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Expression of ΔNp73 is a molecular marker for adverse outcome in neuroblastoma patients

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Abstract

The *p73* gene is a p53 homologue which induces apoptosis and inhibits cell proliferation. Although *p73* maps at 1p36.3 and is frequently deleted in neuroblastoma (NB), it does not act as a classic oncosuppressor gene. In developing sympathetic neurons of mice, p73 is predominantly expressed as a truncated anti-apoptotic isoform (ΔNp73), which antagonizes both p53 and the full-length p73 protein (TAp73). This suggests that p73 may be part of a complex tumor-control mechanism. To determine the role of ΔNp73 in NB we analyzed the pattern of expression of this gene *in vivo* and evaluated the prognostic significance of its expression. Our results indicate that *ΔNp73* expression is associated with reduced apoptosis in a NB tumor tissue. Expression of this variant in NB patients significantly correlates with age at diagnosis and VMA urinary excretion. Moreover it is strongly associated with reduced survival (HR=7.93; $P<0.001$) and progression-free survival (HR=5.3; $P<0.001$) and its role in predicting a poorer outcome is independent from age, primary tumor site, stage and MYCN amplification (OS: HR=5.24, P=0.012; PFS: HR=4.36, P=0.005). In conclusion our data seem to indicate that *ΔNp73* is a crucial gene in neuroblastoma pathogenesis.
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Keywords: neuroblastoma; p73; apoptosis; prognostic factors

Abbreviations: NB, neuroblastoma; OS, overall survival; PFS, progression-free survival; HR, hazard ratio; LOH, loss of heterozygosity

Introduction

Neuroblastoma (NB) is a common extracranial tumor of infancy originating from neural crest cells. This tumor presents remarkable biological and clinical heterogeneity and its likelihood of progression varies widely according to stage, age at diagnosis and to several molecular parameters[1] (reviewed in[2] and[3]). The emerging concept is that NB represents a group of related tumors with different genetic and biological features. *MYCN* gene amplification, chromosome 1p deletion (1pdel), and the gain of chromosome 17q are the most frequent chromosomal alterations found in this tumor and are important prognostic factors associated with disease progression and poor patient survival.[1–4] Although these factors are highly predictive, still unrecognized genetic alterations must be responsible for the rapid tumor progression in a subset of patients without *MYCN* amplification and/or chromosome 1p deletion. Moreover, the molecular mechanisms at the basis of the favorable outcome in patients with disseminated disease are not yet known and their comprehension might have obvious important implications.

The multiplicity of the chromosomal alterations described in NB indicates that the evolution of this neoplasia involves a complex pattern of oncogenes activation and oncosuppressor genes inactivation. On the basis of common deletion patterns, the chromosomal region 1p36 has been suspected to contain a locus that might act as a tumor suppressor gene in a variety of adult and pediatric tumors.[5,6] The discovery of *p73*, a *p53* homologue mapped at 1p36.3, has elicited a considerable interest in the scientific community and this gene was thought to be the oncosuppressor gene located at chromosome 1p (for recent reviews on *p73* see[7] and[8]). *p73* transactivates several *p53* target genes, inhibits cell proliferation and induces apoptosis and neuronal differentiation in NB cell lines,[9] however its contribution to tumor suppression is still unclear. The lack of spontaneous tumors in *p73*-deficient mice indicates that this gene does not belong to the group of classical two-hit Knudson's tumor suppressor genes and that its role in cancer must be clearly different from that of *p53*.[10] A possible link between p73 and tumorigenesis derives from recent reports demonstrating that p73 is a downstream effector of E2F-1 and an essential component of the p53-independent apoptotic pathway.[11–13] Since the inactivation of the p53-mediated apoptosis is generally observed in highly aggressive tumors, the functionality of the p53-apoptotic pathway may have several potentially relevant implications for new therapeutic approaches.

Unlike *p53*, *p73* codes for a variety of isoforms and understanding of the role *p73* in tumor development is complicated by the antagonizing effects exerted by some of the variants encoded by this gene. In developing sympathetic neurons of mice p73 is predominantly expressed as a truncated anti-apoptotic isoform (ΔNp73), that counteracts

p53 and suppresses the transactivation activity of the full-length p73 variant (TAp73) by oligomerization and competition for DNA binding.[10,14] Δ*Np73* is transcribed from an internal promoter in the third intron of the gene upstream of an alternative exon (exon 3′). Δ*N* promoter functionality in NB cells and tumor tissues is, at least in part, regulated by epigenetic mechanisms.[15]

We have investigated the clinical significance of Δ*Np73* expression in human neuroblastoma. Our results indicate that the expression of this variant is associated with reduced apoptosis *in vivo* and is a strong predictor of unfavourable outcome, independently of age, primary tumor site, stage, chromosome 1p deletion and *MYCN* amplification.

Results

We have previously shown that the truncated, antiapoptotic ΔN isoform of the p73 gene is transcribed in most NB cell lines but not in the myeloid cell lines HL60 and U937.[15] Moreover, we did not detect this isoform in a survey of T and B acute lymphocytic leukemia cells and in PBL from healthy donors. Although the complete pattern of expression of this truncated variant in normal and tumor cells still needs to be evaluated, these results and the preliminary analysis of a panel of tumor cell lines and normal tissues suggests that the expression of Δ*Np73* is not ubiquitous (data not shown).

TA and ΔNp73 exert opposite functions in the control of apoptosis *in vitro* and it was shown that ΔNp73 has an anti-apoptotic role on the programmed cell death of neuronal cells.[14] In an attempt to determine if this variant had a similar role *in vivo* we evaluated the expression of the Δ*N* isoform in distinct tumor areas of a NB specimen presenting morphologic and functional differences. The samples utilized for this study derived from a patient with a diagnosis of bilateral adrenal neuroblastoma at stage 2B. The tumor, that did not present *MYCN* amplification or chromosome 1p deletion, rapidly progressed and the patient died for disease dissemination.[16] Detection of apoptosis on tumor sections by the *in situ* TUNEL assay showed that two tumor areas, derived from the same mass, had drastically different levels of apoptosis (Figure 1A,B). RT–PCR analysis showed that Δ*Np73* was expressed only in the tumor section absent of apoptotic staining (C). No definitive conclusions can be derived from the analysis of a single patient, however, the observation that this *p73* variant is expressed in a tumor area that did not present significant levels of apoptosis, suggests that Δ*Np73* expression may be associated with the inhibition of programmed cell death also in NB tumor tissue.

To verify the possibility that this variant is of clinical relevance in NB, we determined the expression of Δ*Np73* mRNA in 52 primary tumors in relation to several clinical parameters (Figure 1D). In an earlier study[17] we reported that *p73* is expressed in approximately 50% of the NB patients without a significant association with age, stage, *MYCN* amplification, chromosome 1p deletion or reduced survival. The experimental approach utilized in that analysis however, could reveal the expression of *TA* but not of Δ*Np73*. In the present study, that included essentially the same set of patients, the reevaluation of the expression of the TA variant under different experimental conditions,

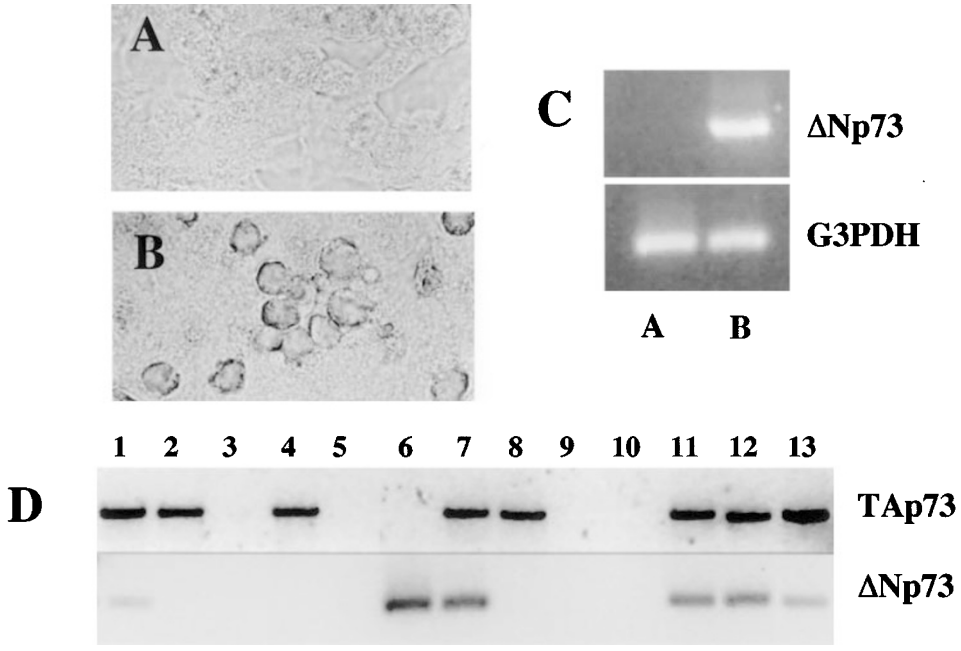

Figure 1 Δ*Np73* expression in NB tumor tissues. (**A**, **B**) *In situ* detection of apoptosis by the TUNEL assay in different areas of the same tumor. Tumor section in (**A**) presents a diffuse and strong staining demonstrating the presence of single- and double-stranded breaks indicative of apoptosis. Note the absence of apoptotic staining in the tumor section of (**B**). (**C**) Total RNA was extracted from corresponding sections and utilized for Δ*Np73* expression analysis. G3PDH expression was utilized as internal control. Expression of the anti-apoptotic Δ*N* variant was detected only in the tumor section not showing apoptotic staining. (**D**) Expression of *TA* and Δ*Np73* in neuroblastoma tumor tissues. In approximately 10% of the cases we have observed that the Δ*N* variant is expressed in the absence of detectable *TAp73*

confirmed our previous findings (Figure 1D and data not shown).

RT–PCR analysis with a set of primers that amplified a cDNA fragment encompassing exons 3′ and 4, showed that the Δ*Np73* isoform is expressed in only 30% of the tumors. Samples expressing both isoforms had a variable ratio of *TA*/Δ*N*, a likely consequence of the heterogeneity of NB tumors (Figure 1D).

In Table 1 we report the pattern of Δ*Np73* mRNA expression in relation to several clinical and biological parameters. This *p73* variant was expressed more frequently in children older than 1 year and in those with advanced disease stage, higher vanillylmandelic acid urinary excretion, *MYCN* amplification and chromosome 1p deletion. Interestingly none of the five tumors derived from patients with disseminated stage 4S disease expressed the Δ*Np73* isoform.

Table 2 lists the OS and PFS rates in relation to Δ*Np73* expression and to main clinical and biological characteristics. Overall the 5-year survival and progression-free survival probabilities were 67 and 57% respectively. A poor prognosis was associated with age, stage and high urinary VMA excretion, however in this group of patients the worst predictor was the expression of the Δ*Np73* isoform.

As shown in Figure 2, the expression of Δ*Np73* is strongly associated with reduced OS (HR=7.93; $P<0.001$) and PFS (HR 5.3; $P<0.001$). Moreover the multivariate analysis demonstrated that the role of Δ*Np73* in predicting

Table 1 *ΔNp73* expression in relation to clinical and biological parameters

		ΔNp73+		
Characteristic	**Total no.**	**No.**	**%**	***P* value**
Study sample	52	16	31	—
Age				
0–11 months	18	2	11	0.031†
⩾1 yr	34	14	41	
INSS Stage				
Localized (1–2)	20	4	20	0.058*
Advanced (3–4)	27	12	44	
4S	5	0	0	
Urine VMA				
<2.5 SD	10	0	0	0.042†
⩾2.5 SD	35	13	37	
Urine HVA				
<2.5 SD	4	1	25	1.0†
⩾2.5 SD	34	9	26	
LDH				
<1000	28	7	25	0.753†
⩾1000	22	7	32	
NSE				
<100	26	7	27	1.0†
⩾100	13	3	23	
MYCN				
Not amplified	41	11	27	1.0†
Amplified	10	5	50	
1p deletion				
Not deleted	32	8	25	0.086†
Deleted	8	5	62	

VMA: vanillylmandelic acid; HVA: homovanyllic acid; LDH: lactic dehydrogenase; NSE: neuronal specific enolase.
*chi-square; †Fisher's exact test

Table 2 Five-year overall survival and progression-free survival in relation to clinical and biological characteristics at diagnosis and *ΔNp73* expression

			OS			PFS	
Characteristic	**No.**	**%**	**HR**	***P* value**	**%**	**HR**	***P* value**
Study sample	52	67	—	—	57	—	—
Age							
0–11 months	18	94	1	0.06	83	1	0.071
⩾1 year	34	56	5.67		46	2.94	
INSS stage							
Localized (1–2)	20	84	1	0.017	80	1	0.086
Advanced (3–4)	27	50	5.79		39	3.12	
4s	5	100	undef.		80	1.34	
Urine VMA							
<2.5 SD	10	100	1	0.058	90	1	0.056
⩾2.5 SD	35	57	undef.		47	5.68	
Urine HVA							
<2.5 SD	4	67	1	0.885	75	1	0.539
⩾2.5 SD	34	65	1.16		52	1.87	
LDH							
<1000	28	80	1	0.071	73	1	0.173
⩾1000	22	55	2.88		44	1.86	
NSE							
<100	26	75	1	0.236	69	1	0.438
⩾100	13	59	2.18		53	1.52	
MYCN amplification							
No	10	72	1	0.079	62	1	0.401
Yes	41	34	2.79		28	1.54	
Chromosome 1p deletion							
No	32	86	1	0.225	70	1	0.348
Yes	8	75	2.32		50	1.72	
ΔNp73 expression							
No	36	84	1	<0.001	82	1	<0.001
Yes	16	0	7.93		27	5.30	

VMA: vanillylmandelic acid; HVA: homovanyllic acid; LDH; lactic dehydrogenase; NSE: neuronal specific enolase.

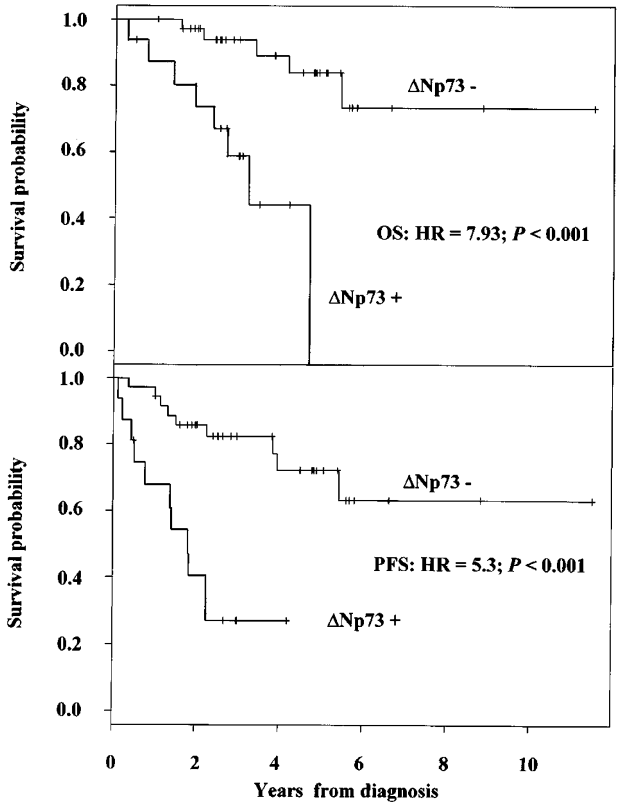

Figure 2 Overall survival (OS) and progression-free survival (PFS) by Δ*Np73* expression in a group of 52 neuroblastoma patients

a significantly poorer outcome was independent from age, primary tumor site, stage and *MYCN* amplification (OS: adjusted HR=5.24, *P*=0.012; PFS: adjusted HR=4.36, *P*=0.005).

Discussion

Neuroblastoma, is a childhood tumor characterized by biological and clinical heterogeneity ranging from spontaneous regression to a dramatic, rapidly progressing disease.[1–3] Advanced stage neuroblastomas, particularly in older children, respond poorly to highly aggressive therapeutic regimens and long-term survival rate for these patients is still below 30%. NB, among human tumors, presents the highest rate of spontaneous regression and the delayed activation of the apoptotic program may be responsible for the initial progression followed by rapid tumor involution observed in neuroblastoma patients at stage 4S.[18]

MYCN amplification and chromosome 1p deletion are strong predictors of unfavorable outcome, however other genetic abnormalities must be responsible for the rapid tumor progression in a subset of patients not presenting these alterations. Our results indicate that the expression of Δ*Np73*, a truncated *p73* variant with a documented antiapoptotic role during sympathetic neuronal development,[14] is a strong predictor of unfavorable outcome independently from other prognostic factors. Interestingly, we did not detect Δ*Np73* in five neuroblastomas at stage 4S, a finding consistent with the proposed role of apoptotic mechanisms in the regression of neuroblastoma at this stage.

Although *p73* was originally considered as a NB oncosuppressor gene,[19] several clinical studies clearly indicated that *p73* does not act as a classic tumor suppressor in this malignancy.[17,20–22] The clinical data reported here however suggest a possible mechanism through which p73 may play a crucial role in neuroblastoma.

TA and ΔNp73 are integral parts of the E2F-1 regulatory network (Figure 3). E2F-1 has intrinsic antagonistic functions and can act as an oncogene or as a tumor suppressor gene. In normal cells the uncontrolled expression of the E2F-1 set of target genes, that activate cell proliferation, is regulated by Rb. In NB cell lines Rb functions may be inhibited by the interaction with Id2 which, in turn, is induced by *MYCN*.[23] The clinical relevance of MYCN-Id2-Rb pathway however has yet to be established.

Unlike most other tumors, mutations inactivating the *p53* gene are rare in neuroblastoma. In this tumor, however, the p53 function is impaired because of cytoplasmic sequestration and transcriptional inactivation within the nucleus.[24,25] In this respect the interaction between p53 and ΔNp73 might be an event contributing to malignancy in the absence of physical damage to the p53 gene. On the other hand the suppression of the transactivating activities of TAp73 by ΔNp73 would eliminate not only an essential anti-tumorigenic safeguard mechanism independent from p53 functionality, but also a neuronal differentiation pathway.[9]

The sample size of this study is relatively limited and probably not fully representative of the entire neuroblastoma tumor population. However in this group of patients Δ*Np73* expression has a clear impact on OS and PFS, as indicated by the high values of hazard ratio estimates derived from uni- and multivariate models and may suggest that the disregulation of *p73* is a critical event in the pathogenesis of this tumor. To better understand the role of p73 in neuroblastoma, a prospective study has been planned within the Italian Neuroblastoma Co-operative Group (INCG).

Materials and Methods

Patients and collection of samples

The 52 tumor samples utilized in this study were retrieved from the Italian Neuroblastoma Tissue Bank. Forty-eight of these patients were included in an earlier analysis on the role of *p73* in neuroblastoma.[17] The specimens were collected, from 1987 to 1998, at the onset of the disease with the approval of the Ethical Committee of the Gaslini Children's Hospital. Informed consent was obtained from the patients or their parents. Tumor cell content was at least 80% for all selected cases. Disease extension was classified according to the International Neuroblastoma Staging System (INSS) criteria.[1] Samples were derived from four patients with stage 1, eight with stage 2, seven with stage 3, 28 with stage 4 and five with stage 4S. The extent of *MYCN* amplification was determined in 51 patients by Southern blot and/or FISH analyses. Chromosome 1p36 deletion

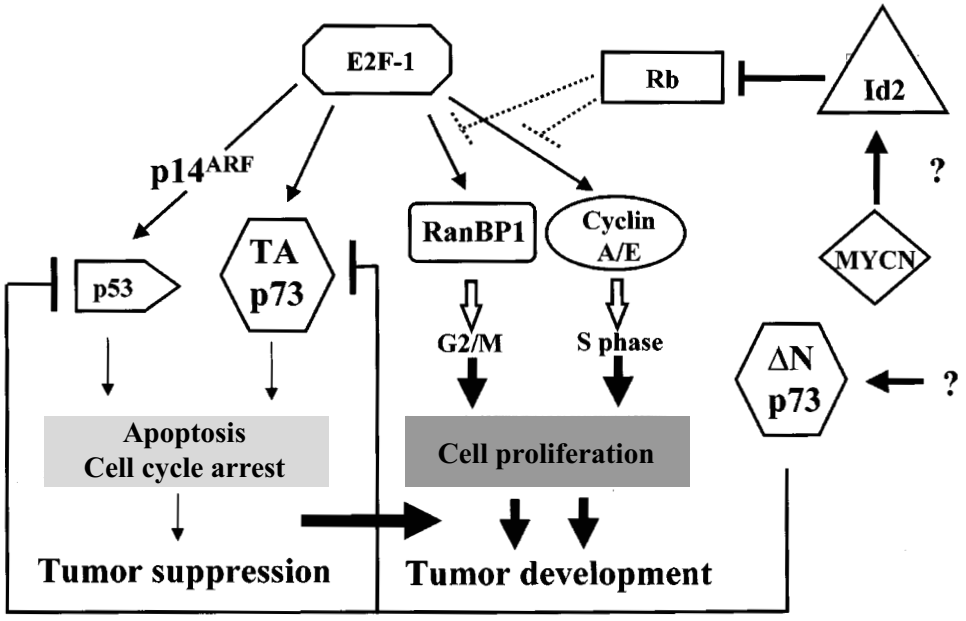

Figure 3 Proposed mechanism through which Δ*Np73*, in co-operation with other genes, might have a central role in neuroblastoma progression. According to this hypothesis the disregulated ΔNp73, interacting with p53 and TAp73, can inactivate the p53-dependent and independent pathways. The expression of the truncated isoform may be finely and rapidly modulated through epigenetic mechanisms.[15] Moreover, in some cases *MYCN* overexpression through Id2 might block the negative regulation on cell proliferation exerted by Rb. The inducers of Δ*Np73* are not yet known

was evaluated in 42 patients and was determined by FISH with 1pter probes and search for LOH by microsatellite analysis at the D1S80 and D1S76 loci. DNA and RNA were extracted from frozen tissues as previously described.[17] DNA and RNA samples from acute lymphocytic leukemia patients were kindly provided by G Basso (University of Padova, Italy).

TUNEL assay

Detection of enzymatically labeled DNA strand breaks in a NB tissue sections has been performed by using the 'In Situ Cell Death Detection Kit' from Roche (Roche Diagnostics, Monza, Italy) according to the manufacturer's instructions as previously described.[26] The clinical report of this case has been previously reported.[16]

RT–PCR for p73 expression

5 μg of total RNA were transcribed with Oligo-dT and 5 U of Superscript II reverse transcriptase (GIBCO, Life Technologies, Milano, Italy) for 1 h at 42°C. PCR amplification with G3PDH primers was utilized as internal control for mRNA integrity and cDNA quantification as described.[17] *p73* expression was determined by semi-quantitative PCR amplification with primers sets designed to discriminate between *TA*- and Δ*Np73*. Primers sequence are: TAp73-FW (exon 1) GGACGGACGCCGATGCC; ΔNp73-FW (exon 3′) ACTAGCTGCGGAGCCTCTCCC; reverse primer for both reactions was: TGCTCAGCAGATTGAACTGG (exon 4). Denaturation, annealing and extension temperatures were 94, 60 and 72°C. Primers were designed with the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Authenticity of the RT–PCR products as human Δ*Np73* was verified by sequencing. Tumor samples were defined as Δ*Np73* positive if a PCR amplification band was detectable after 35 cycles of amplification.

Statistical analysis

The association between ΔNp73 expression and others prognostic factors was assessed by the chi-square or the Fisher's exact test. Overall survival (OS) was defined as the time between diagnosis and death, regardless of the cause. Patients who have not died were censored at the last date they are known to be alive. Progression-free survival (PFS) was calculated from the day of diagnosis until the date of progressive disease or relapse or death is reported, whichever occurs first. Patients who have not experienced any unfavorable event were censored at the last date they were known to be alive. Estimates of OS were calculated according to the Kaplan and Meier product-limit method. Comparison of estimated survival curves were performed by means of the Mantel-Haenszel chi-square test. The uni- and multivariate estimates of the hazard ratio (HR), i.e. the statistic that summarizes over the entire life experience the failure rate in a subgroup of patients compared to that in the reference strata, were calculated by Cox proportional hazards model. All tests were two-sided.

Note added in proof

After acceptance of this paper, Grob *et al*.[27] described a regulatory feedback loop where ΔNp73 is upmodulated by p53 and TAp73 and regulates their functions.

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