracted. □oth cytoplasmic and nuclear fractions were sub□ected to immunoblot analysis using anti-GR antibody. TCPε1 and Lamin □1 were used as markers of the cytoplasmic and nuclear fraction, respectively. Representative images of three reproducible experiments are shown. **D and E,** CO□-1 expression is downregulated by dynein IC2 siRNA treatment. PC12 cells were transfected for 2□h with control siRNA, NF1 (2□□) siRNA, or both NF1 (2□□) and dynein IC siRNAs before treatment with NGF. After □8-h NGF treatment, CO□-1 expression in the cells was analyzed by immunoblotting using anti-CO□-1 antibody. **D,** Representative immunoblot images of CO□1. Actin was used as the internal loading control. Representative images of three reproducible experiments are shown. **E,** Quantification of CO□-1 expression. The normalized CO□-1 intensities obtained from three separate identical experiments are shown in the histogram. Error bars represent S.E. of each set of three experiments. F, G, and H, CO□-1 knockdown recovered the inhibition of neurite outgrowth in NF1-KD PC12 cells. PC12 cells were transfected for 2□h with control siRNA, NF1 (2□□) siRNA, both NF1 (2□□) and CO□-1 (1023) siRNAs, or both NF1 (2□□) and CO□-1 (2618) siRNAs before treatment with NGF. After □8-h NGF treatment, CO□-1 expression in cells was analyzed by immunoblotting with anti-CO□-1 antibody, and the neurite length were measured. **F,** Representative immunoblot images of CO□-1. Actin was used as the internal loading control. **G,** Differential

interference contrast images of PC12 cells treated with control siRNA (**a**), NF1 (2□□) siRNA (**b**), NF1 (2□□) and CO□-1 (1023) siRNAs (**c**), and NF1 (2□□) and CO□-1 (2618) siRNAs (**d**). **H,** 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. The data are expressed as means and S.E. of three independent experiments. For each experiment, more than 50 cells were counted.

Table 1. Pathway-based characterization of up- and downregulated genes and proteins in PC12 cells identified with 2D-DIGE, iTRAQ, and DNA array analysis.

A. Upregulated pathways

rank	keymolnet pathway	score	score (*P*-value)	protein
1	Calcium signaling pathway	16.145	1.38×10^{-5}	**14-3-3**, **Annexin** , cadherin , **Crm1** , DGK , **Importin** , Kinesin , nAChR , **S100** , VILIP1 , **vinculin**
2	Transcriptional regulation by GR	14.298	4.96×10^{-5}	**14-3-3h** , ANXA1 , AP-1 , CGA , collagenase , CRF , GR , GRb , **HSP70** , IBAT , RGS2
3	Wnt signaling pathway	13.59	8.11×10^{-5}	b-TRCP , cadherin , **CRMP** , **MAP**, **MAP1B** , **tub** , Wnt
3	Granzyme signaling pathway	13.59	8.11×10^{-5}	**a2M** , COLIV , **HSP70** , **lamin** , Laminin , PI-9 , **tub**
5	MMP signaling pathway	13.127	1.12×10^{-4}	**a2M** , COLIV, Laminin , laminin5 , LRP , MMP , MMP-10 , MMP-13 , MMP-19 , MMP-3 , PAI-2
6	Intermediate filament signaling pathway	12.449	1.79×10^{-4}	**14-3-3** ,**14-3-3z** , **AP3-C** , **dynein** , **IF-II** , **K8** , Kinesin , **lamin** ,**laminA** , **laminC**, **tub**
7	inflammasome signaling pathway	11.866	2.68×10^{-4}	AIM2 , caspase-1 , inflammasome , IPAF , NALP1 , NALP3 , PI-9
8	CD44 signaling pathway	11.135	4.45×10^{-4}	COLIV , collagen, **ERMP** , **ezrin** , Laminin , MMP , **tub**
9	CYP family	10.729	5.89×10^{-4}	CYP3A , CYP3A4 , CYP3A43 , CYP3A5 , CYP3A7 , CYP4 , CYP4B1
10	LHR signaling pathway	10.609	6.40×10^{-4}	CGA , hCG , LH , LHR

□. Downregulated pathways

rank	keymolnet pathway	score	score (*P*-value)	protein
1	Serotonin signaling pathway	17.213	6.58×10^{-6}	5HT3R , 5HT5AR , 5HT5R , EPAC , Ga(q/11) , Ga(q/11)bg , nNOS , PKC , PLA2
2	CaSR signaling pathway	14.799	3.51×10^{-5}	Ga(q/11) , Ga(q/11)bg , **PI4K** , PKC , PMCA
3	AMPAR signaling pathway	14.213	5.27×10^{-5}	Actin , AKAP5 , Liprin-a , PKC , **Protein 4.1G** ,**tub**
4	Calcium signaling pathway	13.962	6.27×10^{-5}	Actin , CRT , Ga(q/11) , Ga(q/11)bg , **Kinesin** , P2X , PKCg , PMCA4 , troponin
5	GABA signaling pathway	13.363	9.49×10^{-5}	Actin , AKAP5 , GABAAR , GABAARg , GABAARg3 , GABAARq , GABAR , PKC
6	calpain signaling pathway	13.188	1.07×10^{-4}	Actin , calpain , calpain8 , integrin b, **NF-M** , PKC , PKCg , PMCA , TN-T , **tub**
7	HDAC signaling pathway	13.1	1.14×10^{-4}	Ets , HDAC , HDAC11 , **HSP90** , IV-HDAC
8	Melanopsin signaling pathway	11.955	2.52×10^{-4}	Ga(q/11) , Ga(q/11)bg , melanopsin
9	JAM family signaling pathway	11.769	2.87×10^{-4}	Actin , int-aL/b2 , int-aM/b2 , int-aX/b2 , int-b2
10	Guanylate cyclase(receptor type) signaling pathway	10.916	5.18×10^{-4}	CNGC , **HSP70** , **HSP90** , phospholamban , PMCA , **Tyr3MOX**

KeyMolnet software was used to determine statistically overrepresented pathway categories among the up-/downregulated molecules in NF1-KD PC12 cells. The list contained 263 upregulated genes/proteins (iTRAQ, 62 proteins□ 2D-DIGE, 32 proteins□ DNA array, 185 genes) and 23□ downregulated genes/proteins (iTRAQ, 35 proteins□2D-DIGE, 20 proteins□DNA array, 186 genes) (Fig. 2A,□, Table

S6). The top 10 KeyMolnet pathways showing a significant association with up/downregulated genes/proteins are listed with rank, KeyMolnet pathway, score, and *P* value of the score. □old molecules were identified by proteome analysis.