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Short Communication

RNF4 Is a Growth Inhibitor Expressed in Germ Cells but Not in Human Testicular Tumors

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The RING-finger protein RNF4 modulates both steroid-receptor-dependent and basal transcription and interacts with a variety of nuclear proteins involved in cell growth control. RNF4 is expressed at very high levels in testis and at much lower levels in several other tissues. We show that in germ cells RNF4 expression is strongly modulated during progression of spermatogonia to spermatids, with a peak in spermatocytes. Analysis of human testicular germ cell tumors shows that RNF4 is not expressed in all tumors analyzed including seminomas, the highly malignant embryonal carcinomas, yolk sac, and mixed germ cell tumors. We also show that the ectopically expressed *RNF4* gene inhibits cell proliferation of both somatic and germ cell tumor-derived cells. Mutation of critical cysteine residues in the RING finger domain abolished the RNF4 growth inhibition activity. Our results suggest that the lack of RNF4 expression may play a role in the progression of testicular tumors. *(Am J Pathol 2001, 159:1225–1230)*

We have recently identified a new gene, *RNF4*, that maps on human chromosome 4p16.3 and codes for a 21-kd protein containing a RING finger motif.[1] Such cystein-rich motifs have been mostly involved in protein-protein interaction and in the formation of multiprotein complexes.[2] Recent observations suggest that RING fingers have a general function in ubiquitin-mediated proteolysis.[3] It has been proposed that all of the RING proteins act as E3 ubiquitin protein ligases, with implications for a variety of biological areas. RNF4 associates with several nuclear proteins involved in steroid hormone signaling, transcriptional activation, or repression and chromatin modeling.[4–7] RNF4 interacts with the DNA binding domain of the androgen receptor (AR) functioning as a coactivator of AR responsive promoters and can also bind the TATA binding protein (TBP) acting as a coactivator of basal transcription.[4,8] We have shown that RNF4 interacts with PATZ, a novel POZ containing transcriptional repressor and with HMGI-Y, a chromatin modeling factor involved in transcriptional and cell growth regulation, through their AT-hook binding domains.[5] Both PATZ and HMGI-Y play a key role in the control of cell growth. *PATZ* is fused to *EWS* gene in sarcomas as a result of an intrachromosomal rearrangement of chromosome 22, thereby creating a chimeric sequence containing the transactivation domain of EWS fused to zinc finger domain of PATZ.[9] *HMGI-Y* is rearranged in human tumors of mesenchymal origin.[10] Thus, RNF4 represents a potential bridge factor linking different nuclear functions and may play a pivotal role in the regulation of development and cell growth programs.

RNF4, HMGI-Y, and PATZ are expressed at very high levels in the testis[1,4] (our unpublished results) and may thus play a role in the growth and differentiation of germ cells. Here we have analyzed the distribution of RNF4 in the different testicular cells and human testicular tumors and the role of RNF4 in the regulation of cell growth.

Materials and Methods

RNA Preparation and Northern Blot

Total RNA was extracted using the guanidine thiocyanate method. Northern blot analysis was performed as previ-

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ously described.[11] Hybridizations were performed at 42°C with a cDNA probe corresponding to the murine *RNF4* coding region, labeled with [^{32}P] dATP and [^{32}P] dGTP (Amersham Phamacia Biotech, Buckinghamshire, U.K.) by random priming procedure.[11]

Plasmids

To construct the *RNF4* sense and antisense expression vectors (pBP-RNF4 and pBP-RNF4-AS, respectively) a polymerase chain reaction (PCR)-generated full-length *RNF4* cDNA was inserted into the *Eco*RI-*Bam*HI sites of pBabe-puro expression vector harboring the puromycin-resistance gene. Primers used were for pBP-RNF4, SEco (5′-ACGTGAATTCATGAGTACAAGAAAGCGTC-GTG-3′) and SBam (5′-ATCGGGATCCTCAAGGGCA-AGTGTTAGCATTC-3′); for pBP-RNF4-AS, ASEco (5′-ATCGGAATTCTCAAGGGCAAGTGTTAGCATTC-3′) and ASBam (5′-ACGTGGATCCATGAGTACAAGAAAGCGT-CGTG-3′). pEGFP-RNF4 was constructed by cloning a PCR-generated full-length RNF4 fragment into the *Eco*RI site of pEGFP-C1 expression vector (Clontech). Primers used were AA1Eco (5′-ACGT*GAATTC*ATGAGTACAA-GAAAGCGTCGTG-3′) and GREco (5′-CGC*GAATTC*A-TATATAAATGGGGTGG-3′). All plasmid constructs used in this study were subjected to sequence analysis on both strands. pEGFP-RNF4-CS was created using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutagenic oligonucleotides were: 5′-CTCAGG T AC T G T CAG T AG T CCCA T CAG-CATGGACGGATACT-CAG-3′) and 5′-CTGAGTATCC-GTCCATGCTGATGGGACTACTGACAGTACCTGAG). The mutations were confirmed by sequencing.

Immunocytochemistry

Tumor and normal human tissues were obtained from the collection of the Department of Pathology, University of Naples "Federico II," Italy. Normal control tissues were from men undergoing biopsies for infertility (azoospermia). The specimens demonstrated normal spermatogenesis consistent with excurrent ductal obstruction. None of the men biopsied had any other potential causes for infertility, such as hormonal abnormalities or varicoceles. Paraffin embedded sections were dewaxed and rehydrated through graded ethanol rinses. All sections were incubated in a 750 W microwave oven for 15 minutes (3 cycles of 5 minutes) in 10 mmol/L buffered citrate, pH 6.0, to complete antigen unmasking. The endogenous peroxidases were quenched by incubation of sections in 0.1% sodium azide with 0.3% H_2O_2 for 30 minutes at room temperature. The standard avidin-biotin peroxidase complex (Dako Corp., Glostrup, Denmark) procedure was used.[12] Anti-RNF4 was an affinity-purified polyclonal antibody elicited to the RNF4 protein[5] used at a dilution 1:400. The peroxidase activity was developed with the use of a filtered solution of 5 mg of 3–3′-diaminobenzideine tetrahydrochloride (dissolved in 10 ml of Tris buffer 0.05mol/L, pH 7.4) and 0.03% H_2O_2. For nuclear counterstaining, Mayer's hematoxylin was used. Sections were mounted with a synthetic medium. The following controls were performed: omission of the primary antibody; substitution of the primary antiserum with non-immune serum diluted 1:500 in blocking buffer; RNF4 antibodies were preadsorbed with recombinant RNF4 protein. No immunostaining was observed after any of the control procedures.

Cell Culture, Transfections, Colony Assays, and BrdU Incorporation Experiments

Human 293T and NTERA-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, Paisley, U.K.). Cells were plated at a density of 2×10^6 per 100 mm Petri dish 16 hours before transfection. DNA transfections were carried out by calcium phosphate precipitation using Calphos (Clontech, Palo Alto, CA). 293T and NTERA-2 cells were transfected with 5 μg of plasmid DNA. After puromycin selection the plates of transfected cells were fixed and stained with 500 μg/ml of crystal violet in 20% methanol, and counted. Experiments were done in duplicate and repeated at least three times. For BrdU incorporation experiments, NTERA-2 and 293T cells were transiently transfected with 5 μg of the appropriate plasmid. 48 hours after transfection, BrdU (10 μmol/L) was added to the cultures for an additional 5 hours. At this time, cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at 4°C and processed for immunofluorescence using a monoclonal antibody against BrdU (Boehringer Mannheim, Germany) according the manufacturer's recommendations. The number of BrdU positive cells in GFP expressing and nonexpressing cells was determined. Experiments were done in duplicate and repeated at least three times.

Preparation of Testicular Cells

Testes of adult CD1 mice (Charles River, Milan, Italy) were used to prepare germ cells. Germ cells at pachytene spermatocytes, and spermatids were obtained by elutriation of the unfractionated single cell suspension as described previously.[13] Homogeneity of cell populations was routinely monitored morphologically and ranged between 80 and 85% (pachytene spermatocytes) and 95% (spermatids). Mature spermatozoa were obtained from the cauda of the epididymis of mature mice while spermatogonia were obtained from prepuberal mice as previously described.[14]

Western Blot and Antibodies

Immunoblotting experiments were performed according to standard procedures. Anti-RNF4 was an affinity-purified polyclonal antibody elicited to the RNF4 protein.[5] Anti-ERK1 and anti-GFP were commercial rabbit poyclonal antibodies (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA). Immunoblots were stained with anti-

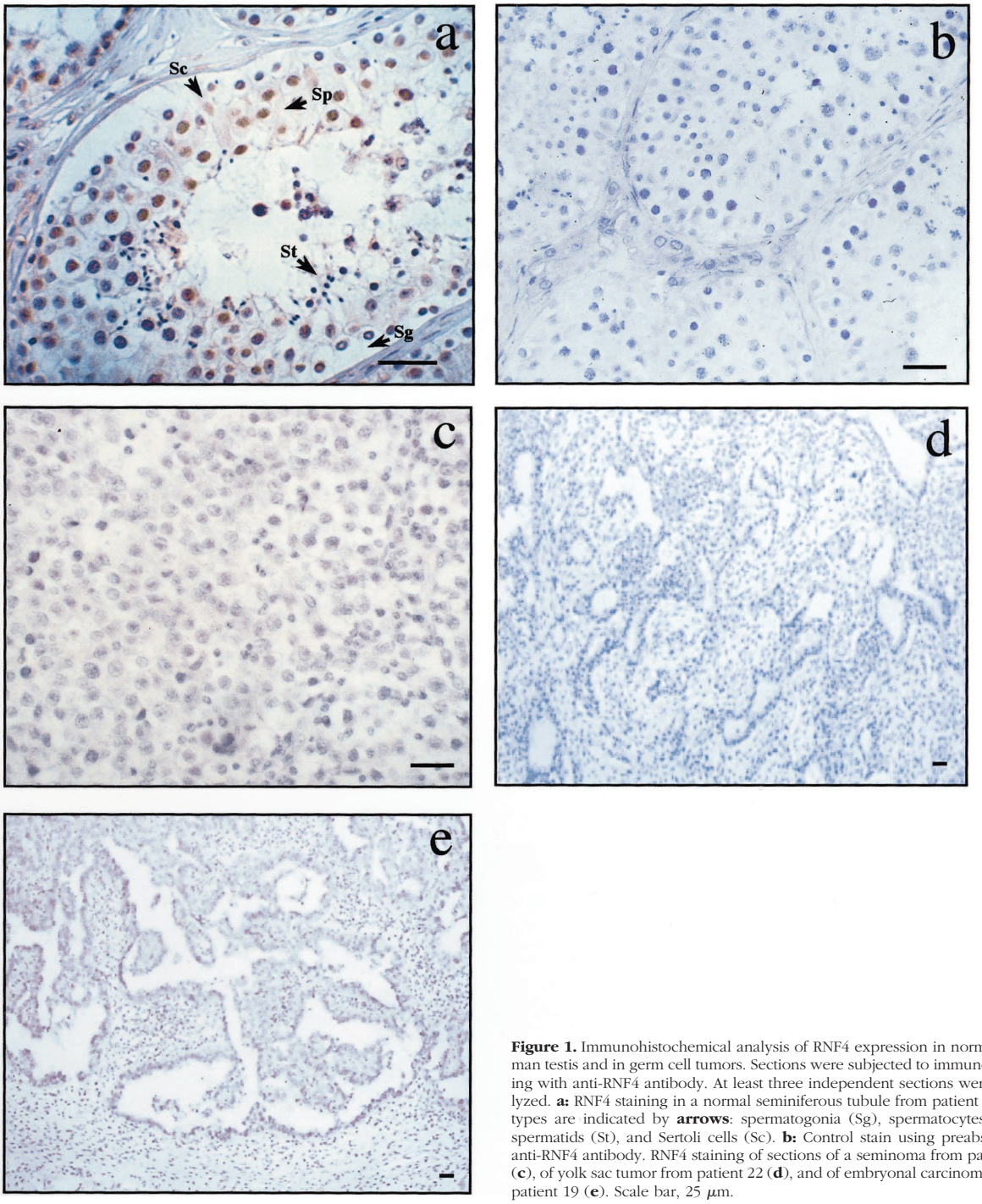

Figure 1. Immunohistochemical analysis of RNF4 expression in normal human testis and in germ cell tumors. Sections were subjected to immunostaining with anti-RNF4 antibody. At least three independent sections were analyzed. **a:** RNF4 staining in a normal seminiferous tubule from patient 2. Cell types are indicated by **arrows**: spermatogonia (Sg), spermatocytes (Sp), spermatids (St), and Sertoli cells (Sc). **b:** Control stain using preabsorbed anti-RNF4 antibody. RNF4 staining of sections of a seminoma from patient 9 (**c**), of yolk sac tumor from patient 22 (**d**), and of embryonal carcinoma from patient 19 (**e**). Scale bar, 25 μm.

rabbit secondary antibodies (Amersham) and revealed with the enhanced chemiluminescence, ECL system (Amersham).

Results and Discussion

Expression of RNF4 in Normal Testis and in Human Testicular Tumors

We performed an immunohistochemical analysis on sections of normal human adult testis (Figure 1a). Undifferentiated spermatogonia in the basal compartment showed little staining, whereas primary and secondary spermatocytes showed a strong nuclear positivity. Spermatids and mature spermatozoa were slightly immunoreactive. Western blot analysis of cell extracts from fractionated mouse germ cells confirmed such differential distribution of RNF4 (Figure 2).

RNF4 expression was examined in a series of testicular cancers. Immunohistochemical analysis was performed in 12 seminomas, 4 embryonal carcinomas, 1 yolk sac tumor, 8 mixed germ cell tumors, and 5 unaffected testis

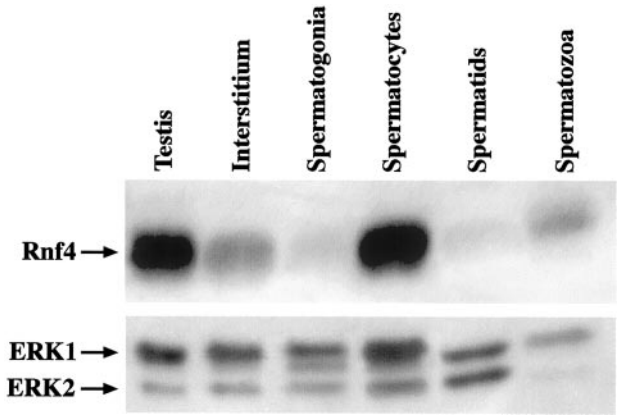

Figure 2. Western blot analysis of RNF4 protein in mouse adult testis and in isolated normal mouse testicular cells. Whole lysates were normalized for equal protein amounts and analyzed by Western blotting with anti-RNF4 polyclonal serum or with anti-ERK1 antibodies used as an internal standard. ERK1 antibodies recognize both ERK1 and ERK2, which are expressed at similar levels in all cell types with the exception of spermatozoa.[20]

tissues (Table 1). Representative experiments are shown in Figure 1, b–d. All neoplasms tested revealed no staining for RNF4. No difference has been found between seminomas and embryonal carcinomas. Northern blot analysis of mRNA extracted from available specimens confirmed that RNF4 mRNA was not expressed in tumors or, in some cases, it was detected at very low levels (Table 1 and data not shown).

Table 1. Histopathological Features and RNF4 Expression in Germ Cell Tumors

Case no.	Age	Histology	Immuno-histochemistry	mRNA
1	18	N*	+++†	n.d.‡
2	24	N	+++	+++
3	22	N	+++	+++
4	34	N	+++	n.d.
5	31	N	+++	+++
6	40	S	−	n.d.
7	25	S	−	−
8	32	S	−	−
9	38	S	−	−
10	39	S	−	−
11	35	S	−	n.d.
12	40	S	−	−
13	25	S	−	n.d.
14	36	S	−	−
15	43	S	−	−
16	45	S	−	−
17	34	S	−	n.d.
18	16	EC	−	n.d.
19	18	EC	−	+/−
20	23	EC	−	−
21	25	EC	−	+/−
22	15	YS	−	−
23	18	EC/S	−	−
24	27	EC/S	−	n.d.
25	29	EC/S/YS	−	−
26	24	EC/S	−	n.d.
27	18	EC/S	−	+/−
28	19	EC/S/YS	−	+/−
29	28	EC/S/YS	−	−
30	30	EC/S	−	n.d.

*N, normal (biopsies from infertile patients); S, seminoma; EC, embryonal carcinoma; YS, yolk sac tumor.
†+++, high reactivity; −, no reactivity.
‡n.d., not determined; +++, strong signal within 12 h exposure; +/−, weak signal after 72 h exposure; −, no signal after 72 h exposure.

We have not addressed the mechanisms leading to the suppression of *RNF4* gene but it will be important to determine whether absence of *RNF4* gene expression in tumors can be attributed to the lack of activating transcription factors, epigenetic modifications such as DNA methylation of the promoter region or to alterations of the gene. In a large study conducted in human germ cell tumors (including seminomas and nonseminomas), loss of chromosome 4p material has been detected in over half of the cases and has been suggested to be of significance.[15]

RNF4 Inhibits Cell Proliferation

The stage-specific expression together with the lack of RNF4 expression observed in human testicular germ cell tumors suggested that RNF4 could be involved in either the establishment of a differentiated phenotype and/or the regulation of germ cell proliferation. Thus, we investigated the effect of ectopically expressed RNF4 on the growth potential of two different cell lines (NTERA-2 and 293T cells) using two independent experimental approaches: colony formation assays and bromodeoxyuridine (BrdU) incorporation experiments. Endogenous RNF4 mRNA was undetectable in 293T and very low in NTERA-2 cells (data not shown). First, both the sense (pBP-RNF4) and antisense (pBP-RNF4-AS) RNF4 expression plasmids were transfected in 293T or in NTERA-2 cells. We found that stable expression of the sense construct, but not of the antisense construct, led to a significant reduction in the proliferative capacity of these cells as measured by a colony formation assay in monolayer culture (Figure 3A). We then subcloned *RNF4* coding region into the pEGFP fusion protein expression plasmid (pEGFP-RNF4) and analyzed, on transfection into NTERA-2 and 293T cells, the ability to enter S phase displayed by the transfected (GFP-positive) and non-transfected cells (GFP-negative). The results are shown in Figure 3B. We found that for both cell lines there is a clear inhibition of BrdU incorporation in transfected cells indicating that RNF4 overexpression led to S phase entry inhibition.

To characterize the importance of the RING finger structure in the RNF4-mediated growth inhibition, two point mutations converting cysteines 132 and 135 of RNF4 to serines were introduced in the EGFP-RNF4 fusion protein (plasmid pEGFP-RNF4-CS). The conversion of the corresponding cysteines of the rat RNF4 homologue (SNURF) has been shown to destroy the RING finger and to abolish the ability of RNF4 to stimulate basal transcription.[4] The influence of C→S mutation on growth regulation was investigated. The EGFP-RNF4-CS mutant was incapable of inducing growth inhibition in NTERA-2 and 293T cells (Figure 3B). These results imply that the integrity of the RING finger structure is required for the RNF4 growth inhibition activity probably because it mediates the interactions with other regulatory proteins. In-

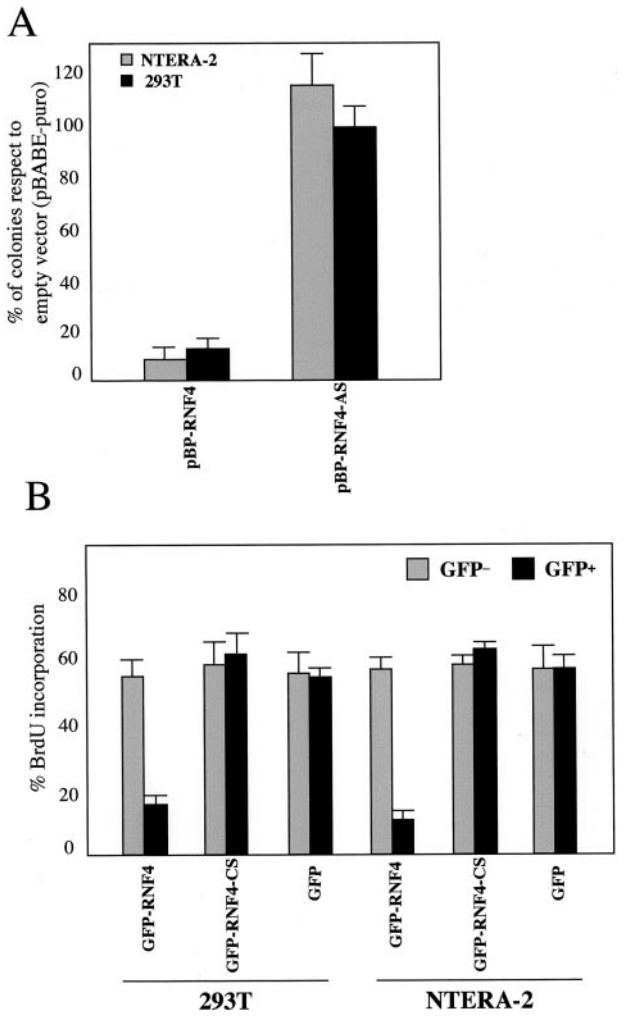

Figure 3. Effects of RNF4 and RNF4-CS on the cell growth. **A:** Colony formation assay. NTERA-2 and 293T cells were transfected with 5 μg of the empty pBabe-puro vector or with sense (pBP-RNF4) or with antisense (pBP-RNF4-AS) RNF4 expression vectors and cultured in selection medium containing puromycin for 2 weeks. Cultures were then stained with crystal violet and the number of puromycin resistant colonies was scored. The number of colonies was referred to that obtained in the same experiments using empty vector. Data represent the mean ± SD of three independent experiments. **B:** BrdU incorporation experiments. 293T and NTERA-2 cells were transfected with the indicated expression vectors (5 μg). The number of BrdU positive cells in GFP expressing and nonexpressing cells was determined. Expression levels and stabilities of GFP-RNF4 and GFP-RNF4-CS were similar as determined by Western blot analysis using anti-GFP antibodies (data not shown). Data come from three independent experiments scoring at least 300 cells on each experiment and are shown as mean ± SD.

terestingly, naturally occurring mutations in the RING finger domain of different tumor suppressors have been detected in human tumors.[3] In their complex, these findings demonstrate that RNF4 inhibits cell proliferation and support the possibility that the lack of RNF4 expression observed in germ cell tumors can play a role in the development of testicular tumors.

The hypothesis that the absence of RNF4 expression could have an important role in the genesis or progression of germ cell tumors is corroborated by the demonstration that, when overexpressed, RNF4 behaves as a growth inhibitor in an embryonal carcinoma cell line (NTERA-2) (Figure 3). RNF4 expression is also increased on retinoic acid treatment of NTERA-2 cells that induces differentiation and growth arrest of these cells (our unpublished results). A similar extent of growth inhibition is induced by RNF4 in somatic 293T cells thus suggesting that such activity is not restricted to germ cell tumor-derived cell lines. Further investigation is required to establish whether the lack or decrease of RNF4, which is ubiquitously expressed in adult tissues,[1] could be a more general phenomenon in human tumors of different origins and to clarify whether RNF4 functions in either E3 ligase pathway or through the association with other transcription factors.

RNF4 interacts with a least two oncogenic products of genes (*HMGI* and *PATZ*) that have been found rearranged in human tumors.[5,9,10] Chromosomal alterations within 4p16.3 are associated with different neoplastic diseases: a translocation breakpoint in malignant cells from patients with hairy cell leukemia,[16] allelic losses in 50% of breast carcinomas (localized at 4p16.3),[17] and in 23% of neuroblastomas (4p).[18] A loss of heterozygosity is detected in 22% of bladder carcinomas, suggesting the presence of a tumor suppressor gene, and involves a 750-kb region between D4S43 and D4S127 where the RNF4 gene is located.[19] Finally, other RING-finger proteins such as BRCA1, mel18, and PML have been shown to possess tumor suppressor activity which is lost by mutation in the RING finger.[3] All these observations indicate that *RNF4* shares some properties with tumor suppressor genes and warrant additional genetic studies to assess the pathogenetic contribution of *RNF4* to cancer.

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