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Analysis of the hypoxia-sensing pathway in *Drosophila melanogaster*

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The mechanism by which hypoxia induces gene transcription involves the inhibition of HIF-1α (hypoxia-inducible factor-1 α subunit) PHD (prolyl hydroxylase) activity, which prevents the VHL (von Hippel-Lindau)-dependent targeting of HIF-1α to the ubiquitin/proteasome pathway. HIF-1α thus accumulates and promotes gene transcription. In the present study, first we provide direct biochemical evidence for the presence of a conserved hypoxic signalling pathway in *Drosophila melanogaster*. An assay for 2-oxoglutarate-dependent dioxygenases was developed using *Drosophila* embryonic and larval homogenates as a source of enzyme. *Drosophila* PHD has a low substrate specificity and hydroxylates key proline residues in the ODD (oxygen-dependent degradation) domains of human HIF-1α and Similar, the *Drosophila* homologue of HIF-1α. The enzyme promotes human and *Drosophila* [^{35}S]VHL binding to GST (glutathione S-transferase)–ODD-domain fusion protein. Hydroxylation is enhanced by proteasomal inhibitors and was ascertained using an anti-hydroxyproline antibody. Secondly, by using transgenic flies expressing a fusion protein that combined an ODD domain and the green fluorescent protein (ODD–GFP), we analysed the hypoxic cascade in different embryonic and larval tissues. Hypoxic accumulation of the reporter protein was observed in the whole tracheal tree, but not in the ectoderm. Hypoxic stabilization of ODD–GFP in the ectoderm was restored by inducing VHL expression in these cells. These results show that *Drosophila* tissues exhibit different sensitivities to hypoxia.

Key words: *Drosophila melanogaster*, hypoxia, prolyl hydroxylase, tissue specificity, von Hippel Lindau.

INTRODUCTION

Oxygen homoeostasis is a primary requirement constraining development, growth and internal organization of animals. At low oxygen tensions, cells activate the expression of genes involved in angiogenesis (e.g. vascular endothelial growth factor), erythropoiesis (erythropoietin), and glucose metabolism (GLUT-1 glucose transporter and glycolytic enzymes) [1]. The transcriptional activation of target genes is mediated by a common transcription factor, HIF-1 (hypoxia-inducible factor-1) [2,3]. HIF-1 consists of a heterodimer of two basic helix-loop-helix-*per-arnt-sim* transcription factors: HIF-1α and the ARNT (aryl hydrocarbon receptor nuclear translocator) [4]. Under normal oxygen conditions (normoxia), newly synthesized HIF-1α is rapidly degraded. Degradation is controlled by PHDs (prolyl hydroxylases) [5,6], a family of 2-OG (2-oxoglutarate)-dependent dioxygenases that hydroxylate conserved proline residues (Pro^{402} and Pro^{564}) in the ODD (oxygen-dependent degradation) domain of HIF-1α [7]. Hydroxylated HIF-1α is recognized by the product of the *VHL* (von Hippel-Lindau) tumour suppressor gene, a component of an E3 ubiquitin ligase complex, and targeted for proteasomal degradation [8–10]. Under hypoxic conditions degradation of HIF-1α is blocked. As a consequence, the protein accumulates, migrates to the nucleus and the HIF-1α–ARNT complex interacts with hypoxia-responsive elements within the 5′ or 3′ transcriptional regulatory regions of target genes [2].

Fly gene homologues of mammalian HIF-1α are *trachealess*, *single-minded*, and *similar* (*sima*). *Trachealess* is a key regulator of tracheal development [11,12]; *single-minded* controls central nervous system midline cell specification [13], and *sima* is the most closely related to the human HIF-1α gene [14,15]. Trachealess, Single-minded and Sima heterodimerize with the *Drosophila* ARNT-like protein Tango [16,17]. The heterodimers bind to a 5′-ACGTG-3′ core consensus sequence, termed the central midline element [16–18], which is identical with mammalian hypoxia response elements, and lead to transcriptional activation of target genes such as *breathless* [*btl*, an FGF (fibroblast growth factor) receptor in *Drosophila*] and *rhomboid* [*rho*, an EGF (epidermal growth factor) pathway activator] that are expressed in the trachea [19], and *branchless* (*bnl*, a *Drosophila* FGF). *Drosophila* orthologues of VHL (*dVHL*) and of PHD (dPHD, encoded by the *hph* gene, also designated CG1114 by genome annotation) have also been identified [20–23].

Recent studies have demonstrated that the oxygen-dependent stabilization of Sima occurs following RNA interference targeted inactivation of the dPHD and requires the presence of a protein domain homologous with the ODD domain of hHIF-1 (human HIF-1α) [23]. These observations suggest that similar mechanisms operate in both hypoxic flies and mammals. However, little is known about the regulation, interactions and substrate specificity of these proteins in flies. Indeed, the enzymatic activity of dPHD and the involvement of dVHL in the hypoxic response have not been documented so far.

The *Drosophila* tracheal system is an epithelial network of branches that sequentially sprout, extend and interconnect to form a ramifying network of tubes that transport oxygen throughout the larval body. Sprouting of the major branches is stereotyped and is controlled by hard-wired developmental cues. The branching

Abbreviations used: ARNT, aryl hydrocarbon receptor nuclear translocator; *bnl*, *branchless*; *btl*, *breathless*; *en*, *engrailed*; FGF, fibroblast growth factor; GFP, green fluorescent protein; GST, glutathione S-transferase; HIF-1, hypoxia-inducible factor-1; ODD, oxygen-dependent degradation; 2-OG, 2-oxoglutarate; PHD, prolyl hydroxylase; *rho*, *rhomboid*; *sima*, *similar*; VHL, von Hippel-Lindau; the prefixes d and h denote *Drosophila* and human respectively.

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process starts at stage 12. At the end of stage 15, the formation of the largest tracheal tubes, the dorsal trunks, are completed, while other branches continue to extend until the embryos mature. At the beginning of the larval period, the tracheal system forms by developing fine terminal branches [24]. Terminal branching is induced by local hypoxia. Hypoxia-mediated Bnl/Btl association guides new terminal branches towards oxygen-starved tissues [25]. This mechanism is comparable with vascular endothelial growth factor-mediated hypoxic angiogenesis in mammals.

In the present study, we first define the biochemical properties of dPHD and demonstrate that dVHL binds to hydroxylated proline residues. We also document hypoxic signalling in different fly tissues and provide evidence for a tissue specificity of hypoxic signalling that is related to VHL expression.

MATERIALS AND METHODS

Materials

Restriction and DNA-modifying enzymes were purchased from Promega. Culture media and fetal calf serum were from Gibco BRL. All chemicals were purchased from Sigma–Aldrich. [5-^{14}C]2-OG (2.07 GBq/mmol) and L-[^{35}S]methionine (37 TBq/mmol) were purchased from Amersham Biosciences.

DNA constructs

The pCMV-ODD–GFP (green fluorescent protein) vector comprising a 222 bp fragment (amino acid residues 530–603) of the ODD domain of hHIF-1α (accession number U22431) was fused in-frame to the 5′ end of the GFP as described previously [26].

The UAS-ODD–GFP transgene was constructed by ligation of the DNA encoding the ODD–GFP fusion protein from pCMV-ODD–GFP into pUAST vector [27]. Accordingly, the ODD–GFP fragment from the pCI-ODD–eGFP2 construct was amplified by PCR using the sense/antisense set of primers: 5′-GAAGATCT-TAATACGACTCACTATAGG-3′/5′-ATCTTATCATGTCTGCT-CGAA-3′, digested by BglII and NotI, and inserted into pUAST vector. Sequences of all products were confirmed by DNA sequencing using the dideoxy chain termination method.

Fly strains

Oregon-R and *white*^{1118} were the control strains. Strains carrying the following Gal4 and UAS transgenes were used: *btl*–Gal4 and *rho*–Gal4 (kindly provided by Dr B. Z. Shilo, Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel), *en*–Gal4 (Bloomington Stock Center), and UAS–eGFP (kindly provided by Dr P. Léopold, CNRS UMR6543, Institute for Signaling, Developmental Biology and Cancer, Nice, France).

UAS-ODD–GFP transgenic strains carrying stable insertions were generated by P-element-mediated transformation. Eight independent UAS-ODD–GFP lines were obtained and analysed under hypoxic conditions. An insertion on chromosome 3 that showed the highest expression of ODD–GFP under hypoxia was selected and used to generate the different crosses.

A *Drosophila* cDNA fragment encompassing 43 bp upstream to 572 bp downstream of the *dVHL* open reading frame [20] was cloned into the pUAST vector. Transgenic flies carrying this construct were generated using standard P-element-mediated transformation.

Sima and HIF peptide hydroxylation assay

Dechorionated Oregon-R embryos (12 h after egg laying) and stage L1 larvae were homogenized at 4 °C in 250 mM sucrose, 20 mM Tris/HCl (pH 7.5), 1 μM leupeptin, 1 μM bacitracin and 0.1 mM PMSF.

Homogenates (0.5 mg of protein/ml) were incubated at 20 °C for 30 min in 40 mM Tris/HCl (pH 7.5), 0.5 mM dithiothreitol, 50 μM ammonium ferrous sulphate, 1 mM ascorbate, 2 mg/ml BSA, 0.4 mg/ml catalase, 50 000 d.p.m. of [5-^{14}C]2-OG, 0.1 mM unlabelled 2-OG and 100 μM peptide substrate. The peptide substrates were: Sima (SFEAFAGRAPYIPIDDD), HIF-1α Pro^{402} (DALTLLAPAAGDTIISLDF) and HIF-1α Pro^{564} (LDLEALA-PYIPADDDFQLRS). They corresponded to amino acid residues 841–857 of Sima, and residues 395–413 and 557–576 of hHIF-1α, respectively. To avoid spurious oxidation events, Met^{847} of Sima was replaced by a glycine residue. Met^{561} and Met^{568} of hHIF-1α were replaced by alanine residues. At the end of the reaction, the radioactivity associated with succinate was determined as described previously [28]. PHD activity was defined as the peptide-dependent 2-OG to succinate conversion rate.

GST (glutathione S-transferase) pull-down assay

GST–ODD fusion protein (50 μM), prepared as previously described [26], was *in vitro* hydroxylated using homogenates from embryos or larvae (0.5 mg of protein/ml) as described above. The reaction products were incubated at 4 °C in 300 μl of buffer [50 mM Tris/HCl (pH 8), 120 mM NaCl and 0.5 % (v/v) Nonidet P40] supplemented with glutathione–Sepharose beads and 100 000 d.p.m. of [^{35}S]VHL. After 2 h of incubation, beads were washed three times with cold buffer [20 mM Tris/HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5 % (v/v) Nonidet P40]. The bound proteins were eluted in SDS running buffer and analysed by SDS/PAGE followed by autoradiography. Human and *Drosophila* [^{35}S]VHL were synthesized using pcDNA3.1/V5–His–VHL (kindly provided by Dr S. L. McKnight, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) and pET15b-VHL [20] as templates and the TNT-coupled reticulocyte lysate system (Promega).

Transient transfection of S2 cells and preparation of cell extracts

S2 Schneider cells were grown in Schneider's medium supplemented with 10 % fetal bovine serum at 25 °C. For transfection, calcium phosphate precipitates were generated by using 10 μg of pCMV-ODD–GFP or pCMV-eGFP total DNA. Typically, 5×10^6 cells were transfected and grown in 5 ml of Schneider's medium and 10 % fetal calf serum. At 36 h after transfection, cells were exposed to hypoxia (1 % O_2) or kept at normoxia (21 % O_2) for 16 h in the presence or absence of the proteasome inhibitor MG132 (10 μM). Cells were lysed in buffer [20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.5 % (v/v) Nonidet P40, 1 mM sodium orthovanadate, 5 mM NaF, and Complete protein inhibitors (Roche Molecular Biochemicals)] for 20 min at 4 °C. The lysates were cleared by a 20 min centrifugation at 13 000 ***g***. The supernatants were collected and protein concentrations were determined.

Collection of embryos and larvae and hypoxic treatment and preparation of proteins

Embryos and larvae were obtained by selecting individuals from developmentally synchronized populations (siblings from 1 h egg lay), collected and reared in room air until the desired developmental stage. Embryos were placed in a sealed chamber maintained at 5 % O_2 (hypoxia) or kept at normoxia (21 % O_2) until stage 16. Freshly hatched L1 larvae were transferred on to agar plates containing a thin layer of yeast paste and exposed to hypoxia

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(5 % O_2) or maintained at normoxia (21 % O_2) for a total of 20 h. At the end of the treatment, stage 16 embryos or L1 larvae were quickly removed from the chambers, and frozen in liquid nitrogen or prepared for immunofluorescence studies. For Western blotting analysis, embryos and larvae were Dounce homogenized in Laemmli sample buffer and boiled for 10 min. Extracts were clarified by centrifugation at 13 000 ***g*** for 15 min. Supernatants were collected and protein concentrations were determined.

Antibodies

Rabbit anti-Hyp (hydroxylated Pro^{564}) antibodies and the Hyp^{564} peptide (LDLEALA**Hyp**YIPADDDFQLRS) were obtained from Genaxis (Nimes, France). The Hyp^{564} peptide was identical with the Pro^{564} peptide except that the proline-564 residue was hydroxylated. The polyclonal antibodies were characterized using a radioimmunoassay as previously described [28]. Briefly the Hyp^{564} peptide was iodinated using chloramine T and purified by HPLC. The radioimmunoassay buffer contained 150 mM NaCl, 1g/l BSA, 0.1 g/l sodium azide and 10 mM potassium phosphate buffer (pH 7.5) supplemented with 20 000 c.p.m./ml of iodinated peptide. After 16 h of incubation at 4 °C, antibody–peptide complexes were precipitated with poly(ethylene glycol).

Mouse monoclonal antibodies were obtained from the following sources: anti-GFP antibody (Roche Molecular Biochemicals) and anti-α-tubulin antibody (Sigma). Bacterially produced, His-tagged dVHL protein [20] was used to generate rabbit polyclonal anti-dVHL antibody. Secondary antibodies used were from Jackson ImmunoResearch Laboratories (horseradish peroxidase-coupled sheep anti-mouse and donkey anti-rabbit antibodies). For immunofluorescence experiments, dVHL was detected with a biotinylated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) using a TSA amplification kit from NEN (PerkinElmer).

Immunoblotting and immunoprecipitation

Protein extracts were separated by SDS/PAGE and transferred on to nitrocellulose membranes (Schleicher and Schuell) using standard procedures. For immunoprecipitations, samples (500 μg) of transiently transfected S2 cell lysates were incubated for 1 h at 4 °C with 4 μg of anti-VHL antibody, followed by addition of Protein A–Sepharose beads (20 μl) and an overnight incubation. Immunoprecipitates were washed four times with lysis buffer, eluted with sample buffer and immunoblotted. The membranes were blocked with 5 % (w/v) instant non-fat milk powder in PBS/0.1 % (v/v) Tween 20 for 1 h at 22 °C, followed by overnight incubation at 4 °C with primary antibodies against GFP (1:1000), Hyp (1:2000), hVHL (1:1000), dVHL (1:5000) or α-tubulin (1:5000). Monoclonal antibodies against GFP (1:1000) or against VHL (1:1000) were used to probe the immunoprecipitates. Blots were washed three times in PBS/0.1 % (v/v) Tween 20 and incubated for 1 h at 22 °C with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit antibodies (1:10 000) in PBS/0.1 % (v/v) Tween 20. After extensive washes in PBS/0.1 % (v/v) Tween 20, chemiluminescence was detected by incubating the membranes with 100 mM Tris/HCl (pH 8.5), 2.65 mM H_2O_2, 0.45 mM luminol and 0.625 mM coumaric acid for 1 min followed by exposure to X-ray films.

Immunofluorescence microscopy

Embryos were dechorionated in 12 % bleach for 3 min, washed with water and then fixed in 4 % formaldehyde in PBS/heptane for 20–25 min. They were devitellinized in 80 % ethanol/heptane. Finally, embryos were extensively washed in absolute ethanol and then sequentially rehydrated before being processed for immunofluorescence. For dVHL experiments, embryos were incubated overnight at 4 °C with anti-dVHL antibody (1:2000). Larvae were fixed for 20 min in PBS, 0.3 % (v/v) Triton, 3.7 % (v/v) formaldehyde and mounted in PBS/glycerol (1:4). Fluorescent images were captured using a Leica DMR TCS_NT confocal microscope.

RESULTS

Biochemical evidence for a PHD activity in *Drosophila*

The ODD domain of human HIF-1α, positioned at the amino acid residues 401–603, is responsible for the proteasomal degradation of the protein in normoxic cells. This region overlaps with two VHL-binding sites [29] and contains two essential proline residues (Pro^{402} and Pro^{564}) that are hydroxylated by PHDs under normoxic conditions [26,30,31]. The corresponding domain in Sima has been determined to be at the amino acid residues 692–863 and contains one conserved proline residue (Pro^{850}) that has been proposed to mediate the oxygen-dependent stabilization of Sima [23].

To characterize the dPHD activity and to assess whether the enzyme hydroxylates the putative relevant residue Pro^{850} in Sima, we performed the decarboxylation assay that we have developed previously [26]. We synthesized three peptides that reproduced the amino acid sequences around Pro^{850} in Sima and around Pro^{402} and Pro^{564} in hHIF-1α (Figure 1A) and used them as substrates for dPHD. The three peptides stimulated, with different efficacies, the decarboxylation of 2-OG into succinate when embryonic and L1 larval homogenates were used as a source of enzyme. Peptides derived from hHIF-1α sequence were between 20 and 50 % less efficient than the Sima peptide (Figure 1B). The dose–response curve for Sima peptide-induced activation of the enzyme is presented in Figure 1(C). Half-maximal activation was observed with 10 μM Sima peptide. The Sima peptide stimulated activity was completely inhibited by 0.1 mM 3,4-dihydroxybenzoate (results not shown). These findings suggested that embryonic and L1 larval homogenates exhibited a PHD activity that recognized both *Drosophila* and human substrates.

The broad substrate specificity of dPHD prompted us to ascertain the hydroxylation reaction using a GST–ODD fusion protein [26] as a substrate and a pull-down assay relying on the ability of VHL to bind hydroxylated proline residues. Figure 1(D) shows that incubation of bacterially expressed GST–ODD with embryonic or L1 larval homogenates promoted the binding of dVHL to GST–ODD. These results validate the assay and clearly demonstrated the presence of a functional PHD in *Drosophila* embryos and L1 larvae.

ODD–GFP fusion protein is stabilized by hypoxia in *Drosophila* S2 cells

Based on the above results we next sought to determine the involvement of *Drosophila* endogenous VHL in the oxygen-dependent responses. To slow down the degradative process of hydroxylated proteins at normoxia, we generated a chimaeric protein consisting of residues 530–603 of the ODD domain of hHIF-1α fused to GFP, thus only containing the Pro^{564} residue and not the Pro^{402} residue [26]. Transiently transfected S2 cells expressing the ODD–GFP chimera were prepared, and protein expression levels were first analysed by Western blotting using an anti-GFP antibody. As expected, under normoxic conditions, the antibody recognized a 35–40 kDa protein in transfected

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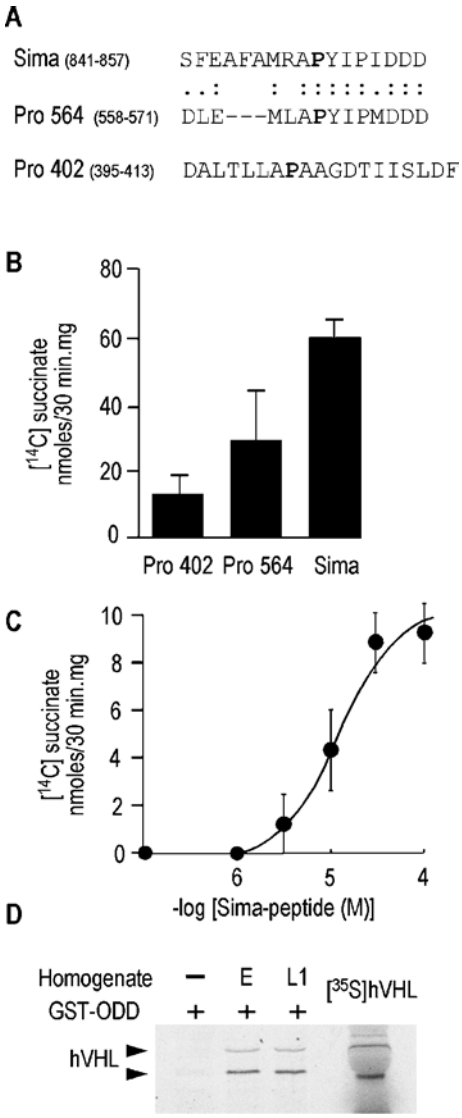

Figure 1 Properties of dPHD

(**A**) Comparison of partial amino acid sequences of Sima and hHIF-1α. Hydroxylation of HIF-1α Pro^{402} and Pro^{564} was previously shown [30,31]. Residues 841–857 of the Sima sequence are aligned with residues 558–571 of the HIF-1α sequence. The hydroxylable Pro residues are shown in bold; hydroxylation of the conserved proline residue in Sima has not been demonstrated. The core motifs are shaded. Identical residues are marked by (:) or (.), according to the degree of similarity. (**B**) Peptides derived from hHIF-1α and Sima induced the decarboxylation of [^{14}C]2-OG. L1 larval homogenates were used as a source of enzyme. The three peptides (100 μM) induced the 2-OG to succinate conversion with different efficacies. (**C**) Dose–response curve for the Sima peptide-induced decarboxylation of 2-OG. Enzyme activity was provided by embryonic homogenates. In (**B**) and (**C**), the means $\pm$ S.E.M. ($n = 3$) are shown. (**D**) GST pull-down assay. Bacterially expressed GST–ODD was *in vitro* hydroxylated using homogenates of embryos and L1 larvae in the presence of cofactors and [^{35}S]hVHL. The bound [^{35}S]hVHL was pulled-down and visualized by autoradiography. The right hand lane shows a band from directly loaded [^{35}S]hVHL. Note the presence of two radioactive bands corresponding to two isoforms of hVHL. Similar results were obtained in two other independent experiments. E, embryos; L1, L1 larvae.

cells (Figure 2A) but not in mock-transfected cells (results not shown). We next exposed the cells to 1 % O_2 for various times. ODD–GFP expression increased in a time-dependent manner (Figure 2A). Figure 2(B) shows a quantitative analysis of the data and shows that 4 and 16 h of low-oxygen exposure resulted in a 3- and 4-fold accumulation of ODD–GFP respectively. A higher expression level of ODD–GFP was also observed when cells were incubated in the presence of MG132 (10 μM), an inhibitor of the proteasomal degradation machinery (Figures 2C and 2D). Thus hypoxia (1 % O_2) stabilized the ODD–GFP reporter protein in *Drosophila* S2 cells. In addition, and in agreement with our previous observations in mammalian cells [26], we observed that forced expression of the ODD–GFP fusion protein containing only one proline hydroxylable (Pro^{564}) results in less labile hydroxylated proteins under normoxia.

We next examined the hydroxylation status of ODD–GFP reporter protein under normoxia or in proteasomally blocked S2 cells. We used a polyclonal antibody specifically directed against the hydroxylated forms of Pro^{564}, the anti-Hyp antibody developed previously [28]. In Western blotting experiments the antibody revealed a band at a position corresponding to ODD–GFP (Figure 2E, lane 1). The inhibition of the proteasome degradation machinery by MG132 resulted in a 3-fold increase in hydroxylated ODD–GFP protein levels (Figures 2E, lane 2, and 2F). No labelling was observed under 1 % O_2 (Figure 2E, lane 5), suggesting that dPHD, was indeed inactive at low-oxygen conditions, as is mammalian PHD. The specificity of the labelling by the anti-Hyp antibody was ascertained by two additional results: (i) no band was observed in mock-transfected cells (results not shown) and (ii) an excess of Hyp^{564} peptide (0.2 μM), to neutralize specific antibodies, abolished the detection of ODD–GFP (Figure 2E, lanes 3 and 4). These results showed that ODD–GFP is hydroxylated by the endogenous dPHD in *Drosophila* S2 cells.

The hallmark of hVHL activity is its ability to bind to Hyp residues in the ODD domain of HIF-1α and to target the protein to the proteasome. Since the amino acid sequence of dVHL shows only 29 % identity with that of both hVHL gene products [20], we tested whether dVHL recognized Hyp residues. As shown in Figure 2(G), dVHL only associated with GST–ODD proteins that had been incubated with embryonic and L1 larval homogenates, i.e. that had been exposed to dPHD. Note that hVHL proteins are encoded by two alternative start codons, resulting in two protein isoforms (see Figure 1D), whereas dVHL contains only one protein species corresponding to the smaller of the two hVHL isoforms.

Finally, we assessed the function of endogenous dVHL in the oxygen-dependent degradation of ODD–GFP in intact S2 cells. Normoxic, proteasomally blocked and hypoxic S2 cell extracts were prepared. dVHL was immunoprecipitated with an anti-dVHL antibody and the associated ODD–GFP proteins were visualized in Western blotting experiments using an anti-GFP antibody. Figure 2(H) shows that dVHL did not bind to hypoxia-stabilized ODD–GFP (lane 3). A weak but detectable association of endogenous dVHL with ODD–GFP was observed under normoxic conditions (Figure 2H, lane 1). This association was further increased in extracts from proteasomally blocked cells (Figure 2H, lane 2). No association was observed in mock-transfected cell extracts, thus demonstrating the specificity of dVHL/ODD–GFP interaction (results not shown). Taken together, these results provide direct evidence for and biochemically characterize the hypoxic signalling pathway in *Drosophila* S2 cells, demonstrating that it involves proline hydroxylation and VHL-mediated proteasomal degradation.

ODD–GFP protein is a reporter of the tracheal response to hypoxia

The previous experiments established that the mechanism of hypoxic response is active in *Drosophila* S2 cells, and that it is conserved between *Drosophila* and mammals. However, cultured-cell responses to hypoxia do not necessarily reflect the tissue response to hypoxia. To gain further insight into the mechanism

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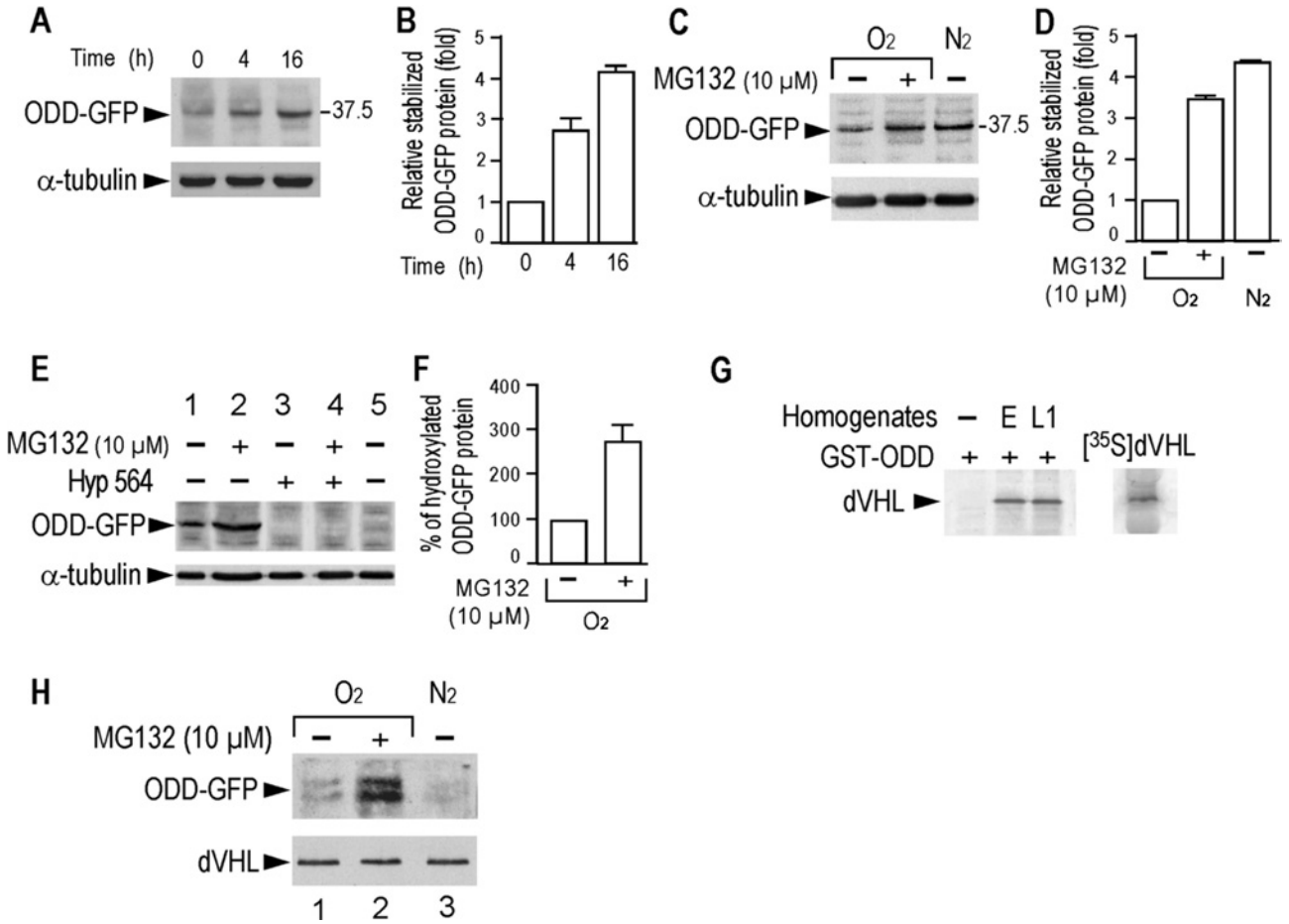

Figure 2 Analysis of the hypoxic signalling cascade in *Drosophila* S2 cells

(**A**)–(**D**). Hypoxia and the proteasomal inhibitor MG132 stabilize ODD–GFP. S2 cells were transiently transfected with an ODD–GFP construct comprising residues 530–630 of the ODD domain of hHIF-1α fused to GFP. Experiments were performed under normoxia (21 % O_2) in the absence or in the presence of MG132 (10 μM), or under hypoxic conditions (4 or 16 h at 1 % O_2). Expression of ODD–GFP and α-tubulin were analysed by Western blotting. (**A**) Representative blots showing the stabilization of ODD–GFP protein in a time-dependent manner, probed with an anti-GFP antibody (upper panel) or with an anti-α-tubulin antibody (lower panel). (**B**) GFP/α-tubulin signals were normalized to the value observed in normoxic cells. The results are expressed as the means $\pm$ S.E.M. ($n = 3$). (**C**) Example of a representative Western blot probed with an anti-GFP antibody (upper panel) or with an anti-α-tubulin antibody (lower panel). (**D**) GFP/α-tubulin signals were normalized to the value observed in oxygenated cells in the absence of MG132. The results are expressed as the means $\pm$ S.E.M. ($n = 3$). (**E**) MG132 induced the accumulation of hydroxylated forms of ODD–GFP. Transiently transfected ODD–GFP expressing cells were incubated under normoxia (21 % O_2) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of MG132 (10 μM) or exposed to 1 % O_2 (lane 5). Whole-cell extracts were resolved by SDS/PAGE and immunoblotted with anti-Hyp polyclonal antibody. Note that the labelling was prevented by addition of 0.2 μM of Hyp^{564} peptide (lanes 3 and 4). The blot was re-probed using anti-α-tubulin antibody as a loading control. Results shown are representative of two independent experiments. (**F**) The anti-Hyp signal was normalized to that of α-tubulin; 100 % represents the ratio observed in control normoxic S2 cells. (**G**) dVHL recognized hydroxylated ODD. Bacterially expressed GST–ODD was *in vitro* hydroxylated using embryonic and L1 larvae homogenates in the presence of cofactors and [^{35}S]dVHL. The bound [^{35}S]dVHL was pulled-down and visualized by autoradiography. The right hand lane shows a band from directly loaded [^{35}S]dVHL. Similar results were obtained in two other independent experiments. E, embryos; L1, L1 larvae. (**H**) Evidence that ODD–GFP is *in vivo* hydroxylated. Transiently transfected ODD–GFP expressing cells were incubated under normoxia (21 % O_2) in the presence (lane 2) or absence (lane 1) of MG132 (10 μM), or hypoxia (16 h at 1 % O_2) (lane 3). Endogenous dVHL was immunoprecipitated using an anti-dVHL antibody, resolved by SDS/PAGE and probed with anti-GFP (upper panel) or anti-dVHL (lower panel) antibodies. Results shown are representative of 2 independent experiments.

of ODD-dependent protein stabilization and to explore the role of hypoxic responses *in vivo*, we focused on the *Drosophila* tracheal (respiratory) system, where sensitivity to hypoxia was previously observed [23,25]. We established independent UAS–ODD–GFP transgenic flies, and targeted the ectopic expression of the fusion protein to the whole tracheal tree with *btl*–Gal4 promoter [32] or to the dorsal trunk with *rho*–Gal4 [33]. The UAS–GFP transgenic line was used as a control.

Stage 16 embryos and L1 larvae were exposed to normoxic or hypoxic conditions. Extracts were prepared and ODD–GFP expression was detected in Western blotting experiments using an anti-GFP antibody. Hypoxia (5 % O_2) induced a 4-fold increase in the *btl*- or *rho*-driven expression of ODD–GFP (Figures 3A–3C). Normoxic and hypoxic expression levels of GFP were comparable. Thus, hypoxic signalling, as shown by the stabilization of ODD–GFP, was active in tracheal tissues.

Immunofluorescense studies were carried out to visualize the consequences of a hypoxic exposure on the developing tracheal system. *btl*-driven expression of ODD–GFP was observed throughout the whole tracheal system. Hypoxia-treated larvae displayed increased fluorescence labelling as compared with normoxic larvae (Figures 3D and 3E). We also observed that hypoxia promoted marked morphological alterations of the tracheal tree as follows: (i) fine terminal branches were more numerous (Figure 3E, arrows); (ii) the dorsal tube followed a more tortuous and circonvoluted shape (Figures 3H and 3I); (iii) dorsal trunks

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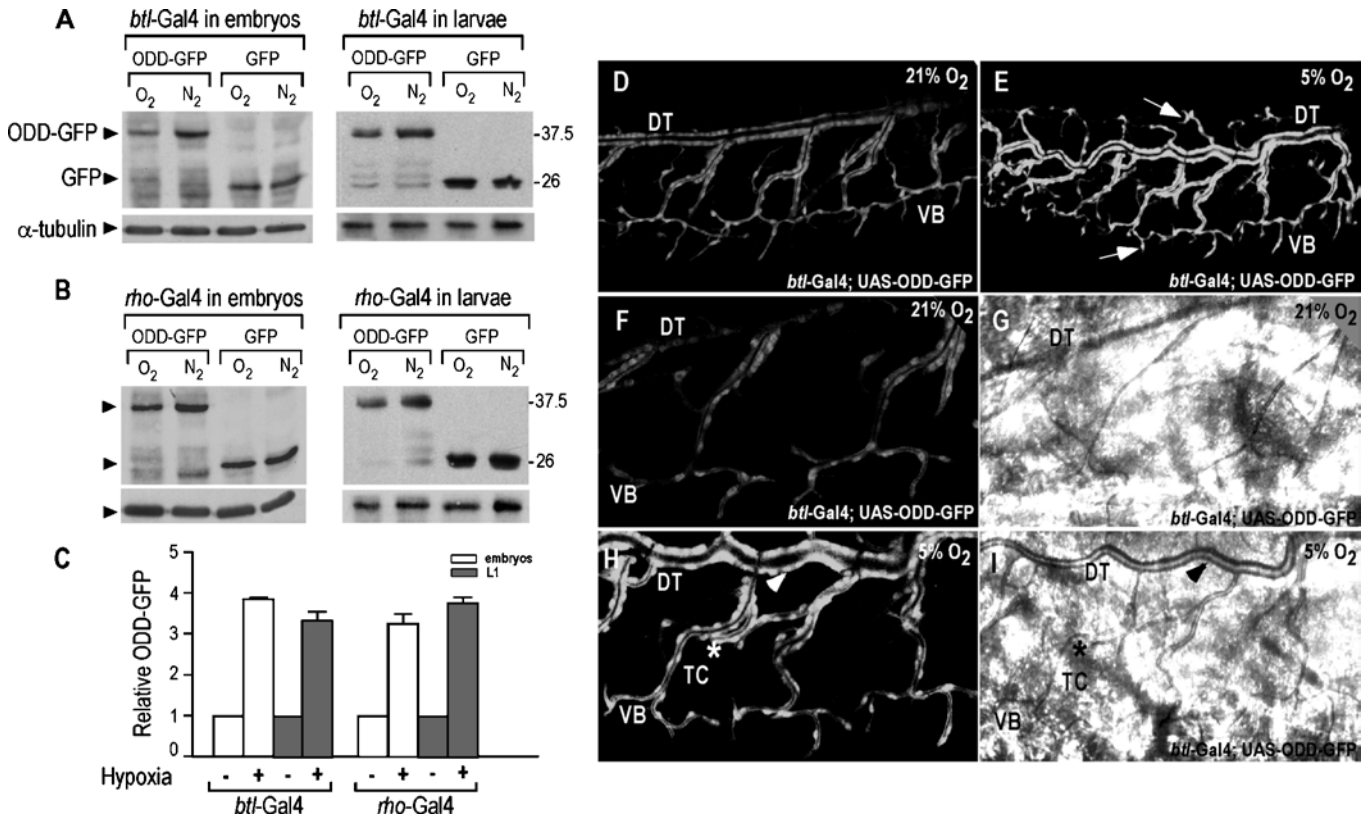

Figure 3 Hypoxia induced ODD–GFP fusion protein stabilization in the tracheal system of embryos and L1 larvae

(**A**) and (**B**) Representative Western blots showing *btl*- and *rho*-driven expression of ODD–GFP and GFP and endogenous α-tubulin in normoxic (21 % O_2) or hypoxic (5 % O_2) embryos and L1 larvae as indicated. (**C**) GFP/α-tubulin signals were normalized to the value obtained for normoxic (21 % O_2) controls. The results are expressed as the means $\pm$ S.E.M. ($n = 3$). (**D**–**I**) Hypoxia induced remodelling of the tracheal tree. Immunofluorescence micrographs of tracheal expression of ODD-GFP protein in L1 larvae (*btl*-Gal4;UAS-ODD-GFP) reared in normoxic (21 % O_2) (**D**) or hypoxic (5 % O_2) (**E**) conditions. Lateral views are shown. (**E**) Hypoxia induced the accumulation of ODD–GFP reporter protein in the whole tracheal system of L1 larvae. Arrows indicate terminal branches corresponding to visceral branches (VB) that have developed in response to low oxygen concentration. Also note that under hypoxia, the dorsal trunk (DT) follows a tortuous path. Magnification, $\times$ 20. (**F**–**I**) Close up of representative regions of DT and transverse connectives (TC) in normoxic (21 % O_2) (**F**, **G**) and hypoxic (5 % O_2) (**H**, **I**) *btl*-Gal4;UAS–ODD–GFP L1 larvae. Immunofluorescence (**F**, **H**) and bright fields micrographs (**G**, **I**) are shown. (**H**, **I**) Hypoxia induced a thickening of the DT and an enlargement of the lumen (arrowheads in **H**, **I**) (compare with **F** and **G**). Asterisks indicate ectopic branching in the TC (**H**, **I**). Magnification, $\times$ 40.

were thickened and their lumen was enlarged (Figures 3H and 3I, arrowheads); (iv) some ectopic branchings were observed in the transverse connective (Figures 3H and 3I, asterisks). Taken together these results indicated that hypoxia (5 % O_2) induced a major remodelling of the whole tracheal tree in L1 larvae. This remodelling was associated with an activation of the hypoxic signalling cascade as shown by the stabilization of the ODD–GFP reporter protein.

Ectodermal tissues show no hypoxic response

To assess the specificity of the hypoxic response in *Drosophila* tissues, we targeted the expression of ODD–GFP to ectodermal cells, which express very low levels of dVHL [20]. This was achieved by using the *en* (*engrailed*)-Gal4 promoter whose expression shows a repeated stripe pattern in the embryonic and larval ectoderm [34]. Embryonic and larval extracts were prepared

Figure 4 Tissue specificity of the hypoxic response is dependent on *dVHL* expression

(**A**–**D**) Ectodermal tissues are insensitive to hypoxia. (**A**). Representative Western blots showing *en*-driven expression of ODD–GFP and GFP (upper panels) and endogenous α-tubulin (lower panels) in normoxic (21 % O_2) or hypoxic (5 % O_2) embryos (left hand panel) and L1 larvae (right hand panel). (**B**) GFP/α-tubulin signals were normalized to the value observed for normoxic (21 % O_2) controls. The results are expressed as the means $\pm$ S.E.M. ($n = 3$). (**C**–**D**) Immunofluorescence micrographs of ectodermal expression of ODD–GFP fusion protein in normoxic (21 % O2) or hypoxic (5 % O_2) (*en*-Gal4;UAS–ODD–GFP) embryos. *en*-driven ODD–GFP expression shows the typical *en* stripe pattern. The fluorescence intensity of ODD–GFP was not affected by hypoxia. Magnification, $\times$ 20. (**E**–**H**) *en*-driven expression of dVHL restored the hypoxic stabilization of ODD–GFP. (**E**) Representative Western blots showing *en*-driven expression of ODD–GFP (upper panel) and endogenous α-tubulin (lower panel) in normoxic and hypoxic embryos. Left hand panel, embryos expressing ODD-GFP protein in *en*-expressing cells (*en*-Gal4; UAS-ODD-GFP); right panel, embryos expressing ODD–GFP and VHL proteins in *en*-expressing cells (*en*--Gal4;UAS–ODD–GFP; UAS–dVHL). (**F**) GFP/α-tubulin signal. The results are expressed as the means $\pm$ S.E.M. ($n = 3$). (**G**–**K**) Immunofluorescence micrographs of ectodermal expression of ODD–GFP and VHL proteins in normoxic (21 % O_2) (**G**–**J**) or hypoxic (5 % O_2) (**K**) embryos. (**G**–**I**) The titre of the anti-dVHL antibody and the staining conditions were adjusted so as to show the ectopically expressed dVHL. (**I**) The merge confocal image shows the that dVHL and ODD–GFP protein co-localized in *en*-expressing cells in 5 segments. Note that ectopic expression of dVHL reduced the fluorescence intensity of ODD–GFP in normoxic embryos (**J**) and restored a hypoxic stabilization of ODD–GFP (**K**). Magnification, $\times$ 20. Intensity parameters for image acquisitions were the same in (**G**) and (**H**). (**L**–**M**) Neither *en*-driven expression of VHL nor hypoxia affect the GFP expression in *en*-expressing cells (*en*–Gal4;UAS–GFP +/– UAS–VHL) (**L**). Representative Western blots showing *en*-driven expressions of GFP and endogenous α-tubulin (lower panels) in normoxic (21 % O_2) or hypoxic (5 % O_2) embryos. Control of dVHL expression is shown. (**M**) GFP/ and dVHL/α-tubulin signals from (**L**) were normalized to the value observed for normoxic (21 % O_2) controls. A 4-fold increase in dVHL expression does not affect the stability of GFP in normoxia and hypoxia. The results shown are representative of those obtained from 4 independent experiments. Normoxic, N_2; hypoxic, O_2.

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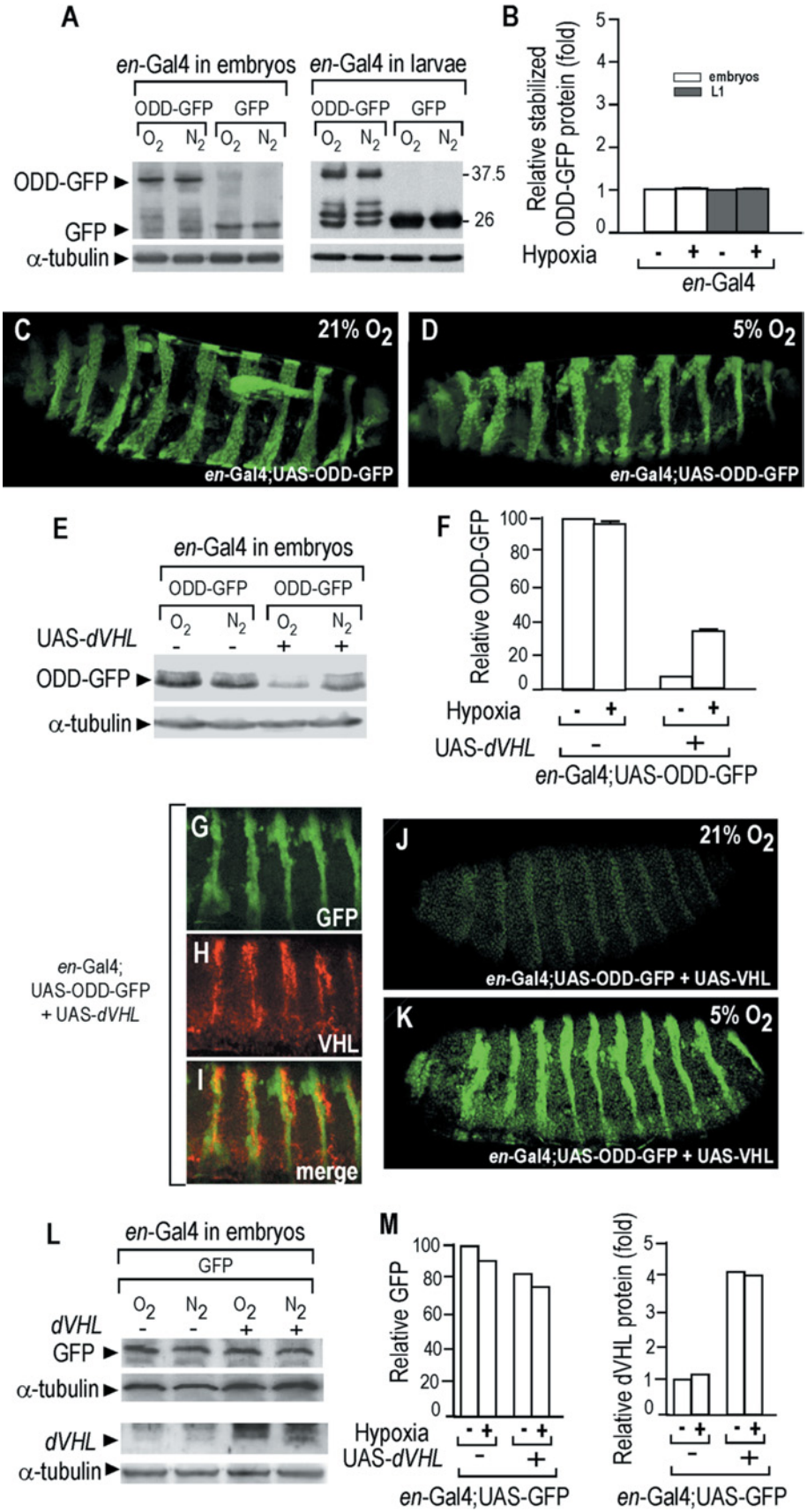

Figure 4 **For legend see facing page**

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and ODD–GFP was detected in Western blotting experiments using an anti-GFP antibody. Figures 4(A) and 4(B) show that the targeted expression of both ODD–GFP and GFP reporter proteins to embryonic and larval ectoderm remained unchanged in normal (21 %) and low (5 %) oxygen levels. Immunofluorescence studies performed on stage 16 embryos showed that expression of ODD–GFP protein had the expected *en*-stripe pattern (Figure 4C). The same *en* expression pattern and a comparable fluorescence intensity were observed in embryos exposed to hypoxic conditions (5 % O_2) (Figure 4D). These results indicated that ectodermal tissues did not respond to hypoxia. They suggest a tissue specificity of the hypoxic responses in *Drosophila* embryos and larvae.

dVHL sensitizes ectodermal tissue to hypoxia

The tissue-specific hypoxic response described above is consistent with the previous report that dVHL expression is enriched in the trachea [20]. We reasoned that the absence of dVHL in ectodermal cells might result in stable expression of ODD-containing proteins, thus precluding an observable up-regulation in hypoxic conditions. That is, in the absence of dVHL, ODD–GFP protein would not be targeted to the proteasome, and would accumulate and stabilize in an oxygen-independent manner. To test this hypothesis we overexpressed dVHL and ODD–GFP in ectodermal tissues and analysed the influence of hypoxia on the expression of ODD–GFP. Western blotting experiments were performed on extracts from embryos expressing ODD–GFP or ODD–GFP and dVHL proteins in *en*-expressing cells (*en*–Gal4; UAS–ODD–GFP −/+ UAS–*d*VHL). The results indicated that *en*-driven expression of dVHL decreased the basal expression of ODD–GFP under normoxic conditions and that hypoxia (5 % O_2) induced a 3.5-fold increased expression of the reporter protein (Figures 4E and 4F). This action of hypoxia was similar to that observed previously in the trachea (Figure 3C). These findings were confirmed by immunofluorescence studies with *en*-Gal4; UAS–ODD–GFP + UAS-*dVHL* embryos (Figures 4G–4K). Figures 4(G)–(4I) show co-overexpression of the *en*-driven ODD–GFP and dVHL. In these embryos, there was very little expression of ODD–GFP in normoxia (21 % O_2; Figure 4J). Exposure to hypoxia (5 % O_2) for 16 h led to ODD–GFP stabilization (Figure 4K). Thus ectopic expression of dVHL in ectodermal tissue restored the oxygen-dependent stabilization of ODD–GFP.

In addition, to confirm that ODD–GFP protein was a target of VHL via the ODD sequence, we performed Western blotting experiments on *en*-Gal4; UAS–GFP −/+ UAS-*dVHL* embryonic extracts. Figures 4(L) and 4(M) show that the targeted expression of GFP reporter protein expression was not altered in normal or low oxygen levels, nor by dVHL overexpression. Taken together our findings clearly demonstrate the requirement for a functional dVHL in ectodermal tissues to restore the tissue oxygen sensitivity.

DISCUSSION

The main goal of the present study was to document and visualize hypoxic responses using the *in vivo* system *Drosophila melanogaster*. It has been shown that key players of the hypoxic response are conserved between mammals and flies. Mammals express three forms of PHD [35] and two biologically active VHL gene products [36]. In that respect, *Drosophila* provides a simpler model to tackle functional analysis: it only expresses one PHD (*hph*, also listed as CG1114 on FlyBase) and one VHL [21–23]. Fly transcription factors related to HIF-1α are *trachealess* [11,12], *single-minded* [14] and *sima* [13]. Deletion mutant analyses have suggested that Pro^{850} in Sima could play the same role as Pro^{564} in the ODD domain of hHIF-1α [23].

This study provides the first direct biochemical evidence that proline hydroxylation and VHL-mediated proteolysis are operating in flies. The dPHD has a broad substrate specificity. It recognizes the Sima peptide, which mimics the Pro^{850} region of *Drosophila* ODD, as well as peptides that mimic the amino acid sequences surrounding Pro^{402} and Pro^{564} in hHIF-1α. A similarly broad substrate specificity has recently been reported for the three human forms of PHDs [37]. This suggests that HIFs/Sima might not be the only substrates of PHD. Recently, it has been reported that the large subunit (Rbp1) of RNA polymerase II binds to VHL in a proline-hydroxylation-dependent manner [38], although it is not yet known whether the PHDs are involved. Finally, we also defined some of the pharmacological properties of dPHD. The enzyme is inhibited by ethyl-3,4-dihydroxybenzoate. This compound has initially been reported as an inhibitor of procollagen proline 4-hydroxylase [39], and recently of hPHD [40].

The *Drosophila* VHL is part of a multiprotein complex that possesses E3 ubiquitin ligase activity. dVHL and hVHL have been shown to target hHIF-1α for ubiquitination and proteasomal degradation [21]. We and others have previously demonstrated that hVHL binds to hydroxylated proline residues in the ODD domain of hHIF-1α [28,41]. We now extend these findings and demonstrate that dVHL recognizes and associates with hydroxylated forms of the ODD domain of hHIF-1α *in vitro* and in *Drosophila* S2 cells that transiently express the ODD–GFP fusion protein. The hydroxylated status of the substrate that was recognized by both hVHL and dVHL was further ascertained by using a newly developed specific anti-Hyp antibody [28]. Taken together, our results provide further biochemical and molecular evidence for a tight conservation of the PHD/VHL signalling pathway between flies and mammals.

We then first took advantage of an ODD–GFP reporter protein that we previously used to analyse hypoxic responses in mammalian cells [26,28], and secondly of the Gal4–UAS binary system [27] to generate transgenic flies and visualize hypoxic responses in different embryonic and larval tissues. *Btl*- or *rho*-driven expression of ODD–GFP allowed us to visualize the tracheal tree and to document the effect of hypoxia (5 % O_2). Our results with L1 larvae confirmed that a major action of hypoxia is to induce secondary tracheal branches [25]. We also observed ectopic and excessive branchings of transverse tracheal branches that led to more tortuous and complex tracheal trees. Similar morphological changes have been observed in dVHL loss of function phenotypes [20]. Finally, we observed that hypoxia also altered the morphology of large tracheal trunks. Dorsal trunks became thicker and their lumen enlarged. Thus hypoxia induced significant remodelling of the tracheal tree. This remodelling is associated with an activation of the hypoxic signalling cascade.

Our results show, for the first time, that the sensitivity to hypoxia is markedly tissue specific. It is highly active in the embryonic and larval tracheal system. It is undetectable in ectodermal tissues. The origin of different sensitivities of tracheal and ectodermal tissues to oxygen concentrations might be dependent on the expression of a functional dVHL. Indeed, we have previously shown that dVHL expression is restricted to the developing trachea [20]. The lack of an active dVHL in the ectodermal tissue could be responsible for the absence of the proteasomal degradation of ODD-containing proteins under normoxic conditions. In the present study we show that ectopic expression of dVHL in the ectoderm re-established the oxygen sensitivity, i.e. it decreased basal expression of ODD–GFP and restored the hypoxia-dependent ODD–GFP protein stabilization. Previous studies have suggested that PHD, which hydroxylates key proline residues in HIF-1α and Sima, is the

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oxygen sensor by which cells sense and adapt to the lack of oxygen. Our results suggest that VHL is also part of the oxygen sensor, since it determines the sensitivity of the cells to the lack of oxygen. Substrates of PHD are stabilized by hypoxia in VHL-expressing tissues, such as trachea. They are not degraded in normoxia in ectodermal tissues that do not express VHL. It is also of interest to note that we previously described that cyclosporine A, which activates mammalian PHD, desensitizes cells to the action of hypoxia [26]. Taken together, these results suggest that the hypoxic sensitivity of cells and tissues is not a fixed property solely determined by the affinity of PHDs for molecular oxygen. Hypoxic sensitivity is more likely to be a property of the whole signalling cascade and involves VHL and possibly other modifying proteins. Indeed, different cells in an organism are exposed to a wide range of different oxygen tensions.

Finally, the results presented here suggest that the function of the hypoxic signalling cascade in *Drosophila* might not be limited to terminal tracheogenesis: (i) ODD domain-dependent protein stabilization is highly active in the whole tracheal system which is directly exposed to oxygen, (ii) stabilization is observed in both embryonic and L1 larval trachea, which develop in an oxygen-independent and -dependent manner respectively [25], (iii) hypoxia induces morphological changes of the main tracheal trunks. These findings suggest that the role of PHD- and ODD domain-dependent protein stabilization might be involved in processes that are unrelated to tracheal development. These observations are fully consistent with the suggestion that the trachea functions as a sensory organ in flies, as the carotid body does in mammals [23]. Physiological evidence in favour of this hypothesis has recently been provided [42].

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