JCB

Yorkie controls tube length and apical barrier integrity in airway development

Kassiani Skouloudaki, Ioannis Christodoulou, Dilan Khalili, Vasilios Tsarouhas, Christos Samakovlis, Pavel Tomancak, Elisabeth Knust, and Dimitrios Papadopoulos

Corresponding Author(s): Dimitrios Papadopoulos, MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK; Elisabeth Knust, Max-Planck-Institute of Molecular Cell Biology and Genetics; and Kassiani Skouloudaki, Max-Planck Institute of Molecular Cell Biology and Genetics

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Re: JCB manuscript #201809121

Prof. Elisabeth Knust
Max-Planck-Institute of Molecular Cell Biology and Genetics
Pfotenhauerstr. 108
Dresden 01307
Germany

Dear Eli,

Thank you for submitting your manuscript entitled "Yorkie controls tube length and apical barrier integrity in the developing Drosophila airways". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, the reviewers are mostly positive about the work, but all have significant concerns about some aspects of the manuscript and propose more experiments to strengthen the conclusions. Unfortunately, for this reason, we are unable to accept the manuscript in its present form. However, we would be willing to consider a revised version of the work that includes the additional data and modifications suggested by the reviewers, along with a point-by-point response to their comments.

Reviewers #1 and #2 are fairly consistent about the areas that need strengthening to bolster the conclusion that Yki modulates tracheal length through transcriptional control of Alas and through a mechanism involving Twinstar. For example, Reviewer #1 asks about defects in heme synthesis when Yki is disrupted, whether other genes directly regulated by Yki are involved, and why, if Tsr negatively regulates Yki transcriptional activity, the loss-of-function mutant has the same phenotype. We do not consider that the LOF Diap1 mutation or GOF mutation analysis is necessary in this context. Reviewer #2 also has a number of additional points, including the lack of EM data showing rescue by trachea-specific Yki cDNA expression in ykiB5 mutant animals, and asking if an Sd mutant presents the same tracheal phenotype as Yki. The reviewers also have a number of minor points, and several points can be addressed by modifications to the text. Overall, they request a significant number of additional experiments but we feel that these are not unreasonable and will greatly strengthen the conclusions to this interesting study.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ian Macara, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The transcriptional coactivator Yorkie (Yki) has been previously reported to be important for proper tracheal tube growth (Robbins et al., 2014). In current manuscript, the authors demonstrated that Yki has a dual role in tracheal development to ensure proper tracheal growth and functionality. They found that Yki controls proper tracheal tube elongation by binding Drosophila Twinstar, the orthologue of the vertebrate actin-severing protein Cofilin, to regulate F-actin levels and apical cell membrane size. In addition, Yki controls water-tightness of tracheal tubes by transcriptional regulation of the enzyme δ-aminolevulinate synthase (Alas). Although this is an interesting study, the current version of this manuscript is not sufficient to deduce the authors' conclusion. There are several concerns as indicated below. This study would be of high significance with several

additional experiments.
Major concerns
1.In Fig. 1E, the authors observed partial rescue of the tube length phenotype of yki mutants upon tracheal expression of Diap1. What's the phenotype of tracheal expression of loss-of-function Diap1 mutation (thj5c8) or gain-of-function Diap1 mutation (th6b) in Yki mutants?
2.In Fig. 2 A, C and Fig. S1, there are obvious differences in SJs markers (Yurt, FasIII, Mega, Dig, Cont) between embryonic trachea of wild type (WT) and ykiB5 mutants. However, the authors concluded that 'SJs did not appear impaired in Yki mutants'. Once again, in Fig. 2 C, D, the author concluded that 'this phenotype was rescued by the tracheal expression of a Yki cDNA'. But embryonic trachea of YkiB5 mutants of stage 17 stained for the core septate junction components in Figs. 2 C and D didn't support the conclusion. The author should address these inconsistences.
3.In Fig. 4, the authors provided evidence that Yki acts through transcriptional activation of Alas. However, Loss of Alas leads to a decrease in the production of chitin, which may also cause defects in heme synthesis. So, are there any defects in heme production when Yki is lacking? And what about the level of δ-aminolevulinate in that situation? The authors cited ChiP-seq data from Nagaraj et al., 2012 which showed that Yki binds the regulatory region of the Alas gene in Drosophila imaginal discs from. However, beside Alas gene, there were some other genes directly regulated by Yki. Are they also involved in air-filling and tracheal impermeability?
4.In Figs.6 A, B, C, Yki mutant embryos and Tsr mutant embryos all exhibited over-elongated tracheal tubes. However, Fig. 6 showed the increased Diap1 (Yki target gene which can regulate tube length) mRNA levels in Tsr mutant embryos, and then the authors concluded that Tsr is a negative regulator of Yki transcriptional activity. The authors should address why Tsr negatively regulats Yki transcriptional activity but their loss-of-function mutants have the same phenotype? In Fig. S9, the authors found a reduction in total Yap and p-Yap after using cofilin (vertebrate orthologue of Drosophila Tsr) siRNA in HEK293T cell. However, these data could not support that Tsr is a negative regulator of Yki nuclear localization.

5.Robbins groups (Robbins et al., 2014) showed that mutations such as Wats, Mats, and Sav can result in a shorter tracheal tube. The authors should test whether overexpression of Alas or Tsr could suppress the air-filling and tracheal impermeability, or return tracheal tube length to normal levels in one of Wats, Mats, and Sav mutants?

Reviewer #2 (Comments to the Authors (Required)):

In this study by Papadopoulos et al. performed a series of experiments to elucidate the function of the Hippo pathway effector yki in the developing Drosophila respiratory system, the trachea. They showed that Yki has a dual function in the trachea by regulating tube size and membrane permeability. It was previously published that yki mutant animals have elongated trachea through a mechanism involving the Yki target diap1 and the Drosophila effector Caspase, Ice (Robbins et al. 2014). In this study, the authors suggest that the role of Diap1 in this process is partial and they proposed a new mechanism. They claim that Yki interacts with the fly homolog of Cofilin called Twinstar (Tsr), to induce F-actin depolymerization at the apical side of the cells and thereby restricts apical domain size. Removal of Yki results in reduced Tsr accumulation at the apical side and this leads to an increased size of the apical domain, which then results in elongated trachea. Moreover, the authors showed that Yki has another important function for the development of trachea in which Yki induces expression of Alas (δ-Aminolevulinate synthase), encoding an enzyme that is important for the formation of the impermeable barrier. This discovery is novel and provides a unique function of Yki in the developing airways.

Most of the findings of this paper provide interesting insights into the function of Yki in the trachea. The rescue of the DT elongation phenotypes by ectopic expression of one gene in animals with a mutation in another gene clearly shows that Yki and Tsr are somehow connected. However, there are a number of concerns that should be addressed by the authors for this study to be published in JCB.

Major points:
• The authors claim that Yki has cytoplasmic and non-transcriptional functions. To strengthen this argument I suggest to also present the tracheal phenotype of a Sd mutant embryo.
• The authors made a new genomically tagged Yki protein (mKate-Yki). The authors need to analyze its localization pattern in tsr mutant embryos. This experiment is obviously missing.
• What is the expression of diap1-lacz in Yki overexpressing embryos and in yki mutant embryos? What about other Yki target genes such as Myc and ex?
• Shorter exposures of Western blots should be used. In supplementary Fig 8A, and 8B, it is impossible to get to a conclusion with such overexposed images. In Fig. S8A, there is no loading control. In Figure S8B, the loading control seems strongly overexposed. The same is true for Supplementary Fig. 9.
• On page 7: "This phenotype was rescued by the tracheal expression of a yki cDNA (Fig. 2, C, D)." However, there is no EM data for trachea-specific Yki cDNA expression in ykiB5 mutant animals showing the rescue.
• In figure 4H, the Alas mRNA downregulation is not convincing. If possible Alas protein should be monitored to test whether Yki-dependent Alas regulation is the main reason for permeability defects in Yki mutant embryos. Also, in the figure legend it is not clear whether the mRNA comes from embryos or trachea.
• The authors suggest that removal of Yki causes reduction in Tsr protein levels and vice versa. In Figure 6Q-R, there is no statistical measurement showing that presence of one protein affects the presence of second protein. Just looking at the blot, I do not think that Yki affects Tsr protein levels. The authors list n=2 in the legend, but where these biological replicates? Two sample points is not enough for robust statistics and it may be that the error bars in 6Q-R are derived from re-imaging or re-analyzing the same blot/exposure. Please clarify and perform biological replicates if this is an important argument to be made. As is, the presented data do not support the claim that Yki regulates Tsr.
• Although the authors suggest that SJ proteins are not affected in ykiB5 animals, it seems that many of the tested proteins are downregulated to some extent in Figure S1. Can this be quantified?
• In Figure S5, aPKC protein levels seem higher and even mislocalized to the basal side in ykiB5 mutants. Is this true and could this be a reason for increased apical size in mutant animals? Please measure and explain.
• Cofilin knockdown in HEK293 cells showed that YAP Ser381 phosphorylation is decreased. This site primes YAP for degradation. Therefore, one would expect an increase of total YAP protein upon Cofilin knockdown if Cofilin induces YAP S381 phosphorylation. However, the authors claim that Cofilin stabilizes YAP, which seems to be up in the HEK293 cell experiment. However, this blot suffers from overexposure and no quantifications are presented. I find this data confusing.

Minor points:
• In different parts of the manuscript, YAP was defined as the sole ortholog of Yki. This is incorrect. Those should be reverted to YAP/TAZ.
• Although Mtf in Fig1B is mentioned in the figure legend, mentioning it inside the text would be helpful for readers that are not familiar to the field just like it was done for Figure 3G.

• On page 6, authors wrote "Drosophila embryos, bearing a deletion of the whole yki locus (Huang et al., 2005) (ykiB5 loss-of function allele, henceforth referred to as yki), die at late embryonic stages with elongated tracheal tubes (Fig. 1, A and B) (Huang et al., 2005; Robbins et al., 2014)." However, there is no data in Huang et al., showing that YkiB5 has such phenotype. Second Huang et al., reference should be omitted.
• Supplementary Figure 3: The conclusion drawn from this figure seems to be true but the Yki-overexpressing disc looks like in different developmental stage compared to the control disc expressing only GFP, which has a completely different di-Tyr staining profile. Please make sure that discs match.
• In figure 3D: how many biological replicates (different discs) were measured?

Reviewer #3 (Comments to the Authors (Required)):

In their manuscript entitled "Yorkie controls tube length and apical barrier integrity in the developing Drosophila airways" Papadopoulos and colleagues identify a novel mechanism of Yki action in which Yki binds to the Drosophila cofilin (Twinstar) and thereby regulates the cortical actin network. Additionally, the authors suggest that this binding likewise regulates Yki activity by sequestration at the cortex. The effect of cofilin/Twin star on Yki target genes has been previously described, but the proposed mechanism is novel. In addition, the authors identify a Yki transcriptional target whose expression is critical for making tracheal tubes impermeable to water.

The results section of the manuscript begins by revisiting a previous report that Yki limits tube length through a septate junction- and chitin-independent mechanism. The authors identify a permeability defect in yki mutant tubes that had previously escaped detection. Taking advantage of a data set of Yki binding sites, authors propose δ-aminolevulinate synthase (Alas) may be a relevant Yki target gene for regulation of permeability, and indeed, expression of Alas in a yki mutant background is shown to rescue paracellular barrier function. In addition, the authors suggest that expression of Diap, another yki target gene, only partially rescues yki tube length defects, suggesting the existence of a Diap-independent mechanism. Alas expression is shown not to influence tube length.

To identify additional potential Yki interactors, authors made a tagged Yki construct and carried out Mass Spec analysis of pull downs. This analysis identified cofilin (Twin star) as the most abundant Yki interactor. The interaction was validated by co-IP and in vivo proximity of the two proteins demonstrated by PLA and BiFC.

In tsr mutants, the authors describe a tube length defect very similar to that observed with yki, but do not observe a permeability defect. In yki tsr double mutants a stronger defect than either single mutant is found, arguing for parallel paths. However, Yki expression in a tsr mutant background, and Tsr expression in a yki mutant background were able to rescue tube length defects. Levels of Yki and Tsr protein were mutually dependent, such that reduction in one resulted in reduction of the other, consistent with formation of a complex stabilizing the proteins. Despite this result, Yki transcriptional targets were elevated in tsr mutant embryos, interpreted as resulting from higher levels of nuclear Yki despite the overall decrease in abundance. Turning to HEK293T cells, authors show that knockdown of cofilin results in both lower YAP and lower phospho-YAP, important because Phospho-Yap is restricted to the cytoplasm.

Authors show that elongation defect in the tracheal system is due to increased size of the apical

domain. Authors show that f-actin staining increases in both mutants. Lastly, authors make a mkate-tagged knockin allele of yki and show that Yki appears to be stably anchored at the apiacal cortex as indicated its relative rate of diffusion.

Clearly a lot of work has gone into this manuscript, and the interaction between Tsr and Yki is of particular interest and likely to appeal to a wide audience. The biology and the writing become a bit complex and repetitive towards the end of the manuscript, so the whole would benefit from some careful re-writing.

My concerns include the following:

* paragraphs on p20 and p21 are nearly identical, including a sentence with a typo ("can be partly be"):
p.20: Absence of Yki lowers Tsr levels and, therefore results in increased apical F-actin accumulation (Fig. 12B). Furthermore, absence of Yki prevents transcription of Diap1 and Alas, thus giving rise to longer and water-permeable tubes. The tracheal growth phenotype of yki mutants can be partly be attributed to increased apical F-actin (via reduction of apical Tsr) and partly to the absence of Diap1 transcription, since expression of Diap1 in yki mutants only partially rescues the tube elongation phenotype.

and

p.21: Similarly, absence of Yki lowers Tsr levels and, therefore results in increased apical F-actin accumulation (Fig. 12C). Furthermore, absence of Yki prevents transcription of Diap1 and Alas, thus giving rise to longer and water-permeable tubes. Thus, the tracheal growth phenotype of yki mutants can be partly be attributed to increased apical F-actin (via reduction of apical Tsr) and partly to the absence of Diap1 transcription.

* authors suggest a direct physical interaction between Yki and Tsr, but data are equally consistent with complex formation dependent on bridging protein(s).

* In the discussion, tracheal expression of yki in tsr mutants is said to only partially restore the tube length, but in the results "To determine whether there is any connection between the two pathways to regulate tracheal tube length, we expressed a yki cDNA in the tracheae of tsr mutant embryos. In these embryos tracheal tube length was restored and comparable to that of control embryos (Fig. 6I).

* In describing how actin stabilization in yki and tsr mutants results in tube lengthening, authors cite literature that argues such changes should increase apical membrane domain and do not mention prior work showing p-Moe or tsr loss of function expand the apical domain in tracheal terminal cells (Schottenfeld-Roames et al).

* for the model, it would be helpful to know how authors view Diap function in the context of actin stability. Does it promote actin severing? or regulate Tsr levels?

* authors point out that actin turn over is required during embryonic apical constriction, but this may not be particularly relevant here given that disruption of actomyosin in the trachea does not seem to affect tube length, but rather tube diameter (see paper from Ochoa-Espinosa et al., 2017).

We would like to thank the reviewers for their constructive comments on our manuscript. We could address most of them and thereby improve the manuscript.

Reviewer #1 (Comments to the Authors (Required)):

The transcriptional coactivator Yorkie (Yki) has been previously reported to be important for proper tracheal tube growth (Robbins et al., 2014). In current manuscript, the authors demonstrated that Yki has a dual role in tracheal development to ensure proper tracheal growth and functionality. They found that Yki controls proper tracheal tube elongation by binding Drosophila Twinstar, the orthologue of the vertebrate actin-severing protein Cofilin, to regulate F-actin levels and apical cell membrane size. In addition, Yki controls water-tightness of tracheal tubes by transcriptional regulation of the enzyme δ-aminolevulinate synthase (Alas). Although this is an interesting study, the current version of this manuscript is not sufficient to deduce the authors' conclusion. There are several concerns as indicated below. This study would be of high significance with several additional experiments.

Major concerns

1.In Fig. 1E, the authors observed partial rescue of the tube length phenotype of yki mutants upon tracheal expression of Diap1. What's the phenotype of tracheal expression of loss-of-function Diap1 mutation (thj5c8) or gain-of-function Diap1 mutation (th6b) in Yki mutants?

The complication of the *Diap1* gene is that different mutants that have been used by Robbins et al. (Robbins et al., 2014) show different phenotypes, which result in difficulty to draw robust conclusions on the role of this gene in trachea development. In Robbins et al., the authors mention: “the genetics of *Diap1* mutations is complex, and further complicated by maternal contribution in the embryo”. Therefore, although this is a valid point, we do not have in our hands the necessary reagents to robustly address this question. To understand the function of *Diap1* would require, in our opinion, the generation of a “clean” loss-of-function allele, which falls beyond the scope of the current study.

2.In Fig. 2 A, C and Fig. S1, there are obvious differences in SJs markers (Yurt, FasIII, Mega, Dig, Cont) between embryonic trachea of wild type (WT) and ykiB5 mutants.

We respectfully disagree with the reviewer’s point that “there are obvious differences in SJs markers”. Therefore, we performed again stainings for two core components of the SJ (Cora and Yurt) in WT and *yki* mutant embryos, by including this time a mutation of another component of the SJ protein complex Mtf, (as a positive control). This analysis shows clear mis-localization of Cora and Yurt in *mtf* mutants (Fig. 1 H-H’). More specifically, Cora and Yurt do not remain restricted to the apical part of the lateral membrane in *mtf* mutants (as in the WT and *yki* mutants), but expand along the basolateral membrane. These findings complement our EM analysis and further indicating that Yki does not affect the localization of SJ proteins. We have added a similar sentence into the legend of Fig. 1H-H’).

However, the authors concluded that 'SJs did not appear impaired in Yki mutants'. Once again, in Fig. 2 C, D, the author concluded that 'this phenotype was rescued by the tracheal expression of a Yki cDNA'.
But embryonic trachea of YkiB5 mutants of stage 17 stained for the core septate junction components in Figs. 2 C and D didn't support the conclusion. The author should address these inconsistences.

We apologize for inserting this argument in the wrong position. This has been a writing mistake. We have removed this sentence.

3.In Fig. 4, the authors provided evidence that Yki acts through transcriptional activation of Alas. However, Loss of Alas leads to a decrease in the production of chitin, which may also cause defects in heme synthesis. So, are there any defects in heme production when Yki is lacking?
And what about the level of δ-aminolevulinate in that situation?

This is a valid point raised by the reviewer. However, we could not find a well-established method for testing heme synthesis in *Drosophila*. Nevertheless, we could find published methodology for testing the abundance of δ-aminolevulinic acid, which is one of the upstream components in the heme biosynthetic pathway (Fig. 3 I). We believe that this experiment sufficiently addresses possible defects in the biosynthesis of heme in the context of the *yki* mutation. Our results are summarized in Fig. 3 I. Briefly, we found that the concentration of δ-aminolevulinic acid in *yki* mutants is significantly lower than in the WT and there is no significant difference between *yki* and *Alas* mutants (positive control). We conclude that Yki regulates the synthesis of δ-aminolevulinic acid through transcription of *Alas*, which is the enzyme that catalyzes the production of δ-aminolevulinic acid.

The authors cited ChiP-seq data from Nagaraj et al., 2012 which showed that Yki binds the regulatory region of the Alas gene in Drosophila imaginal discs from. However, beside Alas gene, there were some other genes directly regulated by Yki. Are they also involved in air-filling and tracheal impermeability?

Although we have not performed an exhaustive study on Yki target genes involved in gas-filling, we have found that *Alas* can rescue the gas-filling defects of *yki* mutant embryos as much as it can rescue gas-filling in the *Alas* mutant itself. This indicates that the there is no major involvement of other Yki target genes in gas-filling, as otherwise the *yki* mutation could not be rescued by re-introducing *Alas* alone. Given this lack of preliminary evidence, we sought to characterize the function of *yki* through *Alas*, which has been the scope of this paper.

4.In Figs.6 A, B, C, Yki mutant embryos and Tsr mutant embryos all exhibited over-elongated tracheal tubes. However, Fig. 6 showed the increased Diap1 (Yki target gene which can regulate tube length) mRNA levels in Tsr mutant embryos, and then the authors concluded that Tsr is a negative regulator of Yki transcriptional activity.
The authors should address why Tsr negatively regulates Yki transcriptional activity but their loss-of-function mutants have the same phenotype?

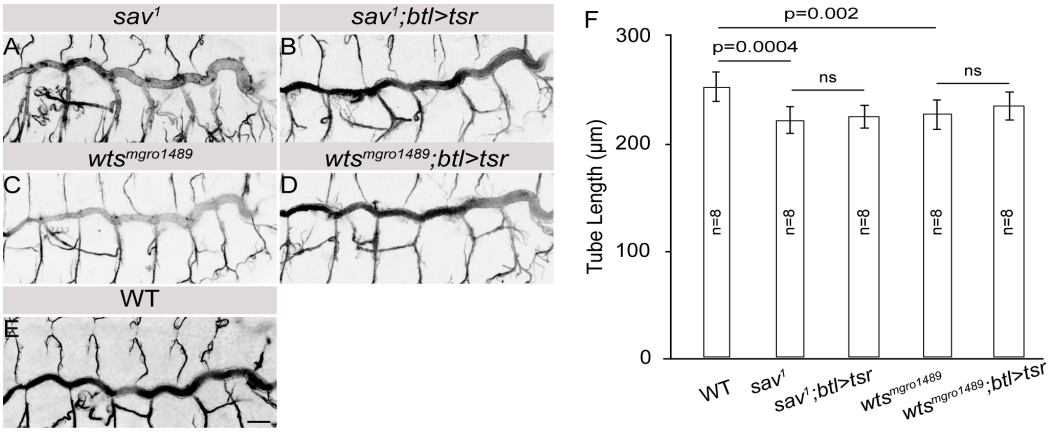
This is a valid point raised by the reviewer. If the tube length phenotype were only dependent on the transcriptional activity of Yki in the nucleus, one should expect that the *tsr* and *yki* mutants (with Tsr being a negative regulator of Yki) would have opposite phenotypes. However, we argue (and our data show) that Tsr additionally regulates Yki's (and Yki regulates Tsr's) protein stability through protein-protein interactions. In the *yki* mutant, Tsr levels are very low (Fig. 5 P-R) and therefore cortical F-actin increases. Similarly, in the *tsr* mutant, Yki is destabilized and cortical F-actin increases. We have visualized these differences by imaging the endogenous Yki protein in WT and *tsr* mutant embryos (Fig. 9 F-H) and also quantified the abundances of apical Yki in these contexts by FCS (Fig. 10 C-J). These experiments argue in favor of additional mechanisms regulating apical membrane size that are not exclusively dependent on Yki's nuclear localization. Therefore, the similarity of the loss-of-function mutant phenotypes between *yki* and *tsr* can be explained by this additional mechanism.

In Fig. S9, the authors found a reduction in total Yap and p-Yap after using cofilin (vertebrate orthologue of Drosophila Tsr) siRNA in HEK293T cell. However, these data could not support that Tsr is a negative regulator of Yki nuclear localization.

As mentioned in the previous point by the same reviewer, we have made two observations. First, Yki and Tsr regulate each other at the protein level and are required for each other's protein stability (see Fig. 5 P-R). Second, Tsr negatively regulates the nuclear localization of Yki. It is well established that the levels of pYAP are a good indicator of YAP/Yki nuclear localization, with low pYAP levels indicating high YAP nuclear abundance (Zhao et al., 2010; Zhao et al., 2007). We believe that this experiment gives us the opportunity to address the dual effect of Tsr/Cofilin on Yki/YAP (i) protein stability and (ii) nuclear localization. Therefore, we replaced the words "negative regulator" by "inhibitor". Please note that Fig. S9 is now Fig. 6D.

5.Robbins groups (Robbins et al., 2014) showed that mutations such as Wats, Mats, and Sav can result in a shorter tracheal tube. The authors should test whether overexpression of Alas or Tsr could suppress the air-filling and tracheal impermeability, or return tracheal tube length to normal levels in one of Wats, Mats, and Sav mutants?

We thank the reviewers for suggesting these experiments. We have attached the results in the Figure below.
Mutations in Hippo pathway components such as *sav* and *wts* cause tube length reductions rather than gas filling defects in embryonic trachea (Fig. A, C, E and F). Therefore, we determined if Tsr acts through the Hippo pathway to regulate Yki in the embryonic trachea. Tsr overexpressed in the *sav* or *wts* mutant background did not modify the tracheal tube length displayed by *sav* or *wts* mutants alone (Fig. B, D and F). These results suggest that Tsr does not act through Hippo pathway to control tracheal tube size.
Fly stocks: the following *Drosophila* stocks were used: sav^{1} kindly provided from Nic Tapon and $wts^{mgro1489}$ kindly provided from Amin Ghabrial.



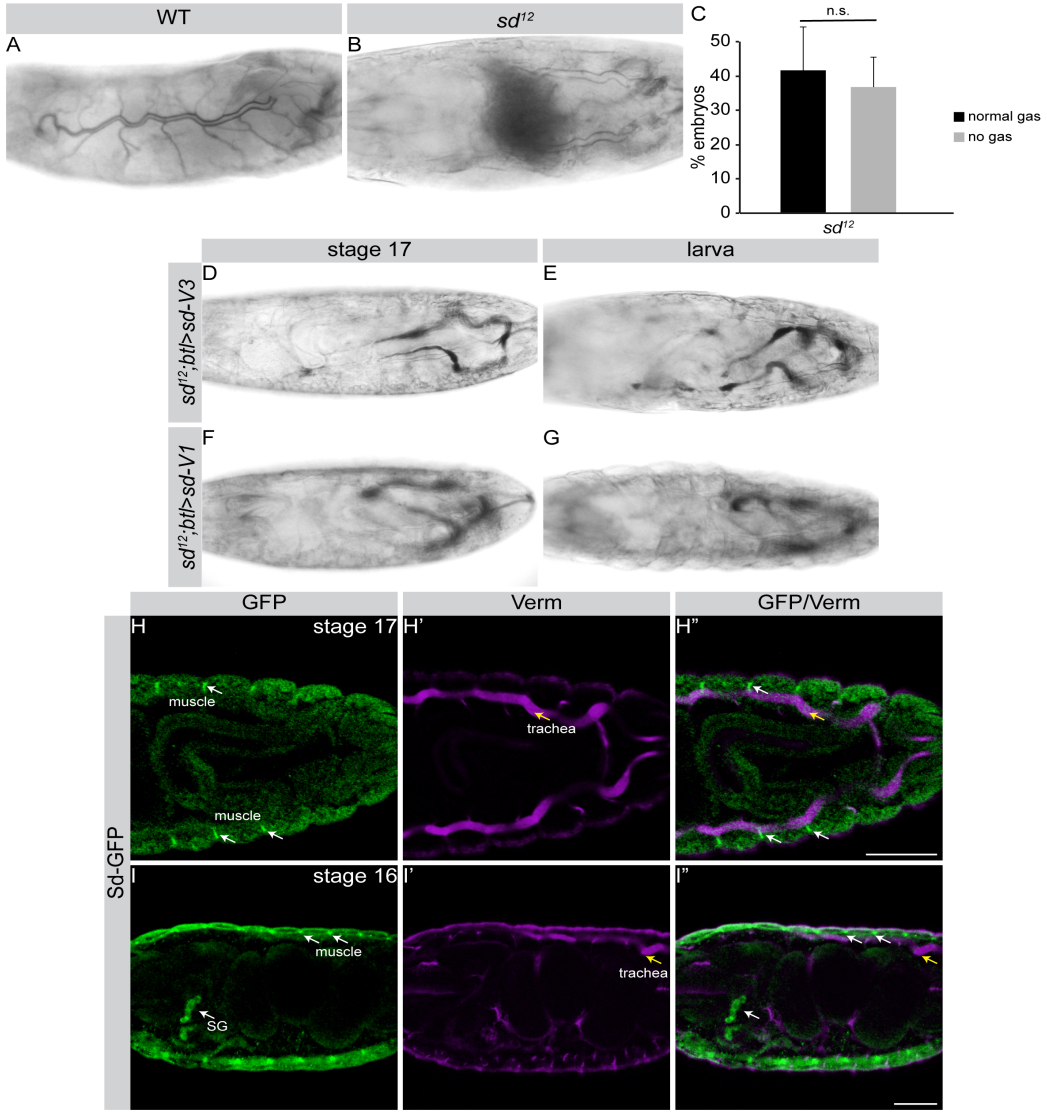
Reviewer #2 (Comments to the Authors (Required)):

In this study by Papadopoulos et al. performed a series of experiments to elucidate the function of the Hippo pathway effector yki in the developing Drosophila respiratory system, the trachea. They showed that Yki has a dual function in the trachea by regulating tube size and membrane permeability. It was previously published that yki mutant animals have elongated trachea through a mechanism involving the Yki target diap1 and the Drosophila effector Caspase, Ice (Robbins et al. 2014). In this study, the authors suggest that the role of Diap1 in this process is partial and they proposed a new mechanism. They claim that Yki interacts with the fly homolog of Cofilin called Twinstar (Tsr), to induce F-actin depolymerization at the apical side of the cells and thereby restricts apical domain size. Removal of Yki results in reduced Tsr accumulation at the apical side and this leads to an increased size of the apical domain, which then results in elongated trachea. Moreover, the authors showed that Yki has another important function for the development of trachea in which Yki induces expression of Alas (δ-Aminolevulinate synthase), encoding an enzyme that is important for the formation of the impermeable barrier. This discovery is novel and provides a unique function of Yki in the developing airways.

Most of the findings of this paper provide interesting insights into the function of Yki in the trachea. The rescue of the DT elongation phenotypes by ectopic expression of one gene in animals with a mutation in another gene clearly shows that Yki and Tsr are somehow connected. However, there are a number of concerns that should be addressed by the authors for this study to be published in JCB.

Major points:
• The authors claim that Yki has cytoplasmic and non-transcriptional functions. To strengthen this argument I suggest to also present the tracheal phenotype of a Sd mutant embryo.

We have attached the results below. *Drosophila* embryos, bearing a 157bp deletion of the *sd* locus (*sd*^{12}), die at late embryonic stages with gas filling defects in the tracheal tubes (see Fig. below, A-C) (Srivastava et al., 2004). The defective gas filling phenotype was not suppressed upon tracheal-specific expression of a *sd* cDNA, which encodes the wild type Sd protein (*sd-V1* and *sd-V3*) (Fig. D-G). Staining in a protein trap line for Sd-GFP in late stage 16 and 17 embryos shows that *sd* is expressed in body muscles and salivary glands rather than in trachea, as indicated by the luminal marker Verm (Fig. H-I"). These results indicate that defects in body muscles can affect the air filling of the tracheal tube, hence the failure to rescue the phenotype by the tracheal expression of Sd.



Fly stocks: The following *Drosophila* stocks were used: UAS-*sd-V1* (BDSC # 9373), UAS-*sd-V3* (BDSC # 9374), *sd*^{12} mutant (BDSC # 9371), *sd*-GFP kindly provided by Alan Spradling.

• The authors made a new genomically tagged Yki protein (mKate-Yki). The authors need to analyze its localization pattern in tsr mutant embryos. This experiment is obviously missing.

We have generated the *mKate2-yki* allele by CRISPR-Cas9 editing, using the PiggyBac transposase approach, in which a 3xP3-dsRed cassette and the mKate2 N-terminal tag were first inserted into the locus, essentially creating a mutant allele with red eye fluorescence as a selectable marker. Subsequently, this line was crossed to a PiggyBac transposase stock to excise seamlessly the 3xP3-dsRed cassette, creating the *mKate2-yki* allele which was bred to homozygosity. This allele was screened for the loss of dsRed and essentially bears no selectable marker. The *tsr* locus physically lies very close to *yki* on the chromosome (a few kilobases apart). With the absence of a selectable marker for *mKate2-yki*, it is very laborious to generate a tsr^{k05633},*mKate2-yki* chromosome (and in fact this can only be screened for by PCR, since the *mKate2-yki* allele is both homozygous viable, precluding non-complementation screening, and the Yki fluorescence is very weak to be used as a selection marker). Our experience with generating the double tsr^{k05633},yki^{B5} mutant (in which case both alleles contained a mini-white marker and it was feasible) was that we had to screen thousands of adult flies (~12,000) to find a single fly that bore both mutant alleles. This means that generating this reagent would be a great challenge.
To overcome this problem we have used the *tsr*,*yki* double mutant in combination with a *yki-GFP* rescuing BAC on the 3rd chromosome. We performed the requested experiments and our results are summarized in Fig. 10. We have tested both the localization of Yki-GFP in the *tsr* mutant (compared to the control) and performed FCS experiments to probe the concentration and mobility of Yki in the presence and absence of a *tsr* mutation. We found that, while Yki localizes apically also in the rescuing allele, in the *tsr* mutant it is expressed at lower levels and dispersed all over the trachea cells. This result is in line with our blot experiments showing reduction of Yki in the *tsr* mutant. We have precisely quantified this reduction and loss of Yki enrichment apically by FCS experiments in the *tsr,yki*;*yki-GFP* line. Our results are summarized in Fig. 10.

• What is the expression of diap1-lacz in Yki overexpressing embryos and in yki mutant embryos? What about other Yki target genes such as Myc and ex?

We performed the requested experiments and included them in Fig. 7 C-E. We observed that in *yki* overexpressing embryos, *Diap1* transcription is increased, whereas in *yki* mutants, transcription is decreased, as shown in Fig. 7 C-D. In addition, we performed qRT-PCR experiments for the following genotypes: WT, yki^{B5} mutant and *btl>yki* (*yki* overexpressing) testing the expression of the suggested genes, such as *ex*, *Myc* and *Diap1*. We found that in *yki* overexpressing embryos *Myc* and *Diap1* transcription is significantly increased whereas *ex* doesn't show any major difference to the WT. In *yki* mutant embryos, transcription of all three target genes is decreased (Fig. 7 E).

• Shorter exposures of Western blots should be used. In supplementary Fig 8A, and 8B,

it is impossible to get to a conclusion with such overexposed images. In Fig. S8A, there is no loading control. In Figure S8B, the loading control seems strongly overexposed. The same is true for Supplementary Fig. 9.

We have performed new experiments with shorter exposures of Western Blots, as well as loading control. Please note that Fig. S8 A is now Fig. 6 A; Fig. S8 B is now Fig. 6 B; and Fig. S 9 is now Fig. 6 D.

• On page 7: "This phenotype was rescued by the tracheal expression of a yki cDNA (Fig. 2, C, D)." However, there is no EM data for trachea-specific Yki cDNA expression in ykiB5 mutant animals showing the rescue.

We apologize for inserting this sentence in the text. There has been no reason for us to do an EM study on the rescuing genotype, since there was no abnormal septa phenotype in the EM experiment of the *yki* mutant, compared to WT. This has been a writing mistake.

• In figure 4H, the Alas mRNA downregulation is not convincing. If possible Alas protein should be monitored to test whether Yki-dependent Alas regulation is the main reason for permeability defects in Yki mutant embryos. Also, in the figure legend it is not clear whether the mRNA comes from embryos or trachea.

Unfortunately, there is no anti-Alas antibody available for us to be able to probe the abundance of Alas protein in the *yki* mutant. We acknowledge that there is a small decrease of the *Alas* mRNA in the yki^{B5} mutant, although this difference is statistically significant among biological replicates. Both *yki* and *Alas* mRNAs are maternally deposited in the egg, which indicates that transcription of *Alas* will not be diminished in either of the mutants. Moreover, as stated in the Methods, the RTqPCR experiments were preformed using whole embryo mRNA and cDNA, which essentially reflects the total *Alas* and *yki* transcripts. Also, the dependence of permeability defects of *yki* mutants on Alas is best shown by the similar efficiency of rescue of the *yki* mutant by both the *yki* and the *alas* cDNAs in the trachea. The same is true for the gas-filling phenotype and the abundance of DiTyr crosslinking.

• The authors suggest that removal of Yki causes reduction in Tsr protein levels and vice versa. In Figure 6Q-R, there is no statistical measurement showing that presence of one protein affects the presence of second protein. Just looking at the blot, I do not think that Yki affects Tsr protein levels. The authors list n=2 in the legend, but where these biological replicates? Two sample points is not enough for robust statistics and it may be that the error bars in 6Q-R are derived from re-imaging or re-analyzing the same blot/exposure. Please clarify and perform biological replicates if this is an important argument to be made. As is, the presented data do not support the claim that Yki regulates Tsr.

We performed three biological replicates using protein extracts from wild-type, yki^{B5} and tsr^{k05633} embryos.
Fig. 6 is now Fig. 5 in which we have included Western Blots (Fig. 5 P), as well as Yki and Tsr protein quantifications with statistics from three replicates (Fig. 5 Q-R).

• Although the authors suggest that SJ proteins are not affected in ykiB5 animals, it seems that many of the tested proteins are downregulated to some extent in Figure S1. Can this be quantified?

We performed quantifications in *yki*^{B5} mutant tracheal tube for each of the SJ proteins in which we observe no significant difference, compared to wild type embryos. The plot with the quantification is included in Fig. S2 E.

• In Figure S5, aPKC protein levels seem higher and even mislocalized to the basal side in ykiB5 mutants. Is this true and could this be a reason for increased apical size in mutant animals? Please measure and explain.

We have collected more data and performed quantifications, which are presented in Fig S4 E-F. We have not observed any significant difference between wild type and *yki*^{B5} mutants in aPKC intensity or localization.

• Cofilin knockdown in HEK293 cells showed that YAP Ser381 phosphorylation is decreased. This site primes YAP for degradation. Therefore, one would expect an increase of total YAP protein upon Cofilin knockdown if Cofilin induces YAP S381 phosphorylation. However, the authors claim that Cofilin stabilizes YAP, which seems to be up in the HEK293 cell experiment. However, this blot suffers from overexposure and no quantifications are presented. I find this data confusing.

First, we have repeated the blot and improved the quality of our images (Fig. 6 D).
Second, Zhao et.al (Zhao et al., 2010) have shown that S381 is required but not sufficient for YAP degradation by showing that: "*Mutation of S127 to alanine did not stabilize YAP. Although mutation of S381 to alanine alone did not substantially stabilize YAP, when S381 was mutated in the S127A mutant background, it dramatically stabilized."* Therefore, our results are not conflicting the results of Zhao et al., because we do not know how much of the Phos-S127-YAP is also Phos-S381 positive and vice versa. All that can be safely concluded from these experiments is: i) that there is a decrease in total YAP; and ii) YAP is more nuclear (based on the Phos-S127 and Phos-S381 levels) in the Cofilin knockdown.

Minor points:
• In different parts of the manuscript, YAP was defined as the sole ortholog of Yki. This is incorrect. Those should be reverted to YAP/TAZ.

We have corrected the text according to the reviewer's suggestion.

• Although Mtf in Fig1B is mentioned in the figure legend, mentioning it inside the text would be helpful for readers that are not familiar to the field just like it was done for Figure 3G.

We followed reviewer's suggestion and corrected the text.

• On page 6, authors wrote "Drosophila embryos, bearing a deletion of the whole yki

locus (Huang et al., 2005) (ykiB5 loss-of function allele, henceforth referred to as yki), die at late embryonic stages with elongated tracheal tubes (Fig. 1, A and B) (Huang et al., 2005; Robbins et al., 2014)." However, there is no data in Huang et al., showing that YkiB5 has such phenotype. Second Huang et al., reference should be omitted.

We prefer to keep the reference of Huang et al., since the *yki*^{B5} loss of function allele was first described in this work.

• Supplementary Figure 3: The conclusion drawn from this figure seems to be true but the Yki-overexpressing disc looks like in different developmental stage compared to the control disc expressing only GFP, which has a completely different di-Tyr staining profile. Please make sure that discs match.

We thank the reviewer for pointing this out. We included in the figure a Yki-overexpressing disc of a similar stage.

• In figure 3D: how many biological replicates (different discs) were measured?

We apologize for having missed this information. We have now included the biological replicates in the Figure S3 D.

Reviewer #3 (Comments to the Authors (Required)):

In their manuscript entitled "Yorkie controls tube length and apical barrier integrity in the developing Drosophila airways" Papadopoulos and colleagues identify a novel mechanism of Yki action in which Yki binds to the Drosophila cofilin (Twinstar) and thereby regulates the cortical actin network. Additionally, the authors suggest that this binding likewise regulates Yki activity by sequestration at the cortex. The effect of cofilin/Twin star on Yki target genes has been previously described, but the proposed mechanism is novel. In addition, the authors identify a Yki transcriptional target whose expression is critical for making tracheal tubes impermeable to water.

The results section of the manuscript begins by revisiting a previous report that Yki limits tube length through a septate junction- and chitin-independent mechanism. The authors identify a permeability defect in yki mutant tubes that had previously escaped detection. Taking advantage of a data set of Yki binding sites, authors propose δ-aminolevulinate synthase (Alas) may be a relevant Yki target gene for regulation of permeability, and indeed, expression of Alas in a yki mutant background is shown to rescue paracellular barrier function. In addition, the authors suggest that expression of Diap, another yki target gene, only partially rescues yki tube length defects, suggesting the existence of a Diap-independent mechanism. Alas expression is shown not to influence tube length.

To identify additional potential Yki interactors, authors made a tagged Yki construct and carried out Mass Spec analysis of pull downs. This analysis identified cofilin (Twin star) as the most abundant Yki interactor. The interaction was validated by co-IP and in vivo proximity of the two proteins demonstrated by PLA and BiFC.

In tsr mutants, the authors describe a tube length defect very similar to that observed with yki, but do not observe a permeability defect. In yki tsr double mutants a stronger

defect than either single mutant is found, arguing for parallel paths. However, Yki expression in a tsr mutant background, and Tsr expression in a yki mutant background were able to rescue tube length defects. Levels of Yki and Tsr protein were mutually dependent, such that reduction in one resulted in reduction of the other, consistent with formation of a complex stabilizing the proteins. Despite this result, Yki transcriptional targets were elevated in tsr mutant embryos, interpreted as resulting from higher levels of nuclear Yki despite the overall decrease in abundance. Turning to HEK293T cells, authors show that knockdown of cofilin results in both lower YAP and lower phospho-YAP, important because Phospho-Yap is restricted to the cytoplasm.

Authors show that elongation defect in the tracheal system is due to increased size of the apical domain. Authors show that f-actin staining increases in both mutants. Lastly, authors make a mkate-tagged knockin allele of yki and show that Yki appears to be stably anchored at the apiacal cortex as indicated its relative rate of diffusion.

Clearly a lot of work has gone into this manuscript, and the interaction between Tsr and Yki is of particular interest and likely to appeal to a wide audience. The biology and the writing become a bit complex and repetitive towards the end of the manuscript, so the whole would benefit from some careful re-writing.

My concerns include the following:

** paragraphs on p20 and p21 are nearly identical, including a sentence with a typo ("can be partly be"): p.20: Absence of Yki lowers Tsr levels and, therefore results in increased apical F-actin accumulation (Fig. 12B). Furthermore, absence of Yki prevents transcription of Diap1 and Alas, thus giving rise to longer and water-permeable tubes. The tracheal growth phenotype of yki mutants can be partly be attributed to increased apical F-actin (via reduction of apical Tsr) and partly to the absence of Diap1 transcription, since expression of Diap1 in yki mutants only partially rescues the tube elongation phenotype.*

and

p.21: Similarly, absence of Yki lowers Tsr levels and, therefore results in increased apical F-actin accumulation (Fig. 12C). Furthermore, absence of Yki prevents transcription of Diap1 and Alas, thus giving rise to longer and water-permeable tubes. Thus, the tracheal growth phenotype of yki mutants can be partly be attributed to increased apical F-actin (via reduction of apical Tsr) and partly to the absence of Diap1 transcription.

We apologize for inserting an identical sentence. This has been a writing mistake. We have removed this sentence.

** authors suggest a direct physical interaction between Yki and Tsr, but data are equally consistent with complex formation dependent on bridging protein(s).*

The reviewer is right that the interaction between Yki and Tsr can be indirect. However, we respectfully disagree with reviewer that we have made implications in the text that the interaction is direct. In fact, there is no instance in our manuscript where we refer to the Tsr-Yki interaction as being direct. We

have now explicitly stated this in the text: “To further confirm that Yki and Tsr are found in the same complex, we examined their interaction by several methods in embryonic tracheae and wing imaginal discs. Overexpressed *Yki-V5* co-precipitated Tsr from protein lysates of embryonic tracheae (Fig. 4 A). Further, in-situ Proximity Ligation Assay (PLA) (Soderberg et al., 2006) (Fig. 4 B-C”) and Bimolecular Fluorescence Complementation (BiFC) (Hu et al., 2002) (Fig. 4 D-D”) both confirmed that the two proteins, when expressed in wing imaginal discs, were found in close proximity. From these results we concluded that Yki and Tsr physically interact (directly or indirectly) to form a protein complex.”

** In the discussion, tracheal expression of yki in tsr mutants is said to only partially restore the tube length, but in the results "To determine whether there is any connection between the two pathways to regulate tracheal tube length, we expressed a yki cDNA in the tracheae of tsr mutant embryos. In these embryos tracheal tube length was restored and comparable to that of control embryos (Fig. 6I).*

We corrected the text in the discussion as follows: *“tracheal expression of yki in tsr mutants restores the tube length”*.

** In describing how actin stabilization in yki and tsr mutants results in tube lengthening, authors cite literature that argues such changes should increase apical membrane domain and do not mention prior work showing p-Moe or tsr loss of function expand the apical domain in tracheal terminal cells (Schottenfeld-Roames et al).*

This is a very good point brought up by the reviewer. We have integrated the following sentence in the discussion: *“Additionally, studies of the role of apical F-actin in seamless tracheal tubes have revealed that, upon depletion of Tsr/Cofilin, abnormal apical cysts form in the tracheal cells. This has been attributed to increased F-actin stability, resulting in apical domain growth (Schottenfeld-Roames et al., 2014).”*

** for the model, it would be helpful to know how authors view Diap function in the context of actin stability. Does it promote actin severing? or regulate Tsr levels?*

We respectfully disagree with the suggestion of the reviewer. We have not performed any experiments of F-actin visualization in the context of the *Diap1* mutant, as *Diap1* has not been the main focus of this manuscript. Therefore, we cannot conclude whether, and, if so, how *Diap1* protein may impact actin polymerization. Therefore, have decided not to include this point in our model.

** authors point out that actin turn over is required during embryonic apical constriction, but this may not be particularly relevant here given that disruption of actomyosin in the trachea does not seem to affect tube length, but rather tube diameter (see paper from Ochoa-Espinosa et al., 2017).*

In the referred paper the authors investigate the dorsal branches of the trachea, which are elongated by cell intercalation. The cell intercalation in tracheal branches is mediated by enhanced trafficking of adhesion molecules regulated by the transcription factor Spalt (Shaye et al., 2008). Failure in cell intercalation

results in short and thick tracheal branches. In contrast, the dorsal trunk lacks this mechanism. Tube elongation in the dorsal trunk doesn't depend on cell movements, rather on cell surface rearrangements, including cytoskeletal changes (e.g. via the formin dDAAM), which direct growth along the longitudinal axis (Nelson et al., 2012) and ECM modifications (secreted Verm, Serp). Therefore, we do not expect the same phenotypes upon impairment of actomyosin in these two types of tracheal tubes. In fact, several authors have shown that cell migration in the trachea is MyoII-independent and actin polymerization-based (Matsubayashi et al., 2011; Serra-Picamal et al., 2012).

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May 24, 2019

RE: JCB Manuscript #201809121R

Dr. Kassiani Skouloudaki
Max-Planck Institute of Molecular Cell Biology and Genetics
Pfotenhauerstrasse 108
Dresden 01307
Germany

Dear Dr. Skouloudaki,

Thank you for submitting your revised manuscript entitled "Yorkie controls tube length and apical barrier integrity in the developing Drosophila airways". We and the returning reviewers find that your significant revisions adequately address the key points raised in the reviews. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: perhaps a shorter version could be considered, since the species will be made clear to the reader in the summary statement or abstract that immediately follow the title?
Yorkie controls tube length and apical barrier integrity in airway development

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
Suggested eTOC:
Skouloudaki et al identify an alternative role of the transcriptional co-activator Yorkie (Yki) in controlling water impermeability and tube size of the developing Drosophila airways. Tracheal impermeability is triggered by Yki-mediated transcriptional regulation of the δ-aminolevulinate synthase (Alas), whereas tube elongation is controlled by binding of Yki to the actin severing factor Twinstar.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to S1A-B'
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 6BD

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- Please abbreviate the names of journals according to PubMed.

7) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.

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9) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

Ian Macara, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

All of my comments/queries have been addressed well.

Reviewer #2 (Comments to the Authors (Required)):

accept