JBC Papers in Press. Published on October 24, 2016 as Manuscript M116.745471
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M116.745471

Forkhead transcription factor 3a (FOXO3a) modulates hypoxia signaling *via* up-regulation of von Hippel-Lindau gene (VHL)

Xing Liu[1, 2], Xiaolian Cai[1,2], Bo Hu[1, 2], Zhichao Mei[1], Dawei Zhang[1], Gang Ouyang[1] , Jing Wang[1], Wei Zhang[1] and Wuhan Xiao[1, 3, *]

[1]The Key Laboratory of Aquatic Biodiversity and Conservation, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, P. R. China; [3]State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, P. R. China.

Running Title: FOXO3a modulates hypoxia signaling

[2]These authors contributed equally to this work.

*To whom correspondence should be addressed: Wuhan Xiao. Ph.D., Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, P.R. China. Fax: 86-27-68780087; E-mail: w-xiao@ihb.ac.cn

Key Words: FOXO3a, pVHL, hypoxia signaling, transcriptional regulation, zebrafish

ABSTRACT

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 (HIF-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. Herein, we show that FOXO3a directly binds to *VHL* promoter and up-regulates *VHL* expression. Using a zebrafish model, we confirmed the up-regulation of *vhl* by *foxo3b*, an orthologue of mammalian *FOXO3a*. Furthermore, by employing the 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 HIF activity *via* up-regulation of *VHL*, *FOXO3a* (*foxo3b*) plays an important role in survival in response to hypoxic stress.

INTRODUCTION

FOXO3a (also known as FKHRL1), together with *FOXO1* (also known as FKHR), FOXO4 (also known as AFX1) and FOXO6, comprise the conserved forkhead box O (FOXO) gene family, which is a subclass of the forkhead family of transcription factors. 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 down-stream targets. Several upstream signaling pathways modulate FOXO activity *via* nuclear-cytoplasmic shuttling and post-translational 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 (MnSOD) (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); 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 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, heman-glioblastomas, 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 multi-protein E3 ubiquitin ligase complex that targets the proline hydroxylated hypoxia-induced factor(HIF)-1/2α for proteasomal degradation under normoxic conditions (19) (18). 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 maintain oxygen homeostasis, organisms use the hypoxia signaling pathway for adapting to low oxygen conditions (22). The hypoxia-inducible factors, HIF-1α and HIF-2α, coordinate the cellular response to hypoxic conditions by inducing gene expressions (23). Prolyl hydroxylase (PHD) gene family and pVHL E3 ligase complex controls HIF activity through oxygen-dependent hydroxylation and subsequent proteasomal degradation (24).

Research has shown that mitochondrial-derived reactive oxygen species (ROS) can activate hypoxia signaling (25). Because FOXO3a, as well as FOXO4, can up-regulate *MnSOD* and other antioxidant enzymes (9,11), thus, FOXO transcription factors can inhibit HIF-1 activation by reducing the levels of mitochondrial-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 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 orthologues of mammalian FOXO3 gene, *foxo3a* and *foxo3b*. Zebrafish *foxo3b* was initially identified and named as *zFKHR/foxo5*, whose protein shared 55% identity to that of human FOXO3a (26). The phylogenetic analysis of vertebrate FOXO protein sequences showed that compared to 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 down-stream 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 Figure 1A, luciferase reporters containing the full length or truncated portions of the human *VHL* promoter (-2101-+66; -989-+66; -599-+66; -499-+66 and -371-+66; the transcription start site is designated as "+1") were transiently transfected into HEK293T cells together with Myc empty vector, or Myc tagged-FOXO3a expression vector. Overexpression of FOXO3a induced activity of promoter reporters including -2101-+66; -989-+66 and -599-+66, but not of promoter reporters including -499-+66 and -371-+66 (Fig.1B). Notably, among three FOXO3a-inducible reporters (-2101-+66; -989-+66 and -599-+66), the reporter -599-+66 had the highest activity, suggesting that FOXO-DNA binding element (DBE) might locate in the region between -599 to +66. After carefully searching, we identified one potential FOXO-DNA binding element (DBE) at position -567 to -561 (TTGTTAC) in *VHL* promoter (9) (Fig. 1 C). A single G to C substitution in the putative FOXO DBE completely abolished FOXO3a and FOXO3a-3A (an active form of FOXO3a with T32A/S253A/S315A triple mutation(2))-mediated up-regulation of the *VHL*

promoter reporter activity (Fig. 1 D).

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 blot 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 wildtype *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 *VHL* promoter.

To determine whether FOXO3a can directly bind to *VHL* promoter. We designed a pair of primers that could amplify a fragment encompassing the potential FOXO-DNA binding element (DBE), and performed chromatin immunoprecipitation (ChIP) using anti-FOXO3a antibody. As shown in Figure 2A and 2B, *in vivo* binding of FOXO3a to the *VHL* promoter region encompassing the FOXO DBE was observed. Further semi-quantitative 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. Firstly, we confirmed the efficiency of shRNA mediated *FOXO3a* knockdown via semi-quantitative RT-PCR assays in HEK293T cells and H1299 cells, respectively (Fig.3A and 3C). By contrast, knockdown of *FOXO3a* reduced *VHL* mRNA level in HEK293T cells and H1299 cells (Fig. 3B and 3D). Moreover, reduction of pVHL protein level and enhancement of HIF-1α protein level when *FOXO3a* was knocked down was validated (Fig.3E and 3F). 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 *VHL*'s function. 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 (two left panels in Fig.4A and 4B).

However, we noticed that under normoxia, the base level of hypoxia inducible genes, including *SLC2A1* and *LDHA* was very low due to lack of HIF-α. So, it was hard 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 partial impact on *HIF-α* function under a certain hypoxic conditions (29,30), thus, *VHL* could affect *HIF-α* function as well under this kind of hypoxic conditions. Because HIF-α protein was enhanced, resulted in higher expression level of hypoxia inducible gene, it became much easier to evaluate the effect on these gene expression(29,31), particularly for evaluating the suppression role. To get a clear picture about the role of *FOXO3a* on

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 (two right panels in Fig.4A and 4B, respectively). In addition, inhibitory effect of FOXO3a on hypoxia-inducible gene expression was more obvious under hypoxia compared to that under normoxia, which might resulted from higher base level of hypoxia-inducible gene under this kinds of hypoxic conditions.

By contrast, knockdown of *FOXO3a* in H1299 cells resulted in up-regulation of *SLC2A1* and *LDHA* under normoxia and hypoxia (Fig.4C and 4D). 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. 4E). Figure 4F 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.4G), further indicating that under the hypoxic condition of our experiments in this study, *VHL* still had some impacts on *HIF-α* function. 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 underling 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-month old) *via* semi-quantitative RT-PCR assays. *Foxo3b* was highly expressed in muscle, then in brain, heart, and liver (Fig.5A). Interestingly, *vhl* was also highly expressed in muscle, then in brain, heart and liver (Fig.5B). The similar expression patterns between *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 *vhl* promoter. Injection of a 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* (1-277aa) (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 Figure 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 semi-quantitative RT-PCR assays (Fig.7B). By contrast, knockdown of *foxo3b* using morpholino oligonucleotides, previously validated 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 a sgRNA targeting a region in exon 2 of *foxo3b* (Fig.8A). After micro-injecting the synthesized *sgRNA* and *Cas9* mRNA into one-cell stage embryos, we initially employed HMA (Heteroduplex mobility assay) to examine the efficiency of sgRNA/Cas9-mediated *foxo3b* disruption in F1 generation (Fig.8B), and then conducted sequencing confirmation in F1 generation. After screening, we obtained one mutant in *foxo3b* gene (thereafter designated as MT) (Fig.8C). We crossed $foxo3b^{+/-}$×$foxo3b^{+/-}$ and obtained offspring with $foxo3b^{+/+}$, $foxo3b^{+/-}$ and $foxo3b^{-/-}$ genetic background at Mendel's ratio (1:2:1). From the embryonic stages to adulthood, $foxo3b^{-/-}$ homozygosity were indistinguishable from their wild-type siblings. Overall, no obvious phenotypes were observed in $foxo3b^{-/-}$ zebrafish under normal condition. Semi-quantitative 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 other FOXO family members in zebrafish might have partial redundant function for inducing *vhl* expression.

Subsequently, we examined whether some common hypoxia-inducible genes were affected following disruption of *foxo3b*. As shown in Figure 9A-9D, expression of *vegf*, *pou5f1*, *pai1,* and *il11a* was enhanced in *foxo3b*-null zebrafish under hypoxia (28,33). These data suggested that *foxo3b* affected expression of hypoxia-inducible genes. Consistently, when the embryos were treated with DMOG, a hydroxylase inhibitor (34), expression of *vegf* and *pou5f1* was also enhanced in *foxo3b*-null zebrafish (Fig.9E-9F). Taken together, these data indicated that disruption of *foxo3b* in zebrafish enhanced expression of hypoxia-inducible genes; and as such, *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 the wild-type sibling embryos, injection with *foxo3b* mRNA inhibited expression of four hypoxia-inducible genes (*vegf*, *ldha*, *cited2*, and *il11a*) significantly compared to those injected with *GFP* mRNA (Fig.10A-10D). By contrast, in *vhl*-null embryos, injection with *foxo3b* mRNA had no obvious effect on expression of the four hypoxia-inducible genes (*vegf*, *ldha*, *cited2*, and *il11a*) compared to those injected with *GFP* mRNA (Fig.10A-10D). Expression of injected *foxo3b* and *GFP* mRNA was further confirmed by Western blot assays (Fig.10E) and fluorescent stereo-microscope respectively (Fig.10F). These data suggested that the suppressive role of *foxo3b* on expression of hypoxia-inducible genes was mediated by up-regulation of *vhl*.

To determine the biological consequence of *foxo3b* in modulating hypoxia signaling, we compared the acute hypoxia tolerance between *foxo3b*-null zebrafish and their wild-type siblings. In a 2% oxygen environmental condition, *foxo3b*-null fries (15dpf) died earlier than their wild-type siblings (Fig.11). Similarly, with adult zebrafish, in a 10% oxygen environmental condition, *foxo3b*-null adults (3-month old) also died earlier than their wild-type siblings (Supplementary video 1 and video 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 are well documented. Under normoxia, prolyl hydroxylases (PHD1-3) use oxygen as a substrate to hydroxylate key proline residues of HIF-1α and HIF-2α, resulting in subsequent proteasomal degradation by pVHL E3 ubiquitin ligase complex (36-38). Under hypoxia, PHD activity is inhibited, resulting in HIF-1α and HIF-2α stabilization and translocation to the nucleus for activating down-stream genes involved in systematic and cellular adaptation to hypoxia (39). Therefore, PHDs (PHD1-3) and pVHL has been recognized as the major regulators for modulating hypoxia signaling through regulating protein stability of HIF-1α and HIF-2α. In fact, it is evident that some factors could affect hypoxia signaling *via* regulation of PHDs and pVHL (31,40-43). 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, exhibit 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 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 in protein level through protein-protein interactions or post-translational 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 transcriptional level has largely remained under-reported (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 on 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 the studies of humans high-altitude adaptation, especially on Tibetan adaptation studies, positive directional selection is identified in HIF pathway and hypoxia-related genes(51). Among these genes, *HIF-2α* and *PHD2* genes are particularly noteworthy because of the consistency with which they have been observed by different investigators and the biochemical evidences that show the adaptive changes of these two genes and their effect on HIF signaling (51-54). These observations further support the vital role of HIF pathway in hypoxic adaptation (51). It is likely that the genes affecting HIF signaling should 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 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, the change of 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-1α and HIF-2α are key factors that orchestrate hypoxic response, further delineation of the hypoxic response phenotypes of these genes directly or indirectly involved in HIF pathway in knockout and overexpression status will uncover animal hypoxic tolerance or hypoxic adaptation mechanistically.

Of note, mitochondrial ROS are required for hypoxic activation of HIFs, indicating a connection between two vital cellular stress signaling (25). FOXO3a activates MnSOD 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 (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. FOXO3a-shRNA targeting sequence was 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: F: 5'-ATATCGGTACCGACTTGGAAGTCTCGGAAGG-3' and R: 5'-ATATCCTCGAGCGTCAAAGACAGGACAGTTCC-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 ATCC. All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). 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). 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 means $\pm$ S.E.M. 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 hrs. Luciferase activity was also determined using the Dual-luciferase Reporter Assay System.

Semi-quantitative 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: F: 5'-TCAGAGATGCAGGGACACACGATG-3' and R: 5'-ACCTGACGATGTCCAGTCTCCTGTA-3'. The following primers were used to assess human FOXO3a mRNA expression: F: 5'-TGCTAAGCAGGCCTCATCTC-3' and R: 5'-CTTGTGTCAGTTTGAGGGTCTG-3'. The following primers were used to evaluate the internal control, 18s RNA: F: 5'-TCAACTTCGATGGTAGTCGCCGT-3' and R: 5'-TCCTTGGATGTGGTAGCCGTTCT-3'.

The following primers were used to assess zebrafish genes: *foxo3b*-RT-F: TCATCTTCAAGGAGGAATGC, *foxo3b*-RT-R: GGATGGAGTTCTTCCAACCA; β-actin-RT-F: TACAATGAGCTCCGTGTTGC, β-actin-RT-R: ACATACAATGGCAGGGGTGTT; *vhl*-RT-F: GTGGGACATCCATGGATGTT, *vhl*-RT-R: CGTCAGCACAGGCAATGTGA; *pou5f1*-RT-F: TGAGGAAGAGGAGACTCTGA, *pou5f1*-RT-R: GACTGAACATTTTGCCATAC; *pai1*-RT-F: ATTCCAAGGTTCTCCATGGA *pai1*-RT-R: GGTTCCTCAGTAGTAATGCG; *ldha*-RT-F: CCTTCTCAAGGATCTGACCG, *ldha*-RT-R: ACACTGTAATCTTTATCCGC; *il11a*-RT-F: CCGGGTGTTTAGTACAGAGATT, *il11a*-RT-R: CATGGAGCTGAGAAAGAGTAGG; *cited2*-RT-F: GTTCCGAGACAGTATCGCTAAG, *cited2*-RT-R: CATCAAGACCTCCTCGTCAATAA; *vegf*-RT-F: TGCTCCTGCAAATTCACACAA, *vegf*-RT-R: ATCTTGGCTTTTCACATCTGCAA.

Data are reported as means ± S.E.M. 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: F: 5'-ATAAGCGTGATGATTGGGTGTTC-3' and R: 5'-CCCGAGTAGTTGGTACTGTAGGC-3'. The primers for amplifying β-actin and the procedure for ChIP were described previously (58).

Western blot assays--The following antibodies were used for Western blot: Myc (9E10, Santa Cruz), α-tubulin (Upstate). Human pVHL polyclonal antibody was generated against synthesized peptides of pVHL (Abmart, Shanghai, China). The pVHL (A0377), FOXO3a (A0102) and HIF-1α (A6265) antibodies were purchased from ABclonal Company. The procedures for Western blot assays were described previously (59). The Fuji Film LAS4000 mini luminescent 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 protocols. 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 previously described (26). The following primers were used for the *vhl* probe: vhl-F: 5'-CTTTAGTCTAACTCGGTGGT-3', and vhl-R: AGGCAATGTGATCTTGG-3'. The *foxo3b* antisense morpholino oligonucleotides (foxo3b-ATG-MO and foxo3b-sp-MO) and its 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 website (http://crispr.mit.edu). The zebrafish-Codon-Optimized Cas9 plasmid (61) was digested with XbaI, purified and transcribed using T7 mMessage Machine Kit (Ambion). pUC19-gRNA vector was used for amplifying sgRNA template(62). The primers for amplifying gRNA template are: 5'-GTAATACGACTCACTATAGGACAACGGCA GCCCAAGCCGTTTTAGAGCTAGAAATAGC -3' and 5'- AAAAGCACCGACTCGGTGCC-3'. sgRNA was synthesized using Transcript Aid T7 High Yield Transcription Kit (Fermentas). Cas9 RNA and sgRNA were mixed and injected into embryos at one cell stage. Cas9 RNA and sgRNA were injected at 0.75-1.25 ng/per embryo and 0.075 ng/per embryo, respectively.

After the injected embryos were incubated at 28.5℃ for 24h, the genomic DNA was extracted from 20-30 embryos by heating the embryos at 94°C for 40min in lysis solution (50mM NaOH) (the reaction was terminated by adding 1M Tris-HCl (PH=8.0)). The mutant detection was followed HMA (Heteroduplex mobility assay) as described previously (63). If the results were positive, the remainder 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 back-crossed with the wild-type zebrafish to obtain F2. The F2 adult zebrafish with the same genotype (+/-) were inter-crossed to generate F3 offspring, which should contain wildtype (+/+), heterozygous (+/-) and homozygous (-/-) offspring. The primers for detecting mutants were: F: 5'-TGGACATTGCCATTGATCCAG-3' and R: 5'-CCATGCATTCCTCCTTGAAGA-3'.

Hypoxic treatments—In pilot experiments, we noticed that zebrafish fries were more resistant to hypoxic treatment than adult zebrafish as judged by survival time in the hypoxia chamber (Ruskinn INVIVO$_2$400). Thus, we used 2% oxygen for zebrafish fries' treatment, and used 10% oxygen for adult zebrafish's treatment.

For zebrafish fries, two flasks were filled with 250 ml water respectively. *Foxo3b*-null fries (15dpf, n=20) were put into one flask and their wild-type siblings (15dpf, n=20) were put in the second flask. Then, two flasks were put into the hypoxia chamber simultaneously. The oxygen concentration of the chamber had been adjusted to 2% O_2 ahead of time. The dead fries were counted once an hour until the death of the last zebrafish with one genotype (*foxo3b*$^{-/-}$). This experiment was repeated for three times.

For adult zebrafish experiment, the zebrafish were initially weighed. During pilot experiments, we noticed that body weight of zebrafish could dramatically affect zebrafish acute hypoxic tolerance. In order to avoid interference by body weight in acute hypoxia tolerance determination, zebrafish of similar weights were chosen for further experiments. Subsequently, two flasks were filled with 250 ml water respectively. Three *foxo3b*-null zebrafish (0.36g, 0.37g, 0.39g) were put into one flask, and three wild-type siblings (0.36g, 0.37g, 0.38g) were put into the second flask. Before immersion into the hypoxia chamber (Ruskinn INVIVO$_2$400), the oxygen concentration in flask water was measured by an HQd portable Meter (HACH Company, USA). The oxygen concentration in *foxo3b*-null zebrafish flask was 7.10 mg/L, and the oxygen concentration in the wildtype sibling flask was 7.04 mg/L. The oxygen concentration in hypoxia chamber had been adjusted to 10% ahead of time. After putting the flasks containing zebrafish into the hypoxia chamber, the behavior of 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 flask water. At that moment, the oxygen concentration in *foxo3b*-null zebrafish flask was 1.48 mg/L, and the oxygen concentration in the wild-type sibling flask was 1.54 mg/L.

ACKNOWLEDGEMENTS

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

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

WX designed the study and wrote the manuscript. XL, XC, ZM and BH designed the study, conducted the experiments and analyzed the data. DZ generated *foxo3b*-null zebrafish. GO, JW and WZ contributed the reagents and analyzed the data. All authors analyzed the results and approved the final version of the manuscript.

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FOOTNOTES

W. X. is supported by the CAS Major Scientific and Technological Project (XDA08010208) and NSFC grant 31461163003, 91631102, 31671315. X.L. is supported by NSFC grant 31401072.

This article contains Supplemental Data.

The abbreviations used as: FOXO, forkhead box O; VHL, von-Hippel-Lindau; HIF, hypoxia-inducible

factor; MnSOD, manganese superoxide dismutase; PHD, prolyl hydroxylase; ROS, reactive oxygen species; ER, estrogen receptor; 4-HT, 4-hydroxy-tamoxifen; DBE, DNA binding element; ChIP, chromatin immunoprecipitation; WISH, whole-mount in situ hybridization; HMA, heteroduplex mobility assay.

Figure legends

Figure 1. VHL is a direct down-stream target of FOXO3a. (*A*) Schematic of VHL promoter constructs. (*B*) Promoter reporter activities were measured after transiently transfection with Myc empty vector or Myc-FOXO3a expression vector in HEK293T cells. (*C*) One sub-optimal FOXO DNA binding element (DBE), TTGTTAC (bold and underlined), was localized to the -567 to -561 region of the VHL promoter. (*D*) Compared to the wildtype 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 blot 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 wildtype or mutant VHL promoter reporters in the absence or presence of 4-hydroxy-tamoxifen (4-HT, 500nM)

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,B*), which was confirmed by semi-quantitative real-time RT-PCR assays (*C*). (*D*) Semi-quantitative real-time RT-PCR assays revealed that activation of FOXO3a with 500nm 4-HT caused VHL mRNA increased. (*E*) Induction of pVHL by FOXO3a was confirmed by Western blot assays.

Figure 3. Knockdown of FOXO3a causes VHL expression reduced. (*A*) Knockdown of FOXO3a by two sets of FOXO3a-shRNA in HEK293T cells was confirmed by semi-quantitative real-time RT-PCR assays. Luciferase-shRNA was used as a control. (B) Knockdown of FOXO3a in HEK293T caused VHL expression reduced. (*C*) Knockdown of FOXO3a by two sets of FOXO3a-shRNA in H1299 cells was confirmed by semi-quantitative real-time RT-PCR assays. Luciferase-shRNA was used as a control. (D) Knockdown of FOXO3a in H1299 cells caused VHL expression reduced. (*E*) Knockdown of FOXO3a resulted in reduction of endogenous pVHL and induction of endogenous HIF-1α in HEK293T cells was confirmed by Western blot assays. (*F*) Knockdown of FOXO3a resulted in reduction of endogenous pVHL and induction of endogenous HIF-1α in H1299 cells was confirmed by Western blot assays.

Figure 4. FOXO3a suppresses hypoxia-inducible gene expression via VHL. (*A*) Overexpression of FOXO3a caused SLC2A1 expression reduced in HEK293T cells under normoxia and hypoxia (2% O2). (*B*) Overexpression of FOXO3a caused LDHA expression reduced in HEK293T cells under normoxia and hypoxia (2% O2). (*C*) Knockdown of FOXO3a caused SLC2A1 expression increased in H1299 cells under normoxia and hypoxia (2% O2). (*D*) Knockdown of FOXO3a caused LDHA expression increased in H1299 cells under normoxia and hypoxia (2% O2). (*E*) Knockdown of endogenous VHL by transiently transfected with two sets of VHL-shRNA was confirmed by Western blot 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.

Figure 5. The expression pattern of vhl in zebrafish and the effect of foxo3b on vhl promoter activity. (*A-B*) Semi-quantitative 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 *foxo3b* expression vector in embryos. (*D*) Injection of a *foxo3b*-3A mutant induced zebrafish *vhl* promoter activity more dramatically (column 3 *vs.* column 2); *vhl* promoter was not activated after injection of a *foxo3b* dominant-negative mutant (dn-foxo3b, p=0.089).

Figure 6. The expression pattern of *vhl* during zebrafish embryogenesis as detected by whole-mount *in situ* hybridization assays (WISH). (*A-F*) *vhl* was ubiquitously expressed before 10-somite stage. (*G-I*) At 25 hpf (hours post fertilization), *vhl* was highly expressed in head and anterior mesoderm. (*J-M*) At 48 hpf, *vhl* was highly expressed in otic vesicle, pectoral fin and posterior mesoderm. (*N-Q*) At 3.5 dpf (days post fertilization), *vhl* was highly expressed in swimming bladder, inner ear and kidney. (*R-T*) At 4.5 dpf, *vhl* was highly expressed in swimming bladder, liver, inner ear and kidney.

Figure 7. Zebrafish *foxo3b* enhances *vhl* expression. (*A*) Ectopic expression of *foxo3b* by mRNA injections induced *vhl* expression in embryos as detected by whole-mount *in situ* hybridization (WISH). *GFP* mRNA was used as a control. (*B*) Ectopic expression of *foxo3b* by mRNA injections enhanced *vhl* expression in embryos as revealed by semi-quantitative real-time RT-PCR assays. *GFP* mRNA was used as a control. (*C*) Knockdown of *foxo3b* in embryos by morpholino oligonucleotides 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*) as compared to 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 respectively. (C) The predicted protein products of *foxo3b* in the mutants and their wild-type siblings. (D-E) The expression of *foxo3b* (*D*) or *vhl* (*E*) in the wild-type (*foxo3b* $^{+/+}$) and *foxo3b*-null(*foxo3b* $^{-/-}$) zebrafish embryos under normoxia and hypoxia conditions.

Figure 9. Zebrafish *foxo3b* suppresses hypoxia-induced gene expression. (*A*) *foxo3b*-null embryos (*foxo3b*$^{-/-}$) exhibit higher *vegf* expression compared to their wild-type siblings (*foxo3b* $^{+/+}$) under normoxia and hypoxia. (*B*) *foxo3b*-null embryos (*foxo3b*$^{-/-}$) exhibit higher *pou5f1* expression compared to their wild-type siblings (*foxo3b* $^{+/+}$) under normoxia and hypoxia. (*C*) *foxo3b*-null embryos (*foxo3b*$^{-/-}$) exhibit higher *pai1* expression compared to their wild-type siblings (*foxo3b* $^{+/+}$) under normoxia and hypoxia. (*D*) *foxo3b*-null embryos (*foxo3b*$^{-/-}$) exhibit higher *il11a* expression compared to their wild-type siblings (*foxo3b* $^{+/+}$) under normoxia and hypoxia. (*E*) *foxo3b*-null

embryos (*foxo3b*$^{-/-}$) exhibit higher *vegf* expression compared to their wild-type siblings (*foxo3b* $^{+/+}$) with or without DMOG (100μM) treatment. (*E*) *foxo3b*-null embryos (*foxo3b*$^{-/-}$) exhibit higher *pai1* expression compared to their wild-type siblings (*foxo3b* $^{+/+}$) with or without DMOG (100μM) treatment.

Figure 10. Zebrafish *foxo3b* suppresses hypoxia-induced gene expression through *vhl*. (*A*) Ectopic expression of *foxo3b* by mRNA injections suppressed *vegf* expression in the wild-type sibling embryos (*vhl* $^{+/+}$), but not in *vhl*-null embryos (*vhl* $^{-/-}$), as revealed by semi-quantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. (*B*) Ectopic expression of *foxo3b* by mRNA injections suppressed *ldha* expression in the wild-type sibling embryos (*vhl* $^{+/+}$), but not in *vhl*-null embryos (*vhl* $^{-/-}$), as revealed by semi-quantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. (*C*) Ectopic expression of *foxo3b* by mRNA injections suppressed *cited2* expression in the wild-type sibling embryos (*vhl* $^{+/+}$), but not in *vhl*-null embryos (*vhl* $^{-/-}$), as revealed by semi-quantitative real-time RT-PCR assays. *GFP* mRNA injections were used as controls. (*D*) Ectopic expression of *foxo3b* by mRNA injections suppressed *il11a* expression in the wild-type sibling embryos (*vhl* $^{+/+}$), but not in *vhl*-null embryos (*vhl* $^{-/-}$), as revealed by semi-quantitative 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 blot assays. (*F*) expression of control GFP mRNA injected into embryos was examined under a fluorescent stereo-microscope (Leica M205).

Figure 11. *Foxo3b* is required for hypoxia tolerance in zebrafish. Three groups of *foxo3b*-null zebrafish fries (15dpf, 20 each group) and their wild-type siblings (15dpf, 20 each group) were placed in the hypoxia chamber (Ruskinn INVIVO$_2$400). The oxygen concentration was adjusted to 2% O_2 ahead of time. The dead fries were counted once an hour until the death of the last fry with one genotype (*foxo3b*$^{-/-}$).

Figure 1

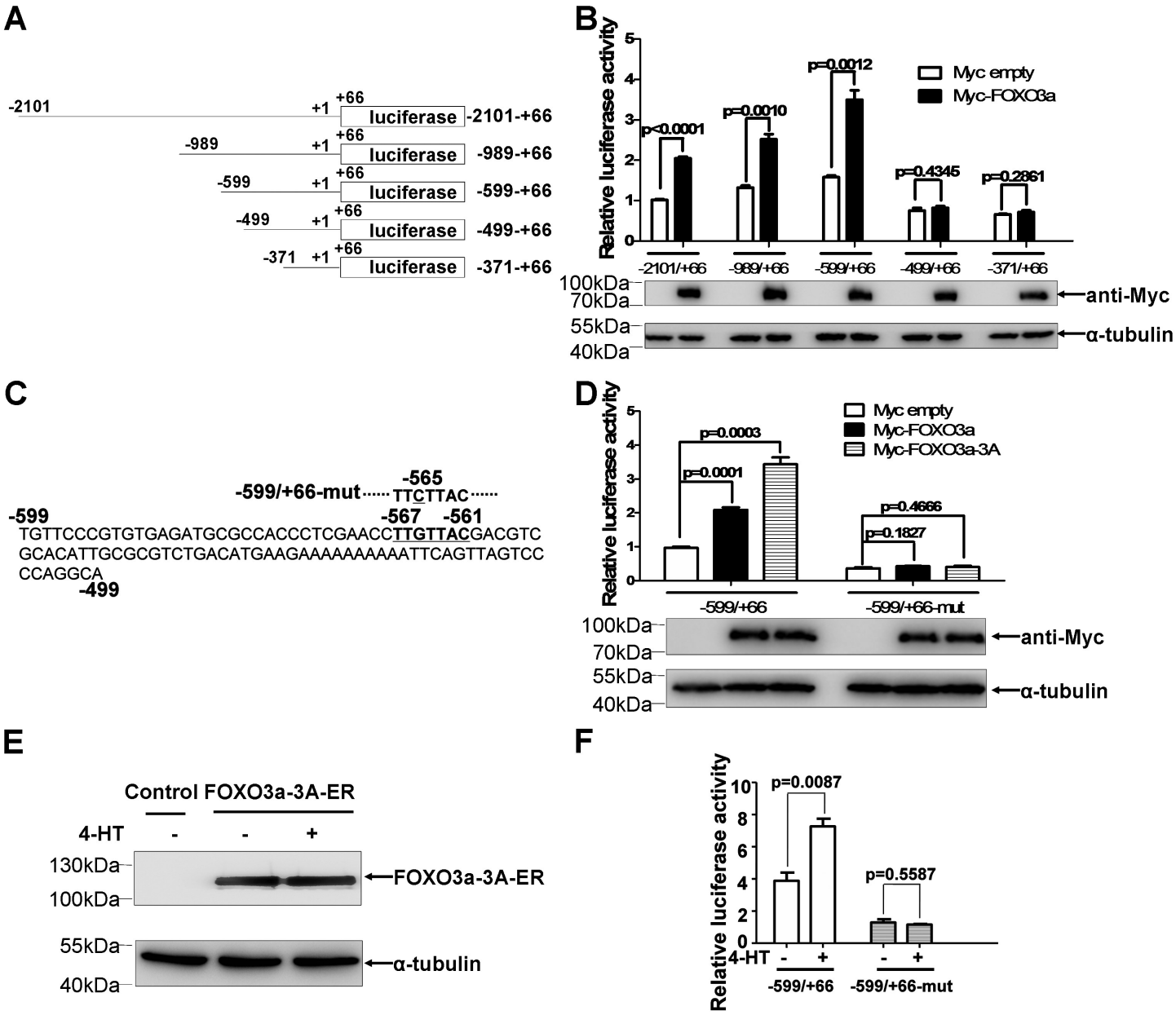
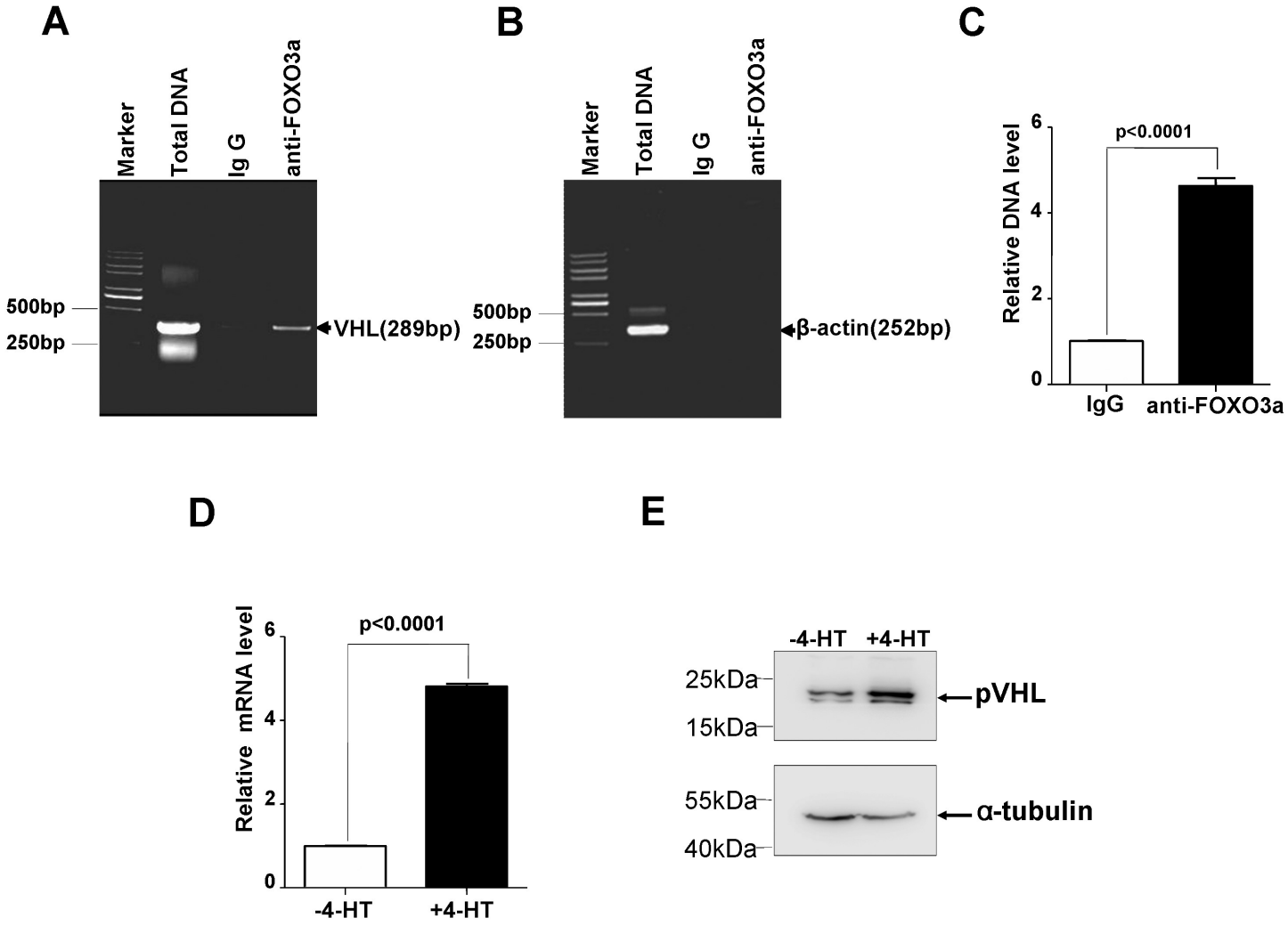


Figure 2



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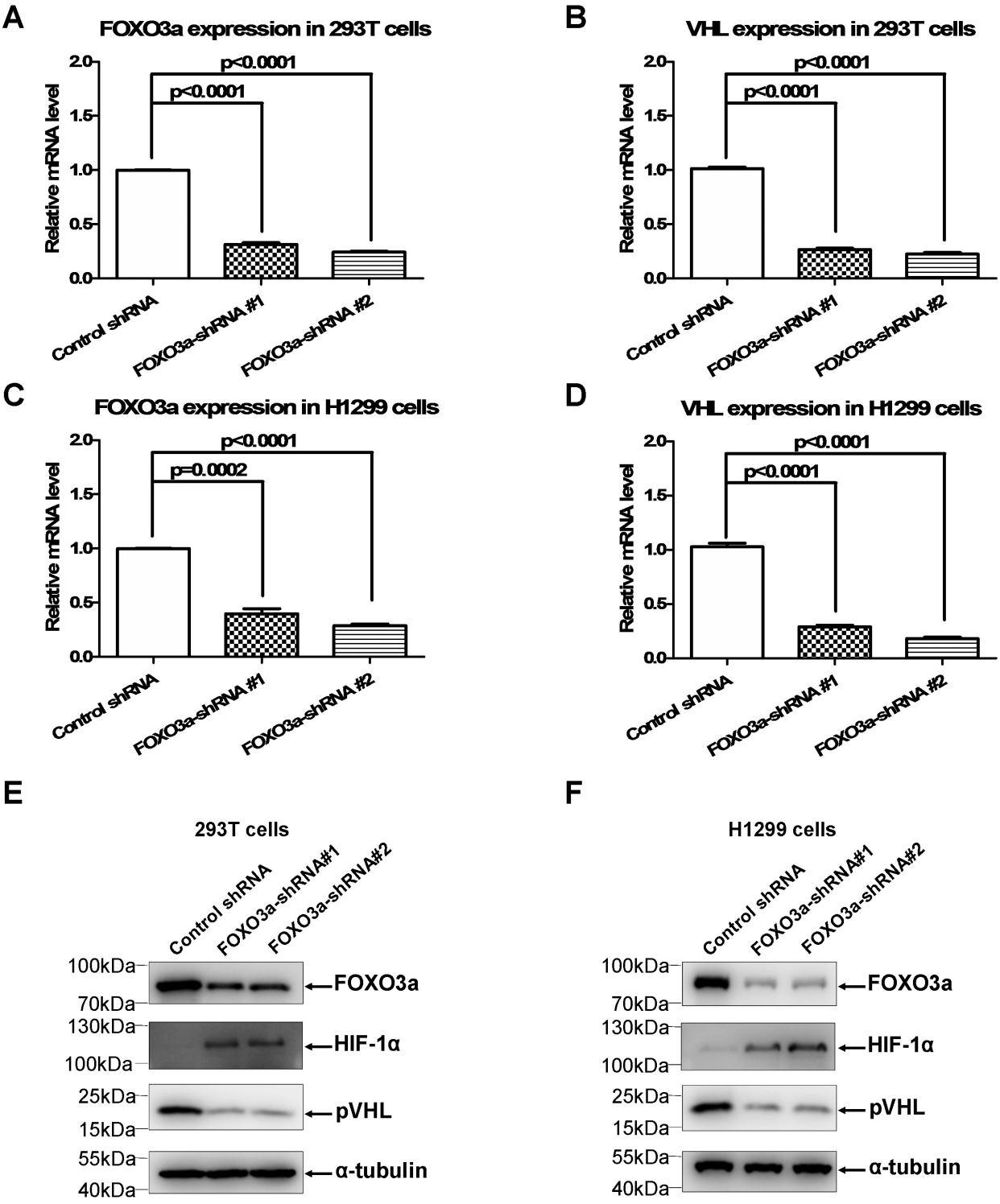

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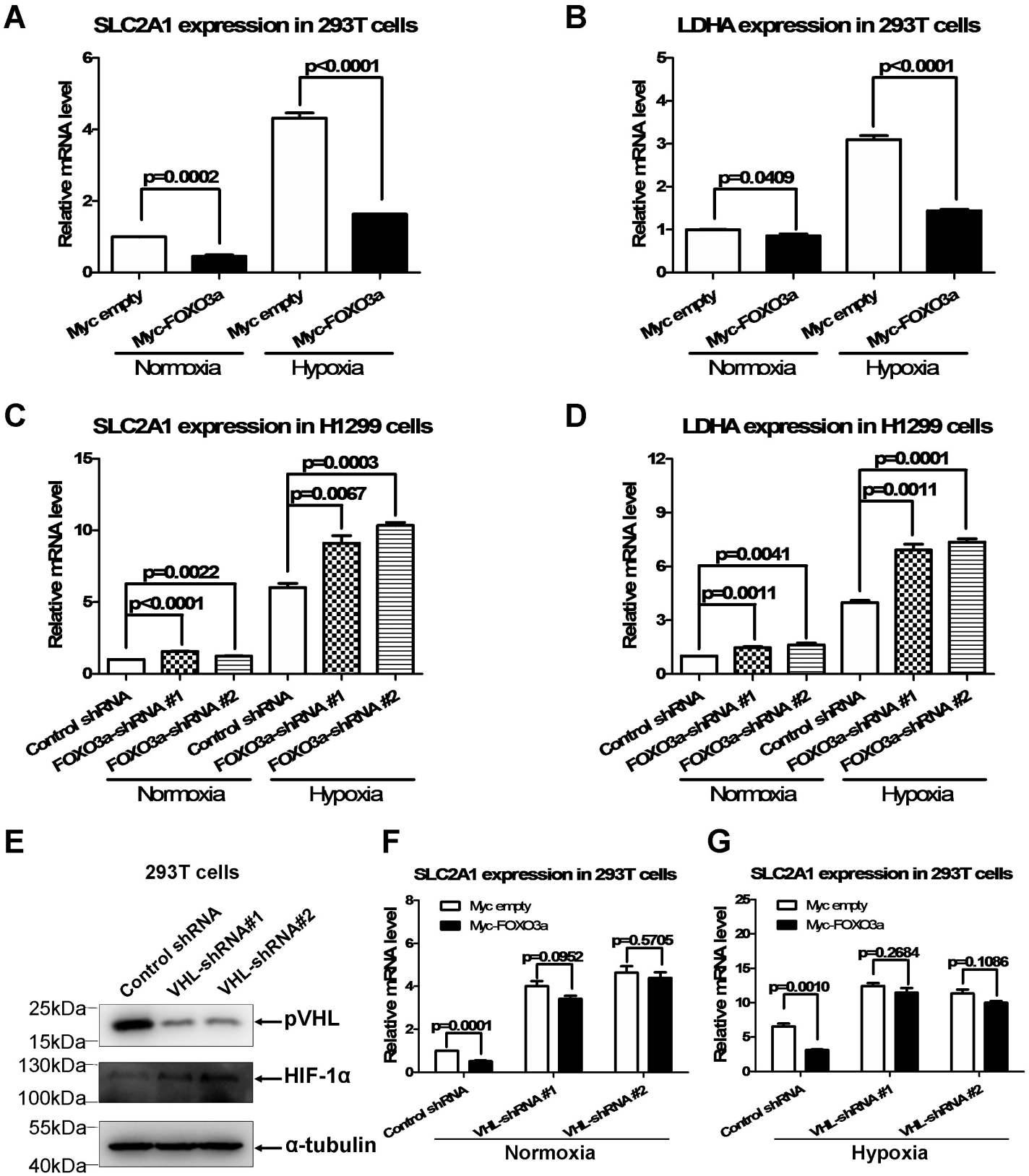

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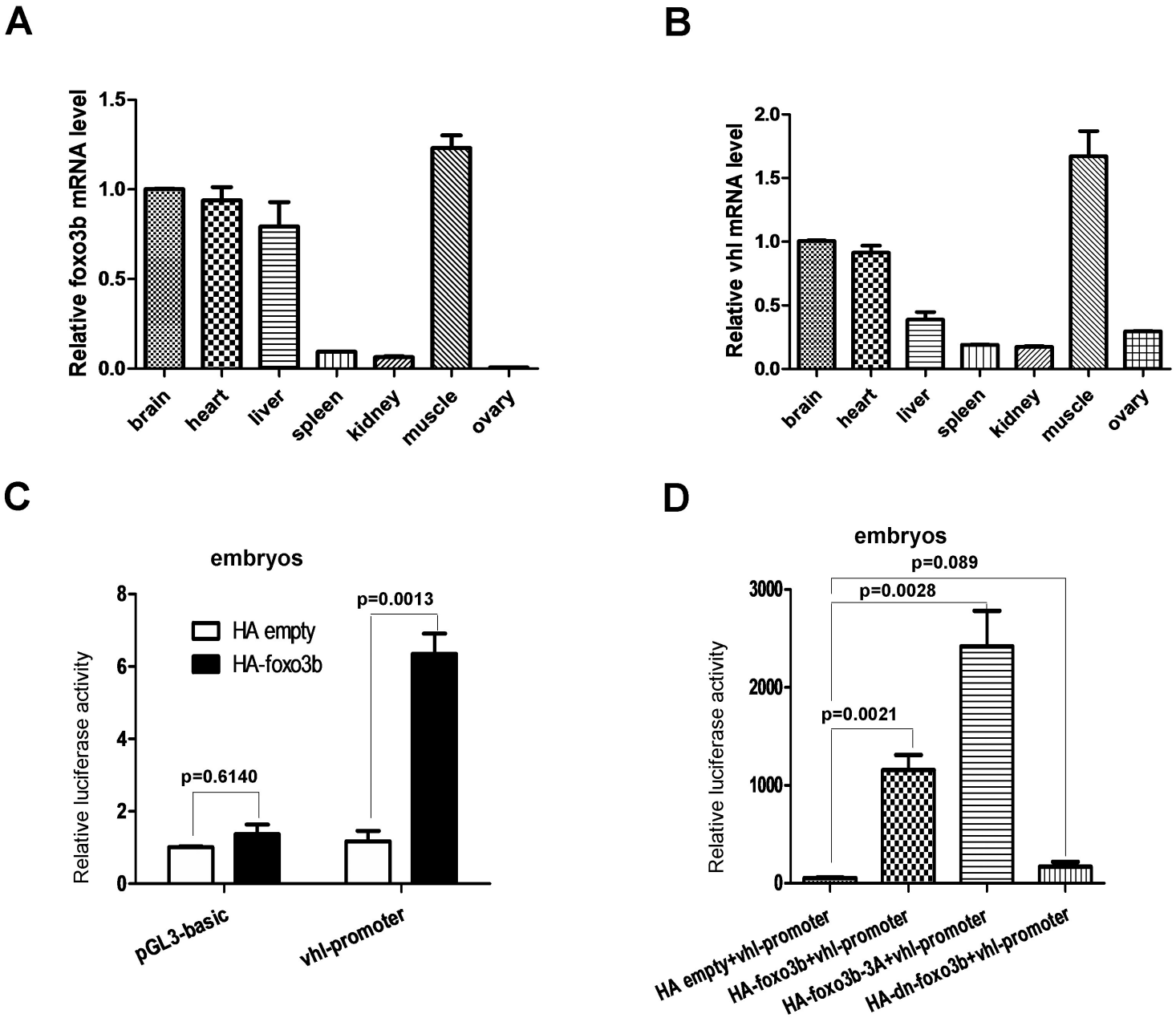

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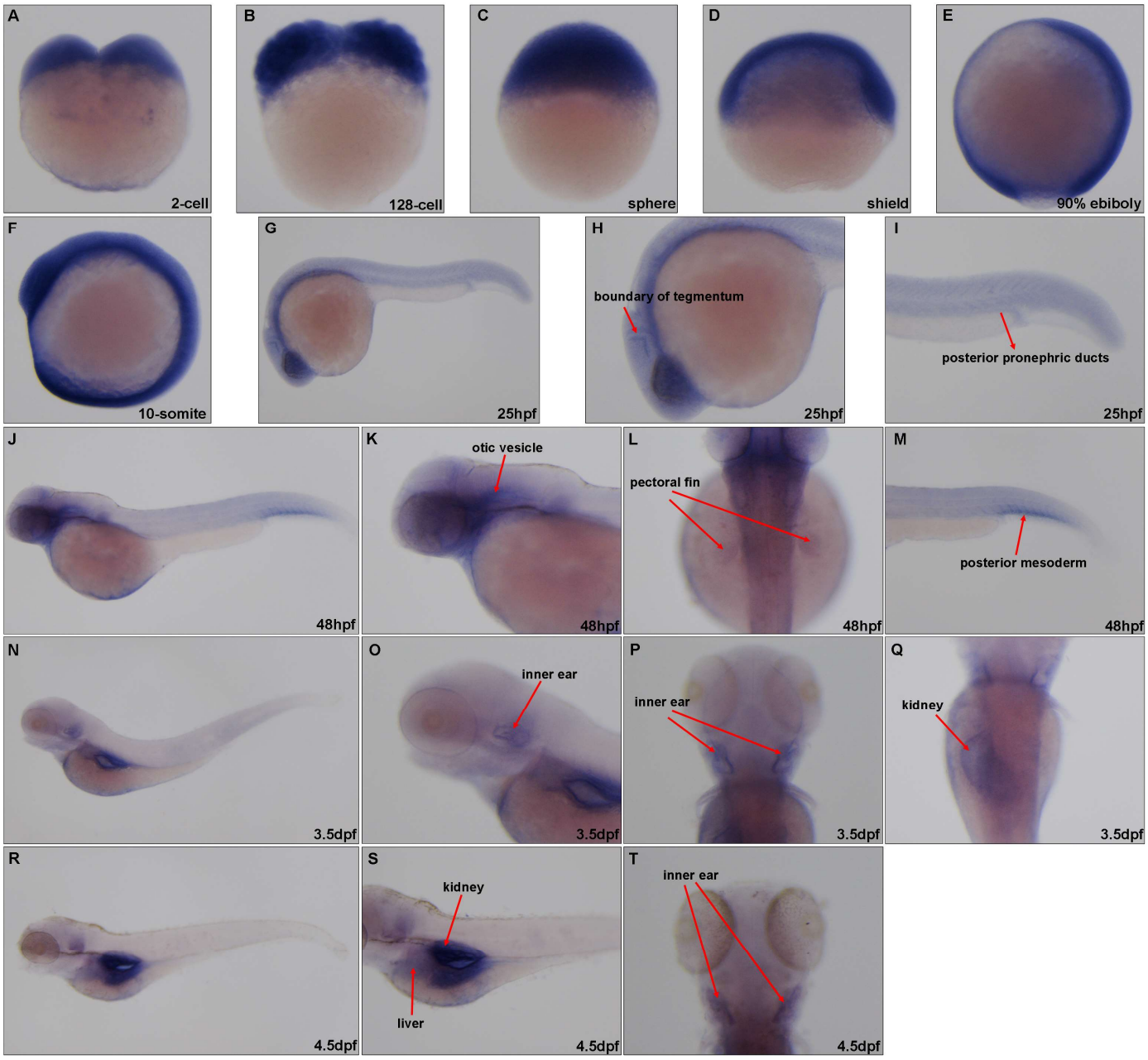

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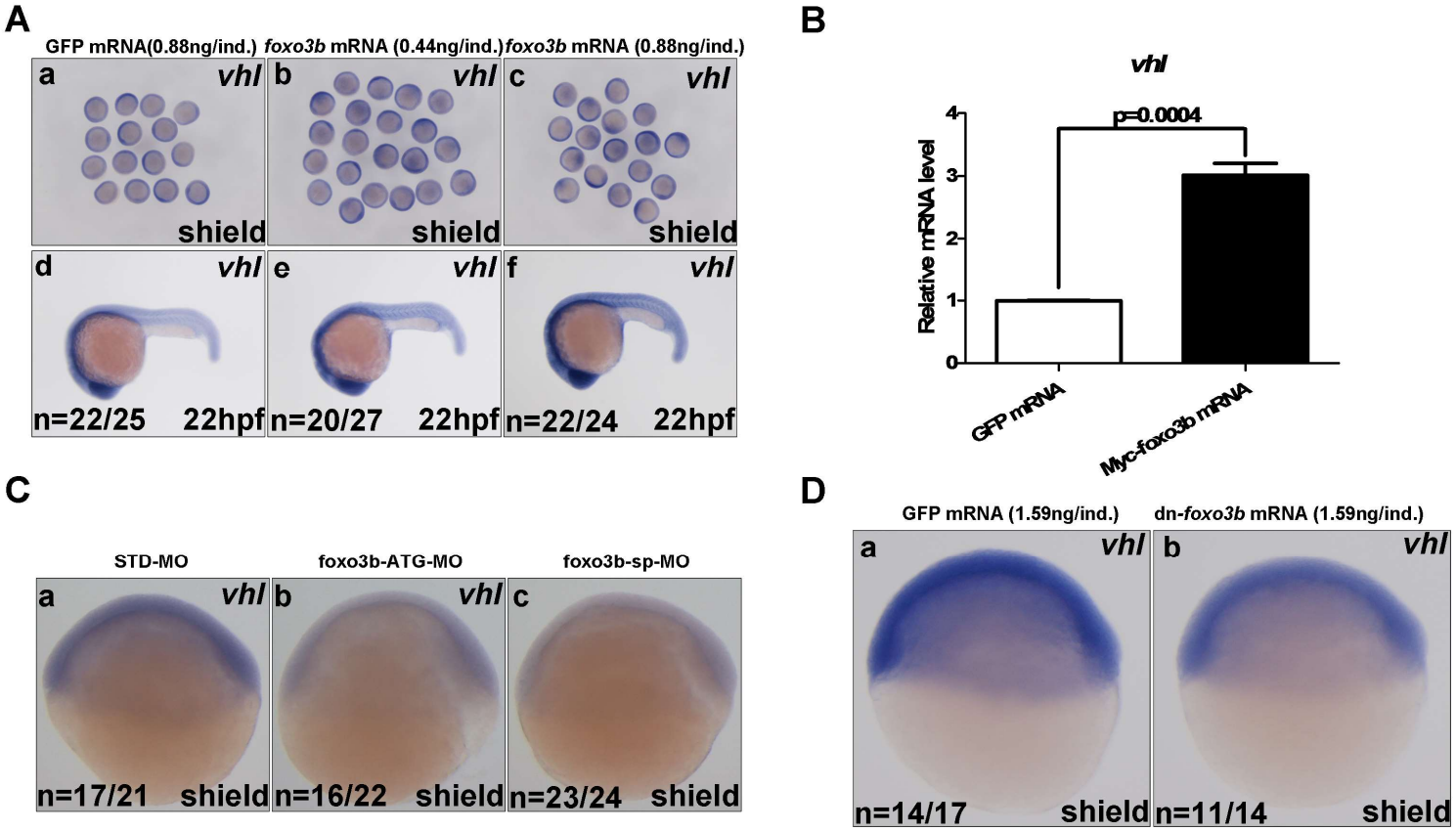

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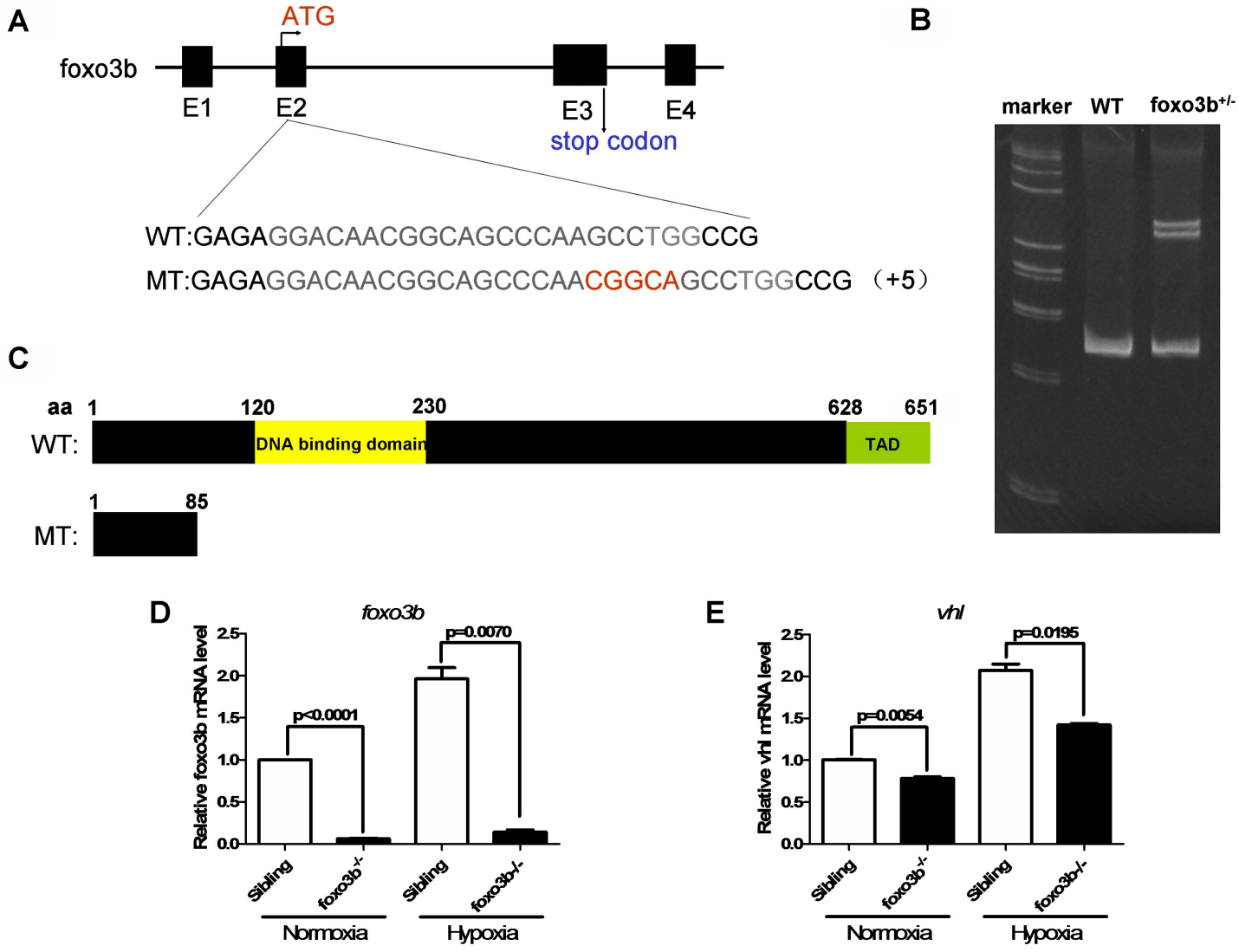


Figure 9

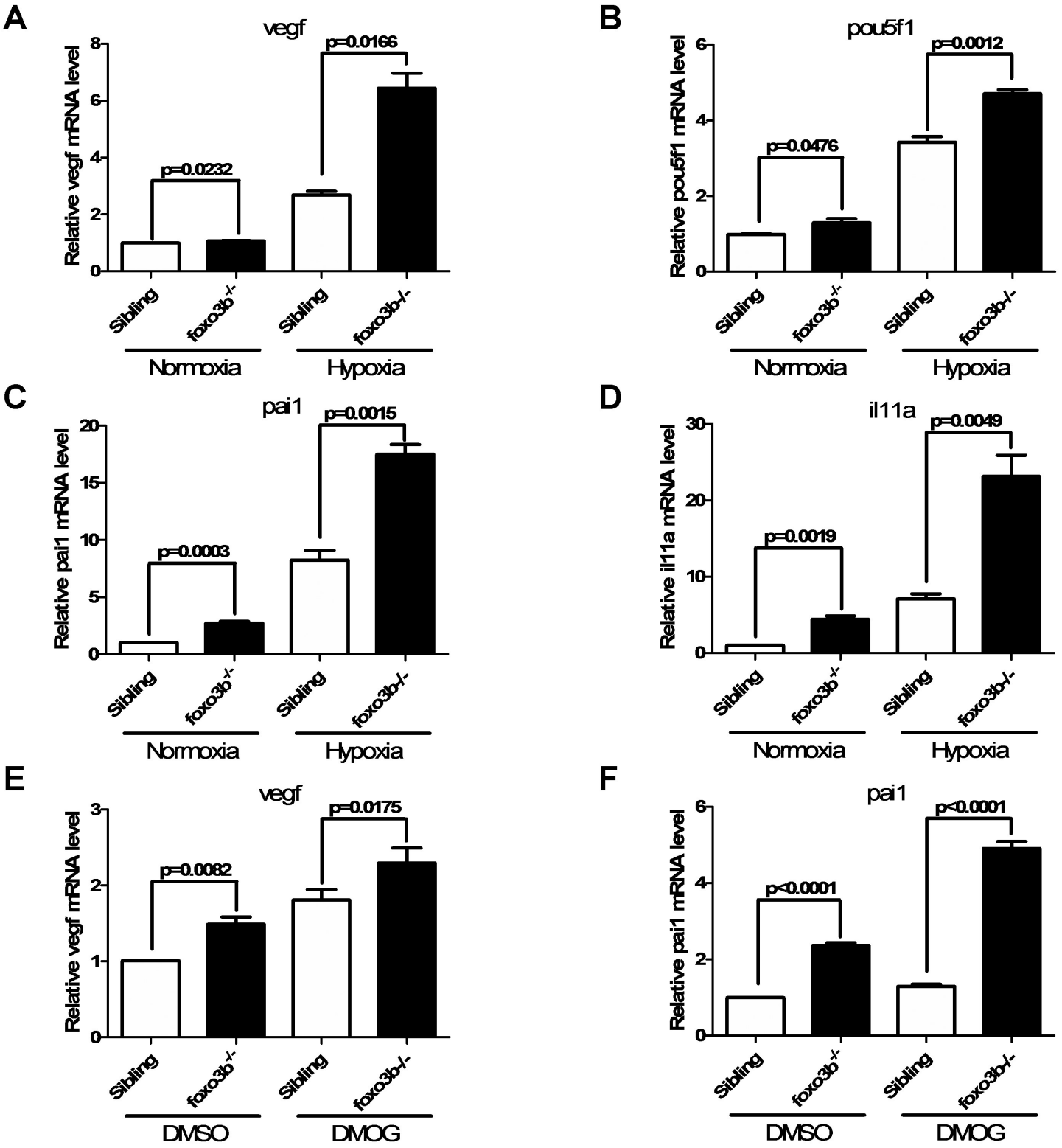
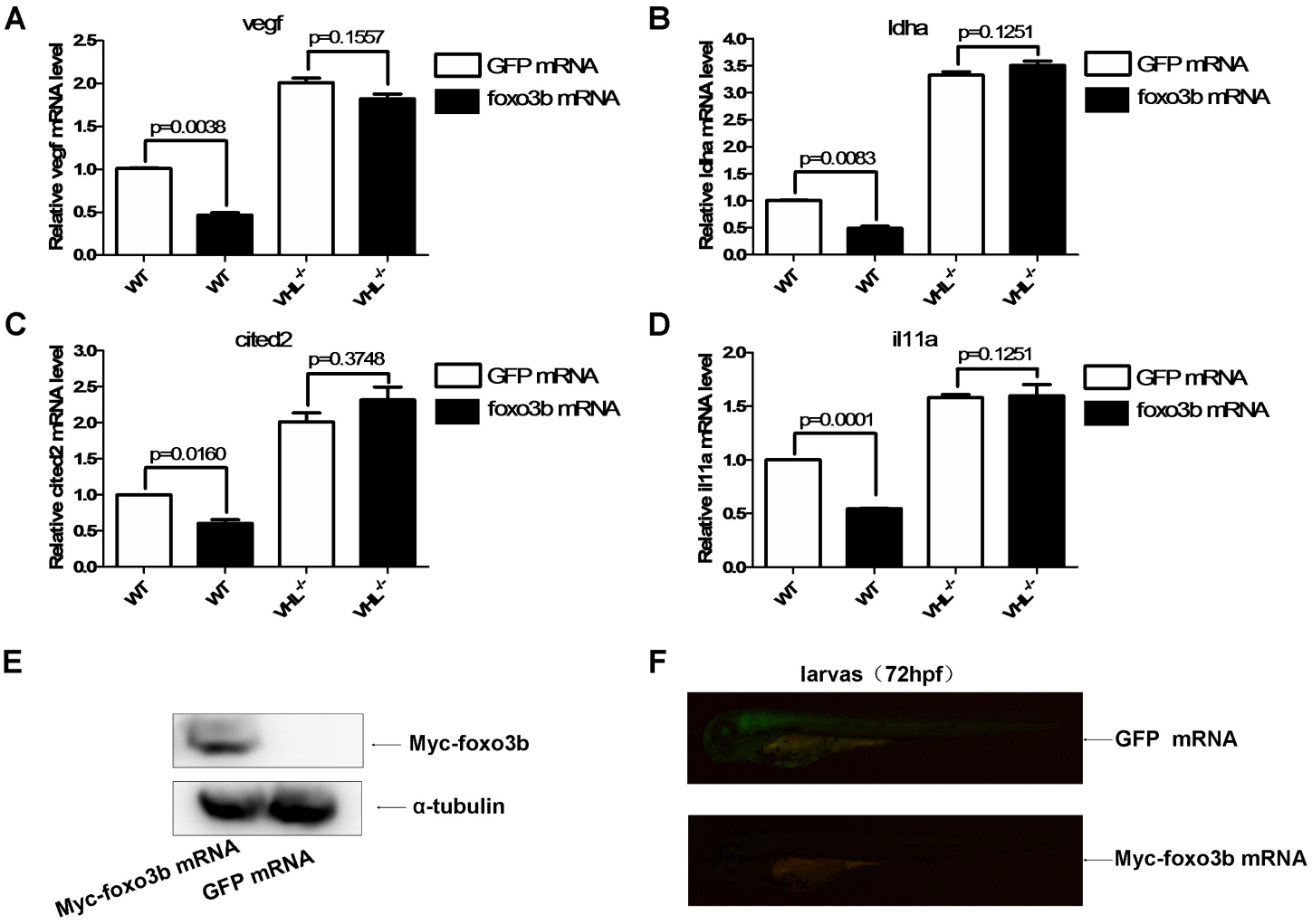


Figure 10



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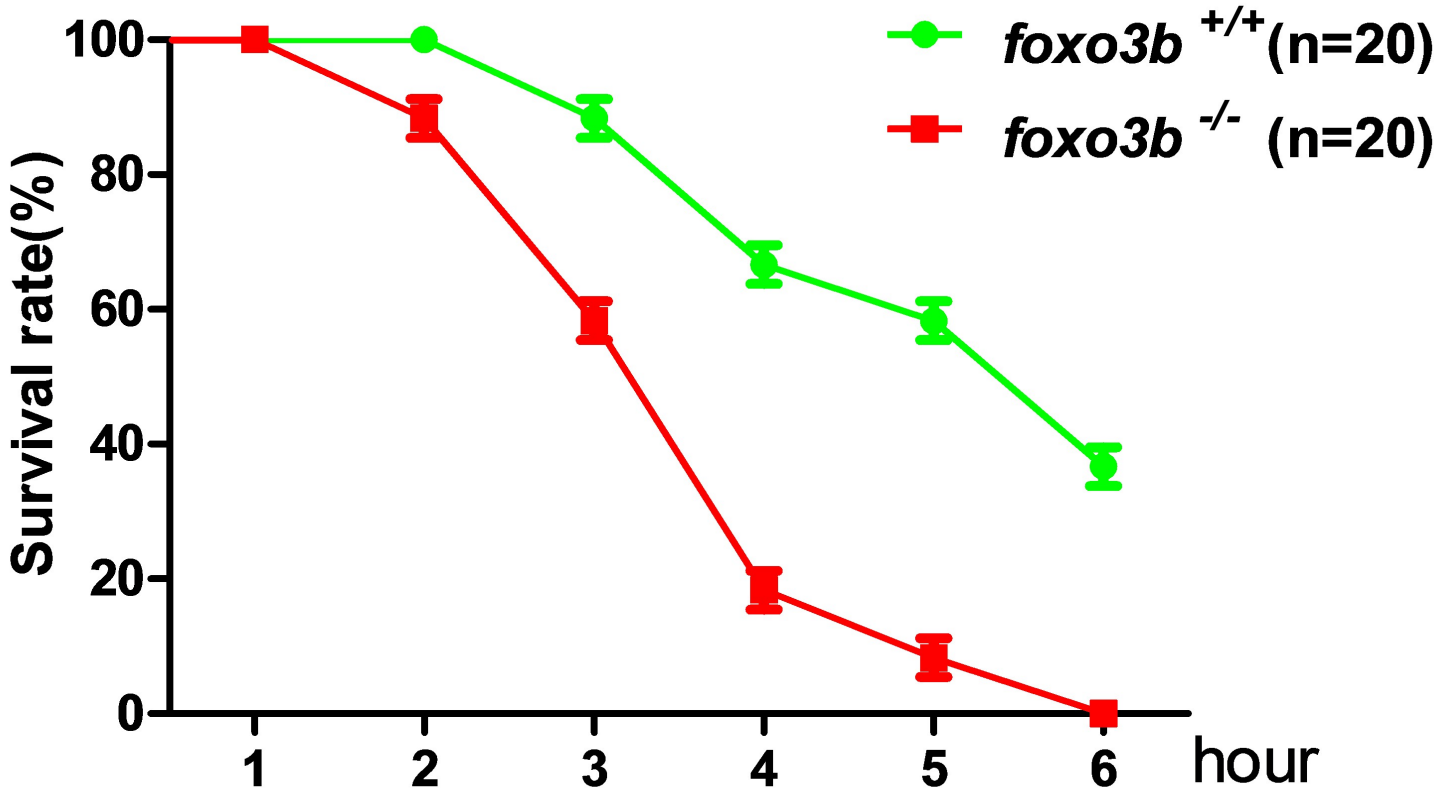

Forkhead transcription factor 3a (FOXO3a) modulates hypoxia signaling via up-regulation of von Hippel-Lindau gene (VHL)

Xing Liu, Xiaolian Cai, Bo Hu, Zhichao Mei, Dawei Zhang, Gang Ouyang, Jing Wang, Wei Zhang and Wuhan Xiao

J. Biol. Chem. published online October 24, 2016
