Developmental Biology 329 (2009) 294–305

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Regulation of *Drosophila* embryonic tracheogenesis by *dVHL* and hypoxia

Nathan T. Mortimer, Kenneth H. Moberg *

Department of Cell Biology, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322, USA

ARTICLE INFO

Article history:
Received for publication 5 September 2008
Revised 27 January 2009
Accepted 3 March 2009
Available online 11 March 2009

Keywords:
Drosophila
Trachea
Hypoxia
dVHL
Archipelago
Sima
FGF signaling

ABSTRACT

The tracheal system of *Drosophila melanogaster* is an interconnected network of gas-filled epithelial tubes that develops during embryogenesis and functions as the main gas-exchange organ in the larva. Larval tracheal cells respond to hypoxia by activating a program of branching and growth driven by HIF-1α/*sima*-dependent expression of the *breathless* (*btl*) FGF receptor. By contrast, the ability of the developing embryonic tracheal system to respond to hypoxia and integrate hard-wired branching programs with *sima*-driven tracheal remodeling is not well understood. Here we show that embryonic tracheal cells utilize the conserved ubiquitin ligase *dVHL* to control the HIF-1 α/*sima* hypoxia response pathway, and identify two distinct phases of tracheal development with differing hypoxia sensitivities and outcomes: a relatively hypoxia-resistant 'early' phase during which *sima* activity conflicts with normal branching and stunts migration, and a relatively hypoxia-sensitive 'late' phase during which the tracheal system uses the *dVHL*/*sima*/*btl* pathway to drive increased branching and growth. Mutations in the *archipelago* (*ago*) gene, which antagonizes *btl* transcription, re-sensitize early embryos to hypoxia, indicating that their relative resistance can be reversed by elevating activity of the *btl* promoter. These findings reveal a second type of tracheal hypoxic response in which Sima activation conflicts with developmental tracheogenesis, and identify the *dVHL* and *ago* ubiquitin ligases as key determinants of hypoxia sensitivity in tracheal cells. The identification of an early stage of tracheal development that is vulnerable to hypoxia is an important addition to models of the invertebrate hypoxic response.

Introduction

The development and survival of an organism are dependent on its ability to adapt to changing environmental conditions. Responses to some environmental changes, for example in nutrient availability, temperature, or oxygen concentration, involve alterations in patterns of gene expression that allow the organism to survive periods of environmental stress. In metazoan cells, the cellular response to reduced oxygen is mediated primarily by the HIF (hypoxia inducible factor) family of transcription factors, which are heterodimers composed of α and β subunits belonging to the bHLH Per-ARNT-Sim (bHLH-PAS) protein family (reviewed in Kaelin and Ratcliffe, 2008). The HIF-1 αβ heterodimer is the primary oxygen-responsive HIF in mammalian cells and binds to a specific DNA sequence termed hypoxia response element (HRE) present in the promoters of target genes involved in energy metabolism, angiogenesis, erythropoiesis, and autophagy (Manalo et al., 2005). HIF-1 activity is inhibited under normoxic conditions by two hydroxylase enzymes that use dioxygen as a substrate for catalysis to hydroxylate specific proline or aspartate residues in the HIF-1α subunit (reviewed in Kaelin and Ratcliffe, 2008). These modifications limit HIF-1 activity by either reducing HIF-1α levels or inhibiting its ability to activate HRE-containing target promoters. One of these inhibitory mechanisms involves the 2-oxoglutarate/Fe(II)-dependent HIF-1 prolyl hydroxylase (HPH), which attaches a hydroxyl group onto each of two conserved proline residues in the oxygen-dependent degradation domain (ODD) of mammalian HIF-1α. These modifications create a binding site in the HIF-1α ODD for the Von Hippel-Lindau (VHL) protein, the substrate adaptor component of a ubiquitin ligase that subsequently polyubiquitinates HIF-1α and targets it for degradation by the proteasome (reviewed in Kaelin, 2005). This degradation mechanism operates constitutively in normoxia and is epistatic to otherwise wide spread expression of HIF-1α mRNA. HIF-1α protein is also modified by a second oxygen-dependent hydroxylase termed Factor Inhibiting HIF (FIH) that hydroxylates an asparagine residue in the HIF-1α C-terminal activation domain (reviewed in Kaelin, 2005). This blocks interaction with the CBP/p300 transcriptional co-factor and thus further restricts expression of HIF-1 responsive genes. These parallel O_2-dependent hydroxylation mechanisms by HPH and FIH ensure that HIF-1α levels and activity remain low in normoxic conditions. However as oxygen levels become limiting in the cellular environment, rates of hydroxylation decline and HIF-1α is rapidly stabilized in a form that dimerizes with HIF-1β, translocates to the nucleus, and promotes transcription of HRE-containing target genes.

Evidence suggests that invertebrate homologs of HIF-1 are also regulated in response to changes in oxygen availability (reviewed in

* Corresponding author.
E-mail address: kmoberg@cellbio.emory.edu (K.H. Moberg).

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doi:10.1016/j.ydbio.2009.03.001

Gorr et al., 2006). In the fruit fly *Drosophila melanogaster*, the HPH homolog *fatiga* (*fga*) has been shown to genetically antagonize the HIF-1α homolog *similar* (*sima*) during development (Centanin et al., 2005). The *Drosophila* VHL homolog dVHL has also been shown to be capable of binding to human HIF-1α and stimulating its proteasomal turnover in vitro (Aso et al., 2000). In addition, the *Drosophila* genome encodes a well-characterized HIF-1β homolog *tango* (*tgo*) (Sonnenfeld et al., 1997), and two potential FIH homologs (*CG13902* and *CG10133*; Berkeley Drosophila Genome Project) that have yet to be analyzed functionally. Spatiotemporal analysis of *sima* activation using *sima*-dependent hypoxia-reporter transgenes has shown that exposure to an acute hypoxic stress induces Sima most strongly in cells of the larval and embryonic tracheal system (Arquier et al., 2006; Lavista-Llanos et al., 2002), while induction of reporter activity in other tissues requires more chronic exposure to low oxygen (Lavista-Llanos et al., 2002). The larval tracheal system is composed of an interconnected network of polarized, epithelial tubes that duct gases through the organism (reviewed in Ghabrial et al., 2003). As the trachea acts as the primary gas-exchange organ in the larva, it is thus a logical site of hypoxia sensitivity. During larval stages, specific cells within the tracheal system called 'terminal cells' respond to hypoxia by initiating new branching and growth that results in the extension of fine, unicellular, gas-filled tubes toward hypoxic tissues in a manner somewhat analogous to mammalian angiogenesis (Guillemin et al., 1996; Jarecki et al., 1999). Studies have shown that *sima* and its upstream antagonist *fga* function within terminal cells to regulate this process (Centanin et al., 2008). *sima* is necessary for terminal cell branching in hypoxia and its ectopic activation, by either transgenic overexpression or loss of *fga*, is sufficient to induce excess branching even in normoxia. These phenotypes have been linked to the ability of *sima* to promote expression of the *breathless* (*btl*) gene (Centanin et al., 2008), which encodes an FGF receptor (Klambt et al., 1992) that is activated by the *branchless* (*bnl*) FGF ligand (Sutherland et al., 1996). This receptor/ligand pair is known to act via a downstream MAP-kinase signaling cascade to promote cell motility and tubular morphogenesis in a variety of systems (reviewed in Lubarsky and Krasnow, 2003). Excessive activation of this pathway within tracheal cells by transgenic expression of *btl* is sufficient to drive excess branching (Lee et al., 1996b). Reciprocally, misexpression of the *bnl* ligand in certain peripheral tissues is sufficient to attract excess terminal cell branching (Jarecki et al., 1999). Indeed production of secreted factors such as Bnl may be a significant part of the physiologic mechanism by which hypoxic cells attract new tracheal growth. Sima-driven induction of *btl* in conditions of hypoxia thus allows larval terminal cells to enter what has been termed an 'active searching' mode (Centanin et al., 2008) in which they are hyper-sensitized to signals emanating from nearby hypoxic non-tracheal cells.

The role of the *btl*/*bnl* pathway in tracheal development is not restricted to hypoxia-induced branching of larval terminal cells. It also plays a critical, earlier role in the initial development of the embryonic tracheal system from the tracheal placodes, groups of post-mitotic ectodermal cells distributed along either side of the embryo that undergo a process of invagination, polarization, directed migration, and fusion to create a network of primary and secondary tracheal branches (reviewed in Ghabrial et al., 2003). *btl* and *bnl* are each required for this process via a mechanism in which restricted expression of *bnl* in cells outside the tracheal placode represents a directional cue for the migration of *btl*-expressing cells within the placode. Accordingly, *btl* expression is normally highest in pre-migratory and migratory embryonic fusion cells (Ohshiro and Saigo, 1997). In contrast to the larval hypoxic response, *sima* does not appear to be required for morphogenesis of the embryonic tracheal system (Ohshiro and Saigo, 1997). Rather, developmentally programmed signals in the embryo dictate a stereotyped pattern of *btl* and *bnl* expression that leads to a similarly stereotyped pattern of primary and secondary tracheal branches (Centanin et al., 2008). The *btl*/*bnl* pathway thus responds to developmental signals to drive a fixed pattern of branching in the embryo, while in the subsequent larval stage it responds to hypoxia-dependent *sima* activity to facilitate the homeostatic growth of larval terminal cells and tracheal remodeling.

Under normal circumstances, developing *Drosophila* tissues do not begin to experience hypoxia until the first larval stage, when organismal growth and movement begin to consume more oxygen than can be provided by passive diffusion alone (Manning and Krasnow, 1993). As a consequence, the first hypoxic challenge normally occurs after the *btl*/*bnl*-dependent elaboration of the primary and secondary embryonic branches is complete. Thus, the ability of the larval tracheal system to drive new branching and remodeling via *sima* and *btl* represents the response of a developed 'mature' tracheal system to reduced oxygen availability. By contrast the effect of hypoxia on embryonic tracheal development, which requires tight spatiotemporal control of Btl signaling to pattern the tracheal network, is not as well understood. Given that the trachea does not function as a gas-exchange organ until after fluid is cleared from the tubes at embryonic stage 17 (Tsarouhas et al., 2007), it may be that the transcriptional response of embryonic tracheal cells to hypoxia (Lavista-Llanos et al., 2002) leads to mainly metabolic changes rather than to a *btl*-driven program of tubulogenesis and remodeling. However, if the embryonic tracheal system does utilize the *sima* pathway to induce hypoxia-dependent changes in *btl* gene transcription, then hypoxic exposure of embryos might be predicted to produce a situation of competing developmental and homeostatic inputs that converge on the *btl*/*bnl* pathway. The ability of tracheal cells to integrate such signals may then determine whether or not the embryonic tracheal system is able to adapt to oxygen stress, or whether embryonic tracheal development represents a sensitive period during which the organism's ability to respond to changes in oxygen levels is inherently limited by a pre-programmed pattern of developmental gene expression.

Here we show that the embryonic tracheal system utilizes the *dVHL*/*sima* pathway to respond to hypoxia, but that the type and severity of resulting phenotypes depend on the developmental stage of exposure. Hypoxic challenge while embryonic tracheal cells are responding to developmentally programmed *btl*/*bnl* migration signals disrupts tracheal development and results in fragmented and unfused tracheal metameres. In contrast, hypoxic challenge at a somewhat later embryonic stage after fusion is complete results in overgrowth of the primary tracheal branches and the production of extra secondary branches. Interestingly, we find that the threshold of hypoxia required to induce tracheal phenotypes in the early embryo is higher than that required to induce excess branching phenotypes in later embryonic stages, indicating that tracheal patterning events in the embryo are relatively resistant to hypoxia. Genetic analysis indicates that both types of hypoxic tracheal phenotypes – stunting and overgrowth – require *sima* and can be phenocopied in normoxia by reducing expression of the HIF-1α ubiquitin ligase gene *dVHL* specifically within tracheal cells. Moreover, we find that reduced *dVHL* expression in the larval trachea leads to excess terminal cell branching in a manner quite similar to that observed in *fga* mutants. Molecular and genetic data indicate that excess *btl* transcription is a major cause of hypoxia-induced tracheal phenotypes. Consistent with this, mutations in the *archipelago* (*ago*) gene, which antagonizes *btl* transcription in tracheal fusion cells (Mortimer and Moberg, 2007), synergize strongly with *dVHL* inactivation to disrupt tracheal migration and branching. Interestingly, *ago* mutations also lower the threshold of hypoxia required to elicit tracheal phenotypes in the 'early' embryo, suggesting that the relative activity of the *btl* promoter can affect hypoxic sensitivity. These findings show that the *dVHL*/*sima* pathway plays an important role in tracheal development, and identify two distinct phases of embryonic development that show different phenotypic outcomes of activating this pathway: an early phase during which *sima* activity conflicts with developmental control of tracheal