A Zebrafish Model to Study and Therapeutically Manipulate Hypoxia Signaling in Tumorigenesis

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Abstract

Hypoxic signaling is a central modulator of cellular physiology in cancer. Core members of oxygen-sensing pathway including the von Hippel-Lindau tumor suppressor protein (pVHL) and the hypoxia inducible factor (HIF) transcription factors have been intensively studied, but improved organismal models might speed advances for both pathobiologic understanding and therapeutic modulation. To study HIF signaling during tumorigenesis and development in zebrafish, we developed a unique in vivo reporter for hypoxia, expressing EGFP driven by prolyl hydroxylase 3 (phd3) promoter/regulatory elements. Modulation of HIF pathway in Tg(phd3:EGFP) embryos showed a specific role for hypoxic signaling in the transgene activation. Zebrafish vhl mutants display a systemic hypoxia response, reflected by strong and ubiquitous transgene expression. In contrast to human VHL patients, heterozygous Vhl mice and vhl zebrafish are not predisposed to cancer. However, upon exposure to dimethylbenzanthracene (DMBA), the vhl heterozygous fish showed an increase in the occurrence of hepatic and intestinal tumors, a subset of which exhibited strong transgene expression, suggesting loss of Vhl function in these tumor cells. Compared with control fish, DMBA-treated vhl heterozygous fish also showed an increase in proliferating cell nuclear antigen-positive renal tubules. Taken together, our findings establish Vhl as a genuine tumor suppressor in zebrafish and offer this model as a tool to noninvasively study VHL and HIF signaling during tumorigenesis and development. Cancer Res; 72(16); 4017–27. ©2012 AACR.

Introduction

Oxygen homeostasis, a physiologic process essential and critical for the normal development and functioning of an organism, requires coordinate regulation of multiple pathways. Reduction in the normal level of oxygen tension in tissues (hypoxia) is observed in a range of disease conditions, including locally in peripheral vascular disease, myocardial infarction and stroke, and systemically in pulmonary disease. In addition, hypoxia is a well-described feature of tumor microenvironment. Intratumoral hypoxia is elicited by uncontrolled proliferation of tumor cells coupled with aberrant growth of tumor vasculature. Under hypoxic conditions 2 distinct mechanisms are activated, which promote cell/tissue survival in low oxygen environments and enhance oxygen delivery to the hypoxic sites. These adaptive responses to hypoxia are induced by a key transcription factor, hypoxia inducible factor (HIF), whose activity is regulated by the availability of oxygen (1). HIF is a heterodimeric protein complex consisting of basic helix-loop-helix Per/Amt/Sim (PAS) domain containing α and β subunits (HIFα and HIFβ). HIF plays a crucial role in altering the cellular metabolism of tumors to thrive under hypoxic conditions and in facilitating malignant transformation of tumors (2). Several HIF target genes such as VEGF, PDGF, TGF, CXCR4, TGF-α, and MMP-1 play important roles in the development of cancer (3–5) and significant correlations have been observed between increased HIF-1α levels and patient mortality in many types of cancer (6).

The stability of HIF-1 is regulated in an oxygen-dependent mechanism by the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is encoded by VHL whose biallelic loss lead to the development of a hereditary cancer syndrome involving multiple hemangioblastomas of CNS, clear cell renal carcinoma and pheochromocytoma (7), which occur when heterozygosity is lost either by mutation on the second allele of VHL or via epigenetic silencing (8). pVHL is an essential component of the E3 ubiquitin ligase complex and interacts with the HIFα subunit under normoxic conditions, targeting them for proteasomal degradation. The pVHL–HIFα interaction requires oxygen-dependent hydroxylation of either of 2 conserved prolyl...
generally healthy and fail to show indications of increased cancer. We therefore sought to test their susceptibility when required to turn a

ic and intestinal tumorigenesis, showing for the first time in zebraﬁsh that the Tg(phd3::EGFP) reporter line was used to generate transgenic lines according to published protocols (25). We selected 2 different lines namely, Tg(phd3::EGFP)I144/þ and Tg(phd3::EGFP)I146/þ with the I144 exhibiting a stronger expression. All the experiments in this study were conducted on the I144 line.

We have observed phd3 transgene activation phenotype in the 2 different vhl mutant allele backgrounds viz. vhlhu2117/hu2117 and vhlC0/C24 as well as in the vhlhu2117/hu2117 transheterozygous embryos.

Hypoxia chamber assay

The embryos were raised in E3 medium in a 5% oxygen supplied sealed chamber (INVIVO2 200; Ruskinn Technology Ltd.) for stipulated periods of time. The chamber was deoxygenized by positive infusion of 5% CO2/95% N2 gas mixture with >90% relative humidity at 28°C. The continuous O2 saturation and the pressure inside hypoxia chamber were maintained during the course of the experiment. The transgenic embryos raised under similar conditions in a well-aerated bag inside the chamber served as controls.

vhl morpholino and mRNA injections

A splice blocking antisense morpholino (Genetools) sequence, namely 5’-GCATAATTTCCAGAACCCACAAAG-3’, designed to the exon1 splice-donor site of the vhl gene was utilized to perturb its function. The morpholino stock solution (10 mg/mL) was diluted appropriately in distilled H2O and the 1-cell stage transgenic embryos were injected with ~3 ng per embryo. Full-length zebraﬁsh vhl gene was subcloned into the pCS2+ expression vector. mRNA from the sequence veriﬁed constructs was transcribed in vitro after NoT linearization, using SP6 mMESSAGE Kit (Applied Biosystems). Dominant active and dominant negative forms of hif-1aa and hif-1ab were synthesized and injected as previously described (26). For the rescue experiments, 6.5 pg vhl mRNA was injected.
Dimethyloxaloylglycine treatment  

The 3-dpf wild-type transgenic embryos were treated with 100-μmol/L dimethyloxaloylglycine (DMOG; Frontier Scientific) or 0.1% dimethylsulfoxide (DMSO; Sigma-Aldrich) for 1 to 2 days as previously described (21).

RNA in situ hybridization  

Whole-mount in situ hybridization was conducted as per standard protocols (27). The phd3 (BC066699) antisense digoxigenin-UTP labeled mRNA probe was synthesized from an expressed sequence tag (EST) clone (RZPD/Imagenes; ref. 21) and the in situ data were collected on a Zeiss Axioplan with a 5× or 20× objective using a Spot4 digital camera.

7,12-Dimethylbenz[a]anthracene treatment of vhl−/− fish  

Wild-type and vhl−/− fry at 3 weeks of age were immersed overnight in aquarium water containing 5 ppm of DMBA (Sigma-Aldrich-Chemie BV) dissolved in DMSO (23). The next day, all fry were rinsed several times in regular aquarium water and subsequently were returned to the aquarium. Fish were carefully grown and regularly monitored for signs of sickness or evidence of tumor formation. Eventually all fish were culled for analysis at 14 months after the DMBA treatment.

Histology and immunostaining  

Adult fish were euthanized, culled, and fixed in 4% paraformaldehyde for 3 to 4 days at 4°C. The samples were decalcified in 0.25M EDTA pH 8 for 2 to 4 days at room temperature, embedded in paraffin, and 5-μm sections were made. For immunostaining, the primary antibodies mouse monoclonal antibody, we observed strong deposition of Hif-1α in both heterozygous and homozygous mutant and vhl−/− sibling larvae, we observed upregulation of several Hif target genes, including epo, vegf, phd1-3 in the mutants, with phd3 consistently exhibiting the strongest increase overall (e.g., 23-fold at 7 dpf) (21, FvE unpubl.). When we did Western blot analysis of vhl−/− embryos using Hif-1α antibody, we observed strong deposition of Hif-1α in the mutants (Fig. 1A). Subsequently, when we conducted qPCR on 3.5 dpf vhl−/− mutant embryos, we observed 216-fold increase in phd3 expression in mutant embryos compared with wild types (Fig. 1B). In comparison, the classical HIF target vegf-a was increased 7-fold (Fig. 1B) in the mutants. To identify the earliest developmental time point at which phd3 expression is stimulated in the vhl−/− mutant embryos, we conducted whole mount in situ hybridization on selected stages of mutant and sibling embryos. In wild-type embryos, phd3 expression could only be weakly detected in the brain around 30 hpf (data not shown). By 48 hpf, the expression became restricted to a population of cells in the tectum and midbrain–hindbrain boundary (MHB) region and the wild-type fish continued to preserve this confined phd3 expression pattern during the subsequent embryonic and larval development (Fig. 1C and Supplementary Fig. S1A). In contrast, vhl mutant embryos showed the strongest phd3 expression as early as 21.5 hpf (25-somite stage) in the brain and pronephros (Fig. 1D), suggesting an induction in Hif activity. By 36 hpf, the mutant embryos showed strong phd3 expression in the heart and other visceral organs indicating a systemic response. These embryos continued to exhibit robust expression of the phd3 gene all through their embryonic (Fig. 1F and Supplementary Fig. S1B) and larval development, until their death at 10 to 12 dpf. Presence of wild-type maternal vhl mRNA and Vhl protein during the early embryonic stages in mutant embryos (21), might explain the delay in the activation of phd3 expression until ~21 to 22 hpf. These observations suggested that, although the physiologic and behavioral responses of vhl mutant only become evident by 3 dpf, the molecular onset of the hypoxic response can be detected from approx. 22 hpf.

Strong induction of a phd3:EGFP transgene in vhl−/− mutants  

In the human PHD3 gene, a hypoxia response element (HRE) was identified in the first intron, but its conservation in fish is
unclear and further HREs could be present (31). Therefore, we utilized a recombinatorial strategy to insert an EGFP with an SV40 3' UTR at the phd3 ATG start site in the BAC clone CHORI73-277E22. This BAC contains the complete phd3 gene in a large genomic context (>25 Kb 5' and >90 Kb 3'). The phd3 start codon was destroyed by the recombinatorial process preventing the transgene construct from interfering itself with the hypoxic signaling pathway. This modified BAC was injected to generate 2 stable transgenic lines and, the stronger line was used in all subsequent experiments.

In wild-type embryos, similar to phd3 in situ staining, phd3::EGFP transgene-mediated EGFP fluorescence commenced weakly in the brain and spinal cord from 30 hpf (data not shown). The transgene expression became restricted to MHB region by around 60 hpf and the EGFP fluorescence was found to adhere to this region during subsequent stages of development (Fig. 1G). In contrast, the vhl mutant embryos commenced transgene expression from 25–somite stage onward in the brain, spinal cord, and pronephros (data not shown). Subsequently, the mutants began exhibiting strong induction of the transgene in several other organs like the heart, pectoral fins, pancreas, and the hatching gland (Fig. 1H). In situ hybridizations for gfp mRNA on phd3::EGFP transgenic embryos correlated well with the endogenous phd3 expression at various stages of development (Supplementary Fig. S2). We also quantified and compared the level of EGFP fluorescence between the vhl mutants and wild-type siblings at 4 dpf, and found a 3-fold increase in the amount of fluorescence in the mutants (Supplementary Table S1).

vhl mRNA rescues phd3::EGFP activation in vhl knockout and knockdown embryos

To prove that phd3::EGFP activation observed in the vhl mutant embryos is specifically affected by the loss of Vhl protein, we tried to rescue this phenotype by injecting 6 pg of capped vhl mRNA into single-cell stage embryos. By around 28 hpf, both the uninjected and injected wild-type embryos showed no activation of the phd3::EGFP transgene. But while the uninjected vhl mutants exhibited strong expression of the transgene at 28 hpf, the mRNA-injected mutant embryos exhibited no EGFP fluorescence at the identical time point (Fig. 2A).

We also stimulated phd3::EGFP expression in the wild-type embryos by knocking down vhl function using antisense morpholino oligonucleotides. Injection of vhl splice morpholino into 1-cell stage Tg(phd3::EGFP)i144/C0 embryos led to strong induction of the transgene from 60 hpf onward and morphant embryos continued to display strong EGFP fluorescence until 120 hpf. But when we injected vhl morpholino and vhl mRNA together, persistence of wild-type expression pattern was observed during embryonic and larval development (Fig. 2B), suggesting a complete rescue of the morphant phenotype.

phd3::EGFP activation is Hif dependent

Our observations suggest that loss of function mutations in vhl can activate phd3::EGFP reporter gene expression possibly through the stabilization of Hifα subunit but Vhl protein is reported to have numerous noncanonical functions as well (32). To verify the role of Hif transcription factor in eliciting transgene expression in vhl−/− embryos, we generated dominant active (DA) forms of Hif-1αa and Hif-1αb by mutating the conserved proline and asparagine hydroxylation sites of Phd and Fih hydroxylases, respectively (26). When the DA hif-1αa and hif-1αb mRNAs were coinjected into 1-cell stage Tg(phd3::EGFP)i144/C0 embryos, we observed activation of the transgene.
in the injected embryos from 24 hpf, as evidenced by strong
EGFP fluorescence, mainly in the brain and CNS. The enhanced
phd3:EGFP expression persisted in the larvae expressing DA
Hif-1α isoforms until at least 48 hpf (Fig. 2C). When the same
amount of wild-type hif-1αa and hif-1αb mRNAs were injected
this level of phd3:EGFP expression was not observed, confirming
that the DA versions of hif-1a have successfully stabilized
the protein. These observations clearly show the direct role of
Hif-1α transcription factor in mediating the phd3:EGFP trans-
genome activation (Supplementary Fig. S3A).

Chemical activation of Hif signaling in wild-type
embryos induces phd3::EGFP expression

To further extend this observation, we activated Hif in the
wild-type transgenic embryos by inhibiting Phd/factor inhibiting
Hif (Fih) functions using the prolyl hydroxylase inhibitor
DMOG, and thus preventing the degradation of the Hifα
subunit even in the presence of functional Vhl. Transgenic
wild-type embryos at 2.5 dpf were treated with 100 μmol/L
DMOG for 2 days displayed an increase in phd3:EGFP expression
in the pronephros, gall bladder, liver, and brain (Supple-
 mentality Fig. S4A) compared with very mild expression of
 transgene observed in the 0.1% DMSO-treated control embryo-
os. Hif activity and hence the transgene induction were observed to be restricted to selected tissues/cells of DMOG-
treated embryos, because limitations in the mode and timing of
the treatment (21). When mRNAs encoding DN forms of
hif-1αa and hif-1αb mRNAs at 1-cell stage led to the enhancement of phd3::EGFP mediated EGFP fluorescence in the transgenic embryos by around 48 hpf. The injection of wild-type isoforms of hif-1αa and hif-1αb mRNAs into Tg(phd3::EGFP)144/144 embryos however did not induce transgene expression.

Tg(phd3::EGFP) zebrafish as a live reporter of hypoxia

Our results predict that the Tg(phd3::EGFP)144/144 embryos
should act as a live reporter of hypoxia, and to validate this we
exposed the transgenic embryos to reduced oxygen environ-
ment in a hypoxic chamber for stipulated periods of time.
When we subjected 12 hpf transgenic embryos to 5% O2
sh as a live reporter of hypoxia
hif-1αa and hif-1αb (26) were injected into Tg(phd3::EGFP)144/144 embryos and subsequently treated with DMOG at 24 hpf, we could block the DMOG-mediated induction in EGFP fluorescence at 48 hpf (Supplementary Figs. S3B and S4B).

Figure 2. vhl mRNA rescues vhl mutant and morphant phenotypes and Hif mediates phd3::EGFP transgene activation. A, embryos obtained from Tg(phd3::
EGFP)144/144; vhl−/− parents were injected with full-length vhl mRNA at the 1-cell stage and the injected embryos were raised individually in 24-well plates. At
28 hpf, although the uninjected vhl−/− mutant embryos showed strong phd3::EGFP mediated EGFP fluorescence, none of the vhl mRNA-injected embryos
showed activation of transgene expression. Genotypes of the experimental embryos were known by culling the embryos at 96 hpf and performing molecular
genotyping assay. B, Tg (phd3::EGFP)144/144 embryos were injected with vhl splice morpholino (MO) at the 1-cell stage. At 60 hpf, although the uninjected
embryos showed EGFP fluorescence only in the MHB region, the morpholino-injected embryos showed strong induction of transgene-mediated EGFP
fluorescence. When the vhl morpholino and vhl mRNA were coinjected into 1-cell stage Tg (phd3::EGFP)144/144 embryos, the injected embryos exhibited no
transgene activation, showing rescue of the morpholino-induced phenotype. C, Tg(phd3::EGFP)144/144 embryos coinjected with DA isoforms of hif-1αa and
hif-1αb mRNAs at 1-cell stage led to the enhancement of phd3::EGFP mediated EGFP fluorescence in the transgenic embryos by around 48 hpf. The injection of wild-type isoforms of hif-1αa and hif-1αb mRNAs into Tg(phd3::EGFP)144/144 embryos however did not induce transgene expression.


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**phd3:EGFP transgene functions as an in vivo fluorescent marker for intratumoral hypoxia**

Because the presence of hypoxic regions is a hallmark of locally advanced solid tumors, we asked whether the phd3:EGFP transgene could be used as a novel reporter for detecting intratumoral hypoxic regions in fish tumor models. To test this hypothesis, we induced melanoma in the Tg(phd3:EGFP)\textsuperscript{144/144} fish by injecting a human oncogenic HRAS\textsuperscript{G12V} (V12RAS::mCherry) construct into single-cell embryos and misexpressing the oncogene specifically in cells of the melanocyte lineage (33). Few days after injections, the V12RAS injected fish developed several clones of ectopic melanocytes and after few weeks they progressed into melanocytic naevi. Subsequently, after 3 months we observed few of these lesions to form small tumor nodules. As these melanoma nodules became expanded into melanomas, we periodically monitored them for phd3:EGFP expression. After 1 year, few fish with large tumors (Fig. 4A) exhibited distinct regions of phd3:EGFP-mediated fluorescence (Fig. 4C). These EGFP\textsuperscript{+} regions are likely to correspond to intratumoral hypoxic areas in these melanomas, essentially revealing a subpopulation of tumor cells that possess activated hypoxia-mediated Hif signaling. Histologic analysis of these samples using proliferating cell nuclear antigen (PCNA), Hif-1α and GFP antibody staining revealed clear demarcation of the hypoxic regions within these tumors (Fig. 4D–G). When taking into account the differences in expected half-lives of EGFP (Supplementary Fig S5) and HIF-1α (34) proteins, we observed good correlation between GFP and Hif-1α staining within the melanoma (Fig. 4F and G), thus validating that the EGFP\textsuperscript{+} regions correspond to intratumoral hypoxic regions with activated Hif signaling. These experiments show the potential of the phd3:EGFP transgene as a novel reporter for tracking hypoxia under in vivo tumor pathologic conditions in zebrafish.

**vhl\textsuperscript{1−/−} zebrafish are highly susceptible to DMBA tumorigenesis**

The vhl\textsuperscript{1−/−} fish are not obviously predisposed to neoplasia formation. One reason for the lack of tumors might be that the lifespan of the fish is too short to accumulate the necessary changes. Hence we asked whether DMBA treatment of Tg (phd3:EGFP)\textsuperscript{144/144};vhl\textsuperscript{1−/−} fish would make them susceptible to tumorigenesis. We treated 4 batches of 21 dpf fish fry with DMBA (23), with each batch consisting of an equal mix of Tg (phd3:EGFP)\textsuperscript{144/144};vhl\textsuperscript{1−/−} and Tg(phd3:EGFP)\textsuperscript{144/144};vhl\textsuperscript{+/+} genotypes. Two months after the treatment, we observed that a subset of fish in every batch possessed EGFP\textsuperscript{+} cell clones, primarily in retina (Supplementary Fig. S6A), skin (Supplementary Fig. S6B), gills and fins because of easy identification of fluorescent cells in these tissues. When we segregated such fish having EGFP\textsuperscript{+} cells and conducted genotyping analysis: 89% (102/114) was found to be vhl\textsuperscript{1−/−} carriers (Supplementary Table S2). The activation of phd3:EGFP-mediated GFP fluorescence in random cell populations in the treated fish suggested DMBA-induced loss of the wild-type vhl allele in these cells. Expectedly, the frequency of occurrence of EGFP\textsuperscript{+} cells is significantly higher in the vhl\textsuperscript{1−/−} heterozygotes where only a single wild-type copy needs to be lost.
All the DMBA challenged fish were grown to adulthood and were constantly monitored for tumor formation. Seven to eight months after the DMBA treatment, we observed a small group of fish in all 4 treated batches to exhibit strong EGFP fluorescence mainly in the trunk region as well as in the region adjacent to cloaca (Fig. 4H and I). At the end of 9 months, genotyping and statistical analysis of fish exhibiting strong EGFP fluorescence in all the 4 batches revealed a significantly
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Table 1. Number of 14-month-old DMBA-treated wt and vhl<sup>+/−</sup> fish with different types of neoplasias

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total fish (n)</th>
<th>Gut</th>
<th>Liver</th>
<th>Bileduct</th>
<th>Ovary</th>
<th>Testis</th>
<th>Percentage of fish with neoplasia (%)</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>vhl&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>DMBA-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vhl&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>77</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>vhl&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>94</td>
<td>13 (7)</td>
<td>20 (13)</td>
<td>11 (3)</td>
<td>6</td>
<td>5</td>
<td>59</td>
</tr>
</tbody>
</table>

NOTE: All the fish were culled and processed for histologic analysis, and subsequently the sections were screened for tumor formation. The numbers within the brackets indicate GFP<sup>+</sup> neoplasias.

(P < 0.002, Chi-square test) greater number (23/27; Supplementary Table S3) of them to be vhl<sup>+/−</sup> heterozygotes.

After growing all the experimental fish for 14 months, they were culled and processed for histologic analysis. Serial sagittal sections were made for every sample and subsequently immunostained for the proliferation marker, PCNA. Screening of the PCNA-stained slide preparations showed a significant increase (P < 0.02, Chi-square test) in the occurrence of neoplastic growth in the DMBA-treated vhl<sup>+/−</sup> samples (39%) than the similarly treated wild-type samples (23%; Table 1). In many cases, the tumor formation was found in the liver, bile duct, and gut tissues. Importantly, the analysis also showed that the fluorescing tissues in all strongly EGFP<sup>+</sup> fish to correlate with neoplasms.

The wild-type adult zebrafish intestine showed a regular arrangement of villi and the proliferating epithelial cells were observed to be strictly restricted to inter villus pockets (Fig. 4J; refs. 35 and 36). However, in the DMBA-treated vhl<sup>+/−</sup> fish, we observed a disorganized pattern of intestinal villi architecture (n = 8; Fig. 4K) and these villi were completely encompassed by PCNA<sup>+</sup> proliferating epithelial cells (Fig. 4K) analogous to adenoma formation in the human gut. A few of the gut tumors showed abnormally proliferating intestinal epithelial cells in one half of the gut whereas the other half showed restricted presence of PCNA<sup>+</sup> cells (Fig. 4L and M). A few samples exhibited lesions wherein PCNA<sup>+</sup> epithelial cells were scattered all through the intestinal villi (Fig. 4M).

The liver tissue in zebrafish consists of hepatocytes, bile ducts, and portal vessels (35). In the wild-type liver tissue usually very few proliferating cells are present (Fig. 4N), but in the DMBA-treated vhl<sup>+/−</sup> fish we observed the presence of highly proliferating hepatocytes (n = 18) in the liver tissue (Fig. 4O). These tumors were identified histologically as primary hepatocellular carcinoma, wherein either the whole liver or selected regions of liver tissue showed strong staining for PCNA (Fig. 4P and Q). These liver neoplasias showed altered hepatic architecture as well as abnormal cell morphology along with apoptotic characteristics such as presence of fragmented nuclei (Fig. 4R). Similarly, compared with the wild-type bile duct tissue, which were not actively proliferating, the DMBA-treated fish possessed enlarged, abnormally shaped bile ducts as well as cysts. These bile ducts exhibited the presence of PCNA<sup>+</sup> epithelial cells as well as showing abnormal growth of these ducts leading to the development of cholangiocarcinoma (Fig. 4S).

In addition, we also observed occurrence of testicular (Supplementary Fig. S7A) and ovarian (Supplementary Fig. S7B) tumors in the DMBA treated wild-type and vhl<sup>+/−</sup> batches at equal frequencies.

**DMBA induces enhanced proliferation of epithelial cells in vhl<sup>+/−</sup> renal tubules**

Because ccRCC originates from renal epithelial cells and the human VHL gene is observed to exhibit strong tumor suppressor activity in renal tubules, we asked whether the DMBA treatment of vhl<sup>+/−</sup> fish could trigger proliferation of renal tubular epithelial cells. Hence, we screened the kidney regions in the PCNA-stained slide preparations of DMBA treated vhl<sup>+/−</sup> and wild-type groups. In the untreated samples (both wild-type and vhl<sup>+/−</sup>), the hematopoietic cells in the kidney strongly expressed the proliferation marker, PCNA, and the epithelial cells of the tubules are found to be mostly quiescent. However, the DMBA treatment is observed to induce the transformation of the epithelial cells of renal tubules to a proliferative state as evidenced by robust PCNA<sup>+</sup> staining (Fig. 5A–F). A normal renal tubule typically consists of a single organized layer of epithelial cells but, in contrast, most of the proliferating renal tubules exhibited abnormal organization of epithelial cells (Fig. 5A–F) as well as altered cellular morphology (Fig. 5C and E). Statistical analysis showed a highly significant increase in the number of proliferative renal tubules in DMBA treated vhl<sup>+/−</sup> fish compared with the controls (26/30; P < 0.0002, Chi-square test; Supplementary Table S4). These observations suggest that DMBA can induce strong proliferation of renal epithelial cells in the vhl<sup>+/−</sup> fish.

**Vhl exhibits tumor suppressor function in zebrafish**

The observation that after DMBA treatment, ~85% of the fish with EGFP<sup>+</sup> tumors belonged to the vhl<sup>+/−</sup> group suggested that such tumors might have originated from vhl null cells and hence most of the tumor cells might be possessing vhl<sup>+/−</sup> genotype. To address this question, we isolated tumor cells, by laser capture microdissection (LCM), from paraffin tissue sections of 5 vhl<sup>+/−</sup> EGFP<sup>+</sup> tumor samples, extracted...
dissected tumor tissue from 5 different strongly EGFP showed aberrant organization (C) and altered morphology (E). Proliferative In addition, epithelial cells in the proliferative renal tubules DMBA treated Normally, the renal tubules are composed of a single layer of quiescent wild-type But, we were unable to identify unambiguously the loss of genomic DNA and conducted standard sequencing analysis. fi
vhl levels of fi
vhl locus or elsewhere in the coding region (Supplementary Figure 5). To extend this analysis further, we wanted to quantify the levels of vhl mRNA in the vhl/Ergetic tumor cells. We dissected tumor tissue from 5 different strongly fluorescing EGFP fish (Supplementary Fig. S9A and S9B) selected from a DMBA treated vhl group, isolated total RNA and conducted quantitative real-time PCR analysis. In 4 of 5 tumor samples, we observed a medium to strong decrease in the vhl mRNA levels compared with control (Supplementary Fig. Fig. S9C), suggesting that in these samples the wild type Vhl function could be lost/reduced either by mutation(s) or by epigenetic inactivation. Because of technical limitations, we could not assay potential molecular lesion(s) on the wild-type vhl allele in these neoplastic cells with decreased vhl mRNA levels. The fact that DMBA treatment led to tumorogenesis in 55 of 94 vhl/– fish compared with neoplasias in only 18 of 77 experimental wild-type fish strongly indicate that the Vhl possesses a conserved tumor suppressor function in zebrafish.

Discussion
In this study, we have generated and validated a novel reporter line, Tg(phd3::EGFP), for tracking VHL/HIF signaling in zebrafish. When we subjected the transgenic embryos to hypoxia induced by either genetic, physical, or chemical conditions, we could observe strong activation of the transgene. We have also showed the potential of the transgenic line as an in vivo reporter for intratumoral hypoxia in a melanoma model system. These phd3::EGFP transgenic embryos would be ideally suited for small molecule screens to identify novel chemicals that activate or suppress Hif activity in the wild-type or vhl/– background, respectively.

Several approaches are being utilized to monitor intratumoral hypoxia such as direct measurements of tumor oxygenation, assaying the levels of hypoxia marker proteins such as carbonic anhydrase IX, lysyl oxidase, etc. and by using small molecule markers such as EF5, pimonidazole, which are derivatives of 2-nitroimidazole, but many of these procedures are invasive and time consuming (37, 38). Moreover, these procedures and/or molecular markers have not been successfully employed for measuring hypoxia in zebrafish (39), indicating the need for novel markers for hypoxic signaling. The low background expression and powerful induction by HIF could make the Tg(phd3::EGFP) a novel fluorescent marker for in vivo monitoring of hypoxic signaling in many zebrafish tumor model systems.

von Hippel-Lindau disease is characterized by formation of a variety of tumor types, most importantly clear cell renal cell carcinoma and retinal and central nervous system hemangioblastomas. Unfortunately, although a variety of mouse models have been made for this disease, a comparable tumor suppressor activity of VHL homologs has been difficult to show in rodent models. Therefore, we tested for such a function of vhl in the zebrafish. Indeed, we report increased susceptibility of the vhl/– zebrafish to hepatic, bileduct, and intestinal tumorigenesis upon exposure to dimethylbenzanthracene (DMBA) compared with the control fish. We also report significantly increased occurrence of proliferating renal tubules in the DMBA-treated vhl/– batch compared with appropriate controls.

We tracked for random occurrence of vhl LOH in live Tg (phd3::EGFP) fish by scoring cells and/or cell clones emitting strong EGFP fluorescence. Although the 6-month-old untreated vhl/– fish had approx. 1 to 2 detectable EGFP cells in the skin and retinal epithelia, as they become older (~1.5–2-yr-old) they accumulated few more LOH events in the skin epithelium (~ 8–10 GFP cells/fish). Upon treatment with DMBA, the vhl/– fish showed a significant increase in the occurrence of vhl/– EGFP cells in the skin epithelium. Probably owing to the low rate of spontaneous vhl LOH, vhl/– heterozygous fish exhibited normal development and showed no detectable signs of tumor or cyst formation. In the case of Vhlh/– heterozygous mice, depending on the type of genetic background, variable susceptibility to spontaneous...
tumorigenesis were observed (refs. 40, 41, and 42). Because many of the zebrafish laboratory strains are not highly inbred (43), the strain differences might be less pronounced in fish. However, the lifespan of the zebrafish is only a few years, so even after the spontaneous vhl LOH at few cells they may not be undergoing enough subsequent mutations in other genes to predispose such fish with a disease phenotype. But, when we challenged the vhl+/− fish with DMBA, we observed a high incidence of tumorigenesis arising from liver, bile duct, and gut.

Interestingly, we observed decreased vhl mRNA levels in a set of vhl+/− EGF+ tumors, suggesting that in these samples the wild-type vhl allele might have undergone either loss-of-function mutation(s) or epigenetic silencing. Interestingly in human VHL patients, an epigenetic mechanism for inactivation of the wild-type VHL allele, mediated through the transcriptional silencing of the gene via hypermethylation of a 5′-CpG island, has been frequently observed (8, 44). DMBA in addition to inducing mutations is also known to cause aberrant methylation of 5′-CpG islands leading to epigenetic silencing of gene(s) (45). Our study exhibit the potential of the vhl+/− zebrafish as a model to characterize molecular mechanisms involved in VHL induced tumorigenesis.

Although occurrence of ccRCC is the classical VHL disease phenotype, we did not observe development of renal cysts or ccRCC in the DMBA-treated vhl+/− fish within the 14-month experimental period of our analysis. However, the presence of a significantly increased number of PCNA+ renal tubules suggests that these proliferating tubules might be at an early stage in the process of renal cyst formation. Some of these proliferating tubules were dysplastic and might be comparable to the VHL mutant foci that are observed in VHL patients (46).

Interestingly, the presence of a duplicated copy of the vhl gene, a vhl-like (vll) gene in zebrafish, indicates a possible segregation of VHL functions between these 2 genes and it would be interesting to assess the combined role of these genes in renal tumor suppression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Tumors in vhl Zebrafish and a Novel Reporter for HIF Signaling


A Zebrafish Model to Study and Therapeutically Manipulate Hypoxia Signaling in Tumorigenesis

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