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The *Drosophila* F-box protein Archipelago controls levels of the Trachealess transcription factor in the embryonic tracheal system

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Abstract

The *archipelago* gene (*ago*) encodes the F-box specificity subunit of an SCF(*s*kp-*c*ullin-*f* box) ubiquitin ligase that inhibits cell proliferation in *Drosophila melanogaster* and suppresses tumorigenesis in mammals. *ago* limits mitotic activity by targeting cell cycle and cell growth proteins for ubiquitin-dependent degradation, but the diverse developmental roles of other F-box proteins suggests that it is likely to have additional protein targets. Here we show that *ago* is required for the post-mitotic shaping of the *Drosophila* embryonic tracheal system, and that it acts in this tissue by targeting the Trachealess (Trh) protein, a conserved bHLH-PAS transcription factor. *ago* restricts Trh levels in vivo and antagonizes transcription of the *breathless* FGF receptor, a known target of Trh in the tracheal system. At a molecular level, the Ago protein binds Trh and is required for proteasome-dependent elimination of Trh in response to expression of the Dysfusion protein. *ago* mutations that elevate Trh levels in vivo are defective in binding forms of Trh found in Dysfusion-positive cells. These data identify a novel function for the *ago* ubiquitin-ligase in tracheal morphogenesis via Trh and its target *breathless*, and suggest that *ago* has distinct functions in mitotic and post-mitotic cells that influence its role in development and disease.

Keywords: *Drosophila*; Tracheal development; Degradation; F-box; Trachealess; Fbw7

Introduction

The morphogenesis of branched networks of cells with a single, fused lumen plays an important role in the development of many metazoan organs, including the vertebrate lungs, vasculature, kidneys, and mammary glands. The cellular architecture of these mammalian organs is quite similar to tubular structures in simpler metazoans, suggesting that the molecular and cellular mechanisms underlying the process of branching morphogenesis are conserved. In the fruit fly *Drosophila melanogaster*, tubular morphogenesis underlies formation of the tracheal system, a network of interconnected tubes that duct air throughout the developing organism. The complete embryonic tracheal network is composed of approximately 1600 polarized epithelial cells that originate in early embryogenesis as 20 ectodermal placodes distributed along either side of the embryo (Samakovlis et al., 1996). Each tracheal placode contains approximately 20 cells, which undergo two rounds of cell division, exit the cell cycle, and complete the subsequent stages of invagination and tracheogenesis without further cell division or cell death. Following invagination, primary branches bud from the tracheal sac and form a continuous lumen within each placode (Lubarsky and Krasnow, 2003). The pattern of placode branching is segmentally repeated and under fixed genetic control (Samakovlis et al., 1996). Budded branches extend toward their target tissues by a process of cell migration and cell extension, and subsequent fusion between adjacent tracheal metameres at later stages of embryogenesis produces a continuous, open tubular system.

Forward genetic screens for mutations that disrupt tracheal development have revealed a central role for fibroblast growth factor (FGF) signaling in promoting the post-mitotic cell migration and extension of the embryonic tracheal arbor (reviewed in Metzger and Krasnow, 1999). The central components of the *Drosophila* FGF pathway are encoded by the *breathless* (*btl*) (Klambt et al., 1992) and *branchless* (*bnl*)

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(Sutherland et al., 1996) genes, which encode an FGF-like receptor and an FGF-like secreted ligand respectively. The role of this receptor/ligand pair in controlling tracheal outgrowth is based upon a simple model in which the restricted expression of *bnl* in cells outside the tracheal placode represents a directional cue for the migration of *btl*-expressing cells within the tracheal placode. Initial induction of *btl* transcription within tracheal cells depends upon the *trachealess* (*trh*) gene, which encodes a basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) domain transcription factor (Isaac and Andrew, 1996; Kuo et al., 1996; Ohshiro and Saigo, 1997; Wilk et al., 1996) most closely homologous to the mammalian NPAS1 and NPAS3 proteins (Brunskill et al., 1999; Zhou et al., 1997). Mutation of any one of these core components – *btl*, *bnl*, or *trh* – produces a failure of tracheal branching. Phenotypic analysis of these and other tracheogenesis genes has shown that Btl/FGF signaling is used to promote successive rounds of primary and secondary tracheal branching during embryonic *Drosophila* development (reviewed in Ghabrial et al., 2003). A similar role has been proposed for the FGF pathway in controlling the branching morphogenesis of tubes in the mammalian lung (Min et al., 1998).

In addition to a positive role in tracheal outgrowth, experimental evidence indicates that inappropriately timed Btl/FGF signaling can also impair tracheal development. First, in the course of normal development *btl* expression is not constant in tracheal cells, but oscillates: initially *btl* mRNA rises in all placode cells at stages 10–12 preceding primary branch formation, subsequently falls during late stage 12/early stage 13, and is re-initiated in a restricted set of cells that define sites of secondary branching at late stage 13/early stage14. Unlike early *btl* expression, this second wave is dependent upon a *bnl*-dependent feedback loop (Ohshiro et al., 2002; Ohshiro and Saigo, 1997). Second, ectopic activation of Btl, either by mutational inactivation of the *btl* inhibitor *abnormal wing disc* (awd) (Dammai et al., 2003), or by constitutive expression of *bnl* (Sutherland et al., 1996) or *btl* (this study and Lee et al., 1996), interferes with directed cell migration in the trachea. Similarly, expression of activated *Ras* or *btl* perturbs the ability of tracheal cells to form proper branching patterns in larval development (Cabernard and Affolter, 2005). These observations have led to the hypothesis that spatial and quantitative restriction of Btl activity is necessary to permit normal patterns of tracheal cell migration and fusion (Lee et al., 1996). While many genes that modulate Btl/FGF signaling have been identified, it is likely that other as yet unrecognized mechanisms are required to restrict Btl/FGF signaling to specific times and places in the developing organism.

Here we identify *archipelago* (*ago*), a gene known primarily for its role in cell proliferation control, as a component of the genetic circuitry that patterns the post-mitotic development of the embryonic tracheal system. *ago* encodes an F-box/WD-repeat protein that recruits target proteins to an SCF-type E3 Ub-ligase for subsequent poly-ubiquitination and proteolytic destruction. *ago* limits the division and growth of *Drosophila* eye epithelial cells by targeting the G1/S regulator Cyclin E and dMyc, the fruit fly ortholog of the human c-Myc proto-oncogene, for proteasome-mediated degradation (Moberg et al., 2001, 2004). A highly conserved mammalian *ago* ortholog (variously termed Fbw7, Fbxw7, hAgo, hCDC4, or hSel-10) also targets Cyclin E and c-Myc and is a mutational target in a rapidly expanding array of human cancers (Balakrishnan et al., 2007; Bredel et al., 2005; Calhoun et al., 2003; Hagedorn et al., 2007; Malyukova et al., 2007; Mao et al., 2004; Maser et al., 2007; Minella et al., 2007; O'Neil et al., 2007; Rajagopalan et al., 2004; Thompson et al., 2007). Our current data indicate that *ago* is required for tracheal morphogenesis via a previously unrecognized target, the Trachealess (Trh) transcription factor. We find that *ago* mutant embryos contain excess Trh protein and ectopically express the *btl* gene, a known Trh target. Alleles of *ago* exhibit strong genetic interactions with *trh* and other known tracheogenesis genes, and the Ago protein is able to bind the Trh protein and regulate its proteasomal turnover via a mechanism that involves a third factor, the bHLH-PAS protein Dysfusion (Dys; Jiang and Crews, 2003). Collectively, these data reveal a previously unappreciated developmental function for the *ago* tumor suppressor in the embryonic tracheal system, and identify the Trh transcription factor as a target of Ago in this process.

Materials and methods

Stocks, genetics, and statistics

The *ago* alleles ago^1 and ago^3 have been previously described (Moberg et al., 2001). Unless indicated, analysis was performed on ago^1/ago^3 *trans*-heterozygotes. Full-length *ago* and *agoΔF*, a version of *ago* lacking the core F-box domain (Moberg et al., 2004), were cloned as PCR products into the *Eco*RI site of the *pUAST* vector (Brand and Perrimon, 1993) and used to generate *UAS-ago* and *UAS-agoΔF* stocks (D. Rennie, Massachusetts General Hospital Transgenic Drosophila Core). Other alleles used in this study were: btl^{dev1}, $btl^{EY01638}$, trh^{10512}, awd^{j2A4}, *Df(3L)Exel9000* and *esg-lacZ* (all from Bloomington Drosophila Stock Center), *1-eve-1* (Perrimon et al., 1991), *btl-Gal4* (Shiga et al., 1996), *UAS-trh* (Jin et al., 2001), *FRT80B*, *UAS-CycE*, *UAS-dMyc*. Crosses involving the temperature-sensitive *UAS-Pros26*^{1} and *UAS-Prosβ2*^{1} transgenes (gift of J. Belote) were performed at 21 °C. Embryos were genotyped using the *TM6B, P{iab-2(1.7)lacZ}6B, Tb*^{1} and *CyO, P{elav-lacZ.H}YH2* 'blue' balancers, or the *TM3, P{Gal4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb*^{1}*Ser*^{1} balancer. Statistical comparisons were made using Student's *t*-test.

Embryo immunohistochemistry and antibodies

Embryos were staged and fixed in 37% formaldehyde-saturated heptane, devitellinized in methanol and stored in ethanol at 4 °C. These samples were rehydrated and washed in PBS with 0.05% Triton-X 100 (PBSTx), blocked in 5% milk powder/5% NGS in PBSTx, and incubated with the following primary antibodies: mouse anti-Tango (1:5, Developmental Studies Hybridoma Bank; DSHB), rat anti-Trh (1:200) a gift of D. Andrew (Henderson et al., 1999), mouse mAb2A12 (1:5; DSHB), rabbit anti-β-Gal (1:300; Cappel), guinea pig anti-full length Ago (1:2500; Pocono Rabbit Farm & Laboratory), rat anti-Dys (1:200) and rabbit anti-Dys (1:800) both a gift of S. Crews (Jiang and Crews, 2003). Secondary antibodies conjugated to HRP, AP, Cy3, and Cy5 were used as recommended (Jackson ImmunoResearch). Embryos from w^{1118} and $ago^1/$*TM3, P{Gal4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb*^{1}*Ser*^{1} strains were collected at stages 13/14 and sorted by absence of GFP fluorescence. Extracts were prepared in sample buffer containing DTT and resolved on 7.5% SDS-PAGE prior to Western blotting with rat anti-Trh (1:2000), or anti-β-tubulin (1:2000; Santa Cruz Biotechnology). Anti-HA and anti-Flag antibodies (Sigma) were used according to manufacturer's instructions.