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CHAPTER 15

Neural crest development and neuroblastoma: the genetic and biological link

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Abstract: Neuroblastoma is one of the most common pediatric solid tumors originating from the sympathoadrenal lineage of neural crest. The tumor shows extremely different clinical phenotypes such as spontaneous regression on one hand and aggressive growth on the other hand. The different biological behavior of neuroblastoma appears to be determined by the genetic abnormalities including amplification of *MYCN* oncogene, DNA ploidy and some allelic imbalances. However, the spontaneous regression of neuroblastoma mimics the programmed cell death normally occurring in developing sympathetic cells expressing both TrkA tyrosine kinase A and $p75^{NTR}$ neurotrophin receptor. Indeed, TrkA expression is the most important factor related to the induction of tumor cell differentiation and/or programmed cell death because without its expression spontaneous regression of neuroblastoma never occurs. Thus, the enigmatic clinical behaviors of neuroblastoma are strictly linked to the molecular mechanism of neural crest development.

Keywords: neuroblastoma; NGF; TrkA; $p75^{NTR}$; *MYCN* oncogene; MYCN oncoprotein; stem cells

Neuroblastoma, a neural crest tumor in childhood

Neuroblastoma is an embyonic tumor originating from the sympathoadrenal lineage of neural crest and one of the most common solid tumors found in children (Bolande, 1974). Its incidence is about 1/8000 births and there is no significant difference among U.S., Europe and Japan. However, after beginning the mass screening to test the urine for the levels of catecholamine metabolites (VMA and HVA) in Japan in 1985 (Sawada et al., 1984), the incidence of neuroblastoma has almost doubled without decreasing the number of the sporadic tumors (Bessho, 1996). This strongly suggested the actual presence of '*in situ* neuroblastoma', which was first proposed by Beckwith and Perrin (1963), during the development of sympathetic neurons in human fetuses. They described the detection of '*in situ* neuroblastoma' in developing human embryos at the incidence of more than 40 times that of sporadic neuroblastomas, but most of them regressed spontaneously. Therefore, it is highly possible that we detect a part of the '*in situ* neuroblastomas' by mass screening, most of which otherwise regress without giving any therapy. However, at this moment it is unclear whether the regression of *in situ* neuroblastoma is due to the developmentally regulated programmed cell death of neuronal cells.

The sporadic neuroblastomas clinically found are divided into several subsets according to the clinical behavior, biological markers and genetic abnormalities (Brodeur, 2003). One of the most important clinical factors is the patient's age. The tumors found in the patients under one year of age are usually favorable and take a good clinical course to cure. On the other hand, many of the tumors symptomatically

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found in the patients over one year of age are poor prognostic and eventually kill the patients. Among the biological markers so far found, expression of the TrkA tyrosine kinase A receptor, as well as p75 neurotrophin receptor ($p75^{NTR}$) expression, is the most important indicator of prognosis (Nakagawara et al., 1992, 1993). TrkA is a high-affinity receptor for nerve growth factor (NGF), and $p75^{NTR}$ is its low-affinity receptor. The high levels of TrkA expression are strongly associated with favorable prognosis, whereas its decreased levels are significantly correlated with poor prognosis (Nakagawara et al., 1993). The important genetic markers include DNA ploidy, amplification of the *MYCN* oncogene and an allelic loss of the distal region of chromosome 1p (1p36) (Westermann and Schwab, 2002; Brodeur, 2003). Contrary to the other cancers, neuroblastomas with hyperdiploid karyotype show a good prognosis, while those with *MYCN* amplification and/or deletion of chromosome 1p36 are strongly associated with poor prognosis. The combination of these strong prognostic indicators segregates the subsets of neuroblastoma with different clinical behavior.

Figure 1 shows three types of neuroblastoma subset. Fig. 1 (left) demonstrates a stage 1 tumor originated from the adrenal gland in a patient under one year of age. The tumor is well encapsulated without metastasis. This type of neuroblastoma usually expresses high levels of TrkA and shows triploid DNA pattern with a single copy of *MYCN*. It clinically regresses spontaneously but very slowly. The baby in Fig. 1 (middle) is the patient with stage 4s neuroblastoma. The immature tumor cells occupy

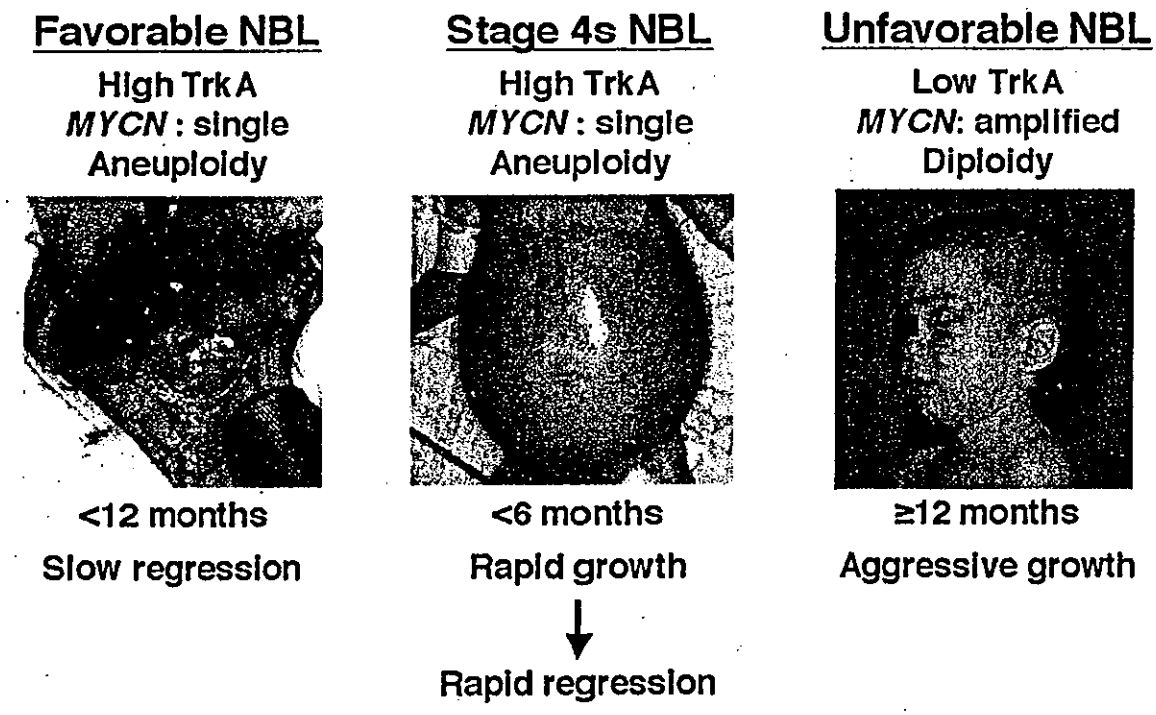

Fig. 1. Three distinct subset of human neuroblastomas with different biology and clinical behavior. Left: stage 1 neuroblastoma in a 7-month-old patient. The tumor originated from the right adrenal gland is small and well encapsulated. This kind of neuroblastoma usually regresses spontaneously. Middle: stage 4s neuroblastoma in a one-month-old baby. The primary tumor is located at the left adrenal gland. The liver is extremely enlarged and occupied by the tumor cells. The neuroblastoma cells are also positive in the bone marrow. The abdominal distension often oppresses the diaphragm to induce dyspnea. In a typical stage 4s neuroblastoma, the rapid tumor growth suddenly stops and starts to regress spontaneously. Right: stage 4 neuroblastoma in a 3-year-old boy. The tumor cells originated from the adrenal gland metastasize to long bones, skull and orbita with protrusion of the eye. The tumor cells show low TrkA expression, amplification of *MYCN*, diploid karyotype and deletion of the distal region of chromosome 1p.

the adrenal gland, liver and bone marrow (sometimes even skin), and rapidly grow at an early clinical stage. However, one day the tumor cells suddenly stop growing and start to regress spontaneously. This seems like just a miracle. The stage 4s tumor also shows high TrkA expression, triploidy and no amplification of *MYCN*. In contrast, the advanced stage of neuroblastoma shown in Fig. 1 (right) usually occurs in the patient over one year of age and metastasizes to bones and distant lymph nodes and eventually kill the patient. In this type of neuroblastoma, TrkA expression is strongly downregulated, the DNA ploidy pattern is diploid, and *MYCN* is amplified.

Genetic abnormalities of neuroblastoma

Neuroblastoma has many types of genetic abnormalities including chromosomal aneuploidy, gene amplifications, deletions, mutations, and deregulated DNA methylations. However, the pattern of the genetic aberration is different among the subsets, especially between those with favorable and unfavorable prognosis (Westermann and Schwab, 2002; Brodeur, 2003). The tumors with a tendency to regress spontaneously usually have triploidy but few abnormalities in the genome. On the other hand, the tumors with aggressive growth show a diploid or tetraploid karyotype, frequent amplification of *MYCN* oncogene, and chromosomal deletion of 1p36. The frequent gain of the chromosome 17q is reported to be associated with poor prognosis, however, it is also commonly observed in the tumors with favorable prognosis (Tomioka et al., 2003). The loss of heterozygosity at the chromosome 11q23 is reported to be frequent in the intermediate type of neuroblastoma in advanced stages with a single copy of *MYCN* and variable levels of TrkA expression (Guo et al., 1999). Thus, the subsets with different clinical behavior may be defined by the combination of the genomic aberrations.

Molecular and biological bases of neuroblastoma

Figure 2 shows a scheme of migration of the developing neural crest-derived cells, which segregate into several lineages such as melanocytes, sensory neurons, enteric neurons, and sympathetic ganglion cells. However, neuroblastoma never occurs in the other tissues than sympathetic ganglion or adrenal medulla. This suggests that the genetic events to cause neuroblastoma occur after the cell fate determination directing to sympathetic differentiation. The most likely candidate molecule to decide the direction of sympathetic differentiation at this moment is a basic helix-loop-helix transcription factor MASH1 which is transiently expressed during the neural development (Guillemot et al., 1993). In human neuroblastomas, MASH1/hASH1 is kept overexpressed (Soderholm et al., 1999; Ichimiya et al., 2001). Interestingly, induction of neuroblastoma cell differentiation in culture by treating with retinoic acid decreases the level of MASH1 mRNA. These suggest that the

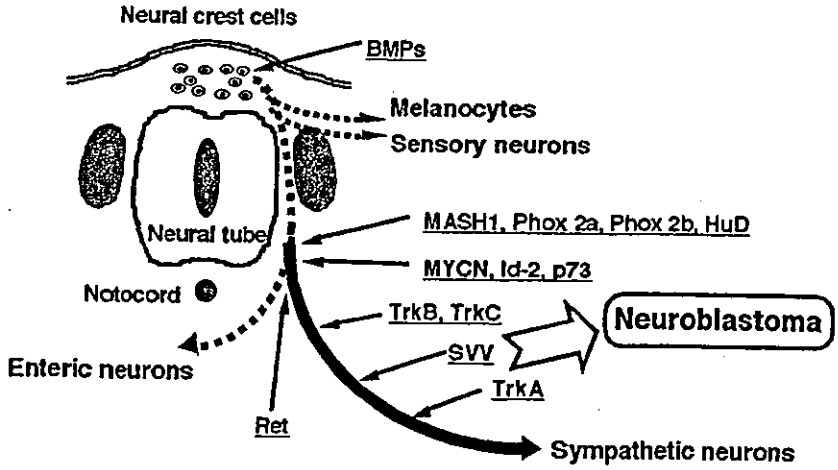

Fig. 2. Neuroblastoma occurs only from the sympathoadrenal lineage of neural crest. The important molecules regulating the sympathetic development are shown.

oncogenic events occurred in the early stage maintain the cells under arrest of differentiation by keeping the MASH1 expression at high levels, although this hypothesis must be proved. Even though the precise mechanism to regulate the oncogenic events of neuroblastoma and normal sympathetic differentiation is still elusive, it may be true that the targeting of the specific genes in such events is strictly controlled by the developmental program.

According to the accumulating evidence, it is clear that many important genes regulating normal development of sympathetic neurons are targeted to cause neuroblastoma or to modulate its biology. They include the *MYCN* gene encoding a basic helix-loop-helix transcription factor (Brodeur et al., 1984; Schwab et al., 1984), Id-2, a target of MYCN and a negative regulator of basic helix-loop-helix transcription factors (Iasorella et al., 2000), MASH1 (Soderholm et al., 1999; Ichimiya et al., 2001), Phox2a and Phox2b, the homeotic proteins functioning with MASH1 (Kuno et al., unpublished data), and the downstream receptors such as Trk family members (Nakagawara et al., 1993) and Ret (Hishiki et al., 1998). These suggest that the important regulators of sympathetic differentiation are targeted to cause or maintain the cancerous status of neuroblastoma. This idea can be extended to the possible link between developmentally regulated programmed cell death of sympathetic neurons and spontaneous regression of neuroblastoma, because in both phenomena, expression of TrkA receptor is necessary (Nakagawara, 1998a, 2001). In other words, TrkA expression is almost exclusively required to induce spontaneous regression of neuroblastoma.

NGF family signaling in neuroblastoma

The NGF signals and their depletion strongly regulate survival and death of the normal sympathetic neurons, respectively. Similarly, it has recently become obvious that the NGF family signals strongly regulate the biology of neuroblastoma. Most neuroblastomas with favorable prognosis express high levels of both TrkA and $p75^{NTR}$ and functionally respond to exogenous NGF by extending neurites and promoting survival in primary culture (Nakagawara et al., 1993). The association between high levels of expression of TrkA and/or $p75^{NTR}$ and favorable outcome is statistically significant in primary human neuroblastomas. On the contrary, in aggressive neuroblastomas with *MYCN* amplification in advanced stages, expression of TrkA is extremely downregulated. The many studies about the role of Trk signaling in neuroblastoma cell lines also suggest that the intracellular TrkA signal is disturbed even though autophosphorylation of TrkA is induced by addition of NGF (Nakagawara et al., 1994). Thus, for the gain of growth advantage, the aggressive neuroblastoma cells appear to shut off the TrkA signal. Instead, they utilize a functional brain-derived neurotrophic factor (BDNF) and/or neurotrophin-4 (NT-4)/TrkB signaling system in an autocrine manner (Nakagawara et al., 1994). This BDNF/TrkB autocrine system also promotes invasion and metastasis in advanced tumors (Matsumoto et al., 1995). These suggest that spontaneous regression occurs only in neuroblastoma with high levels of TrkA expression and is induced by depletion of NGF within the tumor. The aggressive neuroblastoma cells seem to escape from the control by NGF, but to take advantage of the BDNF/TrkB autocrine loop for promotion of survival.

The family of glial cell line-derived neurotrophic factor (GDNF) mediates another important extracellular signal to regulate the survival of sympathetic neurons. Many neuroblastoma cells express the GDNF family receptors (Ret, GFRα-1, -2 and -3) and functionally respond to their ligands (GDNF, neurturin and artemin) in the primary culture (Hishiki et al., 1998). However, their expression and the responsiveness to the ligands are not associated with the disease stages or prognosis.

The other neurotrophic factors, pleiotrophin (PTN) and midkine (MK), may also be important in regulating neuroblastoma biology (Nakagawara et al., 1995). The expression of PTN is high in favorable neuroblastomas, whereas that of MK is high in all primary neuroblastomas. However, their functional roles in neuroblastoma are currently unknown.

Role of p53 and p73 in life and death of neuroblastoma

Pozniak et al. (2000) have recently reported about the crucial role of the tumor suppressor p53 and its

family member p73 in regulating survival and apoptosis during the induction of programmed cell death in mouse sympathetic neurons. Life and death of the sympathetic cervical ganglion (SCG) neurons are regulated by the balance between the levels of p53 and ΔNp73, an NH_2-terminally truncated dominant-negative form of p73. In neuroblastoma, p53 is not mutated but localized in the cellular cytoplasm especially in advanced stage tumors (Moll et al., 1995). Just recently, the anchoring molecule of p53 in the cytoplasm has been identified as Parc which is a structurally E3 ubiquitin ligase but binds to and stabilizes p53 (Nikolaev et al., 2003). It is interesting that the apoptosis-inducing stresses often trigger nuclear translocation of cytoplasmic p53 in neuroblasatoma cell lines (Ostermeyer et al., 1996).

p73 is the first family member of p53 and has occasionally been discovered as a candidate tumor suppressor of neuroblastoma (Kaghad et al., 1997). It is mapped to chromosome 1p36.2-3 which is commonly deleted in many aggressive neuroblastomas with *MYCN* amplification. The extensive mutation search has revealed that p73 is not mutated in many cancers including neuroblastoma (Ikawa et al., 1999). However, we found two mutations of the COOH-terminally located proline residues, one was somatic and the other germline. Nevertheless, most primary neuroblastomas have no mutation of p73 (Ichimiya et al., 1999).

Interestingly, in many malignant solid tumors, p73 has satisfactorily shown to be upregulated, though it has functionally the apoptosis-inducing ability like p53. We and the other investigators have recently found that p73 can bind to the Δ*Np73* proper promoter and induce transcription of which possesses the oncogenic function (Nakagawa et al., 2002). In addition, Δ*Np73* binds to both wild type p53 and p73 to suppress their apoptosis-inducing function (Nakagawa et al., 2002). These observations are very important because they might at least in part explain how the cancers without p53 mutation do develop the tumors with poor prognosis. In neuroblastoma, Casciano et al. (2002) have reported that both *p73* and Δ*Np73* are highly expressed in aggressive rather than favorable tumors.

Figure 3 shows the current summary of the signals for induction of neuronal apoptosis. Both p53 and p73 as well as ΔNp73 might be cooperatively functioning to regulate the programmed cell death of sympathetic as well as neuroblastoma cells.

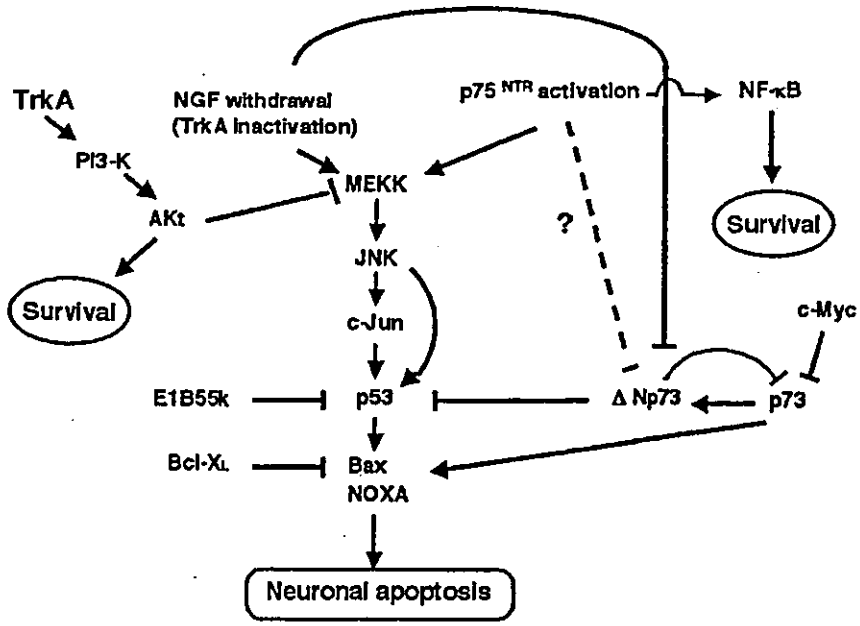

Fig. 3. Intracellular signaling of neuronal survival and apoptosis: the role of p53, p73 and ΔNp73.

Comprehensive genomics to identify the novel genes

To date, several genes functioning as landmark regulators in different subsets of neuroblastoma, such as *MYCN*, *MASH1*, *Trk*, *p53* and *p73*, have been identified. However, in the present postgenome era, we can try comprehensive approach to identify the important genes in a mass scale. For that purpose, we generated oligo-capping cDNA libraries from the primary neuroblastoma tissues of three different subsets as shown in Fig. 1. In total, we obtained 6252 gene clusters from 9729 clones randomly picked up from the cDNA libraries, among which 34% were novel genes with unknown function. The expression profiles of each subset of neuroblastoma were extremely different. By using the semi-quantitative reverse transcriptase (RT)-PCR, we have identified 757 genes differentially expressed between favorable (stage 1, high expression of *TrkA* and a single copy of *MYCN*) and unfavorable (stage 3 or 4, decreased levels of *TrkA* expression and amplification of *MYCN*) neuroblastomas. Among them, 502 are novel genes. [The results of our neuroblastoma cDNA project excluding those obtained from the stage 4s cDNA libraries were published elsewhere (Ohira et al., 2000; Ohira et al., in press).]

The expression profile of known genes was very different among the three subsets of neuroblastoma. The favorable subset frequently expressed neuronal specific genes including those related to neural differentiation, synapse, catecholamine metabolism and protein degradation. On the other hand, the unfavorable subset expressed many genes related to cell cycle control, protein synthesis and transcriptional regulation. The 4s tumor contained apoptosis-related genes, oncogenes and HLA family members which might be derived from the infiltrated lymphocytes into the tumor.

The 757 differentially expressed genes were strongly implicated in understanding of neuroblastoma biology. Of interest, vast majority of those genes was expressed at higher levels in the favorable subset as compared to the unfavorable one. The genes highly expressed in the favorable subset contained those related to neuronal differentiation, migration, cell–cell interaction, protein degradation, synaptic vesicles, catecholamine metabolism and intracellular signaling (Ohira et al., 2000; Ohira et al., in press). Most of them define the neuronal-specific phenotype and maintain the neuronal function. They also included heat shock proteins and ubiquitin/proteasome-related molecules that might sense the stress. On the other hand, only about 10% of the differential genes were expressed at high levels in the unfavorable subset. The protein products of such known genes contained many transcriptional and translational regulators including oncoproteins.

We also applied the primary culture of newborn mouse SCG neurons for screening those genes which change during the NGF-induced differentiation and/or the NGF depletion-induced apoptosis. This approach has identified 33 genes related to the former and 56 genes changeable during the latter (Isogai et al., unpublished data).

Our unique approach has identified more than several interesting genes as well as their products which include Nbla0219/BMCC1, a novel proapoptotic molecule with BCH domain, P-loop and coiled-coil domain, and Nbla0078/NEDL1, a novel E3 ubiquitin ligase with the HECT domain. The other interesting genes whose analyses have been published during our studies also include human *RIM/Nbla0761*, a Rab3-interacting molecule in the synaptic vesicles, *XCE/Nbla3145*, a new endothelin-converting enzyme and *FOG2/Nbla3139*, a coactivator of GATA transcription factor. Currently, a total of 7000 genes we cloned from the primary neuroblastomas are being fixed on the slide glass for cDNA microarray analysis.

Thus, our neuroblastoma cDNA project has provided enormous information and the gene materials for understanding of neuroblastoma biology as well as the molecular mechanism of neural crest development.

Developmental time axis and oncogenic events

Our neuroblastoma cDNA project has provided us with tremendous information about the genes expressed in different subsets with characteristic biology (Ohira et al., 2000; Ohira et al., in press). It suggested the presence of a kind of rule in the expression patterns of the subset-specific genes. Figure 4 shows the groups of genes expressed along the time axis of sympathetic neuron development. During the early

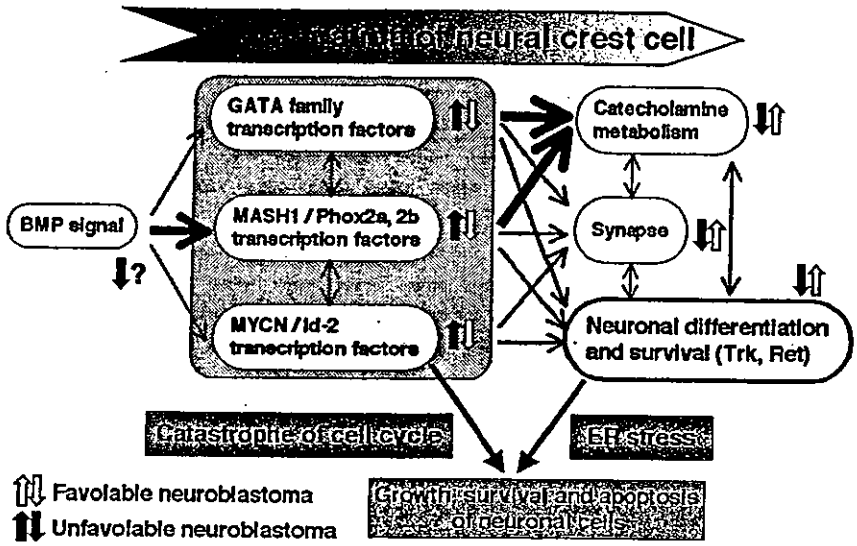

Fig. 4. The gene expression cascade along the time axis during the neural crest development, which is expected from the results obtained from the neuroblastoma cDNA project. Many transcription factors are upregulated in the unfavorable neuroblastoma, whereas the genes related to the terminal differentiation of neuron are upregulated in the favorable neuroblastoma. ER, endoplasmic reticulum.

stages of development, many transcription factors seem to function in deciding the direction of differentiation as well as regulating cell growth and survival of neural crest-derived cells. Interestingly, many genes highly expressed in unfavorable NBLs contain transcription factors and the components of their complexes. They include MYCN and Id family transcription factors that link to the regulation of Rb and p53 and regulate cell growth and apoptosis (Lasorella et al., 2000). The another basic helix-loop-helix transcription factor, MASH1, is constitutively activated in neuroblastoma, and by collaborating with Phox2a and Phox2b, it may regulate the arrest of differentiation in an unfavorable neuroblastomas (Kuno et al., unpublished data). Our neuroblastoma cDNA project has also revealed that there may be a neuronal cassette of GATA transcription factor complex that controls growth and differentiation of sympathetic progenitor cells (Ohira et al., 2003). Some molecules in this complex are upregulated in unfavorable neuroblastomas (Aoyama et al., manuscript in preparation). Thus, many important components in the transcriptional regulators appear to be highly expressed in unfavorable neuroblastomas and function to regulate the tumor cell growth or the status of de-differentiation.

On the other hand, most of a remarkable number of the genes expressed at high levels in favorable neuroblastomas encode the molecules that are necessary to maintain the neuronal function. They may be necessary for keeping catecholamine metabolism, synapse formation, neuronal cell survival, etc. We have also found many genes related to the ubiquitin-proteasome pathway and heat shock proteins in favorable neuroblastomas. They might be involved in induction of apoptosis triggered by endoplasmic reticulum stress.

Thus, the pattern of the differentially expressed genes in neuroblastoma subsets suggests the changes in the developmentally regulated gene expression along the time axis.

The hypothesis of neuroblastoma stem cells

According to the result of neuroblastoma mass screening, it may be true that most of the early stage neuroblastomas do not progress to the advanced tumors. In addition, the study of molecular mechanism linking neural development and neuroblastoma has revealed that the aggressive neuroblastoma occuring in an older patient seems to be arrested at

the earlier stage of differentiation than the favorable tumor found in younger patient (Nakagawara, 1998b). These suggest a difference between the progenitor cells, or the cancer stem cells, of neuroblastomas with dissimilar genetic and biological characteristics. The classic observation by Beckwith and Perrin (1963) suggests that the migrating neural crest cells destined to differentiate to sympathetic neurons first enter the sympathetic ganglion, and a part of them further migrate to reach the adrenal medulla where the concentration of glucocorticoid is kept high. The detailed investigations done by neurobiologists have shown high expression levels of both TrkA and $p75^{NTR}$ in almost all of the neurons in the sympathetic ganglion as well as their dependence on the target-derived NGF for survival (trophic theory) (McMahon et al., 1994). This may be reflected on that neuroblastomas originated from the sympathetic ganglia show better prognosis than those from the adrenal medulla and that the former tumors usually express high levels of TrkA and $p75^{NTR}$. On the other hand, aggressive neuroblastomas with *MYCN* amplification almost exclusively originate from the adrenal medulla in patients older than one year (Nakagawara et al., 1990). The most imporant fact is the hyperdiploid karyotype of more than half of the favorable neuroblastomas (Brodeur, 2003), suggesting that the mitotic instability or dysfunction is the main event during the tumorigenesis. On the other hand, in aggressive tumors occurring in the adrenal medulla, the DNA ploidy is diploid and the regional abnormalities of the chromosome induced by the genetic instability are the main phenotypes of the genome. Those tumor cells usually have an autocrine signaling loop of BDNF and TrkB receptor, the dependency on which has been reported to be the rather immature phenotype of developing sympathetic neurons (Pinon et al., 1996).

Figure 5 shows the hypothetical scheme of the cancer stem cells A and B of neuroblastoma. The cancer stem cells A, which express high levels of TrkA and $p75^{NTR}$ and are dependent on NGF for survival, are present in both sympathetic ganglia and adrenal medulla. They are prone to have a mitotic dysfunction to get a proliferating advantage. However, those cancer stem cells are still dependent on NGF to survive and to differentiate. The hypothesis recently proposed by Kaneko and Knudson (2000) in terms of the DNA ploidy pattern in neuroblastoma may be attractive to explain the mitotic dysfunction found in favorable neuroblastomas. In addition, those stem cells may sustain the function at the late stage

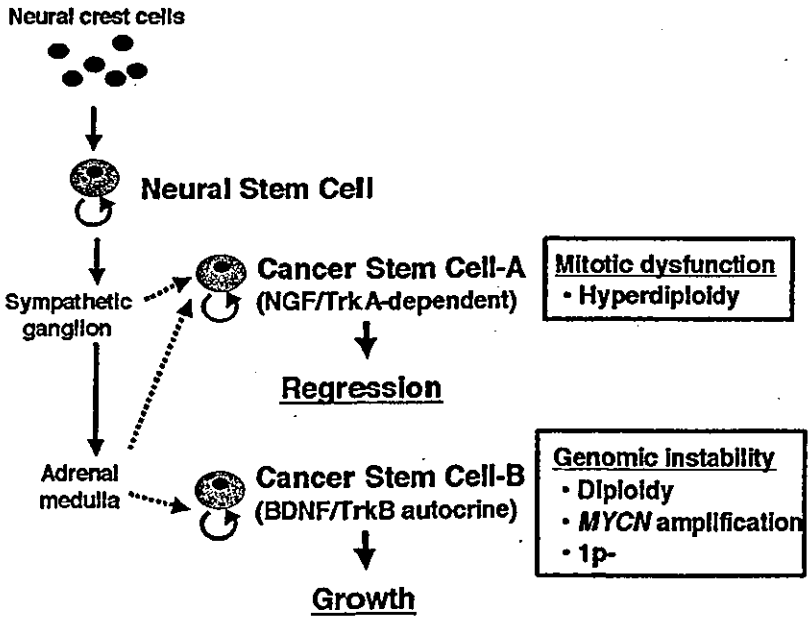

Fig. 5. The hypothesis of cancer stem cells in neuroblastoma.

of sympathetic development by maintaining the NGF-dependency. On the other hand, the cancer stem cells B may be localized in the adrenal medulla and are prone to have a genetic instability to cause *MYCN* amplification and allelic loss of chromosome 1p36. They may retain the phenotype of rather immature stage of sympathetic development by expressing TrkB but not TrkA. The comprehensive approach such as our neuroblastoma cDNA project and an array CGH methodology may help to prove the above hypothesis in the near future.

Conclusion

Neuroblastoma is an enigmatic tumor showing multiple clinical behaviors. However, the recent advances in neuroblastoma research have revealed that the molecular mechanism of neural crest development may strictly regulate the tumorigenesis as well as biology of different subsets of neuroblastoma. Indeed, the Trk family receptors and MYCN oncoprotein appear to link between neural development and cancer. Thus, unveiling the molecular mechanism of neuroblastoma should extremely help to understand how the neurons develop, differentiate, survive and die.

Abbreviations

ARTN	artemin
BDNF	brain-derived neurotrophic factor
GDNF	glial cell line-derived neurotrophic factor
MK	midkine
NGF	nerve growth factor
NT-4	neurotrophin-4
NTRN	neurturin
p75NTR	p75 neurotrophin receptor
PTN	pleiotrophin
SCG	superior cervical ganglion
Trk	tyrosine kinase

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NEDL1, a Novel Ubiquitin-protein Isopeptide Ligase for Dishevelled-1, Targets Mutant Superoxide Dismutase-1*

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Approximately 20% of familial amyotrophic lateral sclerosis (FALS) arises from germ-line mutations in the superoxide dismutase-1 (*SOD1*) gene. However, the molecular mechanisms underlying the process have been elusive. Here, we show that a neuronal homologous to E6AP carboxyl terminus (HECT)-type ubiquitin-protein isopeptide ligase (NEDL1) physically binds translocon-associated protein-δ and also binds and ubiquitinates mutant (but not wild-type) SOD1 proportionately to the disease severity caused by that particular mutant. Immunohistochemically, NEDL1 is present in the central region of the Lewy body-like hyaline inclusions in the spinal cord ventral horn motor neurons of both FALS patients and mutant *SOD1* transgenic mice. Two-hybrid screening for the physiological targets of NEDL1 has identified Dishevelled-1, one of the key transducers in the Wnt signaling pathway. Mutant SOD1 also interacted with Dishevelled-1 in the presence of NEDL1 and caused its dysfunction. Thus, our results suggest that an adverse interaction among misfolded SOD1, NEDL1, translocon-associated protein-δ, and Dishevelled-1 forms a ubiquitinated protein complex that is included in potentially cytotoxic protein aggregates and that mutually affects their functions, leading to motor neuron death in FALS.

Amyotrophic lateral sclerosis (ALS)[1] is a progressive, fatal, neurodegenerative disease that is characterized by selective loss of motor neurons in the spinal cord, brain stem, and motor cortex. The sporadic and familial forms of the disease have similar clinical and pathological features. About 10% of ALS cases are familial, and mutation of superoxide dismutase-1 (SOD1) is found in 20% of familial ALS (FALS) patients (1, 2). Mice that express mutant *SOD1* transgenes develop an age-dependent ALS phenotype independent of levels of dismutase activity, suggesting that FALS pathology is because of a toxic gain of function in SOD1 and that the abnormal protein structure of mutant SOD1 is critical in the pathogenesis of motor neuron death (3–6). Recently, proteasome expression and activity have been reported to decrease with age in the spinal cord (7, 8). Furthermore, mutant SOD1 turns over more rapidly than wild-type SOD1, and an inhibitor of proteasome action inhibits this turnover and thus selectively increases the steady-state level of mutant SOD1 (8). These results suggest the involvement of the ubiquitin-proteasome function in the cause of FALS. However, the biochemical nature of this gain-of-function mutation in SOD1 and the mechanism by which SOD1 mutations cause the degeneration of motor neurons have remained elusive.

We show here the identification of a novel HECT-type ubiquitin-protein isopeptide ligase (E3), NEDL1, which is expressed in neuronal tissues, including the spinal cord, and selectively binds to and ubiquitinates mutant (but not wild-type) SOD1. NEDL1 is physically associated with translocon-associated protein-δ (TRAP-δ), one of the endoplasmic reticulum (ER) translocon components that has previously been reported to bind mutant SOD1 (9, 10). Both NEDL1 and TRAP-δ form a complex with mutant SOD1, with the binding intensity among these proteins being roughly proportionate to the rapidity of progression of the associated FALS phenotype. Immunohistochemical study has shown that NEDL1 is positive in the Lewy body-like hyaline inclusions in the spinal cord motor neurons of both FALS patients and mutant *SOD1* transgenic mice. We have also found that NEDL1 targets Dishevelled-1 (Dvl1) for ubiquitination-mediated degradation and that mutant (but not wild-type) SOD1 affects the function of Dvl1. Our observations suggest that NEDL1 is a quality control E3 that recognizes mutant SOD1 to form a tight complex with the physiological targets of NEDL1 in motor neurons of FALS patients.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human neuroblastoma-derived cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. COS-7 and Neuro2a cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fe-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB048365 (Nbla0078 and human NEDL1), AB002320 (KIAA0322), and AB083710 (mouse Nedl1).

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[1] The abbreviations used are: ALS, amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; SOD1, superoxide dismutase-1; E3, ubiquitin-protein isopeptide ligase; NEDL1, NEDD4-like ubiquitin-protein ligase-1; TRAP-δ, translocon-associated protein-δ; ER, endoplasmic reticulum; Dvl1, Dishevelled-1; RT, reverse transcription; LBHI, Lewy body-like hyaline inclusion; JNK, c-Jun N-terminal kinase; HECT domain, homologous to E6AP carboxyl-terminus.

tal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified 37 °C incubator with 5% CO_2. All transfections were carried out with LipofectAMINE Plus transfection reagent (Invitrogen) according to the manufacturer's instructions. In some experiments, transfected cells were treated with MG-132 for 30 min at a final concentration of 40 μM.

RNA Analysis—A human multiple tissue mRNA blot and a fetal human multiple mRNA blot (Invitrogen) were hybridized with a ^{32}P-labeled ApaI-ScaI restriction fragment of *NEDL1* cDNA under standard conditions. For reverse transcription (RT)-PCR analysis, cDNA derived from adult human neural system (BioChain Institute, Hayward, CA) was subjected to PCR amplification using the following primers: *NEDL1*, 5′-CCGATTTGAGATCACTTCCTCC-3′ (sense) and 5′-CCGCTTTCCATCAGGTTGTT-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase, 5′-ACCTGACCTGCCGTCTAGAA-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense). The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide post-staining. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

In Vitro Ubiquitination Assays—*In vitro* ubiquitination assays were performed as follows. Reaction mixtures containing 0.5 μg of purified glutathione *S*-transferase fusion proteins, 0.25 μg of yeast ubiquitin-activating enzyme (*E1*) (BostonBiochem, Cambridge, MA), 1 μl of crude lysates from *Escherichia coli* expressing ubiquitin carrier proteins (*E2*), and 10 μg of bovine ubiquitin (Sigma) were incubated in 250 mM Tris-HCl (pH 7.6), 1.2 M NaCl, 50 mM ATP, 10 mM $MgCl_2$, and 30 mM dithiothreitol. Reactions were terminated after 2 h at 30 °C by the addition of SDS sample buffer. Samples were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with anti-ubiquitin monoclonal antibody 1B3 (Medical & Biological Laboratories, Nagoya, Japan).

Immunofluorescence Staining—Cells grown on coverslips were processed for immunofluorescence. Briefly, cells were fixed in 3.7% formaldehyde, permeabilized in 0.2% Triton X-100, and finally incubated with anti-NEDL1 antibody (diluted 1:100). The primary antibody was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (diluted 1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Images were taken using an Olympus confocal microscopy system.

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed using the Gal4-based Matchmaker two-hybrid system with the cDNA libraries derived from fetal human brain (first screening) and adult human brain (second screening) (Clontech, Palo Alto, CA). *Saccharomyces cerevisiae* CG1945 cells were transformed with pAS2–1-NEDL1-1 (amino acids 757–1114; first screening) or pAS2–1-NEDL1-2 (amino acids 382–1448; second screening), which did not activate the transcription of *lacZ* alone. The transformants were subsequently transformed with the cDNA library, and the *lacZ*-positive colonies were selected. The plasmid DNAs were extracted from these positive colonies, and their nucleotide sequences were determined.

Immunoprecipitation and Western Blot Analysis—Anti-NEDL1 and anti-TRAP-δ polyclonal antibodies were raised in rabbits against an NEDL1 oligopeptide (amino acids 460–482) and a TRAP-δ oligopeptide (amino acids 93–126), respectively. For immunoprecipitation, COS-7 or Neuro2a cells were cotransfected with the expression plasmids in various combinations and lysed 48 h later in 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride supplemented with protease inhibitor mixture (Sigma). Whole cell lysates were immunoprecipitated with anti-NEDL1, anti-FLAG (M2; Sigma), or anti-Myc (9B11; Cell Signaling Technology, Beverly, MA) antibody. Immune complexes were recovered on protein G-Sepharose beads, eluted by boiling in Laemmli sample buffer, electrophoresed on SDS-polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA) by electroblotting. For ubiquitination experiments, cell lysis was performed in radioimmune precipitation assay buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA), followed by strong sonication and freeze-thaw. The membrane was probed with the indicated primary antibodies and then incubated with the appropriate secondary antibodies labeled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc. and Southern Biotechnology Associates, Inc., Birmingham, AL). Immunoreactive bands were detected by the enhanced chemiluminescence technique (ECL, Amersham Biosciences). For the detection of c-Jun phosphorylation, we used anti-c-Jun (sc-45, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phospho-Ser^{63} c-Jun (Cell Signaling Technology) antibody.

Cloning of Human NEDL1 cDNA—A forward primer (5′-GGTTTTTAGGCCTGGCCGCC-3′) and a reverse primer (5′-CAATGAGGTACATGCCAATCC-3′) were used to amplify the 5′-part of the *NEDL1* cDNA using cDNA libraries derived from human neuroblastoma and fetal human brain (Stratagene, La Jolla, CA) as templates. The full-length human *NEDL1* cDNA was generated by fusion of the PCR-amplified fragment (nucleotides +1 to +68, where position +1 represents the translation initiation site) and the *KIAA0322* cDNA (a gift from T. Nagase, Kazusa DNA Institute). Gel electrophoresis and Western blot analysis were carried out as described above.

Expression Constructs—The mammalian expression plasmids for hemagglutinin-tagged and His_6-tagged ubiquitin were kind gifts of D. Bohmann. The full-length *NEDL1* cDNA was inserted into the mammalian expression plasmid pEF1/His (Invitrogen) or pIRESpuro2 (Clontech). cDNAs encoding wild-type and mutant forms of SOD1 were fused to the FLAG or Myc epitope tag sequence at their C termini and subcloned into pIRESpuro2. Similarly, the FLAG or Myc epitope tag sequence was attached to the C terminus of TRAP-δ. Also similarly, the FLAG or Myc epitope tag sequence was attached to the N terminus of Dvl1. Coding sequences were verified by automated DNA sequencing.

Protein Stability Experiments—Neuro2a cells were transfected with the expression plasmid for the wild-type or mutant form of SOD1 with or without the NEDL1 expression plasmid. Twenty-four hours after transfection, cycloheximide (50 μg/ml) was added to the culture medium, and the cells were harvested at the indicated time points by lysis in radioimmune precipitation assay buffer. The protein concentrations were determined using the Bradford protein assay system (Bio-Rad) according to the instructions of the manufacturer.

Immunohistochemistry—The immunohistochemical studies were performed as described previously using affinity-purified rabbit anti-NEDL1 antibody (11). Patient tissues were obtained at autopsy from two FALS siblings from a Japanese family. The clinical course of the sister, who died at age 46, was 18 months (case 1), and that of the brother, who died at age 65, was 11 years (case 2) (11). The *SOD1* gene was mutated with a 2-bp deletion at codon 126 (11, 12). Normal spinal cord tissues were obtained from three neurologically and neuropathologically normal individuals. The same study was performed on spinal cord tissues from three normal rats and a transgenic ALS rat carrying a mutant allele of the human *SOD1* gene (H46R) (13). These mice were killed at 180 days. As a negative control, some sections were incubated with anti-NEDL1 antibody that had been pre-absorbed with an excess of NEDL1 antigen. Bound antibodies were visualized by the avidin-biotin-immunoperoxidase complex method.

RESULTS

Cloning and Expression of the NEDL1 E3 Gene—To detect novel molecules that are important in regulating neuronal programmed cell death, we constructed oligo-capping cDNA libraries from a mixture of three fresh human neuroblastoma tissues (stages 1 and 2) that were undergoing gradual spontaneous regression, probably by neuronal apoptosis (14). Screening of 1152 novel genes by RT-PCR revealed that 194 genes were expressed differentially in regressing neuroblastomas with favorable prognosis and in aggressive tumors with poor prognosis. Among these genes, we found a partial cDNA sequence with an HECT-like domain (*Nbla0078*) that partially matched the *KIAA0322* gene. Because *KIAA0322* lacks a 5′-coding region, we used a genome-based PCR procedure to clone the corresponding full-length cDNA. This is predicted to encode a protein product of 1585 amino acids with homology to NEDD4 E3 (15, 16), which includes a C2 domain at the N-terminal region supposed to mediate its membrane localization in a calcium-dependent manner, two WW motifs important for protein-protein interaction through binding to specific proline-rich clusters, and a conserved catalytic HECT domain at the C terminus (Fig. 1*A*). We named this novel ligase, which mapped to chromosome 7p13, NEDL1 (NEDD4-like ubiquitin-protein ligase-1). We also cloned the mouse counterpart of *NEDL1* cDNA, whose amino acid sequence is 78% identical to the human sequence. Tissue-specific expression of *NEDL1* mRNA of ~10 and 7 kb in size was observed, with predominant expression in adult and fetal brains as examined by Northern blot analysis (Fig. 1*B*). Its

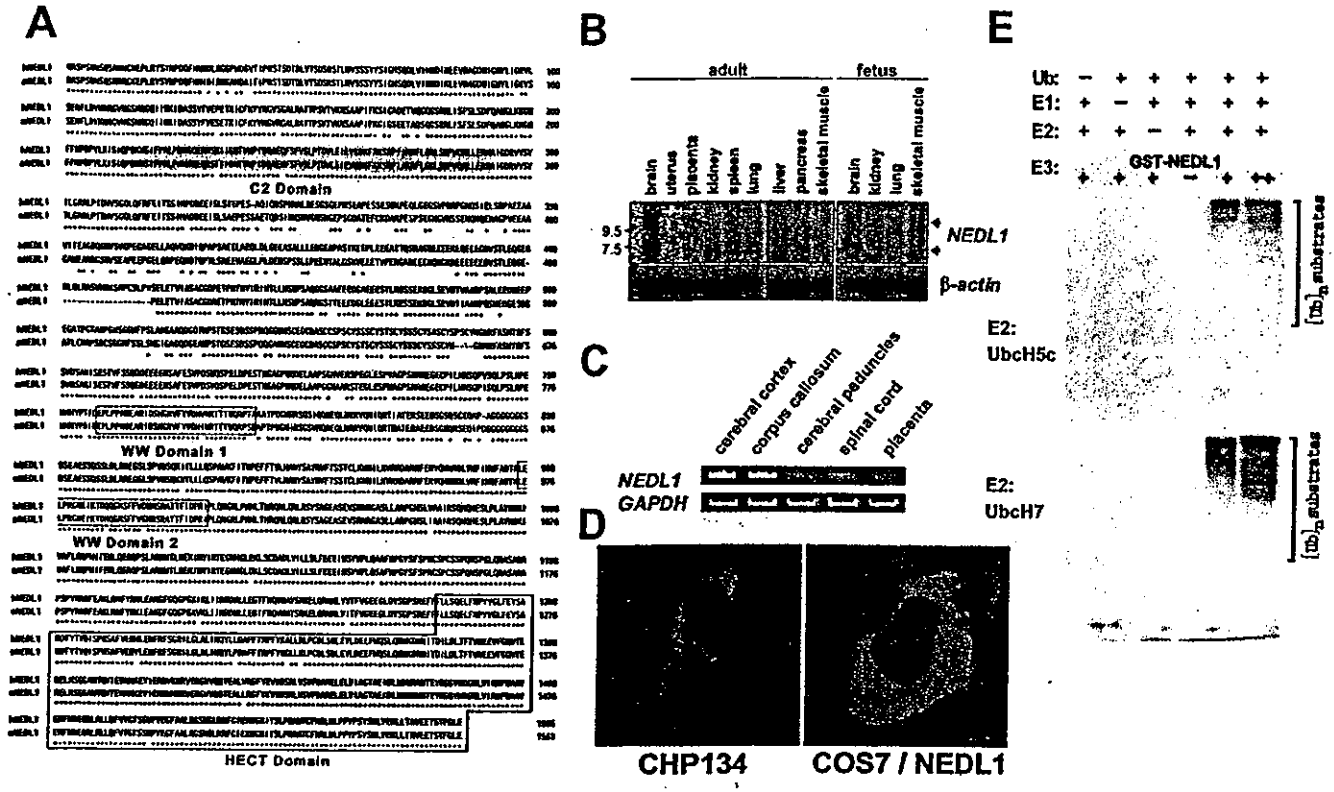

FIG. 1. **Amino acid sequence, brain-specific expression, and subcellular localization of NEDL1 E3.** *A*, alignment of conserved amino acid sequences of human NEDL1 (*hNEDL1*) and its mouse homolog (*mNEDL1*). *Numbers* on the right indicate the number of residues to the initiator methionine. The C2 domain (*shaded*), two WW domains (*dashed boxes*), and the HECT domain (*solid box*) are indicated. *B*, brain-specific expression of *NEDL1* mRNA. Total RNAs derived from the indicated adult (*left panel*) and fetal (*right panel*) human tissues were analyzed by Northern blotting using a ^{32}P-labeled human *NEDL1* cDNA restriction fragment as a probe. Control hybridization with a human β-actin cDNA probe verified the equal amount of RNA loaded. *C*, expression of *NEDL1* in human brain subsections. Total RNA from the cerebral cortex, corpus callosum, cerebral peduncles, spinal cord, or placenta was subjected to RT-PCR using specific primers for *NEDL1* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). RT-PCR analysis for *NEDL1* in the placenta provided a negative control. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. *D*, confocal microscopic images of human neuroblastoma CHP134 cells (*left panel*) and COS-7 cells transfected with an expression plasmid for NEDL1 (*right panel*). Cells were subjected to immunofluorescence analysis using rabbit anti-NEDL1 polyclonal antibody, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. E, *in vitro* ubiquitination assays showing that NEDL1 has a ubiquitin-protein ligase activity. The degree of ubiquitination was increased in an NEDL1-dependent manner. In this assay, yeast ubiquitin-activating enzyme (*E1*), bacterially expressed ubiquitin carrier protein (*E2*; UbcH5C or UbcH7), and bacterial lysates were incubated in the presence or absence of increasing amounts of glutathione *S*-transferase (*GST*)-NEDL1. Polyubiquitinated bacterial proteins appeared to migrate in a high molecular mass complex. *Ub*, ubiquitin.

expression was also weakly detected in adult kidney, where the size of the expressed transcript appeared to be <7 kb. Expression of *NEDL1* in specific regions of the nervous system was further confirmed in the cerebral cortex, corpus callosum, cerebral peduncles, and spinal cord by RT-PCR (Fig. 1*C*). Thus, NEDL1 is a novel HECT-type E3 preferentially expressed in neuronal tissues, including the spinal cord. Using a specific anti-NEDL1 polyclonal antibody that we generated, we localized NEDL1 primarily to the cytoplasm in both intact human neuroblastoma CHP134 cells and COS-7 cells transiently expressing NEDL1 (Fig. 1*D*). The *in vitro* system containing UbcH5c or UbcH7 demonstrated that NEDL1 has a ubiquitin-protein ligase activity (Fig. 1*E*).

NEDL1 Physically Interacts with TRAP-δ and Mutant SOD1—We then sought protein-binding partners of NEDL1 by yeast two-hybrid screening using the region including two WW protein interaction domains (amino acids 757–1114) as bait. Of 96 positive clones subjected to DNA sequencing, one was a full-length cDNA for TRAP-δ; this was of considerable interest, as TRAP-δ was previously reported to bind mutant (G85R and G93A), but not wild-type, SOD1 (9). TRAP-δ is a protein component of the translocon in the ER membrane (10). We therefore examined the interaction among NEDL1, TRAP-δ, and SOD1 by an immunoprecipitation assay after cotransfecting the corresponding expression constructs into COS-7 cells. As shown in Fig. 2 (*A* and *B*), NEDL1 was physically associated with both exogenous and endogenous TRAP-δ probably through the region of two WW domains, as originally suggested by the result of two-hybrid screening. Surprisingly, NEDL1 bound to mutant (but not wild-type) SOD1 (Fig. 2*C*). Furthermore, the degree of binding between NEDL1 and different mutant SOD1 proteins was roughly proportionate to the rapidity of progression (time from clinical onset to death) of the associated FALS phenotype (17–23). For example, two mutant SOD1 proteins associated with an extremely rapid clinical course (C6F and A4V) interacted very strongly with NEDL1. By contrast, the binding of NEDL1 to other mutants was less striking and decreased proportionately to the falloff of disease severity corresponding to those mutants. Of further interest, like the NEDL1-mutant SOD1 interaction, the binding intensity between TRAP-δ and mutant SOD1 was also dependent on the disease severity (Fig. 2*D*). These observations suggest that NEDL1 and TRAP-δ are normally associated with each other, but that misfolded mutant SOD1 makes a complex with them. Such a complex is not formed with wild-type SOD1. The experiments using the *in vitro* translated proteins suggested that association of mutant SOD1 and TRAP-δ was direct (data not shown). It therefore appears that mutant SOD1 forms tightly bound protein complexes with NEDL1 and TRAP-δ and that the tightness of binding in the complex is determined in part by

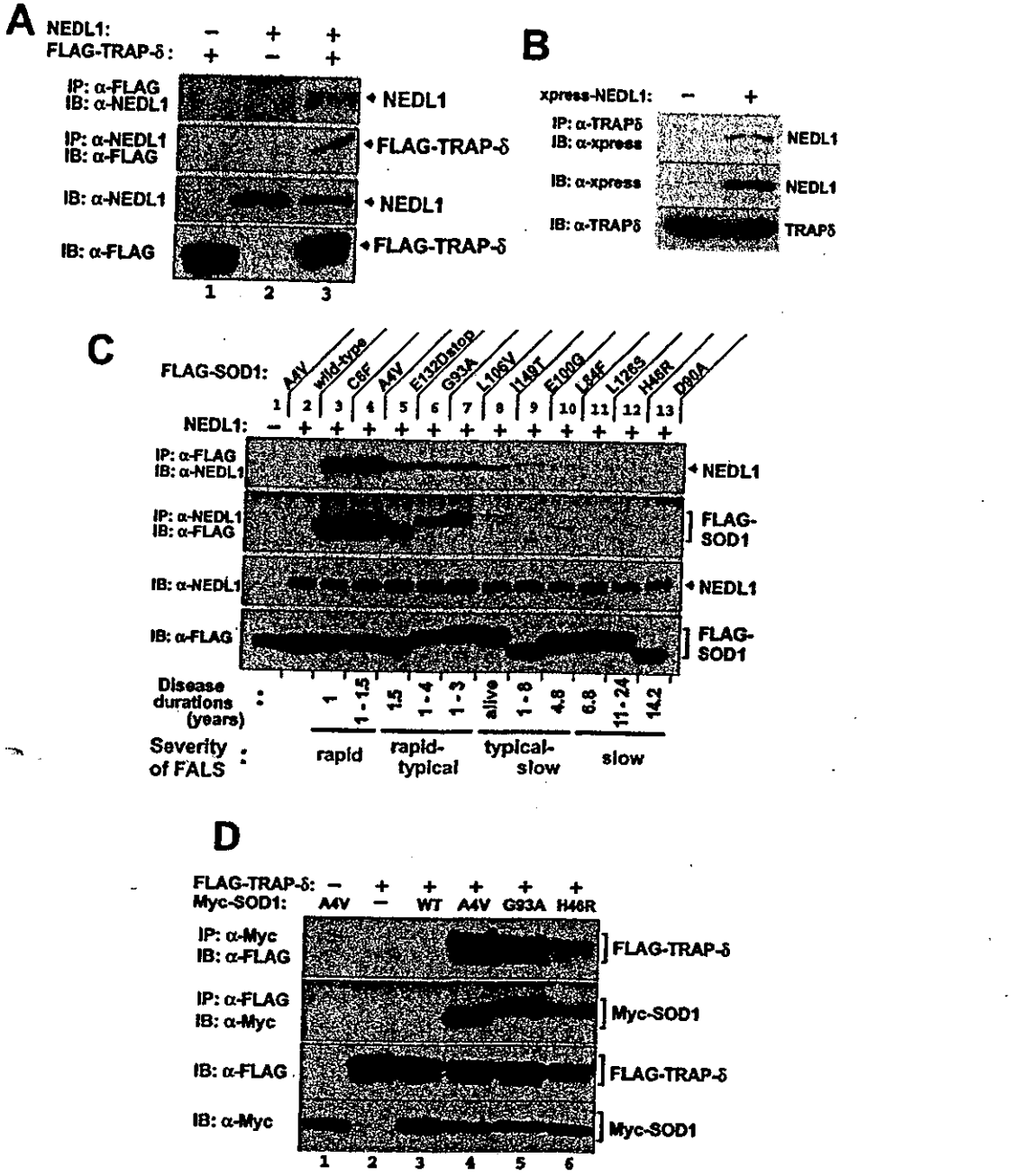

FIG. 2. **NEDL1 interacts with TRAP-δ and FALS-associated mutant forms of SOD1, but not with wild-type SOD1.** *A*, NEDL1 interacts with TRAP-δ. COS-7 cells were cotransfected with the indicated expression plasmids, and whole cell lysates were immunoprecipitated (*IP*) with anti-FLAG (*first panel*) or anti-NEDL1 (*second panel*) antibody. Immunoprecipitates were analyzed by immunoblotting (*IB*) using the indicated antibodies. Whole cell lysates were analyzed for expression levels of each protein by immunoblot analysis (*third* and *fourth panels*). Detection was performed with horseradish peroxidase-conjugated secondary antibodies. *B*, NEDL1 also binds to endogenous TRAP-δ. *C*, interaction between NEDL1 and mutant SOD1. Whole cell lysates from COS-7 cells overexpressing NEDL1 and one of the FLAG-tagged SOD1 mutants or wild-type SOD1 were immunoprecipitated with anti-FLAG (*first panel*) or anti-NEDL1 (*second panel*) antibody and then immunoblotted with anti-NEDL1 or anti-FLAG antibody, respectively. The expression of NEDL1 or FLAG-tagged SOD1 mutants was analyzed by immunoblotting using anti-NEDL1 (*third panel*) or anti-FLAG (*fourth panel*) antibody, respectively. Patients carrying the SOD1(C6F) and SOD1(A4V) mutations have a rapid clinical course, whereas mutant SOD1(L126S), SOD1(H46R), or SOD1(D90A) is associated with a slow clinical course. *D*, interaction of TRAP-δ with mutant SOD1. COS-7 cells were transiently cotransfected with the expression plasmid for FLAG-tagged TRAP-δ and the expression plasmid encoding one of the Myc-tagged SOD1 mutants or wild-type (*WT*) SOD1. Whole cell lysates were immunoprecipitated with anti-Myc (*first panel*) or anti-FLAG (*second panel*) antibody, followed by immunoblotting with anti-FLAG or anti-Myc antibody, respectively. The levels of overexpression of FLAG-tagged TRAP-δ (*third panel*) and Myc-tagged SOD1 (*fourth panel*) were analyzed by immunoblotting using anti-FLAG and anti-Myc antibodies, respectively.

properties of the mutant enzyme that also modulate disease severity of the resulting ALS phenotype. Such complexes do not form in cells with wild-type SOD1.

Determination of the Interaction Domains—We next examined the domains of NEDL1 required for formation of the SOD1·NEDL1·TRAP-δ complex. We generated various constructs of NEDL1 with deletions of each domain. Fig. 3 shows the results of immunoprecipitation assay for the association between deletion mutants of NEDL1 and mutant SOD1(G93A). Mutant SOD1 bound weakly to NEDL1 lacking WW domain-1 (Fig. 3*A*), suggesting that WW domain-1 and its surrounding portion are the region involved in their interaction. Immuno-

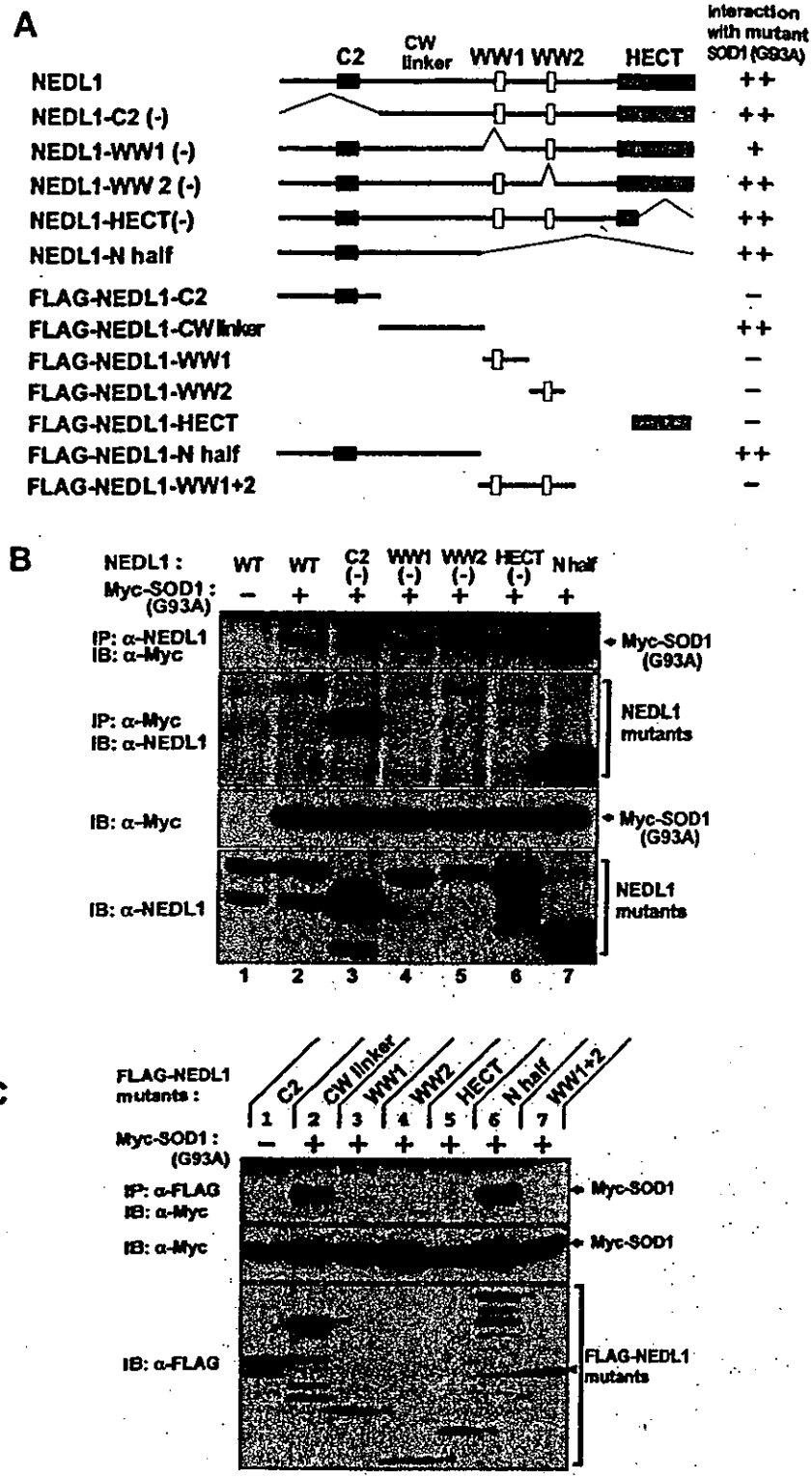

FIG. 3. **The region of NEDL1 between the C2 domain and WW domain-1 is required for interaction with mutant SOD1.** *A*, schematic illustration of wild-type NEDL1 and a series of deletion mutants of NEDL1. *CW linker* indicates the region between the C2 domain and WW domain-1 (*WW1*). *B* and *C*, immunoprecipitation and immunoblot analyses. In *B*, Myc-tagged mutant SOD1(G93A) was overexpressed together with wild-type (*WT*) NEDL1 or the indicated deletion mutants of NEDL1 in COS-7 cells. Whole cell lysates were immunoprecipitated (*IP*) with anti-NEDL1 (*first panel*) or anti-Myc (*second panel*) antibody, followed by immunoblotting (*IB*) with anti-Myc or anti-NEDL1 antibody, respectively. The expression levels of each protein were analyzed by immunoblotting using the indicated antibodies (*third* and *fourth panels*). In *C*, whole cell lysates were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-Myc antibody (*upper panel*). Whole lysates were also analyzed by Western blotting for each protein (*middle* and *lower panels*).

precipitation analysis using the specific regions of NEDL1 clearly showed that the region between the C2 domain and WW domain-1 (CW linker region) is necessary for binding to mutant SOD1(G93A). Mutant SOD1(A4V) was also associated with NEDL1 through the same region, and TRAP-δ bound to the two WW domains of NEDL1 (data not shown).

NEDL1 Ubiquitinates Mutant SOD1 for Degradation Depending on the Disease Severity of FALS—Because NEDL1 is an E3, we next tested whether it ubiquitinates TRAP-δ and mutant SOD1 for degradation. As shown in Fig. 4*A*, NEDL1 clearly ubiquitinated mutant SOD1(A4V), but not TRAP-δ (data not shown). Furthermore, the degree of ubiquitination of mutant SOD1 by NEDL1 was dependent on the disease severity of FALS (A4V > G93A > H46R) (Fig. 4*A*). Fig. 4*B* shows the time course of degradation of wild-type and mutant SOD1 in the presence or absence of NEDL1. As reported previously (46), mutant SOD1 was degraded more rapidly than wild-type SOD1. NEDL1 did not affect wild-type SOD1 degradation. As expected from the co-immunoprecipitation and ubiquitination analyses, degradation of mutant SOD1 was stimulated by NEDL1 proportionately to the disease severity of FALS caused by the particular SOD1 mutant (A4V > G93A > H46R ≥

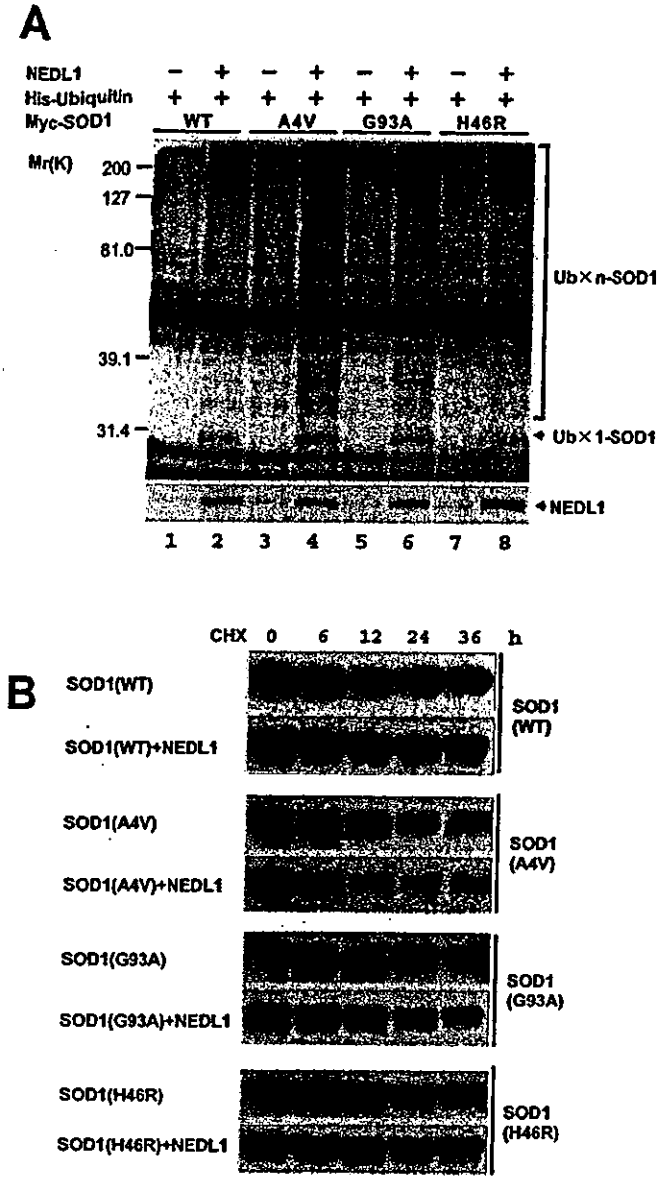

FIG. 4. **NEDL1-dependent ubiquitination and degradation of mutant forms of SOD1 correlate broadly with their respective clinical phenotypes.** *A*, NEDL1 ubiquitinates mutant SOD1 in a mutant type-dependent manner. COS-7 cells were transiently cotransfected with the indicated expression plasmids. Whole cell lysates from transfected COS-7 cells were immunoprecipitated with anti-Myc antibody, and immunoprecipitates were analyzed by Western blotting with anti-ubiquitin (*Ub*) antibody (*upper panel*). The *bracket* indicates slowly migrating ubiquitinated forms of SOD1. Whole cell lysates were analyzed by immunoblotting with anti-NEDL1 antibody to confirm the expression of transfected NEDL1 (*lower panel*). The running positions of molecular weight markers are indicated on the left. *B*, half-lives of wild-type (*WT*) and mutant SOD1 proteins in the presence or absence of NEDL1. Cell lysates were harvested from Neuro2a cells transfected with *SOD1* alone or with *SOD1* plus *NEDL1* at different time points as indicated after the addition of cycloheximide (*CHX*; final concentration of 50 μg/ml) and were analyzed for SOD1 protein levels by Western blotting with anti-FLAG antibody. In the presence of NEDL1, the half-lives of various mutant SOD1 proteins were reduced also roughly dependent on the disease severity of FALS (A4V > G93A > H46R).

wild-type). Thus, NEDL1 targeted mutant SOD1 for ubiquitin-mediated degradation in the cell in parallel with the binding intensity.

Immunohistochemistry—One of the characteristic cytopathological changes of mutant SOD1-linked FALS is the formation of neuronal Lewy body-like hyaline inclusions (LBHIs) that contain aggregates of SOD1 and ubiquitin (24). We therefore

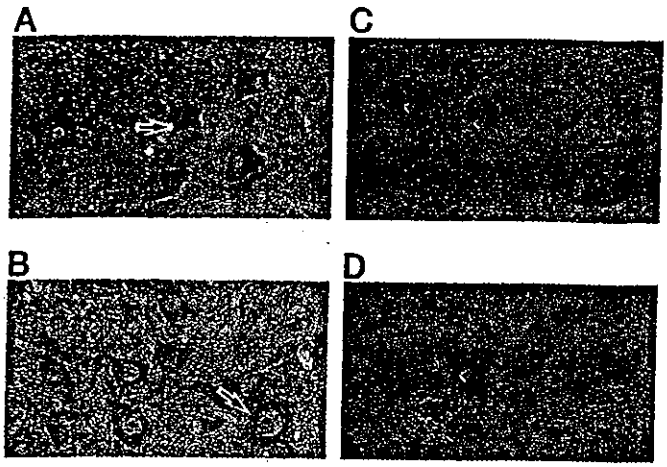

FIG. 5. **NEDL1 immunohistochemical analyses.** *A*, immunohistochemical analysis of NEDL1 in normal human spinal cord. NEDL1-positive anterior horn cells are evident (*arrow*), although the immunoreactivity for NEDL1 is somewhat faint. There was no counterstaining. Magnification ×520. *B*, NEDL1 immunohistochemistry in normal mouse spinal cord. Normal anterior horn cells are positive for NEDL1 (*arrow*). The section was counterstained with hematoxylin. Magnification ×750. *C*, immunostaining for NEDL1 in spinal cord LBHIs from an FALS patient with a frameshift 126 mutation in the *SOD1* gene. The NEDL1-positive reaction products were mostly restricted to the cores of the core and halo-type LBHIs (*arrowheads*). In the LBHI-bearing neurons and residual neurons, the antibody to NEDL1 also stained the neuronal cell body. There was no counterstaining. Magnification ×540. *D*, NEDL1 immunostaining in a spinal cord LBHI from an *SOD1*(H46R) transgenic mouse. An ill defined LBHI in the *SOD1*(H46R) transgenic mouse was positive for NEDL1; this ill defined LBHI shows a diffuse staining pattern (*arrowhead*). The staining intensity in the residual neurons stained by anti-NEDL1 antibody varied from neuron to neuron. The section was counterstained with hematoxylin. Magnification ×770.

performed immunostaining to determine whether the NEDL1 protein is included within the LBHIs of the spinal cord motor neurons obtained from two siblings with FALS caused by frameshift 126 mutation of SOD1 (11, 12). One case had neuropathological findings compatible with FALS with posterior column involvement, whereas the other had multisystem degeneration in addition to motor neuron disturbance. We also performed NEDL1 immunostaining in specimens obtained from mutant *SOD1*(H46R) transgenic mice at 180 days, by which time they show clinical motor signs in the hind limbs (13). The specificity of the NEDL1 staining was confirmed by pretreating the specimens with an excess of NEDL1 antigen. NEDL1 immunoreactivity in the spinal cords of the human control cases was identical to that of normal mice: immunoreactivity was identified predominantly in the cytoplasm of the neurons of the spinal cords (Fig. 5, *A* and *B*). The LBHIs in the anterior horn cells of two FALS patients and transgenic mice showed equivalent immunoreactivity for NEDL1. Although the intensity of NEDL1 immunoreactivity in neuronal LBHIs varied, most of the LBHIs were immunoreactive for NEDL1 (Fig. 5, *C* and *D*). The reaction products were generally restricted to the cores of the core and halo-type LBHIs that showed eosinophilic cores with pale peripheral halos upon hematoxylin and eosin staining (Fig. 5*C*); by contrast, immunopositive NEDL1 in ill defined LBHIs was distributed throughout the inclusions (Fig. 5*D*). NEDL1 immunoreactivity in the residual neurons in humans and mice was identified primarily in cell bodies. Thus, NEDL1 immunostaining was clearly positive in the FALS-related LBHIs that were also positive for ubiquitin and SOD1 (data not shown).

NEDL1 Targets Dishevelled-1 for Ubiquitin-mediated Protein Degradation—We next hypothesized that the physiological function of NEDL1 to mediate ubiquitination is interfered with

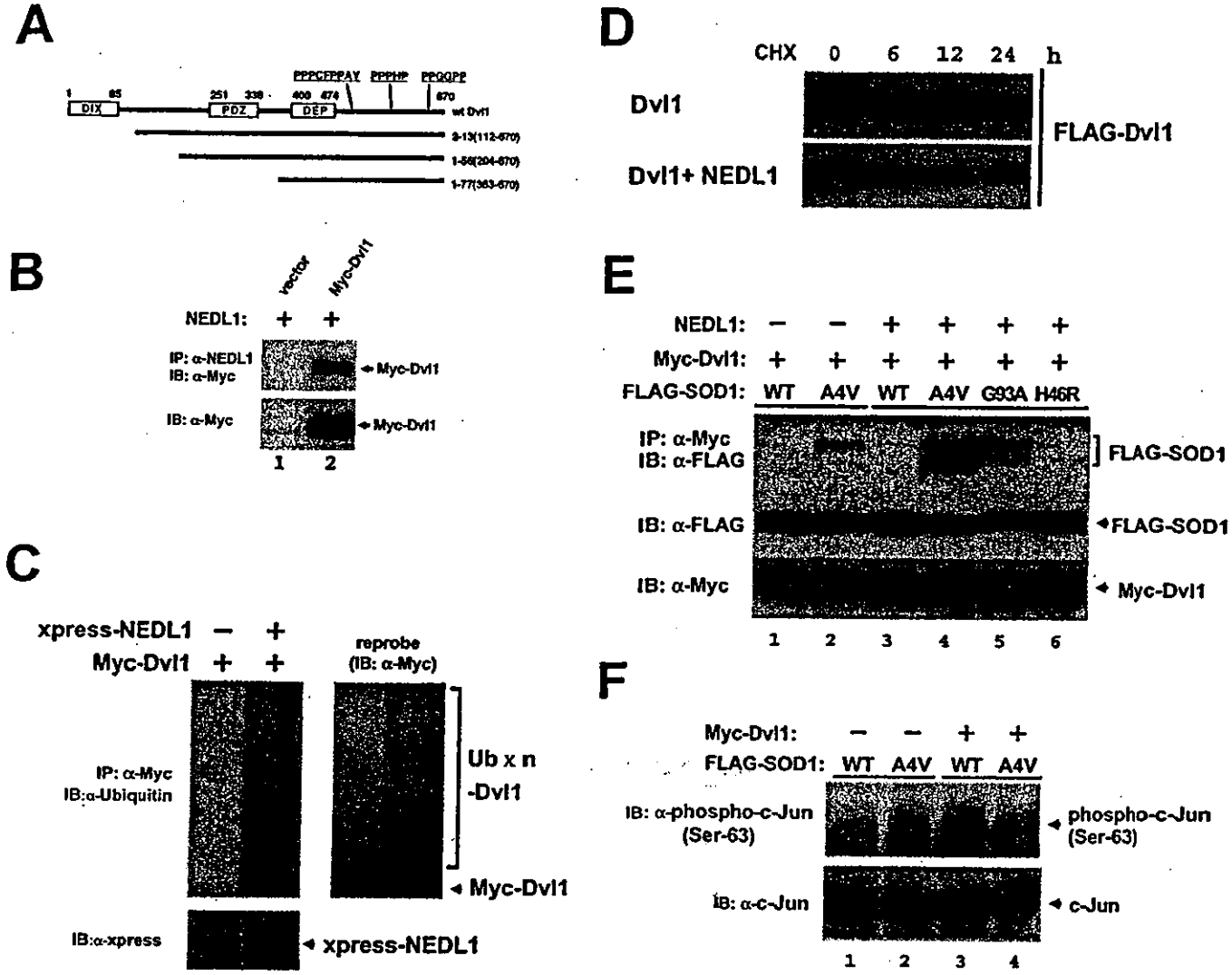

FIG. 6. **Dvl1 is a substrate of NEDL1, and its functions are disturbed by mutant SOD1(A4V).** *A*, schematic illustration of full-length Dvl1 and three clones obtained by yeast two-hybrid screening. Human Dvl1 consists of 670 amino acids and contains three conserved domains, including the DIX, PDZ, and DEP domains. Between the DEP domain and the C-terminal end, there are three proline-rich clusters, which might act as WW domain recognition sites. All three clones (clones 2–13, 1–56, 1–77) contain the DEP domain and these clusters. *B*, NEDL1 interacts with Dvl1. Myc-tagged Dvl1 was overexpressed together with NEDL1 in Neuro2a cells. Whole cell lysates were immunoprecipitated (*IP*) with anti-NEDL1 antibody, followed by immunoblotting (*IB*) with anti-Myc antibody (*upper panel*). The expression levels of Myc-tagged Dvl1 were analyzed by immunoblotting using anti-Myc antibody (*lower panel*). *C*, NEDL1 ubiquitinates Dvl1 in Neuro2a cells. The cells were transiently transfected with the indicated expression plasmids along with the ubiquitin expression plasmid in the presence or absence of the expression plasmid for XPRESS-tagged NEDL1. Whole cell lysates were immunoprecipitated with anti-Myc antibody and then immunoblotted with anti-ubiquitin antibody (*left panel*). The ladder of bands denoted by the *bracket* appeared to be ubiquitinated Dvl1. The expression of XPRESS-NEDL1 was analyzed by immunoblotting using anti-XPRESS antibody. The membrane was reprobed with anti-Myc antibody (*right panel*). *D*, Dvl1 is degraded by NEDL1. Neuro2a cells were transfected with the expression plasmid for FLAG-tagged Dvl1 with or without the NEDL1 expression plasmid. Transfected cells were harvested at different time points as indicated after the addition of cycloheximide (*CHX*; final concentration of 50 μg/ml), and Dvl1 protein levels were analyzed by Western blotting with anti-FLAG antibody. In the presence of NEDL1, the half-lives of FLAG-Dvl1 were significantly reduced. *E*, Dvl1 binds to mutant SOD1(A4V), and the degree of its binding is enhanced in the presence of NEDL1. Whole cell lysates prepared from COS-7 cells transfected with the indicated combinations of expression plasmids were subjected to immunoprecipitation and Western analyses as indicated. *F*, c-Jun phosphorylation by overexpression of Dvl1 is suppressed upon coexpression of mutant SOD1(A4V). Whole cell lysates from COS-7 cells transfected with the indicated combinations of expression plasmids were subjected to Western blotting with antibody against the phosphorylated form of c-Jun (*upper panel*) or with anti-c-Jun antibody (*lower panel*). *wt/WT*, wild-type.

by mutant SOD1. To test this hypothesis, we again performed yeast two-hybrid screening to obtain NEDL1-interacting molecules using the large region of NEDL1 (amino acids 382–1448) as bait. Of 396 His and β-galactosidase double-positive clones, 282 clones were subjected to DNA sequencing, and we identified Dvl1 (three clones). Human Dvl1 is a 670-amino acid protein with three conserved domains: a DIX domain, which is required for canonical Wnt/T-cell factor signaling; a PDZ domain, which is a target of both Stbm and casein kinase I binding; and a DEP domain, which is responsible for Dvl membrane localization during planar cell polarity signaling (25–27). Between the DEP domain and C-terminal end, there are three proline-rich clusters unique to mammalian Dvl1, which presumably act as the WW domain recognition sites. All three clones (clones 2–13, 1–56, and 1–77) contain the DEP domain and proline-rich clusters, suggesting that NEDL1 interacted with Dvl1 in the C-terminal half (Fig. 6*A*). In Neuro2a cells, NEDL1 co-immunoprecipitated with Dvl1 (Fig. 6*B*) and ubiquitinated it for degradation (Fig. 6, *C* and *D*). Thus, Dvl1 may be one of the physiological targets of NEDL1 E3. As recent studies strongly suggest that the cytotoxicity of SOD1 mutants is responsible for their aggregate properties, incorporating other proteins essential for cells into their aggregates (28), we examined the association between mutant SOD1 and Dvl1,

both of which interact with NEDL1. Of interest, Dvl1 bound to mutant SOD1(A4V), and complex formation was increased in the presence of NEDL1 roughly proportionately to the disease severity of FALS caused by the particular SOD1 mutant (Fig. 6*E*). Dvl1 is known to transduce not only the Wnt/β-catenin/T-cell factor pathway, but also the JNK/c-Jun pathway (27). Therefore, we next examined whether the Dvl1-induced phosphorylation of c-Jun at Ser^{63} was affected by the tight complex formation induced by inclusion of mutant SOD1. As shown in Fig. 6*F*, c-Jun phosphorylation induced by overexpression of Dvl1 was significantly suppressed by coexpression with mutant SOD1(A4V) in COS-7 cells.

DISCUSSION

Our present results demonstrate that a novel HECT-type NEDL1 E3, which is preferentially expressed in neuronal tissues, specifically targets mutant forms of SOD1 for ubiquitination-mediated protein degradation. NEDL1 is also associated with TRAP-δ localized at the ER translocon. The TRAP complex has recently been shown to facilitate the initiation of protein translocation in a substrate-specific manner (29). The NEDL1·TRAP-δ complex recognizes mutant (but not wild-type) SOD1, with a binding intensity that broadly parallels the disease severity of FALS. NEDL1 immunoreactivity was detected in the FALS-related LBHIs in the spinal cord ventral horn motor neurons, suggesting that, although mutant SOD1 is ubiquitinated for degradation by NEDL1, the mutant SOD1·NEDL1·TRAP-δ complex aggregates within the LBHIs. It is also conceivable that fragmentation of the Golgi apparatus reported in ALS patients and transgenic mice might be related to this aggregation (30, 31). These findings suggest possible hypotheses for the role of NEDL1 in the pathogenesis of FALS: 1) NEDL1, alone or with TRAP-δ, ubiquitinates and aggregates mutant SOD1, thereby decreasing the function of mutant SOD1; 2) NEDL1 and TRAP-δ form aggregates with mutant SOD1 that induce fragmentation of the Golgi apparatus, leading to neuronal apoptosis; 3) formation of these aggregates causes dysfunction of NEDL1 and/or TRAP-δ, and this, in turn, induces disturbances that ultimately cause motor neuron death; and 4) the mutant SOD1·NEDL1·TRAP-δ aggregates trap and inactivate unknown factor(s) such as molecular chaperones whose normal function is important for motor neuron viability.

To further understand the role of NEDL1 in motor neuron death, we searched for the physiological targets of NEDL1 and identified Dvl1. As expected, Dvl1 is ubiquitinated for degradation by NEDL1. Surprisingly, however, Dvl1 also interacts with mutant SOD1 in the presence of NEDL1 roughly proportionately to the disease severity of FALS caused by the particular SOD1 mutant. Dvl1, an essential multimodule signal transducer localized in the cellular cytosol and cytoskeleton, mediates planar cell polarity signaling as well as canonical Wnt/β-catenin signaling (27, 32). In mammals, three Dvl family members have so far been reported, and the level of Dvl1 expression is high in neuronal tissues (33). As far as we know, NEDL1 is the first E3 for Dvl1, interacting with the C-terminal region containing three proline-rich clusters. A recent report suggests that Dvl1 regulates microtubule stability through inhibition of glycogen synthase kinase-3β (34). Because cytoskeletal abnormalities have been reported in ALS motor neurons (35), it is possible that the effect of mutant SOD1 on NEDL1-mediated Dvl1 degradation is involved in the motor neuron death. Furthermore, Dvl1 is abundant in the postsynaptic membrane region at the neuromuscular junction (36) that is reported to be involved in several neurodegenerative disorders (37, 38). Of interest, *Dvl1* is mapped to chromosome 1p36, which is a commonly deleted region in many human cancers, including neuroblastoma (39). As NEDL1 is highly expressed in neuroblastomas with favorable prognosis, which have a tendency to differentiate and/or regress, NEDL1 may be involved in the regulation of neuronal differentiation and survival possibly by controlling Dvl1.

NEDL1, TRAP-δ, mutant SOD1, and Dvl1 appear to form a complex roughly proportionately to the disease severity of FALS caused by the particular SOD1 mutant. Our present observations strongly suggest that NEDL1 may be a quality control E3 recognizing misfolded mutant SOD1 (40). The association between mutant SOD1 and NEDL1 may induce the conformational change in the NEDL1 protein to increase the binding intensity with other physiological targets such as TRAP-δ (not ubiquitinated) and Dvl1 (ubiquitinated). This may lead to tight complex formation especially when the proteasome activity is impaired. It has been reported that the expression and function of proteasomes decrease with age in the spinal cord (7). Okado-Matsumoto and Fridovich (41) have also found that complex formation between mutant SOD1 and heat shock proteins leads to protein aggregates. Because our data show that the ER translocon component TRAP-δ is involved, aggregate formation may occur at the sites of the ER or Golgi apparatus or even at other cellular sites. The complex formation including NEDL1 and mutant SOD1 may conversely affect the physiological function of NEDL1, as demonstrated by a decrease in Dvl1-induced phosphorylation of c-Jun.

Recently, the RING finger-type E3 Dorfin has been reported to ubiquitinate mutant SOD1 for degradation (42). However, NEDL1 and Dorfin appear to be different in several aspects. First, NEDL1 is expressed specifically in neuronal tissues, including the spinal cord, whereas Dorfin is ubiquitously expressed in most human tissues. Second, both interaction between NEDL1 and mutant SOD1 and ubiquitination of the latter by NEDL1 roughly parallel the disease severity caused by the particular SOD1 mutant, whereas Dorfin similarly ubiquitinates mutant forms of SOD1. In addition, we have identified Dvl1 and TRAP-δ as cellular target proteins of NEDL1, whereas the physiological targets of Dorfin have never been reported. It is probable that there are some other E3 ligases targeting mutant SOD1. However, the molecular characteristics, including tissue-specific expression, subcellular localization, and age-dependent expression, might be important in the development of the FALS phenotype.

In conclusion, we have identified a novel neuronal E3 (NEDL1) that interacts with TRAP-δ and also binds to and ubiquitinates Dvl1 for degradation. Strikingly, NEDL1 targets and ubiquitinates mutant (but not wild-type) SOD1 for degradation. NEDL1 may normally function in the quality control of cellular proteins by eliminating misfolded proteins such as mutant SOD1, possibly via a mechanism analogous to that of ER-associated degradation (43–45). NEDL1 appears to complex tightly with mutant SOD1, Dvl1, and TRAP-δ, forming aggregates with species of mutant SOD1 that have escaped ubiquitin-mediated degradation. The NEDL1 function that affects the activities of the target proteins may also be modulated by mutant SOD1. All of these might contribute to the pathogenesis of FALS; further elucidation of the molecular mechanism of formation of this complex and its pathogenicity may provide insights into motor neuron death in ALS as well as possible new therapeutic strategies for ALS.

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