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Id2 Is Critical for Cellular Proliferation and Is the Oncogenic Effector of N-Myc in Human Neuroblastoma[1]

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ABSTRACT

Perturbation of the function of the retinoblastoma (Rb) protein is found in most human tumors. Id2 is a natural target of the Rb protein that is recruited by Myc oncoproteins to bypass the tumor suppressor function of Rb. Here we report that an "N-Myc-Id2 pathway" persists during late development of the nervous system and parallels the rising levels of active Rb in neuronal precursors withdrawing from the cell cycle. An immunohistochemical analysis of primary neuroblastoma from 47 patients shows that expression of Id2 is strongly predictive of poor outcome, irrespective of other clinical and biological variables. Overexpression of Id2 mediates cellular transformation and is required to maintain the malignant behavior of neuroblastoma cells. Correspondingly, embryonic fibroblasts from Id2-null mice display impaired ability to proliferate. We suggest that Id2 overexpression may be a better prognostic indicator than N-*myc* gene amplification in neuroblastoma. Thus, disrupting Id2 function may lead to new and useful therapeutic strategies for cancer patients.

INTRODUCTION

Id proteins are "integrators" of positive and negative environmental stimuli to the transcriptional machinery that regulates differentiation (1, 2). One member of the Id family, Id2, coordinates inhibition of differentiation and stimulation of cell proliferation by inactivating the Rb[3] tumor suppressor protein (3, 4). Id2 operates, at least in part, under control of Myc proto-oncogenes, which directly bind to and activate the Id2 promoter (5). By raising Id2 levels, Myc proteins circumvent the block on cell cycle progression imposed by the Rb pathway. Not surprisingly, tumor cells with oncogenic activation of Myc, such as the neuroblastoma cell lines carrying N-*myc* gene amplification, overexpress Id2 to constitutively bypass the cell cycle checkpoint imposed by Rb (5).

Previous studies proposed that the different biological properties of Rb and Rb family members result from their ability to regulate E2F transcription factors (6–8). Our recent results from genetic intercrosses of *Rb* and *Id2* knockout mice indicated that negative control of Id2 activity is an additional requirement for Rb function *in vivo* (5). Here we analyzed the expression of N-Myc and Id2 during normal development and in primary neuroblastoma. Because of the crucial importance ascribed to loss of Rb function in human cancer, we investigated whether overexpression of Id2 induced cellular transformation and predicted clinical outcome in children with neuroblastoma. Having found that Id2 expression is a prognostic determinant in neuroblastoma, we asked whether inhibition of Id2 could serve as a point of intervention in cancer by studying the consequences of eliminating Id2 from primary and tumor cells.

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[3] The abbreviations used are: Rb, retinoblastoma; MEF, mouse embryo fibroblast; BrdUrd, 5-bromodeoxyuridine; LI, labeling index; GFP, green fluorescent protein.

MATERIALS AND METHODS

Patient Population and Statistical Methods. We analyzed 47 primary neuroblastoma samples from 47 patients diagnosed and treated between 1991 and 2000 at the Ospedale Pediatrico Bambino Gesu' (Rome, Italy). Median follow-up was 46 months (range, 12–108 months). Staging was performed according to the International Neuroblastoma Staging System criteria. The patient population included 8 stage 1, 8 stage 2, 9 stage 3, 21 stage 4, and 1 stage 4S. Stages 1, 2, and 4S were classified as favorable, whereas stages 3 and 4 were unfavorable. Event-free survival time for each patient was defined as the time from the date of diagnosis to the date of earliest occurrence of recurrence, disease progression, or death resulting from any cause. The Pearson correlation coefficient was used to test the strength of association between the continuous variables. Associations among Id2 expression and other prognostic variables were examined by χ^2 test. Survival analyses were performed according to the method of Kaplan and Meier, and comparisons of outcome between subgroups were performed by the log-rank test for univariate comparisons. For multivariate analysis, Cox's proportional hazard regression model was applied. Statistical analyses were performed using StatView 4.1 (Abacus Concepts, Inc., Berkeley, CA).

Immunohistochemistry. Antibodies used for immunohistochemistry included a previously characterized anti-N-Myc monoclonal antibody at concentration of 10 μg/ml (Ref. 9; Oncogene Research Products, Boston, MA) and the anti-Id2 polyclonal antibody C-20 at concentration of 2 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of Id2 immunostaining was assessed by preabsorption of the antibody with the peptide from which the antibody was raised. This, but not preabsorption with a nonrelevant peptide, abolished Id2 staining. Controls using polyclonal rabbit immunoglobulin instead of primary antibody showed no evidence of staining. Also, tissues from Id2 null mice provided a negative control for Id2 antibody (Fig. 1*c*). Immunohistochemical results of N-Myc and Id2 staining in neuroblastoma were scored independently by two investigators (A. L. and A. I.). At least 600 cells from 5 to 10 high-power fields were scored for each tumor sample.

Immunoblot Analysis. Mouse embryo tissues were collected on dry ice, and proteins were extracted with RIPA buffer in the presence of protease and phosphatase inhibitors. The antibodies used for immunoblotting were anti-Id2 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-N-Myc (Oncogene Research Products), and anti-Rb (G3-245; PharMingen, San Diego, CA).

Growth Experiments and Antisense Oligonucleotide Transfections. Primary MEFs were obtained from 13.5-day-old embryos and cultured as described (5). Passage 4 cells were plated at density of 3×10^4/35-mm dish and counted at the indicated days. Unless specified otherwise, cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co.). Retroviral infection of NIH 3T3 was done as described (5). For saturation density experiments, cells were plated in DMEM containing 5% fetal bovine serum and cultivated for up to 7 days. Cells were counted daily with a hemocytometer. For growth in soft agar of NIH 3T3 derivatives and LAN1, 5×10^4 and 1×10^5 cells, respectively, were plated in 35-mm dishes in 0.33% agar solution (BiTek; Difco) in DMEM containing 5% fetal bovine serum. The bottom layer was prepared using 0.6% agar in growth medium. After 7 days, 1.5 ml of agar mixture were added. Colonies were scored in triplicate wells after incubation for 14 days. Phosphorothioate oligonucleotides complementary to human Id2 and the mismatched control were obtained from Life Technologies, Inc. The sequences of oligonucleotides were as follows: Id2-AS, 5′-AGGCTTTCATGCTGACCGC-3′; and Id2-MSM, 5′-GCGAGTTGTCGCACGGTCT-3′. Oligonucleotides were mixed with Superfect (Qiagen) according to the manufacturer's instructions and used to treat LAN1 cells

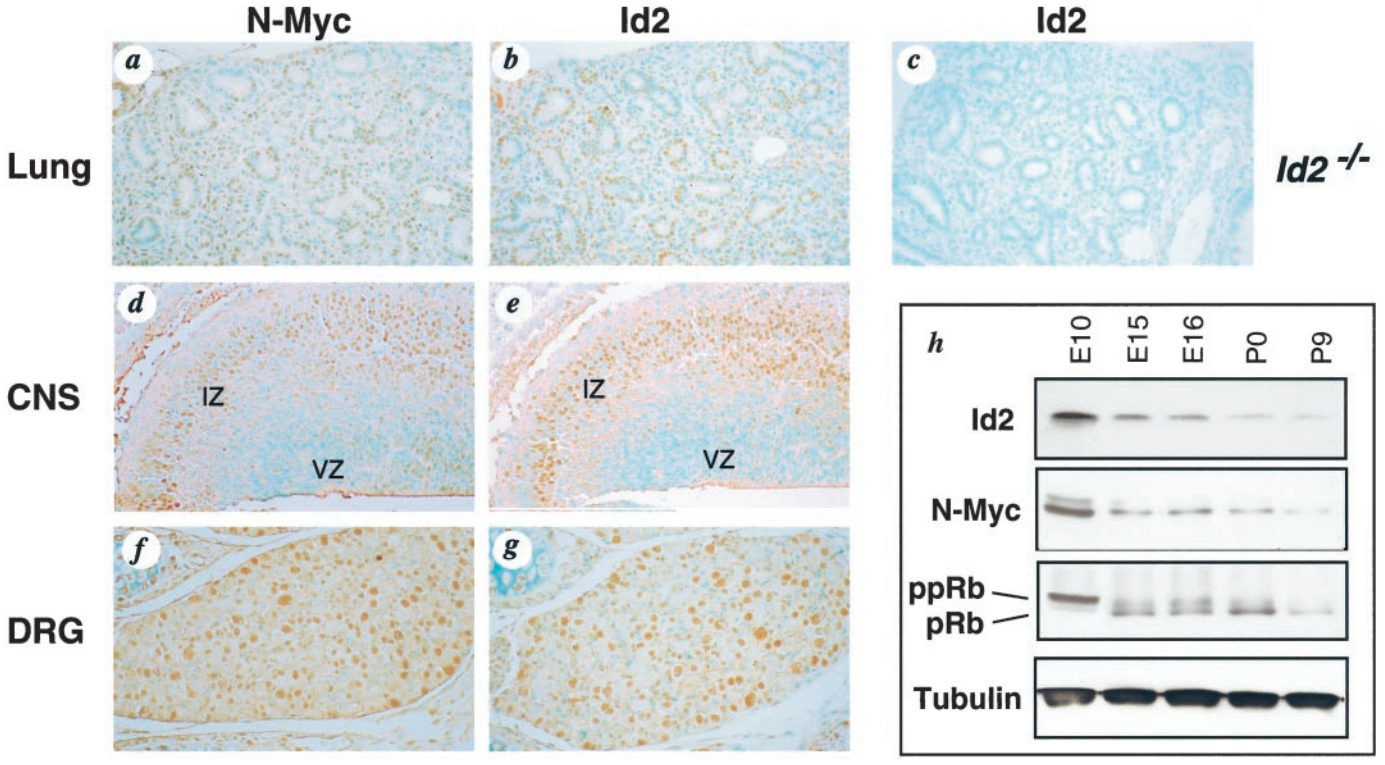

Fig. 1. Immunohistochemical analysis of N-Myc and Id2 in 15-day mouse embryo. N-Myc and Id2 show an overlapping pattern of nuclear staining in the alveolar epithelium of the lung (*a* and *b*) intermediate zone of the telencephalon (*d* and *e*) and dorsal root ganglia (*f* and *g*). Specificity of Id2 staining is demonstrated by the absence of immunoperoxidase activity in the lung of $Id2^{-/-}$ embryo (*c*). ×25. *h*, Western immunoblot analysis of N-Myc, Id2, Rb, and α-tubulin in the mouse brain during development. *pRb*, hypophosphorylated Rb; *ppRb*, hyperphosphorylated Rb. ppRb:pRb ratios from a quantitative densitometric analysis of the Rb blot are: *E10*, 7.0; *E15*, 0.31; *E16*, 0.26; *P0*, 0.10; and *P9*, 0.04.

at the final concentration of 0.8 μM. After incubation for 24 h, cells were analyzed for the ability to incorporate BrdUrd and form colonies in soft agar.

RESULTS

N-Myc and Id2 Display an Overlapping Pattern of Expression during Development. We performed an immunohistochemical analysis of N-Myc and Id2 proteins in the mouse embryo at mid-gestation (embryonic day 15, E15). At this stage of development, Rb is essential for cell cycle withdrawal and differentiation of neural progenitors (10, 11). We found a striking overlap between the pattern of expression of N-Myc and Id2 in epithelial and neural tissues (Fig. 1). Lung is an example of abundant expression of N-Myc and Id2 proteins in the epithelium without any detectable signal in the adjacent mesenchyme (Fig. 1, *a–c*). A similar pattern of expression is present in the small intestine (data not shown). During early neurogenesis (before day E12), N-Myc and Id2 are expressed in the telencephalon throughout the proliferating ventricular zone (12–14). However, at E15, the two proteins appear in the intermediate zone, which predominantly contains postmitotic neurons (Fig. 1, *d* and *e*). Positive staining in the ventricular and subventricular layers is detectable only in small areas of the olfactory lobe and the hippocampus (data not shown). N-Myc and Id2 are also abundant in large, differentiated neurons of dorsal root and trigeminal ganglia, which fail to incorporate BrdUrd at this stage of development (Fig. 1, *f* and *g*, and data not shown). To determine at which time active Rb appears in the brain and how it relates with N-Myc and Id2, we compared expression of N-Myc, Id2, and Rb in embryonic and postnatal brains by Western immunoblot analysis (Fig. 1*h*). Although expression of N-Myc and Id2 decreases with development, significant amounts of the two proteins are found at E15, and they are still detectable after 9 days of postnatal life. Rb undergoes a change from hyperphosphorylated (abundant at E10) to hypophosphorylated, active forms by E15. Thus, expression of N-Myc and Id2 is detectable at midgestation in predominantly postmitotic neuroectodermal tissues and is paralleled by a shift of Rb from inactive to active forms.

Id2 Expression Is Predictive of Poor Survival of Neuroblastoma Patients. To determine whether the "N-Myc-Id2 pathway" is activated in neuroblastoma cells before tissue culture and whether Id2 expression correlates with clinical behavior, we conducted an immunohistochemical analysis for the proteins N-Myc and Id2 in 47 primary neuroblastomas. N-Myc and Id2 were not expressed in postnatal adrenal medulla, the most common site of origin of neuroblastoma in humans (data not shown). N-Myc and Id2 were either absent ("negative" samples; Fig. 2, *a* and *b*) or detectable in the nuclei of 25% or more tumor cells ("positive" samples; Fig. 2, *c–f*). In our series there were 8 tumors with N-*myc* gene amplification (Table 1). All of them showed positive staining for N-Myc and Id2. Among the 37 tumors without N-*myc* gene amplification, 19 had N-Myc protein expression. These findings confirm results from previous studies, which indicated that mechanisms other than gene amplification lead to deregulated expression of N-Myc in a significant number of neuroblastomas (9, 15, 16). With the exception of one case (N-Myc negative/Id2 positive), we found an invariable correlation between the expression of N-Myc and Id2 (28 tumors were N-Myc positive/Id2 positive; 18 tumors were N-Myc negative/Id2 negative). We scored the percentage of neuroblastoma cells in each tumor that stained positive for N-Myc and Id2 (LI) and found that the LIs for N-Myc and Id2 were highly correlated (Pearson correlation coefficient, 0.868; $P < 0.0001$; Fig. 2*g*). Next, we examined the correlation between Id2 expression and clinical behavior of neuroblastoma. On all patients, the cumulative overall and event-free survival of Id2-negative tumors were 0.877 ± 0.082 and 0.889 ± 0.074 *versus* 0.417 ± 0.103 and

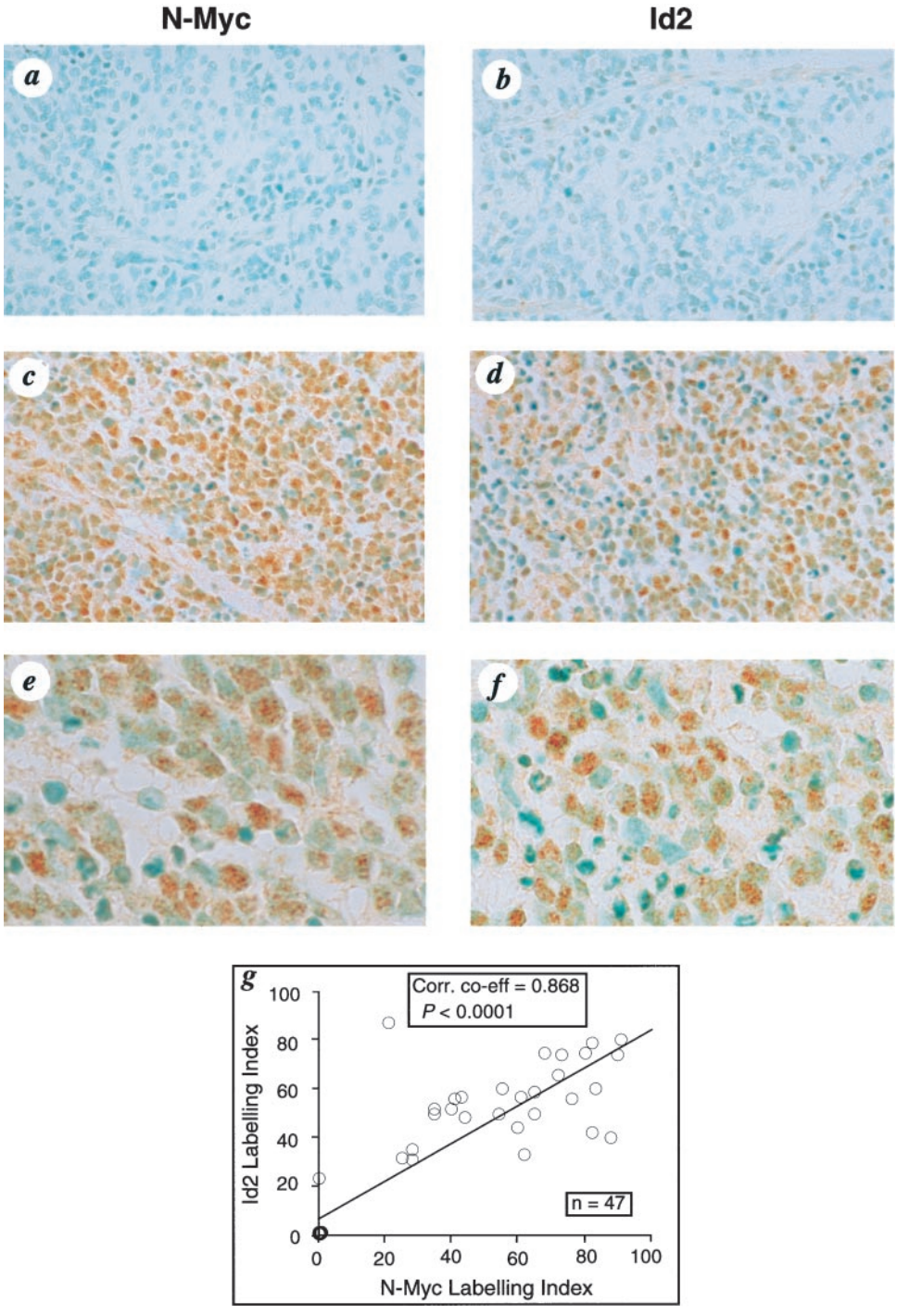

Fig. 2. Analysis of N-Myc and Id2 proteins in neuroblastoma. Immunohistochemistry of serial tumor sections shows the absence of N-Myc (*a*) and Id2 (*b*) in a stage 1 neuroblastoma patient (×25). The majority of cells in a stage 4 neuroblastoma (×25) express high levels of N-Myc (*c*) and Id2 (*d*) proteins. Higher magnification (×100) demonstrates prevalent nuclear localization of N-Myc (*e*) and Id2 (*f*). *g*, scatter plot of N-Myc LI *versus* Id2 LI for neuroblastoma. LI is defined as the percentage of neuroblastoma cells that stained positive after counting at least 600 cells for each tumor. Eighteen samples lacking N-Myc and Id2 immunostaining are reported as a *thick symbol* at the origin of the two axes. The line of best fit is shown with a Pearson correlation coefficient of 0.868.

0.472 ± 0.094 for Id2-positive tumors (Fig. 3, *a* and *b*). The Kaplan-Meier analysis showed that Id2 expression was predictive of increased mortality (Fig. 3, *a* and *b*; log-rank $P = 0.0046$ and 0.0065 for overall and event-free survival, respectively). Outcome of neuroblastoma patients of ages <1 year is good, regardless of other prognostic variables (17). Conversely, patients with N-*myc* gene amplification display an invariably poor prognosis (18). We therefore asked whether Id2 remained a predictive indicator for patients older than 1 year of age and/or lacking N-*myc* gene amplification. Kaplan-Meier plots of event-free survival showed that Id2 expression was associated with increased mortality in each subgroup (Fig. 3, *c–e*). χ^2 analysis demonstrated a positive correlation between Id2 expression and unfavorable clinical stages (Table 1; $P = 0.0051$). When outcome was evaluated in this subgroup of patients, there was a trend toward a correlation between Id2 expression and low event-free survival ($P = 0.12$; Fig. 3*f*). In a multivariate Cox proportional hazard model that included Id2 expression (positive), age (<1 year), and N-*myc* copy number (>3 copies), Id2 expression was the strongest independent predictor of disease-free survival ($P = 0.0264$; relative hazard, 10.996; Table 2).

Id2 Transforms NIH 3T3 and Is a Rate-limiting Factor for Proliferation of Fibroblasts and Neuroblastoma. To determine whether the large amounts of Id2 expressed by neuroblastoma cells are sufficient to induce transformation, we introduced Id2 in NIH 3T3

Table 1 *Patient demographics and statistical analysis comparing Id2 status to clinical features*

		Id2[a]		
	Total *n* (%)	Positive *n* (%)	Negative *n* (%)	*P*[b]
Age				
<1 year	14 (29.8)	9 (19)	5 (10)	
>1 year	33 (70.2)	20 (43)	13 (28)	0.8
Stage				
Favorable	17 (36.2)	6 (13)	11 (23)	
Unfavorable	30 (63.8)	23 (49)	7 (15)	0.0051
N-*myc* gene				
<3 copies	37 (78.7)	20 (43)	17 (36)	
>3 copies	8 (17.8)	8 (17)	0 (0)	0.015
Unknown	2 (4.2)	1 (2)	1 (2)	
Events				
No event	30 (63.8)	14 (30)	16 (34)	
Event	17 (36.2)	15 (32)	2 (4)	0.0048

[a] Id2 positive samples contained ≥25% tumor cells that stained for Id2. Id2 negative samples did not contain tumor cells with Id2 staining. No tumor showed Id2 positivity >0% but <25%.
[b] χ^2 *P*.

cells. NIH 3T3 transduced with LZRS-GFP-Id2 retrovirus (NIH 3T3-Id2) expressed levels of Id2 protein comparable with the endogenous levels of Id2 in neuroblastoma cells carrying amplification and overexpression of the N-*myc* oncogene (Fig. 4*a*). NIH 3T3-Id2 cells overcame the serum requirement of vector-infected NIH 3T3 for entry into S-phase and long-term proliferation (Fig. 4*b* and data not shown). In the presence of high levels of Id2, cells overgrew a monolayer doubling the saturation density of control cells (Fig. 4*c*). As a further sign of transformation, when cultured in soft agar, NIH 3T3-Id2 formed distinct foci (29 ± 5 colonies/well), which were absent in cultures of vector-infected NIH 3T3 (Fig. 4*d*). However, NIH 3T3-Id2 were not tumorigenic when injected in nude mice (data not shown).

To assess the consequences of loss of Id2 function on cell proliferation, we analyzed MEFs derived from $Id2^{+/+}$ and $Id2^{-/-}$ embryos. $Id2^{-/-}$ MEFs showed a noticeably lower rate of division (Fig. 4*e*). The defective ability to grow persisted when we compared 3T3 derivatives from $Id2^{-/-}$ MEFs with their wild-type counterparts, indicating that immortalization was not sufficient to overcome the requirement for Id2 (data not shown). We next sought to determine

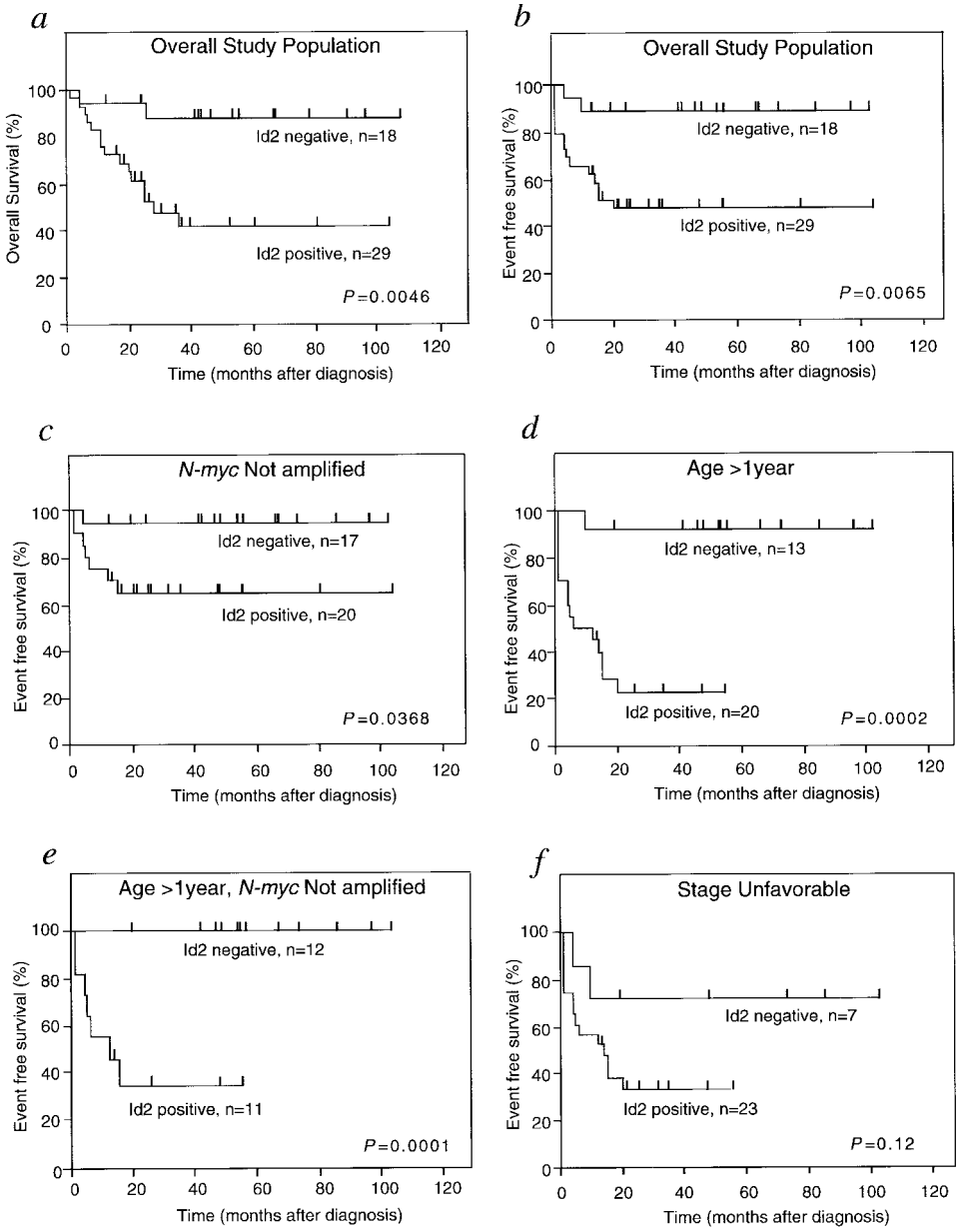

Fig. 3. Kaplan-Meier curves of neuroblastoma patients according to Id2 status. Id2 expression is associated with decreased overall (*a*) and event-free survival (*b*) in the entire series of patients tested. Event-free survival for patients without N-*myc* amplification (*c*), age >1 year (*d*), age >1 year without *N-myc* amplification (*e*), and unfavorable stages (*f*).

Table 2 *Multivariate Cox regression analysis*[a]

Covariate	Relative hazard	Confidence interval	*P*
Id2 expression (positive)	10.996	1.325–91286	0.0264
Age (<1 year)	0.122	0.015–1.010	0.0511
N-*myc* amplification (>3 copies)	1.733	0.583–5.155	0.3288

[a] $n = 47$.

whether opposing the uncontrolled expression of Id2 in neuroblastoma cells could affect their malignant behavior. For these experiments, we used the neuroblastoma cell line LAN1 that carries N-*myc* amplification and Id2 overexpression (5). Treatment of LAN1 with a phosphothioate Id2 antisense oligonucleotide for 24 h led to a decrease of endogenous Id2 by ~60% compared with a mismatch oligonucleotide (Fig. 4*f*). Consistent with reduction of Id2, BrdUrd incorporation showed decreased S-phase entry (15% *versus* 28% in mismatched treated cells; Fig. 4*g*). Interestingly, reduction of Id2 protein levels in LAN1 led to an even stronger effect on the ability of these cells to form colonies in soft agar (from 41 ± 3 colonies/well for mismatched treated LAN1 to 12 ± 2 colonies/well for anti-Id2-treated cells; Fig. 4*h*). These results suggest that lowering Id2 in neuroblastoma may generate antitumor mechanisms involving more than antiproliferative effects.

DISCUSSION

During development of the nervous system, Rb is not required for induction of neuronal determination and migration of neural progenitors from the ventricular zone to postmitotic areas of the brain, but it is essential for neurons to exit the cell cycle and survive (10). In the absence of Rb, neurons undergo ectopic proliferation and apoptosis (11). These defects are rescued by ablation of Id2 *in vivo* and are recapitulated *in vitro* by overexpressing Id2 in normal cortical progenitors (5, 19). Previous studies proposed that loss of Id proteins from the proliferating zones of the brain, which occurs during early neurogenesis, initiates neuronal gene expression and differentiation (13, 14, 20). We suggest that permanent elimination of Ids from the ventricular zone renders these early events insensitive to the genetic inactivation of Rb. However, persistent expression of the "N-Myc-Id2 pathway" in postmitotic areas of the central nervous system and peripheral nervous system may dictate the window of Rb requirement. In this model, Rb is essential during midgestation in postmitotic areas of the nervous system to control Id2 function and execute terminal cell cycle withdrawal, complete differentiation, and secure survival of neuronal cells.

To our knowledge, Id2 is the first transcriptional target of N-Myc, the expression of which correlates with the N-Myc protein during

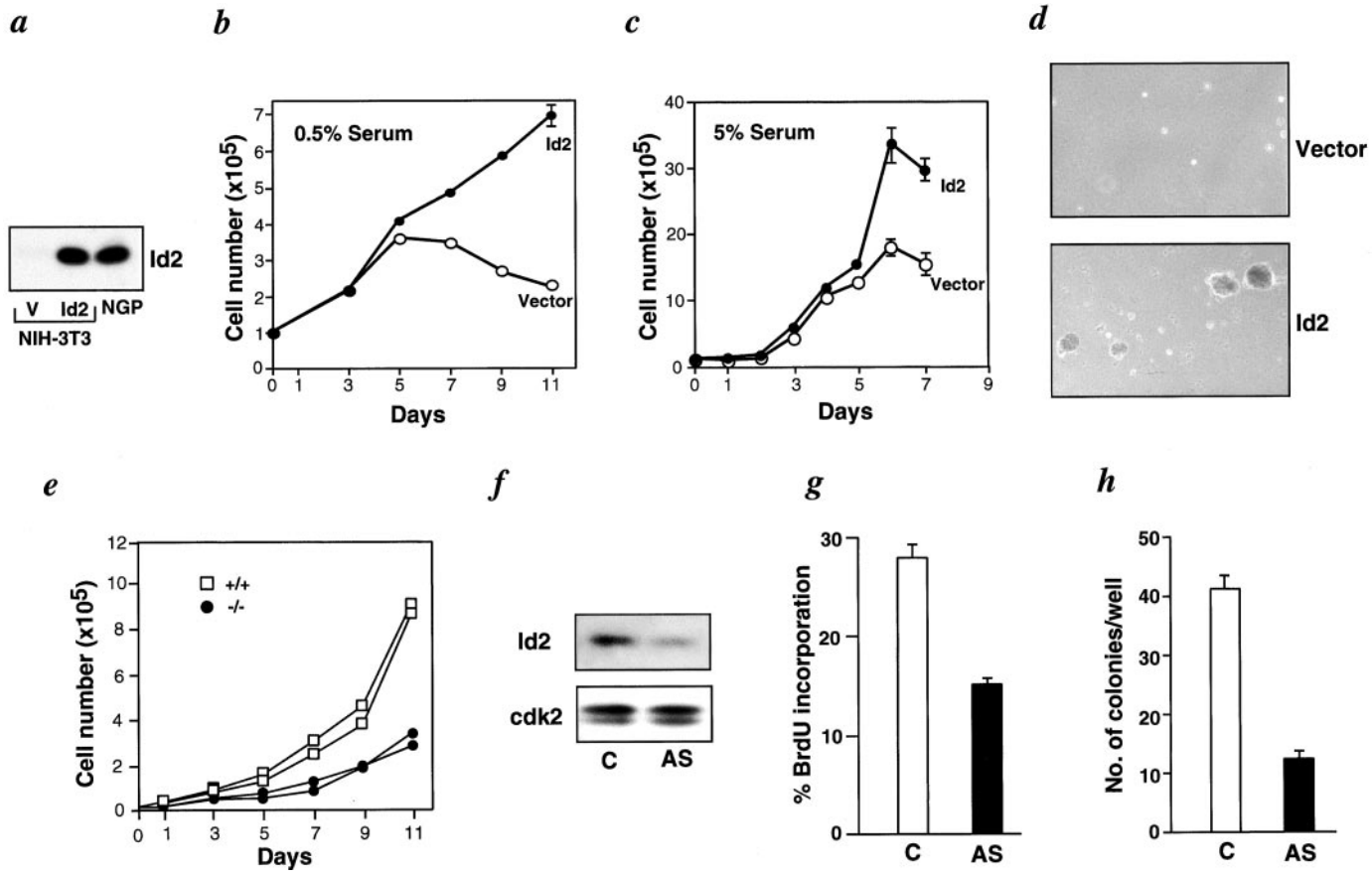

Fig. 4. Id2 transforms NIH 3T3 and is required for cell proliferation in normal and tumor cells. *a*, lysates from NIH 3T3 infected with LZRS-GFP (*V*) and LZRS-Id2-GFP (*Id2*) and from the neuroblastoma cell line NGP (*NGP*) were analyzed by Western immunoblot for expression of Id2. *b* and *c*, proliferation in 0.5% FBS (*b*) and 5% FBS (*c*) of NIH 3T3-vector (○) and NIH 3T3-Id2 (●). Cell number at each time point represents the average of duplicate samples; *bars*, SD. *d*, anchorage-independent growth of NIH 3T3-vector and NIH 3T3-Id2. Cells were assayed for their ability to grow in soft agar and photographed after 14 days in culture. *e*, passage 4 primary MEFs from two independent litters containing wild-type (□) and $Id2^{-/-}$ littermates (●) were plated in triplicates in 10% FBS and counted at the indicated time points. Error bars are entirely contained within each symbol. *f*, Western immunoblot from LAN1 cells treated for 24 h with 0.8 μM mismatch (*C*) or Id2 antisense oligonucleotides (*AS*) shows reduced Id2 protein levels in cells treated with Id2 antisense. A Western immunoblot for cyclin-dependent kinase 2 is shown as control for loading. *g*, BrdUrd incorporation is inhibited in LAN1 cells expressing lower amounts of Id2. *h*, LAN1 cells were treated with Id2 antisense oligonucleotides as in *f* and plated in soft agar. The number of colonies in triplicate wells was scored after 14 days. Data in *f–h* are representative of independent experiments; *bars*, SD.

development and in neuroblastoma. The immunohistochemical analysis of Id2 in neuroblastoma shows that tumor aggressiveness depends, at least in part, on activation of the N-Myc-Id2 pathway. In our series, among the 17 patients who relapsed and/or died, 15 (88%) overexpressed Id2 and 2 (12%) did not. Therefore, the vast majority of prognostically unfavorable neuroblastoma deregulates Id2 expression. Although N-*myc* gene amplification is a well-established adverse prognostic indicator in neuroblastoma, the significance of N-Myc protein expression is still debated (9, 15, 16, 21). In our series, expression of Id2 correlates with N-Myc (Fig. 2*g*). However, the unique feature of assaying Id2 in neuroblastoma is that its expression integrates the effects of N-*myc* activation and possibly other upstream signals to overcome the crucial tumor suppressor function of the Rb pathway. A number of studies suggested that inactivation of the Rb pathway is a determinant of poor prognosis for cancer patients (22–24). We propose that neuroblastoma with favorable outcome may retain a functional Rb pathway, whereas Id2-independent mechanisms for Rb inactivation may be present in the small subgroup of unfavorable, Id2-negative neuroblastoma. The analysis of Id2 expression has considerable potential to be of practical use in the routine assessment of neuroblastoma patients. This is strengthened by the fact that immunohistochemical tests for Id2 expression are inexpensive and could easily be available to most medical centers.

Our studies on the role of Id2 in cellular proliferation show that loss of Id2 significantly reduces the rate of cellular proliferation of primary and immortalized embryonic fibroblasts. These data are consistent with previous reports showing impaired proliferation rate of other cell types from *Id2*$^{-/-}$ mice (25, 26). It is likely that a critical threshold of Id2 determines the rate of proliferation in primary cells. Specular of these findings are our results showing that overexpression of Id2 at levels comparable with those found in neuroblastoma cells renders cells insensitive to extracellular antimitogenic signals such as serum deprivation and contact inhibition, thus conferring oncogenic potential. The role of Id2 as an oncogenic factor in neuroblastoma is specifically supported by reduced entry into S-phase and severely compromised anchorage-independent growth of neuroblastoma cells, where Id2 expression has been forcibly reduced by antisense oligonucleotides.

In human tumors, Rb is functionally inactivated by genetic alterations of the "Rb pathway" or by constitutive activation of the "N-Myc-Id2 pathway." We have now shown that Id2 expression determines the rate of proliferation of primary, immortalized, and tumor cell lines. These observations suggest that Id2 will make a major contribution to the inappropriate cell proliferation that results from loss of the negative control of Rb upon Id2 in tumors with genetic alterations of the "Rb pathway." Anti-Id2 therapeutic approaches might be attractive new tools, even in tumors where inactivation of Rb results from mechanisms different from the activation of the "Myc-Id2 pathway."

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