Non-Canonical Roles for Yorkie and Drosophila Inhibitor of Apoptosis 1 in Epithelial Tube Size Control

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Abstract

Precise control of epithelial tube size is critical for organ function, yet the molecular mechanisms remain poorly understood. Here, we examine the roles of cell growth and a highly conserved organ growth regulatory pathway in controlling the dimensions of the Drosophila tracheal (airway) system, a well-characterized system for investigating epithelial tube morphogenesis. We find that tracheal tube-size is regulated in unexpected ways by the transcription factor Yorkie (Yki, homolog of mammalian YAP and TAZ) and the Salvador/Warts/Hippo (SWH) kinase pathway. Yki activity typically promotes cell division, inhibits apoptosis, and can promote cell growth. However, reducing Yki activity in developing embryos increases rather than decreases the length of the major tracheal tubes; the dorsal trunks (DTs). Similarly, reduction of Hippo pathway activity, which antagonizes Yki, shortens tracheal DTs. yki mutations do not alter DT cell volume or cell number, indicating that Yki and the Hippo pathway regulate cell shape and apical surface area, but not volume. Yki does not appear to act through known tracheal pathways of apical extracellular matrix, septate junctions (SJs), basolateral or tubular polarity. Instead, the Hippo pathway and Yki appear to act downstream or in parallel to SJs because a double mutant combination of an upstream Hippo pathway activator, kibra, and the SJ component sine have the short tracheal phenotype of a kibra mutant. We demonstrate that the critical target of Yki in tube size control is Drosophila Inhibitor of Apoptosis 1 (DIAP1), which in turn antagonizes the Drosophila effector caspase, Ice. Strikingly, there is no change in tracheal cell number in DIAP1 or ice mutants, thus epithelial tube size regulation defines new non-apoptotic roles for Yki, DIAP1 and Ice.

Introduction

Organs comprised of epithelial tubes that transport gases or fluids are essential to life for most multicellular animals. The function of organs, such as the kidneys, lungs and the vascular system is highly dependent on tubes developing to the proper size [1]. Defective tube size control leads to human diseases such as polycystic kidney disease (PKD), in which tubules enlarge to become cysts that severely impair kidney function [2,3]. The Drosophila tracheal system is an excellent model for studying the complex processes underlying epithelial tube morphogenesis (reviewed in [1,4–7]). The Drosophila tracheal system serves as a combined pulmonary and vascular system that directly delivers oxygen to tissues through a ramifying network of epithelial tubes. The tracheal system arises from clusters of cells on the surface of the embryo, and these clusters invaginate, undergo one round of cell division and do not divide again. During invagination, tracheal cells retain their apical surfaces and organize into lumen-containing branches. During later embryonic development (stages 15–17), the large dorsal trunk (DT) tubes elongate by changing cell shape and rearranging cell-cell junctions without increasing cell number [8,9]. Programmed cell death (apoptosis) plays little, if any, role in tracheal morphogenesis as only 1 cell of the ~80 cells in a tracheal metamere (segment) undergoes apoptosis in about half the developing metameres [10].

Several pathways have been identified that regulate tracheal tube size [4,11–13]. For reasons that are not clear, SJs feature in several of these pathways. Insect SJs are claudin-containing cell-cell junctions that have the paracellular barrier function of the vertebrate tight junctions [14,15], but have a basolateral localization and contain conserved basolateral polarity proteins such as Scribbled (Scrib), Discs Large (Dlg), and Yurt (Yrt) [16,17]. SJs have at least two distinct functions in tracheal tube-size control, neither of which involves the paracellular barrier function [12,18]. First, SJs are required for the specialized apical secretion of Vermiform (Verm) and Serpentine (Serp), putative chitin deacetylases that are part of a transient, chitin-based, apical extracellular matrix (aECM) whose organization is required to restrict the length of the trachea [19–24]. Apical secretion of Verm and Serp is lost in SJ mutants such as sine (sineu), coracle (cora), and nervana (nerva), which leads to a disorganized ECM and elongated trachea. Second, the basolateral polarity proteins that localize to the SJs antagonize the apical polarity protein Crumbs (Crb), a transmembrane protein that promotes expansion of the tracheal cell apical surface and tube elongation [12,16].

In addition to the aECM and polarity pathways, it was recently shown that the highly conserved non-receptor tyrosine kinase Src42 is required for the normal surface area growth of the tracheal apical membrane, and to orient apical growth along the
length rather than the circumference of the tube [25,26]. Excessive Src42 activity increases apical surface area in the direction of the length of the tube, which makes tracheal cells more rectangular and increases tracheal length. In contrast, insufficient Src42 activity reduces total apical surface area and remaining membrane growth is misoriented around the circumference of the tube. Thus, Src42 mutant trachea have tracheal tubes that are too short but that are also abnormally large in diameter.

The effectors of ECM, polarity, and Src42 pathways have not yet been determined. However, one possibility is the evolutionarily conserved Hippo/MST pathway, also known as the Salvador/Warts/Hippo (SWH) pathway that controls tracheal cell shape and organ morphogenesis. Full activity of Hippo and Warts requires the scaffolding proteins Sav/Sav1 and Mats/Mob1. Yki/YAP/TAZ are negatively regulated by the Hippo pathway through Warts/LATS phosphorylation of Yki/YAP/TAZ that causes Yki/YAP/TAZ to be localized to the cytoplasm. The core Hippo pathway is regulated by the Hippo kinase cascade in which Hippo and its mammalian homologs MST1/2 phosphorylate and activate the Warts/LATS kinases. Full activity of Hippo and Warts requires the scaffolding proteins Sav/Sav1 and Mats/Mob1. Yki/YAP/TAZ are negatively regulated by the Hippo pathway through Warts/LATS phosphorylation of Yki/YAP/TAZ that causes Yki/YAP/TAZ to be localized to the cytoplasm. The core Hippo pathway is regulated by numerous converging inputs that respond to cell-cell interactions, receptor signaling, polarity proteins such as Crb, and apical actin cytoskeletal-associated proteins such as Kibra (Kib) [29,40,41].

A conserved function of the Hippo pathway and Yki is to regulate cell death via transcriptional regulation of inhibitors of apoptosis. In flies, Yki activates transcription of Drosophila Inhibitor of Apoptosis (DIAP1), which encodes a protein containing two baculovirus IAP repeats (BIR) domains that bind caspases and pro-apoptotic factors, and a RING domain that mediates ubiquitination of DIAP1 itself as well as caspases and cell death regulators that bind the BIR domains of DIAP1. Expression of DIAP1 inhibits cell death by inactivating the caspases Dronc and Ice. Binding of cell death initiators such as Grim, Reaper or Hid to DIAP1 releases the inhibition of Dronc and Ice, which become proteolytically active. Most commonly, activation of caspases leads to cell death, however there are multiple examples of non-apoptotic functions of caspases in Drosophila and mammals [42–45].

In this study, we find that Yki has an unexpected role in controlling cell shape during tracheal morphogenesis. While loss of Yki activity typically decreases organ size, loss of Yki increases tracheal dorsal trunk length without increasing tracheal cell volume or number. Despite this unexpectedly opposite result, Yki nonetheless regulates tracheal tube-size through its well-established downstream mediators thread/DIAP1 and the effector caspase Ice. However, loss of Yki, DIAP or Ice does not alter apoptosis in the tracheal dorsal trunk. Thus, Yki, DIAP and Ice act in their typical “cassette”, but together they act in a non-canonical pathway that controls tracheal cell shape and organ morphogenesis independent of apoptosis.

**Results**

**Yorkie negatively regulates embryonic tracheal tube length**

Yki activity typically promotes cell division and can increase cell size, so we expected that reduced Yki activity would decrease tracheal tube length and/or diameter. Surprisingly, we found that the dorsal trunks (DTs) of stage 16 yki mutant embryos had a convoluted appearance and were 16% longer than DTs of wild-type (WT) embryos (Figure 1A,B, I; p < 0.005). This over-elongated phenotype closely resembles the long tracheal phenotype caused by previously characterized tracheal tube-size control mutants such as sinu [14] and cora [18,46], and yki trachea are indeed as long as sinu or cora mutant trachea (Fig. 1D,F; Fig. 2G, J). We confirmed that loss of yki activity was responsible for this over-elongated phenotype using two approaches. First, embryos homozygous for the small chromosomal deficiency D(2R)BSC356 that completely deletes yki have the same long trachea phenotype as the yki<sup>b5</sup> mutant (Fig. 1D). Second, the long trachea phenotype caused by the yki<sup>b5</sup> mutation is rescued by expressing a WT form of yki in tracheal cells using the tracheal-specific breathless-GAL4 (btl-GAL4) driver in yki mutant embryos (Fig. 1C, I; [47,48]). Significantly, this result also demonstrates that yki is acting in tracheal cells to control tracheal tube length.

To test if Yki was not only necessary but also sufficient for controlling tube size, we expressed Yki-GFP in the trachea of WT embryos. This expression decreased tracheal length, as did overexpression of one of Yki’s transcriptional co-activators, the TEAD-family member Scalloped (Sd) (Fig. 1I, p < 0.05 for both). Co-expression of Yki and Sd further reduced tracheal length compared to expression of Yki alone (p < 0.05, Fig. 1I). Together, these results suggest that Yki has an instructive role in regulating tracheal tube length, even though Yki activity causes the opposite of what would typically be expected for a positive regulator of cell growth.

The Hippo pathway positively regulates embryonic tracheal tube length

To determine if Yki is regulated by the Hippo pathway in the embryonic trachea, we examined zygotic mutants of the core Hippo pathway components Hpo, Warts (Ws), Salvador (Sav), Mob as tumor suppressor (Mats) and Kib [41,49,50]. Consistent with negative regulation of Yki by the Hippo pathway observed in other tissues, mutations in sav, wts, mats and kib had short trachea (Fig. 1F–I). Trachea in homozygous hpo mutants were not short, presumably due to maternal contribution (Fig. 1E, I). However, we were unable to confirm this possibility as embryos lacking both zygotic and maternal hpo arrested development prior to tracheal morphogenesis. Notably, the length reductions caused by mutations in Hippo pathway components were comparable to the reductions caused by overexpression of Yki and Sd, suggesting that the Hippo pathway acts through Yki to control tracheal tube size.

To more directly demonstrate that the Hippo pathway regulates Yki activity, we utilized transcriptional reporters that respond to Yki activity. These reporters are transposable element insertions in the Yki target genes thread (th) and expanded (ex) that produce a nuclear-localized lacZ in response to Yki activity [35]. Quantification of anti-β-gal fluorescence revealed an approximately 2-fold difference in reporter signal between trachea heterozygous or homozygous for the reporter insertions, indicating that the reporters should have sufficient dynamic range to detect changes in Yki activity. (Fig. 3A–E, Material and Methods). We verified that the ex reporter responds to Yki activity in the trachea by determining that reporter activity is decreased by 31% in yki<sup>b5</sup> mutant trachea (p = 0.006, Fig. 3F).

Consistent with the Hippo pathway acting through Yki to regulate tracheal length, Yki reporter activity was increased by 79% (p < 0.0005) in wts mutant trachea (Fig. 3F), which are shorter than WT (Fig. 1A vs. G, I). Reporter activity was not significantly changed in hpo mutant trachea, which have WT length (Fig. 1A
mutation and nrv223B (Fig. 2M, arrowhead). Interestingly, more disruptions of lumenal matrix in the lateral branches components, such as organization of the lumenal matrix. In contrast to mutations in SJ loss of paracellular barrier function (Fig. 2E), in mislocalized around the cell periphery (e.g. Cora in Fig. 2B) and secretion appears normal in the secretion of the lumenal matrix protein Verm (Fig. 2B), Verm function is intact (Fig. 2F). Further, whereas SJ mutations prevent canonical SJ protein Cora is correctly localized to the apical region tracheal phenotype in yki nrv2 regulated in SJ mutants. Null mutations of the SJ components tube length, we tested whether Yki reporter activity was down- disrupted SJs or lumenal matrix formation.

Figure 1. Yorkie and the Hippo Pathway Regulate Tracheal Tube Size. (A–D) Maximum projections of confocal sections showing tracheal lengths. Compared to stage 16 WT embryos (A), ykiB5 mutant trachea (B) have an over-elongated phenotype similar to the SJ mutant sinu (D). Expression of UAS-yki in the trachea of ykiB5 mutants rescues tracheal length defects (C). Yellow lines indicate dorsal trunk measurements. Tracheal lumens visualized by anti-2A12 marker staining. (E–H) Although embryos trans-heterozygous for hpo mutations have WT tracheal length (E), mutations in Hippo pathway components sav, wts and kib shorten tracheal length (F–H, respectively). (I) Quantification of tracheal length shows that decreased Yki activity lengthens trachea, while increased Yki activity or decreased Hpo pathway activity reduces tracheal length. Tracheal length was measured in three dimensions in confocal stacks, normalized to embryo length and then normalized to WT (w1118, red line). Error bars show normalized s.e.m.; *: p<0.05; **: p<0.005; ***: p<0.0005. p-values determined using Student’s t-test. Scale bar for (A–G) in (H), 50 μm. doi:10.1371/journal.pone.0101609.g001

Yorkie acts separately from the SJ, basal polarity, and Src pathways

We next investigated whether yki mutations affect SJs or the organization of the lumenal matrix. In contrast to mutations in SJ components, such as nrv2 [18], that cause other SJ proteins to be mislocalized around the cell periphery (e.g. Cora in Fig. 2B) and loss of paracellular barrier function (Fig. 2E), in yki mutants the canonical SJ protein Cora is correctly localized to the apical region of the lateral cell-cell contacts (Fig. 2C) and paracellular barrier function is intact (Fig. 2F). Further, whereas SJ mutations prevent the secretion of the lumenal matrix protein Verm (Fig. 2B), Verm secretion appears normal in yki mutants (Fig. 2C). Thus, the long tracheal phenotype in yki mutants is not caused by grossly disrupted SJs or lumenal matrix formation.

To determine if SJs could act through Yki to control trachea tube length, we tested whether Yki reporter activity was down-regulated in SJ mutants. Null mutations of the SJ components nrv2, cora and sinu all cause tracheal length increases similar to those caused by mutations in yki (Fig. 1D,I; Fig. 2G,J,K), yet reporter activity was neither consistently nor strongly altered by these SJ mutants (Fig. 3F). Consistent with Yki and SJs acting in separate pathways, double mutant combinations of the ykiB5 null mutation and nrv2218 null mutations have longer trachea than either the ykiB5 or the nrv2218 mutations alone (Fig. 2G,M) and double mutant combination of ykiB5 and nrv2218 had significantly more disruptions of lumenal matrix in the lateral branches (Fig. 2M, arrowhead). Interestingly, cora5, ykiB5 double mutant trachea are somewhat shorter than either cora5 or ykiB5 alone, but still clearly longer than WT trachea (Fig. 2G,J,K). Although unexpected, this result is nonetheless consistent with SJs and Yki not acting in a single linear pathway.

To further test the relationship between the SJ and Hippo pathways, was asked if the kib1 mutation, the Hippo pathway mutation that causes the shortest trachea, could suppress the long tracheal phenotype cause by the sinu mutation. kib1 is completely epistatic to sinu (Fig. 2R-T), demonstrating that the Hippo pathway can profoundly influence tracheal cell shape and that the Hippo pathway acts downstream or in parallel to the SJ pathway in regulating tracheal tube size.

We also investigated whether Yki acted in either of the apicobasal polarity pathways that we had previously showed control tracheal tube length [12,16]. Apicobasal polarity of the tracheal cells was not obviously affected in yki mutants, as SJ junctional components, whose localization depends on cellular polarization and adherens junction formation, were correctly localized to the apicolateral cell-cell contacts in yki mutants (Fig. 2C). Further, the combination of a mutation in the basolateral polarity gene Lethal giant larvae (Lgl) and yki was as long or longer than either single mutant (Fig. 2G,1N–O). Finally, Yki reporter activity was not substantially altered in embryos lacking the basolateral polarity components yurt or scrib (Fig. 3F). Thus, it appears that yki acts distinctly from apicobasal polarity pathways.

We also tested if Yki acted in the pathway containing Src42, which controls both the orientation and amount of growth of tracheal cells and acts distinctly from SJs and apicobasal polarity pathways [23]. Double mutant combinations of ykiB5 and Src4226-7, a putative null allele, have the same short trachea phenotype of Src4226-7 mutants, indicating that Src42 is largely epistatic to yki (Fig. 2G,P-Q). In contrast to SJ mutations, which do not modify
the Src42 length phenotype [25], the ykiB5 mutation slightly lengthens the Src42 mutant trachea (p, 0.05), suggesting that Yki may act in parallel to the Src42 pathway. Consistent with this possibility, Yki reporter activity was reduced by only 10% in Src42 mutant trachea. While it is possible that the ability of yki mutations to affect the Src42 mutant phenotype results from maternal Src42 contributions, the above results suggest that Yki activity represents a distinct input into mechanisms that control tracheal tube size from the known pathways involving SJs, apicobasal polarity and Src42.

Yorkie mutants increase tube length without increasing cell volume or number

As Yki plays a critical role in controlling cell proliferation in many tissues and can also control cell size [30,31,34], we investigated the possibility that loss of Yki alters tracheal cell volume or cell number. To allow comparison with normal development, we first characterized trachea cell volume in WT embryos, which had not been previously done. Tracheal cell bodies and nuclei were visualized by staining for a tracheal-GFP and for the transcription factor Trachealess (Trh) [51] (Fig. 3G and materials and methods). Cell volumes and number measurements revealed several unexpected findings. First, tracheal cell volume steadily increases along the length of the dorsal trunk. Surprisingly, at stage 16, anterior cells (DT2) have 44% less volume than posterior cells (DT8; 32 \( \mu \text{m}^3 \) vs. 57 \( \mu \text{m}^3 \), respectively, p, 0.0005; Fig. 3H). This anterior-to-posterior differential is not observed in epidermal cells that gave rise to the tracheal cells approximately six hours earlier, but does account for anterior tracheal cells having a more squamous (flatter) profile.
than the more cuboidal posterior cells (epidermal cell volume for segments 2, 5 and 8, respectively: 95 ± 3 µm³, 92 ± 11, 103 ± 8). Second, cell volume is not linearly proportional to cell length (Fig. 3H). For example, the posterior segment DT8 is only 22% longer than the anterior segment DT2, yet DT8 cells have a 60% larger volume than DT2 cells, and there are 31% more cells in DT8 than DT2 (Fig. 3H, I). Third, despite the length of tracheal segments increasing over the 4-hour period from stage 14–16, there is a consistent, but not statistically significant, decrease in tracheal volume over this time (Fig. 3H). Thus, while tracheal cell volume is dynamically regulated during development, tube length increases are not driven by increases in cell volume.

Having established a baseline of WT development, we determined tracheal cell number and volume in yki mutants and, for comparison, the SJ mutant cor. Consistent with the almost complete absence of cell death or division during most of tracheal morphogenesis [9,10], cell number was not significantly altered in yki mutants (Fig. 4I). Tracheal cell volume in yki mutants was also not statistically different than in WT (Fig. 3I). Thus, the increased tracheal length in yki mutants is not driven by increased tracheal cell volume. Surprisingly, in the SJ mutant cor, cell volumes were significantly increased compared to WT (Fig. 3H; p < 0.05 for DT2 and DT5, p < 0.005 for DT8), providing further evidence that Yki does not act in the same pathway as SJ components. In combination, these results suggest that while cell volume is dynamically regulated in the tracheal system, cell volume changes do not drive tube length increases.

Yki acts through thread/DIAP to control tracheal tube length

Since Yki does not appear to control tracheal size by regulating cell growth, we examined embryos mutant for the known Yki transcriptional targets: thread (th, which encodes the protein DIAP1- Drosophila Inhibitor of Apoptosis), expanded (ex), and bantam (ban). Tracheal length was not substantially changed in ban or ex mutants (Fig. 4E, F, K). However, embryos homozygous for the reduction-of-function th^{jcs} mutation, which results from a
transposable element insertion in the th 5' UTR [52,53], had dramatically longer trachea, comparable to length increases in ykiB5 mutants (p<0.005; Fig. 4A,K). We attempted to confirm this result using additional loss-of-function alleles of th: th4, which substitutes a tyrosine for an invariant histidine in the BIR2 domain, and th7, which is a small deletion the removes part of the BIR2 domain [54]. Unfortunately, most homozygous th4 embryos had severe morphogenesis defects and it was not possible to meaningfully determine tracheal length of this genotype (data not shown). On the other hand, th7 mutants had normal embryonic morphology, but also had WT tracheal length, presumably

Figure 4. Non-apoptotic functions of th/DIAP1 and Ice Mediate Yki Activity in Tracheal Size Control. (A–C) th/DIAP1 regulates tracheal tube length. The loss-of-function allele thJ5C8 causes an over-elongated trachea (A) that closely resembles the yki phenotype (G). The hypomorphic allele th does not alter length (B) but the gain-of-function allele th6B shortens trachea (C). (D) A loss-of-function allele of Ice, an effector caspase normally inhibited by DIAP1, shortens the trachea, similar to the GOF th allele in (C). (E–F) Mutations in other Yki targets, ex and bantam, do not cause dramatic changes in tracheal length. (G–H) Expression of th in the trachea of ykiB5 mutants is sufficient to rescue tracheal length to WT. (I) Expression of th in WT trachea shortens trachea, similar to the GOF th allele and Ice mutants. Yellow lines indicate dorsal trunk measurements. Lumens visualized by 2A12 staining. (J) Quantification of tracheal cell numbers in w1118, cora2, ykiB5, thJ5C8, and Ice17 mutants. (K) Quantification of tracheal lengths for th, Ice, ex and ban mutants, and th and p35 expression experiments. Tracheal length was measured in three dimensions in confocal stacks, normalized to embryo length and then normalized to WT (w1118, red line). Error bars show normalized s.e.m.; *: p<0.05; **: p<0.005; ***: p<0.0005. p-values determined using Student’s t-test. Scale bar for (A–H) in (H), 50 μm. (L) Model of genetic pathways regulating tracheal tube length. doi:10.1371/journal.pone.0101609.g004
because of a combination of maternal contribution and th7 being a partial loss-of-function mutation.

To further investigate the role of DIAP in tracheal size control, we also examined embryos homozygous for three gain-of-function mutations in the th locus, each of which has a different spectrum of interactions with the pro-apoptotic proteins Grim, Reaper and Hid, and with the caspases Drone and Ice [54–56]. We examined thSL, which is a valine to methionine substitution in the RING domain [54], th21-2s which is a semi-letal allele resulting from a proline to serine change also in the RING domain [54]. Tracheal length was normal in homozygous thSL and th21-2s st. 16 embryos. However embryos homozygous for th6B had shortened trachea that were as short as the strongest SWH mutants (Fig. 4C; [57]). Since the th6B can act as a dominant negative mutation with regard to Hid induced-killing in the eye [54], this result supports the model that DIAP mediates the effects of Yki activity and implicates Hid as a potential regulator of tracheal length. Notably, thSL mutation does not act as dominant negative with regard to Hid [54]. The significance of the semi- viable th21-2s mutation not causing a tracheal phenotype is unclear since the mutation only causes partial lethality and was scored by Goyal et al. as a weak suppressor of Hid in the eye. Although the genetics of DIAP1 mutations is complex [56], and further complicated by maternal contribution in the embryo, these results clearly implicate DIAP as a downstream effector of the Yki in tracheal size control.

To more directly assess the role of DIAP in tracheal size control, we expressed WT th in the trachea of otherwise WT embryos. Consistent with th mediating the effects of yki on tracheal size, th expression resulted in shortened tracheal tubes that were approximately the same length as Hippo pathway mutants (Fig. 4L,K). To confirm the overall relationship of the Hippo pathway and th, we examined kib4, thSCD double mutants (a short kib mutant in combination with a long th mutant), however the mid-body tracheal segments were grossly defective in these embryos and we were unable to determine tracheal length (data not shown). Critically, expressing th in the trachea of yki17 mutants restored tracheal length to WT (Fig. 4L,K), strongly supporting the model that th/DIAP1 is the primary mediator of Yki function in tube size control.

A non-apoptotic function of the caspase Ice mediates tracheal tube size control

How does the th/DIAP1 control tube size? A critical function of DIAP1 is to regulate cell death by binding and inhibiting the pro-apoptotic caspases Drone and Ice [58,59]. We tested if caspases control tracheal size by characterizing the phenotypes of mutations in Drone and Ice. Tracheal length was not altered by the Drone^cl null mutation, however, the loss-of-function Ice^cl and Ice^cl mutations decreased tracheal length as much as the th6B and wts338/1489 mutations (Fig. 4C–D,K). Given that Drone is thought to be required to activate Ice (reviewed in [56]), it is somewhat surprising that Ice but not Drone mutations affect tracheal length. However, it is likely that a role for Drone in tracheal size control is obscured by the significant maternal contribution of Drone [56].

To confirm a role for Ice and its protolytic activity in size control, we used the tracheal specific driver btl-Gal4 to express baculovirus p35, a well-characterized caspase inhibitor that covalently attaches and inhibits Ice. p35 expression decreased tracheal length similar to Ice and th6B mutations. Because p35 blocks proteolytic activity of Ice without necessarily eliminating Ice protein, this result suggests the proteolytic activity of Ice is required for tracheal size control, even though Baer et al. did not observe significant staining of an antisem that binds cleaved caspases in the dorsal trunks of late stage embryos [10]. Strikingly, expressing p35 in yki^cl mutant trachea restored tracheal length to WT, strongly supporting the model that Yki acts through DIAP1 and subsequently Ice to regulate tube size (Fig. 4K).

To confirm that changes in overall tracheal cell number were not influencing tracheal system length, we counted the tracheal cells in yki, th and Ice mutants (Fig. 4J). Tracheal cell numbers in these mutants were within the WT range and not correlated with tracheal length (Fig. 4J and [8,9]). Thus, control of epithelial tube length by Yki-DIAP1-Ice is a non-apoptotic function of this evolutionarily conserved cell death pathway.

Discussion

The Hippo-Yki/YAP/TAZ pathway has been well established as the regulator of organ size via cell proliferation and cell death. However, this pathway also has broader roles, including regulating cell-cycle exit and differentiation, acting as a switch in cell fate decisions, and controlling signaling pathways [60,61]. To date there has been limited investigation of non-proliferative/apoptotic roles of the Hippo pathway or Yki/TAZ/YAP in tubulogenesis and cell shape control, but interesting and unexpected findings are emerging. Mice and zebrafish lacking TAZ develop cystic kidneys due to an abnormal accumulation of polycystin-2, which TAZ normally regulates through an ubiquitin ligase complex [62]. Tissue specific inactivation of YAP in developing mouse nephrons severely disrupts nephron morphogenesis, without affecting either cell proliferation or cell death [63]. While the altered expression of genes that mediate tubule morphogenesis in YAP-deficient cells likely contributes to abnormal nephron morphogenesis, failure to activate non-apoptotic functions of cell death genes may also contribute. The Drosophila tracheal system provides a powerful model system for investigating non-proliferative/apoptotic roles of the Hippo pathway and Yki/YAP/TAZ in morphogenesis.

One puzzling aspect of our results is that previous work by Ghabrial et al. showed that single cell clones of a Wts mutation in the larval dorsal trunk caused what they described as a “general overgrowth” phenotype, and what appears to be a roughly isotropic increase in tracheal apical surface [64]. This increase is in marked contrast to the reduction in apical surface area we observed in the shortened dorsal trunks of homozygous wts338/1489 embryos. Explanations for this discrepancy fall into two general categories. First, it may be the case that the Hippo pathway acts on targets other than, or in addition to, Yki, that differ between embryos and larvae. Similarly, Hippo may regulate Yki equivalently in larvae and embryos, but Yki may have different targets in the two stages. Second, and perhaps more interestingly, the Hippo pathway may mediate cell-cell communication that regulates apical membrane growth, and a single cell lacking Hippo pathway may mediate cell-cell communication that regulates apical membrane growth, and a single cell lacking Hippo pathway activity behaves differently depending on whether its neighbors have a functional Hippo pathway. In this model, similar results would be obtained in embryos and larvae as long as equivalent experiments are performed (single cell clones versus entire tissue). Resolving this discrepancy will require making single cell clones of Hippo pathway components in embryonic trachea, and reducing Hippo pathway activity in all tracheal cells during larval development.

While additional details of the roles of Hippo and Yki remain to be elucidated, our results clearly demonstrate a non-apoptotic function of DIAP1 and Ice in embryonic epithelial tube size control. What is this non-apoptotic function? An attractive possibility is that it would be related to the DIAP1-dependent, but non-apoptotic, process that regulates migration of border cell
migration during oogenesis. However, in contrast to tracheal size control, border cell migration does not require Ice, and is not affected by expression of p35, which binds to and inhibits Ice but not Dronc [65]. Moreover, Lucas et al. recently showed that the Hippo pathway acts through the actin regulatory protein Ena to activate F-actin Capping protein activity on inner membranes and restrict F-actin polymerization [66]. Since our results show that tracheal size control depends on DIAP1 and Ice, these results argue that the Hippo pathway and DIAP have distinct roles in border cell migration and tracheal size control.

A possible function of DIAP1 and Ice to regulate tracheal membrane involves SREBP/HLH106, the sterol response element binding protein that is a transcription factor required for production of membrane lipid. Interestingly, SREBP is translated as a plasma membrane-bound form that is cleaved by Ice to liberate the functional transcription factor [67]. In the tracheal system, non-apoptotic Ice activity may activate SREBP to promote membrane expansion. Another intriguing possibility is that tracheal growth could be related to non-apoptotic functions of caspases-3 that enable melanoma cells to generate tubes via “vascular mimicry”, the process by which non-endothelial tumor cells form tubes that connect the tumor to the vascular system to facilitate tumor growth. However, the role of caspase-3 in mammalian tube formation pathway remains to be determined [68,69]. Plausible pathways that could be regulated by non-apoptotic functions of caspases include apicobasal polarity, which we have previously showed control cell length [12,16], and the endomembrane system, which could potentially mediate changes in apical surface area, cell shape and tube size.

Further work will be required to delineate the targets and logic of caspase-mediated tube-size control in the trachea, but the genetic tractability of the Drosophila trachea system make it an excellent system for investigating the non-canonical functions of Yki, DIAP1 and Ice in cell shape control and organ morphogenesis.

**Materials and Methods**

**Fly reagents**

Fly stocks and alleles were obtained from Bloomington Drosophila Stock Center [70] or were generously provided by A. Bergmann (Ice17), R. Carthew (ye hsFLP, FRT42D, FRT42B ovoD1/MS(2)M1, Sp/CyO), R. Felon (ybPUB:UAS-FLAG-yki), A. Ghabriel [w [muscle-gal4]38, w [muscle-gal4]83, w [muscle-gal4]1889, G. Halder [yb1, ex6], I. Harirhanan [ybMGAT1, sav1], B. Hay [IceA1], R. Holmgren (UAS-p25), J. Kennison (th5), K. Moberg (thPSC, ex697), H. Steller (th21b), H. Stocker (kb1), and Kristin White (th6b, thSL1). Fly stocks were maintained at room temperature and fly experiments: RMR SCG. Analyzed the data: RMR SCG GJB. Conceived and designed the experiments: RMR GJB. Performed the experiments, signal levels of 20–30 nuclei in DT7 and DT8 tracheal cells (3–5 sections/segment) per embryo were measured and averaged. Tracheal metamere volumes were determined from confocal sections of fixed embryos that had expressed a cytoplasmic and nuclear GFP in the trachea. GFP was visualized with a fluorescent secondary antibody. Trachea were automatically detected as “objects” with a fluorescence intensity above a threshold value. To fill missing volumes in the cell bodies due to non-uniform expression of GFP, automatic functions of the Volocity software were used to “dilate”, “close” and “fill” spaces within the confocal stack. This resulted in a contiguous filled object for the cell bodies of the trachea. A representative image of processed trachea is shown in Fig. 3G. Tracheal metameres were manually segmented and the volume of segmented object was calculated. For statistical analyses, p-values were calculated using Student’s t-test with two-tailed unpaired variance.

**Immunohistochemistry**

The following antibodies were used: mouse (ms) anti-2A12 1:2, rabbit (rb) anti-Verm 1:300 (S. Luschnig), rat anti-Tracheless 1:50 (B. Shilo [31]), guinea pig (gp) anti-Cora 1:10000 (R. Fehon), rb anti-β-galactosidase 1:75 (Capel), ms anti-GFP (Molecular Probes, A1120), and rb anti-GFP (Molecular Probes, A1122). Alexa Fluor 488, 546, and 647 were used for secondary antibodies (Molecular Probes). The luminal antibody 2A12, embryo fixation, staining, and staging procedures were performed as previously described [8]. All embryos were imaged with a Leica SP2 or SP5 confocal microscope. Control heterozygous embryos were imaged on the same slide as the experimental homozygous embryos using the same laser settings such that relative signal levels could be directly compared. For Yki reporter activity experiments, lasers were set such that in a control embryo, the maximum signal for pixels in tracheal nuclei did not saturate the detector.

**Dye exclusion assay**

Paracellular barrier function of tracheal SJs was assayed as previously described by injecting a 10 kD Texas Red-conjugated dextran (Molecular Probes) into the body cavities of stage 16 embryos [75]. Injected embryos were imaged on a Leica TCS SP2 confocal microscope within 20 min of injection.

**Acknowledgments**

We thank K. Nelson, W. Rusin, P. Brannon and the Northwestern Biological Imaging Facility for technical and imaging support, the Bloomington Drosophila Stock Center at Indiana University for reagents, and K. Nelson, R. Holmgren and anonymous reviewers for comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: RMR GJB. Performed the experiments: RMR SCG. Analyzed the data: RMR SCG GJB. Contributed to the writing of the manuscript: RMR SCG GJB.

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