Deletion of the von Hippel–Lindau gene causes sympathoadrenal cell death and impairs chemoreceptor-mediated adaptation to hypoxia

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Abstract

Mutations of the von Hippel–Lindau (VHL) gene are associated with pheochromocytomas and paragangliomas, but the role of VHL in sympathoadrenal homeostasis is unknown. We generated mice lacking Vhl in catecholaminergic cells. They exhibited atrophy of the carotid body (CB), adrenal medulla, and sympathetic ganglia. Vhl-null animals had an increased number of adult CB stem cells, although the survival of newly generated neuron-like glomus cells was severely compromised. The effects of Vhl deficiency were neither prevented by pharmacological inhibition of prolyl hydroxylases or selective genetic down-regulation of prolyl hydroxylase-3, nor phenocopied by hypoxia inducible factor overexpression. Vhl-deficient animals appeared normal in normoxia but survived for only a few days in hypoxia, presenting with pronounced erythrocytosis, pulmonary edema, and right cardiac hypertrophy. Therefore, in the normal sympathoadrenal setting, Vhl deletion does not give rise to tumors but impairs development and plasticity of the peripheral O2-sensing system required for survival in hypoxic conditions.

Keywords adult carotid body neurogenesis; intolerance to hypoxia; sympathoadrenal tumorigenesis; Vhl-deficient mouse model; von Hippel–Lindau protein

Subject Categories Cancer; Development & Differentiation; Stem Cells

Introduction

During embryogenesis, most neural crest-derived sympathetic precursor cells undergo c-jun-dependent apoptosis as the availability of trophic factors (particularly nerve growth factor) becomes limiting (Estus et al., 1994). In recent years, in vitro experiments on PC12 cells have suggested that the von Hippel–Lindau (VHL) protein might participate in the molecular cascade leading to apoptosis of sympathetic progenitor cells and that impairment of this protein could predispose to pheochromocytomas, a tumor of the adrenal gland, in adulthood (Lee et al., 2005). Besides pheochromocytomas, carotid body (CB) paragangliomas and other tumors of neural crest lineage are frequently associated with VHL disease, a hereditary syndrome caused by mutations in the VHL gene and characterized by the occurrence of tumors in multiple tissues (Haase, 2005; Kaelin, 2008; Boedeker et al., 2009). The best-known function of VHL is to act as the substrate recognition unit of an ubiquitin ligase complex that targets HIFα-subunits for proteasomal degradation (Maxwell et al., 1999). However, the role of VHL in the development and homeostasis of the sympathoadrenal system is, as yet, poorly studied. Whereas loss of VHL protein can induce tumors in several organs, it also negatively affects cell survival and proliferation in other tissues (Haase, 2005; Young et al., 2008; Li & Kim, 2011). Therefore, inactivation of VHL could have differing effects in cells of diverse embryological origin or developmental stage.

To further elucidate the actions of VHL protein, we have generated conditional Vhl knockout (KO) mouse models (TH-VHLKO and TH-CREERT-VHLKO mice) restricted to catecholaminergic (tyrosine hydroxylase—TH—positive) cells to investigate the role played by Vhl in sympathoadrenal development as well as in maintenance of catecholaminergic cells and CB neurogenesis in adulthood. The CB and adrenal medulla (AM) are part of a homeostatic acute O2-sensing system that is essential for survival upon exposure to hypoxia (Weir et al., 2005). The CB contains neuron-like, O2-sensitive, glomus (type I) cells that acutely respond to hypoxia by the release of neurotransmitters that stimulate afferent nerve fibers, which activate the brain stem respiratory center and increase sympathetic tone (Lopez-Barneo et al., 2001). This organ acts in concert with chromafin cells of the AM. Activation of the chemosensitive CB-AM axis leads to adaptive hyperventilation and increased cardiac output with redistribution of blood flow to the most O2-demanding organs, such as the brain or the heart. During protracted exposure to low PO2, acclimatization to hypoxia depends on CB hypertrophy and the resulting enhancement of the respiratory drive necessary for sustained hyperventilation (Powell et al., 1998; Joseph & Pequignot,
This remarkable proliferative response, uncommon for an organ of neural origin, is achieved thanks to the presence in the adult CB of a resident population of multipotent, neural crest-derived stem cells, which are the glia-like sustentacular (type II) cells (Pardal et al., 2007). Alterations of peripheral neural organs of the sympathoadrenal lineage might lead to dysfunction of the homeostatic acute O$_2$-sensing system with relevant medical impact. Indeed, developmental defects in the CB have been associated with respiratory pathologies in children, such as the sudden infant death or congenital hypoventilation syndromes (see for reviews López-Barneo et al., 2008; Perez & Keens, 2013; Porzionato et al., 2013). Constitutive intolerance to low PO$_2$ could also explain why some individuals are unable to acclimatize to hypoxia and develop complications such as pulmonary hypertension, right heart failure, or brain dysfunction (see Ghofrani et al., 2004; Schou et al., 2012).

Herein, we show that, contrary to generalized beliefs ascribing to VHL a role as tumor suppressor gene (see for references Lee et al., 2005; López-Jiménez et al., 2010; Li & Kim, 2011), Vhl inactivation in rodent catecholaminergic cells in vivo does not lead to tumorigenesis but rather to a marked atrophy of the CB, AM, and sympathetic ganglia. Hypoxia-induced adult CB neurogenesis is also markedly inhibited in mice with the ablation of $Vh_l$ alleles. Vhl-deficient animals live normally under normoxic conditions, but show a striking intolerance to systemic hypoxia leading to impending death.

Results

Selective disruption of the sympathoadrenal system in Vhl-deficient mice

TH-VHL$^{floxed}$ mice, with embryonic ablation of $Vh_l$ alleles (see Materials and Methods), were viable, fertile, and appeared healthy, reaching adulthood without obvious abnormalities (Supplementary Fig S1A and B). CB and AM dissected from adult TH-VHL$^{floxed}$ mice did not present any signs of tumor formation. On the contrary, histological analyses showed atrophy of the CB, superior cervical ganglion (SCG), and AM, with a striking decrease in TH$^+$ cell number. These differences between control and mutant animals were already observed in neonates, but became more apparent during postnatal development (Fig 1A–C; Supplementary Fig S2A and B). CB cells of TH-VHL$^{floxed}$ mice appeared intermingled with SCG neurons and lacked the cluster-like organization (glomeruli) characteristic of this structure. Quantification of the volume of the CB-SCG TH$^+$ area clearly showed a marked decrease in size with respect to normal animals (Fig 1B). As a consequence of cell death, the AM almost disappeared by 2–3 months of age (Fig 1C). Abdominal sympathetic ganglia were also affected in TH-VHL$^{floxed}$ mice (Supplementary Fig S2C). In accord with these structural modifications, the plasma levels of noradrenaline, and particularly adrenaline, were drastically decreased (Fig 1D).

Electron microscope analyses demonstrated profound ultrastructural alterations in CB glomus cells of TH-VHL$^{floxed}$ mice, which showed large vacuoles resembling aberrant autolyosomes, disappearance of the typical dense-core secretory vesicles, and disorganization of nuclear chromatin (Fig 1E). Similar alterations were observed in adrenal chromaffin cells (Fig 1F). Non-catecholaminergic neural crest-derived cells (such as those in the enteric nervous system or dorsal root ganglion—DRG—neurons) were unaffected by TH promoter-directed Vhl deletion (Supplementary Fig S3A and B). Interestingly, dopaminergic and noradrenergic neurons in the ventral mesencephalon and locus coeruleus, respectively, appeared preserved in juvenile mutant animals (Supplementary Fig S4A–C). These observations suggest that Vhl inactivation in TH$^+$ cells selectively impairs the development of sympathoadrenal organs, particularly the CB, AM, and sympathetic ganglia, in a cell-autonomous manner.

Since VHL disease-associated tumorigenesis is triggered when the second Vhl allele is lost in adult life, we also tested the effects of catecholaminergic-specific Vhl deletion in adult $Vh_l^{Flox/–}$ mice (TH-CREER-VHL$^{Flox/–}$ mice). These animals, studied 6 months after deletion of the floxed Vhl allele, did not show tumor formation in the CB or AM but a trend to decreased density of TH$^+$ cells and disorganization of the TH$^+$ cell clusters (Fig 2A–G). Although macroscopically the CB or AM volumes remained unaltered, impairment of CB function was already detectable in TH-CREER-VHL$^{Flox/–}$ mice (see Fig 8B below). These experiments support the notion that homozygous Vhl loss does not induce tumorigenesis in mouse sympathoadrenal cells.

Carotid body neurogenesis from adult stem cells is impaired by Vhl deficiency

We tested whether, in addition to its effects on embryonic development, Vhl influenced differentiation and/or survival of newly generated adult CB glomus (type I) cells. It is known that the adult CB is a neurogenic niche containing GFAP$^+$, glia-like stem cells, that upon exposure to low O$_2$ generate nestin$^+$ intermediate progenitors which proliferate and, eventually, differentiate into new TH$^+$ glomus cells and other cell types (Pardal et al., 2007; Platero-Luengo et al., 2014). The number of GFAP$^+$ CB progenitors was clearly larger in Vhl-ablated animals than in controls and increased progressively with age (Fig 3A). Similarly, the number of proliferating (BrdU$^+$) cells in the CB-SCG area was also augmented in TH-VHL$^{floxed}$ mice with respect to controls (VHL$^{WT}$) (Fig 3B). Ultrastructural studies demonstrated that unlike glomus cells, type II (GFAP$^+$) cells, characterized by the form of their nuclei, lack of secretory vesicles, and long processes embracing glomus cells (Platero-Luengo et al., 2014), remained unaffected in TH-VHL$^{floxed}$ mice (Fig 3C). Therefore, it seems that the loss of differentiated neuron-like glomus cells induced a slow compensatory mechanism that led to an increase in the number of stem cells. In contrast with these findings in the CB, GFAP$^+$ sustentacular cells in the AM (Suzuki & Kachi, 1995) remained unaffected by the loss of chromaffin cells (Supplementary Fig S5A). Despite the existence of a large population of CB stem cells in the TH-VHL$^{floxed}$ mice, they did not show the characteristic CB hypertrophy in response to hypoxia (Platero-Luengo et al., 2014) (Supplementary Fig S5B–D). These findings support the notion that damage of Vhl-deficient glomus cells markedly reduced CB responsiveness to lowering O$_2$. Indeed, as shown below, TH-VHL$^{floxed}$ animals showed a striking intolerance to hypoxia.

To test that GFAP$^+$ CB stem cells in TH-VHL$^{floxed}$ mice are actually multipotent and able to differentiate into glomus cells, we performed clonal neurosphere assays (Platero-Luengo et al., 2014).
Figure 1. Atrophy of sympathoadrenal organs in TH-VHLKO mice.

A–D Immunostaining of TH⁺ cells in the carotid bifurcation (A) and adrenal gland (C) of TH-VHLKO mice compared with control animals (VHLWT). To facilitate comparison, the areas inside the rectangles in (A) are shown in the insets at a higher magnification. ECA, external carotid artery; ICA, internal carotid artery; CB, carotid body; SCG, superior cervical ganglion; AM, adrenal medulla. Scale bars: (A) 100 μm; (C) 200 μm. (B) Quantitative analysis of the CB-SCG TH⁺ volume in VHLWT and TH-VHLKO mice (n = 3 animals per age and genotype). The loss of TH⁺ cells in mutant mice, already clearly evident at birth (P0), was accentuated during the first postnatal months (P60). *P < 0.02, **P < 0.000002 (unpaired two-tailed t-test). (D) Plasma catecholamine levels measured by HPLC (n = 5 animals 8–12 weeks old per genotype). *P = 0.04, **P = 0.008 (unpaired two-tailed t-test).

E, F Electron micrograph illustrating ultrastructural alterations observed in glomus (E) and chromaffin (F) cells of TH-VHLKO mice compared with VHLWT animals (2-month-old mice). The presence of numerous dense-core vesicles (arrowheads) and multiple enlarged vacuoles (asterisks) throughout the cytoplasm of Vhl-deficient type I and chromaffin cells are indicated. Scale bars: 2 μm. This figure is accompanied by Supplementary Figs S2, S3 and S4.
CBs of wild-type (VHLWT) and TH-VHLKO mice were enzymatically dispersed, and typical CB neurospheres were generated from these cells after 8 days in culture (Fig 4A). However, the number of neurospheres was greater (Fig 4B) and their diameter larger (Fig 4C) in TH-VHLKO than in the VHL WT mice, a result compatible with the increased number of stem cells and cell proliferation in the Vhl-deficient CB (see above and Fig 3). Immunocytochemical analysis of sectioned neurospheres produced from VHLWT and TH-VHLKO mice revealed the presence of nestin+ cells inside the core of the neurosphere similar to that previously described for rat CB neurospheres. However, small clusters of TH+ cells, which appeared in the wild-type neurospheres after a few days in culture due to differentiation of progenitor cells into glomus cells (Pardal et al., 2007), were not observed in preparations from TH-VHLKO mice (n=5 experiments) (Fig 4D). These data indicated that, although progenitor cells in the TH-VHLKO neurospheres are...
Figure 3. Increase of GFAP+ CB stem cell number in TH-VHLKO mice.

A  GFAP immunostaining illustrating the large increase of GFAP+ cells in the carotid bifurcation of TH-VHLKO animals compared with VHLWT mice (from P15 to P180). The areas inside the rectangles are shown at a higher magnification in the insets. ECA, external carotid artery; ICA, internal carotid artery; CB, carotid body. Scale bars: 100 μm.

B  Left. Incorporation of BrdU to proliferating cells in the carotid bifurcation of VHLWT and TH-VHLKO mice maintained in normoxia. Right. Number of BrdU+ cells versus total cells inside the area occupied by TH+ cells in the CB (VHLWT) or the CB-SCG region (TH-VHLKO). Scale bars: 100 μm. Cell counting was performed on 4 animals (8 weeks old) per genotype. *P = 0.02 (unpaired two-tailed t-test).

C  Electron micrograph showing the normal appearance of CB type II cells in 2-month-old VHLWT and TH-VHLKO mice. Typical synaptic-like contact between type I and type II cell is shown in the inset at higher magnification. Scale bars: 2 μm. This figure is accompanied by Supplementary Fig S5.
highly proliferative, they seem to be unable to give rise to new TH+ cells, thereby explaining the lack of neurogenesis in vivo described above. This supposition was confirmed by culturing neurospheres on adherent substrate to promote differentiation. In contrast to VHLWT-derived neurospheres, where mature TH+ cells were present in all cultures (n = 15), TH+ cells were not, or very rarely, seen in the neurosphere cultures from TH-VHLKO animals (n = 15). In some cultures (n = 5), we observed cells in mitosis that expressed both nestin and TH (Fig 4E left-bottom). CB progenitor cells in neurospheres from both VHLWT and TH-VHLKO mice were able to give rise to new smooth muscle actin-positive (SMA+) cells (Fig 4E right), another cell type derived from CB stem cells (Pardal et al., 2007). Hence, CB stem cells from TH-VHLKO preserve their multipotency and are able to differentiate into SMA+ and TH+ cells. However, it appears that newly formed glomus cells are unable to differentiate to TH+ cells or die as soon as they express TH, and Hif is inactivated by Cre-mediated site-specific recombination. It would seem therefore that normal Vhl function is thus required not only for proper embryonic development of the sympathoadrenal organs but also for the survival of newly formed adult CB glomus cells.

Vhl deficiency is not compensated for by ablation of Phd3 or Hif modulation

Phd3 is an O2-sensing hydroxylase that has pro-apoptotic actions (Lee et al., 2005; Bishop et al., 2008; reviewed in Schlisio, 2009). We consequently investigated whether the catecholaminergic cell loss due to Vhl deficiency might be as a consequence of Hif-α stabilization and subsequent Phd3 induction. We selected specific shRNA lentiviral vectors (LVs) for Phd3 silencing (Fig 5A) and then transduced with these vectors dispersed CB cells that were used for neurosphere generation and differentiation assays. In all experiments (n = 9 for each condition) with VHLWT neurospheres (with or without LVs), we observed the generation of newly differentiated TH+ cells (Fig 5B top), and the number of these cells slightly increased in response to Phd3 down-regulation (Fig 5C). As indicated in the preceding section, newly generated TH+ cells were practically absent in the experiments performed with TH-VHLKO neurospheres (n = 9) (Fig 5B bottom left). Phd3 silencing in the TH-VHLKO background favored the generation of some TH+ cells in 5 of 9 experiments (Fig 5B bottom), but this effect was quantitatively negligible as the total number of TH+ cells...
cells present was small (Fig SC). Similar differentiation assays were performed in neurospheres with variable HIF levels. Neurospheres were treated with 0.5 mM dimethyloxalylglycine (DMOG) to inhibit prolyl hydroxylases, thus permitting us to test for the effect of HIF stabilization in vitro. In 5 of 6 experiments, incubation of TH-VHLKO neurospheres with DMOG favored the appearance of some TH+ cells; however, this effect was also quantitatively very small (Fig SB and C). Similarly, transduction of cells with lentiviral vectors for Hif-1α and Hif-2α silencing did not produce a significant increase in the number of newly generated TH+ cells (Fig SD). Taken together, these data indicate that the lack of stem cell-dependent glomus cell differentiation, observed in Vhl-deficient animals, is not, or only marginally, compensated for by the Phd3 deficit, generalized prolyl hydroxylase inhibition, or Hif down-regulation.

We further investigated whether Phd3 can compensate for Vhl protein deficiency in a mouse model with general ablation of Phd3 and deletion of Vhl in catecholaminergic cells. As previously described (Bishop et al., 2008), ablation of Phd3 (VHLWT;PHD3KO) resulted in a slight hypertrophy of the CB and AM (Fig 6A and C, top panels). Ablation of Vhl on the Phd3 knockout background (TH-VHLKO;PHD3KO) resulted in strong reduction of TH+ parenchyma in the CB-SCG area (Fig 6A and B) similar to that observed on TH-VHLKO animals. TH-VHLKO;PHD3KO mice also showed higher number of GFAP+ cells in the CB region and marked cell loss in the AM (Fig 6A and C, bottom panels). This is a phenotype qualitatively similar to that described above for TH-VHLKO mice (see Figs 1 and 3). CB neurosphere differentiation assays for the TH-VHLKO;PHD3KO mice yielded TH+ cells in the six experiments performed, thus indicating that Phd3 ablation does not alter multipotency and that it favors the catecholaminergic differentiation of CB stem cells. However, as occurred in the knockdown experiments (Fig 5), the effect of Phd3 deletion on the total number of differentiated TH+ cells, although statistically significant, was quantitatively negligible.

**Figure 5. Phd3, Hif-1α, and Hif-2α down-regulation and prolyl hydroxylase inhibition with DMOG in CB neurosphere cultures.**

A. Phd3 mRNA levels in NIH3T3 cells 48 h after transduction with non-silencing and specific Phd3 shRNA (Phd3 shRNA LV-1 and LV-6) lentiviral vectors (n = 3 for each condition). Non-silencing shRNA versus Phd3 shRNA LV-1, **P = 0.005 and non-silencing shRNA versus Phd3 shRNA LV-6, *P = 0.004** (unpaired two-tailed t-test). r.u., relative units.

B. Immunocytochemical analysis of CB neurospheres from VHLWT and TH-VHLKO mice transduced with non-silencing and Phd3 shRNA lentiviral vectors (Phd3 shRNA LV-1 and LV-6) or treated with 0.5 mM DMOG. Transduced cells were identified by GFP expression. Some differentiated TH+ cells were identified in Phd3 silenced and DMOG-treated TH-VHLKO-derived neurospheres (n = 5 of 9 and n = 5 of 6 experiments, respectively). Scale bars: 10 μm.

C. Quantification of TH+ cells per neurosphere (n = 9 for control and non-silencing conditions, n = 5 for Phd3 shRNA transduction and n = 6 for DMOG administration). *P = 0.02, **P = 0.007 (unpaired two-tailed t-test).

D. Left: Hif-1α and Hif-2α mRNA levels in NIH3T3 cells 48 h after transduction with non-silencing and specific Hif-1α and Hif-2α shRNA lentiviral vectors (n = 3 for each condition). **P = 0.003 (unpaired two-tailed t-test). r.u., relative units. Right. Number of TH+ cells per CB neurosphere (n = 4) transduced with the indicated non-silencing and Hif-α shRNA LV6. NS, non-significant (unpaired two-tailed t-test).
HVR and acclimatization to chronic hypoxia are severely impaired in TH-VHLKO animals

Despite a marked atrophy of peripheral O₂-sensing organs, TH-VHLKO mice exhibited respiratory parameters in normoxic conditions similar to those of controls (see Supplementary Table S1 and Fig 8). Animal responsiveness to acute hypoxia (hypoxic ventilatory response—HVR) was tested by plethysmography. Figure 8A illustrates the changes of respiratory rate during a cycle of normoxia—hypoxia—normoxia in VHLWT and TH-VHLKO mice. After application of hypoxia (10% O₂), VHLWT animals increased their respiration up to a plateau level, which then decreased to basal levels upon returning to normoxia (21% O₂) (Fig 8A, black line). In contrast, TH-VHLKO mice, which exhibited a normal respiratory rate in normoxia, failed to hyperventilate in response to hypoxia (Fig 8A, gray discontinuous line). In about half of the trials, TH-VHLKO mice exposed to hypoxia also showed a transient loss of consciousness and marked respiratory depression (Fig 8A, red line). A summary of average respiratory rates during exposure to hypoxia of the various animal models studied is given in Fig 8B. As expected, VHLWT and VHLWT; PHD3KO animals showed an increased average respiratory rate in response to hypoxia. In contrast, TH-VHLKO and TH-VHLKO; PHD3KO mice failed to show any sign of HVR. TH-CREERTVHLKO mice studied 6 months after tamoxifen treatment (see Fig 2) also showed partial inhibition of the HVR (Fig 8B). Additional respiratory parameters altered by Vhl deletion are presented in Supplementary Table S1. We also measured arterial hemoglobin saturation during acute exposures to mild (14% O₂) and more severe (10% O₂) hypoxia as an indicator of the efficacy of compensatory hyperventilation. Whereas VHLWT and VHLWT; PHD3KO mice were able to maintain hemoglobin saturation at 60–65% during exposure to hypoxia, this value dropped to < 40% in TH-VHLKO and TH-VHLKO; PHD3KO animals (Fig 8C).

The lack of responsiveness to acute lowering of O₂ tension made Vhl-deficient animals intolerant to sustained hypoxia. Consistent with plethysmography and hemoglobin saturation recordings, TH-VHLKO mice showed signs of respiratory distress and loss of consciousness after 30–40 s in response to chronic hypoxia conditions; most of them, however, recovered in 2–3 min, although their long-term survival was severely compromised. While VHLWT mice adapted well to chronic hypoxia (10 or 14% O₂), none of the TH-VHLKO or TH-VHLKO; PHD3KO mice (in which Vhl alleles had been deleted only in catecholaminergic tissues) survived for more than 11 days in either a 10% (Fig 8D) or 14% O₂ environment. When maintained under hypoxic conditions, TH-VHLKO mice showed abnormally high hematocrit and EPO plasma levels on day 7 compared with wild-type mice (Fig 8E and F). These animals, with catecholaminergic cell Vhl deficiency and lack of the hypoxic hyperventilatory response, also exhibited gross anatomical alterations of the heart, lung, and spleen (Fig 9A–F). The most salient pathological feature was a marked increase in heart size due to enlargement of the right ventricle (Fig 9A,B and D), probably due to pulmonary hypertension. We also found evidence of pulmonary edema and parenchyma micro-hemorrhage in the lungs of TH-VHLKO animals (Fig 9E). Spleens also showed a marked increase in size (Fig 9A and C) and exhibited changes in histological architecture characteristic of extramedullary hematopoesis (Fig 9F). None of these histological alterations were seen in TH-VHLKO mice living under normoxic conditions (Supplementary Fig S6A–C).

Discussion

Role of Vhl in sympathoadrenal development, tumorigenesis, and adult CB neurogenesis

Vhl is commonly recognized as a tumor suppressor gene given that its homozygous deletion is known to induce tumors, particularly hemangioblastomas and renal cysts cancers, in a HIF-dependent fashion (see Haase, 2005; Kaelin, 2007 for reviews). A point mutation (R200W) in Vhl that is not associated with tumor development recapitulates in mice Chuvash polycythemia via Hif-2α signaling (Ang et al., 2002; Hickey et al., 2007). These effects are consistent with the most-studied function of VHL as a substrate recognition unit of an ubiquitin ligase complex that targets HIFα for proteasomal degradation (Maxwell et al., 1999). VHL mutations can also produce pheochromocytomas and paragangliomas, but the role of VHL in the development and homeostasis of the sympathoadrenal system is still not well understood. In vitro experiments on PC12 cells have suggested that Vhl participates in the c-jun-dependent cascade that leads to the apoptosis of sympathetic progenitor cells during the late stages of development (Estus et al., 1994; Lee et al., 2005). In this model, Phd3, a prolyl hydroxylase with a well-established pro-apoptotic role (reviewed in Schlissel, 2009), acts downstream of Vhl to regulate sympathoadrenal progenitor survival. It was thus postulated that alterations of the Vhl-Phd3 system during embryogenesis could predispose affected individuals to pheochromocytomas in adulthood (Lee et al., 2005). In contrast to these findings, our in vivo and in vitro observations suggest that Vhl deletion not only results in sympathoadrenal cell loss but also impairs the differentiation and/or survival of stem cell-derived newly generated glomus cells in the adult CB. Moreover, we did not observe any indication of adrenal or CB tumorigenesis after the deletion of a remaining floxed Vhl allele, which produced loss of heterozygosity in adulthood (TH-CREERTVHLKO mice). Embryonic and adult sympathoadrenal cell death observed in Vhl-deficient animals is independent of genetic (Phd3) or pharmacological (DMOG administration) prolyl hydroxylase inhibition and is not mimicked by either Hif-1α or Hif-2α.
Figure 6. In vivo Phd3 ablation does not prevent the effects of Vhl deficiency.
A TH and GFAP immunostaining in carotid bifurcation of control (VHL WT;PHD3 KO) and mutant (TH-VHL KO;PHD3 KO) mice at P60. ECA, external carotid artery; ICA, internal carotid artery; CB, carotid body; SCG, superior cervical ganglion. The regions inside the rectangles are shown in the insets at higher magnification. Scale bars: 100 μm.
B CB-SCG TH + volume quantification in adult (P60) VHL WT;PHD3 KO control mice compared with TH-VHL KO;PHD3 KO mutants (n = 3 mice per genotype). ***P = 0.000002 (unpaired two-tailed t-test).
C Immunofluorescence images of adrenal gland sections stained for TH. AM, adrenal medulla. Scale bars: 200 μm.
D Representative examples of CB neurosphere cultures from VHL WT;PHD3 KO and TH-VHL KO;PHD3 KO mice illustrating the presence of nestin, TH (left panel), and SMA (right panel) cells. Scale bars: 10 μm.
E Number of differentiated TH + glomus cells per neurosphere generated in vitro (n = 6 per genotype). **P = 0.001 (unpaired two-tailed t-test).
F Quantification of TH + cells identified after 3 or 10 days in differentiation culture conditions (n = 6 per genotype). *P = 0.04 (unpaired two-tailed t-test).
Figure 7. Sympathoadrenal effects of HIF-1α and/or HIF-2α over-expression.

A–C Immunohistochemical analysis of the carotid body in mice overexpressing non-degradable variants of HIF-1α (A), HIF-2α (B), or both HIF-1α and HIF-2α (C) restricted to the sympathoadrenal system (TH-HIF1AΔPA, TH-HIF2AΔPA, and TH-HIF1AΔPA;TH-HIF2AΔPA mouse lines, respectively) compared with controls (HIF1AΔPA, HIF2AΔPA, and HIF1AΔPA;HIF2AΔPA, respectively). Scale bars: 100 μm. ECA, external carotid artery; CB, carotid body; SCG, superior cervical ganglion.

D, E Quantification of the carotid body volume (D) and TH+ cell density (E) in the indicated HIF-overexpressing mouse lines compared with controls, respectively. (n = 3 per genotype). *P = 0.03, **P = 0.009 in (D) and *P = 0.02, **P = 0.008 in (E) (unpaired two-tailed t-test).

F–H Adrenal gland thin sections stained for TH detection illustrating the appearance of chromaffin cells with HIF-1α (F bottom), HIF-2α (C bottom), or both HIF-1α and HIF-2α (H bottom) activation compared with controls (F–H upper panels). Scale bars: 200 μm. All animals were 8–12 weeks old.
down-regulation or transgenic Hif-1α and Hif-2α activation. Therefore, it seems that Vhl is not only necessary for the survival of sympathoadrenal cells during development, but it is also required for maintenance (full differentiation and survival) of these cells in adulthood and for neurogenesis in the adult CB in a manner unrelated to the Vhl-Hif-Phd3 pathway. Interestingly, it has been noted that the bi-allelic loss of Vhl seems to be incompatible with pheochromocytoma development and that most cases of type 2 VHL disease (high risk of pheochromocytomas) are caused by missense mutations of the VHL gene. Vhl-null murine embryonic stem (ES) cells generate teratocarcinomas that are smaller than those produced by wild-type ES cells, indicating that the tumor suppressor activity of Vhl is only manifested in a background of other mutations (Mack et al., 2003). As previously suggested (see Lee et al., 2005 for a detailed discussion), it could be that mutations of the VHL-producing pheochromocytoma are gain-of-function mutations that lead to abnormal cell proliferation in the adrenal cell setting. In agreement with these findings, homzygous Vhl deletion has been shown to produce impairment of pancreatic beta cell function (Cantley et al., 2009) and apoptosis in thymocytes and chondrocytes (Haase, 2005). Although the small size of the CB or AM has precluded any detailed biochemical analysis of sympathoadrenal cell death induced by Vhl deficiency, our electron microscope studies suggest that it is compatible with autophagy dysregulation. This idea is in accord with numerous recent reports demonstrating Hif-independent involvement of Vhl in cell senescence, apoptosis, and autophagy (Young et al., 2008; Mazure & Pouysségur, 2010; Li & Kim, 2011). Incubation of neurospheres from TH-VHL KO animals with a cocktail including inhibitors of autophagy, apoptosis, and necroptosis significantly increased the generation of viable TH+ cells (data not shown). However, the small effect of this pharmacological treatment precludes any definitive conclusion on the mechanism(s) of cell death in Vhl-deficient catecholaminergic cells. In any instance, our results stress the critical importance of Hif-independent functions of Vhl for sympathoadrenal cell homeostasis.

Adult CB stem cell population in TH-VHLKO mice

TH-VHLKO mice exhibited a compensatory increase in the number of GFAP+ CB stem cells compared with wild-type mice. This cell population formed multipotent clonal colonies in vitro, thus supporting the role of the CB as a neurogenic niche in the peripheral nervous system (Pardal et al., 2007). However, as indicated above, differentiation and/or survival of newly generated glomus cells from
sustentacular (GFAP +) cells was impaired in the TH-VHL KO mice due to the loss of the floxed Vhl allele in TH + cells. Interestingly, the GFAP + progenitor cells in TH-VHL KO mice did not proliferate in response to sustained hypoxia. This observation fits well with the concept that neurotransmitter release from O2-sensitive glomus cells (lost or profoundly impaired in the TH-VHL KO mice) is the signal that triggers the proliferation of progenitor cells to bring about CB growth during exposure to hypoxia (Platero-Luengo et al., 2014).

Intolerance to hypoxia in mice with impairment of the acute O2-sensing system

Acute HVR, a reflex response necessary for adaptation to hypoxic environments, disappears in patients that have undergone surgical CB resections (most commonly due to tumors or asthma treatment) (Timmers et al., 2003). These patients appear to live unaffected in normoxic environments, although disturbances during sleep and cases of sudden death have been attributed to a lack of functional chemoreceptors (López-Barneo et al., 2008). Alterations of CB development have also been associated with respiratory dysfunction in neonates and children (for recent reviews see Perez & Keens, 2013; Porzionato et al., 2013; Gozal et al., 2013). TH-VHL KO mice, which also have a blunted acute HVR, seemed to live unaffected by this condition in normoxia, although their respiratory functions were not systematically analyzed here.

The CB is thought to play an essential role in acclimatization to chronic hypoxia, an environmental or medical condition affecting millions of people worldwide. Nevertheless, this process has been poorly investigated due to a lack of appropriate experimental models. The CB is generated during embryogenesis by the migration of sympathetic precursor cells from the SCG to the primordial carotid artery (see Hempleman & Warburton, 2013 for a detailed review). Mutations of genes that prevent either carotid artery formation or sympathetic development are known to result in CB defects.
However, these mutations are embryologically lethal or animals die shortly after birth due to major respiratory alterations, meaning that the animals cannot be studied in adulthood (Dauger et al., 2003; Kameda et al., 2008). The TH-VHLKO is a novel animal model in which the consequences of functional inhibition of peripheral chemoreceptors can be studied throughout the normal life span of mice. When maintained under normoxic conditions, TH-VHLKO mice show full development of the brain and other organs and normal physiological functions. Nonetheless, they exhibit a striking intolerance to sustained hypoxia. Even exposure of TH-VHLKO mice to mild hypoxia (14% O2), caused strong hemoglobin desaturation, which within a few days was followed by splenomegaly, severe pulmonary hypertension, and right cardiac hypertrophy leading to death. Therefore, these data demonstrate the absolute necessity of peripheral chemoreceptors for acclimatization and survival during exposure to hypoxia. These observations make TH-VHLKO mice an ideal model to study the early signs of hypoxia intolerance or to identify biomarkers sensitive to maladaptation to hypoxia. This could help prevent hypoxia-associated morbidities affecting the brain or cardiorespiratory system, which are highly prevalent in susceptible individuals (Sutherland & Cherniack, 2004; Schou et al., 2012; Gozal et al., 2013).

General pathophysiological consequences of sympatoadrenal atrophy

Besides a lack of functional peripheral chemoreceptors, the TH-VHLKO mice had also atrophy of the peripheral sympathetic nervous system and decreased catecholamine (particularly adrenaline) secretion. These animals also showed hypoglycemia, which was particularly prominent in the fasting state, along with other signs of sympathetic dysfunction (data not shown). TH-VHLKO mice could thus serve as an excellent model to test the role of the CB-AM axis in adaptation to situations involving an elevated O2 demand (such as physical exercise) or the function of organs devoid of autonomic innervation such as, for example, the endocrine pancreas (Borden et al., 2013; Muñoz-Bravo et al., 2013) or bone marrow (Méndez-Ferrer et al., 2008). These animals could also help provide further insight into the role of the CB-AM axis in glucose homeostasis and blood pressure regulation. Experiments performed in animals (Koyama et al., 2000; Pardal & López-Barneo, 2002) and in man (Wehrwein et al., 2010; Ortega-Sáenz et al., 2013) have suggested that CB glomus cells are glucose sensors that participate in the acute counter-regulatory response to hypoglycemia; however, this function of the CB is under debate. On the other hand, it is well established that CB activation leads to increased sympathetic tone. CB inhibition produces marked effects on blood pressure and counter-regulatory response to hypoglycemia; however, this function of the CB is under debate. On the other hand, it is well established that CB activation leads to increased sympathetic tone. CB inhibition produces marked effects on blood pressure and...
shRNA LV was validated by transducing a mouse embryonic fibroblast cell line (NH3T3) at a multiplicity of infection (MOI) of 5 and subsequent use of qRT-PCR (see for details online Supporting Information). Dispersed CB cells were transduced (MOI = 10) with selected Phd3, Hif-1α or Hif-2α shRNA LVs and then used for neurosphere formation and differentiation assays. For GFP detection on transduced flat neurosphere colonies, neurospheres were incubated with Alexa-Fluor 488-conjugated rabbit anti-GFP (1:500; Molecular Probes, A21311).

**Plethysmography and measurement of hemoglobin saturation**

Respiratory parameters were measured in conscious, unrestrained mice using whole-body plethysmography (Emka Technologies) according to the manufacturer’s recommended configuration of the apparatus for use with mice. Animals were maintained in a hermetic chamber with controlled normoxic airflow until they were settled, following which they were exposed to hypoxic air (10% O2) for 5 min, with normoxia again reinstated after this period. Each animal was subjected to this cycle of normoxia–hypoxia–normoxia twice per session. Control (VHLWT and VHLWT;PHD3KO) and mutant (TH-VHLKO and TH-VHLKO;PHD3KO) mice were routinely alternated between both chambers to avoid any intrinsic variability in the equipment. Real-time respiratory data were acquired and stored using iox2 software (Emka Technologies). Raw data obtained from plethysmography recordings were filtered by score rate, and only those that fully complied with the experimental protocol were analyzed. For the calculation of average respiratory parameters, we took into account values during the first 2 min once a 10% O2 level had been reached inside the chamber, as determined by an oxygen probe (Greisinger Electronic). This corresponds to an O2 level just above that before mutant TH-VHLKO and TH-VHLKO;PHD3KO mice suffer respiratory depression or loss of consciousness. Arterial blood hemoglobin saturation was measured in conscious, slightly anesthetized unrestrained mice using MouseOx Plus (Starr Live Sciences Corp.) linked to the Emka plethysmography system to monitor real-time percent oxygen saturation while a stable normoxic or hypoxic (14% O2 or 10% O2) airflow was applied. Raw data were acquired and stored with iox2 software (Emka Technologies). Average percentages of hemoglobin saturation levels were determined throughout the first minute after a 14% O2 or 10% O2 had been reached.

**Chronic hypoxia and physiological parameters**

Mice (2–3 months old) were chronically exposed to a 14% O2 or 10% O2 environment by using a specially designed hermetic chamber with controlled O2 and CO2 levels and temperature and humidity monitoring (Coy Laboratory Products). After 7 days of exposure, animals were weighed, anesthetized with ketamine/xylazine (100 mg/kg body weight and 8 mg/kg body weight, respectively), and bled for subsequent hematocrit measurement and plasma collection. Plasma EPO levels were determined using the Quantikine Mouse EPO ELISA kit (R&D Systems) according to the manufacturer’s protocol. Heart, lung, and spleen tissues were removed, and their wet weights measured. Next, tissues were histologically processed as described above. Analogous procedures were followed for mice maintained in normoxia.

**Statistical analysis**

Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was assessed by the Student’s t-test with a Levene test for determining the homogeneity of variances in cases of normal distribution, or by the nonparametric Mann–Whitney U-test in cases of non-normal distribution. Kaplan–Meier survival curves’ statistical significance was analyzed by log-rank test. PASW18 software was used for all statistical analyses.

**Supplementary information** for this article is available online: http://embomolmed.embopress.org

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Author contributions
DM and JL-B designed the experiments and wrote the manuscript; DM, MCF-A, and VB-H performed the experiments; and JL-B supervised the project.

Conflict of interest
The authors declare that they have no conflict of interest.

References


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