

Forkhead Transcription Factor 3a (FOXO3a) Modulates Hypoxia Signaling via Up-regulation of the von Hippel-Lindau Gene (VHL)^{*[5]}

Received for publication, June 27, 2016, and in revised form, October 20, 2016. Published, JBC Papers in Press, October 24, 2016, DOI 10.1074/jbc.M116.745471

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Edited by Xiao-Fan Wang

FOXO3a, a member of the forkhead homeobox type O (FOXO) family of transcriptional factors, regulates cell survival in response to DNA damage, caloric restriction, and oxidative stress. The von Hippel-Lindau (VHL) tumor suppressor gene encodes a component of the E3 ubiquitin ligase complex that mediates hypoxia-inducible factor α degradation under aerobic conditions, thus acting as one of the key regulators of hypoxia signaling. However, whether FOXO3a impacts cellular hypoxia stress remains unknown. Here we show that FOXO3a directly binds to the VHL promoter and up-regulates VHL expression. Using a zebrafish model, we confirmed the up-regulation of *vhl* by *foxo3b*, an ortholog of mammalian FOXO3a. Furthermore, by employing the clustered regularly interspaced short palindromic repeats (CRISPR)-associated RNA-guided endonuclease Cas9 (CRISPR/Cas9) technology, we deleted *foxo3b* in zebrafish and determined that expression of hypoxia-inducible genes was affected under hypoxia. Moreover, *foxo3b*-null zebrafish exhibited impaired acute hypoxic tolerance, resulting in death. In conclusion, our findings suggest that, by modulating hypoxia-inducible factor activity via up-regulation of VHL, FOXO3a (*foxo3b*) plays an important role in survival in response to hypoxic stress.

FOXO3a (also known as FKHL1), together with FOXO1 (also known as FKHR), FOXO4 (also known as AFX1), and FOXO6 comprise the conserved forkhead box O (FOXO)⁴ gene family, which is a subclass of the forkhead family of transcrip-

tion factors. The FOXO family is an evolutionarily conserved group of proteins and of vital importance in the control of cell and organism growth, development, metabolism, and longevity (1). FOXO proteins affect various cellular processes, including cell cycle regulation, cell survival, proliferation, and apoptosis (1). In their functional capacity as transcription factors, FOXO proteins bind to the consensus sequence (5'-TTGTTTAC-3') and induce expression of their downstream targets. Several upstream signaling pathways modulate FOXO activity via nuclear-cytoplasmic shuttling and posttranslational modifications of FOXO proteins (2–4).

Multiple lines of evidence have shown that FOXO transcription factors are key players in stress signaling (1, 5, 6). The critical role of FOXO transcription factors in oxidative stress has been well defined (7, 8). For example, FOXO3a protects quiescent cells from oxidative stress by inducing expression of manganese superoxide dismutase (Mn-SOD) (9). Under oxidative stress, the function of FOXO3a is regulated by SIRT1 and SIRT2 deacetylation (10, 11), FOXO3a can regulate reactive oxygen metabolism by inhibiting mitochondrial gene expression (12), and association of β -catenin with FOXO4 or FOXO3a enhances FOXO transcriptional activity (13). In addition, redox-dependent formation of cysteine-thiol disulfide modulates FOXO activity (14, 15). It is also evident that the role of FOXO family members in oxidative stress signaling might account for their function in aging and cancer (16, 17).

The von Hippel-Lindau (VHL) gene is a classic tumor suppressor, and its inactivation is linked to the development of clear-cell renal cell carcinomas, hemangioblastomas, pheochromocytomas, and tumors in other organs (18). The best characterized function of pVHL is its ability to function as a substrate recognition subunit of a multiprotein E3 ubiquitin ligase complex that targets proline hydroxylated hypoxia-induced factor (HIF) 1/2 α for proteasomal degradation under normoxic conditions (18, 19). Therefore, pVHL acts as a key regulator in hypoxia signaling.

Despite cellular stress induced by reactive oxygen, cellular oxygen is a fundamental factor that controls developmental processes as well as normal tissue homeostasis. Oxygen is required for metabolism, ATP production, and cell survival (20, 21). To

* The authors declare that they have no conflicts of interest with the contents of this article.

[5] This article contains supplemental Movies 1 and 2.

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² Supported by National Natural Science Foundation in China (NSFC) Grant 31401072.

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⁴ The abbreviations used are: FOXO, forkhead box O; Mn-SOD, manganese superoxide dismutase; VHL, von Hippel-Lindau; HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; ROS, reactive oxygen species; DBE, DNA binding element; ER, estrogen receptor; 4-HT, 4-hydroxy-tamoxifen; WISH, whole-mount *in situ* hybridization; HMA, heteroduplex mobility assay; MT, mutant; dpf, days post-fertilization; hpf, hours post-fertilization; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats (CRISPR)-

associated RNA-guided endonuclease Cas9; FKHR, forkhead in rhabdomyosarcoma; sgRNA, single guide RNA; DMOG, dimethyl-oxalyglycine.

maintain oxygen homeostasis, organisms use the hypoxia signaling pathway for adapting to low-oxygen conditions (22). The hypoxia-inducible factors HIF-1 α and HIF-2 α coordinate the cellular response to hypoxic conditions by inducing gene expression (23). The prolyl hydroxylase (PHD) gene family and the pVHL E3 ligase complex control HIF activity through oxygen-dependent hydroxylation and subsequent proteasomal degradation (24).

Research has shown that mitochondrially derived reactive oxygen species (ROS) can activate hypoxia signaling (25). Because FOXO3a, as well as FOXO4, can up-regulate *Mn-SOD* and other antioxidant enzymes (9, 11), FOXO transcription factors can inhibit HIF-1 activation by reducing the levels of mitochondrially derived ROS, suggesting a connection between FOXO transcription factors and hypoxia signaling. Moreover, FOXO3a has been shown to up-regulate *CITED2* for inhibiting HIF1-induced apoptosis in response to hypoxic stress (2) and mediates the suppressor role of the tumour-suppressor phosphatase with tensin homology (PTEN) on p300-dependent HIF-1 activity (10). In addition, FOXO3a has been shown to promote metabolic adaptation to hypoxia by antagonizing Myc function (18). Although these observations preliminarily outline the role of FOXO3a in hypoxia signaling, the subtle process and the underlying mechanisms are still largely unknown.

In zebrafish, there are two orthologs of the mammalian FOXO3 gene, *foxo3a* and *foxo3b*. Zebrafish *foxo3b* was initially identified and named as *zFKHR/foxo5*, whose protein shared 55% identity with that of human FOXO3a (26). The phylogenetic analysis of vertebrate FOXO protein sequences showed that, compared with *foxo3a*, *foxo3b* was much closer to mammalian FOXO3a (26).

To elucidate the function of *FOXO3a* in hypoxia signaling, we examined the regulation of *VHL* by FOXO3a. In this study, we found that *VHL* was a direct downstream target of *FOXO3a*. Using a zebrafish model, we not only corroborate that *vhl* is regulated by *foxo3b* but also reveal that *foxo3b* could modulate hypoxia signaling through regulating *vhl*.

Results

VHL* Is a Direct Downstream Target of *FOXO3a—To investigate whether *VHL* was transcriptionally regulated by *FOXO3a*, promoter assays were undertaken. As shown in Fig. 1A, luciferase reporters containing the full-length or truncated portions of the human *VHL* promoter (−2101 to +66, −989 to +66, −599 to +66, −499 to +66, and −371 to +66; the transcription start site is designated +1) were transiently transfected into HEK293T cells together with a Myc empty vector or Myc tagged-FOXO3a expression vector. Overexpression of FOXO3a induced activity of promoter reporters including −2101 to +66, −989 to +66, and −599 to +66 but not of promoter reporters including −499 to +66 and −371 to +66 (Fig. 1B). Notably, among three FOXO3a-inducible reporters (−2101 to +66, −989 to +66, and −599 to +66), the reporter −599 to +66 had the highest activity, suggesting that the FOXO DNA binding element (DBE) might locate in the region between −599 to +66. After carefully searching, we identified one potential FOXO DBE at position −567 to −561 (TTGT-

TAC) in the *VHL* promoter (9) (Fig. 1C). A single G-to-C substitution in the putative FOXO DBE completely abolished FOXO3a- and FOXO3a-3A-mediated up-regulation of *VHL* promoter reporter activity (Fig. 1D) (FOXO3a-3A is an active form of FOXO3a with a T32A/S253A/S315A triple mutation (2)).

To further confirm the above observations, we established an inducible FOXO3a-expressing human embryonic kidney cell line (HEK293T) using a lentivirus expressing a conditionally active FOXO3a-A3-ER fusion protein that consisted of a T32A/S253A/S315A triple mutant fused with the ligand-binding domain of the estrogen receptor (ER) (2, 9). Expression of FOXO3a-A3-ER fusion protein in HEK 293T cells was confirmed by Western blotting assays (Fig. 1E). Treatment of the cells with 4-hydroxy-tamoxifen (4-HT), a modified ligand for the ER, for 16–20 h (27) significantly increased luciferase activity of the wild-type *VHL* promoter reporter ($p < 0.01$) but not of the mutated *VHL* promoter reporter (Fig. 1F), further validating the induction and binding region of FOXO3a on the *VHL* promoter.

To determine whether FOXO3a can directly bind to the *VHL* promoter, we designed a pair of primers that could amplify a fragment encompassing the potential FOXO DBE and performed ChIP using anti-FOXO3a antibody. As shown in Fig. 2, A and B, *in vivo* binding of FOXO3a to the *VHL* promoter region encompassing the FOXO DBE was observed. Further semiquantitative RT-PCR assays validated this binding ability of FOXO3a (Fig. 2C).

Subsequently, we determined the induction of *VHL* by FOXO3a in the inducible HEK293T cell line. After treatment of cells with 4-HT for 24 h, a 5-fold increase in *VHL* mRNA was observed (Fig. 2D). The induction of pVHL protein by activating FOXO3a was further verified (Fig. 2E).

To determine the effect of endogenous FOXO3a on *VHL* expression, we used two sets of *FOXO3a* shRNA to knock down endogenous FOXO3a via lentivirus infection. First, we confirmed the efficiency of shRNA-mediated *FOXO3a* knockdown via semiquantitative RT-PCR assays in HEK293T and H1299 cells (Fig. 3, A and C). By contrast, knockdown of *FOXO3a* reduced the *VHL* mRNA level in HEK293T and H1299 cells (Fig. 3, B and D). Moreover, reduction of the pVHL protein level and enhancement of the HIF-1 α protein level when *FOXO3a* was knocked down was validated (Fig. 3, E and F). Taken together, these results suggest that *VHL* might be a direct downstream target of *FOXO3a*.

FOXO3a* Suppresses Hypoxia-inducible Gene Expression via *VHL—To understand the biological consequence of FOXO3a up-regulating *VHL*, we examined whether FOXO3a has impacts on the function of *VHL*. To date, the well defined function of *VHL* is to modulate hypoxia-inducible gene expression via mediating oxygen-dependent HIF- α proteasomal degradation (19). Therefore, we examined the effect of *FOXO3a* on hypoxia-inducible gene expression. Overexpression of *FOXO3a* could inhibit expression of typical hypoxia-inducible genes, *SLC2A1* and *LDHA* (28), in HEK293T cells under normoxia (Fig. 4, A and B, left columns).

However, we noticed that, under normoxia, the base level of hypoxia-inducible genes, including *SLC2A1* and *LDHA*, was

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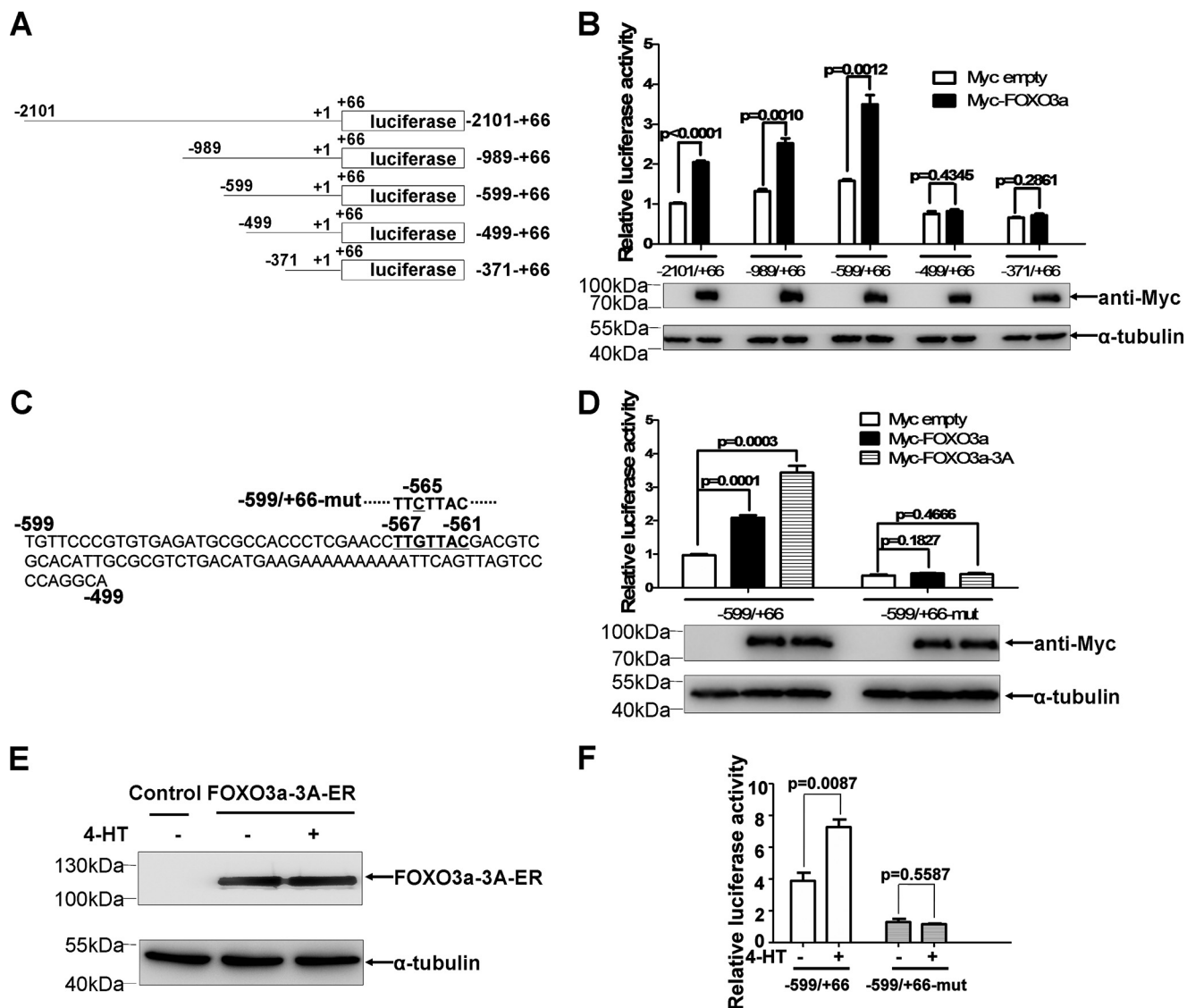


FIGURE 1. VHL is a direct downstream target of FOXO3a. *A*, schematic of VHL promoter constructs. *B*, promoter reporter activities were measured after transient transfection with a Myc empty vector or Myc-FOXO3a expression vector in HEK293T cells. *C*, one suboptimal FOXO DBE, TTGTAC (*bold* and *underlined*), was localized to the -567 to -561 region of the VHL promoter. *D*, compared with the wild-type VHL promoter reporter ($-599/+66$), the mutant VHL promoter reporter (single G-to-C substitution in the putative FOXO DBE) was not up-regulated by overexpression of FOXO3a and FOXO3a-3A in HEK 293T cells. *E*, expression of FOXO3a-3A-ER was confirmed by Western blotting assays. *F*, the mutant VHL promoter reporter (single G-to-C substitution in the putative FOXO DBE) was not up-regulated by FOXO3a activation. HEK 293T cells that constitutively express FOXO3a-A3-ER fusion protein were transfected with the wild-type or mutant VHL promoter reporters in the absence or presence of 4-HT (500 nM).

very low because of lack of HIF- α . Therefore, it was difficult to evaluate the effect of FOXO3a on hypoxia-inducible gene expression because of the low values detected. Of note, in some cases, it appeared that PHD still had a partial effect on HIF- α function under certain hypoxic conditions (29, 30); thus, VHL could affect HIF- α function as well under these kinds of hypoxic conditions. Because HIF- α protein was enhanced, resulting in a higher expression level of hypoxia-inducible genes, it became much easier to evaluate the effect of these gene expressions (29, 31), particularly for evaluating the suppression role. To get a clear picture of the role of FOXO3a in hypoxia-inducible gene expression, we further examined the effect of FOXO3a under hypoxia. Similar to the tendency detected under normoxia, overexpression of FOXO3a also inhibited expression of SLC2A1 and LDHA under hypoxia (Fig. 4, *A* and *B*, right columns). In addition, the inhibitory effect of FOXO3a on hypox-

ia-inducible gene expression was more obvious under hypoxia compared with normoxia, which might result from a higher base level of hypoxia-inducible genes under this kind of hypoxic condition.

By contrast, knockdown of FOXO3a in H1299 cells resulted in up-regulation of SLC2A1 and LDHA under normoxia and hypoxia (Fig. 4, *C* and *D*). To validate whether the suppression of FOXO3a on expression of hypoxia-inducible genes is mediated by VHL, we knocked down VHL by shRNA (32) and then conducted further assays (Fig. 4*E*). Fig. 4*F* shows that, when VHL was knocked down, the inhibitory role of FOXO3a on SLC2A1 expression was abrogated under normoxia. Moreover, knockdown of VHL also abolished the inhibitory role of FOXO3a on SLC2A1 expression under hypoxia (Fig. 4*G*), further indicating that, under the hypoxic conditions of our experiments in this study, VHL still had some effects on HIF- α func-

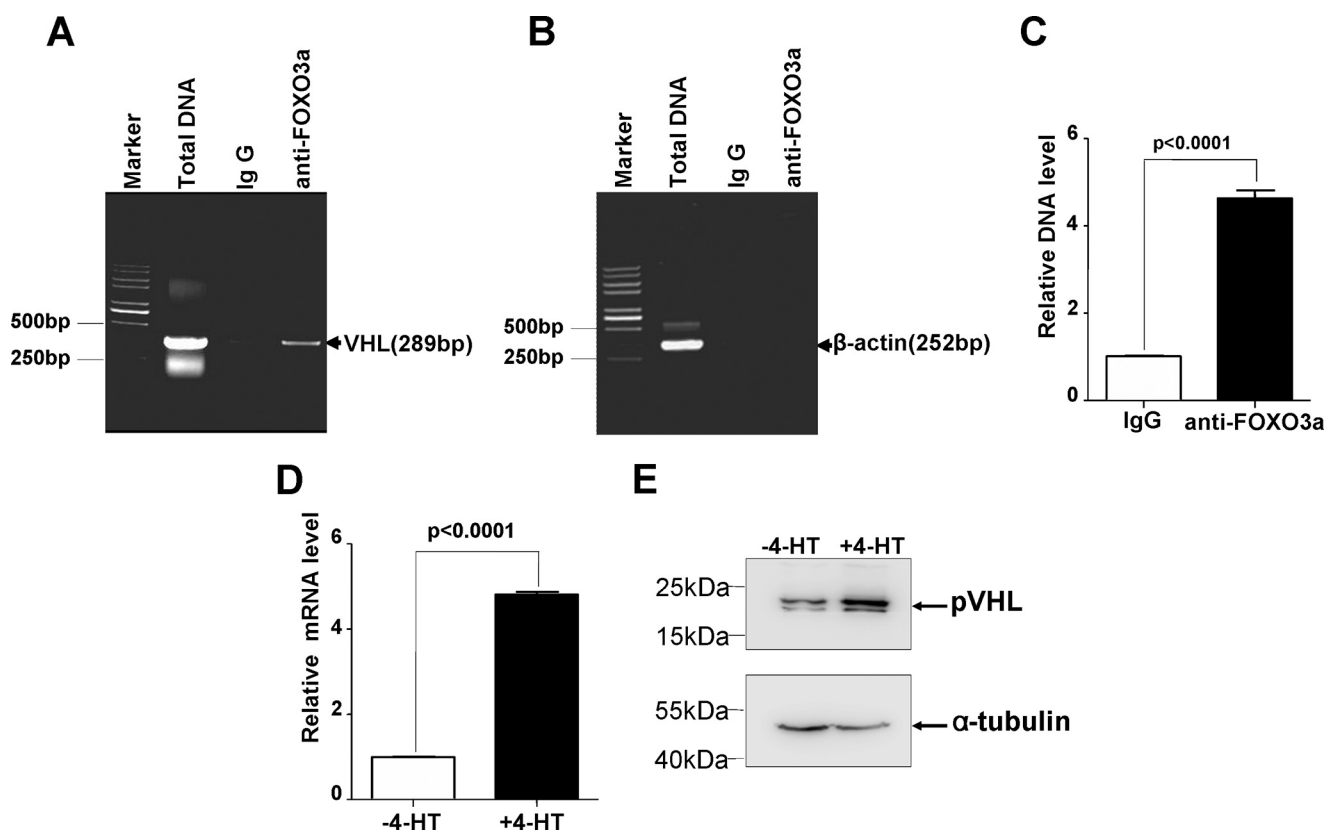


FIGURE 2. **FOXO3a directly regulates VHL expression.** A–C, chromatin immunoprecipitation analysis confirmed that FOXO3a directly interacted with the VHL promoter region harboring the FOXO DBE (A and B), which was confirmed by semiquantitative real-time RT-PCR assays (C). D, semiquantitative real-time RT-PCR assays revealed that activation of FOXO3a with 500 nM 4-HT caused VHL mRNA to be increased. E, induction of pVHL by FOXO3a was confirmed by Western blotting assays.

tion. These data suggest that *FOXO3a* might modulate hypoxia signaling via regulation of *VHL*.

Zebrafish vhl Is a Downstream Target of foxo3b—To further determine the regulation of *VHL* by *FOXO3a* and the underlying biological consequences *in vivo*, we took advantage of the zebrafish model. Initially, we examined the expression patterns of both *foxo3b* mRNA and *vhl* mRNA in different organs or tissues of adult zebrafish (3 months old) via semiquantitative RT-PCR assays. *Foxo3b* was highly expressed in muscle and then in the brain, heart, and liver (Fig. 5A). Interestingly, *vhl* was also highly expressed in muscle and then in the brain, heart, and liver (Fig. 5B). The similar expression patterns in *foxo3b* and *vhl* suggested that these two genes might be functionally correlated.

Subsequently, we conducted promoter assays in embryos to determine whether ectopic expression of *foxo3b* could activate the *vhl* promoter. Injection of an HA-*foxo3b* expression vector in zebrafish embryos induced zebrafish *vhl* promoter reporter activity (Fig. 5C). Moreover, the T30A/S223A/S289A *foxo3b*-3A mutant stimulated *vhl* promoter reporter activity to a greater extent than that observed for wild-type *foxo3b*, which was similar to a mammalian FOXO3a-3A mutant (Fig. 5D) (2). By contrast, *vhl* promoter reporter activity was not induced by the dominant-negative *foxo3b* (1–277 amino acids) (Fig. 5D).

Next, we employed whole-mount *in situ* hybridization (WISH) assays for determining the regulation of *vhl* by *foxo3b*. The expression pattern of *foxo3b* by WISH has been reported

previously (26). The expression patterns of *vhl* by WISH during zebrafish embryogenesis are shown in Fig. 6. During early embryogenesis, expression of *vhl* was ubiquitous. However, in the later stages of embryogenesis, *vhl* was expressed specifically in the otic vesicle, pectoral fin, inner ear, swimming bladder, kidney, and liver (Fig. 6). Ectopic expression of the zebrafish *foxo3b* by mRNA injection increased *vhl* expression in a dose-dependent manner (Fig. 7A). The induction of *vhl* by ectopic expression of *foxo3b* was further validated by semiquantitative RT-PCR assays (Fig. 7B). By contrast, knockdown of *foxo3b* using morpholino oligonucleotides, validated previously for specificity and efficiency (26), reduced *vhl* expression (Fig. 7C). Similarly, injection of dominant-negative *foxo3b* mRNA reduced *vhl* expression (Fig. 7D).

Targeted Disruption of foxo3b in Zebrafish Enhances Hypoxia-inducible Gene Expression and Impairs Zebrafish Hypoxia Tolerance—To further determine the regulation of *vhl* by *foxo3b* and the function of *foxo3b* in response to hypoxia, we deleted *foxo3b* in zebrafish via CRISPR/Cas9 technology. To disrupt the *foxo3b* gene in zebrafish, we designed an sgRNA targeting a region in exon 2 of *foxo3b* (Fig. 8A). After microinjecting the synthesized sgRNA and Cas9 mRNA into one-cell-stage embryos, we initially employed a heteroduplex mobility assay (HMA) to examine the efficiency of sgRNA/Cas9-mediated *foxo3b* disruption in the F1 generation (Fig. 8B) and then conducted a sequencing confirmation in the F1 generation. After screening, we obtained one mutant in the *foxo3b* gene

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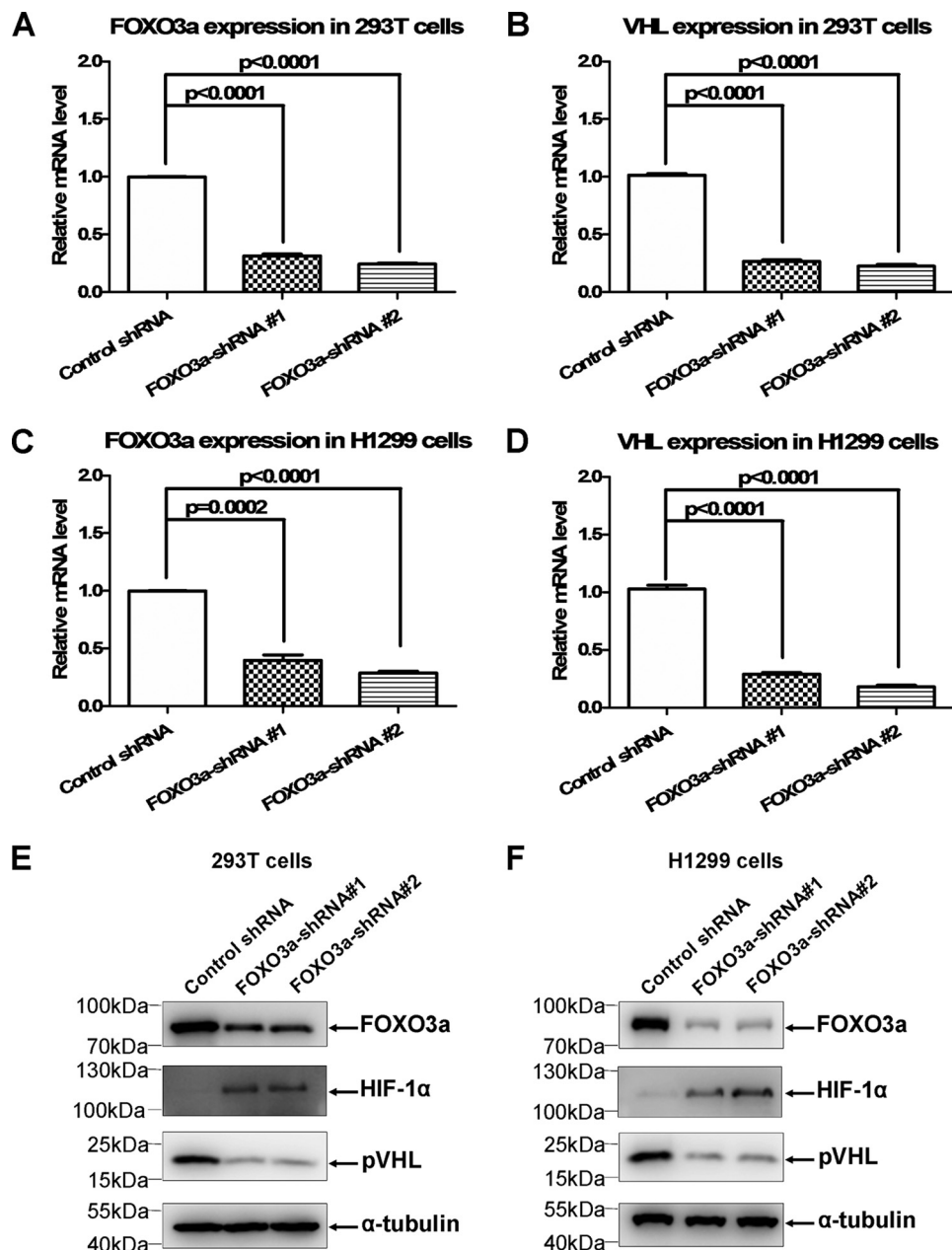


FIGURE 3. Knockdown of FOXO3a causes VHL expression to be reduced. *A*, knockdown of FOXO3a by two sets of FOXO3a shRNA in HEK293T cells was confirmed by semiquantitative real-time RT-PCR assays. Luciferase shRNA was used as a control. *B*, knockdown of FOXO3a in HEK293T caused VHL expression to be reduced. *C*, knockdown of FOXO3a by two sets of FOXO3a shRNA in H1299 cells was confirmed by semiquantitative real-time RT-PCR assays. Luciferase shRNA was used as a control. *D*, knockdown of FOXO3a in H1299 cells caused VHL expression to be reduced. *E*, knockdown of FOXO3a resulted in reduction of endogenous pVHL and induction of endogenous HIF-1 α in HEK293T cells, as confirmed by Western blotting assays. *F*, knockdown of FOXO3a resulted in reduction of endogenous pVHL and induction of endogenous HIF-1 α in H1299 cells, as confirmed by Western blotting assays.

(hereafter designated MT) (Fig. 8C). We crossed *foxo3b*^{+/-} × *foxo3b*^{+/-} and obtained offspring with the *foxo3b*^{+/+}, *foxo3b*^{+/-}, and *foxo3b*^{-/-} genetic background at Mendel's ratio (1:2:1). From the embryonic stages to adulthood, *foxo3b*^{-/-} homozygosity was indistinguishable from wild-type siblings. Overall, no obvious phenotypes were observed in *foxo3b*^{-/-} zebrafish under normal conditions. Semiquantitative RT-PCR analysis indicated that *foxo3b* mRNA was largely reduced in *foxo3b*-null zebrafish under either normoxia or hypoxia (Fig. 8D), which suggested that *foxo3b* was effectively disrupted. Similarly, *vhl* mRNA was also reduced in *foxo3b*-null zebrafish under either normoxia or hypoxia, but the reduction

rate was not as dramatic as that of *foxo3b* (Fig. 8E). These data suggested that *vhl* was transactivated by *foxo3b* *in vivo* and that other FOXO family members in zebrafish might have a partially redundant function for inducing *vhl* expression.

Subsequently, we examined whether some common hypoxia-inducible genes were affected following disruption of *foxo3b*. As shown in Fig. 9, *A–D*, expression of *veg*, *pou5f1*, *pai1*, and *il11a* was enhanced in *foxo3b*-null zebrafish under hypoxia (28, 33). These data suggested that *foxo3b* affected the expression of hypoxia-inducible genes. Consistently, when the embryos were treated with DMOG, a hydroxylase inhibitor (34), expression of *veg* and *pou5f1* was also enhanced in *foxo3b*-null zebrafish (Fig.

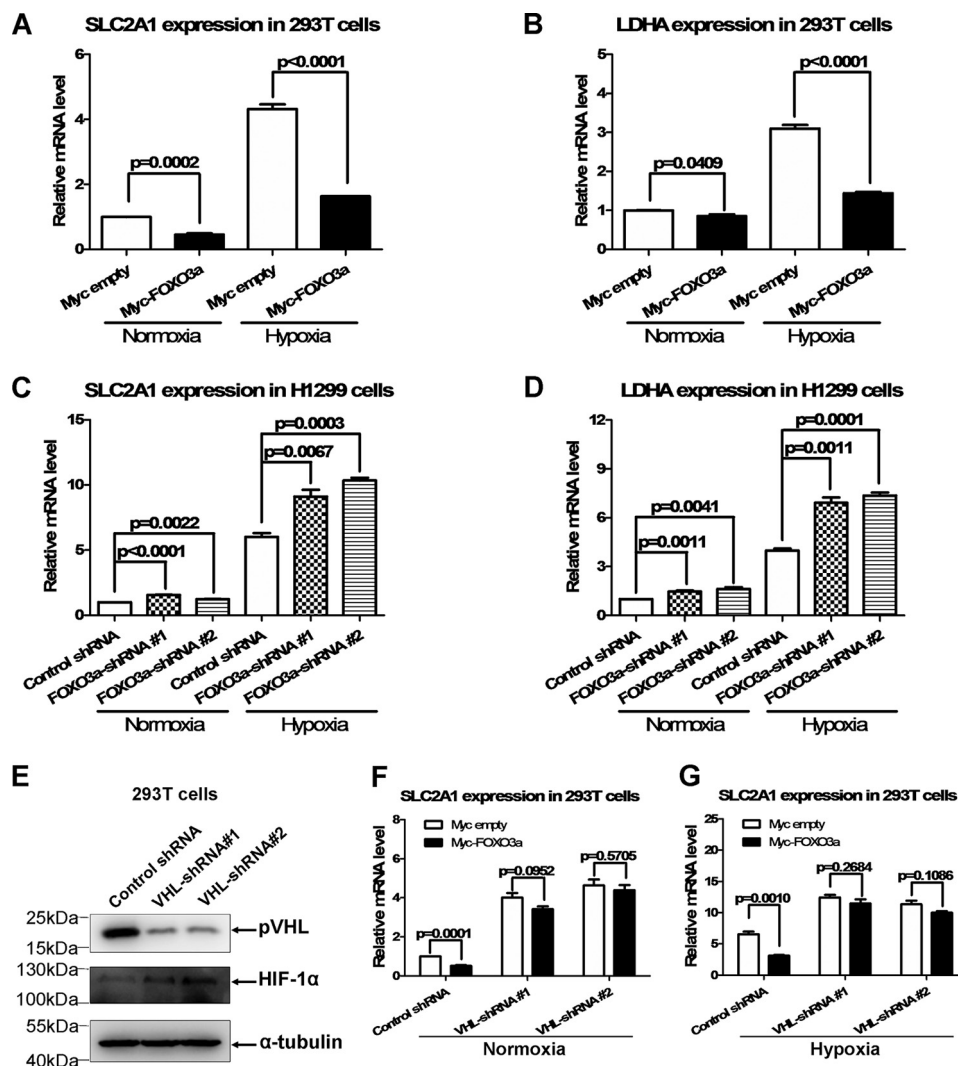


FIGURE 4. FOXO3a suppresses hypoxia-inducible gene expression via VHL. *A*, overexpression of FOXO3a caused SLC2A1 expression to be reduced in HEK293T cells under normoxia and hypoxia (2% O₂). *B*, overexpression of FOXO3a caused LDHA expression to be reduced in HEK293T cells under normoxia and hypoxia (2% O₂). *C*, knockdown of FOXO3a caused SLC2A1 expression to be increased in H1299 cells under normoxia and hypoxia (2% O₂). *D*, knockdown of FOXO3a caused LDHA expression to be increased in H1299 cells under normoxia and hypoxia (2% O₂). *E*, knockdown of endogenous VHL by transient transfection with two sets of VHL shRNA was confirmed by Western blotting assays. *F*, when VHL was knocked down, the suppressive effect of FOXO3a on SLC2A1 expression was abrogated under normoxia. *G*, knockdown of VHL abolished the suppressive effect of FOXO3a on SLC2A1 expression under hypoxia.

9, *E* and *F*). Taken together, these data indicated that disruption of *foxo3b* in zebrafish enhanced the expression of hypoxia-inducible genes and that *foxo3b* could modulate hypoxia signaling.

To determine whether the effect of *foxo3b* on hypoxia-inducible gene expression was indeed mediated by the regulation of *vhl*, we took advantage of *vhl*-null zebrafish embryos (35). In wild-type sibling embryos, injection with *foxo3b* mRNA inhibited the expression of four hypoxia-inducible genes (*veg*, *ldha*, *cited2*, and *il11a*) significantly compared with those injected with *GFP* mRNA (Fig. 10, *A–D*). By contrast, in *vhl*-null embryos, injection with *foxo3b* mRNA had no obvious effect on expression of the four hypoxia-inducible genes (*veg*, *ldha*, *cited2*, and *il11a*) compared with those injected with *GFP* mRNA (Fig. 10, *A–D*). Expression of injected *foxo3b* and *GFP* mRNA was further confirmed by Western blotting assays (Fig. 10*E*) and fluorescence stereomicroscope, respectively (Fig. 10*F*). These data suggested that the suppressive role of *foxo3b* in

expression of hypoxia-inducible genes was mediated by up-regulation of *vhl*.

To determine the biological consequences of *foxo3b* modulating hypoxia signaling, we compared the acute hypoxia tolerance in *foxo3b*-null zebrafish and their wild-type siblings. Under 2% oxygen environmental conditions, *foxo3b*-null fry (15 dpf) died earlier than their wild-type siblings (Fig. 11). Similarly, with adult zebrafish, under 10% oxygen environmental conditions, *foxo3b*-null adults (3 months old) also died earlier than their wild-type siblings (supplemental Movies 1 and 2). These data suggested that *foxo3b* potentially affected zebrafish survival via modulation of hypoxia signaling.

Discussion

Low oxygen tensions activate hypoxia signaling by promoting the protein stability of HIF-1α and HIF-2α (19, 21). As the key transcriptional regulators of hypoxia signaling, the regulation of HIF-1α and HIF-2α is well documented. Under nor-

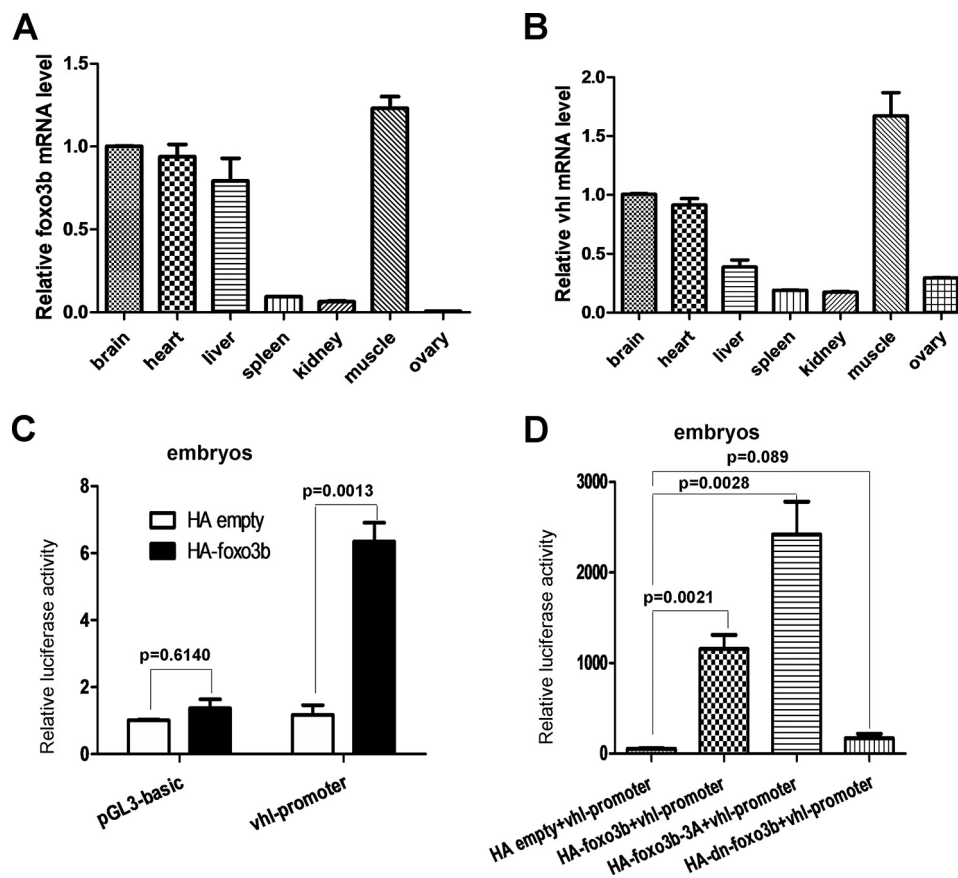


FIGURE 5. **The expression pattern of *vhl* in zebrafish and the effect of *foxo3b* on *vhl* promoter activity.** *A* and *B*, semiquantitative real-time RT-PCR assays demonstrated the expression pattern of *foxo3b* (*A*) and *vhl* (*B*) in different tissues of adult zebrafish. *C*, zebrafish *vhl* promoter activity was induced after injection of the *foxo3b* expression vector in embryos. *D*, injection of a *foxo3b*-3A mutant induced zebrafish *vhl* promoter activity more dramatically (third column versus second column). The *vhl* promoter was not activated after injection of a *foxo3b* dominant-negative mutant (*dn-foxo3b*, $p = 0.089$).

moxia, PHD1–3 use oxygen as a substrate to hydroxylate key proline residues of HIF-1 α and HIF-2 α , resulting in subsequent proteasomal degradation by the pVHL E3 ubiquitin ligase complex (36–38). Under hypoxia, PHD activity is inhibited, resulting in HIF-1 α and HIF-2 α stabilization and translocation to the nucleus for activating downstream genes involved in systematic and cellular adaptation to hypoxia (39). Therefore, PHDs (PHD1–3) and pVHL have been recognized as the major regulators for modulating hypoxia signaling through regulating the protein stability of HIF-1 α and HIF-2 α . In fact, it is evident that some factors could affect hypoxia signaling via regulation of PHDs and pVHL (29, 31, 40, 41, 43). Although FOXO3a has been reported to affect hypoxia signaling, it has not been shown to directly modulate the major proteins involved in hypoxia signaling, including PHDs and pVHL. Here we show that FOXO3a (*foxo3b* in zebrafish) transactivates pVHL (*vhl* in zebrafish) expression, resulting in suppression of hypoxia signaling, which provides additional evidence for supporting the role of FOXO3a in hypoxia signaling. Intriguingly, FOXO3a, together with other FOXO family members, exhibits tumor-suppressive functions (17, 44). It is noteworthy that pVHL is a well known classic tumor suppressor (45). Thus, FOXO3a, and other related FOXO proteins, might function as tumor suppressors by up-regulating pVHL expression. Confirmation of whether pVHL is transactivated by other FOXO family genes

will further expand our knowledge about the function of the FOXO family in tumor suppression.

As a component of an E3 ubiquitin ligase complex, the targets of pVHL other than HIF- α have been identified (35, 46–48). Notably, the regulation of pVHL at the protein level through protein-protein interactions or posttranslational modifications has been revealed, which can affect VHL function in mediating its targets for proteasomal degradation (15, 31, 49). However, the regulation of VHL at the transcriptional level has largely remained underreported (50). Here we provide data to show the transcriptional regulation of VHL by FOXO3a. Future studies to identify additional transcription factors that can transactivate VHL will further our understanding of the regulation of VHL and the underlying mechanism thoroughly.

Even though many genes have been reported to affect hypoxia signaling directly or indirectly, it is still unclear whether these genes have any impact on animal hypoxic tolerance (acute hypoxia) or hypoxic adaptation (chronic hypoxia) (43). In addition, a key question regarding the functionality of these genes under animal hypoxic tolerance or hypoxic adaptation remains unanswered. Based on studies of human high-altitude adaptation, especially Tibetan adaptation studies, positive directional selection is identified in the HIF pathway and hypoxia-related genes (51). Among these genes, HIF-2 α and

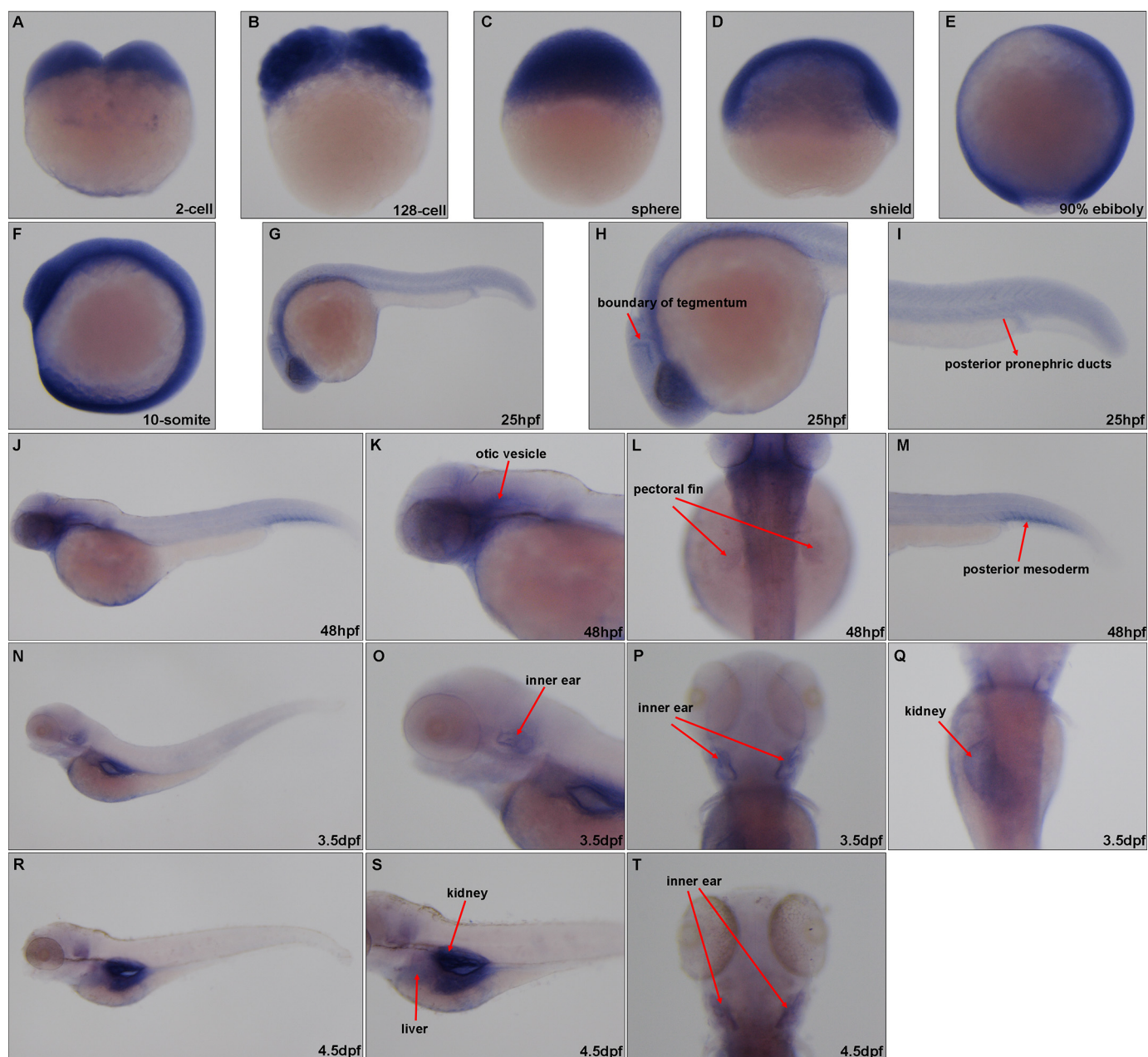


FIGURE 6. The expression pattern of *vhl* during zebrafish embryogenesis as detected by WISH. A–F, *vhl* was ubiquitously expressed before the 10-somite stage. G–I, 25 hpf, *vhl* was highly expressed in the head and anterior mesoderm. J–M, 48 hpf, *vhl* was highly expressed in the otic vesicle, pectoral fin, and posterior mesoderm. N–Q, 3.5 dpf, *vhl* was highly expressed in the swimming bladder, inner ear, and kidney. R–T, 4.5 dpf, *vhl* was highly expressed in the swimming bladder, liver, inner ear, and kidney.

PHD2 are particularly noteworthy because of the consistency with which they have been observed by different investigators and the biochemical evidence that shows the adaptive changes of these two genes and their effect on HIF signaling (51–54). These observations further support the vital role of the HIF pathway in hypoxic adaptation (51). It is likely that the genes affecting HIF signaling contribute to hypoxic tolerance or hypoxic adaptation (51). In this study, we showed that disruption of *foxo3b* in zebrafish led to impaired hypoxic tolerance, which might also be due to the influence of *foxo3b* on HIF activity via regulation of *vhl*. However, it should be acknowledged that the correlation between HIF activity and hypoxia tolerance or adaptation is undoubtedly complicated (51). The change of

the *PHD2* allele resulting in loss of function has been suggested to account for either hypoxic adaptation or hypoxic sensitivity (55, 56). On the other hand, change of the allele resulting in gain of function has also been suggested to respond to either hypoxic adaptation or hypoxic sensitivity (56, 57). In this regard, predicting the consequence of disruption of the genes involved in hypoxia signaling may not be simple. Given that HIF-1 α and HIF-2 α are key factors that orchestrate the hypoxic response, further delineation of the hypoxic response phenotypes of genes directly or indirectly involved in the HIF pathway in knockout and overexpression status will uncover animal hypoxic tolerance or hypoxic adaptation mechanistically.

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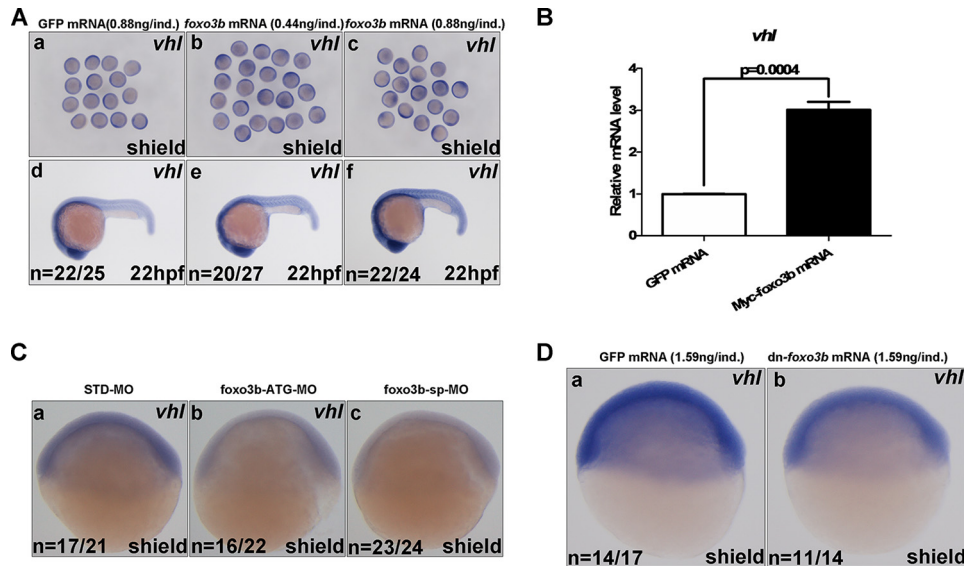


FIGURE 7. Zebrafish *foxo3b* enhances *vhl* expression. A, ectopic expression of *foxo3b* by mRNA injection induced *vhl* expression in embryos, as detected by WISH. GFP mRNA was used as a control. B, ectopic expression of *foxo3b* by mRNA injection enhanced *vhl* expression in embryos, as revealed by semiquantitative real-time RT-PCR assays. GFP mRNA was used as a control. C, knockdown of *foxo3b* in embryos by morpholino oligonucleotide injection reduced *vhl* expression. a, standard morpholino oligonucleotide control (STD-MO, 8 ng/individual embryo). b, *foxo3b* translation-blocking (ATG code) morpholino oligonucleotide (*foxo3b*-ATG-MO, 8 ng/individual embryo). c, *foxo3b* splicing-blocking morpholino oligonucleotide (*foxo3b*-sp-MO, 8 ng/individual embryo). D, injection of dominant-negative *foxo3b* mRNA (*dn-foxo3b*) in zebrafish embryos reduced *vhl* expression (b) compared with those injected with GFP mRNA (a).

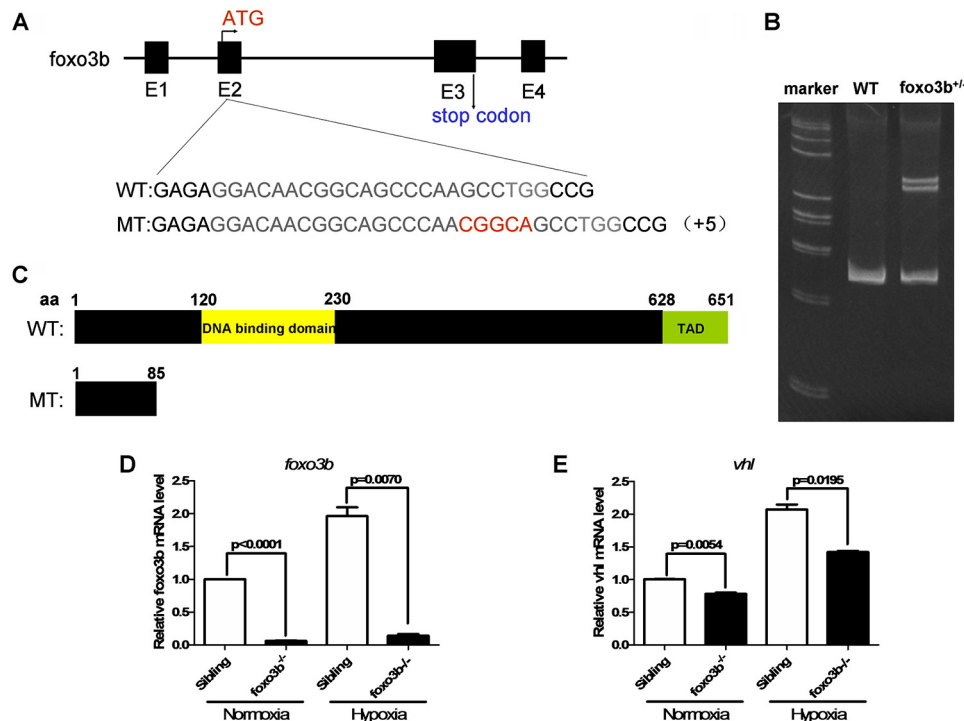


FIGURE 8. Generation of *foxo3b*^{-/-} zebrafish via CRISPR/Cas9 technology. A, targeting strategy for generating mutations in exon 2 of *foxo3b*. The sequence difference in *foxo3b* between the mutants and their wild-type siblings is indicated. B, verification of zebrafish *foxo3b* disruption by HMA. Genomic DNA was prepared from the mutants and their wild-type siblings. C, the predicted protein products of *foxo3b* in the mutants and their wild-type siblings. TAD, transactivation domain. D and E, expression of *foxo3b* (D) or *vhl* (E) in wild-type (*foxo3b*^{+/+}) and *foxo3b*-null (*foxo3b*^{-/-}) zebrafish embryos under normoxia and hypoxia.

Of note, mitochondrial ROS are required for hypoxic activation of HIFs, indicating a connection between two vital cellular stress signals (25). FOXO3a activates Mn-SOD expression to protect cells from oxidative stress (8, 9). In this study, we found that FOXO3a (*foxo3b*) could activate VHL (*vhl*) expression to enhance zebrafish hypoxic tolerance. Therefore, FOXO3a may

serve as a vital factor that orchestrates cells in response to reactive oxygen stress and hypoxic stress conditions.

Experimental Procedures

Plasmid Construction—The human VHL promoters were amplified by PCR and subcloned into the pGL3-Basic vector

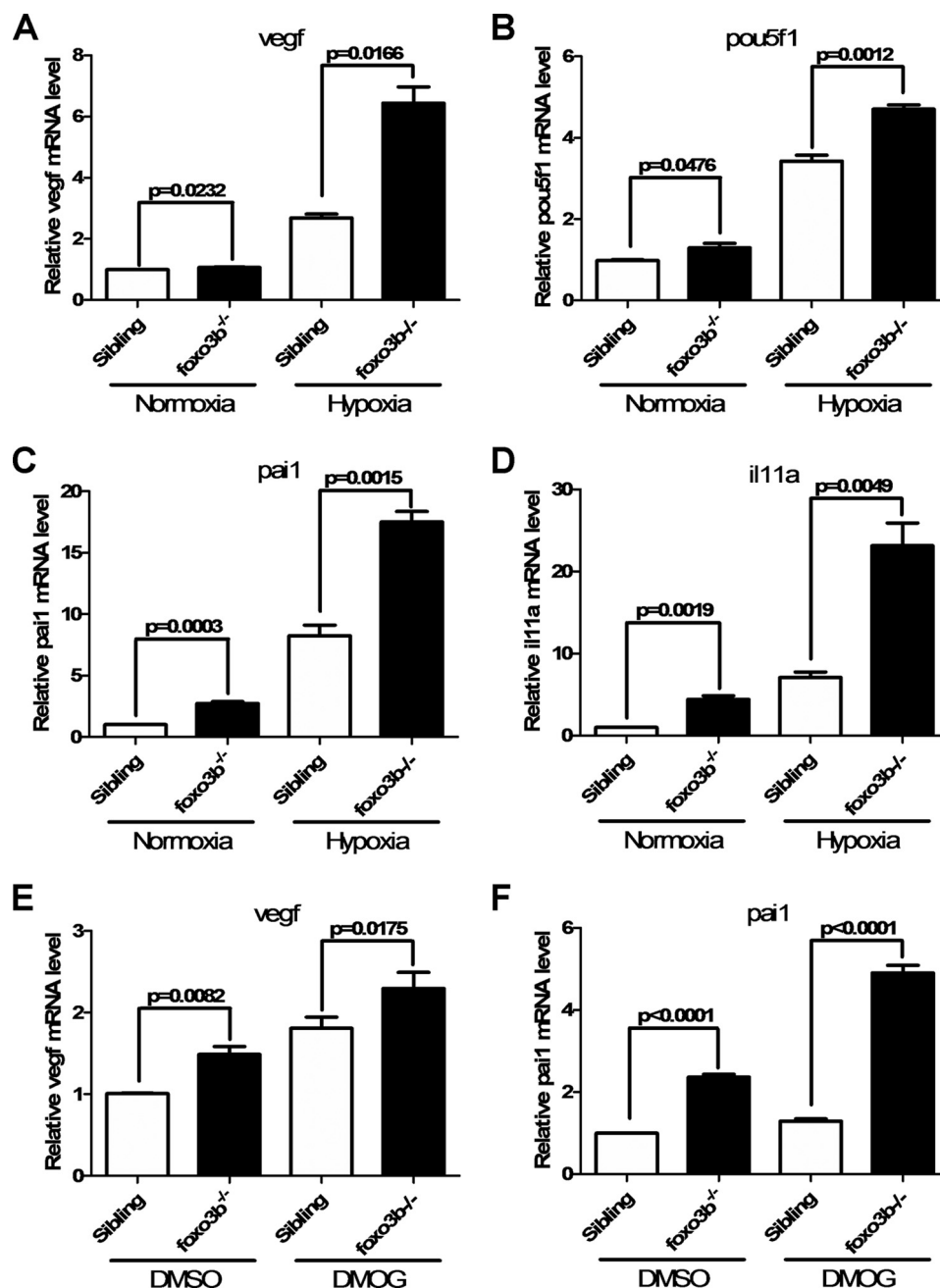


FIGURE 9. Zebrafish *foxo3b* suppresses hypoxia-induced gene expression. A, *foxo3b*-null embryos (*foxo3b*^{-/-}) exhibit higher *vegf* expression compared with their wild-type siblings (*foxo3b*^{+/+}) under normoxia and hypoxia. B, *foxo3b*-null embryos (*foxo3b*^{-/-}) exhibit higher *pou5f1* expression compared with their wild-type siblings (*foxo3b*^{+/+}) under normoxia and hypoxia. C, *foxo3b*-null embryos (*foxo3b*^{-/-}) exhibit higher *pai1* expression compared with their wild-type siblings (*foxo3b*^{+/+}) under normoxia and hypoxia. D, *foxo3b*-null embryos (*foxo3b*^{-/-}) exhibit higher *il11a* expression compared with their wild-type siblings (*foxo3b*^{+/+}) under normoxia and hypoxia. E, *foxo3b*-null embryos (*foxo3b*^{-/-}) exhibit higher *vegf* expression compared with their wild-type siblings (*foxo3b*^{+/+}) with or without DMOG (100 μ M) treatment. F, *foxo3b*-null embryos (*foxo3b*^{-/-}) exhibit higher *pai1* expression compared with their wild-type siblings (*foxo3b*^{+/+}) with or without DMOG (100 μ M) treatment.

(Promega). The deletion and DBE mutants of the VHL promoter were obtained by PCR and subcloned into the pGL3-Basic vector. FOXO3a-A3-ER was cloned into the lentivirus vector pHAGE-CMV-MCS-IZsGreen. FOXO3a shRNA was cloned into the lentivirus vector LentiLox3.7. The FOXO3a shRNA targeting sequences were 5'-GAGCTCTTGGTGGATCATC-3' and 5'-GCACAGAGTTGGATGAAGT-3'. Two sets of VHL shRNA targeting human VHL and GFP shRNA targeting GFP have been described previously (32).

The zebrafish *vhl* promoter was amplified by PCR from zebrafish genomic DNA using the following primers: forward, 5'-ATATCGGTACCGACTTGGAAAGTCTCGGAAGG-3'; reverse, 5'-ATATCCTCGAGCGTCAAAGACAGGACAGTTC-3'. The resultant product was subcloned into the pGL3-Basic vector. All plasmids were verified by sequencing.

Cell Line and Culture Conditions—HEK293T and H1299 cell lines were originally obtained from the ATCC. All cell lines were cultured in DMEM supplemented with 10% FBS.

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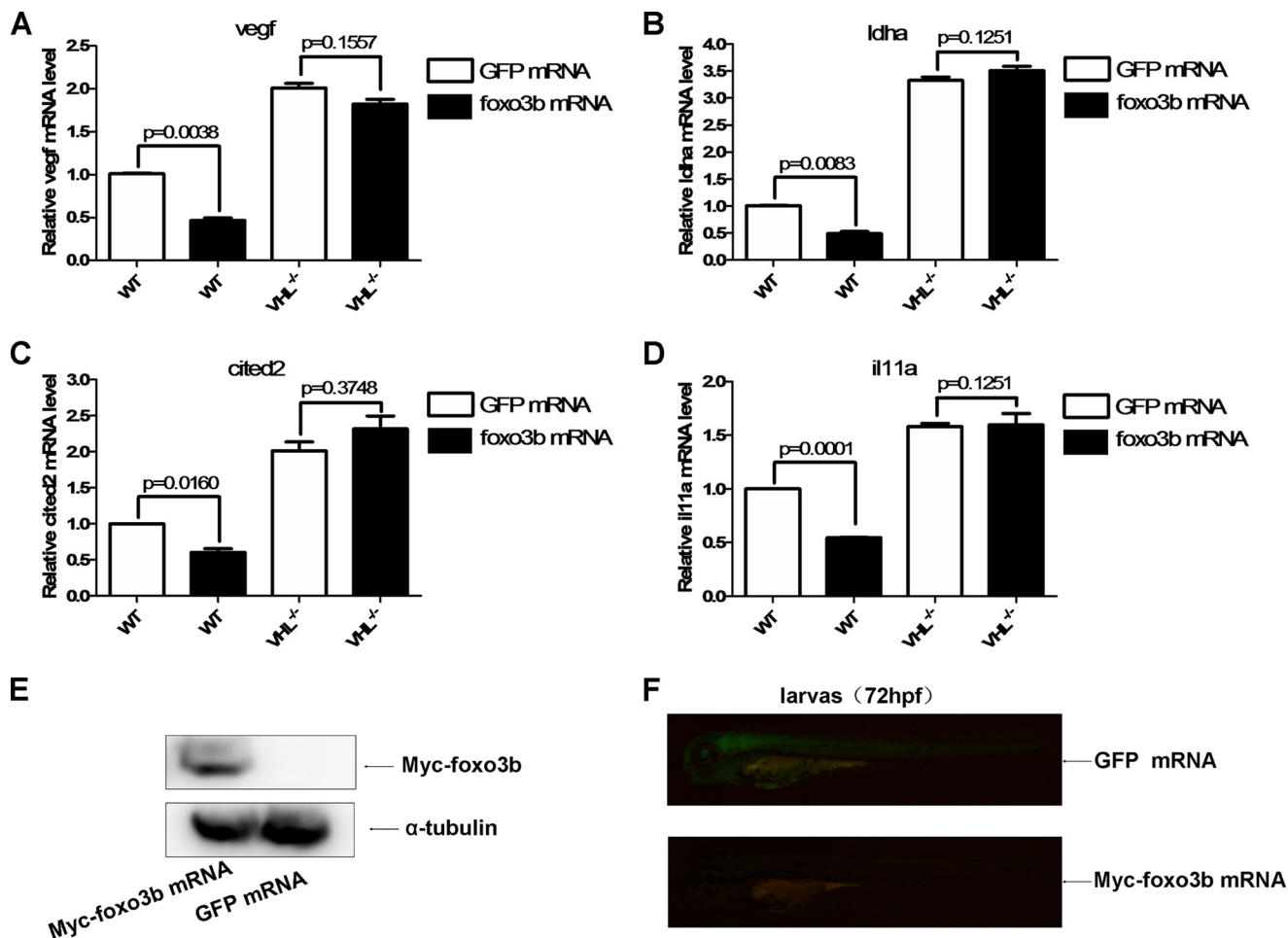


FIGURE 10. Zebrafish *foxo3b* suppresses hypoxia-induced gene expression through *vhl*. *A*, ectopic expression of *foxo3b* by mRNA injection suppressed *vegf* expression in wild-type sibling embryos (*vhl*^{+/+}) but not in *vhl*-null embryos (*vhl*^{-/-}), as revealed by semiquantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. *B*, ectopic expression of *foxo3b* by mRNA injection suppressed *ldha* expression in the wild-type sibling embryos (*vhl*^{+/+}) but not in *vhl*-null embryos (*vhl*^{-/-}), as revealed by semiquantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. *C*, ectopic expression of *foxo3b* by mRNA injection suppressed *cited2* expression in wild-type sibling embryos (*vhl*^{+/+}) but not in *vhl*-null embryos (*vhl*^{-/-}), as revealed by semiquantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. *D*, ectopic expression of *foxo3b* by mRNA injection suppressed *il11a* expression in wild-type sibling embryos (*vhl*^{+/+}) but not in *vhl*-null embryos (*vhl*^{-/-}), as revealed by semiquantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. *E*, expression of myc-tagged *foxo3b* mRNA injected into embryos was confirmed by Western blotting assays. *F*, expression of control *GFP* mRNA injected into embryos was examined under a fluorescence stereomicroscope (Leica M205).

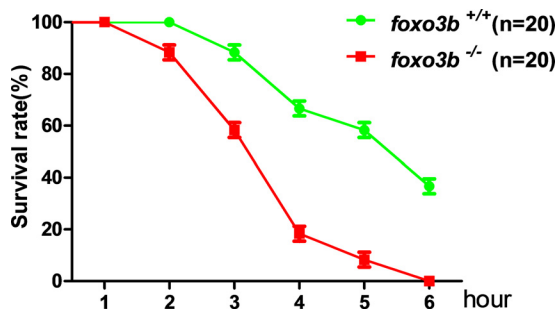


FIGURE 11. Foxo3b is required for hypoxia tolerance in zebrafish. Three groups of *foxo3b*-null zebrafish fry (15 dpf, 20/group) and their wild-type siblings (15 dpf, 20/group) were placed in the hypoxia chamber (Ruskin INVIVO₂400). The oxygen concentration was adjusted to 2% O₂ ahead of time. The dead fry counted once an hour until the death of the last fry with one genotype (*foxo3b*^{-/-}).

HEK293T cells that constitutively expressed the FOXO3a-A3-ER fusion protein were obtained by infecting HEK293T cells with a lentivirus expressing FOXO3a-A3-ER.

Luciferase Reporter Assay—HEK293T cells were grown in 24-well plates and transfected with the indicated luciferase

reporter, including pTK-*Renilla* as an internal control, using VigoFect reagent (Vigorous Biotech, Beijing, China). Luciferase activity was assayed 16–28 h after transfection using the Dual-Luciferase reporter assay system (Promega). Data were normalized to *Renilla* luciferase. Data are reported as mean + S.E. of three independent experiments performed in triplicate. The statistical analysis was performed using GraphPad Prism 5 (unpaired *t* test) (GraphPad Software Inc.).

For luciferase reporter assays in zebrafish, embryos were injected with the indicated plasmids and homogenized after 8–10 h. Luciferase activity was also determined using the Dual-Luciferase reporter assay system.

Semiquantitative Real-time RT-PCR—Total RNA was extracted by TRIzol reagent (Invitrogen), and cDNA synthesis was carried out using a first strand cDNA synthesis kit (Fermentas). The following primers were used to assess human VHL mRNA expression: forward, 5'-TCAGAGATGCAGGG-ACACACGATG-3'; reverse, 5'-ACCTGACGATGTCCAGT-CTCCTGTA-3'. The following primers were used to assess

human FOXO3a mRNA expression: forward, 5'-TGCTAAG-CAGGCCTCATCTC-3'; reverse, 5'-CTTGTGTCAGTTT-AGGGTCTG-3'. The following primers were used to evaluate the internal control, 18s RNA forward, 5'-TCAACTTCGATG-GTAGTCGCCGT-3'; reverse, 5'-TCCTTGGATGTGGTAG-CCGTTCT-3'. The following primers were used to assess zebrafish genes: *foxo3b*-RT, TCATCTTCAAGGAGGAATGC (forward) and GGATGGAGTTCTTCCAACCA (reverse); β -actin-RT, TACAATGAGCTCCGTGTTGC (forward) and ACATACAATGGCAGGGGTGTT (reverse); *vhl*-RT, GTGG-GACATCCATGGATGTT (forward) and CGTCAGCACAG-GCAATGTGA (reverse); *pou5f1*-RT, TGAGGAAGAGGAG-ACTCTGA (forward) and GACTGAACATTTTGCCATAC (reverse); *pai1*-RT, ATTCCAAGGTTCTCCATGGA (for-ward) and GGTTCCCTCAGTAGTAATGCG (reverse); *ldha*-RT, CCTTCTCAAGGATCTGACCG (forward) and AACT-GTAATCTTTATCCGC (reverse); *il1a*-RT, CCGGGTGT-TAGTACAGAGATT (forward) and CATGGAGCTGAGAA-AGAGTAGG (reverse); *cited2*-RT, GTTCCGAGACAGTAT-CGCTAAG (forward) and CATCAAGACCTCCTCGTCA-ATAA (reverse); and *vegf*-RT, TGCTCCTGCAAATTCAC-ACAA (forward) and ATCTTGGCTTTTCACATCTGCAA (reverse).

Data are reported as mean + S.E. of three independent experiments performed in triplicate. The statistical analysis was performed using GraphPad Prism 5 (unpaired *t* test) (GraphPad Software Inc.).

Chromatin Immunoprecipitation—An anti-FOXO3a antibody was purchased from Epitomics. The following primers were used for amplifying the VHL promoter region: forward, 5'-ATAAGCGTGATGATTGGGTGTTTC-3'; reverse, 5'-CC-CGAGTAGTTGGTACTGTAGGC-3'. The primers for ampli-fying β -actin and the procedure for ChIP were described previ-ously (58).

Western Blotting Assays—The following antibodies were used for Western blotting: Myc (9E10, Santa Cruz Biotechnol-ogy) and α -tubulin (Upstate). The human pVHL polyclonal antibody was generated against synthesized peptides of pVHL (Abmart, Shanghai, China). The pVHL (A0377), FOXO3a (A0102), and HIF-1 α (A6265) antibodies were purchased from ABclonal Co. The procedures for Western blotting assays were described previously (59). A Fuji Film LAS4000 mini lumines-cent image analyzer was used to image the blots.

Zebrafish Embryo Manipulation and Whole-mount *In Situ* Hybridization—Zebrafish (*Danio rerio*) strain AB was raised, maintained, reproduced, and staged according to standard pro-tocols. The probes for *foxo3b* and *vhl* were amplified by PCR from cDNA pools using the appropriate sets of primers. The primers for *foxo3b* probe were described previously (26). The following primers were used for the *vhl* probe: *vhl* forward, 5'-CTTTAGTCTAACTCGGTGGT-3'; *vhl* reverse, AGGCA-ATGTGATCTTGG-3'. The *foxo3b* antisense morpholino oligo-nucleotides (*foxo3b*-ATG-MO and *foxo3b*-sp-MO) and their validation were also described previously (26). The procedure for whole-mount *in situ* hybridization was described previously (60).

Generation of *foxo3b*-null Zebrafish—Disruption of *foxo3b* in zebrafish was accomplished *via* CRISPR/Cas9 technology.

Zebrafish *foxo3b* sgRNA was designed using the tools provided in the CRISPR Design web site (<http://crispr.mit.edu>). The zebrafish-Codon-Optimized Cas9 plasmid (61) was digested with XbaI, purified, and transcribed using the T7 mMessage Machine Kit (Ambion). The pUC19-gRNA vector was used for amplifying the sgRNA template (62). The primers for amplifying gRNA template were 5'-GTAATACGACTCACTATAG-GACAACGGCAGCCCAAGCCGTTT'TAGAGCTAGAAAT-AGC-3' and 5'-AAAAGCACCGACTCGGTGCC-3'. sgRNA was synthesized using the Transcript Aid T7 High Yield Tran-scription Kit (Fermentas). Cas9 RNA and sgRNA were mixed and injected into embryos at the one-cell stage. Cas9 RNA and sgRNA were injected at 0.75–1.25 ng/embryo and 0.075 ng/em-bryo, respectively.

After the injected embryos were incubated at 28.5 °C for 24 h, the genomic DNA was extracted from 20–30 embryos by heat-ing the embryos at 94 °C for 40 min in lysis solution (50 mM NaOH) (the reaction was terminated by adding 1 M Tris-HCl (pH 8.0)). Mutant detection was followed by HMA as described previously (42). If the results were positive, the remaining embryos were raised to adulthood and treated as F0, which were backcrossed with the wild-type zebrafish for generating F1, which were genotyped by HMA initially and confirmed by sequencing of target sites. The F1 zebrafish harboring the mutations were backcrossed with the wild-type zebrafish to obtain F2. The F2 adult zebrafish with the same genotype (+/–) were intercrossed to generate F3 offspring, which should contain wild-type (+/+), heterozygous (+/–), and homozy-gous (–/–) offspring. The primers for detecting mutants were 5'-TGGACATTGCCATTGATCCAG-3' (forward) and 5'-C-CATGCATTCTCCTTGAAGA-3' (reverse).

Hypoxia Treatments—In pilot experiments, we noticed that zebrafish fry were more resistant to hypoxic treatment than adult zebrafish, as judged by survival time in the hypoxia cham-ber (Ruskinn INVIVO₂400). Thus, we used 2% oxygen for zebrafish fry treatment and 10% oxygen for adult zebrafish treatment.

For zebrafish fry, two flasks were filled with 250 ml of water. *Foxo3b*-null fry (15 dpf, *n* = 20) were put into one flask, and their wild-type siblings (15 dpf, *n* = 20) were put in the second flask. Then, two flasks were put into the hypoxia chamber simultaneously. The oxygen concentration of the chamber was adjusted to 2% O₂ ahead of time. The dead fry were counted once an hour until the death of the last zebrafish with one geno-type (*foxo3b*^{–/–}). This experiment was repeated three times.

For the adult zebrafish experiment, the zebrafish were ini-tially weighed. During pilot experiments, we noticed that the body weight of the zebrafish could dramatically affect their acute hypoxic tolerance. To avoid interference by body weight in acute hypoxia tolerance determination, zebrafish of similar weight were chosen for further experiments. Subsequently, two flasks were filled with 250 ml of water. Three *foxo3b*-null zebrafish (0.36, 0.37, and 0.39 g) were put into one flask, and three wild-type siblings (0.36, 0.37, and 0.38 g) were put into the second flask. Before immersion in the hypoxia chamber (Ruskinn INVIVO₂400), the oxygen concentration in the flask water was measured by an HQd portable meter (HACH Co.). The oxygen concentration in the *foxo3b*-null zebrafish flask was

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7.10 mg/liter, and the oxygen concentration in the wild-type sibling flask was 7.04 mg/liter. The oxygen concentration in the hypoxia chamber was adjusted to 10% ahead of time. After putting the flasks containing zebrafish into the hypoxia chamber, the behavior of the zebrafish was closely monitored. When one genotypic zebrafish was expired, we took out the flasks from the chamber immediately and measured the oxygen concentration in the flask water. At that moment, the oxygen concentration in the *foxo3b*-null zebrafish flask was 1.48 mg/liter, and the oxygen concentration in the wild-type sibling flask was 1.54 mg/liter.

Author Contributions—W. X. designed the study and wrote the manuscript. X. L., X. C., Z. M., and B. H. designed the study, conducted the experiments, and analyzed the data. D. Z. generated the *foxo3b*-null zebrafish. G. O., J. W., and W. Z. contributed the reagents and analyzed the data. All authors analyzed the results and approved the final version of the manuscript.

Acknowledgments—We thank Drs. Peter Ratcliffe, William Kaelin, Frank Lee, Navdeep Chandel, William Tansey, Katja Knauth, Boudewijn Burgering, and Linda Penn for the generous gift of reagents.

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