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Hypoxia-Inducible Factor 3 Is an Oxygen-Dependent Transcription Activator and Regulates a Distinct Transcriptional Response to Hypoxia

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SUMMARY

Hypoxia-inducible factors (HIFs) play key roles in the cellular response to hypoxia. It is widely accepted that whereas HIF-1 and HIF-2 function as transcriptional activators, HIF-3 inhibits HIF-1/2α action. Contrary to this idea, we show that zebrafish Hif-3α has strong transactivation activity. Hif-3α is degraded under normoxia. Mutation of P393, P493, and L503 inhibits this oxygen-dependent degradation. Transcriptomics and chromatin immunoprecipitation analyses identify genes that are regulated by Hif-3α, Hif-1α, or both. Under hypoxia or when overexpressed, Hif-3α binds to its target gene promoters and upregulates their expression. Dominant-negative inhibition and knockdown of Hif-3α abolish hypoxia-induced Hif-3α-promoter binding and gene expression. Hif-3α not only mediates hypoxia-induced growth and developmental retardation but also possesses hypoxia-independent activities. Importantly, transactivation activity is conserved and human HIF-3α upregulates similar genes in human cells. These findings suggest that Hif-3 is an oxygen-dependent transcription factor and activates a distinct transcriptional response to hypoxia.

INTRODUCTION

Hypoxia, i.e. reduced oxygen availability, has a broad impact on human and animal physiology, and elaborate adaptive mechanisms have evolved to respond to hypoxic stress (Prabhakar and Semenza, 2012; Semenza, 2012). Hypoxia-inducible factors (HIFs) play key roles in the transcriptional response to hypoxia. HIFs are heterodimers, consisting of a unique oxygen-dependent α subunit and a common constitutively expressed β subunit. There are three HIFαs in humans and mammals. Most of our current knowledge about these transcription factors is based on studies of HIF-1α and to a lesser degree HIF-2α. HIF-1α and HIF-2α share high sequence identity. They both contain a basic helix-loop-helix (bHLH) domain, two PAS domains, a PAC domain, an oxygen-dependent degradation (ODD) domain, an N-terminal transactivation domain (N-TAD), and a C-terminal TAD (C-TAD) (Pugh et al., 1997). Under normoxia, HIF-1α is prolyl hydroxylated at P402 and P564 in its ODD. This leads to its binding to the E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) at L574 and its targeting to the proteasome for degradation (Huang et al., 2002; Kageyama et al., 2004). Homologous proline and leucine residues in HIF-2α play similar roles (Masson et al., 2001). The transactivation activity of HIF-1/2α is further inhibited by asparaginyl hydroxylation under normoxia (Simon and Keith, 2008). Under hypoxia, HIF-1α or HIF-2α is stabilized due to reduced prolyl hydroxylation. The stabilized HIF-1α dimerizes with the HIFβ subunit and binds to the hypoxia response element (HRE)/HIF binding site in the promoter regions of its target genes and upregulates their expression (Manalo et al., 2005). These HIF-1 and/or HIF-2 target genes participate in many biological processes, including erythropoiesis, angiogenesis, metabolic reprograming, cell-cycle regulation, and tumor progression (Gordan and Simon, 2007; Rankin and Giaccia, 2008). Genetic studies using knockout mice revealed that HIF-1α and HIF-2α are essential for fetal development and survival (Weidemann and Johnson, 2008).

Although it has become evident that HIF-1 and HIF-2 function as master regulators of the transcriptional response to hypoxia, the role of HIF-3 under hypoxia and its mode of action are far less clear. Available evidence suggests that HIF-3α may be functionally distinct. Its sequence identities with HIF-1α/2α are relatively low (Gu et al., 1998). Whereas HIF-1α and HIF-2α have two TADs (Pugh et al., 1997; Tian et al., 1997), HIF-3α has only one TAD. HIF-3α has a unique leucine zipper domain (LZIP) and an LXXLL motif (Gu et al., 1998), and these unique structural features are evolutionarily conserved (Zhang et al., 2012). The HIF-3α genes are subjected to complex regulation and produce a large number of mRNA variants due to the utilization of different promoters, different transcription initiation sites, and alternative splicing in mammals (Prabhakar and Semenza, 2012). Many short HIF-3α isoforms lack any TAD (Hara et al., 2001). When some of these HIF-3α isoforms were tested in vitro in cultured human/mammalian cells, they had either weak or no transcriptional activity (Gu et al., 1998; Hara et al., 2001; Pasanen et al., 2010). Moreover, overexpression of human HIF-3α isoform 4,

an isoform lacking a TAD, suppressed HIF-1α and HIF-2α-induced reporter construct expression in cultured cells (Maynard et al., 2005). Likewise, mouse inhibitory PAS domain protein (IPAS), an alternatively spliced variant of mouse HIF-3α, inhibited HIF-1α activity by forming a dimer with HIF-1α (Makino et al., 2001). Neonatal and embryonic PAS protein (NEPAS), another alternatively spliced mouse HIF-3α isoform, was found to be able to dimerize with HIFβ and to inhibit HIF-1α activity by competing for available HIFβ (Yamashita et al., 2008). Collectively, these studies led to the prevailing view that HIF-3α acts as a negative regulator of HIF-1α and HIF-2α. This dogma, however, is derived from overexpression experiments performed in cell culture systems with artificial reporter constructs. Although these studies are informative about the biological capability of the particular HIF-3α isoforms tested, they do not necessarily provide insight into the functions of the endogenous protein(s) in vivo. Furthermore, the human and mouse HIF-3α genes produce a large number of transcripts, and only some variants have been studied to date.

In this study, we investigated the role of Hif-3α in the hypoxic response in zebrafish embryos. Zebrafish embryos develop externally, which makes it possible to manipulate environmental oxygen levels and observe changes in gene expression in real time. Recent studies indicated that the oxygen-sensing and HIF-signaling pathways are conserved in zebrafish (Kajimura et al., 2005, 2006). We discovered that Hif-3α functions as a transcriptional activator in zebrafish embryos. Under hypoxia, Hif-3α stabilizes and binds to HREs in the promoters of its target genes and upregulates their expression. We performed transcriptomics analyses and identified a large number of Hif-3 target genes. There are three distinct groups of Hif-3α-regulated genes: (1) those that are upregulated by Hif-3α only, (2) those that are regulated by both Hif-1α and Hif-3α with similar potencies, and (3) those that are regulated by both Hif-1α and Hif-3α but with different potencies. Importantly, we show that the transcriptional activity is conserved, and human HIF-3α-1 and HIF-3α-9 isoforms upregulate similar target genes in human cells.

RESULTS

Zebrafish Hif-3α Has HRE-Dependent Transcriptional Activity, and this Activity Is Conserved in Human HIF-3α Isoforms

Zebrafish Hif-3α-GFP is localized in the nuclei when transfected into U2 osteosarcoma (U2OS) cells (Figures 1A and 1B). Cotransfection of Hif-3α-GFP with p2.1, a well-established HIF-1 reporter construct (Semenza et al., 1996), resulted in a 23-fold, highly significant increase in the reporter activity (Figure 1C). The transcriptional activity of Hif-3α is HRE dependent, because it was abolished when the HRE was mutated (Figure 1C). This effect was concentration dependent (Figure 1D). Zebrafish Hif-1α and human HIF-1α caused a 12-fold and 31-fold increase, respectively (Figures 1C and 1E). Cotransfection of zebrafish Hif-3α and Hif-1α did not cause any further change (Figure 1F). The expression of these Hif-GFP fusion proteins was confirmed by western blot analysis (see Figure S1). When several human HIF-3α isoforms were tested, HIF-3α-1 and HIF-3α-9 (two longer isoforms) had highly significant activity, whereas HIF-3α-4 had no activity. The lack of activity of HIF-3α-4 is in agreement with the fact that this short isoform lacks a TAD (Pasanen et al., 2010). However, these human HIF-3α plasmids are not tagged, and their expression levels could not be compared. These results suggest that Hif-3α is a nuclear protein that possesses HRE-dependent transcriptional activity, and that this activity is conserved in human HIF-3α-1 and HIF-3α-9.

Zebrafish Hif-3α Is Degraded In Vivo under Normoxia, and Three Conserved Residues Are Critical for this Oxygen-Dependent Degradation

To investigate the action of Hif-3α in vivo, we injected GFP or Hif-3α-GFP mRNA into zebrafish embryos together with p2.1 plasmid DNA. No increase in luciferase activity was observed in the Hif-3α-GFP group, and western immunoblot analysis suggested an absence of intact Hif-3α-GFP in the injected zebrafish embryos (Figures 2A and 2B). Zebrafish Hif-3α has an ODD domain and degrades rapidly under normoxia (Zhang et al., 2012), but which structural motif(s) is responsible for its oxygen-dependent degradation is unknown. Zebrafish Hif-3α P393, P493, and L503 are analogous to P402, P564, and L574 in human HIF-1α. These three residues are conserved in all known vertebrate HIFα proteins (see Figure S2). To determine whether P393, P493, and L503 in Hif-3α are involved in its oxygen-dependent regulation, and to generate a stabilized Hif-3α for in vivo functional studies, we mutated these residues individually or in combination (Figure 2C), which resulted in five Hif-3α mutants. The P393A mutation (i.e., mutant H3-393) had little effect on Hif-3α protein abundance (Figure 2D) or transactivation activity (Figure 2E). The P493A mutation (mutant H3-493) resulted in a significant increase in Hif-3α protein levels (Figure 2D) and a 5.6-fold increase in transcription activity compared with the wild-type Hif-3α group (Figure 2E). The double PP mutant behaved essentially the same as the H3-493 single mutant (Figures 2D and 2E), suggesting that P493 is critical for Hif-3α stability. Mutant H3-PL, in which P493 and L503 were mutated, had levels of accumulated Hif-3α protein and transcriptional activity comparable to those of H3-493 (Figure 2D and 2E). In comparison, mutant H3-PPL (P393A/P493A/L503S) had the highest Hif-3α levels (Figure 2D) and caused an approximately 12.2-fold increase in transcription activity compared with the wild-type Hif-3α group ($p < 0.01$; Figure 2E). This increase was greater than that observed for H3-493, H3-PP, and H3-PL ($p < 0.05$; Figure 2E). These results suggest that although P493 is the most critical, P393 and L503 also contribute to the instability of Hif-3α under normoxia.

Hif-3α Has Transcriptional Activity and Upregulates Gene Expression In Vivo

Using the stabilized Hif-3α (i.e., mutant H3-PPL, hereafter referred to as Hif-3α'), we tested whether Hif-3α has transactivation activity and can upregulate endogenous genes in vivo. For this purpose, capped Hif-3α' mRNA was microinjected into zebrafish embryos with the p2.1 reporter plasmid DNA. This resulted in a 42.4 ± 7.8-fold ($n = 4$) increase in p2.1 reporter activity (Figure 3A). Likewise, forced expression of Hif-1α', a stabilized form of Hif-1α (Zhang et al., 2012), resulted in a 59.0 ± 15.8-fold ($n = 4$) increase in p2.1 reporter gene activity

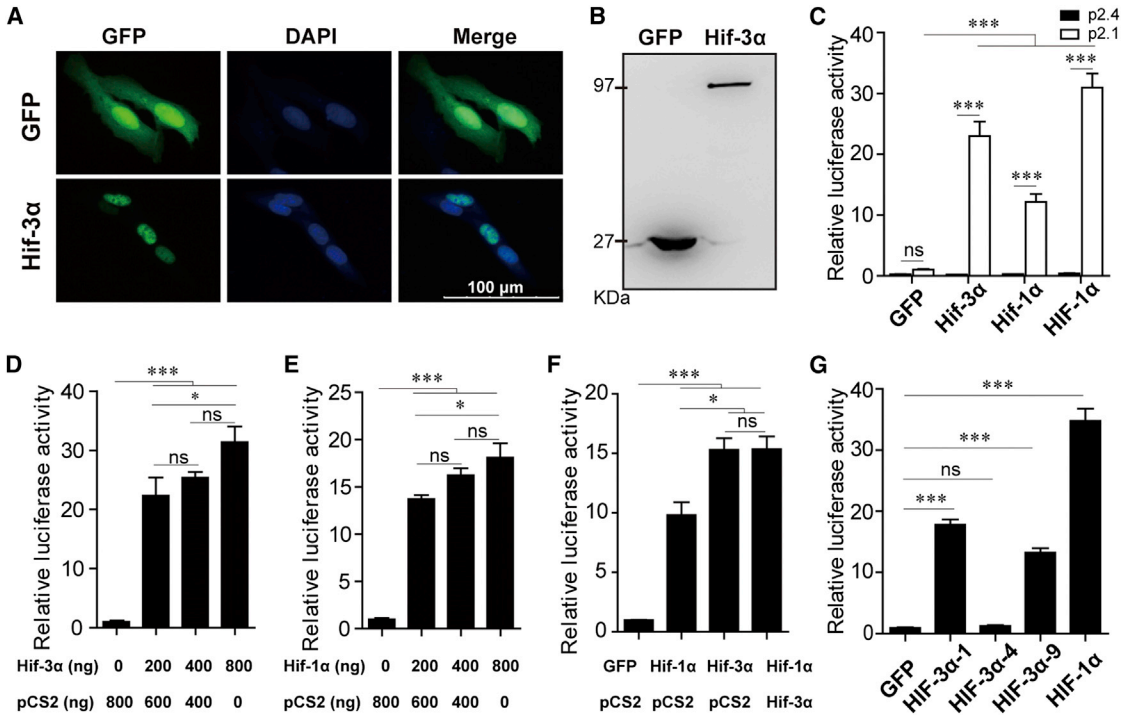

Figure 1. Zebrafish Hif-3α Is a Nuclear Protein and Has HRE-Dependent Transactivation Activity

(A) Nuclear localization of zebrafish Hif-3α. U2OS cells were transfected with pCS2-Hif-3α-GFP (Hif-3α) or pCS2-GFP (GFP). The subcellular localization of the GFP signal was visualized 24 hr after transfection (left panels). Cells were counterstained with DAPI (middle panels). Merged views are shown in the right panels.

(B) Western blot analysis of the above-mentioned cells using a GFP antibody.

(C) HRE-dependent activities of zebrafish Hif-3α, Hif-1α, and human HIF-1α. Cells were transfected with the indicated plasmid together with p2.1 (open bar) or p2.4 (filled bar). pRL-SV40, a *Renilla* luciferase plasmid, was cotransfected as an internal control. The results are normalized and expressed as the fold increase over the GFP p2.1 group. n = 5.

(D and E) Dose-dependent effects of zebrafish Hif-3α (D) and Hif-1α (E). n = 3.

(F) Hif-3α does not inhibit Hif-1α activity. Cells were transfected with p2.1, pRL-SV40, and the indicated plasmid at the same concentration (200 ng). n = 4.

(G) Transactivation activities of human HIF-3α-1, HIF-3α-4, and HIF-3α-9. n = 3.

In this and all subsequent figures, all data shown are means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant. See also Figure S1.

(Figure 3A). Although forced expression of Hif-1α' resulted in significant increases in the mRNA levels of *glut1* and *vegfAb*, two well-known HIF-1 target genes (Chen et al., 2001; Tian et al., 1997), Hif-3α' had no such effect (Figures 3B and 3C). In contrast, both Hif-1α' and Hif-3α' caused significant increases in *redd1* mRNA levels (Figure 3D). There are two duplicated *igfbp-1* genes in zebrafish and they are both regulated by hypoxia (Kajimura et al., 2005; Kamei et al., 2008). Hif-1α' and Hif-3α' both upregulated *igfbp-1a* expression, although Hif-1α' caused a greater increase ($p < 0.01$; Figure 3E). In comparison, expression of Hif-3α', but not Hif-1α', significantly increased *igfbp-1b* expression levels (Figure 3F). These results suggest that Hif-3α is capable of upregulating genes in vivo. In addition, forced expression of Hif-3α or Hif-1α decreases embryo growth and development (see Supplemental Results; Figure S3).

Dominant-Negative Inhibition and Knockdown of Endogenous Hif-3α Reduce Hypoxia-Induced Gene Expression

To investigate the role of endogenous Hif-3α, we engineered a dominant-negative form of Hif-3α (dnHif-3α) and validated its ability to inhibit Hif-3α action in cultured human embryonic kidney 293T (HEK293T) cells (see Supplemental Results; Figure S4). Although forced expression of dnHif-3α in zebrafish embryos completely blocked Hif-3α-stimulated *igfbp-1b* (Figure 4A) and *redd1* (Figure 4B) mRNA expression, it had little effect in inhibiting Hif-1α-induced *glut1* expression (Figure 4C) and caused only a modest reduction of Hif-1-induced *vegfAb* and *igfbp1a* expression (Figures 4D and 4E).

Next, wild-type embryos and dnHif-3α mRNA-injected embryos were subjected to hypoxia treatment. Hypoxia treatment resulted in a 2.9 ± 0.3-fold increase ($p < 0.01$) in *igfbp-1b* mRNA levels (Figure 4F) and a 9.6 ± 0.9-fold increase in *redd1* mRNA levels (Figure 4G) in wild-type embryos. Dominant-negative inhibition abolished the hypoxic induction of *igfbp-1b* expression (Figure 4F) and significantly decreased the magnitude of the hypoxia-induced increase in *redd1* mRNA levels (Figure 4G). In comparison, inhibition of Hif-3α did not change the hypoxia-induced increase in *igfbp1a* expression and had a weaker effect in inhibiting the hypoxic induction of *glut1* and *vegfAb* expression (Figures 4H–4J). These data suggest that while overexpression of dnHif-3α has a stronger effect in

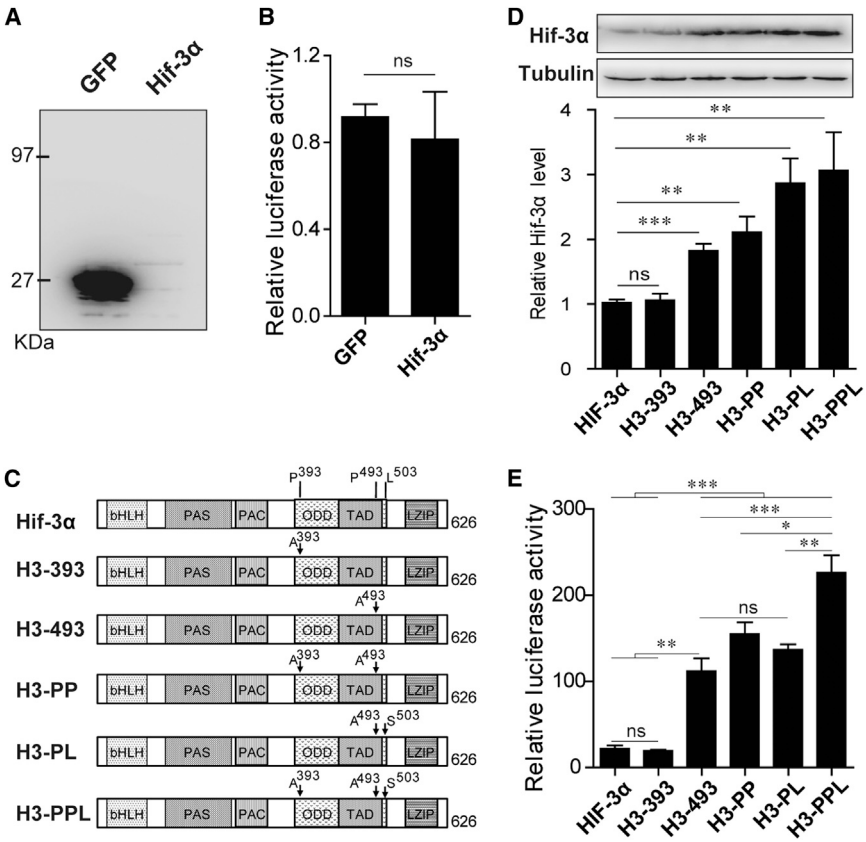

Figure 2. Hypoxia Regulates Hif-3α Stability via Three Conserved Amino Acids in its ODD Domain

(A and B) Hif-3α is rapidly degraded in zebrafish embryos under normoxia. GFP or Hif-3α-GFP capped mRNA was injected into zebrafish embryos together with p2.1 and pRL-SV40 plasmid DNA. The embryos were raised to 12 hpf under normoxia. The embryos were lysed and analyzed by western immunoblot (A) and luciferase assays (B). The levels of luciferase activity were normalized and expressed as the fold increase over the GFP control group. n = 4.

(C) Schematic diagrams of the Hif-3α mutants.

(D and E) Protein stability and transcriptional activity of Hif-3α and its mutants. Capped mRNA encoding Hif-3α-GFP and the indicated mutants was injected into zebrafish embryos together with p2.1 and pRL-SV40 plasmid DNA. The embryos were raised to 12 hpf under normoxia and subjected to western immunoblot.

(D) A representative western blot result is shown in the upper panel. Quantification of Hif-3α-GFP levels, normalized by the Tubulin level, is shown in the lower panel.

(E) The levels of luciferase activity were normalized and expressed as the fold increase over the control group. Results are from three independent microinjection experiments.

See also Figure S2.

inhibiting Hif-3α-regulated genes, it can also affect the Hif-1α target gene expression to a lesser degree.

Because dnHif-3α may also inhibit other Hif-αs, we next used an antisense morpholino (MO)-based targeted gene knockdown approach to further examine the role of endogenous Hif-3α. The efficacy and specificity of the MOs was verified (see Supplemental Results; Figure S5). In the control MO-injected embryos, hypoxia treatment resulted in a highly significant increase in *igfbp-1b* and *redd1* mRNA levels (Figures 4K and 4L). The Hif-3α-targeting MOa significantly reduced the hypoxia-induced increase in *igfbp-1b* and *redd1* gene expression, but it had no effect on their levels under normoxia (Figures 4K and 4L). In comparison, MOa did not change the basal or hypoxia-induced expression of *glut1*, *vegfAb*, and *igfbp1a*, three Hif-1α target genes (Figures 4M–4O). Similar results were obtained with MOb (see Figure S5). These results suggest that Hif-3α functions as a transcriptional activator in zebrafish embryos and mediates hypoxia-induced *igfbp-1b* and *redd1* expression. Data shown in Figure S3 also indicate that Hif-3α plays a role in hypoxia-induced growth and developmental retardation (see Supplemental Results for details).

Identification and Characterization of the Hif-3-Regulated Transcriptional Program

We performed DNA microarray analysis using RNA samples extracted from Hif-1α', Hif-3α', or GFP mRNA-injected embryos. Using a fold change of ≥ 2 and a cutoff line of $p < 0.05$, we found 155 upregulated genes in the Hif-3α' group and 690 genes in the Hif-1α' group (Figure 5A), and 97 of these genes were overlapping. Among the most highly Hif-3α-upregulated genes were caspase 6 (*casp6l2*), myosin (*myhz2*), the developmental gene zp3v2, claudin (*cldn3l*), and calcium channel (*cacng7b*) (see Table S1). Gene Ontology (GO) enrichment and pathway enrichment analyses suggested that Hif-3α and Hif-1α enriched genes in many overlapping processes/pathways, including glucose metabolism, hexose metabolism, arginine and proline metabolism, ubiquitin-mediated proteolysis, apoptosis, p53 signaling, and PPAR signaling (Figures 5B and 5C). There are, however, clear differences. Expression of Hif-3α, but not Hif-1α, enriched genes involved in nitrogen metabolism, methane metabolism, the Jak-STAT signaling pathway, and NOD-like receptor signaling. Hif-1α, but not Hif-3α, enriched genes involved in gluconeogenesis, arachidonic acid metabolism, glycosphingolipid biosynthesis, response to zinc, inositol phosphate metabolism, peroxisome, lysosome, VEGF signaling, insulin signaling, and MAPK signaling (Figures 5B and 5C). Although only Hif-1α expression resulted in the enrichment of known hypoxia response genes (Figure 5B), this is likely due to the fact that most previous studies of HIF target genes focused on HIF-1α.

Quantitative RT-PCR (qRT-PCR) assays were performed to validate the DNA microarray results. Significant increases were confirmed in 32 out 51 genes tested (see Tables S1 and S2). These genes can be categorized into three categories: (1) genes that are upregulated by Hif-3α only, (2) genes that are regulated

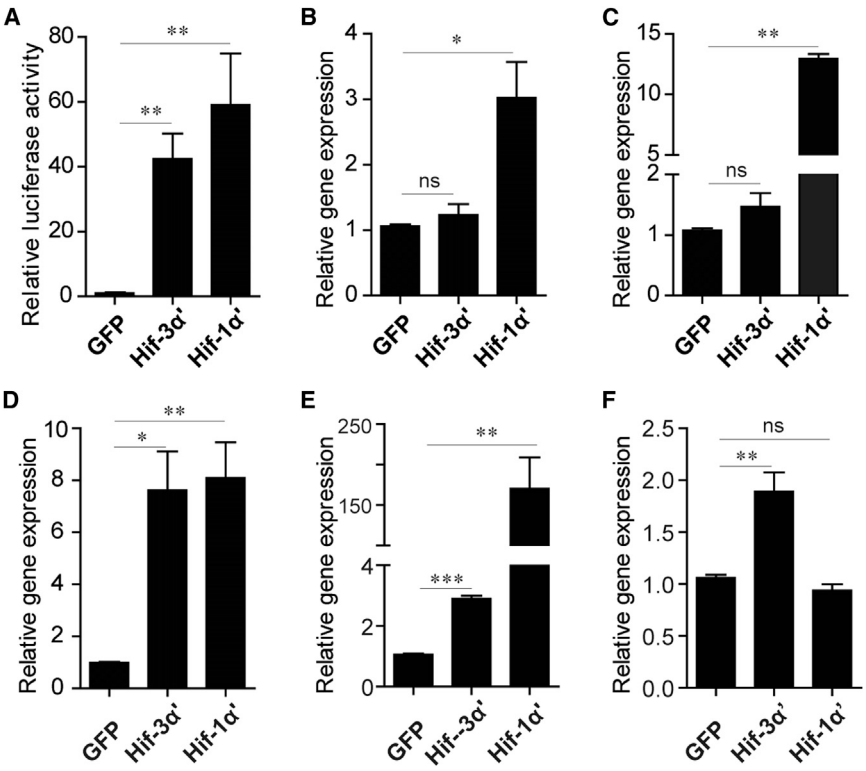

Figure 3. Forced Expression of Hif-3α Increases Hypoxia-Responsive Gene Expression In Vivo

(A) Effect of forced expression of Hif-3α (Hif-3α' = H3-PPL mutant in Figure 2C) and Hif-1α (Hif-1α') on p2.1 reporter activity. GFP, Hif-3α'-GFP, or Hif-1α'-GFP capped mRNA was injected into zebrafish embryos together with p2.1 and pRL-SV40 DNA. The luciferase activity was measured, normalized, and expressed as the fold increase over the GFP control group. n = 4.

(B–F) Effect of Hif-3α' and Hif-1α' expression on *glut1* (B), *vegfAb* (C), *redd1* (D), *igfbp-1a* (E), and *igfbp-1b* (F) mRNA expression. Embryos injected with GFP, Hif-3α', or Hif-1α' capped mRNA were raised to 12 hpf under normoxia and total RNA was isolated. The mRNA levels of the indicated genes were determined by qRT-PCR and normalized by the *β-actin* levels. n = 3.

See also Figure S3.

by both Hif-1α and Hif-3α with similar potencies, or (3) genes that are regulated by both Hif-1α and Hif-3α but with different potencies. We also identified a number of genes that are regulated by Hif-1α, but not by Hif-3α (see Tables S1 and S2). In addition to the upregulated genes, microarray analysis found 100 genes that were downregulated in the Hif-3α group (see Table S3). However, when 12 genes in this group were randomly selected and tested by qRT-PCR, none of them was significantly decreased by Hif-3α expression, and three were actually upregulated by Hif-1α expression (see Figure S6).

To determine whether Hif-3α and Hif-1α upregulate these genes with different kinetics in vivo, we carried out time-course experiments with selected genes in each category. Forced expression of Hif-3α' or Hif-1α' increased their respective candidate target gene expression at 8 hr postfertilization (8 hpf) and reached the maximum effect at 12 hpf (Figures 5D–5J). By 24 hpf, there were no longer detectable increases, with the exception of *igfbp-1a* (Figures 5D–5J). Taken together, these results suggest that Hif-3α expression upregulates a transcriptional program that is distinct from the Hif-1α-regulated gene-expression program in zebrafish embryos.

Hif-3α Binds to the *redd1*, *mlp3c*, and *sqrdl* Promoters and Stimulates their Expression under Hypoxia

We carried out chromatin immunoprecipitation (ChIP)-qPCR experiments using Hif-3α'-GFP mRNA-injected zebrafish embryos to determine whether Hif-3α binds to the promoter sequences of *redd1*, *mlp3c*, and *sqrdl*. Because these genes all contain multiple consensus HREs in the promoter regions, several primer sets were designed and used (Figure 6A). Significant enrichment of *redd1* DNA was detected in both the GFP antibody and Hif-3α antibody ChIP complexes when primer set 2 was used (Figure 6B). No *redd1* enrichment was found using primer set 1, which amplified a more distal region (Figure 6B). Likewise, ChIP-qPCR revealed specific binding of Hif-3α to the *mlp3c* promoter region, but not to distant regions (Figure 6C). These results suggest that Hif-3α specifically binds to the proximal promoter region of *redd1* and *mlp3c*. In the case of *sqrdl*, we detected a 6-fold enrichment in the promoter region near the ATG site (primer set 2) and a smaller enrichment in the region amplified by primer set 4 (Figure 6D), suggesting that Hif-3α binds to more than one HRE in the *sqrdl* promoter.

Western immunoblot analysis indicated that hypoxia treatment resulted in a robust increase in the levels of endogenous Hif-3α (Figure 6E). ChIP-qPCR analysis showed significant enrichment in *redd1* (Figure 6F), *mlp3c* (Figure 6G), and *sqrdl* (Figures 6H and 6I) in the ChIP complexes under hypoxia. In contrast, there was no increase in the normoxia group (Figures 6F–6I). To show that Hif-3α is indeed required for hypoxic induction of *redd1*, *mlp3c*, and *sqrdl* expression, zebrafish embryos injected with GFP mRNA, dnHif-3α mRNA, control MO, MOa, or MOb were raised in normoxic or hypoxic water. The *redd1*, *mlp3c*, and *sqrdl* mRNA levels were determined by qRT-PCR. Whereas hypoxia treatment resulted in highly significant increases in *redd1*, *mlp3c*, and *sqrdl* mRNA levels in the GFP control group and the control MO group, these increases were significantly reduced in the dnHif-3α, MOa, and MOb groups (Figures 7A–7C). These results suggest that Hif-3α is stabilized and binds to the target gene promoters, and increases their expression under hypoxia.

Human LC3C, REDD1, and SQRDL Genes Are Regulated by HIF-3α-9 and Induced by Hypoxia in Human Cells

To determine whether human HIF-3α regulates the expression of similar genes in human cells, we engineered a human HIF-3α

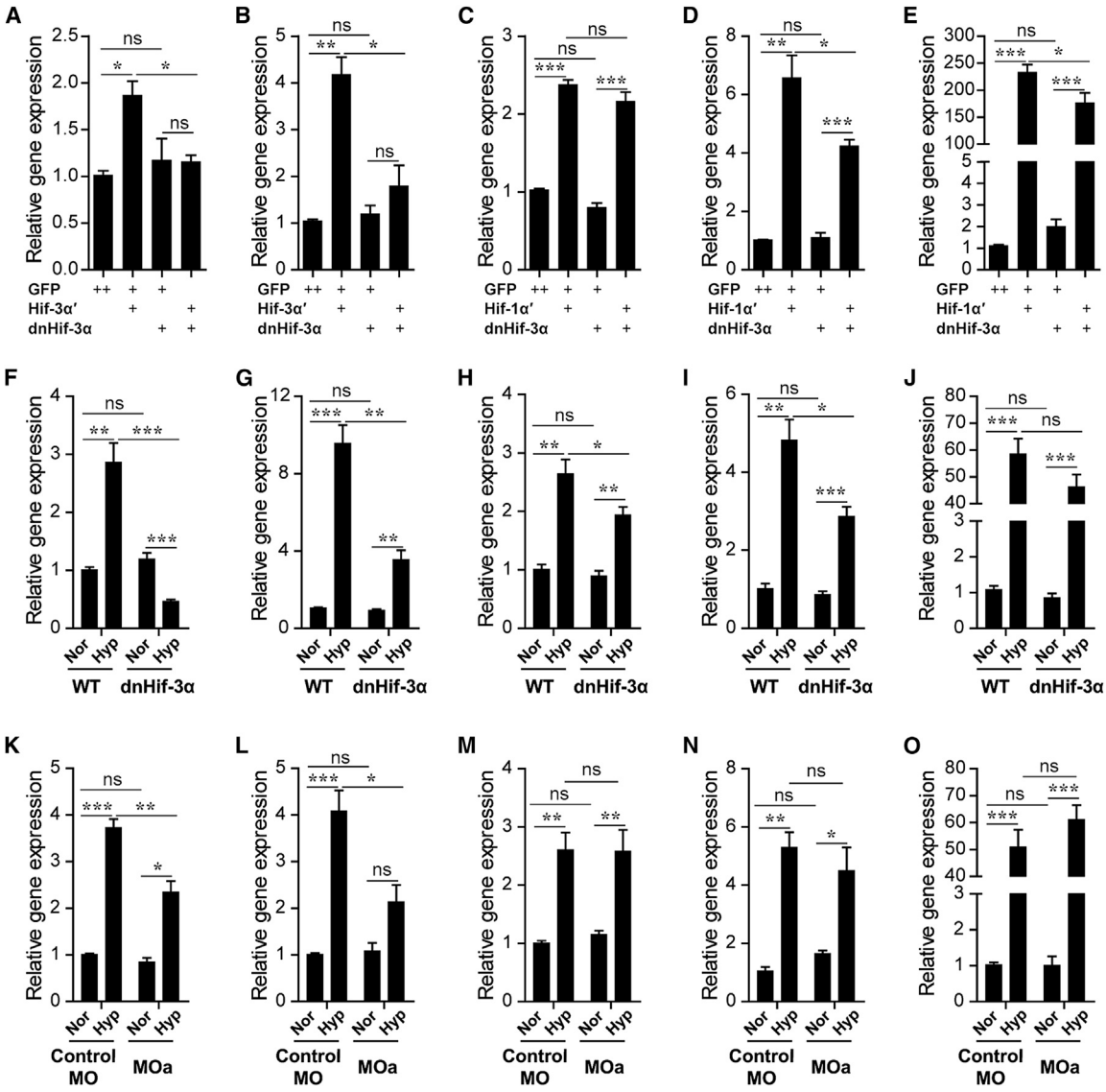

Figure 4. Dominant-Negative Inhibition and Knockdown of Hif-3α Inhibit Hypoxia-Induced Gene Expression

(A–E) Expression of dnHif-3α inhibits Hif-3α'- but not Hif-1α'-stimulated gene expression in vivo. Embryos injected with GFP, Hif-3α', dnHif-3α, or Hif-3α' mRNA^{+}dnHif-3α mRNA were raised to 12 hpf. RNA was isolated and the mRNA levels of *igfbp-1b* (A), *redd1* (B), *glut1* (C), *vegfAb* (D), and *igfbp1a* (E) were determined by qRT-PCR and normalized by the *β-actin* levels. n = 3.

(F–J) Expression of dnHif-3α blunts hypoxic induction of *igfbp-1b* and *redd1* expression. Noninjected or dnHif-3α mRNA-injected embryos were raised in normoxic (Nor) or hypoxic water (Hyp). The mRNA levels of *igfbp-1b* (F), *redd1* (G), *glut1* (H), *vegfAb* (I), and *igfbp1a* (J) were determined by qRT-PCR and normalized by the *β-actin* levels. n = 3.

(K–O) Knockdown of Hif-3α inhibits Hif-3α- and hypoxia-induced gene expression. Control MO or *hif-3α* targeting MOa-injected embryos were raised in normoxic (Nor) or hypoxic (Hyp) water and sampled at 12 hpf. The mRNA levels of *igfbp-1b* (K), *redd1* (L), *glut1* (M), *vegfAb* (N), and *igfbp1a* (O) were determined by qRT-PCR and normalized by the *β-actin* levels. n = 3.

See also Figures S4 and S5.

mutant by changing the conserved P406, P492, and L502 in HIF-3α-9 into A406, A492, and S492 (i.e., HIF-3α'). This mutant had significantly higher transcriptional activity compared with wild-type HIF-3α-9, likely resulting from increased protein stabilization (see Figure S7A). A stabilized human HIF-1α plasmid (i.e., HIF-1α' thereafter) was used as a control (Yan et al., 2007). As in the case with zebrafish embryos, overexpression of human HIF-3α' and HIF-1α' both significantly increased the REDD1 mRNA expression in HEK293 cells, although human HIF-3α' had greater activity compared with HIF-1α' in HEK293 cells (Figure 7D). In HEK293 cells, overexpression of human HIF-3α' significantly increased the levels of LC3C and SQRDL mRNA (Figure 7D), whereas human HIF-1α' had no such effect (Figure 7D). Overexpressed zebrafish Hif-3α' in these human cells

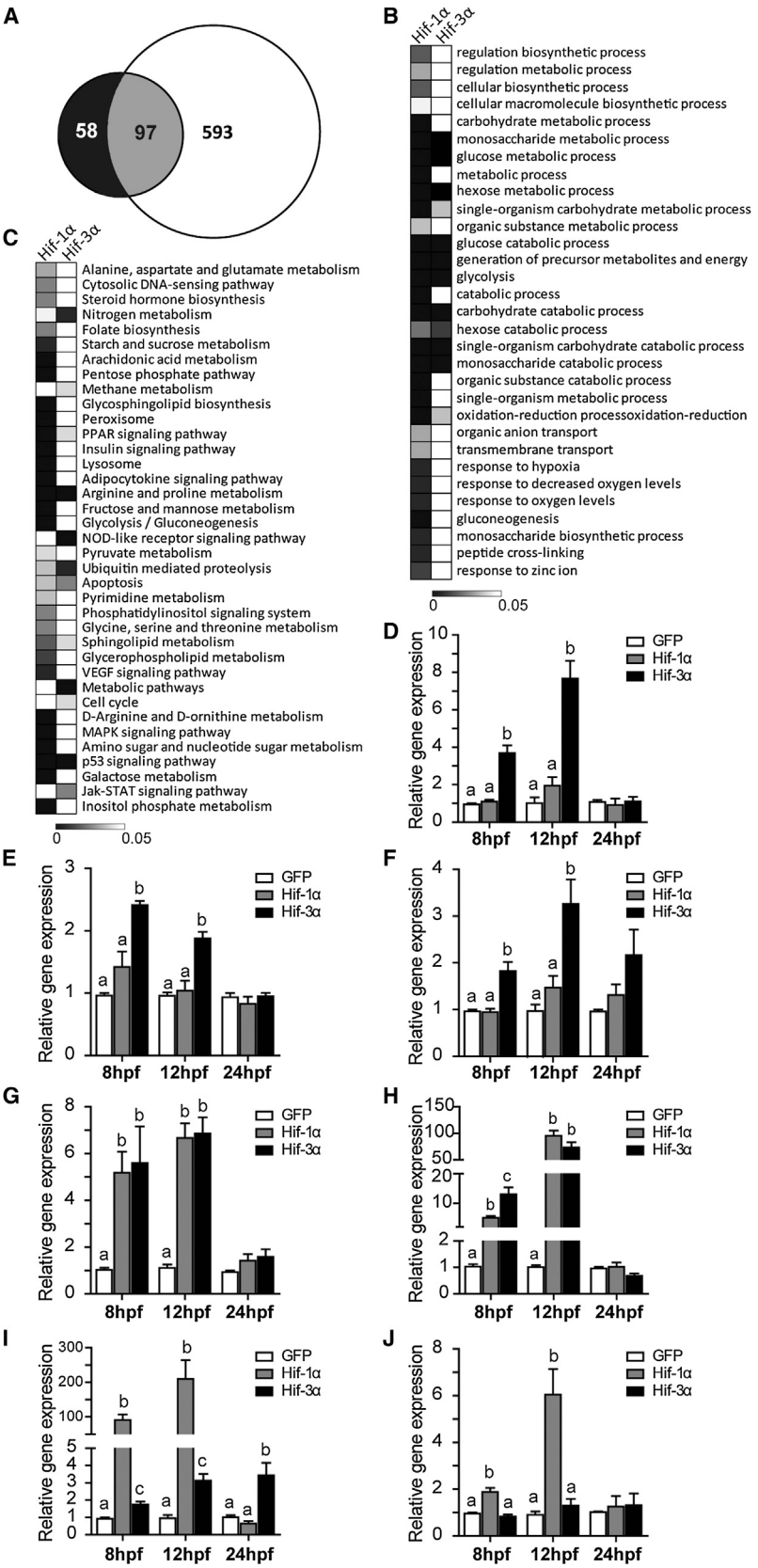

Figure 5. Transcriptomics Analyses of Hif-3α- and Hif-1α-Regulated Genes in Zebrafish Embryos

(A) Venn diagrams represent the number of Hif-1α and Hif-3α upregulated genes identified by gene-expression microarrays. Differentially expressed genes were identified by comparing the Hif-1α' or Hif-3α' mRNA-injected embryos with the GFP mRNA-injected control embryos (fold change value ≥ 2, $p < 0.05$). The official symbols of the Hif-1α and Hif-3α upregulated genes are shown in Table S1.

(B) Heatmaps of GO analysis. Genes were analyzed by SAS and submitted to Gitools. GO terms were selected to display in heatmaps according to their statistical significance and locations in the GO tree. Columns and rows in the heatmaps indicate treatments and enriched biological process GO terms, respectively. Color scales indicate p values of enrichment tests, and open cells represent an empty value or a value ≥ 0.05.

(C) Heatmaps of pathway enrichment analysis. Genes were subjected to analysis based on KEGG by SAS and Gitools. Columns and rows in heatmaps indicate treatments and enriched pathway terms, respectively. Color scales represent p values of enrichment tests, and open cells indicate an empty value or a value ≥ 0.05.

(D–J) qRT-PCR results. The expression of *sqrdl* (D), *mclb* (E), and *zp3v2* (F) is upregulated by Hif-3α, but not Hif-1α. The expression of *redd1* (G) and *mlp3c* (H) is upregulated by both Hif-1α and Hif-3α, with similar potencies and kinetics. The expression of *igfbp1a* (I) is regulated by Hif-1α and Hif-3α, but with different potencies and kinetics. The expression of *vegfAb* (J) is regulated by Hif-1α, but not Hif-3α. Capped mRNA of GFP, Hif-1α', and Hif-3α' was injected into embryos. The injected embryos were kept under normoxia and sampled at the indicated time points after injection. The mRNA levels of the indicated genes were measured by qRT-PCR, normalized by the *β-actin* levels, and shown as values relative to the GFP control group. n = 3. Groups labeled with different letters are significantly different from each other ($p < 0.05$).

See also Figure S6.

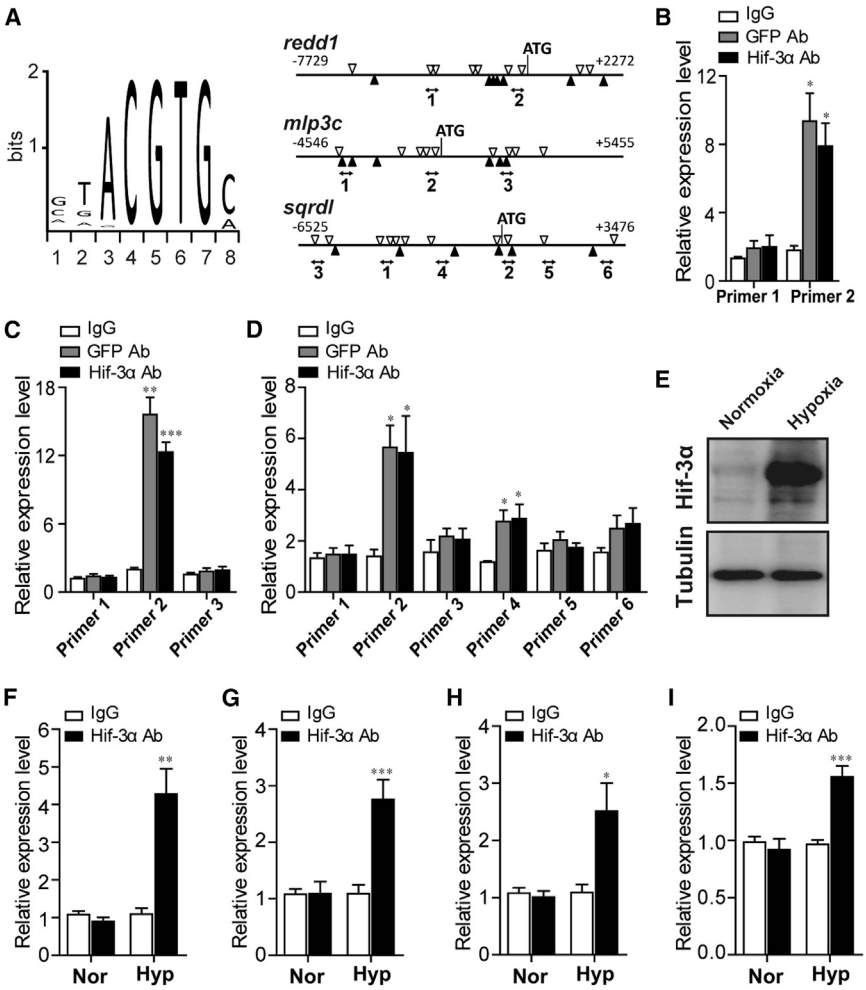

Figure 6. Hif-3α Is Stabilized and Binds to the Target Gene Promoters

(A) Position weight matrix for HREs identified in the promoter region of the indicated genes. Forward HREs are indicated by filled triangles, and reverse HREs are indicated by open triangles. Several primer sets (shown as number in the bottom) were designed and used to perform ChIP-qPCR analysis.

(B–D) ChIP-qPCR analysis of Hif-3α binding to *redd1* (B), *mlp3c* (C), and *sqrdl* (D). Embryos injected with Hif-3α'-GFP mRNA were subjected to ChIP using the indicated antibodies followed by qPCR using the primer sets shown in (A). n = 3.

(E) Hypoxia increases Hif-3α levels. Zebrafish embryos raised in normoxic or hypoxic water were analyzed by western immunoblot using the indicated antibodies.

(F–I) Hypoxia induces Hif-3α binding to the *redd1* (F), *mlp3c* (G), and *sqrdl* (H and I) promoters. Wild-type zebrafish embryos raised in normoxic or hypoxic water were subjected to ChIP-qPCR analysis using the Hif-3α antibody and primer sets described above. n = 3.

had similar effects (Figure 7D). We next tested whether these HIF-3α-9-regulated genes are indeed induced by hypoxia in human cells. Hypoxia treatment resulted in significant increases in expression of LC3C, REDD1, and SQRDL in HEK293 cells (Figure 7E). Similar results were obtained in human osteosarcoma cells, suggesting that hypoxic induction of LC3C, REDD1, and SQRDL occurs in many cell types (Figure 7F). These results suggest that HIF-3α is capable of upregulating gene expression in human cells under hypoxia, and the transcriptional activity of HIF-3α/Hif-3α is evolutionarily conserved.

DISCUSSION

In this study, we provide direct evidence that zebrafish Hif-3α functions as a transcriptional activator and plays an indispensable role in regulating gene expression in response to hypoxia. This HRE-dependent transactivation activity is also found in several human HIF-3α isoforms. When tested in human cells, human HIF-3α-9 and zebrafish Hif-3α were found to be capable of upregulating target gene expression, suggesting that this is an evolutionarily conserved function of Hif-3α/HIF-3α. These findings strongly challenge the dogma that HIF-3α functions a negative regulator of HIF-1α and HIF-2α.

Multiple lines of evidence support our conclusion. First, zebrafish Hif-3α is in the nucleus and possesses HRE-dependent transactivation activity when overexpressed in cultured human cells. Human HIF-3α isoform 1 and isoform 9 have similar activity. Second, expression of a stabilized Hif-3α in zebrafish embryos increases the expression of an HRE reporter construct, as well as endogenous genes such as *igfbp-1b*, *sqrdl*, *mclb*, and *zp3v2*. Expression of stabilized Hif-1α did not alter the expression of the above genes, although it increased the expression of *glut1* and *vegfAb*, two HIF-1 target genes (Chen et al., 2001; Tian et al., 1997). In addition, *redd1*, a hypoxia-responsive gene in zebrafish and in human cells (Brugarolas et al., 2004; Feng et al., 2012), showed elevated expression in response to both Hif-3α and Hif-1α expression. The third line of evidence came from loss-of-function experiments. Under hypoxia, there was a robust increase in endogenous HIF-3α protein levels and a concomitant increase in *sqrdl*, *mclb*, *zp3v2*, *igfbp-1b*, *redd1*, and *mlp3c* mRNA expression. Dominant-negative inhibition and knockdown of Hif-3α abolished or reduced their expression under hypoxia. These data argue strongly that endogenous HIF-3α is required for the hypoxic induction of these genes. The most direct evidence came from the ChIP-qPCR experiments. When Hif-3α was expressed, it bound to specific region(s) in the *sqrdl*, *mclb*, and *redd1* promoters and upregulated their expression. Furthermore, endogenous Hif-3α bound to these promoter regions under hypoxia, but not normoxia. When Hif-3α was knocked down or inhibited, hypoxia-induced Hif-3α-DNA binding was reduced. Taken together, these results

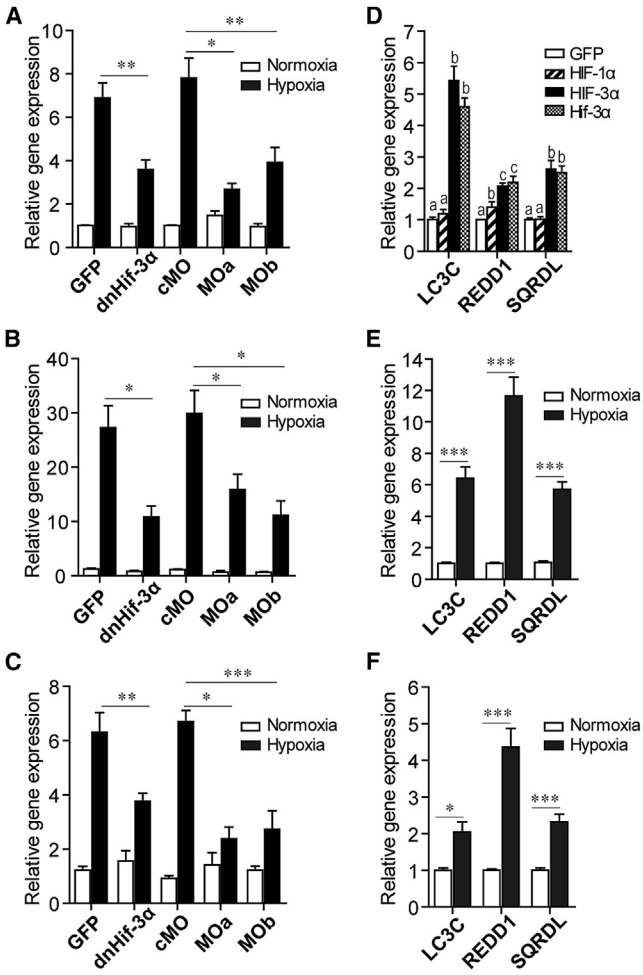

Figure 7. Zebrafish Hif-3α and Human HIF-3α9 Upregulate the Expression of *redd1*/REDD1, *mlp3c*/LC3C, and *sqrdl*/SQRDL

(A–C) Hif-3α is required for the hypoxic regulation of *redd1* (A), *mlp3c* (B), and *sqrdl* (C) expression in zebrafish embryos. GFP mRNA, dnHif-3α mRNA, control MO, *hif-3α*-targeting MOa, and MOb-injected embryos were raised under normoxia (Nor) or hypoxia (Hyp) and sampled at 12 hpf. The mRNA levels of these Hif-3α target genes were determined by qRT-PCR. n = 3.

(D) Overexpression of HIF-3α9' upregulates the expression of REDD1, LC3C, and SQRDL in HEK293 cells. Cells were transfected with the indicated plasmid. Total RNA was isolated 12 hr after transfection. The mRNA levels of the indicated genes were determined by qRT-PCR. n = 3.

(E and F) Hypoxic induction of REDD1, LC3C, and SQRDL expression in human HEK293 (E) and U2OS (F) cells. Cells were grown under ambient air (21% O_2) or hypoxia (1% O_2) for 12 hr. Total RNA was isolated. The mRNA levels of the indicated genes were determined by qRT-PCR and shown as relative value of the control group. n = 3.

See also Figure S7.

provide unequivocal evidence that Hif-3α functions as an oxygen-dependent transcriptional activator in vivo. Importantly, we show that human HIF-3α-1 and HIF-3α-9 have similar transcriptional activity, and overexpression of HIF-3α-9 increases the expression of LC3C, REDD1, and SQRDL in human cells. Hypoxia treatment also induced the expression of LC3C, REDD1, and SQRDL in human HEK293 and osteosarcoma cells. These findings support the notion that LC3C, REDD1, and SQRDL are HIF-3α-9 target genes in human cells. Future studies will be needed to determine which HIF-3α isoforms are expressed in these human embryonic and tumor cells, and whether knockdown of one or more of these HIF-3α isoforms will alter the hypoxic induction of LC3C, REDD1, and/or SQRDL expression.

In this study, we characterized and compared the transcriptional programs induced by Hif-3α and Hif-1α in zebrafish embryos. This study identifies the Hif-3α-regulated gene-expression program and compares it with those regulated by Hif-1α in an in vivo setting. Although more in-depth analyses are needed, several interesting themes have already emerged. It is evident that Hif-3α and Hif-1α regulate overlapping yet distinct sets of genes in vivo. Compared with Hif-3α, Hif-1α regulates a larger set of genes. Based on the DNA microarray and qRT-PCR assay results, there are three distinct groups of Hif-3α-regulated genes: (1) those that are upregulated by Hif-3α only, (2) those that are regulated by both Hif-1α and Hif-3α with similar potencies, and (3) those that regulated by both Hif-1α and Hif-3α but with different potencies. Based on GO enrichment and pathway enrichment analyses, we conclude that both Hif-3α and Hif-1α can increase the expression of genes involved in glucose and amino acid metabolism, apoptosis, proteolysis, p53 signaling, and PPAR signaling. Hif-3α, but not Hif-1α, increases the expression of genes in the Jak-STAT signaling and NOD-like receptor signaling pathways. Likewise, only Hif-1α enriches genes in VEGF signaling, insulin signaling, MAPK signaling, and inositol phosphate metabolism. It is also clear that Hif-3α and Hif-1α increase their respective genes with similar kinetics in vivo. Although approximately 100 genes showed decreased expression resulting from Hif-3α expression in the microarray analysis, we do not think these genes are directly suppressed by Hif-3α, for the following reasons: When 12% of the genes in this group were tested by qRT-PCR, none of them was significantly decreased in the embryos in which Hif-3α was overexpressed. In previous microarray analyses performed in human and mammalian cells, hundreds of mRNAs were decreased by hypoxia treatment (Prabhakar and Semenza, 2012). Subsequent ChIP assays, however, showed that HIF-1 does not bind directly to these hypoxia-repressed genes (Mole et al., 2009). It was suggested that hypoxia/HIF-1 represses these genes by upregulating a transcriptional repressor(s) and/or miRNA(s) (Prabhakar and Semenza, 2012). It has also been proposed that HIF-1 may repress gene expression by binding to the reverse HREs (Jeong et al., 2007; Lee et al., 2010; Mazure et al., 2002). Future studies are needed to determine whether Hif-3α directly represses gene expression and whether it binds to a reverse HRE.

Oxygen-dependent degradation is an important mechanism by which hypoxia regulates HIFα abundance and activity. This is best understood in human HIF-1α (Ivan et al., 2001; Masson et al., 2001). In this study, we show that P393, P493, and L503 in zebrafish Hif-3α, and P406, P492, and L502 in human HIF-3α are involved in the oxygen regulation of their stability. This is supported by the finding that the zebrafish PPL mutant had greater transcriptional activity and increased protein levels. We tested the effect of mutation of P406, P492, and L502 on human HIF-3α protein stability indirectly using the transcriptional

activity assay because these plasmids have no protein tag. These residues are homologous to P402, P564, and L574 in human HIF-1α, suggesting that a similar PHD-pVHL-dependent mechanism is responsible for the oxygen-dependent degradation of Hif-3α/HIF-3α. We noted several differences between our study of Hif-3α/HIF-3α and previous reports on human HIF-1α. Although both P402 and P564 are hydroxylated in human HIF-1α and involved in its degradation under normoxia (Ivan et al., 2001; Masson et al., 2001), P493 is clearly the most important residue in zebrafish Hif-3α. Mutation of this residue significantly increased the stability and biological activity of the protein. In comparison, mutation of P393 alone or together with P493 had little effect. Consistent with the mutational analysis results, we found that the LAPYIXXXDFQL motif containing P493 is conserved in all known vertebrate HIF-1αs, HIF-2αs, and HIF-3αs. The LXXLAP motif is not present in human HIF-3α even though P393 is conserved in mammalian HIF-3αs. pVHL recognizes human HIF-1α at L574 (Huang et al., 2002; Kageyama et al., 2004), which is required for human HIF-1α instability (Bruick and McKnight, 2001; Ivan et al., 2001). The corresponding residue in Hif-3α is L503. Mutation of L503 together with P493 and P393 resulted in a further increase in Hif-3α protein levels and biological activity, suggesting that L503 contributes to the instability of Hif-3α under normoxia.

In conclusion, the present study provides multiple lines of evidence supporting the notion that Hif-3α is an oxygen-dependent transcription activator, and that it plays an important role in the transcriptional response to hypoxia by binding to target gene promoters and stimulating their expression. Our study also identifies numerous Hif-3α target genes. We also provide evidence that human HIF-3α-9 is capable of upregulating unique target gene expression, and these HIF-3α target genes are induced by hypoxia in human cells. The identification of these Hif-3α target genes provides critical information for future studies. It should be noted that our current study focuses on the full-length Hif-3α. As in the case of the human HIF-3α gene, our preliminary analysis showed that the zebrafish *hif-3α* gene generates multiple alternatively spliced transcripts, but the full-length transcript is the predominant form expressed in zebrafish embryos. It will be important to determine the structure and function of these other isoforms and clarify their functional relationship to the full-length Hif-3α. It is evident from the present study that Hif-3α and Hif-1α regulate the expression of overlapping yet distinct sets of genes in vivo. It will be of interest to elucidate why and how some genes are regulated by either Hif-3α or Hif-1α, or both. Understanding the molecular mechanisms underlying the specificity of HIF-1, HIF-2, and HIF-3 actions will provide critical insights into the transcriptional response to hypoxia.

EXPERIMENTAL PROCEDURES

Reagents and Animals

Chemicals and reagents were purchased from Fisher Scientific unless noted otherwise. Human HIF-3α plasmids (pcDNA-Zeo(−)-HIF-3α-1/4/9) were kindly provided by Dr. Johanna Myllyharju (University of Oulu). Stabilized human HIF-1α plasmid (HA-HIF1alpha P402A/P564A-pcDNA3) was obtained from Addgene. The construction of pCS2-Hif-3α-GFP and pCS2-Hif-1α'-GFP (a stabilized form of zebrafish Hif-1α) plasmids was described previously (Zhang et al., 2012). Zebrafish (*Danio rerio*) were maintained on a 14/10 hr light/dark cycle at 28°C and fed twice daily. Fertilized eggs obtained by natural cross were raised and staged as described previously (Kajimura et al., 2005). To inhibit pigmentation, embryo medium was supplemented with 0.003% (w/v) 2-phenylthiourea. All experiments were conducted in accordance with guidelines approved by the University of Michigan Committee on the Use and Care of Animals.

Plasmid Construction

To generate a stabilized form of Hif-3α for in vivo functional analysis, its P393, P493, and L503 were changed into Ala or Ser individually and in combination in the pCS2-Hif-3α-GFP background using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Likewise, the corresponding P406, P492, and L502 in HIF-3α isoform 9 (which has a structure similar to that of zebrafish Hif-3α) were changed into Ala or Ser in a similar manner. The primers used are shown in Table S4. To engineer a dominant-negative form of Hif-3α, DNA encoding a fragment of Hif-3α (14–390 aa) was amplified by PCR and subcloned into the pCS2-GFP vector, resulting in the pCS2-dnHif-3α-GFP plasmid (primers shown in Table S4).

To verify the efficacy of the MOs used (see below), a 373 bp DNA fragment corresponding to the 5′ UTR and exon1 and exon2 of *hif-3α* was amplified by RT-PCR using the primers shown in Table S4. The amplified DNA was subcloned in frame into pCS2-GFP. To generate a control GFP reporter construct lacking the MO target sequence, the target sequences of MOa (GATGGTG AACTCTACGTCGAAAAGG) and MOb (CTGCGACCTTTTCAAACATTACTGA) were mutated into (GATGGTCAATAGCACCAGCAAGCGC) and (CACCCTCG ATTTCTCAGTTGCCACA) by site-direct mutagenesis using the primers shown in Table S4. All of the above plasmids were verified by sequencing.

Cell Culture, Transfection, Subcellular Localization, and Luciferase Reporter Assay

Human U2OS cells and HEK293T cells were purchased from American Type Culture Collection. Cell culture, transfection, and subcellular location of Hif-3α were performed as described previously (Zhang et al., 2012). The transactivation activities of various Hif isoforms and mutants were determined in vitro using HEK293 cells, and in vivo in zebrafish embryos as described in Supplemental Experimental Procedures.

Hypoxia Experiment, mRNA, and MO Injection

Hypoxia treatment of zebrafish embryos was conducted as described previously (Kajimura et al., 2005). Cultured cells were subjected to hypoxia (1% O_2) or normal oxygen (21% O_2) in a humidified modular incubation chamber as previously reported (Ren et al., 2010). Capped mRNA synthesis, MOs, and microinjection are described in Supplemental Experimental Procedures.

Western Blot and Real-Time qRT-PCR

Western blot and real-time qRT-PCR assays were performed as described in Supplemental Experimental Procedures.

DNA Microarray Analysis

Three biological replicates were collected from three independent microinjection experiments. Each RNA sample was extracted from a pool of 60 embryos using Trizol. DNA was removed by RNase-Free DNase I treatment. The RNA samples were subjected to DNA microarray analysis using the SBC Analysis System (http://www.ebioservice.com/). The microarray was conducted using the commercially available Agilent 4 × 44 K (V3) Zebrafish Microarray Chip (Shanghai Biochip). Microarray data were analyzed using the SBC Analysis System. Heatmaps describing enriched GO terms and pathways were generated using Gitools (Perez-Llamas and Lopez-Bigas, 2011). The Diffgene program was used to identify Hif-3α- and/or Hif-1α-regulated genes.

ChIP Analysis

ChIP assays were performed using a previously described procedure (Peng and Westerfield, 2006) with some modifications. Briefly, embryos were injected with capped mRNA encoding Hif-3α'-GFP or Hif-1α'-GFP at the one-cell stage and fixed with 1% formaldehyde at 12 hpf. GFP mRNA-injected

or intact embryos were treated in parallel and used as controls. Crosslinked chromatin samples were fragmented to an average length of 1 kb by sonication. Rabbit GFP antibody or the Hif-3α antibody was added to each chromatin sample. DNA samples from the immunoprecipitated chromatin preparations were purified and then analyzed by qPCR (primers shown in Table S4). The ChIP-PCR results were normalized by no antibody control.

Statistics

The data shown are means ± SE. Differences among groups were analyzed by one-way or two-way ANOVA followed by Tukey's multiple comparison test or by unpaired t test using GraphPad Prism version 5.01. Significance was accepted at $p \leq 0.05$.

ACCESSION NUMBERS

The GEO accession number for the data reported in this paper is GSE54318.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Results, Supplemental Discussion, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.011.

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