

# Aberrant Huntingtin Interacting Protein 1 in Lymphoid Malignancies

Sarah V. Bradley,<sup>1</sup> Mitchell R. Smith,<sup>3</sup> Teresa S. Hyun,<sup>1</sup> Peter C. Lucas,<sup>2</sup> Lina Li,<sup>1</sup> Danielle Antonuk,<sup>1</sup> Indira Joshi,<sup>3</sup> Fang Jin,<sup>3</sup> and Theodora S. Ross<sup>1</sup>

Departments of <sup>1</sup>Internal Medicine and <sup>2</sup>Pathology, University of Michigan Medical School, Ann Arbor, Michigan and <sup>3</sup>Fox Chase Cancer Center, Philadelphia, Pennsylvania

## Abstract

**Huntingtin interacting protein 1 (HIP1) is an inositol lipid, clathrin, and actin binding protein that is overexpressed in a variety of epithelial malignancies. Here, we report for the first time that HIP1 is elevated in non-Hodgkin's and Hodgkin's lymphomas and that patients with lymphoid malignancies frequently had anti-HIP1 antibodies in their serum. Moreover, p53-deficient mice with B-cell lymphomas were 13 times more likely to have anti-HIP1 antibodies in their serum than control mice. Furthermore, transgenic overexpression of HIP1 was associated with the development of lymphoid neoplasms. The HIP1 protein was induced by activation of the nuclear factor- $\kappa$ B pathway, which is frequently activated in lymphoid malignancies. These data identify HIP1 as a new marker of lymphoid malignancies that contributes to the transformation of lymphoid cells *in vivo*. [Cancer Res 2007;67(18):8923-31]**

## Introduction

Huntingtin interacting protein 1 (HIP1) is a clathrin, actin, and inositol lipid binding protein that may be involved in neurodegeneration by virtue of its interaction with huntingtin, the protein mutated in Huntington's disease (1, 2). The original association of HIP1 with hematopoietic malignancies occurred when the oncogenic HIP1/platelet-derived growth factor  $\beta$  receptor fusion protein was identified from a t(5;7) chromosomal translocation in a patient with chronic myelomonocytic leukemia (CMML; ref. 3). Expression of the HIP1 protein has since been found to be elevated in multiple epithelial cancers (4), its overexpression transforms fibroblasts (5), and its deficiency inhibits the progression of murine prostate cancer (6). Although the HIP1 family contains interacting domains for inositol phosphate lipids (7), endocytic molecules (8-11), and actin (12), the activities of these proteins in normal and neoplastic cell biology need much experimental clarification.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

S.V. Bradley participated in the design, execution, and interpretation of the majority of the experiments. In brief, she tested all human tissues and sera for HIP1 abnormalities. She also maintained and analyzed the p53-mutant mice, hHIP1 transgenic mice, and the aged mice. M.R. Smith participated in the design, execution, and interpretation of the cyclin D1 transgenic experiments and provided sera from the human patients with lymphoid malignancies. T.S. Hyun designed, executed, and interpreted the osteoclast experiments. P.C. Lucas did the pathologic analysis of hHIP1 transgenic mice. L. Li analyzed tumors from p53-deficient mice for genetic alterations at the Hip1 locus. D. Antonuk participated in the HIP1 expression analysis in aged mice. I. Joshi and F. Jin carried out and assisted in the analysis of the cyclin D1 transgenic mouse experiments. T.S. Ross wrote the manuscript with S.V. Bradley and participated in the design and interpretation of all of the described experiments except for the generation and analysis of the cyclin D1 transgenic mice.

**Requests for reprints:** Theodora S. Ross, University of Michigan, 6322 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0942. Phone: 734-615-5509; Fax: 734-647-9271; E-mail: tsross@umich.edu.

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However, our pursuit of HIP1 as a cancer marker has progressed over the last few years. In fact, presence of anti-HIP1 antibodies in blood, presumably a surrogate marker for HIP1 overexpression or mutation in cancer cells, has shown use in detecting prostate cancer in mice and humans (6). More recently, we found that patients with glial brain tumors tested positive for anti-HIP1 antibodies more frequently than normal, age-matched controls (13). Further testing of larger cohorts of different types of cancer patients and controls from different backgrounds and institutions will be required to confirm the usefulness of this test. To our knowledge, with the exception of our original discovery of HIP1 as part of the t(5;7) translocation in CMML, patients with hematopoietic malignancies, such as Hodgkin's and non-Hodgkin's lymphomas, remain to be thoroughly evaluated for HIP1 abnormalities.

In 2004, 7,880 new cases of Hodgkin's disease and 54,370 cases of non-Hodgkin's lymphoma were diagnosed in the United States (14). Hodgkin's disease originates in the lymph nodes and may involve the spleen, liver, and bone marrow. Hodgkin's disease is characterized by the Reed-Sternberg cell, which is considered the neoplastic cell and is derived from a B-cell progenitor (15, 16). Currently, no mouse models of Hodgkin's disease are available because the molecular cause of this neoplasm is not well defined. Non-Hodgkin's lymphoma is composed of many subtypes, which can be divided into two main categories of B-cell or T-cell origin (14). There are several mouse models for non-Hodgkin's lymphoma, two of which were used in this study. The first is the aged, pristane-treated E $\mu$ -cyclin D1 transgenic mouse that develops a B-cell lymphoma reflective of the aggressive mantle cell lymphoma (17); the second is the p53 knockout mouse that develops both B-cell and T-cell lymphomas (18).

Mantle cell lymphomas are a particularly interesting type of non-Hodgkin's lymphoma because they are molecularly diagnosed by the presence of a t(11;14) translocation involving the *BCL-1* (cyclin D1) gene, leading to abnormally high levels of cyclin D1 protein in tumor cells (19). Because cyclin D1 stimulates cell cycle progression, it might promote tumorigenesis. However, the overexpression of cyclin D1 in murine models alone is not sufficient to induce mantle cell lymphoma but requires both aging and treatment with a mitogen, such as the isoprenoid alkane pristane (17).

Here, we report the identification of HIP1 abnormalities as an additional oncogenic event that can occur in cyclin D1 transgenic and p53 knockout mice. Humans and mice with different types of lymphomas were systematically evaluated for HIP1 abnormalities. These studies showed that HIP1 abnormalities are common in a wide variety of lymphomas, suggesting the possibility that the HIP1 pathway represents a new area for diagnostic testing and therapeutic target development.

## Materials and Methods

**Animals.** Bcl-1 (E $\mu$ -cyclin D1) transgenic mice were obtained from Dr. Alan Harris (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia)

and bred and housed in the Fox Chase Laboratory Animal Facility, and lymphomas were induced with pristane as described previously (17). Pure C57BL/6 p53-null mice (18) were obtained from The Jackson Laboratory and mated on to a mixed *Hip1*<sup>null/null</sup> background to generate the following genotypes: *p53*<sup>+/-</sup>;*Hip1*<sup>+/+</sup>, *p53*<sup>+/-</sup>;*Hip1*<sup>null/null</sup>, *p53*<sup>-/-</sup>;*Hip1*<sup>+/+</sup>, and *p53*<sup>-/-</sup>;*Hip1*<sup>null/null</sup>. An additional cohort of 1.5- to 2-year-old aged mice that developed spontaneous lymphomas and their non-lymphoma-bearing littermates was on a mixed 129Svj:C57BL/6 background. p53, *Hip1* mutant, and aged mice were housed at the University of Michigan Animal Facility.

**Genotyping.** Mouse tail DNA was genotyped for the cyclin D1 transgene as described (17) and for the p53 alleles [wild-type (WT) or mutant] by PCR using the following primers: Neo (5'-CGCCTTCTATCGCCTTCTTGACGAG-3'), ex5F (5'-CGGAGGTCGTGAGACGCTGC-3'), and in6R (5'-GGCC-TGGGGGAAGACACAGG-3'). The p53 WT DNA was represented by a 647-bp product using primers ex5F and in6R. The p53-mutant DNA was represented by a 546-bp product using Neo and in6R primers.

**Identification of lymphoma in p53-deficient mice.** p53-null mice were examined for tumor development at 5 months of age. Spleens and thymi were weighed and fixed in 10% (v/v) buffered formalin, embedded in paraffin, serially sectioned, and stained with H&E. Splenomegaly in these 5-month-old mice was defined as a spleen weighing >0.15 g. Splenomegaly frequently accompanies peripheral B-cell lymphoma in p53-deficient mice as described previously (20).

**Acquisition of serum samples.** Mice were bled monthly from the saphenous vein of the hind leg or at necropsy as described previously (6). Serum samples from human patients with lymphoid malignancies were obtained from Fox Chase Cancer Center lymphoma clinic under an Institutional Review Board-approved protocol. Normal control sera were obtained from non-cancer-bearing individuals that were seen in the urology clinics at the University of Michigan. All sera were aliquoted and immediately stored at -80°C. Serum was assayed for anti-HIP1 antibodies using the immunoblot method as described previously (6).

**Tissue arrays.** The lymphoma tissue samples were from the following arrays from Cybrdi: CS20-00-002, CS20-00-003, and CS20-01-003.

**Quantitation of HIP1 family member protein levels in tissues from young and old mice.** WT (C57BL/6) mice were purchased from The Jackson Laboratory and sacrificed at 3 months ( $n = 4$ ) and 17 months ( $n = 4$ ). Western blot analysis of the tissues was done using the following antibodies: HIP1 (UM354, 1:5,000), HIP1r (UM374, 1:5,000), and actin (1:2,000; Sigma).

**Bone marrow differentiation with macrophage colony-stimulating factor and receptor activator of nuclear factor- $\kappa$ B ligand.** Mouse bone marrow cells were flushed from dissected femurs with complete RPMI 1640 using a 3 mL syringe and 27-gauge needle. Cells from each femur were divided into three wells of a six-well plate and treated with macrophage colony-stimulating factor (M-CSF; 1 ng/mL) for 1 week to generate macrophages. Fresh medium containing M-CSF (30 ng/mL; PeproTech, Inc.) and soluble receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B) ligand (RANKL; 300 ng/mL; PeproTech) was added on day 7 and cells were cultured an additional 7 days to generate osteoclasts (21). The protocol for fixation and TRAP stain of osteoclasts was obtained from the BD BioCoat Osteologic Bone Cell Culture System (BD Biosciences).

**RAW-OC differentiation.** The mouse macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection and maintained according to their directions. Generation of osteoclasts (RAW-OC) from RAW cells was described previously (22).

**Quantitative PCR.** Total RNA was isolated from osteoclast cultures collected at various time points and stored in 200 to 300  $\mu$ L Trizol (Invitrogen). Extraction of mRNA and quantitative PCR for HIP1 mRNA levels were done as reported previously (23).

## Results

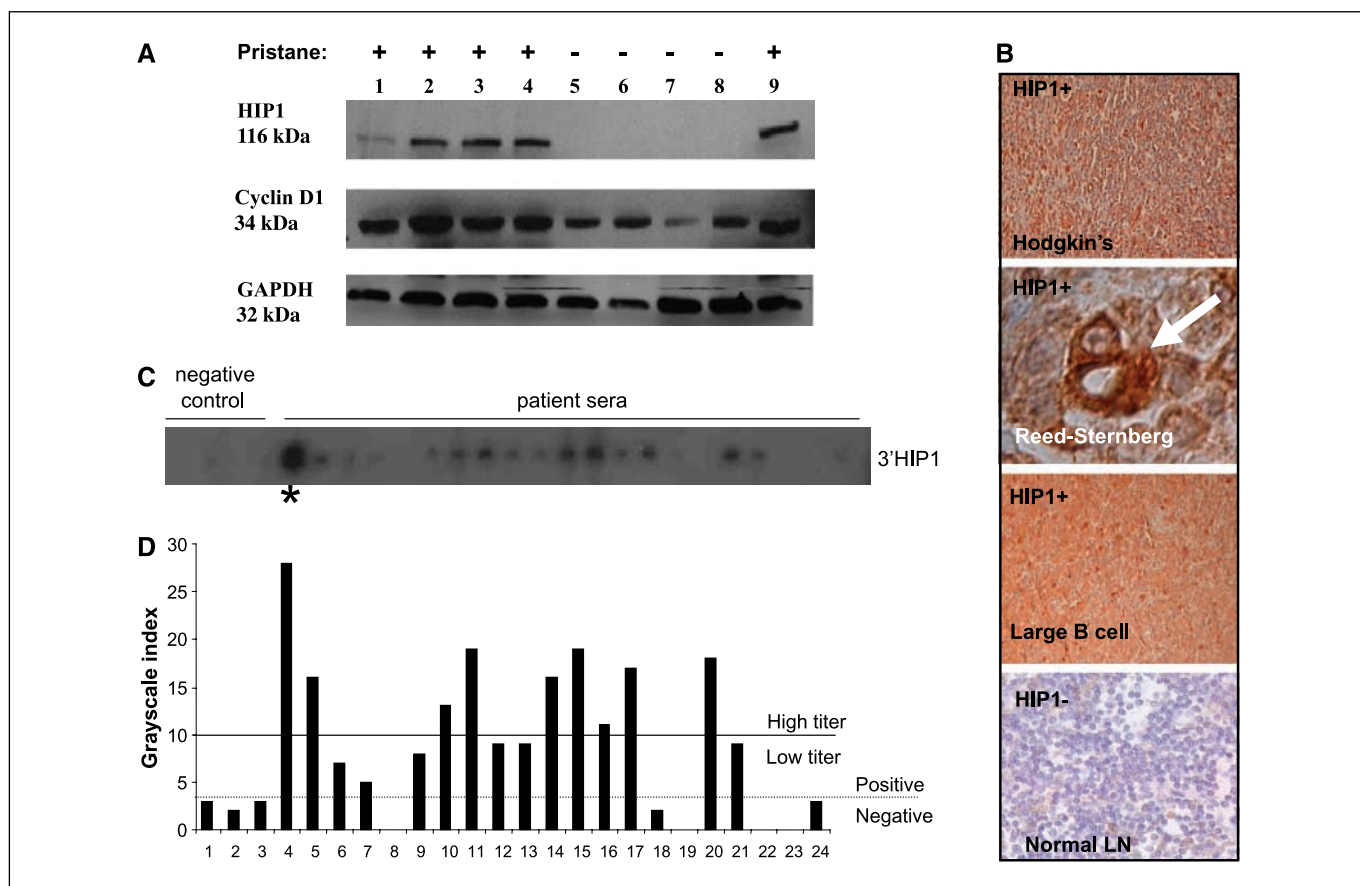
**HIP1 is overexpressed in tumors derived from a murine model of mantle cell lymphoma.** Mantle cell lymphoma is a particularly aggressive B-cell non-Hodgkin's lymphoma defined by a t(11;14) translocation that places the cyclin D1 coding region

from chromosome 11 downstream of the immunoglobulin heavy chain promoter on chromosome 14 (19, 24, 25). Although mantle cell lymphomas uniformly aberrantly express cyclin D1 as a result of the t(11;14) translocation, overexpression of cyclin D1 alone is not sufficient for lymphomagenesis in mice (26). E $\mu$ -cyclin D1 transgenic mice overexpress cyclin D1 in the B-cell lineage, are healthy, and do not develop spontaneous tumors. Three monthly i.p. injections of pristane into 1-year-old E $\mu$ -cyclin D1 transgenic mice on a C57BL/6 genetic background, however, results in generalized adenopathy, splenomegaly, and ascites in all of the mice within 1 month of the third pristane injection. Histologic and immunohistochemical analysis of the lymphomatous tissue from these mice indicates that these tumors are very similar to mantle cell lymphomas in humans (17).

Although it is clear from these prior results that both aging and mitogenic stimulation are required to cooperate with cyclin D1 overexpression for lymphoma induction, the actual molecular defects that result from the aging/pristane processes have not been defined. To address this, Seegene GeneFishing Technology was used to identify genes that are expressed differentially in neoplastic pristane-treated mouse spleens compared with spleens derived from non-pristane-treated littermates. We sequenced the most prominent band with altered expression levels in the neoplastic compared with the normal spleens and this was identified to be HIP1 (data not shown). The differential elevation of HIP1 expression was confirmed by Western blot analysis of an independent group of tumors, lymph nodes, and/or spleens from pristane-injected or non-pristane-injected (vehicle) cyclin D1 transgenic mice (Fig. 1A). Because up-regulation of HIP1 required pristane treatment and aging, we also can conclude that cyclin D1 overexpression on its own does not lead to increased HIP1 levels.

**HIP1 is overexpressed in human Hodgkin's and non-Hodgkin's lymphomas.** Based on the observation that HIP1 overexpression in cyclin D1 transgenic mice occurred in the resultant B-cell lymphomas, we asked whether HIP1 protein was also overexpressed in human lymphomas. Therefore, tissue microarrays that contained specimens from 151 different lymphoma patients were stained for human HIP1 using the HIP1/4B10 monoclonal antibody (Fig. 1B). Overall, HIP1 was overexpressed in 72% of lymphomas ( $n = 151$ ; Table 1; Supplementary Table S2). Interestingly, HIP1 protein was found most frequently in Hodgkin's lymphomas (examples shown in Fig. 1B, *top* and *middle top*), where 86% of Hodgkin's lymphoma tissue tested positive (total  $n = 35$ ; Table 1) compared with 68% of non-Hodgkin's lymphoma tissue (total  $n = 116$ ;  $P < 0.05$ ; Table 1). Normal lymph nodes did not significantly stain for HIP1 protein (Fig. 1B, *bottom*). Importantly, the pathognomonic Reed-Sternberg giant cells of Hodgkin's disease tissue contained high levels of HIP1 (Fig. 1B, *middle top, arrow*). The Reed-Sternberg cell is currently thought to be a B-cell-derived neoplastic cell (15, 16). The fact that high levels of HIP1 are found in the Reed-Sternberg cell and because HIP1, when overexpressed, can transform cells, these data support the assertion that the Reed-Sternberg cell is the tumorigenic cell in Hodgkin's disease. This observation, together with the fact that the majority of the remaining spots on the arrays were derived from B-cell non-Hodgkin's lymphomas, indicated that human B-cell neoplasms frequently express elevated levels of HIP1 compared with nonneoplastic lymph nodes.

**Increased frequency of anti-HIP1 antibodies in sera from non-Hodgkin's lymphoma patients.** Because HIP1 was overexpressed in both mouse and human lymphomas and we previously found that serum from prostate cancer patients more frequently



**Figure 1.** Expression of HIP1 in lymphoma and increased anti-HIP1 antibodies in sera from human non-Hodgkin's lymphoma patients. *A*, increased HIP1 in mouse B-cell lymphoma model. Pristane injection of aged cyclin D1 transgenic mice induces the development of mantle cell lymphoma (17). *Lanes 1, 2, 3, 4,* and *9*, extracts from lymphomas from cyclin D1 transgenic mice injected with pristane; *lanes 5 to 8*, extracts from lymph nodes and spleens from cyclin D1 transgenic mice without pristane injection. HIP1 levels were determined using the HIP1 antibody from BD Sciences. Mouse number and tissues are as follows: (1) #387 lymph node, (2) #387 spleen, (3) #414 lymph node, (4) #421 tumor, (5) #427 abdominal lymph node, (6) #427 spleen, (7) #430 abdominal lymph node, (8) #430 spleen, and (9) #433 spleen. *B*, increased HIP1 expression in human lymphomas. Examples from HIP1/4B10-stained tissue microarrays that contained specimens from 151 different lymphoma patients (Cybrdi): HIP1-positive lymphocyte-depleted Hodgkin's lymphoma in spleen (magnification,  $\times 10$ ), Reed-Sternberg cell from a HIP1-positive lymphocyte-depleted Hodgkin's lymphoma in the mediastinum (*arrow*; magnification,  $\times 100$ ), HIP1-positive large B-cell lymphoma in a cervical lymph node (LN; magnification,  $\times 10$ ), and HIP1-negative normal lymph node (magnification,  $\times 10$ ). *C*, anti-HIP1 antibodies in different human sera were measured by Western blot analysis as described previously (6). Normal control sera from non-cancer-bearing individuals that were seen in the urology clinics here at the University of Michigan were used as normal controls. Three negative control sera were run on each blot. These negative control sera were samples previously found to test negative for HIP1 antibodies (6). *Asterisk*, designates serum from a patient in remission from T-cell ALL whose cancerous bone marrow previously was shown to contain a partial trisomy of 7q, the region that includes the *HIP1* gene locus of 7q11.2. *D*, example of quantification of HIP1 antibody levels in serum samples from each lymphoma patient. Samples with values  $>3$  were considered HIP1 antibody positive. Twenty-three of 39 (59%) lymphoma serum samples were positive. Patients with a "high" titer tested higher than a value of 10.

tested positive for anti-HIP1 antibodies than serum from age-matched controls (6), serum samples from human patients with different types of lymphoid malignancies were tested for the presence of anti-HIP1 antibodies (Fig. 1C and D). Fifty-nine percent

Diagnosis	Positive	Negative	% positive
All lymphomas	109	42	72
Hodgkin's lymphoma	30	5	86*
Non-Hodgkin's lymphomas	79	37	68*

\*Significant difference between Hodgkin's lymphoma and non-Hodgkin's lymphoma ( $P < 0.05$ ,  $\chi^2$  analysis).

( $n = 39$ ) of lymphoma patient serum samples tested positive for HIP1 antibodies, which was a significant increase compared with the 31% ( $n = 49$ ) in normal, healthy controls ( $P < 0.01$ ,  $\chi^2$  analysis; Table 2; Supplementary Table S3). Only one patient serum sample in Fig. 1C was not from a B-cell malignancy-bearing patient but instead was drawn from a patient with a T-cell acute lymphoblastic leukemia (ALL) in remission. The sample from this patient had extremely high levels of anti-HIP1 antibodies (Fig. 1C, *asterisk-marked lane*). Before treatment, karyotypic analysis of this patient's leukemic bone marrow showed a deletion of chromosome 5 and trisomy of the area of chromosome 7q marked by D7s522 (Genzyme Genetics). This area is a region of chromosome 7 that includes the *HIP1* gene. There is no remaining neoplastic sample available to test this further. As a result of this observation, we will analyze neoplasms for genetic abnormalities in the *HIP1* gene whenever future patients with extremely high levels of anti-HIP1 antibodies are identified. In addition, because of the extremely high levels of antibody to HIP1, we considered the possibility that

**Table 2.** Anti-HIP1 antibodies in sera from patients with lymphoid malignancies

Patient category	Positive	Negative	% positive
Normal controls	15	34	31
All lymphoma patients	23	16	59*
Disease present (new/recurrent)	13	12	52
Newly diagnosed disease	9	5	64 <sup>†</sup>
Recurrent disease (relapse)	4	7	37
Disease-free (remission)	10	4	71*

\*Significant difference compared with normal controls ( $P < 0.01$ ,  $\chi^2$  analysis).

<sup>†</sup>Significant difference compared with normal controls ( $P < 0.025$ ,  $\chi^2$  analysis).

antibody frequency might predict whether a patient remained in remission or not. Indeed, as shown in Table 2 in this small cohort of patients, the presence of an anti-HIP1 antibody-positive test correlated with patients with lymphoid malignancies in remission but not relapse. As one might expect, patients in this cohort with splenectomy tested negative for HIP1 antibodies ( $n = 2$ ; data not shown). We also found that patients with a low titer of HIP1 antibodies (defined in Fig. 1D) and a diagnosis of a lymphoid malignancy invariably were relapsed patients (Fig. 1D; Supplementary Table S1). These data not only raise the possibility that the presence of antibodies to HIP1 is indicative of the presence of cancer but also suggest that high titers may be reflective of the host's defense against cancer, although other explanations, such as immunosuppression from prior therapy, could account for this observation. Nonetheless, immunization with HIP1 antigen might therefore be of therapeutic benefit and should be tested in the future.

**Increased anti-HIP1 antibodies in sera from mice with lymphoma.** Sera from p53-mutant mice (18) with different types of lymphomas were screened for the presence of anti-HIP1 antibodies. The p53 knockout mice (18) are extremely useful for controlled studies because the mice develop tumors by 6 months of age, the majority of which are malignant B-cell or T-cell lymphomas (27). The thymic lymphomas are always of T-cell origin (20). The B-cell lymphomas in these mice usually are accompanied by splenomegaly because they arise from either the spleen or major lymph node groups with a proclivity to spread to the spleen (20). Controlled screening/analysis in mouse models is important in the evaluation of the use of tests, such as the HIP1 antibody test. In addition, the availability of mouse anti-HIP1 antibodies, such as 1B11, allows for the generation of standard curves to rigorously quantitate the amount of mouse anti-HIP1 antibodies in test sera (Fig. 2A) unlike the human situation where standard curves can only be derived from previously positive human sera with unlimited and unknown quantities of specific human anti-HIP1 antibodies.

As expected from previous results with p53 knockout mice, by the age of 5 months, 50% (total  $n = 37$ ) of our p53-null cohort of mice developed either B-cell lymphomas characterized by splenomegaly and/or T-cell lymphomas with enlargement of the thymus. Sixty-eight percent (total  $n = 19$ ) of the p53-deficient mice with splenomegaly were positive for anti-HIP1 antibodies compared with only 5% (total  $n = 18$ ) of the p53-null mice with normal size

spleens (13-fold difference;  $P < 0.001$ ,  $\chi^2$  analysis; Fig. 2B). We also tested a cohort of normal, aged mice ( $n = 63$ ; age span, 1.5–2 years), some of which developed spontaneous follicular lymphomas common to aged mice, and found that 60% of the mice with spontaneous lymphomas ( $n = 29$ ) tested positive for anti-HIP1 antibodies compared with only 20% of their unaffected littermates ( $n = 34$ ; 3-fold difference;  $P < 0.01$ ,  $\chi^2$  analysis; Fig. 2C).

It is noteworthy to point out that the 1.5- to 2-year-old control mice in the spontaneous lymphoma experiment had a trend toward increased percentage of positive tests compared with the 5-month-old control mice in the p53 experiment ( $P < 0.2$ ,  $\chi^2$  analysis). This slight difference might indicate that levels of HIP1 or its only known mammalian relative, HIP1r, increase with age in the organs of mice. However, we found no variation in HIP1 and HIP1r levels with age in organs from young (3 months) and old (17 months) WT mice (Supplementary Fig. S1). This result indicated that the HIP1 family of proteins is under tight control under normal physiologic conditions and that underexpression or overexpression could be deleterious to the life of the cell.

To begin to determine the necessity of HIP1 expression in lymphomagenesis, HIP1-deficient mice were crossed with p53-mutant mice to test for susceptibility to p53 deficiency-mediated tumorigenesis. In this small cohort, a comparison of *Hip1* "null/null" mice (23) and *Hip1* WT mice both in the p53-mutant background showed no significant differences in the tumor frequency or distribution (Supplementary Fig. S2A). Because of this, we screened the resultant tumor tissues from p53;*Hip1* double-mutant mice ( $n = 10$  tumors) for large mutations in the *Hip1* locus using Southern blot analysis. Remarkably, an obvious mutation/amplification was indeed found in DNA derived from a widespread lymphoma found in a *Hip1*<sup>null/null</sup>;p53<sup>+/-</sup> mouse (labeled "mutation" in Fig. 2D). Multiple copies of the *Hip1* mutation allele compared with the "null" allele in the cheek, arm, and spine tumors (lanes 2–4) became very apparent when comparing these "homogeneous" tumors with the infiltrated liver (lane 1), which displayed only a 1:1 ratio of mutation allele and null allele compared with a 30:1 ratio in the tumor tissue. These altered ratios make sense as the cheek, arm, and spine tumors consisted mainly of neoplastic tissue, whereas the infiltrated liver tissue consisted of both tumor and normal liver tissue (Supplementary Fig. S2B, top left).

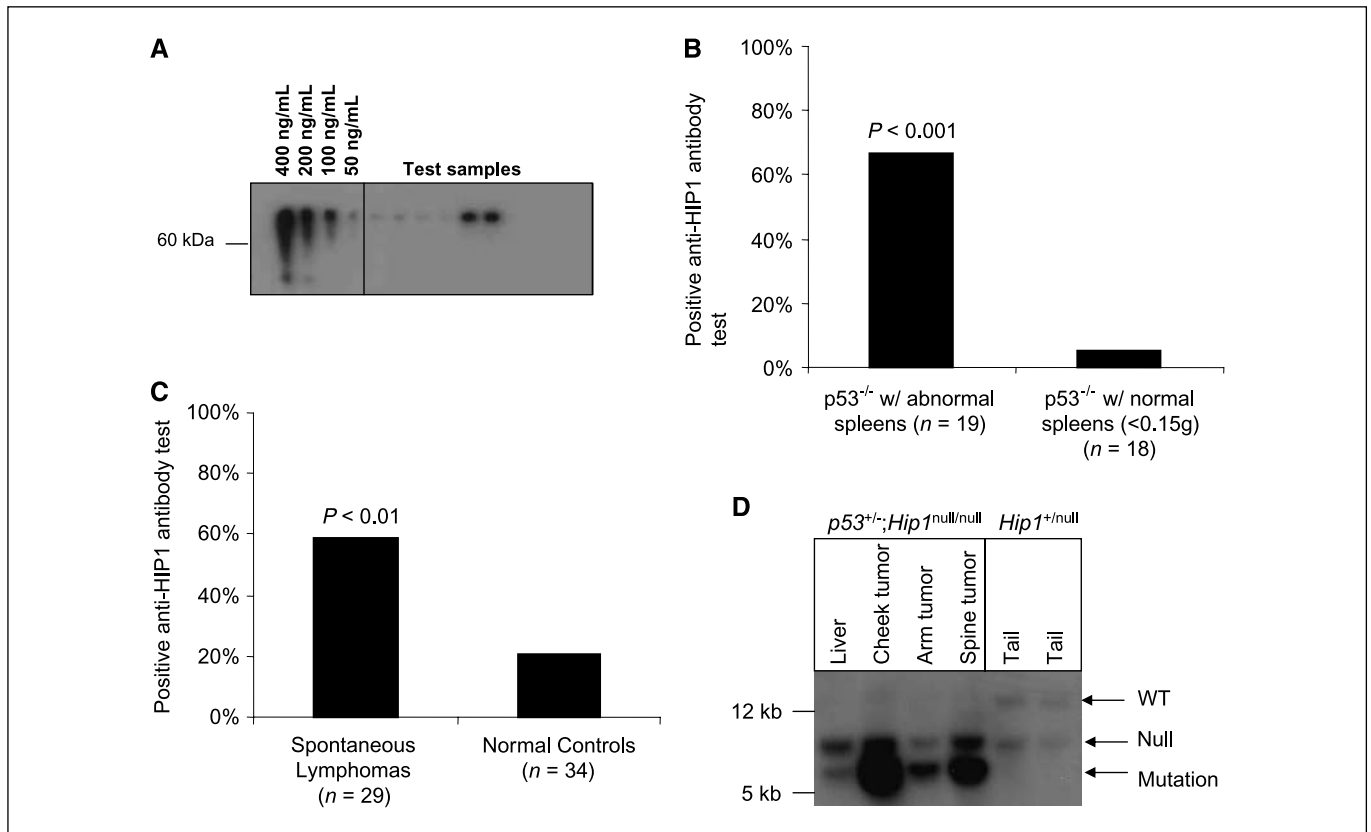
**In vivo overexpression of human HIP1 leads to plasma cell neoplasms.** Because we have observed that HIP1 is overexpressed in a variety of human tumors, transgenic mice with "ubiquitous" overexpression of human HIP1 (hHIP1<sup>hi</sup>) were generated to further study the role of HIP1 in tumorigenesis (28). These mice ubiquitously express human HIP1 driven from a chimeric cytomegalovirus  $\beta$ -actin promoter. The transgenic vector had loxP sites surrounding an enhanced green fluorescent protein (EGFP) transgene upstream of the human HIP1 sequences. Therefore, expression of the human HIP1 cDNA was expected only if EGFP was excised by the presence of Cre recombinase. The hHIP1<sup>hi</sup> transgenic mice were generated from a fluorescent green founder mouse (mouse #395) that was crossed with an EIIa-cre transgenic mouse (28). A cohort of these hHIP1<sup>hi</sup> mice and age-matched nontransgenic mice was sacrificed between 8 months and 1 year of age and necropsied. As shown in Supplementary Table S4, hHIP1<sup>hi</sup> mice frequently displayed splenomegaly [50% hHIP1<sup>hi</sup> ( $n = 12$ ) versus 18% WT ( $n = 34$ );  $P < 0.01$ ]. On histologic analysis, a neoplastic proliferation of dysplastic HIP1-positive plasmacytoid cells replacing lymph nodes from three hHIP1<sup>hi</sup> mice <1 year of age

was found (Fig. 3; Supplementary Table S4). In these mice, multiple lymph nodes had nearly complete effacement of nodal architecture by sheets of atypical plasma cells (Fig. 3, #583, #717, and #200). In the spleen of one of these hHIP1<sup>hi</sup> mice (mouse #200), both the red and white pulp were extensively replaced by expansive sheets of plasma cells, many with atypical nuclear features. Germinal centers and mature lymphoid aggregates were markedly reduced in the white pulp, having been displaced by plasma cells (Fig. 3). Furthermore, these neoplasms, which stained for human HIP1 expression (Fig. 3, #200), were likely clonal in nature as they stained for  $\kappa$  light chains but not  $\lambda$  light chains (Supplementary Fig. S3). On careful examination of lymph nodes from the group of the hHIP1<sup>hi</sup> and control mice, an additional hHIP1<sup>hi</sup> mouse and three WT mice were found to have subtle noneffacing plasmacytoid infiltrates in portions of their lymph nodes. However, these mice did not display a clearly abnormal proliferation of atypical plasmacytoid cells similar to that seen in the original three hHIP1<sup>hi</sup> mice (Supplementary Table S4).

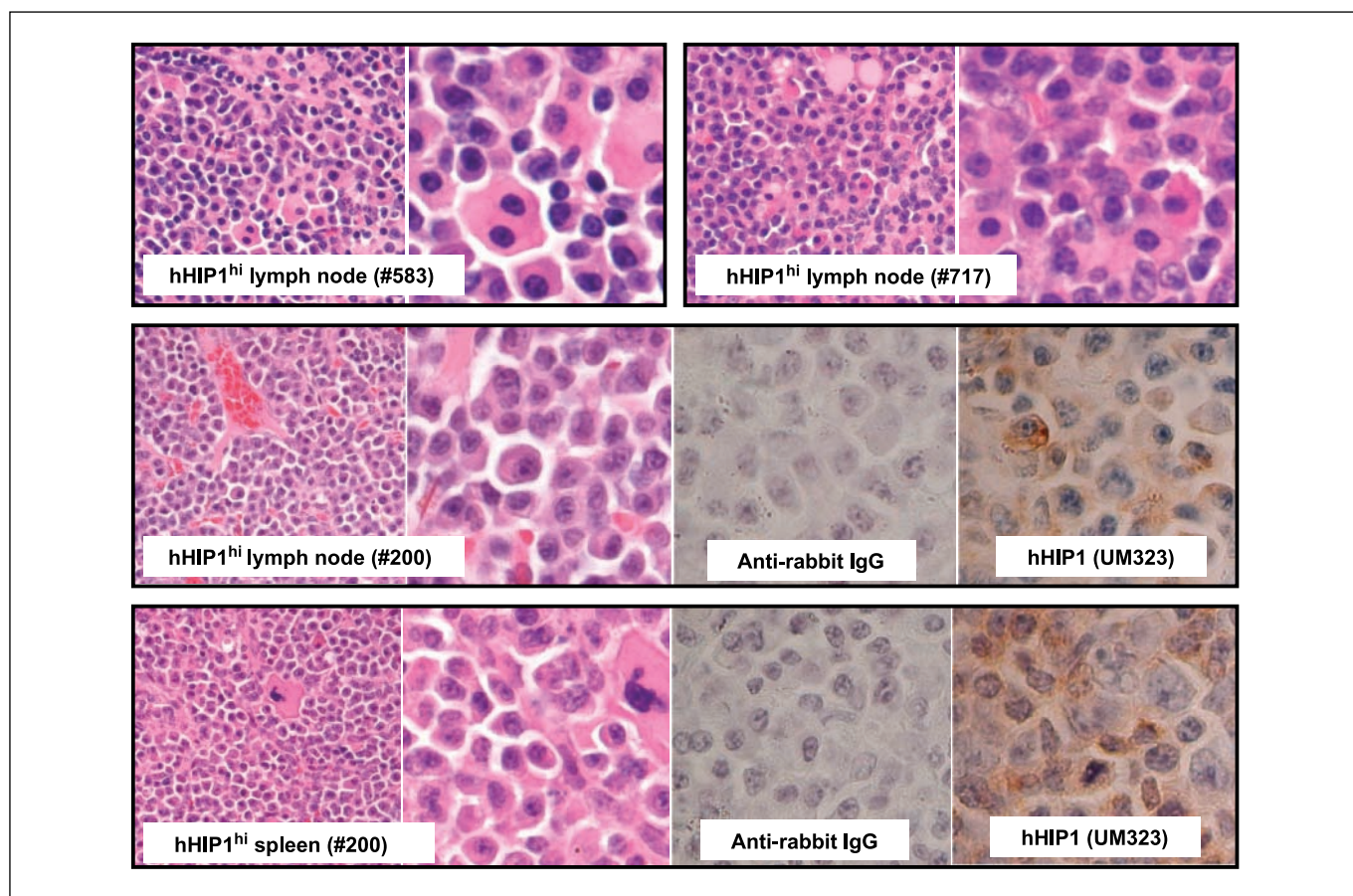
Interestingly, the green founder mouse (mouse #395) was necropsied at 2 years of age, and an atypical plasmacytic infiltrate was also found infiltrating the liver, spleen, and lymph nodes

(Supplementary Fig. S4). This was the only non-hHIP1<sup>hi</sup> mouse found with an overt plasma cell neoplasm (atypical cells with striking nuclear pleomorphism and effacement of lymph nodes). The plasma cell neoplasm was negative for EGFP expression both visually and by immunohistochemistry (data not shown), and more importantly, the tumor cells stained positive for human HIP1 (Supplementary Fig. S4), suggesting that in the tumor recombination had "spontaneously" occurred over 2 years. Alternatively, because plasma cell neoplasms do rarely occur spontaneously in aged C57BL/6 mice, it is possible that the tumor in this aged mouse was spontaneous and not secondary to the HIP1 overexpression in tumorigenic cells.

**Regulation of HIP1 levels.** We next inspected the sequence of the human HIP1 promoter to determine if HIP1 RNA expression might be regulated directly by activation of the cyclin D1 pathway or by other pathways known to be abnormal in lymphoma, such as the NF- $\kappa$ B or p53 pathway. In the dysregulated cyclin D1 pathway, Rb is phosphorylated and does not bind E2F transcription factors, allowing the unbound E2F to induce cell cycle progression (Fig. 4D). Therefore, we analyzed the sequence of the 5' flanking region of the *hHIP1* gene (nucleotides -958 to -1).



**Figure 2.** Increased frequency of anti-HIP1 antibodies in sera from mice with lymphoma. *A*, quantitation of murine anti-HIP1 antibodies. A standard curve was generated as an internal control for immunoblot quantitation of the concentration of HIP1 antibody in mouse sera. The mouse monoclonal antibody 1B11 at known concentrations of 400, 200, 100, and 50 ng/mL was used for this purpose. *B*, mice with p53 deficiency and B-cell lymphomas test positive for anti-HIP1 antibodies. p53-null mice were sacrificed and necropsied at 5 mo of age, and their sera were analyzed for anti-HIP1 antibody levels. A HIP1 antibody level >30 ng/mL was considered positive. Spleens from these 5-months-old mice were classified as abnormal if weights were >0.15 g or the spleen showed grossly visible lymphoma. Thirteen of 19 (68%) p53-null mice with splenomegaly tested positive for anti-HIP1 antibodies compared with only 1 of 18 (5%) of the p53-null mice with normal spleens ( $P < 0.001$ ,  $\chi^2$ ). *C*, aged mice with lymphomas test positive (>30 ng/mL) for anti-HIP1 antibodies. Sera from 1.5- to 2-year-old mice with spontaneous follicular lymphomas common in aged mice were examined for the presence of HIP1 antibodies. Seventeen of 29 (59%) of the mice with spontaneous lymphomas were positive for anti-HIP1 antibodies compared with 7 of 34 (21%) of their normal littermates ( $P < 0.01$ ,  $\chi^2$ ). *D*, Southern blot of tissue and tail DNA from a *p53*<sup>+/-</sup>;*Hip1*<sup>null/null</sup> mouse with a stage IV lymphoma that infiltrated liver and spleen and a *Hip1*<sup>+/-</sup> control, respectively. *Hip1*<sup>+/-</sup> control tail DNA showed the WT and originally targeted *Hip1*-null allele. DNA was digested with *Eco*RI and probed for mouse *Hip1* using the HIP1 3' probe (23). There was amplification of an aberrant form of *Hip1* (labeled mutation) in the tumor tissue from the *p53*<sup>+/-</sup>;*Hip1*<sup>null/null</sup>-mutant mouse, indicating that the *Hip1* locus had both a deletion and amplification in the tumor tissue. Ten *p53*<sup>+/-</sup>;*Hip1*<sup>null/null</sup> tumors were put through this gross Southern blot screening. Additional less obvious mutations have therefore not been ruled out.



**Figure 3.** Development of plasma cell neoplasms in young hHIP1<sup>hi</sup> transgenic mice. Plasma cell neoplasms in hHIP1<sup>hi</sup> transgenic mice at <1 y of age. Lymph nodes from mouse #200, mouse #583, and mouse #717 and the spleen from mouse #200 were stained with H&E. Magnifications,  $\times 40$  and  $\times 100$ . Pictures of human HIP1 staining and negative control anti-rabbit IgG staining are also shown for the lymph node and spleen from mouse #200. Magnification,  $\times 100$ . The lymph nodes from mouse #583 and mouse #717 displayed similar human HIP1 staining.

This region was rich in GC residues and included a CAAT box but lacked a typical TATA box or obvious E2F binding sites (Supplementary Fig. S5). The lack of E2F binding sites in the HIP1 promoter, and the fact it takes up to a year and a half for lymphomas to develop in the cyclin D1 transgenic mice, together with the lack of elevated HIP1 levels in the nonneoplastic spleens of cyclin D1 transgenic mice (Fig. 1A) led us to conclude that abnormal HIP1 expression likely contributes to lymphomagenesis in a cyclin D1-independent pathway (Fig. 4D).

Analysis of the HIP1 promoter sequence also showed the presence of one NF- $\kappa$ B binding site located in the promoter that was conserved between mouse and human sequences (Supplementary Fig. S5). This observation is relevant due to the increase in NF- $\kappa$ B activity in most lymphomas (29), including Hodgkin's disease and B-cell non-Hodgkin's lymphomas (30, 31). As an example, the Reed-Sternberg cell has constitutive activation of the NF- $\kappa$ B pathway due to coexpression of both RANK and its ligand (RANKL; refs. 32, 33). This is thought to induce autocrine stimulation resulting in a positive feedback loop that promotes tumorigenesis.

**HIP1 protein levels are increased in response to NF- $\kappa$ B pathway activation.** To begin to determine if elevated transcript levels of HIP1 up-regulate HIP1 protein in response to NF- $\kappa$ B activation in normal and neoplastic tissue, we analyzed HIP1

protein and mRNA levels after NF- $\kappa$ B activation in two well-defined cell types, primary macrophages and the RAW cell line, both of which respond to NF- $\kappa$ B pathway activation via RANKL stimulation of RANK to differentiate into osteoclasts. First, we collected primary bone marrow cells from dissected femurs, subjected them to osteoclast culture conditions (Fig. 4A), and collected them for Western blot analysis. The results of this experiment showed induction of a second, higher molecular weight HIP1 species as the cells differentiated in response to RANKL treatment (Fig. 4B, day 14). Although not relevant to this discussion, we also observed that HIP1 levels increased in response to M-CSF treatment of bone marrow cells (Fig. 4B, day 4 versus day 7). To test whether HIP1 was transcriptionally regulated under these conditions, RNA was also isolated from primary osteoclast cultures and subjected to quantitative reverse transcription-PCR for HIP1. HIP1 mRNA levels were relatively constant throughout differentiation, with at most an inconsistent 2-fold increase in HIP1 mRNA levels at day 14 of culture (data not shown). This result indicated that alteration of HIP1 in these cells was due mainly to posttranscriptional effects. Cultures at day 14 were stained for TRAP, a marker of osteoclast differentiation and biological response to RANKL. TRAP staining confirmed that the cells did differentiate into multinucleated osteoclasts (Fig. 4B).

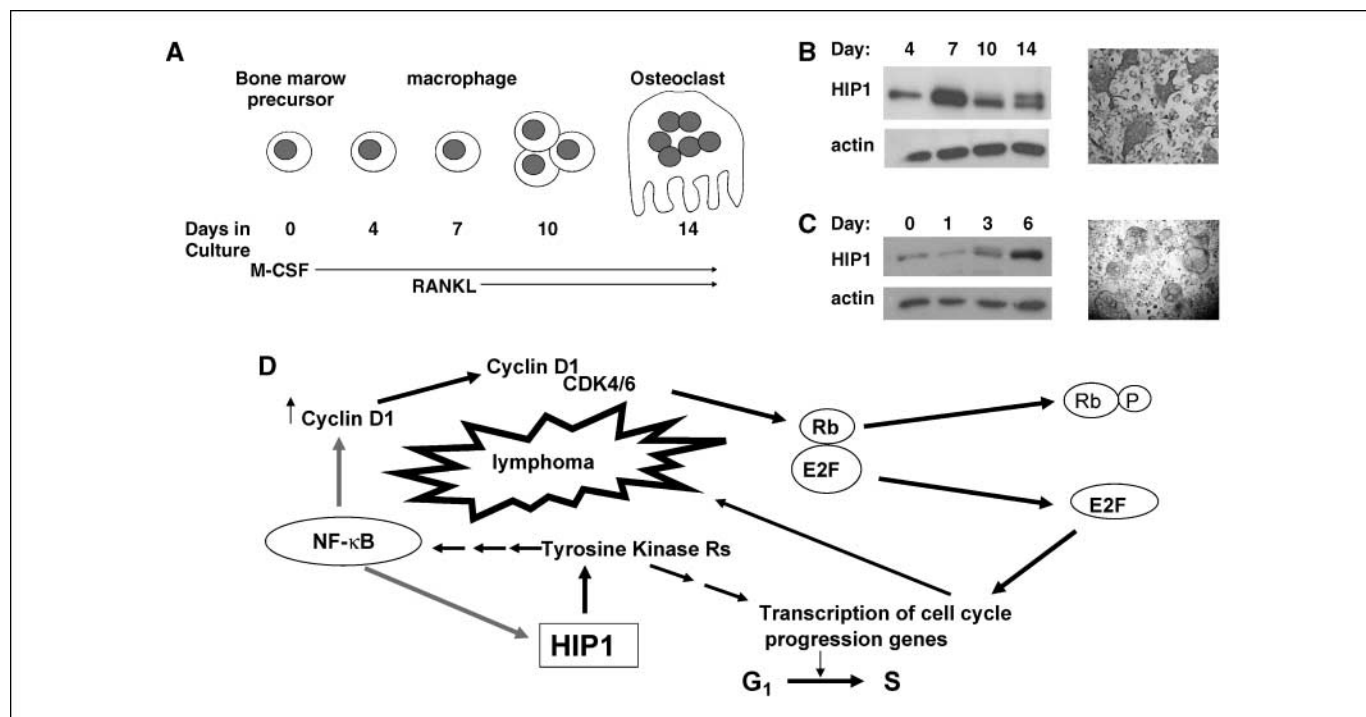
A complicating issue associated with assessing the response of HIP1 to NF- $\kappa$ B activation in primary bone marrow cells is that their treatment requires both the presence of M-CSF for cellular survival as well as RANKL for differentiation from monocytic precursors to multinucleated osteoclasts. To further test the role of RANKL stimulation alone on HIP1 protein levels, we tested the RAW cell line for HIP1 response to NF- $\kappa$ B activation. RAW cells are a murine macrophage line that does not require M-CSF for survival in culture (22) and can be differentiated into osteoclasts (RAW-OC) after 5 to 6 days in culture with the addition of RANKL (35 ng/mL). Analysis of HIP1 levels at days 0, 1, 3, and 6 of differentiation showed that RAW cells had a HIP1 expression pattern similar to the primary osteoclast cultures. Day 0 of RAW-OC culture corresponds to day 7 of primary osteoclast culture and days 5 to 6 of RAW-OC culture correspond to day 14 of primary osteoclast culture. As in primary osteoclast cultures, the higher molecular weight HIP1 band also appeared at later time points during osteoclast differentiation. TRAP staining of RAW-OC cultures at day 6 also showed formation of multinucleated osteoclasts much like those formed in primary cultures (Fig. 4C).

## Discussion

The morbidity and mortality of malignant lymphomas are due to the spread of a clonal growth of neoplastic lymphoid progenitor cells that invade multiple hematopoietic and nonhematopoietic

organs. Although Hodgkin's and non-Hodgkin's lymphomas are distinct in several ways, such as their organ specificity, histology, subtype classification, and epidemiology, they share their origins with a lymphoid progenitor cell that ultimately gives rise to different B-, T-, or null-lymphoid neoplasms. Although the last 2 decades have seen a revolution in the molecular diagnosis, classification, and treatment of lymphomas with bone marrow transplant and targeted agents (immunologic and small molecule), significant challenges including relapse, drug sensitivity, and resistance remain. Obtaining additional tissue and blood tests that can provide the earliest detection of lymphoma will have important prognostic and therapeutic implications.

To our knowledge, the present study is the first study to evaluate the role of HIP1 in lymphoma diagnosis and management. Our data show that HIP1 expression is increased consistently in B-cell lymphomas that arise in cyclin D1 transgenic mice that have been aged and treated with pristane. In addition, human Hodgkin's lymphomas and non-Hodgkin's lymphomas contain high levels of HIP1 protein compared with nonneoplastic lymph nodes. Interestingly, the pathognomonic Hodgkin's lymphoma giant cell, the Reed-Sternberg cell, is positive for HIP1 expression. This cell is clonal and of B-cell origin (15, 16). The characterization of this cell in Hodgkin's lymphoma biology has been the "holy grail" of the Hodgkin's disease field because under the current theory it is the neoplastic cell that needs to be therapeutically targeted in the midst of inflammatory cells that make up the bulk of the tumor.



**Figure 4.** Modulation of HIP1 levels by NF- $\kappa$ B pathway activation. **A**, schematic of how NF- $\kappa$ B activation is reflected (and scored) by osteoclast differentiation from bone marrow precursors. Bone marrow cells were isolated from four 5-month-old mice and treated with M-CSF for 1 wk to induce macrophage differentiation. Cells were then treated with M-CSF and RANKL for the 2nd week to induce osteoclast formation. Cells were harvested at days 4, 7, 10, and 14. **B**, HIP1 levels during osteoclast differentiation. Twenty micrograms of protein from each time point were run on SDS-polyacrylamide gels and blotted for HIP1. Bone marrow precursors were harvested from four mice, two of which were deficient for HIP1. Actin was used as a loading control. *Right*, TRAP stain of day 14 bone marrow cultures showing the presence of multinucleated osteoclasts. This confirms successful NF- $\kappa$ B pathway activation. **C**, HIP1 protein levels increase in RANKL-stimulated RAW 264.7 cells. The murine macrophage cell line RAW 264.7 was treated with RANKL to induce osteoclast differentiation (RAW-OC). Day 0 of RAW-OC culture is considered similar to day 7 of primary osteoclast culture, and day 6 of RAW-OC culture is considered similar to day 14 of primary osteoclast cultures. Twenty micrograms of protein from each time point were separated on SDS-PAGE and Western blotted for HIP1. *Right*, TRAP stain from day 6 of RAW-OC culture. **D**, schematic of the proposed position of HIP1 in the cyclin D and NF- $\kappa$ B pathways as they relate to lymphomagenesis. Based on the data herein, HIP1 was placed downstream of NF- $\kappa$ B and parallel to cyclin D1.

We also present the finding that increased antibodies against HIP1 are present more frequently in the sera of mice and humans with a variety of B-cell lymphoid neoplasms. There is a precedent for the expression of autoantibodies with lymphoma development. Anti-p53 antibodies were found in the sera of 21% of children with B-cell lymphoma (34) and in the sera of 7% of a variety of non-Hodgkin's lymphoma patients (35). Anti-ssDNA antibodies were detected in the serum of 16 of 55 patients (29%) with non-Hodgkin's lymphoma (36). These studies, as well as the current study showing the high frequency of HIP1 "autoantibodies" in individuals with lymphoma, suggest that lymphoma may be associated with immune dysfunction in general. In favor of this is the fact that patients with organ transplants who are chronically immunosuppressed have a strikingly high risk of subsequent non-Hodgkin's lymphoma. However, it is not known if it is a lack of immune surveillance or aberrant immune stimulation that is the cause of immunosuppressant-associated cancers.

Interestingly, in the cohort of human patients with lymphoid cancers studied here, HIP1 antibodies were more frequent and of higher titer in patients who have undergone remission than in relapsed patients (Table 2; Supplementary Table S1). This raises the question of whether induction of these anti-HIP1 antibodies with immunization might represent a novel method for generating a specific and therapeutic immune response to tumors. The fact that generalized autoimmunity does not correlate with cancer prevalence in humans (37) further supports the specificity and potential therapeutic efficacy of the HIP1 antibody. In addition, p53-deficient mice have lower levels of autoantibodies against dsDNA, chromatin, and rheumatoid factor (38). These observations in humans and in p53-deficient mice indicated that increased levels of HIP1 antibodies were likely not due to a generic increase in autoimmunity concurrent with lymphoma and/or p53 deficiency.

Whether the HIP1 antibodies are only a marker of the lymphoma or have some role in lymphomagenesis or lymphoma prevention remains to be experimentally determined. To begin to test this, we did analyze a cohort of mice that were mutant for both *Hip1* and p53. Although we found that tumors still developed in *Hip1*-null mice, we found that the *Hip1* locus was altered in tumor tissue from the double-mutant mice. This result indicates that *in vivo* analysis using mutant alleles may not answer the apparently simple question of tumorigenic necessity, especially in the case of relatively nonredundant oncogenes. In fact, based on our data, "negative" results in such experiments might ultimately reveal how important a protein is in tumorigenesis by selecting for cells where novel mutations in the gene of interest are discovered.

In parallel to carrying out the above tests for HIP1 necessity in tumorigenesis, we have generated a cohort of hHIP1 transgenic mice (28), which were observed for tumor formation to address the question of *in vivo* sufficiency. Remarkably, 25% of hHIP1<sup>hi</sup> mice were afflicted with plasmacytomas at <1 year of age. These are reminiscent of the tumors that develop in cyclin D1 transgenic mice and suggest that the HIP1 protein promotes tumorigenesis in the intact organism. Because these neoplasms only developed in a fraction of the mice and took time to develop, it is likely that additional oncogenic mutations are cooperating with the overexpression of HIP1 to result in cancer.

To begin to understand how HIP1 expression might be dysregulated in lymphomas and other cancers, we analyzed the HIP1 promoter sequence. Because E2F is well known to operate downstream of cyclin D1 activation, we expected that the promoter would have several E2F binding sites. The HIP1 promoter was

found to have a typical CAAT box and was noteworthy for the absence of E2F binding sites. There was one NF- $\kappa$ B site in the promoter that was conserved between mice and humans (Supplementary Fig. S5), suggesting that NF- $\kappa$ B may induce HIP1 mRNA expression in some lymphoid neoplasms. Further analysis of the promoter and determination of whether inhibitors of NF- $\kappa$ B activation can down-regulate the promoter activity or the levels of HIP1 in lymphomas will be important future steps.

Because the HIP1 promoter does not contain E2F binding sites, cyclin D1 overexpression in the cyclin D1 transgenic mice does not likely up-regulate HIP1 transcription directly. In addition, how pristane or the aging process independently or together contributed to the elevated HIP1 expression was not delineated. However, because the NF- $\kappa$ B pathway is the most activated pathway in both Hodgkin's disease and non-Hodgkin's lymphoma, we evaluated the effect of the NF- $\kappa$ B pathway on HIP1 protein levels by determining whether activation of the NF- $\kappa$ B pathway in bone marrow cells via the RANK receptor altered HIP1 levels during differentiation to osteoclasts. This system is relevant because the differentiated osteoclast cells are multinucleated like Reed-Sternberg cells, osteoclasts are of hematopoietic origin, and the biological outcome of NF- $\kappa$ B activation in these cells is easy to score. We found that up-regulation of an isoform of the HIP1 protein occurs on RANKL stimulation. It is interesting to note that the higher molecular weight species of HIP1 appear when mononuclear progenitors are fusing to form multinucleated osteoclasts in response to RANKL. Like endocytosis, cell fusion requires reorganization of the cell membrane. Because we have found HIP1 alterations in both multinucleated Reed-Sternberg cells and osteoclast cells and have shown that HIP1 interacts with lipids, clathrin, and actin, we propose the HIP1 family may play a role in the process of cell fusion. Increased levels of the HIP1 protein in primary bone marrow cells occurred without concomitant alterations in total HIP1 RNA levels. Although it remains formally possible that different splice forms of HIP1 encode for different HIP1 isoforms that are differentially regulated by NF- $\kappa$ B, it is likely that identifying mechanisms for the regulation of HIP1 via post-translational modification and/or RNA stability will prove fruitful.

Although aging per se does not alter steady-state levels of HIP1 or its only known mammalian relative HIP1r (Supplementary Fig. S1), it remains to be determined if mutations in the *HIP1* gene occur with aging. Interestingly, HIP1 levels have been found up-regulated in fibroblasts from progeria patients (39) and double-deficient HIP1/HIP1r mice display a subset of the characteristics of premature aging, such as spinal curvatures and cataracts, as early as 2 weeks of age (28). In fact, we have found that embryonic fibroblasts from HIP1/HIP1r-deficient mice either senesce or survive 3T3 immortalization by invariably losing expression of the INK4 family member, p15INK4b.<sup>4</sup> These data in aggregate have led us to speculate that the cyclins and their inhibitors function in parallel to the HIP1 pathway in the cell. Rather than significant pathway overlaps or cross-talks, we propose that HIP1 tumorigenic pathways collaborate in parallel with the cyclin D1 pathway (Fig. 4D).

In summary, we present the first study of abnormal HIP1 expression associated with mouse and human lymphomas and suggest that testing for anti-HIP1 antibodies in the sera of the

<sup>4</sup> L. Li and T.S. Ross, unpublished observations.

lymphoma patients could allow for improved clinical management of patients with lymphoma. In addition, we show for the first time that the overexpression of HIP1 *in vivo* leads to the promotion of lymphoid malignancies in human HIP1 transgenic mice. We also show that NF- $\kappa$ B pathway activation is associated with altered levels of HIP1 protein in both primary bone marrow macrophages and the corresponding RAW cell line. Further studies of the role of HIP1 and anti-HIP1 antibodies in lymphoma diagnosis and therapy using larger cohorts of humans will be an exciting next step.

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