Identification of oleoylethanolamide as an endogenous ligand for HIF-3α

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Hypoxia-inducible factors (HIFs) are α/β heterodimeric transcription factors modulating cellular responses to the low oxygen condition. Among three HIF-α isoforms, HIF-3α is the least studied to date. Here we show that oleoylethanolamide (OEA), a physiological lipid known to regulate food intake and metabolism, binds selectively to HIF-3α. Through crystallographic analysis of HIF-3 α/β heterodimer in both apo and OEA-bound forms, hydrogen-deuterium exchange mass spectrometry (HDX-MS), molecular dynamics (MD) simulations, and biochemical and cell-based assays, we unveil the molecular mechanism of OEA entry and binding to the PAS-B pocket of HIF-3α, and show that it leads to enhanced heterodimer stability and functional modulation of HIF-3. The identification of HIF-3α as a selective lipid sensor is consistent with recent human genetic findings linking HIF-3α with obesity, and demonstrates that endogenous metabolites can directly interact with HIF-α proteins to modulate their activities, potentially as a regulatory mechanism supplementary to the well-known oxygen-dependent HIF-α hydroxylation.
Hypoxia-inducible factors (HIFs) belong to the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) family of transcription factors, which sense physiological changes or environmental stimuli and respond by modulating target gene expression. Like other members of this family, transcriptionally active HIFs are heterodimeric proteins consisting of one a subunit and one β subunit. There are three HIF-α isoforms in mammals (HIF-1α, HIF-2α, and HIF-3α), each encoded by a different gene. The β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is a Class II member within the bHLH-PAS family, and it acts as a common heterodimerization partner for certain Class I members that include the HIF-α proteins, aryl hydrocarbon receptor, neuronal PAS (NPAS) proteins and single-minded (SIM) proteins.

The biological functions of HIF-1α and HIF-2α have been investigated extensively, since the initial cloning and early characterization of these two genes in 1995 and 1997, respectively. The oxygen-dependent hydroxylation of specific proline residues on HIF-α by hydroxylases, leads to its proteasomal degradation under normoxia. In addition, an asparagine at the C-terminus of HIF-α can be hydroxylated under normoxia, which blocks its interaction with coactivators necessary for the transcriptional activation. These two oxygen-dependent protein modifications both restrict HIF-α activities at normal oxygen levels. But under oxygen deprivation, HIF-α proteins escape degradation, dimerize with ARNT and initiate the transcription of gene programs leading to increased angiogenesis, erythropoiesis and glycolytic metabolism, in an isoform- and cell type-specific manner. Besides oxygen level, the HIF pathway is also regulated by other cellular conditions, such as the metabolic status. For example, the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate is an obligate substrate required for the hydroxylase-dependent HIF-α modification. However, the clear-cut cavities inside the PAS domains of HIF-α and ARNT proteins raise the key question of whether endogenous small-molecule ligands, such as metabolites, can directly bind to and modulate the activity of HIFs.

Compared with HIF-1α and HIF-2α, HIF-3α is a much less studied isoform, despite having its gene first identified in 1998. A complication to understanding the full range of HIF-3α functions is that multiple variants of different sizes appear to be produced from its gene locus, due to alternative mRNA splicing and utilization of different promoters or transcription initiation sites. At least seven such HIF-3α variants were confirmed in human cells, and three variants found in mouse. Among these variants, two types have been investigated most. The first type includes the longer HIF-3α variants that share a similar domain composition with full-length HIF-1α and HIF-2α proteins, except that a C-terminal leucine zipper (LZIP) domain replaces the C-terminal transactivation domain. With this LZIP substitution, these HIF-3α variants can dimerize with ARNT properly but exhibit a relatively lower transcriptional activity. Recent studies have revealed that certain long variants of human HIF-3α function as transcription activators and upregulate their own specific target genes. For example, overexpression of HIF-3α2 in Hep3B cells induces the expression of a group of genes, including erythropoietin (EPO) and heat shock protein family A (Hsp70) member 6 (HSPA6). In addition, overexpression of HIF-3α9 in HEK293 cells upregulates the expression of REDDI, LC3C, and SQDLE. The second type of HIF-3α variants are quite distinct. Through alternative splicing, their polypeptide chains are only about half the size (e.g., HIF-3δ4 in Supplementary Fig. 1), with a special sequence in the PAS-B domain that has been suggested to impart a unique ability to inhibit the HIF pathway activity through direct binding to HIF-1α and HIF-2α proteins.

Interestingly, the potential role of HIF-3α as a lipid sensor was suggested mainly based on the crystal structure of its PAS-B domain in complex with bacterial lipids, which remained bound within the cavity during protein expression, purification and crystallization. Here, we began by examining the overall heterodimeric structure of HIF-3α-ARNT complex, consisting of the N-terminal segments (including the bHLH, PAS-A and PAS-B domains as shown in Fig. 1a) from both proteins. We then conducted a biochemical high-throughput screening campaign using a compound library of cellular metabolites, leading to the identification of an endogenous lipid oleylthanolamide (OEA) as a ligand capable of binding to HIF-3α selectively. OEA is a naturally occurring ethanolamide produced in the small intestine following feeding, and regulates satiety and body weight. We further studied the molecular mechanisms of how OEA enters the closed PAS-B pocket of HIF-3α, and how that binding enhances HIF-3α-ARNT heterodimer stability and transcriptional activity in cells.
Results

Quaternary structure of HIF-3α-ARNT heterodimer. To fully visualize the ligand-binding potential of HIF-3α, we crystallized the multi-domain mouse HIF-3α-ARNT complex and solved its structure at 2.3 Å resolution (Fig. 1b). Each asymmetric unit of the crystal contained a single heterodimer (Supplementary Table 1). The overall structure of HIF-3α-ARNT proved to be fairly similar to that of the HIF-2α-ARNT complex17, with a root mean squared deviation (RMSD) of 1.8 Å between their Ca atoms. Meanwhile as shown in the superimposition of these two structures (Fig. 1c), the N-terminal a helix in the bHLH domain of HIF-3α is slightly longer and rotated about 10° relative to that of HIF-2α. However, it remains unclear if this distinction is real or artificial, as it may have risen only due to the differences in their respective crystal packings that impact this region.

In the previously reported high-resolution structure of the single human HIF-3α PAS-B domain22, its inner cavity was occupied by a mixture of lipids that derived from the Escherichia coli expression system. In our study, protein production of the multi-domain HIF-3α-ARNT heterodimer in E. coli only yielded an apo form with no discernible ligand present in the PAS-B domain of HIF-3α or elsewhere in the complex. The contrasting finding about the pocket contents of overexpressed HIF-3α proteins suggests that although bacteria-derived lipids can bind to HIF-3α, their ease of removal during purification is consistent with their reversible binding capability. As calculated by Fpocket program29, we didn’t find a sizeable pocket in the HIF-3α PAS-A domain, but observed an empty cavity of about 280 Å3 within the PAS-B domain, comparable in size to the corresponding HIF-2α pocket (Fig. 1d). This vacancy provided us with a clear opportunity to screen small-molecule libraries to identify unique molecules capable of binding to HIF-3α selectively.

Identification of OEA as a direct-binding ligand for HIF-3α. To discover novel ligands interacting with HIF-3α, we conducted a biochemical direct-binding assay using the affinity selection-mass spectrometry (AS-MS) technique30 with purified multi-domain HIF-3α-ARNT heterodimer proteins. Aiming at potential intrinsic HIF-3α ligands, we screened an in-house collection of more than 2000 cellular endogenous molecules, with >7% representing fatty acids and lipids. The screen identified OEA, a natural metabolite of oleic acid to be a highly selective binder of HIF-3α proteins suggests that although bacteria-derived lipids can bind to HIF-3α, their ease of removal during purification is consistent with their reversible binding capability. As calculated by Fpocket program, we didn’t find a sizeable pocket in the HIF-3α PAS-A domain, but observed an empty cavity of about 280 Å3 within the PAS-B domain, comparable in size to the corresponding HIF-2α pocket (Fig. 1d). This vacancy provided us with a clear opportunity to screen small-molecule libraries to identify unique molecules capable of binding to HIF-3α selectively.

In our chemical screens, we did not detect OEA binding to either HIF-1α-ARNT or HIF-2α-ARNT heterodimers, which both served as counter-screening targets, indicating the binding of OEA to HIF-3α-ARNT to be selective and further suggesting that this binding was directed to the HIF-3α subunit (since all three heterodimers contained an identical ARNT protein). No other selective ligands whose binding could be reconfirmed using an orthogonal assay were identified in our screening, for any of the three HIF-α subunits. To confirm that OEA indeed binds to HIF-3α directly, we first conducted a thermal shift assay with the HIF-1α-ARNT, HIF-2α-ARNT and HIF-3α-ARNT complex proteins, respectively (Fig. 2b). OEA increased the melting temperature ($T_m$) of the HIF-3α-ARNT complex by about 1.7 °C, much higher than its effects on the other two isomers. Subsequently, we employed the surface plasmon resonance (SPR) method to confirm and measure the binding affinity of OEA to the isolated HIF-3α PAS-B domain, and obtained an approximate $K_D$ value of 14.0 μM (Fig. 2c). These findings pinpointed the PAS-B domain pocket of HIF-3α as the binding site of OEA.

HIF-2α antagonists targeting its PAS-B domain have been found to allosterically disrupt the interactions between HIF-2α and ARNT33-37. We previously discovered HIF-2α agonists and revealed the molecular mechanism of how ligands binding to HIF-2α’s PAS-B pocket bidirectionally modulate its dimerization with ARNT37. To test for this possibility in the context of the HIF-3α-ARNT complex, we used a time-resolved fluorescence resonance energy transfer (TR-FRET)-based in vitro assay that we established and utilized for heterodimeric HIF proteins37. As shown in Fig. 2d, OEA enhanced the interaction between HIF-3α and ARNT, while it had no discernible effect on the interaction of HIF-2α with ARNT. These results suggest that OEA specifically binds to HIF-3α and promotes the physical dimerization between HIF-3α and ARNT proteins, and thus potentially functions as a HIF-3α agonist.

We then sought to test if there is an agonistic effect of OEA on the transcriptional activity of HIF-3α. As mentioned earlier, the human HIF-3α gene undergoes extensive alternative splicing, resulting in a number of variants with different lengths or sequences (namely HIF-3α1 ~ HIF-3α10, Supplementary Fig. 1). In this study we focused on the representative variant HIF-3α1, which possesses the bHLH, PAS-A, PAS-B, ODDD (oxygen-dependent degradation domain), NTAD (N-terminal transactivation domain) and LZIP regions in its polypeptide chain. First, we overexpressed HIF-3α1 in HEK293 cells and checked the mRNA expression level of several reported target genes, including EPO, HSPA6, REDD1, LC3C and SQORDL. Among them, the expression of HSPA6 was clearly elevated by HIF-3α1 overexpression in the HEK293 cells (Fig. 2e), as well as in the Hep3B and HepG2 cells (Supplementary Fig. 2a, b), suggesting that HSPA6 might be a downstream gene positively regulated by the HIF-3α1 variant. Next, we treated the HEK293 cells with OEA, and found that OEA further increased the HSPA6 level with HIF-3α1 overexpression (Fig. 2e), indicating that OEA may function as an agonist to enhance the activity of HIF-3α. To explore for more HIF-3α target genes, we conducted a preliminary RNA-seq using HEK293 cells and confirmed the mRNA expression of several genes by real-time PCR. At least three genes, TCF20, PSME2 and IFIT1, were identified as potential downstream genes of the HIF-3α pathway, as their mRNA levels were elevated by HIF-3α1 overexpression and further by OEA treatment (Supplementary Fig. 2c).

Since the above-mentioned cell-based experiments were performed under a normal oxygen condition, we wanted to know whether or to what extent the overexpressed HIF-3α1 proteins would be degraded in cells. Therefore, we tested the HIF-3α protein level within HEK293 cells that were transfected with an empty vector, the full-length wide-type HIF-3α1 and its corresponding degradation-resistant P490A mutant38, respectively. According to the Western blotting results (Supplementary Fig. 2d), the intrinsic HIF-3α protein was barely detectable under normoxia in these cells. Surprisingly, the overexpressed wild-type HIF-3α1 reached a similar level as the P490A mutant, indicating that the oxygen-dependent degradation was not decisive for HIF-3α in our experimental conditions. Moreover, OEA treatment showed no discriminable effect on the HIF-3α protein level in cells, despite its facilitation on the dimerization between HIF-3α and ARNT biochemically (Fig. 2d).

Agonistic mechanism of OEA through allosteric effects. To further explore the mechanism of binding and HIF-3α transcriptional modulation by OEA, we pursued and obtained the crystal structure of HIF-3α-ARNT in complex with OEA at a
Supplementary Fig. 3c). As a result, the size of PAS-B pocket of HIF-3 showing clear conformational changes. One locates at the PAS-B pocket of ARNT PAS-A and PAS-B domains (A/B loop), which is connecting ARNT PAS-A and PAS-B domains (A/B loop) and the Fα from HIF-3 PAS-B, which is reported as only 510 Å³ even in the presence of bound lipid. For a direct comparison, we calculated the PAS-B pocket of their structure using Fpocket and obtained a similar value of 700 Å³. Since the cavity size measurement would depend on the algorithms of different programs, we adopted another program called PyVOL for a recalibration. The 3.3Å PAS-B pocket of our structure in the apo and OEA-bound forms was measured respectively as 225 Å³ and 516 Å³, which again showed that this cavity expands substantially upon ligand binding. The second region affected is the loop connecting ARNT PAS-A and PAS-B domains (A/B loop), which is more stable and continuous in the OEA-bound complex structure (Fig. 3d), as evidenced by the differences in their “omit” density maps (Supplementary Fig. 3f, g). This stabilization effect of OEA on the A/B loop is consistent with its ability to stabilize the HIF-3α-ARNT heterodimer (Fig. 2d).

To further compare the intrinsic dynamics of HIF-3α-ARNT in the OEA-bound and apo forms, we conducted MD simulations on three systems (Supplementary Fig. 4a–c): HIF-3α-ARNT in complex with OEA (HIF-3αOEA), HIF-3α-ARNT-OEA complex excluding OEA (HIF-3αOEA-ARNT) and apo HIF-3α-ARNT structure (HIF-3αap). The root mean square fluctuation (RMSF) profile of ARNT subunit suggests that besides the modelled segments, the ARNT A/B loop shows a high degree of flexibility, especially in the context of the HIF-3αOEA system (Fig. 3e). We further monitored the RMSD values for ARNT A/B loop in three systems (Fig. 3f), which revealed that A/B loop in HIF-3αOEA is more stable than that in the other two systems, validating this region is stabilized by OEA binding.

We also found that majority of HIF-3α residues within the PAS-B pocket undergo only subtle conformational changes upon OEA binding, except for the three residues G237, F239 and L338 that gate the inner cavity and enlarge the pocket by conformational changes (Fig. 3c, Supplementary Fig. 3c). Mainly through hydrophobic interactions, OEA contacts with multiple residues in the HIF-3α PAS-B pocket, including F239, H243, M247, F249, I276, V284, I288, L291, Y302, F304, and A318 (Fig. 3b). In addition, OEA forms two hydrogen bonds with the main chain atoms of H336 and L338 near the pocket entrance (Fig. 3b).

To learn how OEA binding changes the conformation of HIF-3α-ARNT heterodimer, we compared the OEA-bound and apo structures directly (Supplementary Fig. 3b). By superimposition, their overall dimeric structures are very similar with a RMSD of only 0.4 Å for all Ca atoms. However, there are two regions showing clear conformational changes. One locates at the PAS-B pocket of HIF-3α, where residues G237, F239 and L338 move their positions substantially to accommodate OEA binding (Fig. 3c, Supplementary Fig. 3c). As a result, the size of PAS-B pocket dramatically increased after the binding of OEA, from about 280 Å³ in the apo form, to more than 700 Å³ in the complex bound to OEA, suggesting that OEA binding increases the cavity size by 150% (Supplementary Fig. 3d, e). We noticed that the cavity size within the single PAS-B domain structure of HIF-3α was reported as only 510 Å³ even in the presence of bound lipid. For a direct comparison, we calculated the PAS-B pocket of their structure using Fpocket and obtained a similar value of 700 Å³. Since the cavity size measurement would depend on the algorithms of different programs, we adopted another program called PyVOL for a recalibration. The 3.3Å PAS-B pocket of our structure in the apo and OEA-bound forms was measured respectively as 225 Å³ and 516 Å³, which again showed that this cavity expands substantially upon ligand binding. The second region affected is the loop connecting ARNT PAS-A and PAS-B domains (A/B loop), which is more stable and continuous in the OEA-bound complex structure (Fig. 3d), as evidenced by the differences in their “omit” density maps (Supplementary Fig. 3f, g). This stabilization effect of OEA on the A/B loop is consistent with its ability to stabilize the HIF-3α-ARNT heterodimer (Fig. 2d).

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Next, to experimentally monitor conformational changes of HIF-3α-ARNT in the solution state, we employed the HDX-MS assay and examined the altered protein dynamics in the presence of OEA. HDX-MS data indicated that OEA binding stabilized multiple residues within the Gβ and Fα regions of HIF-3α PAS-B domain (Fig. 3i, Supplementary Fig. 5a), consistent with results
from the above MD studies. To be more specific, OEA stabilized residues F304, Y302 and V284 by hydrophobic interactions, and thus immobilized Gβ and Fα. It is possible that the stabilized Gβ and Fα function as an "interlayer" to further contact with the AB-loop of ARNT. As shown in Fig. 3j, stabilized R303 at Gβ forms hydrogen bonds with ARNT A/B-loop. Therefore, results from HDX-MS, crystallographic and MD studies, together indicate that OEA allosterically enhances the interactions between HIF-3α and ARNT, by stabilizing key residues within the Gβ and Fα regions of HIF-3α PAS-B domain.

**Ligand entry route into the PAS-B pocket.** In the OEA-bound HIF-3α-ARNT co-crystal structure, we find that OEA was not completely shielded inside the HIF-3α PAS-B pocket. While its hydrophobic fatty acid chain was well buried in the pocket, the ethanolamine group of OEA was partially exposed to solvent outside of the cavity (Fig. 4a). This arrangement suggests a highly favorable binding mode whereby hydrophobic and hydrophilic portions are ideally disposed for enhanced overall binding energetics. An enlarged tunnel was formed within the HIF-3α PAS-B domain to accommodate the unique shape and size of the mono-
unsaturated OEA molecule (Supplementary Fig. 3d, e), and this tunnel also suggests a clear entry route for OEA binding. As shown in Fig. 4b, the tunnel entrance was mainly surrounded and sealed by four residues, G237 at Aβ, R255 at Ca helix, V259 at Ca helix, and L338 at Iβ strand, in the apo HIF-3α structure. But upon OEA binding, the side chain of L338 flipped 90°, Ca helix moved 1.2 Å outward, and Aβ flipped by 35° to the outside of the pocket, thereby providing access to OEA within this entrance tunnel (Fig. 3c, Fig. 4c).

To verify the hypothesis that OEA enters the HIF-3α PAS-B pocket through the above route, we made a G237W mutation on HIF-3α, expected to block the entrance due to the bulky side chain of tryptophan. As implied by the decreased ΔTm value in the thermal shift assay (Fig. 4d), the binding ability of OEA to this HIF-3α mutant was indeed reduced, compared with the wild-type protein. Moreover, the G237W mutation diminished the ability of OEA to enhance the stabilization of HIF-3α-ARNT heterodimer as monitored by TR-FRET assay (Fig. 4e), as well as its ability to further induce the mRNA expression of HSPA6 as measured by PCR (Fig. 4f). These results suggested that the loop region prior to Aβ, where G237 is located, is crucial for the entering and binding of OEA into the HIF-3α PAS-B pocket.

As shown in the comparison of superposed structures (Fig. 3c), the binding position of OEA overlaps with the Aβ of PAS-B domain from the apo HIF-3α structure. Therefore, the opening of Aβ is likely a key step for OEA to enter the pocket. RMSF values were calculated for the HIF-3α subunit in each of the three MD systems (Fig. 4g), indicating that besides modelled linkers and terminal positions, the Aβ with its prior loop (loop-Aβ, residues 227–244) and Ca helix regions, which surround the entrance of HIF-3α PAS-B pocket, exhibit changes in flexibility between different systems.

We then examined snapshots isolated from the trajectories of HIF-3α^OEA (h) and HIF-3α^noOEA (i). Error bars, mean ± SD.; n = 3 (biological replicates) for (d–f).
Fig. 5 A possible common mechanism in ligand binding shared by HIF-2α. a The residues of Fα and Gβ with reduced deuteration levels are mapped on the crystal structure in blue, and the R308 in Gβ of HIF-2α forms a hydrogen bond with the A/B loop of ARNT. Hydrogen bonds are shown in red dotted lines. b Two routes previously proposed for ligands to enter or leave the HIF-2α PAS-B pocket are indicated by black arrows, and enlarged views of two routes are shown on the left and right sides, respectively. The possible new entry route of ligands into HIF-2α, corresponding to that of OEA into HIF-3α, is shown by a green arrow, with the residues surrounding the entrance also colored green. M252 and Y281 are shown in magenta in the apo structure, and in blue in co-crystal structures. M252 and Y281 were flipped outwards (as shown by dotted arrows) by antagonist PT2385 and agonist M1001, respectively. The PDB codes of HIF-2α-ARNT-PT2385 and HIF-2α-ARNT-M1001 are 6E3S and 6E3U. c Ca RMSF plots for HIF-2α in a monomeric system. The highly flexible regions including modeled linkers (black) and loop-Aβ (magenta) are highlighted. d The conformational change of the loop-Aβ region in snapshot structures extracted from the trajectories of HIF-2α during the MD stimulations.

These above MD results imply that the loop-Aβ region can sweep between different conformational states, leading to the generation of a small pocket at the entrance of HIF-3α PAS-B domain, which can then become subsequently enlarged and merged into the inner cavity during the entry of ligands.

A common allosteric mechanism of HIF-α isoforms. Since the protein sequences of three HIF-α isoforms are highly conserved (Supplementary Fig. 7), and their overall structural arrangements as heterodimers with ARNT are also similar17 (Fig. 1c), it is possible that they all share a similar allosteric mechanism triggered by ligand binding to their PAS-B domains. We previously discovered novel agonists for HIF-2α, and identified the key residue Y281 that mediates the enhanced interaction at the dimer interface of HIF-2α and ARNT37. Interestingly, the HIF-2α agonist M1001 was also found to promote the stability of ARNT A/B loop in the structure of HIF-2α-ARNT complex37. And the HDX-MS data of this heterodimer revealed that M1001 enhanced the stability of Gβ and Fα in solution (Supplementary Fig. 5b). In addition, the HIF-2α residue R308 at Gβ, which is conserved in all three HIF-α proteins (corresponding to the HIF-3α R303), formed a hydrogen bond with the ARNT A/B loop in the complex structure (Fig. 5a, Supplementary Fig. 7). Therefore, we speculate that agonists binding to the HIF-α PAS-B pockets can allosterically enhance interactions between the Gβ and Fα regions of HIF-α and the A/B loop of ARNT, which might be a common mechanism for agonists to stabilize HIF-α, ARNT heterodimers.

Because both agonists and antagonists of HIF-2α were previously found to be totally encapsulated in the PAS-B domain in the co-crystal structures37, it was unclear what the actual ligand-entry pathway might be for the HIF-2α PAS-B pocket. A previous study using MD stimulations suggested two possible primary routes for ligands to enter or leave the HIF-2α PAS-B domain40. One is between Gβ and Fα (Route 1), and the other is between Fα, Ea and AB-loop (Route 2) (Fig. 5b). We previously identified two critical residues, Y281 and M252 within the PAS-B pocket of HIF-2α, whose side chains can be flipped out toward the PAS-B/PAS-B dimer interface by ligand binding, and directly mediate the stabilizing or destabilizing effects on the dimerization by agonists or antagonists, respectively37. However, we noticed that side chains of Y281 and M252 locate right in the middle of above two proposed routes, making it unfavorable for ligands to flip them out during entry (Fig. 5b). On the other hand, within the PAS-B domain these two residues locate on the opposite side of the position corresponding to the OEA entrance in HIF-3α (between the Aβ and Cα helix as shown in Fig. 4c). This spatial distribution implies that if HIF-2α ligands enter the PAS-B pocket through a similar route as OEA into HIF-3α, it would be much easier for them to allosterically modulate the dimerization by flipping key interface residues out of the pocket (i.e., Y281 and M252). To test this hypothesis of HIF-2α possessing a similar ligand entry route, we examined whether the loop-Aβ region of HIF-2α could also switch its conformations as the corresponding region of HIF-3α by MD stimulations. RMSF values were calculated for the HIF-2α monomeric system (Fig. 5c). As shown in Fig. 5d, the loop-Aβ region of apo HIF-2α also moves outwards during the simulation, leading to a bigger and deeper pocket at the corresponding entrance position of HIF-2α PAS-B domain (Supplementary Fig. 6e, f). These MD results and above data indicated that HIF-2α may share a similar mechanism as HIF-3α.
for the entry of ligands into their PAS-B pockets and consequent allosteric effects on the dimerization.

Discussion

The bHLH-PAS family proteins are versatile transcription factors that help living organisms to sense and respond to environmental changes, including the oxygen supply, day/night cycles, toxicants and so on. The physiological functions of various members of this family have been extensively studied in the past decades. For example, the oxygen-sensitive post-translational modification of HIF-α proteins by the hydroxylases, was proven as the key regulatory step for cells to adapt to different oxygen levels. Meanwhile, whether any endogenous ligands can selectively bind to the HIF-α proteins and modulate their activities, has not been clear. Since the identification of a water-bound cavity within the crystal structure of HIF-2α PAS-B domain, artificial small-molecule inhibitors targeting this pocket have been discovered and developed into an FDA-approved anti-cancer drug belzutifan (also known as MK-6482 and PT2977), highlighting the regulatory potential of HIF-2α by ligands. Recently, we conducted a high-throughput compound screening on HIF-2α, and discovered novel HIF-2α agonists. These findings suggest that potential endogenous HIF-α ligands may act as agonists or antagonists, depending on their structures and physiological roles.

Here with a screening strategy utilizing three HIF-α-ARNT heterodimer proteins, we identified an endogenous metabolite, OEA as a ligand binding selectively to the PAS-B domain of HIF-3α. OEA is a derivative of oleic acid with known effects on food intake control and lipid metabolism. Notably, the OEA concentration was reported to be around 200-400 nM in the rat small-intestine mucosa. As OEA production by enterocytes elevates after feeding, the transient intracellular concentration of OEA may reach a micromole range. Correlating with the intestinal mobilization of OEA, HIF-3α is also highly expressed in the small intestine, especially in the enterocytes experiencing hypoxia due to the coverage of intestinal mucus. Interestingly, an epigenome-wide study revealed that increased DNA methylation at the HIF3α locus was associated with elevated body mass index, suggesting a link between HIF-3α and body weight. Moreover, a recent genome-wide association study provided evidences that HIF-3α may function as a key regulator of lipolysis by modulating the expression levels of several related genes (e.g., LIPE, PLIN1, and PNPLA2) in the adipocytes, where OEA is also biosynthesized. In addition, it was reported that OEA-containing supernatants from the cultured chronic lymphocytic leukemia cells induced lipolysis in isolated adipocytes. These findings all imply the physiological importance of OEA as a strong candidate for endogenous HIF-3α ligands. More investigations are warranted to reveal exactly how the effects of OEA on obesity and lipolysis derive from HIF-3α relative to peroxisome proliferator-activated receptor alpha, a nuclear receptor which can be bound and activated by OEA to induce satiety. The other proteins reported to interact with OEA are transient receptor potential vanilloid 1 and G protein-coupled receptor 119 (GPR119), Therefore, OEA may link multiple signaling pathways together and coordinate various comprehensive physiological processes besides metabolism.

Our discovery of OEA as an endogenous ligand for HIF-3α further supports the possible existence of endogenous ligand-dependent regulatory mechanisms for the HIF pathways, adding a new aspect of transcriptional modulation on top of the well-recognized oxygen-dependent hydroxylation of HIF-α subunits. Moreover, given the variation in residues outlining the PAS-B pockets of three HIF-α isoforms (Supplementary Fig. 7), each isoform is expected to recognize different endogenous ligands selectively to regulate its activity. Furthermore, we also compared OEA with several related N-acyl ethanolamines (NAEs) in term of their interactions with HIF-3α, using the thermal shift assay (Supplementary Fig. 8). The results suggested that HIF-3α may not recognize palmitoylethanolamide (PEA, 16:0), arachidonoyl-ethanolamine (AEA, 20:4) or docosahexaenoylethanolamine (DHEA, 22:6), and among the 18-carbon NAEs, HIF-3α prefers OEA (18:1) to stearoylethanolamide (SEA, 18:0) and linoleoyl-ethanolamide (LEA, 18:2). This ligand specificity might be critical for the supplement to the more general oxygen-dependent mechanism that broadly controls the enzymatic activities of several hydroxylases, which can efficiently catalyze HIF-α proteins but with a relatively low selectivity. Therefore, these above two mechanisms working in concert would provide cells with a fine-tuned responsiveness to both oxygen level and metabolic status, emphasizing the master regulator role of HIF pathway in many biological processes.

As mentioned above, the previously reported single PAS-B domain structure of human HIF-3α came from E. coli-produced proteins, which captured 11Z-octadecenoic acid (i.e., cis-vaccenic acid; 18:1, cis-11) derivatives in the hydrophobic cavity. With efforts on the removal of bacterial lipids from the purified proteins, this study also conducted a fluorescence-based binding assay that implied a preference of the HIF-3α PAS-B domain for unsaturated 18-carbon fatty acids. These findings laid the groundwork for our ongoing efforts pursuing the physiological function of HIF-3α as a specific lipid sensor, and also implied a potential mediator role for HIF-3α in connecting gut microbes and the human host. In this work, we obtained multi-domain heterodimeric HIF-3α-ARNT structures in both apo and OEA-bound forms, suggesting that lipids may not serve as mandatory structural cofactors for HIF-3α as originally proposed, but rather as free regulatory ligands. By directly comparing these ligand-bound HIF-3α structures (Supplementary Fig. 9a-c), we found two PAS-B domains possessing almost identical overall structures (Ca RMSD of 0.89 Å). Moreover, OEA and 1-(11Z-octadecenoyl)-sn-glycerol showed a similar shape and location within the pocket, although their carbon-carbon double bonds are indeed at different positions (cis-9 vs. cis-11) for these two monounsaturated (18:1) lipids, suggesting an accommodation of the HIF-3α PAS-B domain for various specific ligands.

Several previous studies revealed that the longer HIF-3α variants can promote tumor growth or metastasis in colorectal and pancreatic cancers, suggesting HIF-3α inhibitors might be beneficial for certain tumors. However, it has been shown that different HIF-α isoforms can either promote or suppress the tumor progression in different cancer types, such as the well-studied sibling rivalry between HIF-1α and HIF-2α isoforms. Since the physiological functions of HIF-3α (especially its complicated relationship with the other isoforms) have not been fully revealed, more investigations are urgently needed to evaluate the strategy of targeting HIF-3α in tumor and other diseases. The discovery of small-molecule ligands would facilitate these studies by providing tool compounds. Therefore, we will continue to look for new HIF-3α ligands with a better selectivity and potency. Since all the three HIF-α isoforms dimerize with ARNT in a very similar way, it is not very surprising to find that their ligands may share a common allosteric mechanism at the dimer interface. For example, here we show that for both HIF-3α and HIF-2α, agonists can stabilize the G8 and Fα of the PAS-B domain to enhance their interactions with the ARNT A/B loop. Moreover, the ligand entry route revealed by OEA in HIF-3α might also exist in other isoforms. If so, it would provide some informative clues to design proper compounds to efficiently enter and enlarge the relatively small cavities of HIF-α, such as the one within HIF-1α PAS-B domain. Secondly, pocket entry route is one of the key
factors to consider in the design of proteolysis-targeting chimera (PROTAC) molecules. Our findings may shed light on the future development of PROTAC drugs targeting HIF-a proteins.

Methods

Plasmid construction and site-directed mutagenesis. For the protein overexpression in E. coli, mouse ARNT (GenBank AAH12870.1, residues 82-464) was cloned into the pMKH vector with or without a GFP-tag at its C-terminus as previously described. Meanwhile, mouse His3a (GenBank AAI02588.1, residues 4-358) was cloned into the pSJ2 vector. For the direct binding assay, the PAS-B domain of HIF-3a (residues 235-363) fused by a maltose-binding protein (MBP-HIF-3a-PAS-B) was cloned into the pSJ2 vector. For the cell-based experiments, full-length human HIF-3α (GenBank RA60698.1, residues 1-660) and its mutants G237W and P490A were cloned into the pCMV-Tag4 vector. Site-directed mutagenesis was performed as instructed by the kit manufacturer, and confirmed by DNA sequencing.

Protein expression and purification. To obtain HIF-3a-ARNT complex proteins, the recombinant plasmids pSJ2-HIF-3α was co-transformed along with pMKH-ARNT into BL21-CodonPlus (DE3)-RIL competent cells (Agilent Technologies). The bacteria were cultured for another 24 h. Then the cells were harvested, and RNA was extracted using Trizol (Life technologies) according to the manufacturer’s instructions. The concentration of RNA in the sample was measured using a NanoDrop spectrophotometer (ThermoFisher Scientific), and the purity was determined by the 260/280 nm absorbance ratio. The RNA was then treated with DNase I (Invitrogen) to remove any contaminating DNA and then purified using the Qiagen RNeasy mini kit (Qiagen). The purified RNA was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. The cDNA was then subjected to quantitative real-time PCR using a LightCycler 480 system (Roche) and the SYBR Green Master Mix (Yeasen). The primer sequences for the real-time PCR are as follows:

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ACTB: (F: 5′-GCTGTTCCTCCAGACAGCTT-3′, R: 5′-GTTTCCTGGAAGAGTTGCTC-3′);
PSME2: (F: 5′-GGTTTTCAGGGTCCACTTCA-3′, R: 5′-GACAGCGCTCCTCGGCTCTT-3′);
TCF20: (F: 5′-CTTCCTGGGAAATCTTG-3′, R: 5′-GTTGCTGGTTAGAATTTTGAC-3′);
FHL1: (F: 5′-GGTTCCTGCAGGTCACAGCTG-3′, R: 5′-GGTTCCTGCAGGTCACAGCTG-3′);
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The mRNA expression levels of the target genes were normalized to the expression level of β-actin (ACTB) or PSME2 and then calculated using the 2^−△△CT method.

Time-resolved fluorescence energy transfer (TR-FRET)-based in vitro binding assay. The protein complex of GFP-tagged ARNT and His-tagged HIF-3α was dispersed into 384-well plates with OEA of serial concentrations (0.01–200 µM). After addition of the Mab Anti-6HIS-Tb cryptate Gold (Cisbio 61H12TLF) into each well at 1.05 ng for 1 min and kept in dark for 1 h. Then the protein interactions were monitored via the energy transfer signals using a Time-resolved Fluorescence Spectrometer (Tecan). The temperature was controlled at 22 °C with a flow rate of 30 s. The time to reach the maximum value was 23 s after injection start. The raw data were analyzed in BIA evaluation 3.02 software. The equilibrium dissociation constant Kd was calculated by steady state analysis using a 1:1 Langmuir binding model.

Surface plasmon resonance (SPR) binding assay. To assess the binding affinity between the HIF-3a and OEα (Sigma-Aldrich O0383), the SPR assay was performed on a Biacore T200 (GE Healthcare) using commercially available CM5 sensing chips at 25 °C. The Mab in the buffer was injected at 200 µM (pH 7.4), 400 µM NaCl, 0.02% P-2 and 5% DMSO. According to the manufacturer’s instructions, MBP and MBP-HIF-3a-PAS-B proteins diluted in 10 mM NaAc (pH 5.5) were immobilized onto the surface of flow cells 1 and 2 of the chip, respectively. Subsequently, eight different concentrations (30 µM, 25 µM, 20 µM, 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM) of OEA were prepared and injected to the flow cells, and the responses at the baseline were subtracted from each one. The flow rate was a constant 30 µL·min⁻¹. After the binding equilibrium was reached, the flow was switched to the injection buffer for 1 min, then the injection buffer was used for stabilization for another 1 min, and the flow rate was switched back to 30 µL·min⁻¹ until the baseline was reached. The resonance unit (RU) value of DOPE score was further refined using Rosetta Energy score (M. Jones, personal communication). The surface plasmon resonance data were analyzed using GraphPad Prism 7 as described previously.

Western blotting. HEK293 cells were cultured in DMEM medium supplemented with 10% FBS in 6-well plates at 37 °C in 5% CO2. For the Western blotting experiment, the medium was refreshed with or without OEA (25 µM), and the protein concentration was measured using the BCA method. The proteins were then separated by SDS-PAGE and transferred to a PVDF membrane. The membranes were probed with anti-HIF-3α and anti-ARNT antibodies (Biolegend). The bands were visualized using an enhanced chemiluminescence (ECL) system (Amersham) and quantified using ImageJ software.

Protein stability assay. The protein stability was assessed by incubating the protein samples with 0–100 µM OEA in 0.1% DMSO. The protein concentration was measured using the BCA method at 0, 2, 4, and 6 h.

2.5% agarose gel electrophoresis. The samples were loaded onto a 2.5% agarose gel and run at 100 V for 1 h. The gel was stained with ethidium bromide and visualized under UV light. The molecular weight markers were run in parallel.

The concentration of HIF-3α protein was measured using the BCA method.
The HIF-3\textsuperscript{3noOEA} system was constructed by removing OEA from holo HIF-3\textsubscript{\alpha}-ARNT with modelled segments. For the structure of apo HIF-3\textsubscript{\alpha}-ARNT, the bHLH domain (residues 179–416) was cleaved by using a 2.1 × 5 mm Acclaim PepMap 300 C18 (Thermo Hypersil Gold column C18) with a 0.1% formic acid in 80% acetonitrile flow rate of 50 μl/min with 0.1% formic acid in water then delivered by the loading pump on a Thermo Dionex Ultimate 3000 NCS-5500RS system (Sunnyvale). The digested peptides were trapped and desalted using a 2.1 × 5 mm Acclaim PepMap 300 C18 μ-pellet (300 Å, 5 μm). The precolumn was connected to a 1.0 × 50 mm Thermo Hypersil Gold column C18 (175 Å, 1.9 μm). Peptides were eluted and separated by a linear gradient of Buffer B (0.1% formic acid in 80% acetonitrile) at a flow rate of 45 μl/min using the nano-LC-MS/MS-5500RS system. Specifically, the gradient was 4–10% over 3 min, 10–30% over 8 min, 30–90% over 1 min followed by isocratic flow with 90% Buffer B for 1 min. The online digestion, trapping, desalting process was performed at 4 °C and separation process was performed at 0.5 °C in the temperature-controlled compartment of the HDX PAL system. Data were acquired using a Thermo LTQ Orbitrap XL mass spectrometer with a HESI II probe. For HDX-LM identification, mass spectra were acquired in a data-dependent scan using FTMS mode in MS1 (one microscan, 100 ms injection time, 60 k resolution at 400 m/z) at the m/z range of 300–1500 followed by ten CID MS2 scans in the ion trap with a ±2.0 m/z isolation width. Once the peptides were identified, the deuterium uptake in HDX experiments was conducted using FTMS mode in MS1.

The spectra generated were searched in PEAKS Studio X against a homemade database including target protein with a precursor mass tolerance of ≤20 ppm and MS/MS fragment tolerance ≤0.02 Da. Retention time and sequence information for each peptide were exported to Excel for HDX data processing. HDX data analysis was carried out using the deuterium exchanger 2.0 (Sierra Analytics Inc.). The number of D taken up (D-uptake) by each peptide at each exchange time was calculated by the software algorithm for matching the best theoretical isotope distribution pattern to the observed isotope distribution pattern. D-uptake was plotted as a function of exchange time. Triplicate runs were compared using Student’s t-test at the 95% confidence level to confirm the consistency of the analytical results obtained. D-uptake was converted to %D for each peptide based on the theoretical number of D; %D was used to generate heat maps, butterfly comparisons, and difference plots. In HDX-MS analysis was also carried out on non-deuterated and fully deuterated samples to correct back-exchange\textsuperscript{83}.

**Statistical analysis**

All statistical data were calculated using GraphPad Prism version 7.0. The sequence alignment figure of three HIF-α proteins was generated by ESPript 3.0. The pocket volume calculation was conducted using Fpocket 4.0 and PyVOL 1.76. Unpaired two-tailed t-test was used to compare the means of two groups. Significance of mean comparison is annotated as follows: *p<0.05; **p<0.01; ***p<0.001, and p value of <0.05 was considered to be statistically significant.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Coordinates and structure factors of the HIF-3\textsubscript{\alpha}-ARNT protein complexes in apo and OEA-bound forms, have been deposited in the Protein Data Bank under accession codes 7Y7L and 7Y7W, respectively. The accession codes for the previously released structures HIF-2α-ARNT, HIF-2α-ARNT-PT2385, HIF-2α-ARNT-M1001 and HIF-3α-3ZP4, 6E5D, 6E3U and 4WNS, respectively. The HDX-MS data are available under accession code PXD033376 in the PRIDE database. Source data are provided with this paper.

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Author contributions
X.D. conducted experiments including clone construction, protein purification, crystallization, X-ray diffraction, structure refinement and binding assays; F.Y. performed the MD simulations; M.Z. and J.Z. conducted the cell-based experiments; X.R. and X.C. participated in the protein purification and crystallization; X.T. and C.P. executed the HDX-MS analysis; J.L. handled the compound screening. X.S. participated in the plasmid construction; Z.H. and H.D. assisted in the binding assays; F.L. participated in the diffraction data collection and structure refinement; D.W., C.L., and F.R. conceived the study, supervised experiments, and wrote the paper with inputs from all authors.

Competing interests
F.R. is a founder and consultant for Flare Therapeutics. The remaining authors declare no competing interests.

Additional information
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