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Prep1 (pKnox1)-deficiency leads to spontaneous tumor development in mice and accelerates *EμMyc* lymphomagenesis: A tumor suppressor role for *Prep1*

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ARTICLE INFO

Article history:
Received 25 November 2009
Received in revised form
28 December 2009
Accepted 4 January 2010
Available online 7 January 2010

Keywords:
Prep1
Pbx1
Meis1
Tumor suppressor
EmuMyc

ABSTRACT

The *Prep1* homeodomain transcription factor is essential for embryonic development. 25% of hypomorphic *Prep1*$^{i/i}$ embryos, expressing the gene at 2% of the normal levels, survive pregnancy and live a normal-length life. Later in life, however, these mice develop spontaneous pre-tumoral lesions or solid tumors (lymphomas and carcinomas). In addition, transplantation of E14.5 fetal liver (FL) *Prep1*$^{i/i}$ cells into lethally irradiated mice induces lymphomas. In agreement with the above data, haploinsufficiency of a different *Prep1*-deficient (null) allele accelerates *EμMyc* lymphoma growth. Therefore *Prep1* has a tumor suppressor function in mice.

Immunohistochemistry on tissue micrroarrays (TMA) generated from three distinct human cohorts comprising a total of some 1000 human tumors revealed that 70% of the tumors express no or extremely low levels of Prep1, unlike normal tissues. Our data in mice are thus potentially relevant to human cancer.

1. Introduction

The TALE (three amino acid loop extension) class homeodomain proteins Prep1, Meis1 and Pbx are developmentally essential transcription factors that regulate gene expression in the embryo and adult (Azcoitia et al., 2005; De Florian et al., 2004; Ferretti et al., 2000, 2006; Hisa et al., 2004; Selleri et al., 2001). Dimeric Prep1-Pbx and Meis1-Pbx complexes can also interact with other proteins, such as Hoxb1, to expand their DNA sequence selectivity (Berthelsen et al., 1998a,b; De Florian et al., 2004; Ferretti et al., 2000; Knoepfler et al., 1997; Jacobs et al., 1999). In addition to their developmental

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doi:10.1016/j.molonc.2010.01.001

functions, Pbx and Meis1 have oncogenic properties. In man, *Pbx1* fusion to *E2A* in the t(1; 19) translocation causes acute pre-B lymphoid leukemia (Kamps et al., 1990; Nourse et al., 1990), while Meis1 has been demonstrated to contribute directly to the pathogenesis of human and mouse acute myeloid leukemia (Calvo et al., 2001; Kroon et al., 1998; Lawrence et al., 1999; Moskow et al., 1995; Nakamura et al., 1996; Zeisig et al., 2004; Wang et al., 2005; Wong et al., 2007, 1999).

Down regulation of *Prep1.1* in zebrafish is embryonic lethal (De Florian et al., 2004), and, in mouse, *Prep1*-null embryos die before gastrulation (Fernandez et al., submitted for publication). To overcome the embryonic lethality of these mutations and to study the role of *Prep1* in the adult, we took advantage of a hypomorphic *Prep1*$^{i/i}$ mutation with a variable phenotype (expressing 2% mRNA, 3–7% protein). Seventyfive percent of *Prep1*$^{i/i}$ embryos die at about E17 with alterations in hematopoiesis, angiogenesis and eye development (Di Rosa et al., 2007; Ferretti et al., 2006) but the remaining 25% reach term and have an almost normal life-span (Ferretti et al., 2006; Penkov et al., 2005). The hematopoietic defects of the *Prep1*$^{i/i}$ embryos include a deficiency in long term repopulating hematopoietic stem cells and a block in erythroid, B- and T-lymphoid differentiations (Di Rosa et al., 2007; Ferretti et al., 2006; Penkov et al., 2005).

No information is available on the role of *Prep1* in cancer. Here we show that the few *Prep1*$^{i/i}$ hypomorphic mice that escape embryonic lethality develop spontaneous tumors or pretumoral lesions, and that transplantation of *Prep1*$^{i/i}$ fetal liver (FL) cells into lethally irradiated normal mice induces lymphomas. Moreover, loss of a *Prep1* allele in the *EμMyc* transgenic mouse model sharply accelerates tumor formation and death rate. Prep1 is absent or strongly down-regulated in about 70% of some 700 human cancers. These results were validated in a large cohort of non small cells lung cancers (NSCLC). Overall, our data indicate that Overall, our data indicate that *Prep1* isa novel tumor suppressor gene.

2. Materials and methods

2.1. Mice

Prep1$^{i/i}$ mice and embryos have been described (Di Rosa et al., 2007; Ferretti et al., 2006). *Prep1*$^{+/-}$ mice deleted in the homeodomain, will be described elsewhere (Fernandez et al., submitted for publication). *Prep1*$^{+/-}$ mice (C57Bl/6 × 129/svj, backcrossed >10 times to C57Bl/6) were bred with congenic C57Bl/6 *EμMyc* transgenic mice (provided by B. Amati). The *Prep1*$^{+/+}$ and *Prep1*$^{+/-}$ *EμMyc* transgenic littermates offspring were monitored twice a week for morbidity and tumors (Institutional Committee for Animal Care, project # 01/06 and 03/09).

2.2. Cells

E14.5 wild-type and *Prep1*$^{i/i}$ Mouse Embryonic Fibroblasts (MEFs) were cultured in DMEM -10% heat-inactivated FBS under low oxygen tension.

The TT1 tumor derived cells were cultured for 1 month in RPMI, 5% FBS, 20 mg/ml gentamicin, 50 mM beta-mercaptoethanol and 0.5 ng/ml IL7 (Peprotech).

Splenic B cells were isolated from two month-old littermate mice by incubation with CD19 MicroBeads followed by magnetic cell sorting (MACS), according to the manufacturer's instructions (Miltenyi Biotec).

2.3. Antibodies

A monoclonal antibody (CH12.2) specifically recognizing human Prep1 was produced by standard methods. Meis and Pbx antibodies were a kind gift of Dr. M. Torres and M. Clearly, respectively.

Cell surface marker antibodies for flow cytometry were from BD Biosciences (Franklin Lakes, NJ).

2.4. Immunohistochemistry (IHC)

IHC on microarrays was performed using stringent conditions. Slides were pre-treated with 0.25 mM EDTA pH 8.0 for 50 min at 94.5 °C, 2 h at room temperature with 1:20 CH12.2 and detected with DAKO EnVision system peroxidase. An arbitrary scale (scores 0–3) of expression was used during the evaluation of samples (0: no; 1, 2 and 3: low, intermediate and high expression, respectively). However, similar results (not shown) were obtained with lower pre-treatment temperature and shorter antibody incubation, using sections from fresh tissues.

2.5. Transplantations

Wild type or *Prep1*$^{i/i}$ CD45.2^{+} fetal liver (FL) cells were transplanted in lethally irradiated CD45.1^{+} mice according to standard protocol (Di Rosa et al., 2007). Details are described within the main text.

2.6. mRNA extraction and QT-PCR

For quantitative PCR, total RNA was extracted from MEFs and splenic B cells according to standard procedures using the TRIZOL Reagent (Life Technologies) followed by clean-up using RNeasy Mini Kit (QIAgen). Quantitative PCR was carried out using the Taqman Gene Expression Assay of Applied Biosystems (Foster City, CA, USA) with pre-designed, gene-specific Taqman probe and primer sets and the ABI-Prism 7900HT sequence detection system (Applied Biosystems).

2.7. Tissue microarray (TMA)

General tissue microarrays were specifically designed with normal and malignant tumor tissues and prepared as described with minor modifications (Kononen et al., 1998) using two representative normal (when available) and tumor areas (diameter, 0.6 mm) from each sample, identified previously on H&E-stained sections, and cut with a custom-built precision instrument (Tissue Arrayer, Beecher Instruments, Sun Prairie, WI) (2 μm sections).

Confirmatory analysis used 445 consecutive NSCLC cases having undergone surgical resection at the European Institute

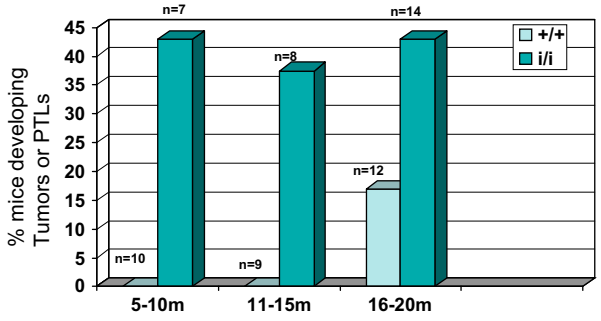


Figure 1 – Development of tumors and pre-tumoral lesions in *Prep1*$^{i/i}$ mice. The graph shows the percentage of wt (blue) and *Prep1*$^{i/i}$ (green) mice that develop a tumor or a PTL. Mice were sacrificed within the indicated age windows (in months) and autopsied. *n* indicates the number of mice within each group.

of Oncology (IEO) from June 1998 to December 2002 (ethical approval obtained from the IEO Institutional Review Board after written informed consent from all patients).

2.8. Statistical analysis

Statistical significance of the survival differences in the Eμ*Myc* transgenic mice was determined with a log-rank test. A one-tailed Fisher's exact test was used to assess the significance of tumorigenesis in the hypomorphic genetic background. All other statistical analyses were done using a two-tailed Student's *t*-test.

3. Results

3.1. *Prep1*$^{i/i}$ mice develop spontaneous pre-tumoral lesions and tumors

Only 25% of the hypomorphic *Prep1* (*Prep1*$^{i/i}$) embryos survive to birth. These animals express extremely little Prep1 (Ferretti et al., 2006; Penkov et al., 2005). Heterozygous embryos and mice show the expected about 50% reduction in *Prep1* expression (see immunoblot of Prep1 in wt, *Prep1*$^{+/i}$, *Prep1*$^{i/i}$ mouse embryonic fibroblasts or in *Prep1*$^{+/-}$ tissues in Suppl. Figure 1). Thus, no compensation of Prep1 levels occurs in this mutant.

We sacrificed littermate wild type and homozygous *Prep1*$^{i/i}$ mice and performed autopsy and histopathology at various age-windows (5–10, 11–15 or 16–20 months) looking for tumors or pre-tumoral lesions (PTLs, i.e. splenomegaly with disrupted organ structure (Suppl. Figure 2). The *Prep1*$^{i/i}$ mutation was generated by insertion of a retroviral enhancer trap in the first intron of *Prep1* (Ferretti et al., 2006). To exclude the possibility that any tumoral phenotype was dependent on a rearrangement at the *Prep1* locus before tumor formation (for example caused by the loss of the enhancer trap), each tumor-bearing mouse was genotyped twice, at birth and on the tumor itself (see below).

Figure 1 shows that a high (40%) percentage of *Prep1*$^{i/i}$ mice develop, at various ages, tumors or PTLs, while wt mice only develop PTLs and these only occur late in life. Table 1 shows the details of the experiment with the total number of animals of a given genotype and their distribution between groups developing tumors and PTLs during the various time windows. Homozygous *Prep1*$^{i/i}$ mice developed tumors or PTLs (12/29, of which 4 tumors and 8 PTLs). Tumors were observed after the 5–10 months window, although PTLs were found earlier. Wild type mice developed no tumors, but two out of 31 (6.5%) showed a PTL at late age. The number of *Prep1*$^{i/i}$ mice developing tumors is clearly underestimated since they were sacrificed at fixed time-points and no survival analysis was carried out. In any case, the results are statistically significant with a *p*-value of 0.002. Thus, *Prep1* deficiency is associated with the development of spontaneous tumors and PTLs.

The 4 spontaneous tumors (ST) in 10 *Prep1*$^{i/i}$ mice included one lymphoma of a lymph node, one of the spleen and one of the thymus, plus one ovarian carcinoma (Table 2). All tumors diagnosed by the pathologists were further characterized for genotype (PCR) and immunoblotting (not shown). In addition to using hematoxylin and eosin staining, lymphomas were characterized by immunohistochemistry with specific anti-B (B220) and anti-T (anti-CD3) antibodies (Suppl. Figures S3 and S4). The solid ovarian carcinoma was diagnosed by H&E staining at pathology (Suppl. Figure S5). In all cases, infiltration of tumor cells into nearby organs was observed, for example salivary gland and lymph-nodes in the case of thymic T-cells lymphoma and liver and lung in the case of spleen B-cell lymphoma.

3.2. *Transplantation of* E14.5 Prep1$^{i/i}$ *fetal liver cells induces lymphomas*

The T- and B cell deficiencies of the *Prep1*$^{i/i}$ mice can be reproduced in wt lethally irradiated mice by transplantation of E14.5 fetal liver (FL) cells (Di Rosa et al., 2007; Penkov et al., 2005). We transplanted CD45.1 lethally irradiated mice with

Table 1 – *Prep1*$^{i/i}$ mice develop spontaneous tumors and pre-tumoral lesions within 20 months.

Genotype	Tumors + PTL (5–10 m)[a]	Tumors + PTL (11–15 m)[a]	Tumors + PTL (16–20 m)[a]	Total Tumors	Total PTL	N Mice with lesions[b]	*p*-value[c]
Wt	0/10	0/9	2/12 (2 PTL)	0/31	2/31	2/31	–
Prep1$^{i/i}$	3/7 (3 PTL)	3/8 (2 PTL)	6/14 (3 PTL)	4/29	8/29	12/29	0.002

a Groups of about 10 mice of the indicated genotype were sacrificed at 5–10, 11–15 or 16–20 months. The number of tumors plus pre-tumoral lesions, PTL, is indicated.
b Number of mice showing lesions (tumors or PTL) observed in each genotype.
c One tail Fisher's exact test.

Table 2 – Tumors spontaneously arisen in Prep1$^{i/i}$ mice.

Genotype	Age months	Tumor Type	Tumor Symbol
i/i	18	LN-B cell lymphoma[b]	ST1
i/i	18	B, T-cell lymphoma (Spleen, LN)[b]	ST2
i/i	17	NC lymphoma[a]	ST5
i/i	13	Ovarian adenocarcinoma[a]	ST8

a NC: diagnosis by an experienced pathologist (HE staining), not characterized by cytofluorimetry or immunohistochemistry.
b Characterized by flow cytometry and immunohistochemistry, after the pathologist's diagnosis.

150,000–500,000 genetically tagged (CD45.2$^+$) wt or *Prep1*$^{i/i}$ E14.5 FL cells (six littermate embryos/each genotype) supplemented with 200,000 wt CD45.1$^+$/CD45.2$^+$ bone marrow cells (to ensure viability of the transplanted mice). In one case we inoculated one million *Prep1*$^{i/i}$ FL cells without supplementing with wt cells, as at this dose the mice mostly survive (Di Rosa et al., 2007). Mice were sacrificed when they showed signs of disease, or at 19 months, and autopsied (Table 3). A tumor (lymphoma) developed in only 1/6 wt embryonic donor transplantations. Conversely, tumors were observed in 4/6 *Prep1*$^{i/i}$ embryonic donors. In some of these tumors were observed already at 7–9 months (Table 3).

In order to establish unambiguously that the tumors originated from the transplanted embryonic cells, we first performed flow cytometry with CD45 markers. In all tumors, 80% or more of the tumor cells had a CD45.2$^+$, donor, haplotype (Table 3). Tumors from *Prep1*$^{i/i}$ FL cells transplantations were genotyped and presented the hypomorphic allelic DNA band (example in Figure 2B) while immunoblotting confirmed the expected extremely low Prep1 protein levels (Example in Figure 2C). Therefore the tumors originated from the transplanted *Prep1*$^{i/i}$ FL cells that have repopulated the bone marrow, i.e. from a *Prep1*$^{i/i}$ stem/progenitor cell. Suppl. Figure S6 shows an example of one of these tumors (tumor TT1 of Table 3), with enlarged spleen and thymus, presence of very large, abnormal cells (May Grunwald Giemsa). These cells were also present in the blood. Measurement of forward and side scatter by flow cytometry confirmed that the tumoral thymus was rich in large CD45.2$^+$ cells (see Figure 2). Finally, flow cytometry showed in the thymus a reduction of CD4, CD8 double positives cells and an increase (25%) of double negative earlier progenitors (Table 4). The blood also contained an abnormally high percent of CD4, CD8 double positives (3.96 v. 1.48) (Table 4). In the spleen, on the other hand, over 50% of the cells were CD4, CD8 double positives compared to wt, in which they were only 0.26% (Table 4). B cells (B220$^+$) were also much less represented than in wt. This and other data led us to conclude that the tumor is a T-cell lymphoma which also invades spleen, bone marrow and blood.

A second thymic T-cell lymphoma generated by transplantation of *Prep1*$^{i/i}$ FL cells was characterized by flow cytometry. The tumor was over 90% CD45.2$^+$ and was characterized by 43% CD4/CD8 double negative cells (mostly the earliest progenitors DN1) and 37% double positive cells compared to about 3% and over 80% (respectively) in transplanted non tumoral thymi (not shown).

3.3. *Prep1 haploinsufficiency accelerates the development of tumors in EμMyc transgenic mice*

The above data suggest that *Prep1* might be a tumor suppressor and predict that the development of oncogene-dependent tumors should be accelerated in the absence of *Prep1*. To test this point and to gather additional evidence from a second, different, *Prep1* mutation, we used a mouse in which the *Prep1* gene is deleted in the homeodomain. Since this mutation (*Prep1*$^-$) is homozygous lethal (Fernandez et al., submitted for publication) we introduced the *Prep1*$^-$ allele in the heterozygous state.

EμMyc transgenic mice constitutively express *Myc* in lymphatic cells causing B-cell lymphomas (Adams et al., 1985;

Table 3 – Transplantation of lethally irradiated wt mice with *Prep1*$^{i/i}$ FL cells induces the development of lymphomas.

Genotype	N. embryos generating tumors[a]	N. of transplanted FL cells[b]	Time of tumor development[c]	Type of tumor	CD45 haplotype[e]
Prep1$^{+/+}$	1/6	500,000	17 months	NC lymphoma	CD45.2+
Prep1$^{i/i}$	4/6	150,000	7, 9 months[d]	T lymphomas	CD45.2+
		250,000	19 months	Mantle cell lymphoma	CD45.2+
		500,000	18 months	NC lymphoma	CD45.2+
		1,000,000	19 months	NC spleen lymphoma	CD45.2+

Fetal liver cells from wt or *Prep1*$^{i/i}$ CD45.2 embryos were transplanted into CD45.1 lethally irradiated two month-old mice. Each embryo was transplanted into 5 different mice. Surviving mice were then sacrificed when they showed signs of bearing advanced state tumors or in any case when they reached 19 months of age. In all positive cases 1/5 animals developed a tumor, with the exception of one *Prep1*$^{i/i}$ embryo (this one transplanted with 150,000 cells) in which 2/5 mice developed tumors.
NC = uncharacterized.
a N. of embryos from which tumors were generated, compared to total number of transplanted embryos.
b Number of cells transplanted. With the exception of the case in which one million *Prep1*$^{i/i}$ cells were transplanted, all other lethally irradiated mice were supplemented with 200,000 wild type bone marrow cells.
c Age of the mice at the time of their sacrifice.
d Tumors were present in 2/5 mice. One of the two is called TT1 (see text). NC = uncharacterized.
e At least 80% of tumor cells were CD45.2-positive.

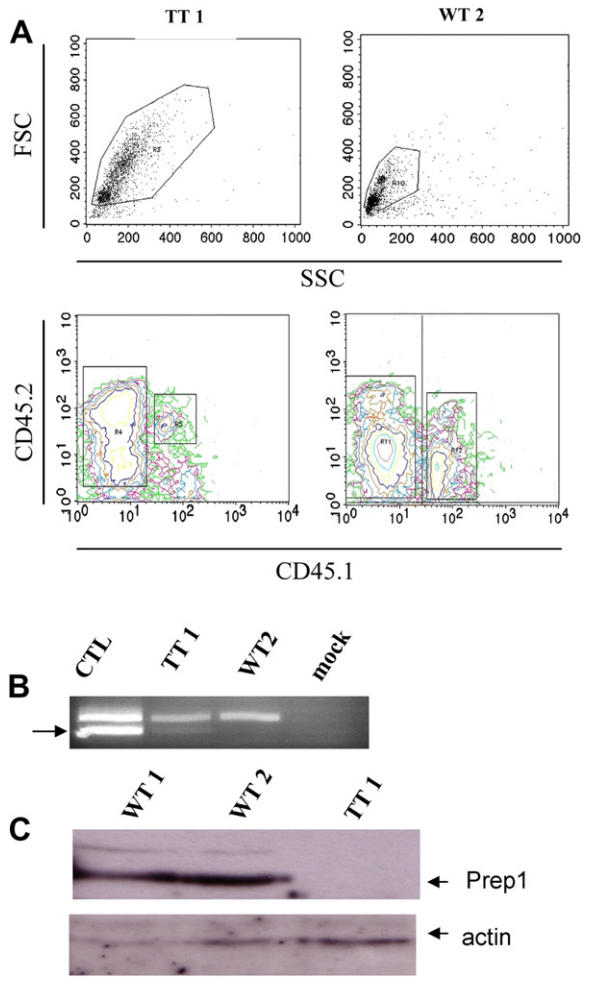


Figure 2 – Cytofluorimetry, genotyping and immunoblotting of the TT1 tumor. A: Flow cytometry analysis of tumor TT1, a T-cell lymphoma generated by transplantation of *Prep1*$^{i/i}$ embryo FL cells into wt mice. Thymus tumor cells (*i/i*) are compared with the cells from the same organ from a mouse transplanted with wt (+/+) FL cells. FSC and SSC parameters show an abnormal cell population in the hypomorphic thymus. B: PCR genotyping (see Methods) of the DNA extracted from tumor TT1. CTL is an heterozygous control thymus showing the wild type and the hypomorphic allele bands. The arrow identifies the hypomorphic band. WT2 cells are thymus cells from a mouse transplanted with wt littermate FL cells. Mock, is a reaction in the absence of reverse transcriptase. C: Immunoblotting analysis for Prep1. Extracts of thymus of two mice (wt1 and wt2) transplanted with wt FL cells are compared with those of the TT1 lymphoma. Immunoblotting of actin is used as loading control.

Harris et al., 1988) which develop faster in the absence of p53 or Tip60 (Eischen et al., 1999; Gorrini et al., 2007). On the other hand, *Prep1*$^{+/-}$ mice do not develop tumors in the first 6 months (not shown). Survival curves (Figure 3A) of the EμMyc mice show that *Prep1*$^{+/-}$ *EμMyc* mice ($n = 51$) had a significantly reduced survival time compared to *Prep1*$^{+}$/+/*EmMyc* ($n = 39$) with a median 21 vs. 58 weeks, $p < 0.01$, a significant reduction that is however not as strong as that observed for p53 and Tip60. This result strongly supports the hypothesis that *Prep1* is a tumor suppressor. Figure 3B shows that the level of Prep1 mRNA (real-time PCR) in pre-tumoral spleen cells follows the *Prep1* gene dosage in both wt and *EμMyc* transgenic mice.

3.4. *Tumor development in Prep1-deficient mice does not depend on the alteration of expression of its direct partners or competitors*

Prep1 transcription factor binds DNA only as a dimer with one of the four Pbx1 proteins (Berthelsen et al., 1998a,b; Knoepfler et al., 1997). These proteins bind Prep1 or its homolog Meis1 through the same interaction surface (Berthelsen et al., 1998a,b; De Florian et al., 2004; Ferretti et al., 2000; Knoepfler et al., 1997; Thorsteinsdottir et al., 2001). Therefore tumor development in *Prep1*-deficient mice might depend on altered expression of the *Pbx* or *Meis* genes, tilting the balance towards the more tumorigenic members of the class. However, we observed no major differences in the levels of expression of the Pbx1-4 and Meis1-3 measured by qPCR in wt vs *Prep1*$^{i/i}$ mouse embryonic fibroblasts (MEFs) (Table 5A). Only a minor decrease of Pbx1 and 2 is observed at the protein level (Figure 4A), as expected (Ferretti et al., 2006; Penkov et al., 2005). The levels of Meis1 protein are also not particularly changed in MEFs (Figure 4A). We measured the levels of Meis1 and Pbx1-3 in pre-tumoral B cells of EμMyc mice bearing a wt or a *Prep1*$^{+/-}$ allele. As shown in Table 5B, haploinsufficiency of Prep1 had little or no effect on the expression of these genes, in either wt or EμMyc transgenic animals. These data confirm our results in MEFs and show no relevant effect of Prep1 haploinsufficiency on the expression of the more oncogenic members of the family, i.e. Meis1 and Pbx1.

3.5. *Prep1 is absent or down-regulated in a large fraction of human tumors*

Tumor suppressors are found often deleted, mutated or silenced in human tumors. To analyze the relevance of our findings for human cancer, we first examined the expression of

Table 4 – A. Flow cytometric phenotype of the TT1 T-cell lymphoma of the thymus and spleen.

	Wild Type $CD4^+CD8^+$ %	TT1 $CD4^+CD8^+$ %	Wild Type $CD4^-CD8^-$ %	TT1 $CD4^-CD8^-$ %	Wild Type $B220^+$ %	TT1 $B220^+$ %
Blood	1.48	3.96	81.7	70	ND	ND
Thymus	76.6	44.0	3.05	25.1	ND	ND
Spleen	0.26	56.0	84.7	18.4	57.4	11.9

The numbers indicate the percent of the $CD45.2^+$ cells, i.e. of the cells originating from the transplant, showing the indicated phenotype. ND: Not Determined.

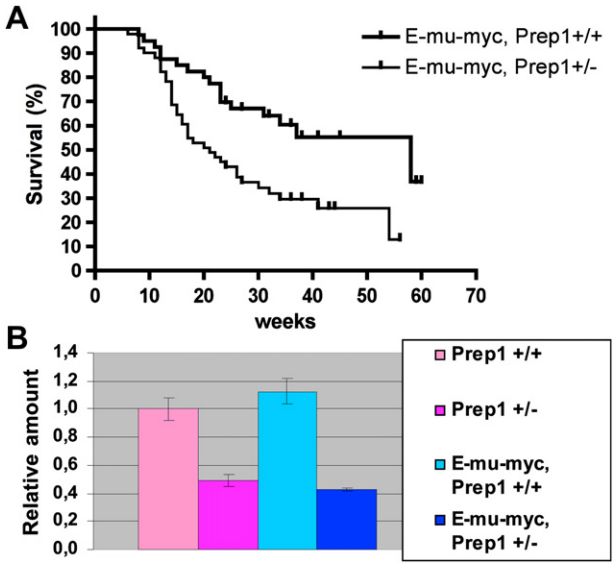


Figure 3 – Prep1 haploinsufficiency accelerates the death of transgenic *EμMyc* mice. A: Kaplan Meyer curves for *EμMyc* C57BL/6 transgenic mice carrying two wild type (heavy line) or one wild type and one deleted (fine line) allele for *Prep1*. Median survival: 21 weeks for *Prep1*$^{+/-}$ *EμMyc*, 58 weeks for *EμMyc* mice (*p*-value 0.0022). In the absence of the *EμMyc* transgene all the *Prep1*$^{+/-}$ mice survive and show no signs of disease for at least 25 weeks (not shown). B: Quantitative (real-time) PCR analysis on CD19 + splenic cells purified from pre-tumoral (two months old) littermate mice of the indicated genotype.

Table 5 – A. TALE PROTEINS mRNA analysis in MEFs.

Gene	n. analyzed embryos	mRNA level (% of wt)
Prep1	4	8.3 +/−3.2
Meis1	4	118.0+/−8.7
Meis2	4	83.0+/−12.0
Meis3	4	93.0+/−9.95
Pbx1	4	95.0+/−13.0
Pbx2	4	106.0+/−8.1
Pbx3	4	104.0+/−9-0
Pbx4	4	87.0+/−13.0

B. TALE PROTEINS mRNA analysis in pre-tumorsl splenic B-cells.

Genotype	mRNA level (% of wt)		
	Prep1$^{+/-}$ ($n=1$)	EμMyc Prep1$^{+/+}$($n=1$)	EμMyc Prep1$^{+/-}$ ($n=2$)
Meis1	102.3+/−2.3	156.9+/−2.8	155.6+/−7.9
Pbx1	75.4+/−1	55.7+/−0.1	57.9+/−4.1
Pbx2	114.3+/−2.1	255.8+/−1.2	200.8+/−13.2
Pbx3	71.8+/−2.6	74+/−5.6	58.3+/−3.9

Data represent quantitative PCR measurements (compared to actin and 18S RNA) of the indicated genes. Data are expressed as % of wild type cells +/− standard deviation.
Data represent quantitative PCR measurements (compared to β-actin) of the indicated genes. Data are expressed as % of wild type cells +/− standard deviation. Determinations were performed in triplicate.
Cells were isolated from two month-old mice as described in the Methods section.

Prep1 in a large number of normal human tissues by immunohistochemistry (IHC) on tissue microarrays (TMA), using a highly specific (Suppl. Figure S7) anti-Prep1 monoclonal antibody, CH12.2. We established an arbitrary, semi-quantitative 0–3 score (0, negative; 3 high level expression) to compare different cells and tissues. Prep1 was expressed at various levels in almost all cells and tissues (Suppl. Figure S7). Prep1 expression was high (score 3) in breast and skin, intermediate (score 2) in colon, larynx, lung bronchial epithelium, uterus stroma, and testicular germinal cells; low (score 1) in lymph-nodes, stomach, kidney tubules, endometrial epithelium, uterine endo- and exocervical epithelia and placenta. No staining (score 0) was detected in the alveolar lung epithelium, renal glomeruli and thyroid gland. Suppl. Figure S7 also shows some examples of immunohistochemical-positive tissues, the intestinal cripts, the testis, where Prep1 is expressed in many but not all cells and the tonsil, where Prep1 is strongly and uniformly expressed. Other examples of positive tissues are shown in Figure 5 (see below).

Next we analyzed by IHC the expression of Prep1 in a tissue microarrays (TMA) of more than 300 different human tumors. Overall (Figure 5A) *Prep1* was absent in 177/324 tumors (score = 0) and expressed at a low level in 71/324 tumors (score = 1). Therefore, Prep1 is undetectable or barely detectable in most tumors (248/324, i.e. 76%). Figure 5B shows examples of Prep1 expression in normal and tumoral breast and larynx. Figure 5C shows an example of a section from a skin carcinoma in which the tumor cells are clearly Prep1-negative or Prep1-low (thin arrows) while normal cells are Prep1-positive (thick arrows). Similar results were obtained with another TMA containing sections from 358 tumors, in which 65.8% were scored 0–1 (data not shown).

We confirmed this overall result with a third TMA containing sections from 443 consecutive non small cells lung adenocarcinomas and squamous carcinomas patients. Again, Prep1 was absent or very low in a substantial fraction (313/445, i.e. 70%) of the lung tumors (Figure 6). No correlation was found between Prep1 expression and its histogenetic derivation (i.e. alveolar v. bronchial), or with patient survival (not shown).

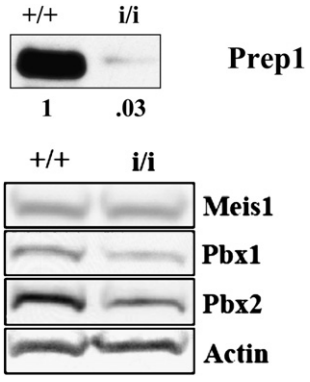
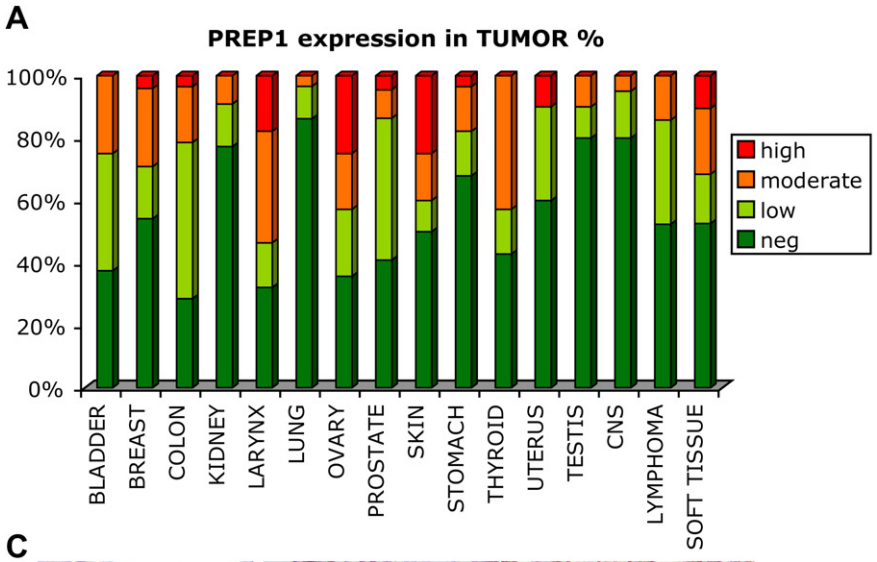
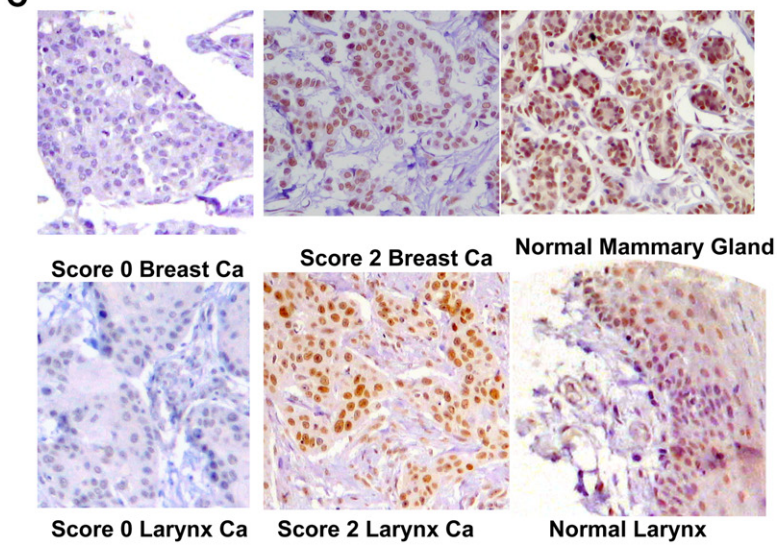


Figure 4 – Immunoblotting analysis of Pbx1, Pbx2 and Meis1 in wt or homozygous *Prep1*$^{i/i}$ MEFs. On top the immunoblotting with an anti-Prep1 antibody is shown. In the middle, the immunoblots with specific anti Meis1, Pbx1 and Pbx2 antibodies; loading control is an anti-actin antibody. At the bottom is the quantitation of the bands by densitometry.



B

ORGAN	0-1 score	2-3 score	Total
Lung	28	1	29
Uterus	9	1	10
Testis*	9	1	10
CNS	19	1	20
Bladder	6	2	8
Kidney	20	2	22
Prostate	19	3	22
Lymphoma	18	3	21
Stomach	23	5	28
Colon*	22	6	28
Soft Tissue	13	6	19
Breast*	17	7	24
Skin*	12	8	20
Ovary	16	12	28
Larynx*	13	15	28
Thyroid	4	3	7
TOTAL	248	76	324



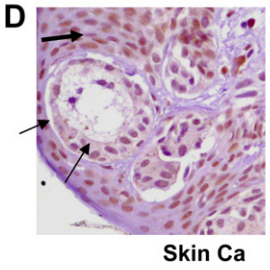


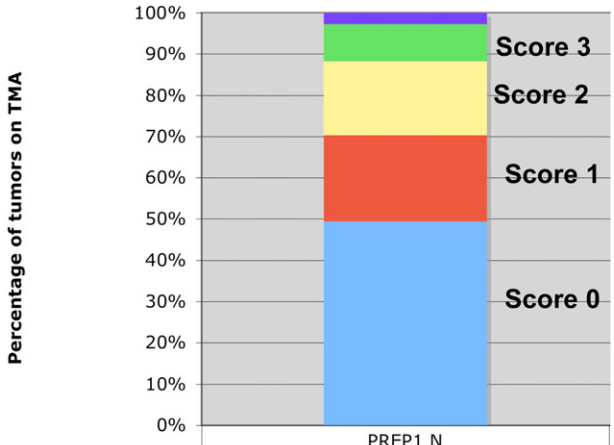
Figure 5 – Prep1 is absent or decreased in most human tumors. Human tissue microarrays (TMA) analyzed by immunohistochemistry for Prep1 (see Methods and Suppl. Figure S3). The scores of Prep1 expression in tumors was defined as: 0 = negative; 1 = low expression; 2 = moderate; 3 = high. A. Histogram reporting the data of the Table in panel B. B. The table reports the number of the different tumors analyzed and their subdivision according to their IHC score. C: Examples of Prep1 immunohistochemistry in normal human mammary gland and breast cancer, as well as in normal and cancerous larynx. Representative negative and positive images (score 0 and 2) of respectively breast and larynx tumors are shown. Prep1 level were high in normal breast and larynx epithelia. D: One example of a skin cancer, showing normal cells (thick arrow) expressing Prep1 in the nucleus and cancer cells (thin arrows) expressing no or low Prep1.

In conclusion, a large fraction of human cancers, especially lung cancers, do not express Prep1. Our results are compatible with a tumor suppressor function of Prep1.

4. Discussion

Analysis of *Prep1*-deficient mice indicates that Prep1 is a novel tumor suppressor. First, the few surviving $Prep1^{i/i}$ mice develop spontaneous tumors and embryonic $Prep1^{i/i}$ FL cells can transplant the tumor phenotype. Second, a haploinsufficient $Prep1^{+/-}$ allele accelerates the appearance of lymphomas and the death rate of *EμMyc* transgenic mice. Thus, two different mutations of *Prep1*, both of which reduce *Prep1* levels, have an oncogenic effect. The hypomorphic $Prep1^{i/i}$ mutation was generated by insertion of an enhancer trap in the first intron of the gene (Ferretti et al., 2006) while the deletion of the homeodomain was obtained by homologous recombination (Fernandez et al., submitted for publication). Both resulting mice have been crossed back to C57BL/6 over 10 times. The confirmation of our results in two mouse strains provide good evidence that tumors are induced by the reduction of *Prep1* eexpression and not a by specific allele that induces tumors. We conclude that *Prep1* is a tumor suppressor in mice.

The acceleration of the death rate of the *EμMyc* mice when Prep1 is reduced to 50% is absolutely clear and statistically significant. Yet, it is not as powerful as that of *p53* or *Tip60*. However,



	PREP1 N
ND	12
HIGH (>=2)	40
INT (>=1 and <2)	80
LOW (<1)	93
NEG (0)	220

Figure 6 – Summary of Prep1 levels determination in 445 human NSCLC. A cohort of lung cancer patients was analyzed by immunohistochemistry with the anti-Prep1 antibody. The tumors were scored as described in the text and in the legend of Figure 5. In this group, 50% of the patients showed no Prep1 expression, and 20% a very low expression. Below the histogram the numbers of patients in each score group is indicated. ND: sections in which the score could not be determined.

one must take into account that Prep1 deficiency is in a haploinsufficient state, since the homozygous deletion is lethal.

The reduction of *Prep1* expression in a large proportion of a general human cancer collection and in a large cohort of lung tumors lends support to a tumor suppressor role for *Prep1* in humans. *Prep1*-deficient mice develop mainly lymphomas, although more carcinomas have been observed in a second experiment (data not shown). We have observed the presence of both lymphomas and carcinomas that are Prep1-negative (or reduced) in human tissues (Figure 5). *Prep1* expression was never found to be absent in the normal counterparts of these tumors (Suppl. Figure 7) including a number of additional "normal" spleen, thymus and lymph nodes (data not shown).

Further studies will be needed to ascertain whether and to what extent this result depends on promoter silencing, gene deletion or mutation and whether the presence/absence of Prep1 correlates with clinical prognosis. An analysis of the literature also supports the tumor suppression function of *Prep1*. Deep sequencing analysis of human colon cancers has identified driver mutations in about 80 genes, including *Prep1* (Wood et al., 2007). Furthermore, *Prep1* (which maps on chromosome 21 from 43.267712–43.326757 Mb) (Berthelsen et al., 1998a,b) is found in a genomic region that undergoes loss of heterozygosity in 50% of informative gastric cancers (Park et al., 2000).

Prep1 is a member of the TALE (Three Amino acids Loop Extension) class of homeodomain proteins and is homologous to the oncogene *Meis1* (Moskow et al., 1995; Nakamura et al., 1996; Wong et al., 2007). Wild type Meis1 and Prep1 proteins compete for binding to the same site of Pbx1 to regulate transcription. *Pbx1* is also involved in cancer since its fusion with E2a causes Pre-B cells leukemia (Kamps et al., 1990; Nourse et al., 1990). Since Meis1 oncogenic activity requires the interaction with Pbx1 (Wong et al., 2007) and since all known functions of Prep1 also require an interaction with a Pbx family member, tumorigenesis in *Prep1*-deficient mice may depend on an imbalance between Meis1 and Prep1 binding to Pbx1. In the absence of Prep1, Meis1 is not differentially expressed but nevertheless its binding to Pbx1 and its oncogenic functions (i.e. transactivation of its target genes) might be favored.

Our transplantation experiments (Table 3) indicate that in the case of lymphomas the *Prep1*-deficient cancer-susceptible cell is possibly a hematopoietic stem/precursor cell. Indeed both B- and T-cell lymphomas developed upon transplantation of $Prep1^{i/i}$ FL cells, suggesting that the decreased expression of *Prep1* modifies a B- or T-cell, or common progenitor, making it susceptible to cancer. This result is remarkable because embryonic $Prep1^{i/i}$ FL cells have a very low competitive repopulation activity in lethally irradiated mice (Di Rosa et al., 2007). The variable latency of tumors moreover indicates that neoplastic transformation requires further hits in addition to *Prep1* deficiency.

Finally, preliminary experiments show that the tumorigenic phenotype is cell autonomous since in at least one case (TT1 T-cell lymphoma) tumor cells can be re-implanted successfully into nude mice (data not shown).

In conclusion, our data support a tumor suppressor role for *Prep1*. However, they give no information on which tumorigenic mechanism is negatively controlled by *Prep1*. While we have no definitive demonstration, preliminary data indicate that *Prep1* deficiency renders cells prone to genetic instability, which in itself is a tumorigenic mechanism. We are currently investigating the molecular basis of the *Prep1* tumor suppression function.

Acknowledgements

Work was supported by AIRC (Italian Association for Cancer Research), the Italian Ministry of Health, the Italian Ministry of University and Research and the Cariplo Foundation to FB and by the Italian Ministry of University and Research, the European Community (VI Framework), the CARIPLO Foundation, the Ferrari Foundation, and the Monzino Foundation to PPDF. We are very grateful to B. Amati, R. Pardi, F. D'Adda di Fagagna, S. Casola and S. Pece for many scientific discussions and to Pascale Romano for her careful editing. G. Iotti was a recipient of an AIRC fellowship. We acknowledge the generous gift of Meis and Pbx antibodies from Drs. Miguel Torres and Michael Cleary. We are grateful to M. Bianchi, L. Gerasi and A. Gobbi for their help.

Appendix. Supplementary information

Supplementary information related to this article can be found at doi: 10.1016/j.molonc.2010.01.001.

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