Heterozygosity for Hypoxia Inducible Factor 1α Decreases the Incidence of Thymic Lymphomas in a p53 Mutant Mouse Model

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Abstract
Hypoxia inducible factors (HIFs) are critical mediators of the cellular response to decreased oxygen tension and are over-expressed in a number of tumors. Although HIF1α and HIF2α share a high degree of sequence homology, recent work has shown that the two α subunits can have contrasting and tissue-specific effects on tumor growth. To directly compare the role of each HIFα subunit in spontaneous tumorigenesis, we bred a mouse model of expanded HIF2α adaptive responses to O2 deprivation that are largely mediated by the role of each HIF

Introduction
Hypoxia, or decreased oxygen (O2) availability, is a common characteristic of solid tumors. Cells have developed a number of adaptive responses to O2 deprivation that are largely mediated by the hypoxia inducible factors (HIFs). HIFs function as α/β heterodimeric transcription factors that regulate over 150 genes involved in metabolism, cell cycle regulation, erythropoiesis, cell survival, and angiogenesis (1, 2). The β subunit is constitutively expressed, whereas the stability of the three α subunits is regulated by O2 availability. Two HIFα subunits, HIF1α and HIF2α, have drawn considerable interest since their discovery. HIF1α and HIF2α share a high degree of sequence homology but display distinct expression patterns in the adult organism. HIF1α is expressed ubiquitously in the adult, whereas HIF2α expression is restricted to the endothelium, kidney, heart, and lung (3–5). In addition, the two subunits have both shared (such as Vegf) and unique transcriptional targets. For example, HIF1α exclusively regulates glycolytic enzymes, whereas HIF2α preferentially regulates genes involved in differentiation (Oct4) or proliferation (Epo, Tgf-α; refs. 6–8).

The observation that HIF1α and HIF2α are highly expressed in a number of human tumors fueled early studies to evaluate the role of each subunit in tumor initiation and progression. Previous attempts to explore the role of HIFs in tumorigenesis used subcutaneous models in immunocompromised mice. When injected into mice, transformed Hif1α−/− mouse embryonic fibroblasts grow more slowly and form less vascularized tumors than wild-type fibroblasts, suggesting a role for HIF1α in both tumor growth and angiogenesis (9). In contrast, HIF2α seems to promote the growth of human neuroblastoma and renal clear cell carcinoma tumors in nude mice, whereas HIF1α does not (10–14). Furthermore, HIF2α actually inhibits tumor growth and promotes apoptosis in rat gliomas (15). These studies suggest that the two HIFα subunits have distinct effects on tumorigenesis. They also indicate that either the HIFs have tissue-specific functions or that xenografts do not accurately replicate the microenvironment for tumor formation in vivo. Introduction of established tumor cell lines subcutaneously in nude mice assesses the capacity of these cells to proliferate and stimulate angiogenesis but does not evaluate tumor initiation, progression, and metastasis, or the effects of the local microenvironment on tumor growth. A recent study by Liao and colleagues (16) in a mouse breast cancer model showed that HIF1α is not necessary for tumor initiation, but HIF1α loss results in increased tumor latency and decreased proliferation, angiogenesis, and metastatic potential. Because HIFα effects are likely to be tissue- and tumor stage–specific, studies in spontaneous tumor models will be critical to determine the role of each HIFα subunit in cancer.

To this end, we used our previously described “knock-in” mouse model in which the HIF2α coding sequence is under the control of the Hif2α locus (Hif2α2KES), thereby broadening HIF2α expression to all tissues (6, 17). In fact, HIF2α has been detected in tumors derived from tissues in which it is not normally expressed (18). This genetic manipulation not only allows us to compare the effect of each subunit directly, as they are expressed in the same tissues, but also avoids nonspecific effects from dramatic overexpression encountered in some transgenic models. Homozygous Hif2α2KES embryonic stem cells generate more proliferative and vascularized teratomas than their wild-type counterparts, further supporting a role for HIF2α in promoting tumor growth (17). Unfortunately, Hif2α2KES mice die before embryonic day 8.5, precluding their use in a tumor study, but heterozygotes are viable (6). Because adult Hif2α2KES animals do not develop tumors spontaneously,5 we induced tumor formation by crossing them to mice bearing an
arginine to histidine mutation in codon 270 of p53 (p53R270H; ref. 19). The equivalent human mutation (R273H) is commonly detected in patients with Li-Fraumeni syndrome, where patients with a R270H mutation have increased the age at onset for thymic lymphomas in these mice. A large fraction of p53R270H mice developed carcinomas in a similarly mixed background. Mice were evaluated daily for signs of morbidity or tumor growth. Distressed mice were euthanized by CO2 asphyxiation and dissected. All soft tissues were fixed in 4% paraformaldehyde and processed as previously described (19). Tumors were then identified by veterinary pathologists (A.C.D. and M.H.G.).

Expression profiling of tumors in both Hif1αK/O p53H/H and Hif1αK/O p53H/H mice revealed reduced Notch activity in tumors heterozygous for Hif1α, providing the first demonstration of an interaction between HIF1α and Notch during tumorigenesis.

Materials and Methods

Aging study. Cohorts were produced by mating p53R270H mice to p53H/+ or p53H/− mice. Because the p53H/− mice were enriched for 129S/ SvJae (19), the p53H/− allele was in a mixed C57BL/6-129/Sv background (23), and Hif1αK/O and Hif1αK/O mice were 129SvEvTac enriched (6), all mice were of a similarly mixed background. Mice were evaluated daily for signs of morbidity or tumor growth. Distressed mice were euthanized by CO2 asphyxiation and dissected. All soft tissues were fixed in 4% paraformaldehyde and processed as previously described (19). Tumors were then identified by veterinary pathologists (A.C.D. and M.H.G.).

Immunohistochemistry. Immunohistochemistry was performed following manufacturer’s guidelines and developed using diaminobenzidine (Vector Laboratories). Primary antibodies used in this study are listed in the supplement. Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the ApopTag Peroxidase In situ Apoptosis Detection kit (Millipore) as per manufacturer’s instructions. Positive-staining cells were counted in eight to nine fields per tumor using ImageJ (NIH). Standard techniques were used for immunoblotting.

RNA extraction and QRT-PCR. Tissues were stored in RNA Later (Qiagen). For RNA extraction, tissues were homogenized in Trizol (Invitrogen) and purified using Qiagen RNaseasy columns. cDNA synthesis was performed as described previously (24). Taqman primer/probe sets were purchased from Applied Biosystems. Microarray analysis was performed at the University of Pennsylvania Microarray Core using the Affymetrix MOE430Av2 array. Raw data for expression profiling are available through the National Center for Biotechnology Information Gene Expression Omnibus with the accession number GSE14336.

Sequencing. The PEST domain of Notch1 was amplified for sequencing from cDNA using the following primers: 5'GCCTCTGGAATGTGGGTGAT-3', 5'GAGGTGTGGCTGTGATGGTG-3', and 5'AGGACCTCAAGGCAGGAG-3' and 5'AGAGTGTTGCTTGATGGTG-3' (25).

Results

Generation of tumor-prone mice with expanded expression of HIF2α. To assess the effect of HIF2α on spontaneous tumorigenesis in vivo, we crossed Hif2α knock-in (Hif1αC50) mice (17) with p53R270H animals (19), which are prone to spontaneous tumor formation. As a control for loss of one Hif1α allele, we crossed Hif1α−/− mice to the same p53 mutant strain. To generate mice homozygous for the R270H mutation and either wild-type for Hif1α (Hif1α+/−; n = 23), heterozygous for Hif1α (Hif1α−/−; n = 20), or carrying an additional Hif2α allele (Hif1α−/−; n = 24), we intercrossed heterozygotes for the R270H mutation from each Hif1α genotype (Fig. 1A). The Hif1αC50 animals were generated in the 129SvEvTac background; therefore, Hif1α−/− mice were backcrossed into the 129SvEvTac strain to control for strain differences (6). Successful generation of each cohort was verified by PCR on tail DNA within the first weeks after birth (Fig. 1B) and confirmed by repeating all four PCRs on tail DNA after animal sacrifice (Emark Scientific). To quantify Hif2α expression in normal Hif1αC50 tissues, QRT-PCR for HIF1α and HIF2α was performed on duodenum and skeletal muscle samples taken from Hif1α−/−, Hif1α−/−, and Hif1αC50 mice on a wild-type p53 background (Fig. 1C). As expected, Hif2α mRNA levels were increased in normal tissues. Given that our mouse model is one of expanded HIF2α expression and not HIF2α overexpression, these changes (~2-fold)
were in the expected range and were similar to levels measured in
$Hif1a^{R270H}$ embryos (6).

**Dosage of the $p53^{R270H}$ mutation affects tumor spectrum.**
Upon dissection, each mouse organ was fixed and prepared for
histologic analysis, which was conducted by a board-certified
veterinary pathologist (A.C.D.). Because the tumor spectrum in
mice homozgyous for the R270H mutation had not been previously
examined, we compared tumor incidence in $p53^{R270H}$ (n = 23) mice
with $p53^{+/+}$ (n = 19) animals in a $Hif1a^{+/+}$ background.
Interestingly, $p53^{R270H}$ mice developed substantially more thymic
lymphomas ($P = 0.0015$) and tended to present with these tumors at
an earlier age than mice in the $p53^{+/+}$ cohort, although the age
difference was not statistically significant (Fig. 2A–B). Instead,
$p53^{R270H}$ mice developed sarcomas ($P = 0.03$), brain tumors,
and teratomas. Why carcinomas (as described by Olive and colleagues;
ref. 19) were not observed in our $p53^{R270H}$ cohort is unclear but may
be due to subtle changes in growth conditions provided by
different animal barriers. Gross and histologic findings are
provided in Supplementary Figs. S3 and S6. The incidence of
thymic lymphomas in the $p53^{R270H}$ cohort was consistent with
published studies (19). Our $p53^{R270H}$ mice were generated in the
129S6/SvEv background (19), a strain less susceptible to lymphomas
than C57BL/6 (26). By contrast, the $p53^{+/+}$ mice were of a
mixed C57BL/6-129/Sv background (23), which should increase
their tendency to develop thymic lymphomas (see Materials and
Methods). Thus, the higher incidence of lymphomas in $p53^{R270H}$ mice
is likely to be a specific effect of the mutant allele.

As noted previously in $p53^{R270H}$ mice (19), we observed the
accumulation of mutant p53 protein by immunohistochemistry on
tumor sections obtained from $p53^{R270H}$ and $p53^{+/+}$ animals (Fig. 2C).
The dramatic increase in thymic lymphoma incidence in $p53^{R270H}$
mice indicates that gain-of-function effects of mutant p53 protein
may uniquely or more potently affect thymocytes. Both the
increase in number and decrease in age of onset of thymic
lymphomas in the $p53^{R270H}$ cohort further suggest that R270H is not
simply a loss-of-function allele.

**Loss of one $Hif1a$ allele reduces the incidence of thymic
lymphomas detected in $p53^{R270H}$ mice.** $Hif1a^{+/+}$ $p53^{R270H}$ mice
exhibited an abrupt decrease in survival between 100 and
130 days (Fig. 3A). These animals were sacrificed due to obvious
signs of respiratory distress and displayed large thymic lymphomas
upon dissection. $Hif1a^{+/+}$ $p53^{R270H}$ mice had a significantly different
($P = 0.026$) Kaplan-Meier survival curve with a more gradual
decrease in survival, whereas $Hif1a^{+/+}$ mice showed an intermediate
phenotype. When compared with each other, all three curves
differed significantly ($P = 0.046$), with $Hif1a^{+/+}$ $p53^{R270H}$ and
$Hif1a^{+/+}$ $p53^{R270H}$ animals typically dying later than $Hif1a^{+/+}$ $p53^{R270H}$
mice. The $Hif1a^{+/+}$ $p53^{R270H}$ and $Hif1a^{+/+}$ $p53^{R270H}$ survival curves were
not significantly different ($P = 0.174$).

The age at sacrifice for all tumors combined (Fig. 3B) and tumor
burden (Fig. 3C) were not significantly different between geno-
types; however, careful gross and histologic analysis of each mouse
tissue revealed differences in tumor spectra (Supplementary
Fig. S1; Fig. 4). $Hif1a^{+/+}$ $p53^{R270H}$ mice exhibited ~30% more CD3+
thymic lymphomas (Supplementary Figs. S2, S3–S5; Figs. 3D
and 4) than $Hif1a^{+/+}$ $p53^{R270H}$ or $Hif1a^{+/+}$ $p53^{R270H}$ animals. Instead,$Hif1a^{+/+}$
$p53^{R270H}$ and $Hif1a^{+/+}$ $p53^{R270H}$ mice were more likely to develop
generalized lymphomas (both B220+ B-cell and CD3+ T-cell)
infiltrating lymph nodes and other tissues, carcinomas, or
teratomas (Supplementary Fig. S2B; Fig. 3D). These data are
consistent with the difference in Kaplan-Meier curve slopes and
suggest that $Hif1a$ haploinsufficiency, rather than expansion of
HIF2α expression, reduces the incidence of thymic lymphoma in
$p53^{R270H}$ mice, allowing other types of tumors to be detected.
Pathologic findings are tabulated in Supplementary Figs. S3 to S5,
and images of tumor types observed during pathologic evaluation
of $p53^{R270H}$ mice are provided in Fig. 4 and Supplementary Fig. S1.
Although these data were not the expected outcome of our
experimental design, they suggested an important role for HIF1α in
T-cell malignancy.

**Age at presentation with thymic lymphomas is significantly
increased in $Hif1a^{+/+}$ $p53^{R270H}$ and $Hif1a^{+/+}$ $p53^{R270H}$ mice.**
In addition to decreased incidence of thymic lymphomas in
$Hif1a^{+/+}$ $p53^{R270H}$ and $Hif1a^{+/+}$ $p53^{R270H}$ mice, the age at which these
animals presented with tumors was also significantly increased
(Fig. 5A). This difference in latency could be caused by changes in
tumor initiation or tumor growth rates. To determine if changes in
proliferation rates contributed to altered onset of thymic
lymphoma symptoms, we performed immunohistochemistry for
the mitotic marker phospho-histone H3 (Ser10) on tumor sections
(Fig. 5B). However, no significant change in tumor mitotic index
was observed. In contrast, TUNEL staining showed increased levels
of apoptosis in both $Hif1a^{+/+}$ $p53^{R270H}$ and $Hif1a^{+/+}$ $p53^{R270H}$ tumors
relative to controls (Fig. 5C). Because all tumors were harvested
after the mice became symptomatic, it is difficult to determine
whether the proliferation and apoptosis rates observed in
histologic sections directly affect tumor progression. Nevertheless, increased apoptosis in Hif1α−/− p53−/− and Hif1α−/− p53−/− tumors suggests that HIF1α may be important for thymocyte survival, specifically within the stressful environment of a rapidly growing tumor.

To elucidate the molecular basis for the decreased incidence and increased latency of thymic lymphomas in Hif1α−/− p53−/− and Hif1α−/− p53−/− mice, we next evaluated changes in gene expression between these tumors. QRT-PCR for Hif2α in thymic lymphoma tissue collected at the time of sacrifice indicated that Hif2α expression is increased in Hif1α−/− p53−/− mice (Fig. 5D). Hif1α mRNA levels were decreased in tumors from Hif1α−/− p53−/− mice but were surprisingly even lower in the Hif1α−/− p53−/− cohort. This suggests cross-regulation between the HIFα subunits and may have contributed to the intermediate phenotype of Hif1α−/− p53−/− mice relative to the Hif1α−/− p53−/− and Hif1α−/− p53−/− cohorts. HIF2α overexpression has been shown to suppress HIF1α levels in renal clear cell carcinoma (14). To determine if increased HIF2α levels alter the expression of HIFα target genes, we also performed QRT-PCR for Vegf and Tgfα, two genes preferentially regulated by HIF2α in renal tumors. Increases in Vegf and Tgfα mRNA levels in Hif1α−/− p53−/− mice suggest that HIF2α activity was elevated in these tumors.

To evaluate global changes in gene expression, we performed microarray analysis of five thymic lymphomas from each cohort. One dramatic difference between Hif1α−/− p53−/− and Hif1α−/− p53−/− and Hif1α−/− p53−/− tumors was IL-2 receptor-α (CD25), which exhibited a >30-fold change in expression (Fig. 6A). Because CD25 is a developmental marker whose expression is closely correlated with Notch activity during T-cell maturation, we hypothesized that differences in CD25 levels could correspond to differential Notch pathway activation among Hif1α−/− p53−/−, Hif1α−/− p53−/−, and Hif1α−/− p53−/− tumors. Therefore, we assessed Notch activity in these tumors both by QRT-PCR to measure mRNA levels of Deltex-1 (Dtx1) and Notch-regulated ankyrin repeat protein (Nrarp), two Notch transcriptional targets, and Western blot of cleaved, activated Notch1 protein (Fig. 6A–B). Both Dtx1 and Nrarp transcript levels were decreased in Hif1α−/− p53−/− and Hif1α−/− p53−/− thymic lymphomas relative to Hif1α−/− p53−/− tumors, suggesting decreased Notch activity in these tumors (Fig. 6A).

**Figure 3.** Characterization of survival, tumor spectrum, and tumor burden in p53−/− mice. A, Kaplan-Meier curve of Hif1α−/− p53−/− (n = 23; green), Hif1α−/− p53−/− (n = 20; black), and Hif1α−/− p53−/− (n = 24; red) mice. Significance was calculated using a Log-rank test over all three genotypes. When comparing the Hif1α−/− p53−/− and Hif1α−/− p53−/− groups, P = 0.024. B, average age at sacrifice of Hif1α−/− p53−/−, Hif1α−/− p53−/−, and Hif1α−/− p53−/− mice. C, tumor burden for Hif1α−/− p53−/−, Hif1α−/− p53−/−, and Hif1α−/− p53−/− mice. D, tumor spectrum in Hif1α−/− p53−/−, Hif1α−/− p53−/−, and Hif1α−/− p53−/− mice. *, P = 0.055; #, P = 0.073 (Student’s two-tailed t test for pair-wise comparisons).
During normal T-cell differentiation, Notch1 expression is high at the β-selection checkpoint, when cells receiving appropriate signals through the T-cell receptor begin to proliferate rapidly (Fig. 6C). These early stages of development also correspond to CD25 expression. The Notch pathway is then down-regulated, cell division ceases, and T cells continue to mature (27, 28). Given that Notch1 is important for T-cell commitment and proliferation during β-selection, aberrant Notch1 expression after this stage could promote tumorigenesis.

Notch mutations are common in mouse T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) and occur in the PEST domain of Notch1, a region important for degradation of activated Notch (27). To determine if Notch1 was mutated in our study, we sequenced the Notch1 PEST domain in the thymic lymphomas but only found mutations in 1 tumor (n = 16; data not shown). However, Western blots of tumor lysates showed increased stabilization of cleaved Notch1 in Hif1a+/+p53H/H tumors relative to most Hif1a+/-p53H/H and Hif1a+/-+/C0 samples (Fig. 6B). Furthermore, Notch stabilization directly correlated with Dtx1 and Nrarp expression levels. Because Notch promotes T-cell lymphoma growth, decreased Notch activity in Hif1a+/-+/C0 and Hif1aKI/+p53H/H tumors could contribute to the delayed presentation observed in these cohorts. These results suggest that HIF1α promotes the stability of activated Notch, thereby contributing to thymic lymphoma development (Fig. 6D).

An alternate mechanism for Notch stabilization is deletion of the E3 ubiquitin ligase Fbw7 or up-regulation of Notch ligands Jagged 1 (Jag1), Jag2, A-like-1 (Dll1), Dll3, or Dll4. However, we did not observe decreases in the expression of Fbw7 or any Notch ligands in our study (data not shown). Instead, it is probable that HIF1α is acting directly to promote Notch stability. Indeed, Gustafsson and colleagues (29) recently showed in neural and myogenic precursor cells that HIF1α can bind and stabilize activated Notch, promoting its transcriptional activity.

**Discussion**

Xenograft experiments (9, 11, 12, 15) indicate that the HIFα subunits can have differential effects on tumor growth. To evaluate the relative contribution of each HIFα subunit in a more physiologic model of spontaneous tumorigenesis, we analyzed the effect of varying HIFα levels on tumor latency and spectrum in mice homozygous for the R270H mutation in p53. Although p53^H+/H+ and p53^H/- mice were previously characterized by Olive and colleagues (19), p53^H+/H+ homozygotes have not been described. We found that p53^H+/H+ mice developed substantially more thymic lymphomas than p53^H+/H+ animals. Consistent with our observations in mice carrying the R270H mutation, Terzian and colleagues (30) describe a very high incidence of lymphomas and sarcomas in p53^H+/H+/H+ mice when compared with heterozygotes for the R172H mutation. p53^H+/H+ mice also present with thymic lymphomas at a younger age. These observations suggest that, at least in thymocytes, the R270H mutant may actively promote tumorigenesis. One potential mechanism for this effect is the observation that p53 mutants disrupt DNA damage-response pathways (31). Mutations in other DNA damage-response genes, such as H2ax and 53bp1, have also been shown to accelerate thymic lymphoma formation in a p53-null background, illustrating the importance of this pathway as a tumor suppressive mechanism in T cells (32, 33). Moreover, when activated, mutant p53 has the potential to accumulate to higher levels in p53^H+/H+ thymocytes compared with p53^H+/H+ thymocytes. Excess stabilized mutant p53 may then promote lymphomagenesis. Of note, mutant p53, stabilized by deletion of MDM2, accelerates tumorigenesis and promotes metastasis in a dose-dependent manner (30). The decreased
numbers of brain tumors and sarcomas observed in p53<sup>H/H</sup> mice was likely to be a consequence of this shift toward increased numbers and earlier incidence of thymic lymphomas. Because mice die quickly from respiratory distress once thymic lymphomas have reached a threshold size, it may preclude their living long enough to succumb to other tumor types. The age at which mice had to be sacrificed was greater than that for thymic lymphomas (189 versus 117 days; Supplementary Fig. S3), suggesting that these tumors have a longer latency period. Because fewer p53<sup>H/−</sup> mice developed thymic lymphomas, other tumor types could be observed.

Using the p53<sup>H/H</sup> mouse model, we then asked if expanded HIF2α expression could alter tumor spectrum or growth given that many human tumors express high levels of HIF2α. To our surprise, the Hif2α knock-in allele had no effect on any of these parameters. This result suggests either that HIF2α is not important for spontaneous tumorigenesis in the p53<sup>H/H</sup> mouse model, or that HIF2α expression levels achieved in susceptible tissues were insufficient to promote tumor formation. There are also several important differences between the spontaneous tumors observed in p53<sup>H/H</sup> mice and xenograft models used in previous studies (6, 19). Increased HIF2α-mediated Tgfα expression contributes to the growth of both renal clear cell tumors and ES cell–derived teratomas by activating epidermal growth factor receptor signaling; however, a role for this pathway in thymic lymphomas has not been described. Thus, the progrowth pathways activated by HIF2α may not play an important role in lymphomagenesis, explaining why the Hif1α<sup>−/−</sup> allele did not promote thymic lymphoma formation in p53<sup>H/H</sup> mice. It should also be noted that homozygous Hif1α<sup>−/−</sup> ES cells were used in previous subcutaneous tumor models, where the dosage of HIF2α achieved was significantly higher (6). Moreover, the incidence of teratomas in p53<sup>H/H</sup> mice was low (only two were observed), so the effect of increased HIF2α expression on spontaneous teratoma formation cannot be adequately determined. The short latency period and limited spectrum of tumors observed in p53<sup>H/H</sup> mice may also have precluded the detection of subtle HIF2α-mediated effects. A similar study was therefore conducted in p53<sup>H/−</sup> mice, which develop a wider spectrum of tumors, but the number of mice examined (n = 20) was not large enough to find significant changes in spectrum and latency.

To further study the role of HIF2α in spontaneous tumorigenesis, a conditional knockout model using global, postnatal Hif2α deletion may be more valuable (8). Similarly, conditional Hif1α deletion will be important to characterize its role at various stages of tumorigenesis, from initiation to growth and metastasis, as well as tissue-specific effects. These studies should reveal the aggregate effect of each of the α subunits on tumor promotion or suppression, clarifying potentially conflicting in vitro findings. For instance, although HIF1α acutely inhibits proliferation in response to hypoxia by inhibiting c-Myc activity, it also promotes tumor growth by increasing angiogenesis and reprogramming cell metabolism (1). Inhibition of proliferation is likely to be a transient phenomenon that allows cells to adapt to hypoxic stress, and may not translate to reduced tumor growth in vivo. The effect of HIF1α on physiologic levels of c-Myc is also different than its effect on overexpressed, oncogenic c-Myc (34). Indeed, in a c-Myc–dependent B-cell lymphoma model, HIF1α promotes tumor growth (35). Another important consideration is the difference between constitutive stabilization of the HIFα subunits, as occurs in VHL-deficient renal carcinomas, and more transient oxygen-regulated accumulation.

In p53<sup>H/H</sup> mice, loss of one Hif1α allele significantly reduced the incidence of thymic lymphomas, thus uncovering an
important role for HIF1α in thymic lymphomagenesis in vivo. In addition, the age at which these mice presented with thymic lymphomas was significantly increased by Hif1α haploinsufficiency, suggesting that tumor onset or tumor growth was delayed by decreasing levels of HIF1α. Although thymic lymphomas observed in Hif1α<sup>+/−</sup>p53<sup>+/−</sup> and Hif1α<sup>−/−</sup>p53<sup>−/−</sup> mice exhibited similar proliferation rates, increased cell death was noted, indicating that HIF1α is important for the survival of thymocytes during tumor growth. To better understand mechanism(s) whereby HIF1α promotes thymic lymphomagenesis, we conducted an unbiased search for gene expression changes correlated with decreased HIF1α. Microarray analysis on thymic lymphoma mRNA from each genotype uncovered a dramatic decrease in CD25 expression, a marker for Notch pathway activity, in Hif1α<sup>+/−</sup>p53<sup>+/−</sup> and Hif1α<sup>−/−</sup>p53<sup>−/−</sup> tumors. Furthermore, the expression of Notch target genes Nrarp and Dtx1 was also significantly reduced in these tumors. These transcriptional changes suggested that Hif1α<sup>+/−</sup>p53<sup>+/−</sup> and Hif1α<sup>−/−</sup>p53<sup>−/−</sup> tumors had reduced Notch activity relative to wild-type controls. Although mutations in the PEST domain of Notch1, a common feature of mouse T-cell lymphomas, were not observed in our study, loss of one Hif1α allele correlated with decreased stabilization of cleaved Notch1. This indicates that HIF1α may be directly involved in stabilizing cleaved Notch, or that heterozygosity for Hif1α selects against Notch activation. In either case, it seems that differential Notch activation may explain the changes in tumor onset and frequency observed in Hif1α<sup>+/−</sup>p53<sup>+/−</sup> and Hif1α<sup>−/−</sup>p53<sup>−/−</sup> mice, as Notch is a critical factor for T-cell lymphoma growth and survival.

Notch pathway activation contributes significantly to the pathogenesis of acute T-ALL in both mice and humans (27). Mouse models have shown that constitutively activated Notch induces murine T-ALL (36–38), and inhibition of Notch in mouse T-ALL cell lines causes cell death (25, 39), suggesting that Notch is critical both for induction of leukemogenesis as well as for leukemic cell survival. In addition, overexpression of activated Notch1 is evident in human T-ALL, and is frequently due to mutations in Notch1 that lead to constitutive stabilization (25, 27, 40). As stated above, activating Notch1 mutations have been observed in multiple mouse models of T-ALL and commonly occur in the PEST domain (26). Although we failed to detect Notch1 PEST domain mutations in most of our tumors, we did observe significantly increased expression of activated Notch1 and downstream Notch transcriptional targets in Hif1α<sup>+/−</sup>p53<sup>+/−</sup> tumors compared with tumors deficient in Hif1α, implicating HIF1α in Notch pathway activation.

![Figure 6](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-08-4223)
HIF1α has previously been proposed to increase the stability of activated Notch1 and to promote Notch target activation in myogenic and neural precursor cells (29). However, a role for HIF1α in Notch-driven tumorigenesis had not been shown. Here, we identify an important and novel role for HIF1α in thymic lymphoma development. Mice with normal HIF1α levels exhibit thymic lymphoma–associated morbidities earlier and at a substantially higher incidence than HIF1α heterozygous mice. In addition, the tumors exhibit less cell death and higher levels of activated Notch and Notch targets than thymic lymphomas arising in HIF1α heterozygous mice, implicating HIF1α in Notch pathway regulation during tumorigenesis. This study is also the first to characterize the phenotype of p53/H/H mice and, in doing so, provides further support for a gain-of-function effect of the R270H mutation.

Disclosure of Potential Conflicts of Interest

M.C. Simon: Consultant, Merck Research Laboratories. None of the other authors disclosed any potential conflicts of interest.

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