

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 important 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

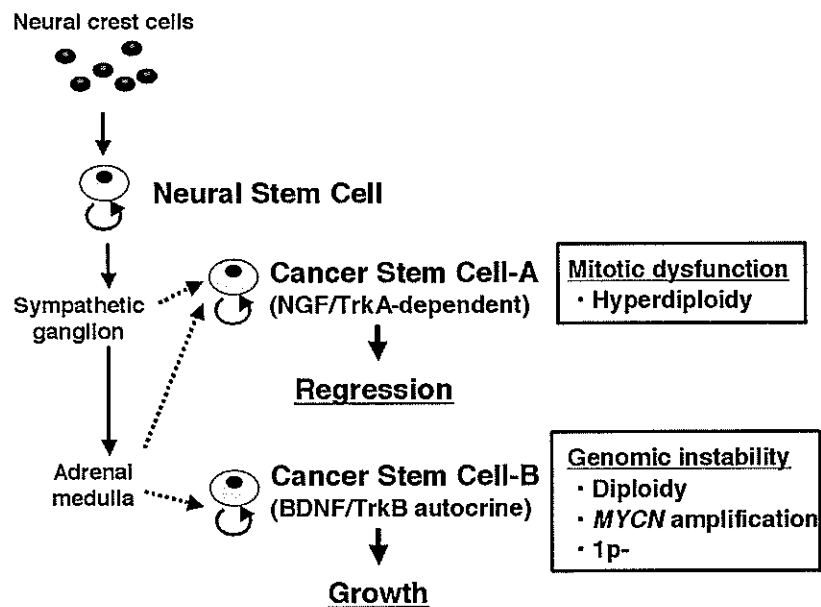


Fig. 5. The hypothesis of cancer stem cells in neuroblastoma.

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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Comprehensive genomics linking between neural development and cancer: neuroblastoma as a model

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Abstract

Cancer cells are derived from their precursor cells, which normally develop to the matured cells to form individual organs. Neuroblastoma, one of the most common pediatric solid tumors, originates from possible cancer stem cells derived from the neural crest. During the development, neural crest cells segregate into several lineages such as sensory, enteric and sympathetic neurons. However, the genetic events to cause neuroblastoma occur only in the sympathetic precursor cells or cancer stem cells. Furthermore, spontaneous regression of a subset of neuroblastoma found in patients under one year of age mimics a developmentally programmed neuronal cell death that occurs in normal sympathetic neurons during the perinatal period. Thus, the genetic events to cause neuroblastoma may be programmed to occur in a lineage-specific as well as developmentally regulated manner. In this review, we discuss about the molecular link between neural development and the genesis of neuroblastoma based on our comprehensive genomics approach.

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Keywords: Comprehensive genomics; Neuroblastoma cDNA project; Neural development

1. Introduction

Neuroblastoma (NBL) is one of the most common childhood cancers and is originated from the sympathetic precursor cells derived from the neural crest [1]. The clinical behavior of NBL is unique and enigmatic: the tumor cells spontaneously regress in patients under one year of age by undergoing differentiation and/or apoptosis, whereas the tumor often grows aggressively and eventually

kills the patient when it occurs after one year of age [2,3]. It is thus mysterious why NBL regress or grow in an age-dependent manner. In 1963, Beckwith and Perrin have reported an interesting observation that only one of 40 microscopic fetal neuroblastomas has later become symptomatic, sporadic tumors. The remaining tumors disappeared spontaneously, suggesting that one of 250 (0.4%) fetuses has an 'in situ neuroblastoma' [4]. This observation may at least in part explain the result of NBL mass-screening performed in infants at the age of six months in Japan [5], because most of those tumors regress or take a favorable clinical course without decreasing the incidence of aggressive NBLs which are usually found in

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the patients over one year of age [6,7]. These have suggested that regression of NBL, which mimics the developmentally regulated 'programmed cell death (PCD)' of neurons [8,9], is still able to occur for about 12 months after birth. However, this idea cannot simply explain why NBLs found in the patients over one year of age do not regress.

It is highly possible that the molecular mechanism of normal development of sympathetic neurons is closely related to the regulation of NBL biology. Some important molecules of NBL, such as MYCN [10] and Trk [11], are already known to be the key players to regulate maturation of neural crest cells during development. However, we still miss a large number of genes or molecules playing important roles in the regulation of NBL biology and its genesis. In this review, we introduce a current knowledge about the molecular link between NBL and normal development of sympathetic cells, and discuss the future approaches to further understanding of the enigmatic tumor, NBL.

2. Neuronal lineage and oncogenic events

The oncogenic events to cause NBL appear to be strictly regulated and lineage-dependent, because NBL never occurs from the neural precursors of

other than sympathoadrenal cell lineage (Fig. 1). The cell fate determination may be regulated by multiple transcription factors and their target genes which may include those of growth factors and their receptors [12,13]. The possible candidate genes to decide the direction of sympathetic differentiation include a human homolog of *Drosophila Acaete-Scute* proneural gene (hASH1) [14,15] and *Phox2a* [16,17] and *Phox2b* [18] of homeodomain genes. However, the precise mechanism is still elusive.

Another aspect of neuronal lineage specificity of the oncogenic events is shown by the mutation pattern of the *Trk* family genes [19]. *Trk* genes are specifically or preferentially expressed in neuronal tissues and cells. Nevertheless, the oncogenic *Trk* genes derived from translocation or somatic mutation have been exclusively observed in human malignancies with non-neuronal origin (colon cancer, papillary thyroid cancer, and acute myeloid leukemia). On the other hand, the expression levels of prototype *Trk* regulate the biology in cancers originated from neuronal precursor cells (neuroblastoma, medulloblastoma, thyroid C-cell hyperplasia and medullary thyroid cancer). Therefore, it should be interesting to know how the precursor cells choose the way to cause or regulate the cancer in a lineage-specific manner, that is absolutely unclear at this time.

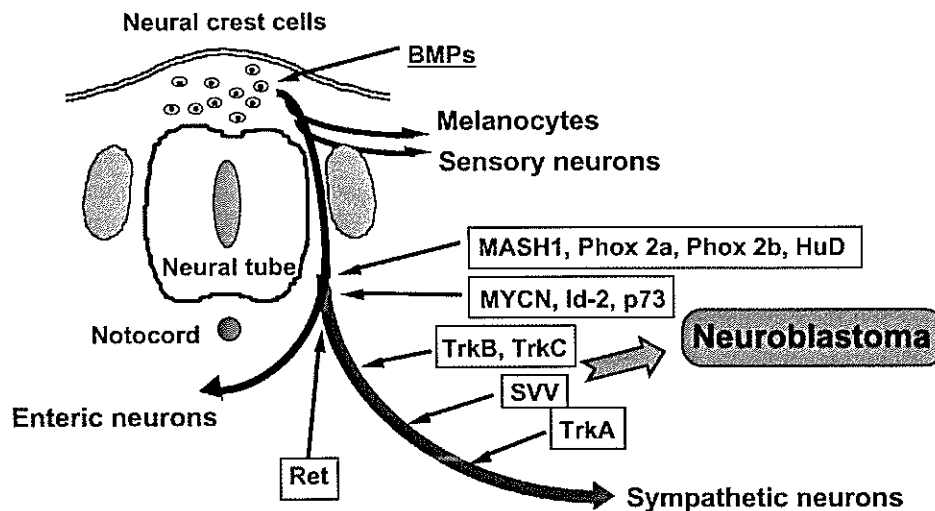


Fig. 1. Developmental lineages derived from the neural crest cells and the genesis of neuroblastoma.

3. Neural crest development and the molecules related to neuroblastoma

Neuroblastomas express many genes that are involved in the regulation of neural crest development [20–22]. The bone morphogenetic protein (BMP) signals regulate early stage of neural crest cell migration and differentiation before the decision to enter the sympathetic lineage [23], from which NBL exclusively arises. Of interest, the BMP signal is still functioning in many NBL cell lines because the treatment of the cells with BMP2 induced both phosphorylation of Smad1 and neurite outgrowth (Y. Nakamura, unpublished data). This suggests that NBLs still possess the ability to respond to the ligands that have worked at the early stage of normal neural development. Similarly, Delta/Notch signaling appears to function in NBLs by regulating neurite outgrowth [24]. Hypoxia, which induces dedifferentiation in NBL cells, decreased the expression of Notch1 [25].

hASH1 continues to be expressed at high levels in many neuroblastomas [26,27]. It is normally down-regulated after transient expression during the development of neural crest cells, that in turn promotes differentiation to the mature sympathetic neurons [28]. Targeted disruption of mouse homolog MASH1 has demonstrated the absence of sympathetic neurons [29], suggesting the important role of MASH1 expression in deciding the direction of sympathetic differentiation. Of interest, hASH1 is down-regulated during the NBL differentiation induced by retinoic acid [26,27]. hASH1 also directly represses expression of PACE4, a mammalian subtilin-like proprotein convertase that activates transforming growth factor (TGF)- β -related proteins such as BMPs [30]. This repression may shut off the BMP signaling and other factors in NBL cells. Expression of HES-1, a neuronal basic helix-loop-helix protein, represses hASH1 expression and leads the NBL cells to the status of de-differentiation [31]. As reported previously, MYCN targets Id-2 to induce its expression and the induced Id-2 inhibits the Rb tumor suppressor [32]. The still unidentified tumor suppressor(s) residing at the distal region of short arm of chromosome 1 might also be involved in this regulation because the allelic loss of the region is

well correlated with amplification of MYCN [33,34]. The homeodomain transcription factors, Phox2a and Phox2b, are also essential for differentiation of noradrenergic neurons [35]. They may play a role in regulating the biology of NBL.

Other transcriptional regulators, that affect both neural crest development and NBL, include MYCN [36], hypoxia-inducible factor 1 (HIF-1) [37] and the tumor suppressors p53 [38] and p73 [39]. MYCN is frequently targeted to amplify in aggressive NBLs [40,41]. Since the importance of MYCN in NBL is invaluable, it is precisely discussed by M. Schwab in the separate chapter of this special issue.

4. NGF family signaling and the role of p53 and p73 in neuroblastoma

The downstream regulators of neural crest cell differentiation include neurotrophic factors and their receptors. Expression of neurotrophin receptors, TrkA and TrkB tyrosine kinases, strongly affects the biology of NBL [2,9]. TrkA, a high-affinity receptor for nerve growth factor (NGF), is expressed at high levels in NBLs of the patients with favorable prognosis, whereas it is extremely down-regulated in the tumors of those with unfavorable outcome [8,42–44]. In contrast, TrkB, a high-affinity receptor for brain-derived neurotrophic factor (BDNF), is preferentially expressed in aggressive NBLs especially with MYCN amplification [45]. The NBLs with high expression of TrkA frequently regress spontaneously by undergoing neuronal apoptosis as well as differentiation [8]. On the other hand, the NBLs with low expression of TrkA and amplification of MYCN often kill the patients. In such tumor cells, BDNF and TrkB promote tumor cell growth and metastasis in an autocrine manner [46]. Thus, the neurotrophic factors and their receptors, that normally regulate terminal differentiation of neural crest cells, may manipulate the differentiation and survival of the NBL cells [19].

Recent investigations have revealed that p53 and its family member p73 play a pivotal role in the developmentally regulated PCD of sympathetic neurons in mice [47]. The p73-deficient mice show neurological and immunological defects [48]. Δ Np73,

an N-terminally truncated form of p73 lacking transactivation ability, is predominantly expressed in developing brain and sympathetic neurons in mice and inhibits the neuronal PCD by blocking the proapoptotic function of p53 [47]. This observation has suggested p53, p73 and $\Delta Np73$ to be the key regulators in the determination of neuronal differentiation and apoptosis. Interestingly, we and other investigators have recently found that p73 directly target $\Delta Np73$ for its expression by binding to the $\Delta Np73$ promoter. The induced $\Delta Np73$ then physically interacts with both p73 and wild type p53 to inhibit their function [49,50]. These relationships have suggested the presence of a negative feedback regulation of TAp73 by its target $\Delta Np73$ in modulating cell survival and death of sympathetic neurons as well as NBL cells. c-Myc has also been involved in this regulatory system [51]. Since it is well recognized that the PCD of sympathetic neurons is strongly regulated by NGF signaling, elucidation of the downstream regulatory mechanism of the signaling by p53, p73 and $\Delta Np73$ may become more important than ever. This may also help to understand the NBL biology regulated by NGF/TrkA signaling (Fig. 2). In NBL, the chromosome 1p36 region, to which p73 is mapped, is frequently deleted in

advanced stage tumors. Furthermore, $\Delta Np73$ is expressed at higher levels in unfavorable NBLs than in favorable ones [52]. In addition, according to the accumulating evidence, p73 may be one of the key factors of neural stem cells. Therefore, p73 could also be an important gene linking between neural development and cancer.

5. Comprehensive genomics of neuroblastoma

The rapid progress of the human genome project has enabled us to challenge the dynamic approaches to understanding of molecular genetic as well as cellular mechanism of cancers. It has also opened the door to unveil the molecular mechanism of neural development and neurogenic cancers. In NBL, the first trial to collect a large number of genes expressed in the CHP134 cell line was performed by K. Matsubara's group in Japan more than 10 years ago [20]. The cDNAs thus identified were published in 'Bodymap', the human gene expression database (<http://bodymap.ims.u-tokyo.ac.jp/>), including the information on gene expression in various tissues or cell types. Recently, R. Versteeg's group in Holland has introduced the method of serial analysis of gene

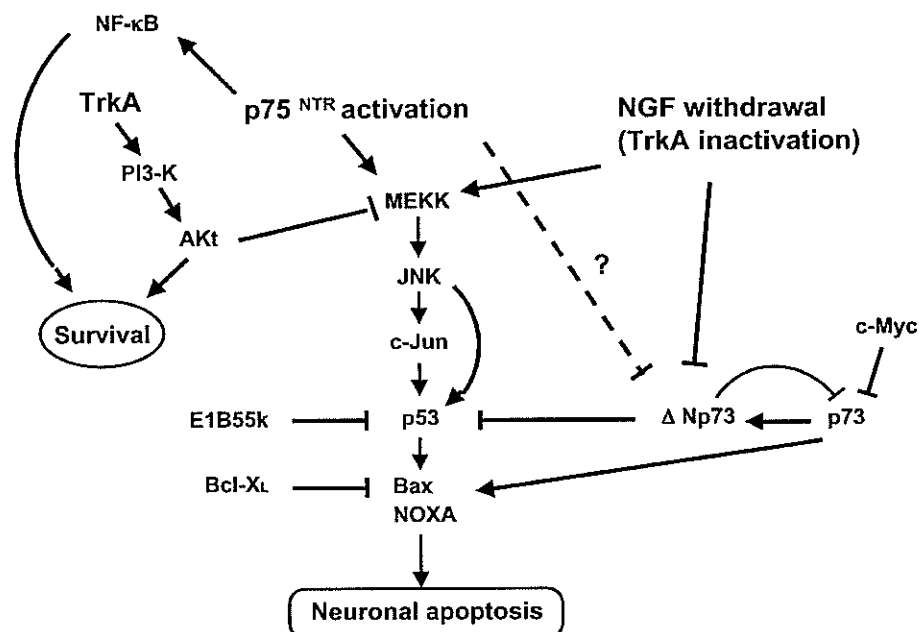


Fig. 2. The role of p53, p73 and $\Delta Np73$ in intracellular signaling of neuronal survival and apoptosis mediated by NGF and its receptors.

Table 1
Summary of the neuroblastoma cDNA project

Number of the total clones	9,729
Number of the gene clusters	6,252
<i>Differentially expressed genes between favorable and unfavorable subsets</i>	
Function, known	255/1,325 (19%)
Function, unknown	502/2,115 (24%)
Total	757/3,440 (22%)
Nervous system-specific genes	156/2,297 (7%)

expression (SAGE) to identify the genes expressed in primary NBLs and cell lines in a large scale [53]. They have successfully found MEIS as an amplified gene in NBL and also identified the MYCN target genes [54]. Thus, the SAGE procedure is powerful for identifying the already known genes. However, it is not suitable for the approach to determine the novel genes whose functions are elusive. Therefore, we have decided to identify the individual genes expressed in the typical subsets of primary NBLs by starting the 'NBL cDNA project' which directly clones the expressed genes in a large scale from the NBL oligo-capping cDNA libraries [21,22]. In this review, we briefly present the results of and discuss about 6,252 gene clusters identified from the screening of 9,729 clones randomly picked up from three different NBL cDNA libraries: three favorable (F: stage 1, high expression of *TrkA* and a single copy of *MYCN*), three unfavorable (UF: stage 3 or 4, decreased levels of *TrkA* expression and amplification of *MYCN*) and a typical stage 4s (4s: high expression of *TrkA* and a single copy of *MYCN*) NBL libraries. This is the extended result of the previous report [22] that analyzed 4,243 cDNA clones randomly picked up from the F and UF NBL cDNA libraries.

5.1. A large scale cloning of the expressed genes from different subsets of primary neuroblastomas (Neuroblastoma cDNA project)

We randomly obtained 2,410, 2,244 and 5,075 cDNA clones from F, UF and 4s cDNA libraries and successfully sequenced the both or either end of 2,134, 2,109 and 5,004 clones, respectively (Table 1). We identified an extremely high number of 2,115 genes with unknown function compared with that

(8%) found in the genes obtained from the cDNA libraries of childhood hepatoblastomas (HBLs) made by the same procedure (unpublished data). Interestingly, the average size of the genes obtained from NBLs (neuronal) was significantly larger than that from HBLs (hepatic) with an obvious tendency. We screened the genes for differential expression between F and UF NBLs by using semi-quantitative RT-PCR (16 F and 16 UF NBL samples) and found that 255 out of 1,325 known genes as well as 502 out of 2,115 novel genes were differential. In addition, RT-PCR analysis for expression in multiple human tissues showed that 156 of 2,297 genes, most of which were novel, displayed specific expression in neuronal tissues.

5.2. Expression profile and identification of the differentially expressed genes among the subsets

The expression profile of known genes was very different among the three subsets of NBL. The F subset frequently expressed neuronal specific genes including those related to neural differentiation, synapse, catecholamine metabolism, and protein degradation. On the other hand, the UF subset expressed many genes related to cell cycle control, protein synthesis and transcriptional regulation. The 4s tumor just before starting rapid regression also showed an extremely unique expression profile. It contained many apoptosis-related genes (*Bcl-10*, *NIP1*, *NIP3L*, *BAG-1*, *BID*, *FADD*, etc.), oncogenes (*c-Abl*, *c-Fos*, *K-Ras*, *c-Raf*, etc.), the other tumor-related genes (*TrkA*, *TGF- β* , *EXT2*, *LEU5*, *TNFR1*, *TRAP-1*, *ING-1*, *TRAIL*, etc.) and HLA family members that might be derived from the infiltrated lymphocytes into the tumor. Since the number of the genes cloned in our system was so small as compared to that of the SAGE method, we decided to examine whether or not the individual gene was expressed differentially between F and UF NBLs by using semi-quantitative RT-PCR.

The significance of the 757 differentially expressed genes was strongly implicated in understanding of NBL biology. Surprisingly, most of the genes were expressed at higher levels in F than UF subset [22]. The genes highly expressed in F subset contained those related to neuronal differentiation, migration, cell–cell interaction, protein degradation, synaptic

vesicles, catecholamine metabolism and intracellular signaling. 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 UF subset. The protein products of such known genes contained many transcriptional and translational regulators including oncoproteins. Notably, downregulation of dopamine- β -hydroxylase (DBH) and monoamine oxidase (MAO), the genes involved in the regulation of catecholamine metabolism, in UF as compared to F tumors could explain the previous observation that aggressive NBLs with *MYCN* amplification were dopaminergic [55].

5.3. Screening of the genes regulated by NGF in the newborn mouse SCG neurons

It is well accepted that both membrane receptors for NGF, TrkA and p75, are highly expressed in the F subset of NBL whereas they are strongly downregulated in the UF tumors with *MYCN* amplification [8,9]. The spontaneous regression only occurs in F type of NBLs, suggesting that the molecular mechanism of NBL regression is closely related to that of the PCD occurring in the late embryonic stage of sympathetic neurons. Indeed, the F type NBL expresses high levels of TrkA and p75 receptors but only a trace amount of NGF which might be supplied from the stromal cells such as Schwannian cells and fibroblasts within the tumor tissue [9]. The possible hypothesis is that only the tumor cells, which have obtained the limited amount of NGF, could survive and differentiate to ganglion-like mature cells, whereas the most cells, which have not been able to get enough amount of NGF to survive, might die. This attractive hypothesis prompted us to examine whether the differentially expressed genes between F and UF change their expression levels during the NGF-induced differentiation and the NGF-depletion-induced apoptosis in the newborn mouse superior cervical ganglion (SCG) neurons which are extremely sensitive to NGF for survival and death [56]. Among 353 genes we selected from the novel genes with

differential expression, we could find 234 mouse counterparts, of which 181 primer pairs worked. They were subjected to screen for the change of expression by using semi-quantitative RT-PCR. Interestingly, seven and six genes were up- and down-regulated after NGF-induced differentiation, respectively, while one and 35 genes were up- and down-regulated after NGF depletion-induced apoptosis, respectively. Eight genes were up-regulated during NGF-induced differentiation and subsequently down-regulated after depletion of NGF, whereas 12 genes were down-regulated during the differentiation and then up-regulated after induction of apoptosis. However, 112 genes did not show any change in their expression in this system. The further analyses of those genes should give important insights into understanding of the differentiation and regression of NBL.

5.4. Chromosomal mapping of the differentially expressed genes

NBL is a cancer with extensive genomic aberrations [33,34]. According to the previous investigations, it is evident that the developmentally important genes like *MYCN* are targeted to cause NBL. Therefore, in order to identify more genes related to the NBL genesis, we mapped the 719 differentially expressed genes to human chromosomes (Fig. 3). It is obvious that there are some clusters of the differential genes in many loci on the chromosomes. The gene density mapped to each chromosome is also not the same. The genes receiving epigenetic regulations such as methylation and acetylation could also be included. The indicated mapping may give some hints for the future researches identifying important genes of NBL as well as those functioning in neural development.

5.5. cDNA microarray and array comparative genomic hybridization (array CGH)

We have recently developed our own cDNA microarray for investigation of NBL biology and mechanism of neuronal development. As many important genes of transcriptional regulator are involved, it may be useful to understand the complicated neural network during the normal

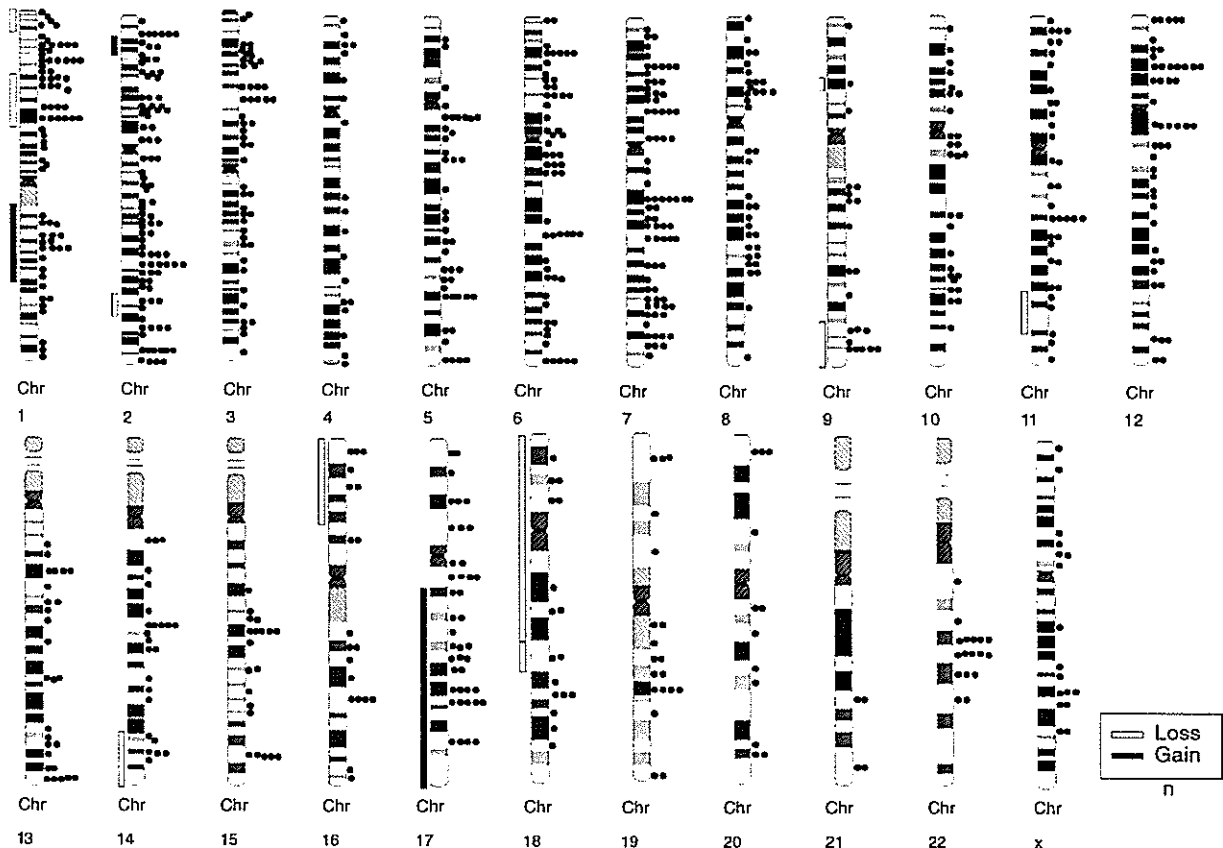


Fig. 3. Chromosomal mapping of the genes differentially expressed between favorable and unfavorable subsets of neuroblastoma. The gray and black lines indicate the regions of loss of heterozygosity (LOH) and those of chromosomal gain, respectively.

development as well as its aberrations in cancers such as NBL. Furthermore, the NBL cDNA microarray should be important for the clinical use to develop it as a diagnostic tool. The comparative and parallel studies between the results obtained from the cDNA microarray analysis and the array comparative genomic hybridization (array CGH) [57] might also be very helpful to identify the genes and to understand the molecular biology of NBL.

6. Developmental clock and the genes of neuroblastoma

Our NBL cDNA project has provided us with tremendous information about the genes expressed in different subsets with characteristic biology. Though the project is still ongoing, a temporary

view so far obtained suggests the presence of a kind of rule in the expression patterns of the subset-specific genes. Fig. 4 shows the groups of genes expressed along the time axis of sympathetic neuron development. During the early stages of development, many transcription factors and their regulators may play important roles in deciding the direction of differentiation as well as in regulating cell growth and survival of neural crest-derived cells. It is interesting that many genes highly expressed in unfavorable NBLs contain transcription factors and the components of their complexes. They involve MYCN and Id family transcription factors that link to the regulation of Rb and p53 and regulate cell growth and apoptosis. The basic helix-loop-helix transcription factor, hASH1, is constitutively activated in NBL, and, by collaborating with Phox2a and Phox2b, it may regulate the arrest of

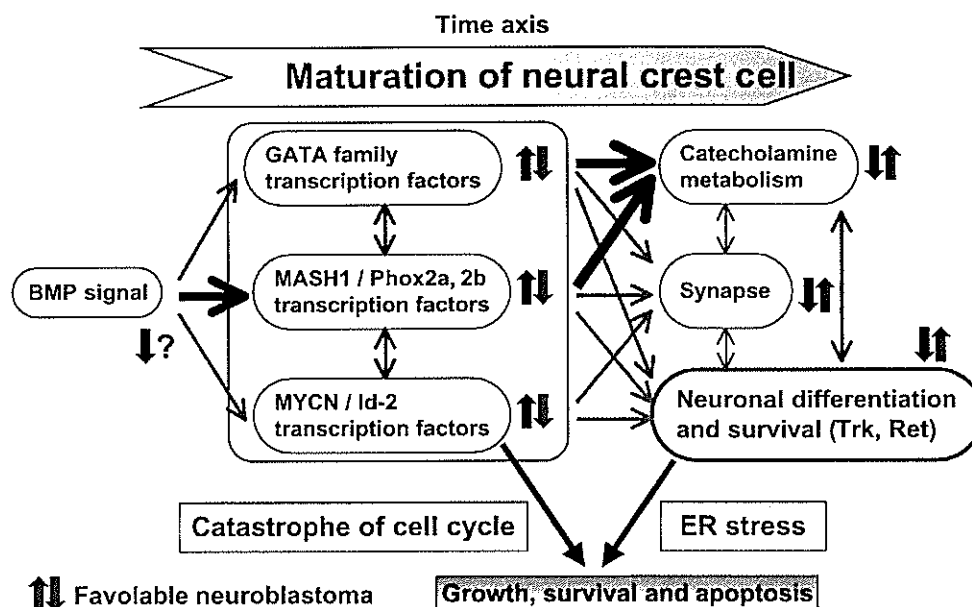


Fig. 4. The developmental time axis and the gene expression cascade during maturation of the neural crest cells. Many transcription factors are up-regulated in the unfavorable neuroblastoma, whereas the genes related to the terminal differentiation of neuron are up-regulated in the favorable neuroblastoma. ER: endoplasmic reticulum.

differentiation in unfavorable NBLs (T. Kuno et al., unpublished data). Our NBL cDNA project has also revealed that there may exist a neuronal cassette of GATA transcription factor complex that controls growth and differentiation of sympathetic progenitor cells. Some molecules in this complex are up-regulated in unfavorable NBLs (M. Aoyama et al., manuscript in preparation). Thus, many important components in the transcriptional regulators appear to be highly expressed in unfavorable NBLs and function to regulate the tumor cell growth or the status of de-differentiation.

On the other hand, a remarkable number of the genes expressed at high levels in favorable NBLs seem to encode the molecules that are necessary to maintain the neuronal function of the matured neuronal cells. They may be needed to maintain catecholamine metabolism, synapse formation, neuronal cell survival, etc. We have also identified many genes related to the ubiquitin-proteasome pathway and heat shock proteins in favorable NBLs,

suggesting that they might be involved in induction of apoptosis triggered by endoplasmic reticulum (ER) stress [58].

Thus, our NBL cDNA project which is still on-going has clearly demonstrated that favorable NBL arrests its differentiation at late stage of neural development, whereas unfavorable NBL arrests at the early and immature stage.

7. From comprehensive to functional genomics of neuroblastoma aiming at the drug discovery

All genes and their products are no doubt necessary to accomplish embryonal differentiation and to maintain homeostasis of the whole human body. However, aberration of even one gene can cause disease or cancer, albeit many cancers including NBL have multiple genetic abnormalities. Therefore, we need to try to select the truly important genes that

affect the genetics and biology of NBL as key regulators.

During the functional analyses of the novel genes we selected for the last three years, several genes have been reported as important regulators especially in the neuronal system. They include Nogo (Nbla00271) [59], a negative regulator of axonal guidance, FOG2 (Nbla03139) [60], a cofactor of the GATA transcription factor complex, small GTPase RAB6B (Nbla00086) [61], Mlt1 (Nbla00106) [62], homeotic regulator homolog MAB21 (Nbla00126) [63], GTP-binding protein RAB3C (Nbla00494) [64], neurexophilin (Nxph1, Nbla00697) [65], RAB3 effector protein RIM1 (Nbla00761) [66], cell recognition molecule Caspr2 (Nbla00831) [67], endothelin converting enzyme-like 1 (ECE1, Nbla03145) [68], doublecortin- and calmodulin kinase-like 1 (DCAMKL1, Nbla10919) [69] and aczonin (Nbla11270) [70]. Our current analyses of the individual genes selected also suggest that the present cDNA resources derived from the primary NBLs contain many novel genes involved in NBL, neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, and other diseases. The progress of the functional genomics of the NBL genes may lead to the discovery of the molecular targets for the therapeutic strategy.

8. Conclusion

The enigmatic tumor, neuroblastoma, is changing not to be enigmatic, because the molecular bases are now rapidly being unveiled. Indeed, the clinical and biological enigma of NBL may suggest a tight link between the cancer and the development of neural crest cells from which NBL is derived. The comprehensive genomics and future proteomics approaches may accelerate further understanding of the biology of NBL which in turn helps to clarify the molecular mechanism of normal neural development. It is important and interesting that the key regulators during the normal neural development are usually the targets of the NBL genesis. We need to intensively search for those target genes which could also be important for the drug discovery leading to the cure of the patients.

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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

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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.

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 μ g/ml streptomycin. COS-7 and Neuro2a cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fe-

tal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. All cells were maintained in a humidified 37 °C incubator with 5% CO₂. 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 μ M.

RNA Analysis—A human multiple tissue mRNA blot and a fetal human multiple mRNA blot (Invitrogen) were hybridized with a ³²P-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'-CCGCTTCCATCAGGTTGTT-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 μ g of purified glutathione S-transferase fusion proteins, 0.25 μ g of yeast ubiquitin-activating enzyme (*E1*) (BostonBiochem, Cambridge, MA), 1 μ l of crude lysates from *Escherichia coli* expressing ubiquitin carrier proteins (*E2*), and 10 μ g of bovine ubiquitin (Sigma) were incubated in 250 mM Tris-HCl (pH 7.6), 1.2 M NaCl, 50 mM ATP, 10 mM MgCl₂, 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-Ser⁶³ c-Jun (Cell Signaling Technology) antibody.

Cloning of Human *NEDL1* cDNA—A forward primer (5'-GGTTT-

TAGGCTGGCCGCC-3') and a reverse primer (5'-CAATGAGGTA-CATGCCAATCC-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 His₆-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 μ g/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. 1A). 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. 1B). Its

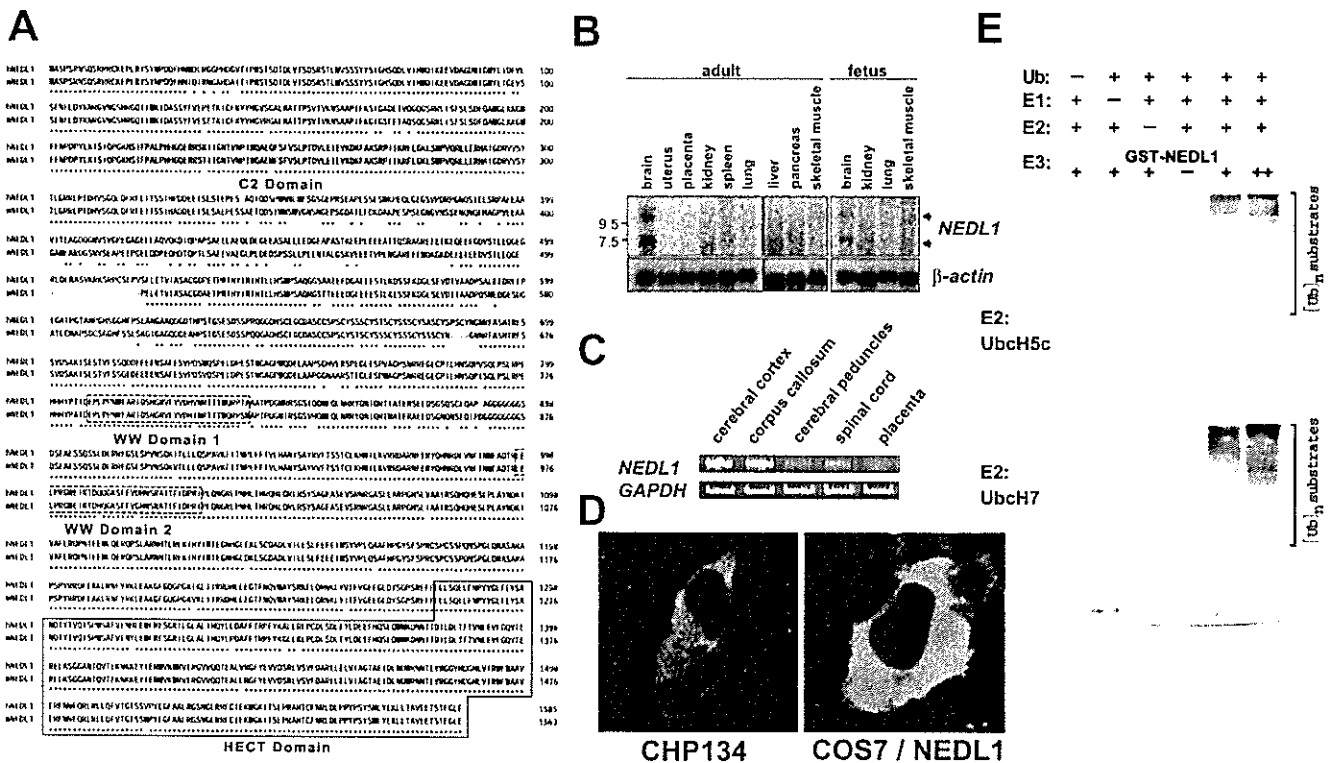


FIG. 1. Amino acid sequence, brain-specific expression, and subcellular localization of NEDL1 E3. **A**, alignment of conserved amino acid sequences of human NEDL1 (*hNEDL1*) and its mouse homolog (*mNEDL1*). Numbers on the right indicate the number of residues to the initiator methionine. The C2 domain (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 ³²P-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. 1C). 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. 1D). The *in vitro* system containing UbcH5c or UbcH7 demonstrated that NEDL1 has a ubiquitin-protein ligase activity (Fig. 1E).

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. 2C). 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. 2D). 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

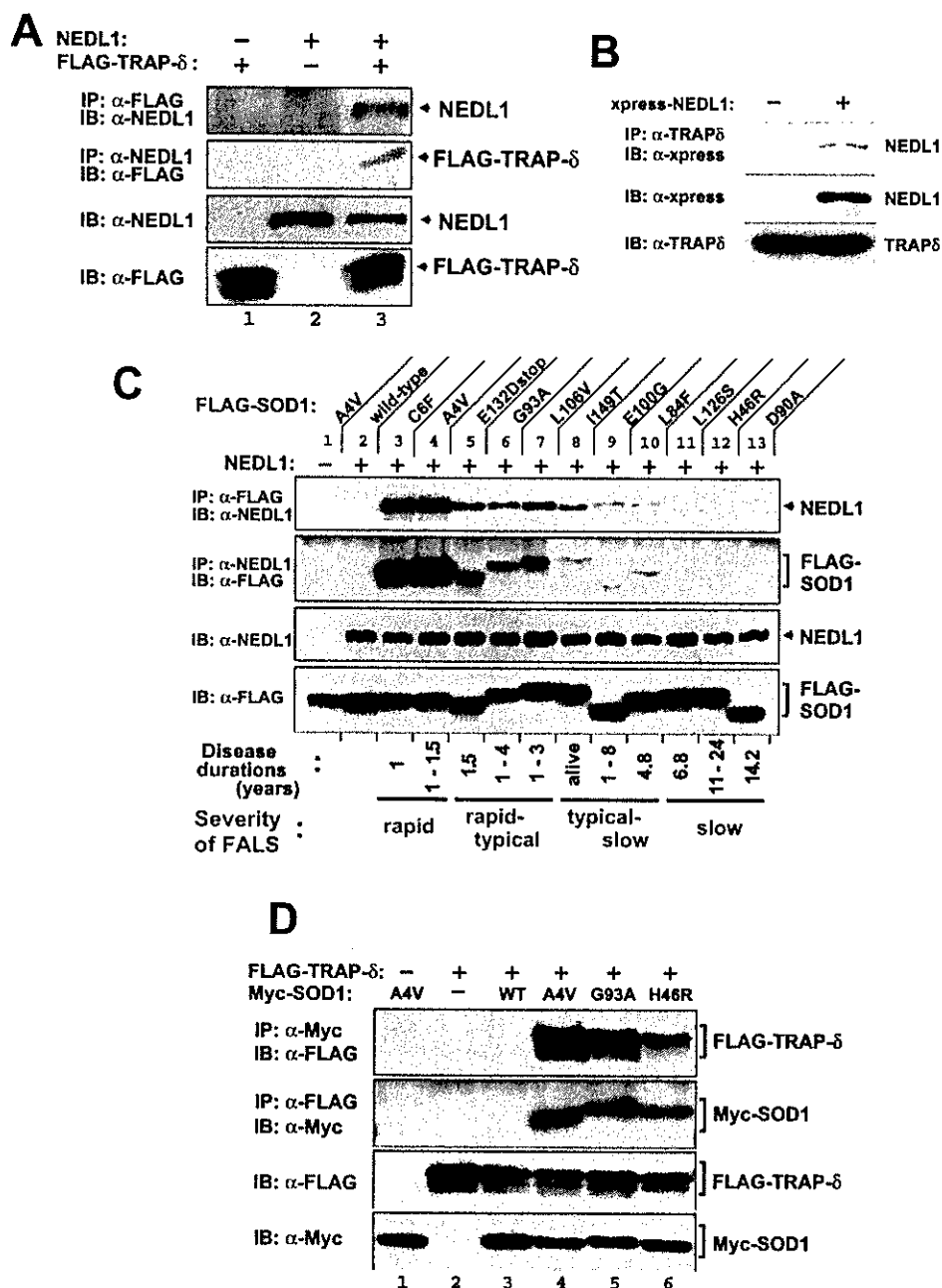


FIG. 2. NEDL1 interacts with TRAP- δ and FALS-associated mutant forms of SOD1, but not with wild-type SOD1. *A*, NEDL1 interacts with TRAP- δ . COS-7 cells were cotransfected with the indicated expression plasmids, and whole cell lysates were immunoprecipitated (IP) with anti-FLAG (*first panel*) or 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 con-

structs 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. 3A), suggesting that WW domain-1 and its surrounding portion are the region involved in their interaction. Immuno-

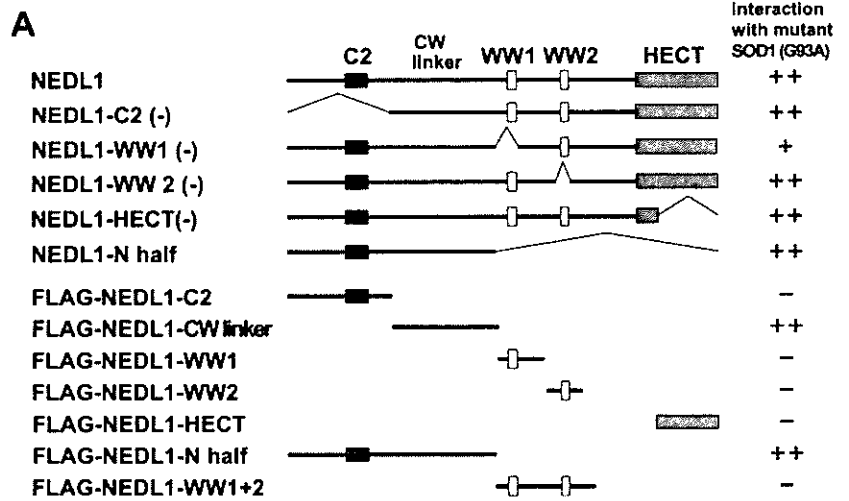
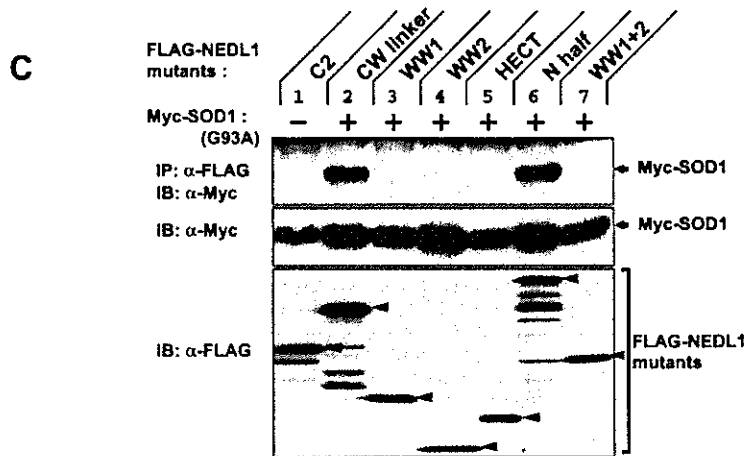
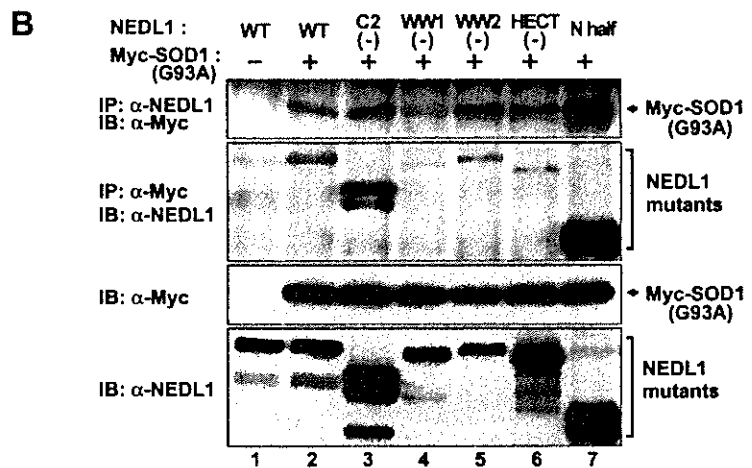


FIG. 3. The region of NEDL1 between the C2 domain and WW domain-1 is required for interaction with mutant SOD1. *A*, schematic illustration of wild-type NEDL1 and a series of deletion mutants of NEDL1. *CW linker* indicates the region between the C2 domain and WW domain-1 (*WW1*). *B* and *C*, immunoprecipitation 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-8 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-8 and mutant SOD1 for degradation. As shown in Fig. 4A, NEDL1 clearly ubiquitinated mutant SOD1(A4V), but not TRAP-8

(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. 4A). Fig. 4B 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 ≥