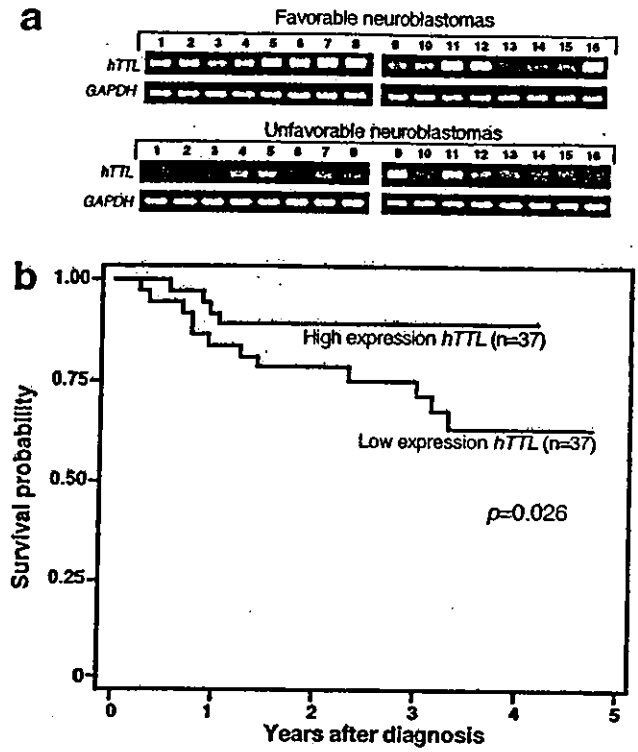


FIGURE 5 – Expression of *hTTL* mRNA is associated with unfavorable prognosis of neuroblastoma. (*a*) Total RNA was purified from the indicated favorable (top) and unfavorable NBL tissues (bottom) and subjected to semiquantitative RT-PCR. Sixteen favorable cases used in this study were classified as stage 1 NBL with a single copy *MYCN* as well as a high expression of *TrkA*. Sixteen unfavorable cases were in stages 3 and 4 NBL with *MYCN* amplification as well as a low *TrkA* expression. *GAPDH* expression was also examined as an internal control. (*b*) Association of *hTTL* mRNA expression levels with favorable prognosis of NBL. Total RNA was prepared from 74 NBL tissues, and *hTTL* mRNA levels were assayed by quantitative real-time RT-PCR as described in text. The values of *hTTL* mRNA were normalized by *GAPDH*. The survival of *hTTL* relatively high-expression group ($n = 37$) and *hTTL* low-expression group ($n = 37$) was compared using the Kaplan-Meier procedure.

TABLE I – RESULTS OF LOG-RANK TESTS FOR CONVENTIONAL PROGNOSTIC FACTORS AND EXPRESSION OF *hTTL* IN 74 PRIMARY NEUROBLASTOMAS

Variable	*n*	*hTTL* expression[1]	*p*-value
Age (year)			0.1
<1	43	117 ± 14	
≥1	31	77 ± 10	
Tumor stage			0.0069
1, 2, 4s	40	127 ± 14	
3, 4	34	69 ± 9	
TrkA expression			0.002
High	36	125 ± 17	
Low	38	77 ± 8	
MYCN			<0.00005
Single	52	123 ± 11	
Amplified	22	46 ± 9	
Mass screening			0.0042
+	37	128 ± 14	
−	37	72 ± 10	
Origin			0.0042
Adrenal gland	47	85 ± 11	
Others	27	127 ± 16	
Prognosis			0.023
Alive	58	113 ± 11	
Dead	16	54 ± 11	

[1]Mean ± SEM.

TABLE II – COX REGRESSION MODELS USING DICHOTOMOUS FACTORS OF AGE, *MYCN* AMPLIFICATION, MASS SCREENING, ORIGIN AND EXPRESSION OF *hTTL*

Factor	*p*-value	Hazard ratio (95% confidence interval)
hTTL expression (log)	0.024	0.64 (0.44, 0.94)
Age (> 1 *vs.* < 1 year)	0.005	5.04 (1.61, 15.8)
MYCN (1 copy *vs.* > 1 copy)	<0.0005	0.06 (0.017, 0.22)
Mass screening (+ *vs.* −)	0.004	0.05 (0.007, 0.38)
Origin (adrenal gland *vs.* others)	0.31	1.79 (0.58, 5.57)

hTTL encoded by the *hTTL* gene in the cells. Interestingly, hTTL is induced during neurite extension in RTBM1 NBL cells treated with BMP2 or RA, suggesting that hTTL expression is associated with neuronal differentiation in human NBL. Immunohistochemically, favorable NBLs are positive for hTTL, Tyr-tubulin, Glu-tubulin and Δ2-tubulin, whereas unfavorable tumors with *MYCN* amplification are positive only for Δ2-tubulin, suggesting that deregulation of tyrosination/detyrosination cycle contributes to malignant progression of NBL. This hypothesis has been further supported by a significant decrease of the levels of *hTTL* expression in the patients with poor prognosis.

The dynamics of microtubule regulates many cellular functions, including migration, motility, differentiation, cell division and cellular cap formation. Though posttranslational modifications of tubulin and their enzymatic regulation have long been studied, the precise mechanisms are still largely unknown. It is interesting that no orthologs of highly conserved mammalian TTL have so far been reported in *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*, suggesting that the tyrosination/detyrosination cycle of tubulin may be related to evolution of the cellular functions, including neuronal differentiation. In newborn rats, TTL expression is found in skeletal muscle at high levels and is developmentally regulated by rapidly decreasing its level during early postnatal period.[31] It is interesting that both BMP2 and RA, which have increased levels of hTTL expression, function as regulators to induce differentiation during neural development.

The tyrosination/detyrosination of tubulin may be regulated by the activities of both TTL and tubulin carboxypeptidase (TCP). Until now, however, the *TCP* gene has never been identified in vertebrates, although biochemical TCP activity has been reported to be present in some subcellular fractions.[18] Tubulin is also posttranslationally modified by nitrotyrosination. Eiserich *et al.*[32] showed that free 3-nitrotyrosine (NO_2Tyr) is transported into mammalian cells and selectively incorporated into the Glu-tubulin posttranslationally, which is catalyzed by TTL. Cellular injury such as microtubule disorganization has consequently been induced. Kalisz *et al.*[33] also showed that nitrotyrosine can be incorporated into α-tubulin by *in vitro* assays. Those reports demonstrated that carboxypeptidase A is incapable of cleaving nitrotyrosine from the modified α-tubulin. On the other hand, Bisig *et al.*[34] reported that nitrotyrosinated tubulin is a good substrate of physiologic TCP, and that it has a similar capability to that of the tyrosinated tubulin to assemble into microtubules, suggesting that incorporation of nitrotyrosine is not injurious at least to dividing cells. Therefore, whether nitrotyrosinated tubulin is harmful or not is still controversial. Nevertheless, as increased nitrotyrosination is reported in Alzheimer's disease and amyotrophic lateral sclerosis,[35–37] the functional analysis of the role of hTTL and tubulin tyrosination/detyrosination cycle should be important for understanding the pathogenesis of these disease. The treatment of cells with methylmercury (MeHg) is also reported to induce perturbation of cellular activities associated with the tubulin/microtubule system by altering the status of tubulin tyrosination in the rat

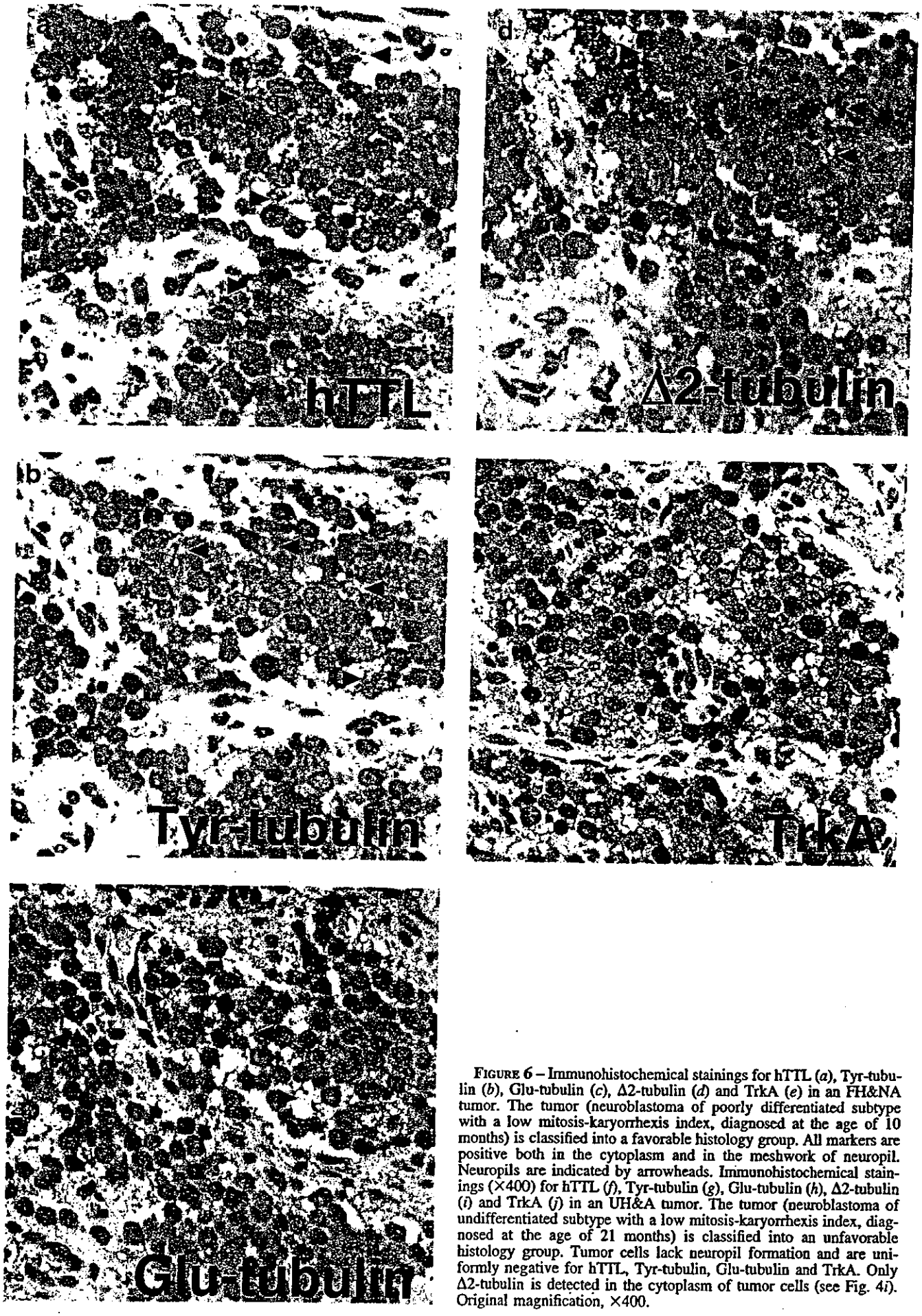
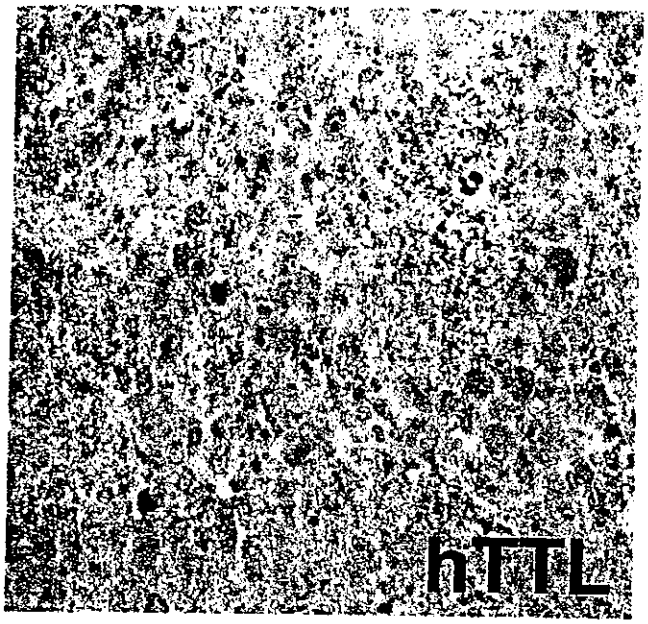
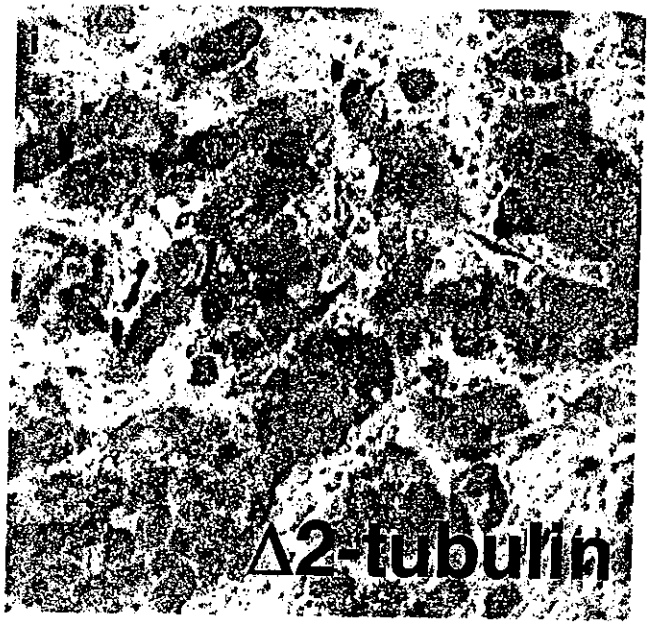
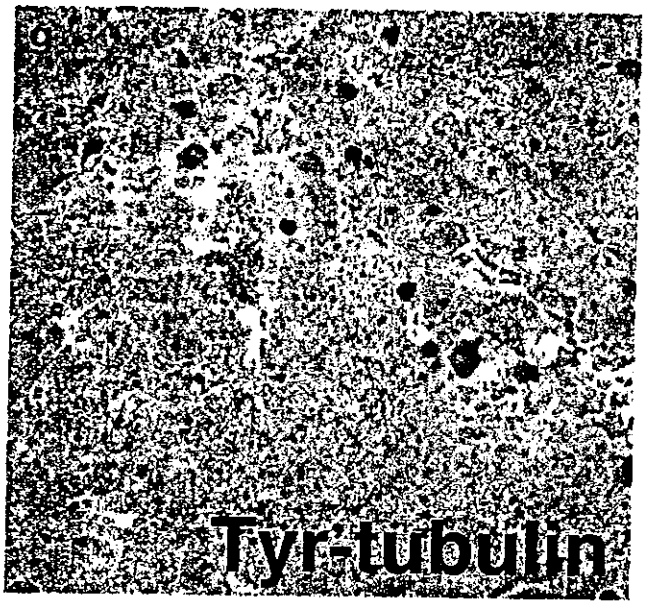
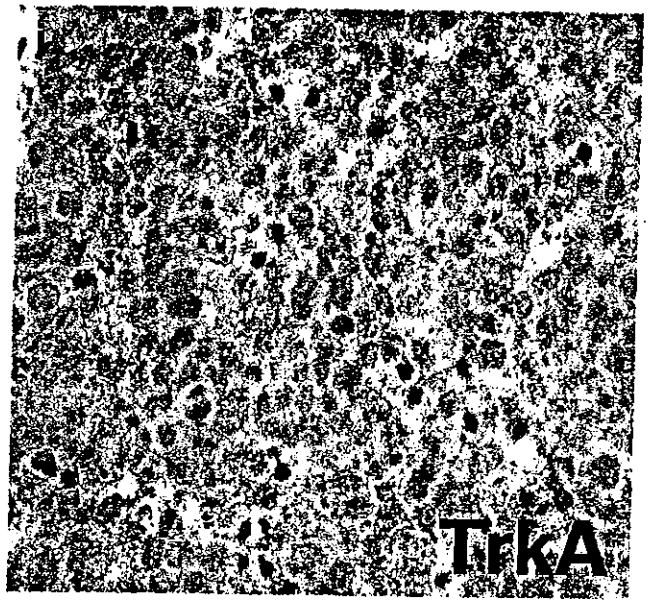


FIGURE 6 – Immunohistochemical stainings for hTTL (*a*), Tyr-tubulin (*b*), Glu-tubulin (*c*), Δ2-tubulin (*d*) and TrkA (*e*) in an FH&NA tumor. The tumor (neuroblastoma of poorly differentiated subtype with a low mitosis-karyorrhexis index, diagnosed at the age of 10 months) is classified into a favorable histology group. All markers are positive both in the cytoplasm and in the meshwork of neuropil. Neuropils are indicated by arrowheads. Immunohistochemical stainings (×400) for hTTL (*f*), Tyr-tubulin (*g*), Glu-tubulin (*h*), Δ2-tubulin (*i*) and TrkA (*j*) in an UH&A tumor. The tumor (neuroblastoma of undifferentiated subtype with a low mitosis-karyorrhexis index, diagnosed at the age of 21 months) is classified into an unfavorable histology group. Tumor cells lack neuropil formation and are uniformly negative for hTTL, Tyr-tubulin, Glu-tubulin and TrkA. Only Δ2-tubulin is detected in the cytoplasm of tumor cells (see Fig. 4*i*). Original magnification, ×400.









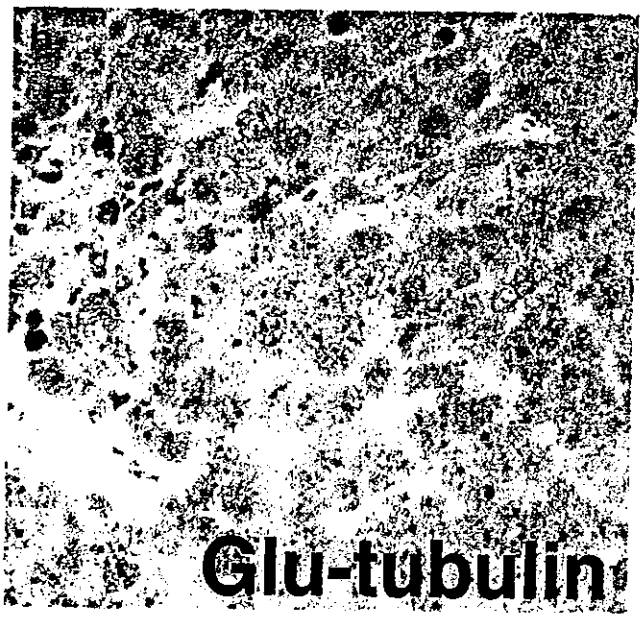


FIGURE 6 – (CONTINUED)

brain.[38] Therefore, many cellular stresses such as oxidative damage may trigger dysfunction of the tubulin/microtubule cytoskeletal system.

Our present study has shown that the decreases in Tyr-tubulin and Glu-tubulin are associated with relatively low levels of hTTL expression in unfavorable NBLs, which have lost a potency of neuronal differentiation and/or apoptosis. They are also correlated with decreased levels of TrkA, a high-affinity receptor for nerve growth factor, whose activation induces morphologic differentiation of NBL cells.[39] In addition, gradual upregulation of hTTL has been observed during induction of neuronal differentiation in RTBM1 cells treated with BMP2 or RA. These suggest that the induction of neuronal differentiation in NBL is accompanied with the activated tyrosination/detyrosination cycle regulated by increased level of hTTL enzyme, while the cycle is arrested by downregulation of hTTL in proliferating NBL cells, resulting in accumulation of Δ2-tubulin within the cells. Indeed, the expression levels of *hTTL* mRNA and Δ2-tubulin are significantly correlated with the prognosis of primary NBLs. This is consistent with the observation that TTL activity is lost, and conversely Δ2-tubulin is upregulated during the tumor cell growth.[19] Lafanechere *et al.*[19] have demonstrated by using mouse TTL null cells both *in vitro* and *in vivo* that mouse TTL activity is strongly decreased during tumor growth. Mas *et al.*[15] have also reported that, using rat *TTL* dominant negative mutant and an antisense cDNA of rat *TTL*, suppression of TTL activity induces 2- to 3-fold faster cell proliferation. Moreover, in human breast cancers, the accumulation of Glu-tubulin and Δ2-tubulin is correlated with poor prognosis by immunohistochemical approach.[28] It is noteworthy that our preliminary data using the microarray hybridized with total RNA obtained from 136 primary NBLs have shown that the gene with the highest score to predict prognosis of NBLs is α-tubulin (data not shown). Thus, the role of microtubule and its component, α-tubulin, is very important to define the biology as well as the aggressiveness of cancer cells.

In conclusion, we have identified a *human tubulin tyrosine ligase* gene and demonstrated its tissue distribution and correlation with neuronal differentiation. Since our data have suggested that the tyrosination cycle of α-tubulin is activated in differentiating NBLs but is inactivated in proliferating tumors, the cycle-related molecules including hTTL could be the targets for developing novel therapeutic strategies against advanced stages of NBL.

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Molecular and Developmental Biology of Neuroblastoma

Akira Nakagawara

Contents

5.1 Neural Crest Development and Neuroblastoma

Cancer has its own face reflecting the characteristics of the tissue from which it is derived. This can be demonstrated by histopathologic examination, by immunohistochemistry, and/or by in situ hybridization. Recent advances in molecular biology and genetics have also revealed that these morphological distinctions among cancers are associated with differences in gene expression profiles within tumor cell and stromal cell components. Furthermore, the patterns of gene expression unique for each cancer are dictated by genetic abnormalities which have occurred in progenitors of the specific developmental lineage. Neuroblastoma originates from the sympathoadrenal lineage, and its biology is closely related to that of normal sympathetic neurons. In this chapter, the molecular and cellular bases for the genesis and biology of neuroblastoma are summarized.

5.1.1 Genes of Neural Development and Molecular Targets of Neuroblastoma

During neural development, neural crest cells migrate and differentiate into several cell lineages, e.g., melanocytes, sensory neurons, enteric ganglion cells, and sympathetic neurons (Fig. 5.1). The first signaling molecules which trigger crest cells to differentiate or migrate are bone morphogenetic proteins (BMPs) and their receptors (Huber et al. 2002). The commitment to differentiate into sympathetic neurons is associated with the transient expression of (a) basic helix-loop-helix transcription factors, e.g., *MASH1* (a proneural gene homologous to *drosophila achaete-*

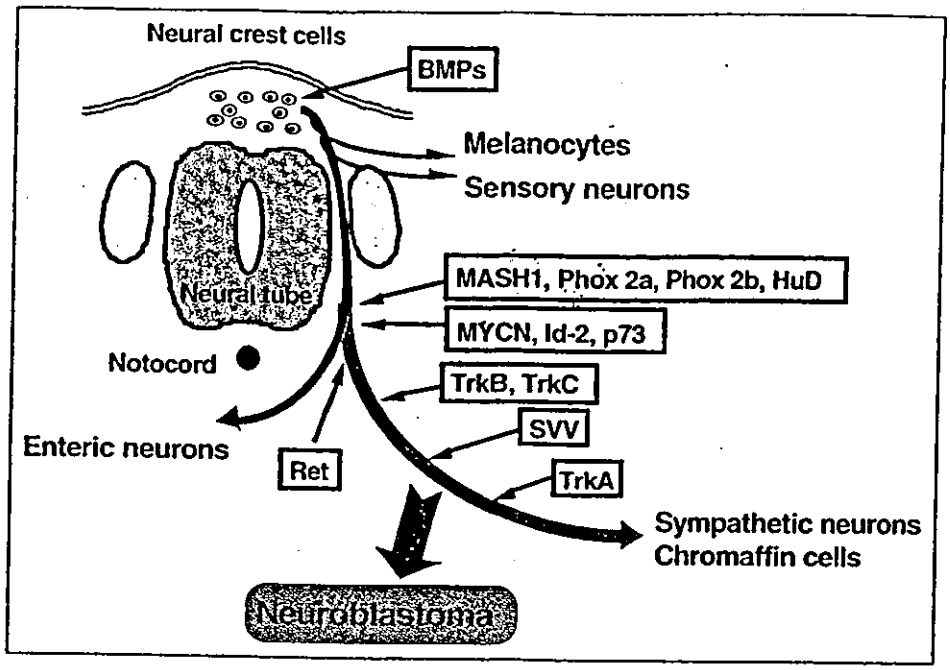


Figure 5.1

Neuroblastoma originates from the sympathoadrenal lineage of neural crest. The bone morphogenetic protein (BMP) signals may be important at the early stage of differentiation of neural crest cells. *MASH1* (*hASH1*) may function as one of the key transcription factors which define the direction of differentiation to sympathetic neurons. The other important nuclear factors, e.g., Phox2a, Phox2b, HuD, *MYCN*, Id2, and p73, may also be involved in the cell-fate determination. Some of those genes are often upregulated or amplified in aggressive neuroblastomas (Nakagawara 2004). At the stage of terminal differentiation of sympathetic neurons followed by programmed cell death, the signals through neuronal tyrosine kinase receptors, e.g., Ret, TrkB, TrkC, and TrkA, are necessary sequentially and/or in a form of crosstalk. The many genes involved in regulation of neuronal terminal differentiation or programmed cell death are often expressed at high levels in favorable neuroblastomas.

scute), *HES1*, *MYCN*, HIF1α and HuD, (b) homeobox genes, e.g., Phox2a and Phox2b, and (c) p73 (a family member of the tumor suppressor gene p53; Nakagawara 2004). Several lines of investigation support the importance of these genes. *MASH1* null mice lack sympathetic ganglion cells (Guillemot et al. 1993). Notch signaling, through its intracellular domain translocation into the nucleus, stimulates the transcriptional activation of the *HES1* and *HES5* genes whose products in turn inhibit transcription of the *MASH1* gene (Radtke and Raj 2003). *MYCN* is indispensable for the normal neural development. It induces Id2 which is a negative regulator of *HES1* and pRb, a retinoblastoma suppressor (Lasorella et al. 2000). p73 knockout mice also show abnormalities in cell survival in both the nervous and immune systems (Yang et al. 2000). Gene targeting of HIF2α disturbs the catecholamine metabolism in sympathetic neurons (Tian et al. 1998). All these genes regulate each other in an orchestrated manner to drive the correct differentiation of neural crest cells into sympathetic neurons.

Further downstream, terminal differentiation to mature sympathetic cells is strongly regulated by the signaling of neurotrophin family members and their receptors (Nakagawara 2001, 2004). In addition, other genetic aberrations associated with neuroblastoma have been mapped to specific genomic regions or genes well known to be important in regulating the normal development of neurons (Nakagawara 2001, 2004). It seems obvious that a relationship should exist between the genetic or biological targets of neuroblastoma and the key molecules involved in the normal development of neural crest cells.

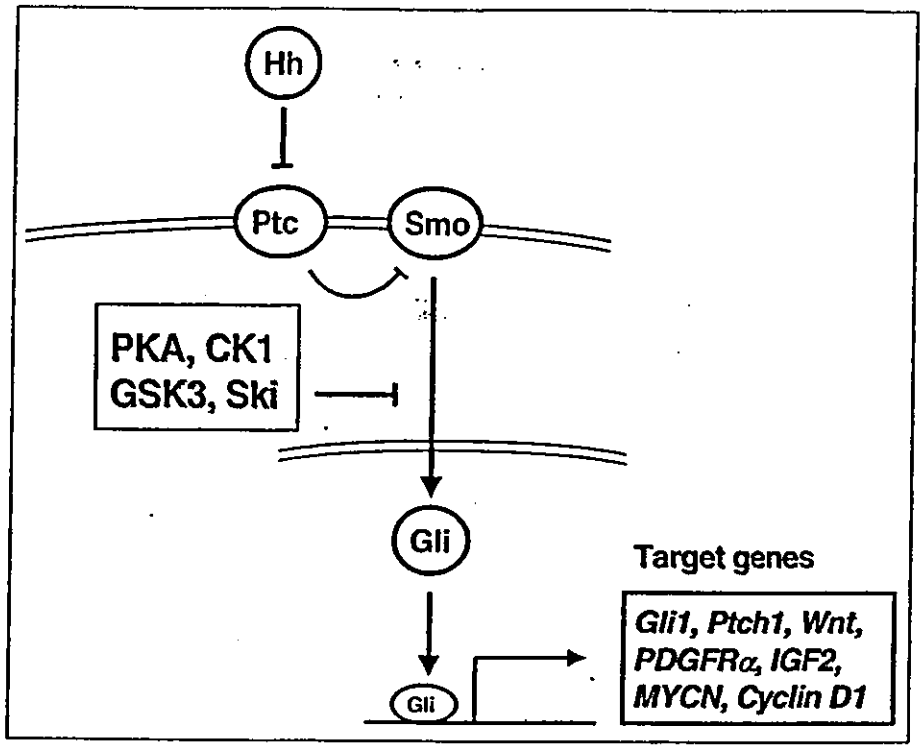


Figure 5.2
Hedgehog-Gli signaling in neural development and tumorigenesis. Sonic hedgehog (Hh) signaling activates Gli transcription factors which then induce the target genes important for regulating neural differentiation as well as neuronal tumorigenesis. They include *MYCN*, *cyclin D1*, *IGF2*, and *PDGFRα*, all of which are known to be players characterizing neuroblastoma biology. *T bars* show inhibitory interactions. *Arrows* show positive interactions

5.1.1.1 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, may be the first signal that defines the early phase of differentiation and migration of neural crest cells during development (Oppenheim 1991). The ligand-dependent activation of BMP receptors transduces its signal into the nucleus through the sequential activation of Smad signaling molecules by phosphorylation. Although the role of BMPs in neuroblastoma has long been elusive, Nakamura et al. (2003) have recently reported that SH-SY5Y and RTBM1 neuroblastoma cell lines are responsive to BMP2 leading to growth arrest and differentiation. Of interest, BMP treatment also induces the downregulation of p53 family members including p53 and p73, as well as their target gene, $p21^{WAF1}$. In contrast, a similar cyclin-dependent kinase inhibitor, $p27^{KIP1}$, is markedly induced at the protein level by downregulation of Skp2, a component of its E3 ubiquitin ligase complex. BMP is also a direct transcriptional target of retinoic acid which induces neuroblastoma differentiation (see Chap. 15; Rodriguez-Leon et al. 1999). The DAN family members are inhibitors of BMP, and are also expressed in neuroblastomas (Enomoto et al. 1994). The DAN gene itself, which is mapped to chromosome 1p36, is a transcriptional target of BMP (Nakamura et al. 2003; Shinbo et al. 2002), suggesting that the BMP signaling network may be important in the differentiation and survival of neuroblastoma (Nakamura et al. 2003). The role of other important signals which function during neuronal development, including Sonic Hedgehog (Shh) and Wnt, is less well known in neuroblastoma. Interestingly, the Shh downstream signaling molecule, Gli, can transactivate *MYCN* and cyclin D1 (Altaba et al. 2004) (Fig. 5.2).

5.1.1.2 *MASH1/hASH1*

Achaete-Scute homolog-1 (*MASH1* in rodents and *hASH1* in humans) is a basic helix-loop-helix transcription factor which plays an important role in the early development of neural and neuroendocrine progenitor cells (Ball 2004). Helix-loop-helix proteins include achaete-scute homologs, E proteins, *MYCN*, *Math*, *NeuroD*, *neurogenin*, *Id*, and *HES*. Targeted disruption of *MASH1* in mice has led to the absence of

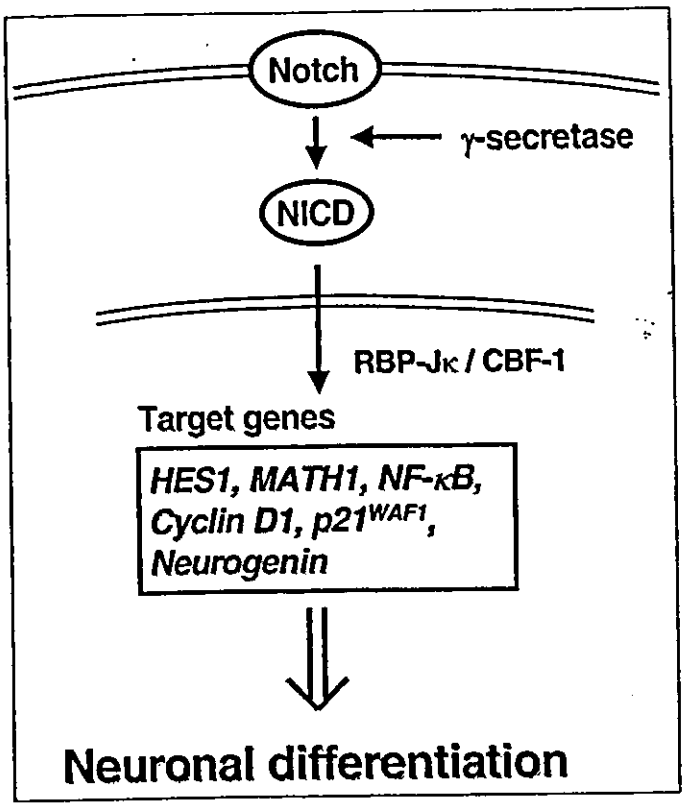


Figure 5.3
Notch signaling transactivates gene expression to induce neuronal differentiation. Binding of the ligand delta to its receptor notch triggers intramembrane proteolytic cleavage by γ-secretase. This results in the release of the notch intracellular domain (NICD), which then translocates to the nucleus where it associates with the CSL family of DNA binding proteins and transactivates gene expression. The target genes include *HES1, MATH1, NF-kB, cyclinD1, p21*, and *neurogenin*. *HES1* then inhibits transactivation of *MASH1* (*hASH1*)

sympathetic neurons, suggesting the important role of *MASH1* in sympathetic differentiation (Guillemot et al. 1993). *MASH1* is transiently induced during neural development to promote neuronal cell differentiation; however, high *hASH1* expression persists in neuroblastoma tumors and cell lines (Soderholm et al. 1999; Ichimiya et al. 2001). Retinoic acid treatment decreases the expression of *hASH1* and induces neurite extension (Ichimiya et al. 2001). *hASH1* also directly represses the expression of PACE4, a mammalian subtilin-like proprotein convertase that activates TGF-β-related proteins (e.g., BMPs) in neuroblastoma cell lines (Yoshida et al. 2001). The Notch signaling pathway also plays a key role during neuronal development (Axelson 2004). One of the important regulators of *hASH1* is a basic HLH protein, *HES1* (Fig. 5.3). *HES1* is regulated, at least in part, by Notch signaling and is induced at the transcription level. *HES1* directly binds to the promoter of *hASH1* and inhibits its transcriptional activation. A constitutively active form of Notch could block neurite extension during the induced differentiation of human neuroblastoma cells, possibly by inhibiting *hASH1* through the induction of *HES1* (Radtke and Raj 2003).

5.1.1.3 Phox2a and Phox2b

Phox2a and Phox2b are paired-like homeodomain transcription factors with complete conservation in their homeodomain. They are specifically expressed in noradrenergic neurons and activate the tyrosine hydroxylase and dopamine-β-hydroxylase genes (Schneider et al. 1999; Stanke et al. 1999; Ernberger 2000). While the expression of Phox2a is regulated by *MASH1*, Phox2b is not (Lo et al. 1999) (Fig. 5.4). The genetic disruption of either Phox2a or Phox2b gene de-monstrated that both genes are essential for the development of autonomic neural crest derivatives (Morin et al. 1997; Pattyn et al. 1999). Interestingly, Trochet et al. (2004) reported that the Phox2b gene was mutated in a family case of neuroblastoma and in a neuroblastoma patient with Hirschsprung's disease.

5.1.1.4 Id

Id proteins generally function as inhibitors of differentiation and as positive regulators of proliferation in neuronal development (Lavarone and Lasorella 2004). Id is a protein with the helix-loop-helix domain without a basic region and forms heterodimers with bHLH proteins, e.g., *MASH1* and *HES1* to inhibit their transactivation function (Massari and Murre 2000). In pediatric cancers, *MYC* oncoproteins and EWS-Ets fusion proteins are targeted to induce Id2 which in turn inhibits Rb and other target proteins including bHLH proteins, Ets and Pax. In neuroblastoma, *MYCN* has been shown to induce Id2 which stimulates cell proliferation by inhibiting Rb function (Lasorella et al. 2000).

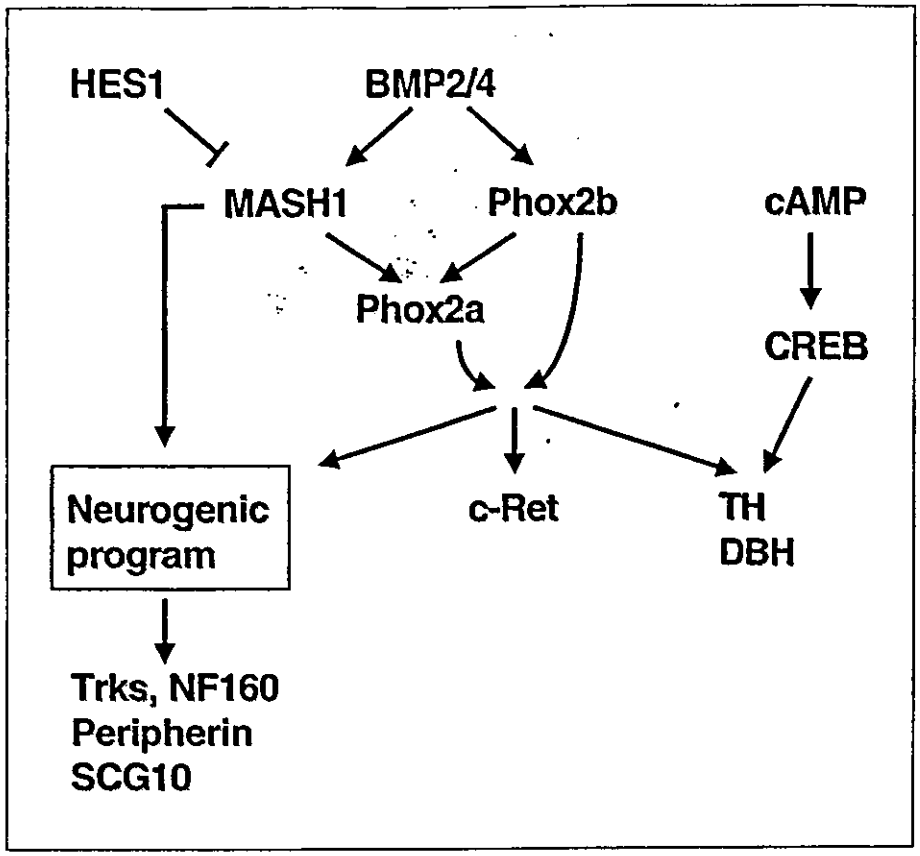


Figure 5.4

Regulatory network controlling sympathetic neuron development. BMP2 and BMP4 are required for the expression of *MASH1* and *Phox2b*. *HES1* induced by notch signaling inhibits expression of *MASH1*. *MASH1* and *Phox2b* are genetically upstream of *Phox2a*, and *Phox2b* is genetically upstream of *Gata3*. Expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) depends on *MASH1*, Phox2b, and Gata3. Cyclic AMP also controls expression of TH and DBH. Phox2a and Phox2b may affect induction or maintenance of *MASH1* expression. *MASH1*, Phox2a, and Phox2b regulate the downstream neurogenic program, leading to terminal differentiation of sympathetic neurons by inducing the genes, e.g., Trks, NF160, peripherin, and SCG10

5.1.1.5 *MYCN*

MYCN is a member of the group of *MYC*-box genes, and its product is a bHLH protein (Schwab et al. 2003). *MYCN* is transiently expressed during normal neural development and defines the direction of neuronal differentiation. *MYCN* is frequently amplified in advanced-stage neuroblastoma (Schwab et al. 1983, 1984; Brodeur et al. 1984; Seeger et al. 1985), and the biology of high-risk neuroblastoma is influenced by the subsequent overexpression of *MYCN* oncoprotein and its targets including telomerase and those functioning in ribosome biogenesis and protein synthesis (Mac et al. 2000; Boon et al. 2001).

5.2 Molecular Bases of Differentiation and Programmed Cell Death

5.2.1 Molecular Aspect of Spontaneous Regression

It is well known that some subsets of neuroblastoma can regress spontaneously. One of the most important hints to understand the mechanism of spontaneous regression is age of the patient at the onset of neuroblastoma. Regression rarely occurs when the tumor is found in patients over 1 year of age. The dramatic regression of the stage 4s tumor after its rapid growth usually occurs within 6 months after birth; therefore, it is plausible that epigenetic regulations, timed with the development of sympathetic neurons, might also control neuroblastoma regression. It is well known that massive death of sympathetic neurons is induced during the perinatal period – a process called developmentally regulated neuronal programmed cell death following deprivation of tar-

get tissue-derived neurotrophins (Oppenheim 1991). This same death mechanism appears to be conserved in primary neuroblastomas found in infants, leading to the induction of their spontaneous regression (Nakagawara 1998b).

5.2.2 Neurotrophic Factors and Their Receptors

5.2.2.1 Neurotrophins and Their Receptors in Neuroblastoma

The neurotrophin family of growth factors consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5; Huang and Reichardt 2003). The corresponding high-affinity neurotrophin receptors with tyrosine kinase activity have been identified as TrkA, TrkB, and TrkC (Snider 1994) (Fig. 5.5 a, b). TrkA is a preferred receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower-affinity neurotrophin receptor $p75^{NTR}$, a member of the tumor necrosis factor receptor (TNFR)/Fas family (Snider 1994). The targeted disruption of neurotrophins and their receptors has demonstrated that NGF/TrkA signaling supports the survival and differentiation of sympathetic and sensory neurons responsive to temperature and pain, while BDNF/TrkB, NT-4/TrkB, and NT-3/TrkC signaling supports those of sensory neurons responsive to tactile stimuli and motor and sensory neurons responsive to limb movement and position, respectively (Klein 1994). These results suggest that neural development and maintenance of the neural network are spatiotemporally controlled by neurotrophin signaling with or without some redundancy in both peripheral and central nervous systems.

In neuroblastoma, high levels of TrkA are expressed in subsets of tumors with good prognosis, often showing spontaneous regression (Nakagawara et al. 1992, 1993; Suzuki et al. 1993; Kogner et al. 1993). Such tumors usually occur in patients under 1 year of age, and their DNA ploidy is aneuploid. A very limited amount of NGF may be supplied from stromal cells, e.g., Schwannian cells and fibroblasts, which at least partly regulate the differentiation and programmed cell death of neuroblastoma cells (Nakagawara 1998a). On the other hand, TrkA expression is strongly downregulated in tumors with aggressive behavior that usually possess amplification of the *MYCN* oncogene and allelic loss of chromosome 1p36 (Nakagawara et al. 1992, 1993). TrkB is preferentially expressed in aggressive neuroblastomas together with its preferred ligands BDNF and NT-4/5 which stimulate in an autocrine/paracrine manner, conferring an enhanced malignant phenotype to the tumor cells (Nakagawara et al. 1994; Matsumoto et al. 1995). TrkC is expressed in favorable neuroblastomas at variable levels (Yamashiro et al. 1996), but its preferred ligand, NT-3, is nearly undetectable by RT-PCR in primary neuroblastomas (Nakagawara 1998a); thus, in regressing neuroblastomas, tumor cells expressing the TrkA receptor may be dependent on a limited amount of NGF supplied from stromal cell. In the presence of NGF the cells mature, whereas they will die in the absence of this ligand (Nakagawara 1998a,b); however, in clinically aggressive neuroblastomas, the TrkA is downregulated and the downstream signaling cascades are disturbed, and these cells utilize the BDNF or NT-4/TrkB autocrine system for efficient growth. Neurotrophin signaling may also regulate tumor metastasis (Matsumoto et al. 1995), proliferation (Matsumoto et al. 1995), and angiogenesis (Canete et al. 2000). The role of $p75^{NTR}$ in neuroblastoma is unclear. The $p75^{NTR}$ receptor is expressed in both neuroblastoma cell lines (Azar et al. 1990) and primary neuroblastomas (Nakagawara et al. 1993). Interestingly, the expression levels of $p75^{NTR}$ mRNA are significantly higher in favorable neuroblastomas (stages 1, 2 and 4s) as compared with the advanced stage tumors, especially those with *MYCN* amplification (Nakagawara et al. 1993).

5.2.2.2 Neurotrophin Signaling in Neuroblastoma

In a rat pheochromocytoma cell line PC12, differentiation signals by NGF may be mediated through the tyrosine phosphorylation of the Trk receptor and through the subsequent activation of Shc/Grb2/SOS, Ras, Raf, MEK, and ERKs, while survival signals in the same cells may be transduced through the direct

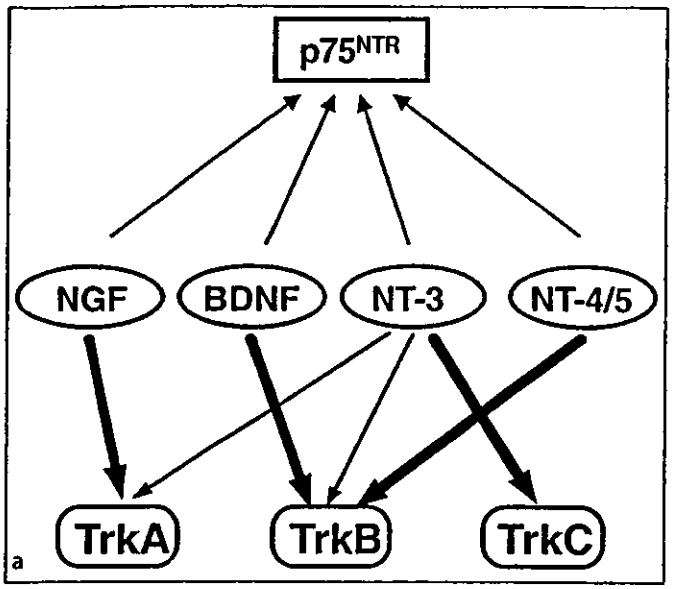
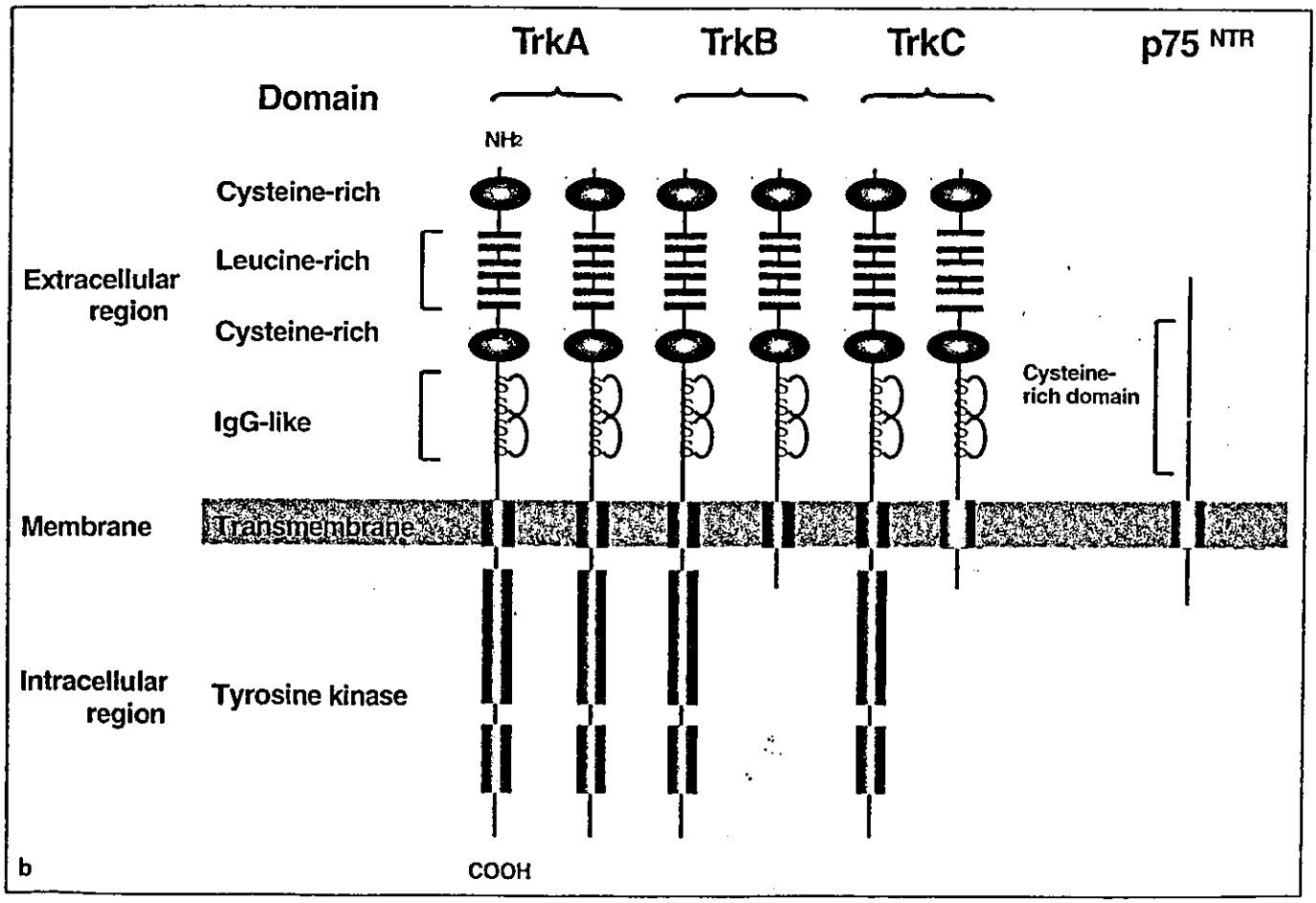


Figure 5.5 a, b

Neurotrophins and their receptors. **a** TrkA is a preferred high-affinity receptor for NGF, TrkB for BDNF, and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower affinity neurotrophin receptor $p75^{NTR}$. **b** The structures of neurotrophin family receptors. The extracellular domains of TrkA, TrkB, and TrkC have high structural similarity. The intracellular domain of Trks possesses tyrosine kinase activity. TrkB and TrkC receptors have truncated forms which lack the tyrosine kinase domain. The low-affinity receptor, $p75^{NTR}$, has a short intracellular region containing the death domain, and belongs to the Fas/TNFR family of the receptors



activation of PI3-kinase which in turn activates downstream molecules, e.g., Akt and Bad (Klesse and Parada 1999). On the other hand, in normal sympathetic neurons, the activation of PI3-kinase is mediated not by the tyrosine phosphorylation of the receptor but by the Ras activation which promotes neuronal survival, suggesting that the Trk intracellular signaling pathway might be deregulated in cancer cells. This is also the case in neuroblastoma. In the neuroblastoma cell lines with a single copy of *MYCN*, NGF can induce differentiation when exogenous TrkA is overexpressed (Eggert et al. 2000). In the cell lines with *MYCN* amplification, however, the NGF-stimulated TrkA receptors which were overexpressed cannot normally activate downstream signaling molecules, resulting in unresponsiveness to the ligand. Furthermore, it is surprising that BDNF/TrkB signaling appears to be functioning in the same cells by promoting survival (Nakagawara et al. 1994; Hishiki et al. 1998), although the signaling pathway might be different from that of sympathetic neurons (Klesse and Parada 1999).

5.2.2.3 GDNF Family Receptors

Neurotrophic factors of the glial cell line-derived neurotrophic factor (GDNF) family, which include GDNF, artemin and neurturin, are secreted by neuroblastoma cells as well as stromal cells and activate their receptor complex composed of Ret tyrosine kinase and the GFRα co-receptors expressed in neuroblastoma cells (Hishiki et al. 1998; Ichikawa et al. 2004). In contrast to NGF/TrkA and BDNF/TrkB, however, the GDNF/Ret/GFRα autocrine system is functioning in both favorable and unfavorable neuroblastomas to enhance the survival and differentiation of tumor cells (Hishiki et al. 1998).

5.2.2.4 Other Factors and Receptors

Neuroblastoma cells express other growth factors and receptors. Both pleiotrophin (PTN) and midkine (MK) are factors in the same family with neurotrophic function (Kadomatsu et al. 1990; Li et al. 1990; Kadomatsu and Muramatsu 2004). PTN is expressed significantly at high levels in favorable neuroblastomas, while MK is highly expressed in almost all neuroblastomas with a tendency to be expressed at high levels in tumors in advanced stages (Nakagawara et al. 1995). Neuroblastoma also expresses many other receptors, e.g., fibroblast growth factor receptor (FGFR; Schweigerer et al. 1991), insulin-like growth factor (IGFR; El-Badry et al. 1991), DCC (deleted in colon cancer) (Reale et al. 1996), and neuronal leucine-rich repeat receptors (NLRRs; Hamano et al. 2004), as well as a novel plasma membrane enzyme ECEL1, which is significantly highly expressed in favorable neuroblastomas (Kawamoto et al. 2003). The biological significance of these factors and receptors in neuroblastoma are not currently known.

5.2.3 Functional Role of p53 Family Genes

Recent lines of evidence suggest that both the p53 tumor suppressor protein and its related protein p73 are involved in the induction of programmed cell death and growth arrest in neuronal cells (Pozniak et al. 2000). p73 is a recently identified candidate tumor suppressor gene mapped to chromosome 1p36.2, a frequently deleted region in many human cancers including neuroblastoma and oligodendroglioma (Ichimiya et al. 1999; Billon et al. 2004). In cultured neonatal sympathetic neurons, p53 protein levels are increased in response to NGF withdrawal as well as $p75^{NTR}$ activation, and it functions downstream of c-Jun NH_2-terminal kinase (JNK) and upstream of Bax to induce apoptosis (Aloyz et al. 1998) (Fig. 5.6). Indeed, in $p53^{-/-}$ mice, naturally occurring sympathetic neuron death is inhibited. Pozniak et al. (2000) have also reported that p73 is primarily present in developing neurons as ΔNp73, an NH_2-terminally truncated isoform, whose level is decreased when sympathetic neurons undergo apoptosis after NGF withdrawal, and that p53 becomes activated to be pro-apoptotic. In contrast to the truncated form of p73, full-length p73 has induced neuronal differentiation in a mouse neuroblastoma cell line N1E115 (Laurenzi et al. 2000). These data suggest that the neuronal apoptosis induced by NGF withdrawal is at least partly regulated by a reciprocal balance between levels of pro-apoptotic p53 and anti-apoptotic ΔNp73.

Figure 5.6

A model of signaling pathway for survival and death in sympathetic neurons regulated by NGF. NGF depletion may induce activation of JNK/p53 pathway which could be modified by p73/ΔNp73 regulatory system. $p75^{NTR}$ activation, which sends signals of both survival and death, may also regulate downstream p53/p73/ΔNp73 pathway.

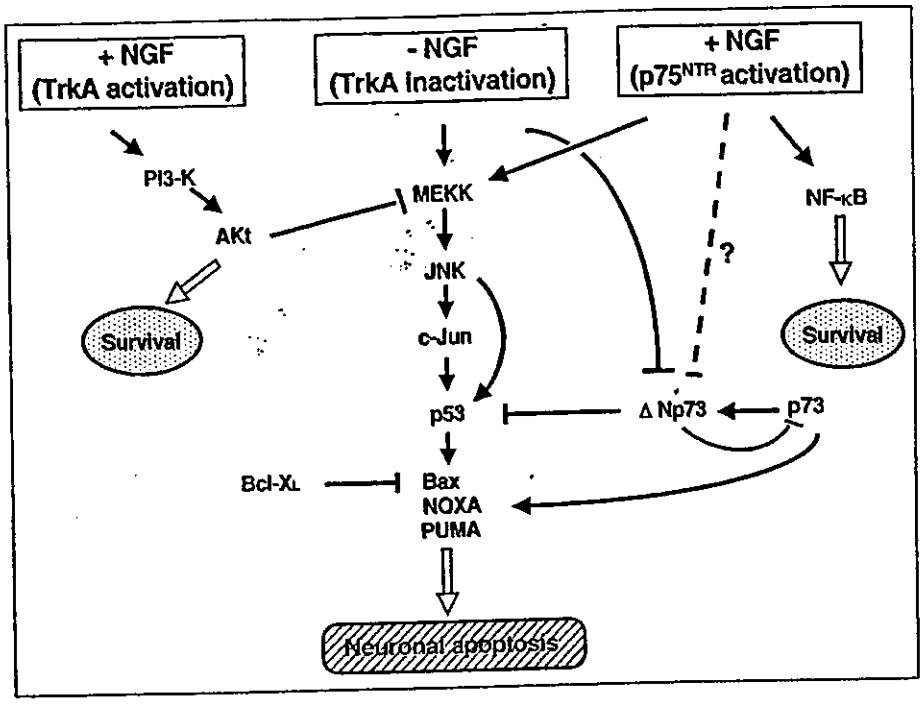
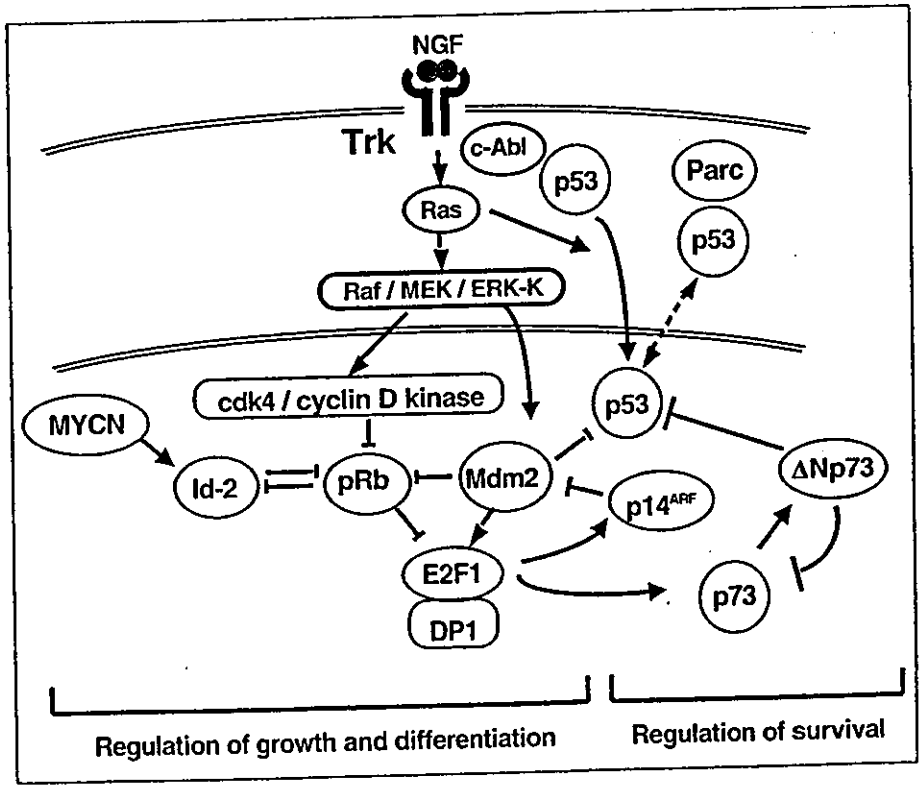


Figure 5.7

A possible signaling pathway regulating growth, differentiation and survival in neuroblastoma cells or sympathetic neurons. The NGF-triggered autophosphorylation of TrkA tyrosine kinase receptor induces activation of Ras/MAPK pathway, which in turn regulates nuclear pRB and Mdm2. In some poor-outcome neuroblastomas, p53, which is shuttling between cytosol and nucleus, is trapped in the cytosol by Parc, an anchoring protein of p53. *MYCN* induces expression of *Id-2* whose protein product in turn inhibits pRB. E2F1 negatively regulated by pRB directly induces expression of p73. p73 is regulated by ΔNp73 in a negative autoregulatory manner (Nakagawa et al. 2002), and ΔNp73 also inhibits p53.



The importance of p53 and p73 has also been emphasized by the important observation that, in cultured neuroblastoma and other cancer cells, p73 directly transactivates the ΔNp73 gene by binding to its promoter after treating the cells with genotoxic reagents, e.g., cisplatin (Nakagawa et al. 2002). The induced ΔNp73 protein in turn interacts with either wild-type p53 or TAp73 and inhibits their proapoptotic function; thus, ΔNp73 can act as an oncogene and as an inhibitor of wild-type p53 and TAp73. The presence of this autoinhibitory feedback loop among p53, TAp73, and ΔNp73 may at least in part explain why there is no mutation of the p73 gene in cancers.

p53 is associated with TrkA via the proto-oncogene product c-Abl as an adaptor or bridging molecule, suggesting that it may also play a role in Trk signaling (Yano et al. 2000) (Fig. 5.7). The activation of Ras by NGF stimulation of the TrkA receptor induces p53 nuclear translocation and growth arrest in PC12 cells (Hughes et al. 2000). The c-Ha-Ras gene could be a target of p53, and protein products induce a positive feedback loop by activating $p14^{ARF}$ which counteracts the negative feedback loop mediated by mdm2 (Deguin-Chambon et al. 2000). These observations strongly suggest that p53 and p73 tumor suppressors function in neurotrophin signaling and modulate the growth, differentiation, and apoptosis of neurons.

In neuroblastoma and some other human cancers, wild type p53 is often localized in the cytoplasm (Moll et al. 1995). Although the regulatory mechanism of cellular localization of p53 and p73 is still unknown, activated Ras in NGF/TrkA signaling stimulates the nuclear translocation of p53 and leads to growth arrest by the induction of $p21^{WAF1}$ in PC12 cells (Hughes et al. 2000). Furthermore, some fractions of recurrent neuroblastomas and neuroblastoma cell lines acquire mutation of the p53 gene (Tweddle et al. 2001).

5.2.4 Apoptotic Signals in Neuroblastoma

To date, the spontaneous regression of neuroblastoma, has occurred only *in vivo*. Although this makes the analysis difficult, there are some important reports. An anti-apoptotic protein, Bcl-2, is expressed in primary neuroblastomas and neuroblastoma cell lines. The expression levels of Bcl-2 and Bcl-X_L are high in aggressive tumor cells but are low in regressing cells (Ikeda et al. 1995; Ikegaki et al. 1995). Caspase-1 and caspase-3 are expressed at significantly higher levels in favorable neuroblastomas (Nakagawara et al. 1997), and caspase-8 is silenced in aggressive neuroblastomas by the methylation of its promoter as one of mechanisms (Teitz et al. 2000). Silencing of caspase-8 is observed in 25–35% of primary neuroblastomas with a high frequency in more aggressive tumors (Teitz et al. 2000; Eggert et al. 2001; van Noesel et al. 2003). Survivin, a member of the inhibitors of apoptosis protein (IAP), is mapped to the long arm of chromosome 17. In neuroblastoma, survivin is highly expressed in high-risk tumors, and its overexpression inhibits cellular apoptosis (Islam et al. 2000). Kitanaka et al. (2002) have recently reported an interesting observation that "autophagy" may be involved in the regression of neuroblastoma cells.

5.3 Conclusions

Development of neuroblastoma may be triggered by a genetic event(s) that leads to chromosome and/or the genomic DNA abnormalities such as amplification of the *MYCN* gene and deletions or gains in chromosomal regions including 1p, 11q, and 17q. Together with other epigenetic mechanisms of gene activation or gene silencing, they affect gene and protein expression which in turn deregulate cellular signaling. In neuroblastoma the normal biology of developing neuronal cells and cancer biology appear to overlap. A further understanding of the mechanisms involved in the transformation of progenitors or the stem cells into neuroblastoma with significant cellular heterogeneity may provide clues for the development of novel therapeutic strategies for this often aggressive lethal disease.

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Protein stability and function of p73 are modulated by a physical interaction with RanBPM in mammalian cultured cells

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Upon a certain DNA damage including cisplatin treatment, p73 is stabilized and exerts its growth-suppressive and/or proapoptotic function. However, the precise molecular basis by which the intracellular levels of p73 are regulated remains unclear. In the present study, we have identified RanBPM as a novel binding partner of p73α by yeast-based two-hybrid screening, and also found that RanBPM has an ability to stabilize p73α. GST pull-down assays and co-immunoprecipitation experiments revealed that RanBPM directly bound to the extreme COOH-terminal region of p73α, whereas it failed to interact with p53. Co-expression of RanBPM with p73α resulted in the nuclear translocation of RanBPM, and both proteins co-localized in cell nucleus as examined by indirect immunofluorescent staining. It is worth noting that the expression of RanBPM inhibited the ubiquitination of p73α, and thereby prolonged its half-life. Subsequent studies demonstrated that the proapoptotic activity of p73α was significantly enhanced in the presence of RanBPM. Taken together, our present findings implicate a novel role for RanBPM in the regulation of p73 stability and function.
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p73 is a newly identified p53-related nuclear transcription factor, and functions to promote cell cycle arrest and/or apoptosis (Kaghad *et al.*, 1997). These cellular roles of p73 are largely attributed to its ability to transactivate specific target genes. In contrast to p53, p73 is expressed as multiple isoforms arising from either alternative splicing or alternative promoter usage (Melino *et al.*, 2002). Although functional differences among the splicing isoforms with different COOH-termini remain unclear, NH_2-terminally truncated forms of p73 (ΔNp73) have an oncogenic potential and exhibit a dominant-negative behavior toward wild-type p73 as well as p53 (Pozniak *et al.*, 2000; Nakagawa *et al.*, 2002; Stiewe *et al.*, 2002).

Steady-state levels of p73 are kept extremely low under normal conditions, however, p73 is significantly induced at protein level in response to a certain genotoxic stress including cisplatin treatment, which is mediated by a nuclear nonreceptor tyrosine kinase c-Abl (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). c-Abl binds to the PXXP motif of p73 and phosphorylates p73 at Tyr-99. Alternatively, Ren *et al.* (2002) reported that protein kinase Cδ catalytic fragment phosphorylates p73 at Ser-289, and increases its stability, suggesting that post-translational modification such as phosphorylation might contribute to increase the stability of p73. Protein phosphorylation has been shown to be involved in the initiation of protein ubiquitination by E3 ubiquitin ligase (Carrano *et al.*, 1999; Ganoth *et al.*, 2001). As described previously (Balint *et al.*, 1999; Lee and La Thangue, 1999), p73 is regulated at least in part by the protein degradation process through the ubiquitin–proteasome system. Additionally, Lee and La Thangue (1999) described that the COOH-terminal region of p73α might have a regulatory role in the proteasome-dependent degradation of p73. Recently, we have found that MM1 and RACK1 interact with the extreme COOH-terminal region of p73α, and regulate its transcriptional activity as well as proapoptotic function (Watanabe *et al.*, 2002; Ozaki *et al.*, 2003). However, these interactions did not have a detectable effect on the intracellular levels of p73α.

To identify the possible cellular protein(s) involved in the regulation of p73 protein stability, we screened a cDNA library derived from human fetal brain using the extreme COOH-terminal region of p73α (amino-acid residues 551–636) as a bait in a yeast-based two-hybrid system. After screening of approximately 5×10^5 transformants, 12 independent clones exhibited a high level of β-galactosidase activity, and subsequent sequence analysis revealed that three out of them encoded the overlapping regions of RanBPM (Figure 1a). RanBPM was initially identified as a cellular protein that can interact with Ran nuclear–cytoplasmic transport protein (Nakamura *et al.*, 1998; Nishitani *et al.*, 2001), and contained the putative SPRY domain which might be involved in protein–protein interactions (Ponting *et al.*, 1997). Although most of the Ran-binding proteins play an important role in nucleocytoplasmic transport, it is

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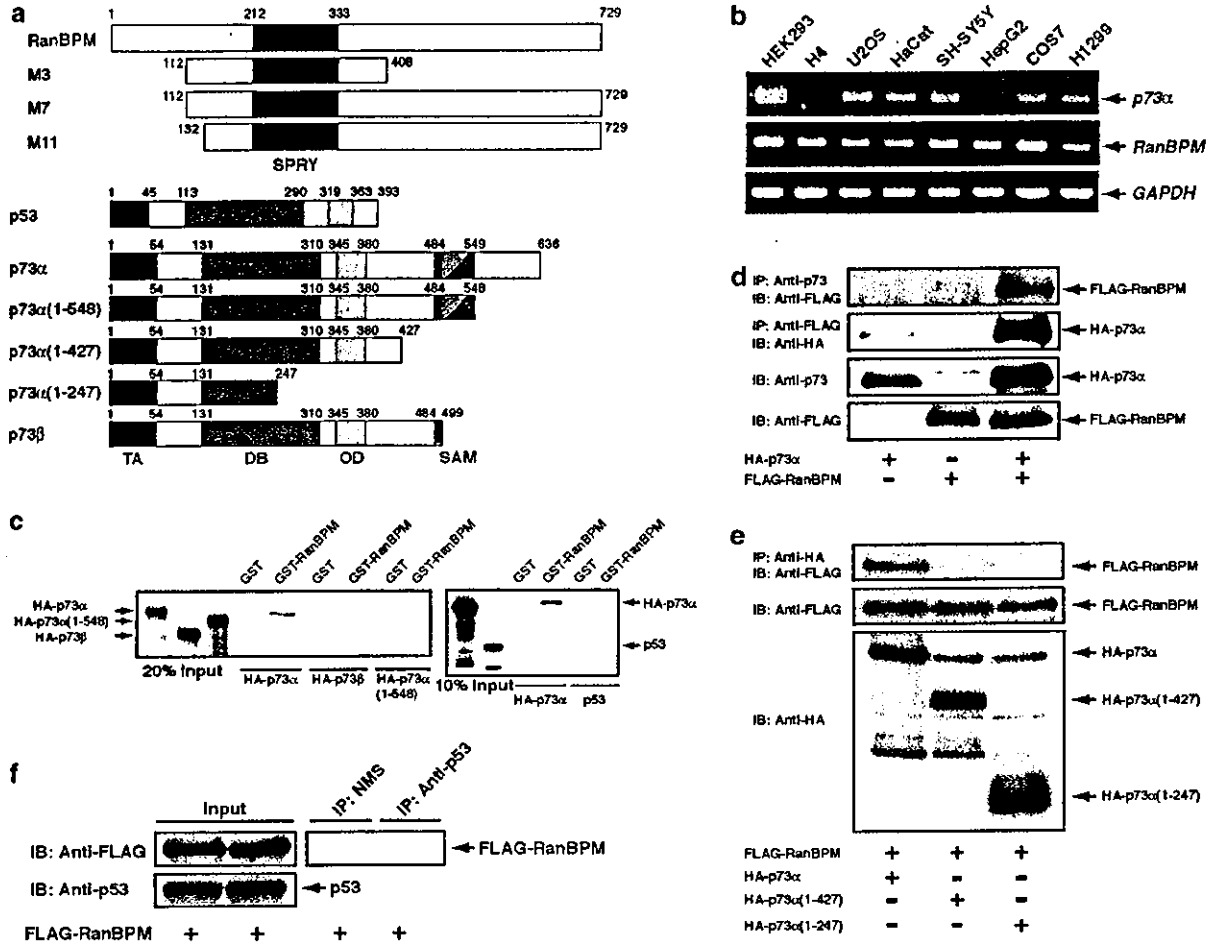

Figure 1 Identification of RanBPM as a binding partner of p73. (a) The three overlapping RanBPM clones (M3, M7 and M11) isolated from the yeast two-hybrid screening along with the full-length RanBPM are shown. The putative SPRY domain (amino-acid residues 212–333) is indicated. Structures of p73 and p53 are also shown. TA, transactivation domain; DB, DNA-binding domain; OD, oligomerization domain; SAM, sterile α motif domain. Amino-acid numbering was relative to first methionine, which represents position +1. (b) Expression of *RanBPM* and *p73*. Total RNA prepared from the indicated cell lines were incubated with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and generated cDNAs were amplified by PCR in the presence of primers specific for *p73* (top panel), *RanBPM* (middle panel) or *GAPDH* (bottom panel). (c) GST pull-down assay. *In vitro* translated ^{35}S-labeled p73α, p73β, p73α (1–548) or p53 was incubated with bacterially expressed GST or GST-RanBPM(112–408) for 2 h at 4°C. Bound complexes were recovered on the glutathione–sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA), washed extensively with the binding buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and then boiled in SDS sample buffer. Bound proteins were resolved by 10% SDS–polyacrylamide gel, and analysed by autoradiography. The input of the radio-labeled proteins used in the binding reaction is also shown. (d) p73α interacts with RanBPM in mammalian cultured cells. COS7 cells transfected with the indicated expression plasmids were lysed in 25 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. Whole-cell lysates were immunoprecipitated with anti-p73 antibody (Ab-4, NeoMarkers, Fremont, CA, USA) or anti-FLAG (M2, Sigma, St Louis, MO, USA), and subjected to immunoblotting with anti-FLAG (first panel) or with anti-HA (12CA5, Roche Molecular Biochemicals, Indianapolis, IN, USA) antibody (second panel), respectively. Separate aliquots of the lysates were immunoblotted with anti-p73 (third panel) or anti-FLAG antibody (fourth panel) to confirm the expression of FLAG-RanBPM or HA-p73α, respectively. (e) COOH-terminal region of p73α is required for the interaction with RanBPM. COS7 cells were co-transfected with the indicated combinations of the expression plasmids, and whole-cell lysates were immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-FLAG antibody (top panel). Cell lysates were immunoblotted as a control for FLAG-RanBPM (middle panel), HA-p73α and HA-p73α derivatives (bottom panel) in the input lysate. (f) p53 does not bind to RanBPM. Cell lysates prepared from COS7 cells transfected with FLAG-RanBPM were immunoprecipitated with the normal mouse serum (NMS, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or anti-p53 antibodies (DO-1 plus PAb1801, Oncogene Research Products, Cambridge, MA, USA). Immunoprecipitates were analysed by immunoblotting with anti-FLAG antibody (right panel). Left panels show the Western blotting with anti-FLAG, or anti-p53 antibody to monitor the expression level of FLAG-RanBPM or the endogenous p53, respectively

unlikely that RanBPM is involved in this process (Nishitani *et al*., 2001). Alternatively, Nakamura *et al*. (1998) reported that RanBPM might be involved in reorganization of the microtubule network; however, the precise function of RanBPM remains unknown.

Consistent with the previous observations (Rao *et al*., 2002), *RanBPM* was expressed in various cell lines (Figure 1b). To confirm the interaction between RanBPM and p73, we performed GST pull-down assays using a GST fusion protein containing RanBPM