Hydroxylated sphingolipid biosynthesis regulates photoreceptor apical domain morphogenesis

Sarita Hebbar, Kai Schuhmann, Andrej Shevchenko, and Elisabeth Knust

Corresponding Author(s): Sarita Hebbar, Max-Planck-Institute of Molecular Cell Biology and Genetics; Elisabeth Knust, Max Planck Institute of Molecular Cell Biology and Genetics; and Sarita Hebbar, Max-Planck-Institute of Molecular Cell Biology and Genetics

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January 3, 2020

Re: JCB manuscript #201911100

Dr. Sarita Hebbar
Max-Planck-Institute of Molecular Cell Biology and Genetics
Pfotenhauerstr. 108
Dresden 01307
Germany

Dear Sarita -

We have now received three external reviews of your manuscript on "A role for hydroxylated sphingolipids in apical domain morphogenesis of Drosophila photoreceptors" from experts in the field. As you will see from their appended comments, they all found the work to be interesting and potentially important. However, they all raised issues with some of the experiments and, crucially, two of the reviewers felt that your model for the mechanism by which reduced Crb results in defective rhabdomeric structures is not convincingly supported by your data. Unfortunately, therefore, we are unable to accept the manuscript for publication in its present form. However, given the interest in this area, we would be willing to consider a suitably revised version that addresses the comments of each of the reviewers. We appreciate that this will involve additional experimental work, and you may elect to instead transfer the manuscript elsewhere.

We believe that the criticisms of all three reviewers have merit, and need to be addressed in a point-by-point rebuttal and in modifications to the manuscript. In particular, however, there are problems with the proposed model that need to be tackled. For example, there seem to be no data actually demonstrating that the amount of fa2h affects Rab11-dependent trafficking to the rhabdomeres; or that reducing inducing oxidative stress independently of Crb loss induces fa2h expression. Moreover, as reviewer #3 points out, "The model in 6B refutes their data. If Crb normally blocks oxidative stress and oxidative stress normally blocks fa2h synthesis, then reducing Crb function would increase oxidative stress, which would further block fa2h synthesis. This is not what was observed - fa2h levels went up in the crb mutants." There are also a number of technical problems with some of the data, listed in the reviewer comments.

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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 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

Andrea L. Marat, Ph.D.
Scientific Editor
Journal of Cell Biology

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

In this study, Hebbar et al. have deciphered a molecular mechanism leading to PRC morphogenesis defects in crb mutant eyes. First, they confirmed that Crb prevents oxidative stress in PRC. They further showed that crb mutations induce fa2h expression, thereby increasing the abundance of hydroxylated sphingolipids. In addition, they provided evidence suggesting that increased fa2h expression results in impaired Rh1 trafficking, likely explaining how Fa2H impacts on rhabdomere morphogenesis. The data presented in the paper are of high quality, and most results are convincing. This study provides significant insights into the morphogenesis of the specialized apical membrane of PRC, and may help understanding retinal pathologies associated with CRB1/2 mutations. In a broader perspective, this work could influence the study of epithelial cell polarity in general, as Fa2h could influence apical trafficking of proteins other than Rh1. In addition, their

findings emphasize the crucial role of lipids, which is often overlooked, in cell polarization.

Prior to publication, a few concerns need to be addressed:

1. Four different crb alleles are used in Fig. 1A and B. However, one or two allele(s) is/are used in Fig. 1D-I. Is it because the other alleles were no tested, or because the missing alleles (e.g. crb4 in D, F; crb11A22 in E) had no impact on the investigated phenotypes? This needs to be clarified. The authors would need to provide an explanation if some alleles showing rhabdomere morphogenesis defects have no impact on fa2h expression and/or oxidative stress.

2. Although the genetic interaction between fa2h and crb8F105 is clear, the data showing the genetic interaction between fa2h and crb11A22 are not convincing. A statistical analysis is required to support Table 1.

3. The authors make a connection between the oxidative stress resulting from the loss of Crb and the expression of fa2h. However, other Crb-dependent signalling events could normally restrict fa2h expression. To clarify this issue, the authors should reduce oxidative stress in crb mutant PRC and look at fa2h expression levels, and test the impact of alternative way(s) to increase oxidative stress in the eye on fa2h expression.

4. Data presented in the paper show that fa2h overexpression impairs Rh1 trafficking. However, fa2h overexpression had no impact on rhabdomere morphogenesis, in contrast to Rh1 deficiency. What is the impact of fa2h overexpression on Rh1 distribution and levels at steady state? Is the impact on Rh1 really explains the role of Fa2h?

5. It is worth showing representative images of the % of overlap of Rh1 and Rab11.

6. The authors concluded that Rh1 trafficking is altered in the absence of Crb owing to increased fa2h expression. To support this conclusion (and their model), they should try to rescue Rh1 trafficking defects in crb mutant PRC by knocking-down fa2h.

7. It could be interesting to discuss how hydroxylated sphingolipids could impact Rab11-dependent trafficking.

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

In this manuscript, authors used Drosophila photoreceptors as a model system to investigate the relationship between the apical membrane expansion and lipid metabolism. Late pupal photoreceptor massively expands their apical membrane, the rhabdomere. Many proteins involving this process have been identified, but little is known for the contribution of lipids and their metabolism.
Authors performed an unbiased lipidomic screening using mutant alleles of crb with altered rhabdomeric structure to elucidate the link between lipids and apical plasma membrane differentiation and growth, and found hydroxylated sphingolipids has increased about 2-fold in crb mutants with altered rhabdomeric structure. Authors also found crb mutants with altered rhabdomeric structure express fa2h at the level for 5-6 times more than the wild type. Authors claimed Fa2h overexpression impaired post-Golgi trafficking of Rh1 and concluded hydroxylated sphingolipids regulate apically-directed delivery of Rhodopsin and hence in the growth of the

rhabdomeres.
It is timely and very important to investigate the contribution of lipid and their metabolism for apical membrane expansion. Increasements of hydroxylated sphingolipids and fa2h expression level are quite interesting, but the connection between the change of lipid metabolism and Rh1 transport is not clearly supported by the data presented here.
Below I list the major points.

Figure 1:
These data give the readers the good understanding of the background of this study, however, Figure 1 does not include any new findings, as these results have already published elsewhere. Author said in lane143: In the second half of pupal development, crb is required for the extension of the rhabdomeres along the proximo-distal axis.
However, the failure of the extension of the rhabdomeres along the proximo-distal axis is likely caused by the breakup of the ZA in crb mutants (Tepass and Harris, 2007 Trends in Cell Biology). Crb is not directly required for extension of the rhabdomeres along the proximo-distal axis in the second half of pupal development.

Figure 2:
All of data presented here are quite new and very interesting.
It is very interesting finding that a 5.9-fold increase in fa2h transcripts at pupal stages (60-80h APF) of crb8F105 animals (Fig. 2E). Indeed, this period coincides with massive rhabdomere growth, as authors described. However, in crb mutants, rhabdomeres grow well (more than the wild type) in distal half of photoreceptors. Thus, the increasement of fa2h transcripts is hard to be explained by the failure of rhabdomere growth in crb mutant.

Figure 3:
3I) Apical membrane extension to the retinal floor occurs around 35-45% pupal development (pd), and the photoreceptor extension along the proximo-distal axis occurs from 50%pd to adult stage. Rh1Gal4 induces the expression from 70%pd and to get the effect of RNAi need the time. Thus, the strong effect of Rh1Gal4-drived Fa2h RNAi for rhabdomere elongation is amazing and surprising. Does Fa2h express in the pigment cells too? If Fa2h expresses mainly in the photoreceptors, please check Fa2h mRNA reduction by qPCR. If Fa2h expresses both in the photoreceptors and pigment cells, please check Fa2h mRNA reduction by in situ hybridization: pigment cell would give a good control. Or please use mosaic retinas.
Figure 4:
I wonder why Rh1 does not localize in the rhabdomeres. Whole mount staining sometimes gives this penetration problem and it is sometimes hard to detect Rh1 in the rhadbomeres, however, the authors used cryo-sections. Please use better experimental conditions to show Rh1 staining in the rhabdomeres.

Figure 5:
Again, there is no Rh1 staining within the rhabdomeres in the wild type cells at 3 hours after BLICS. It is difficult to judge whether Rh1 transport is affected or not affected by these staining data. Please use better experimental conditions to show Rh1 staining in the rhabdomeres. Moreover, please use mosaic eyes for BLICS experiments: because BLICS is sometimes difficult to start Rh1 transport well even in the wild type flies, probably because it is difficult to control how much all-trans-retinal the flies eat or how much 11-cis retinal is formed by blue light. Mosaic retinas containing both the wild type cells and mutant cells (or cells expressing proteins) would help better judgement (Pocha et al., JCB 2011 is the good example).

Which step of Rh1 transport is inhibited by overexpression of fa2h? It is not clear from the pictures in current Figures (there is no Rab11 staining). If post-Golgi trafficking is impaired, Rh1 will be massively accumulated in the cytoplasmic vesicles (see Otsuka et al., JCS 2019). If this is the case, please show EM pictures of crb mutant photoreceptors overexpressing fa2h.

There is no figures of Rab11 staining. How we can judge the colocalization of Rab11 and Rh1 and where do they colocalize? Please show the original pictures for Rh1 and Rab11 staining.

Figure 6,
Figure 5 might be able to include this model.

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

Review

In this paper, the authors explore the contributions of lipidomics to the defects in rhabdomere structure known to be associated with certain alleles of crumbs (crb).
Their lipid profiling revealed an increase in levels of the hydroxylated sphinglolipid ox-CerPE with only the crb mutant alleles associated with aberrant rhabdomere morphologies. They link these changes to increases in expression of the gene encoding Fatty acid-2 hydroxylase (fa2h), an enzyme in the pathway to Ox-CerPE synthesis, and show that knock down of fa2h can partially rescue the rhabdomere defects in certain crb mutants. Since the work of others suggests that fa2h expression is induced by oxidative stress and crb mutant rhabdomeres undergo oxidative stress, they examined levels and localization of the stress marker - gstD1 - in crb mutants and saw expected changes with the crb alleles associated with aberrant rhabdomere morphologies. Finally, the authors explored a potential link between the changes in lipids with trafficking of a major rhabdomere protein - Rhodopsin 1 - to the apical surface membrane, potentially explaining how changes in the lipid profile is linked to the altered rhabdomere morphologies.

Specific findings in the paper:

1. Rhabdomeres fail to fully extend their apical domains along the PD axis in three of four loss-of-function crb alleles examined. Levels of the hydroxylated sphingolipid ox-CerPE go up in the crb alleles affecting PD apical domain elongation. They show that it is the long chain fatty acid versions that are affected by loss of crb. These data are strong - although it would be helpful to know the molecular lesions associated with each of the crb alleles they examine.
2. RNA levels for fa2h, which encodes fatty acid 2 hydroxylase, an enzyme in the pathway leading to ox-CerPE production, and gstD1, a marker for oxidative stress, are higher in crb alleles that affect PD elongation of rhabdomeres. A GFP reporter for gstD1 is observed in retinal cells of crb mutants with affected rhabdomeres. The GFP reporter data are not convincing.
3. Altering levels of fa2h by overexpression or RNAi can alter crb rhabdomere phenotypes with reasonable rescue of one allele with RNAi knockdown of fa2h. Quantification of phenotypes is provided in Table 1. These data are convincing.
4. Rhabdomere expansion is known to be linked to the amount of Rhodopsin making it to the apical surface. So the authors show that they can affect rhabdomere structure by limiting carotenoids in diet (which limits maturation and trafficking of Rhodopsin) and they show that the defects caused by this treatment are exacerbated in a crb mutant. These data are convincing.
5. The authors show that too much fa2h limits recovery of rhabdomeres following provision of the

cis retinal required to make rhodopsin. These data are convincing.
6. The authors suggest that the amount of fa2h affects Rab11 dependent Rh1 trafficking to the rhabdomeres. We don't see this data (other than quantification) in the core figures in the paper. Thus, I am not convinced by these data.

Issues:

Authors have shown that in crb mutant eyes, a marker of oxidative stress goes up based on quantification of transcripts and with some less convincing immunostaining of crb mutant eye sections stained with a GFP tagged version of this marker. They have also shown that mRNA levels of the gene encoding an enzyme involved in production of ox-Cer (fa2h) go up as do levels of ox-CERPE. From this they conclude that reducing Crb increases oxidative stress, which in turn induces expression of the FA synthase gene. Although they show that two things change in crb mutants, they have not demonstrated that one change causes the other. It could be the other way around or the changes could be independent. If they are going to push this idea, they should show that induction of oxidative stress (independent of loss of crb) can induce increased expression of fa2h.

The model in 6B refutes their data. If Crb normally blocks oxidative stress and oxidative stress normally blocks fa2h synthesis, then reducing Crb function would increase oxidative stress, which would further block fa2h synthesis. This is not what was observed - fa2h levels went up in the crb mutants. As suggested above, the other problem with this model is that they provide no evidence that oxidative stress is affecting fa2h levels, only that crb affects levels of both an oxidative stress marker and of fa2h.

I would like to see what they were quantifying regarding vesicles with the staining of both Rab11 and Rh1 (Figure 5E). Where is the raw data or a sample image one could use to judge how easily this quantification was done?

Also, I am assuming that since the authors are looking at pupal and adult eyes that the alleles of crb they are examining are not lethal alleles. So, what kind of alleles are they? What is the molecular lesion in each allele and how might that be linked to the variability in rhabdomere defects?

The gstD-GFP staining is not so convincing given how abnormal these eyes are (Figure 2 H and I). How do the investigators know where the retinal cells are relative to the pigment cells? It would be helpful to know this. Are there other independent markers that could be used? This should be resolved in a convincing manner.

Responses to Editor's and Reviewers' comments

Editorial comment1: For example, there seem to be no data actually demonstrating that the amount of fa2h affects Rab11-dependent trafficking to the rhabdomeres.

Authors' response: See response to reviewer 1 comment 5 on page 3

Editorial comment 2: or that reducing inducing oxidative stress independently of Crb loss induces fa2h expression.

Authors' response: See response to reviewer 1 comment 3 on page 2

Editorial comment 3: Moreover, as reviewer #3 points out, "The model in 6B refutes their data. If Crb normally blocks oxidative stress and oxidative stress normally blocks fa2h synthesis, then reducing Crb function would increase oxidative stress, which would further block fa2h synthesis. This is not what was observed - fa2h levels went up in the crb mutants."

Authors' response: See response to reviewer 3 comment 7 on page 11

There are also a number of technical problems with some of the data, listed in the reviewer comments.

Authors' response: Please read the point-by-point rebuttal below.

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

In this study, Hebbar et al. have deciphered a molecular mechanism leading to PRC morphogenesis defects in crb mutant eyes. First, they confirmed that Crb prevents oxidative stress in PRC. They further showed that crb mutations induce fa2h expression, thereby increasing the abundance of hydroxylated sphingolipids. In addition, they provided evidence suggesting that increased fa2h expression results in impaired Rh1 trafficking, likely explaining how Fa2H impacts on rhabdomere morphogenesis. The data presented in the paper are of high quality, and most results are convincing. This study provides significant insights into the morphogenesis of the specialized apical membrane of PRC, and may help understanding retinal pathologies associated with CRB1/2 mutations. In a broader perspective, this work could influence the study of epithelial cell polarity in general, as Fa2h could influence apical trafficking of proteins other than Rh1. In addition, their findings emphasize the crucial role of lipids, which is often overlooked, in cell polarization. Prior to publication, a few concerns need to be addressed:

1. Four different crb alleles are used in Fig. 1A and B. However, one or two allele(s) is/are used in Fig. 1D-I. Is it because the other alleles were no tested, or because the missing alleles (e.g. crb4 in D, F; crb11A22 in E) had no impact on the investigated phenotypes? This needs to be clarified. The authors would need to

provide an explanation if some alleles showing rhabdomere morphogenesis defects have no impact on fa2h expression and/or oxidative stress.

Authors' response: As stated in the results, the altered rhabdomere shape and size is associated with increased *fa2h* levels and altered oxidative stress signalling. As suggested by the reviewer we have included data on the missing alleles (e.g. crb^{4} in Fig. 2D, F; crb^{11A22} in Fig. 2E). See Figure 2.

2. Although the genetic interaction between fa2h and crb8F105 is clear, the data showing the genetic interaction between fa2h and crb11A22 are not convincing. A statistical analysis is required to support Table 1.

Authors' response: We have now provided a statistical analyses of the genetic interaction in Fig. 4N. We show that reducing *fa2h* by RNAi in both alleles (*Rh>fa2h RNAi & *crb^{8F015} and *Rh>fa2h RNAi & *crb^{11A22}) results in significantly increased abundance of longer rhabdomeres as compared to the mutants alone (crb^{8F015} and crb^{11A22}). See Fig. 4N. The converse relation is observed with *fa2h* overexpression in the crb^{8F015} background. However, the data shows that overexpression of *fa2h* does not worsen the crb^{11A22} phenotype any further. This is in agreement with earlier data showing that crb^{11A22} is a protein null allele.

3. The authors make a connection between the oxidative stress resulting from the loss of Crb and the expression of fa2h. However, other Crb-dependent signalling events could normally restrict fa2h expression. To clarify this issue, the authors should reduce oxidative stress in crb mutant PRC and look at fa2h expression levels, and test the impact of alternative way(s) to increase oxidative stress in the eye on fa2h expression.

Authors' response: We now provide this data in Figure 3. We altered the function of the cytoplasmic *Sod1* gene by RNAi mediated knockdown (*Sod1 IR*; (Missirlis et al., 2003)) or by using a heterozygous mutant allele ($Sod1^{n1}$/+), which is associated with a reduced function of *Sod1* (Phillips et al., 1995). We observed increased expression of *GstD1* mRNA under this condition of increased oxidative stress signalling, and increased *fa2h* mRNA and an incomplete extension of the rhabdomeres. These data support the connection between increased oxidative stress, altered *fa2h* levels, and abnormal rhabdomeric extension independent of *crumbs*.

4. Data presented in the paper show that fa2h overexpression impairs Rh1 trafficking. However, fa2h overexpression had no impact on rhabdomere morphogenesis, in contrast to Rh1 deficiency. What is the impact of fa2h overexpression on Rh1 distribution and levels at steady state? Is the impact on Rh1 really explains the role of Fa2h?

Authors' response: We have demonstrated that *fa2h* overexpression has an effect in a sensitized background (either in the background of *crb* mutants and in the context of the

BLICS assay in which flies are largely reared on a carotenoid-deficient diet and then exposed to retinal only in adulthood).
We show in Supplemental Fig. S4 that Rh1 distribution is indeed altered at steady state upon *fa2h* overexpression and knockdown in the *crb*^{8F015} allele. However, this snapshot does not give us an impression if Rh1 delivery to the rhabdomere or its turnover via the formation of RLVs (Rhodospin Loaded Vesicles) is affected. To address the role of *fa2h* in Rh1 delivery, we used the BLICS assay as described in Figure 6.
We do not expect *fa2h* manipulation to have the same magnitude of effect as Rh1 deprivation since Rh1 itself has an important role in rhabdomere development (Kumar and Ready, 1995). We further elaborate in the discussion that Rh1 delivery is orchestrated by many factors including MyoV, the exocyst complex, Rab 1, Rab11, and their effector GEFs and interacting proteins. We propose that *fa2h* dependence of rhabdomeric growth, described here, is only one aspect of the regulated delivery of Rh1 to the rhabdomeres.

5. It is worth showing representative images of the % of overlap of Rh1 and Rab11.

Author Response: Figure 6E-F" shows representative images of Rh1-Rab11 overlap in control and *fa2h* overexpressing conditions (for the quantification shown in Figure 6G). Suppl Fig. S5 also shows representative images used for quantification to better explain the method.

6. The authors concluded that Rh1 trafficking is altered in the absence of Crb owing to increased fa2h expression. To support this conclusion (and their model), they should try to rescue Rh1 trafficking defects in crb mutant PRC by knocking-down fa2h.

Authors' response: We show in Supplemental Fig. S4 that Rh1 distribution is indeed altered at steady state upon *fa2h* overexpression and *fa2h* knockdown in the *crb*^{8F015} allele. This supports our model that Rh1 in rhabdomeres is dependent on *fa2h* levels. However, we have not attempted to quantify, following a BLICS assay, a rescue in Rh1 trafficking in *crb* mutant PRC by knocking-down *fa2h*.

7. It could be interesting to discuss how hydroxylated sphingolipids could impact Rab11-dependent trafficking.

Authors' response: We have included a few sentences in the discussion:
"We rather favour the conclusion that *fa2h* and thereby hydroxylated sphingolipids play a role as regulators of apical trafficking. This conclusion is based on two findings: i) less newly synthesised Rh1 is delivered to the rhabdomeres upon overexpression of fa2h, as revealed by BLICS assays, which mimic Rh1 'pulse-chase' experiments. ii) A similar defect in Rh1 trafficking has been observed in crb mutant PRCs (Pocha et al., 2011), which have increased levels of fa2h expression. In particular, we identified an effect on post-Golgi trafficking of Rh1 via the Rab11-mediated pathway. Accumulation of cytoplasmic vesicles is observed upon genetic perturbation of Rab11 and/or genes that encode for Rab11 interacting proteins (Li et al., 2007a; Otsuka et al., 2019; Satoh et al., 2005). However, no obvious accumulation of similar vesicles was observed in crb mutant PR (data not shown). Thus, the trafficking defect observed upon *fa2h*

overexpression cannot be attributed to a general increase/decrease in Rab11 compartments, but rather alludes to a defect in sorting apical cargo. These results are consistent with studies in C. elegans, where apical compartments labelled with Rab11 and Rab7 are affected upon loss of fath-1(Li et al., 2018). Hydroxylated sphingolipids, as glycosphingolipids, are proposed to regulate the sorting of apically directed vesicles by combining different polarity cues (Zhang et al., 2011). In line with this, we propose that increased *fa2h* transcription (and hence an increase in hydroxylated sphingolipids) prevents apical membrane (rhabdomere) growth by inhibiting trafficking of Rh1 via the apical Rab11 compartments."

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

In this manuscript, authors used Drosophila photoreceptors as a model system to investigate the relationship between the apical membrane expansion and lipid metabolism. Late pupal photoreceptor massively expands their apical membrane, the rhabdomere. Many proteins involving this process have been identified, but little is known for the contribution of lipids and their metabolism.
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It is timely and very important to investigate the contribution of lipid and their metabolism for apical membrane expansion. Increasements of hydroxylated sphingolipids and fa2h expression level are quite interesting, but the connection between the change of lipid metabolism and Rh1 transport is not clearly supported by the data presented here. Below I list the major points.

1. Figure 1:
These data give the readers the good understanding of the background of this study, however, Figure 1 does not include any new findings, as these results have already published elsewhere.

Authors' response: We have changed Figure 1 following the reviewer's suggestion. To better explain rhabdomeric growth to the readers with no experience in *Drosophila* eye development, we have aspects of Figure 1 now as Supplemental Figure S1. However, we left the cartoon (Fig. 1A) in order to explain to the reader the three dimensions of growth we are talking about, an essential aspect of our work.

2. Author said in lane143: In the second half of pupal development, crb is required for the extension of the rhabdomeres along the proximo-distal axis.
However, the failure of the extension of the rhabdomeres along the proximo-distal axis is likely caused by the breakup of the ZA in crb mutants (Tepass and Harris, 2007

Trends in Cell Biology). Crb is not directly required for extension of the rhabdomeres along the proximo-distal axis in the second half of pupal development.

Authors' response: In (Tepass and Harris, 2007), the authors note that the "The function of Crumbs and its complex partners in regulating apical membrane size appears to be independent of the function of these proteins in ZA biogenesis". In *crb* mutants, only the apical and sub-apical membranes are improperly extended while the basal membranes (marked by Na^+ K^+ ATPase; see Figure I, below, not included in the manuscript) reach the retinal floor. By 70h APF adherens junctions (AJs) are largely normal in appearance and position and there is a substantial recovery of the AJ by the adult stage (Pellikka et al., 2002). This is also evident in *crb*^{11A22} with staining of E-cadherin (a component of the AJ) during the growth phase (Figure II below, not included in the manuscript). Thus, despite a recovery of AJs from 70 hAPF to adulthood, the rhabdomeric extension is abnormal in *crb* mutants. Our study largely focusses on this growth phase between 70h APF to adulthood and we have targeted this using Rh1- Gal4.

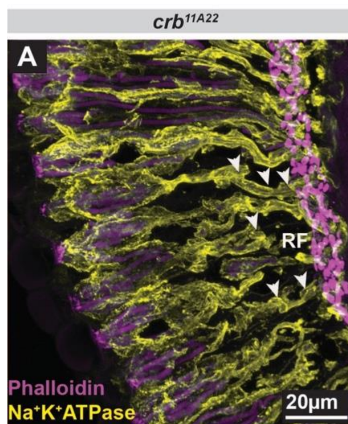

Figure I: Apical domain (rhabdomere) is specifically affected in *crb*^{11A22} mutant.
Confocal projection of 12µm thick sections of *crb*^{11A22} mosaic adult eye. Sections are labelled with phalloidin to mark rhabdomeres and with an antibody against Na^+ K^+ ATPase (yellow) to mark the basal membrane of the photoreceptor cell (PRC). In adult PRCs of *crb*^{11A22}, rhabdomeres (magenta) are improperly extended as opposed to basal membrane (yellow). Basal membrane of PRC is visible proximally (arrowheads), closer to the retinal floor (RF) unlike the rhabdomeres.

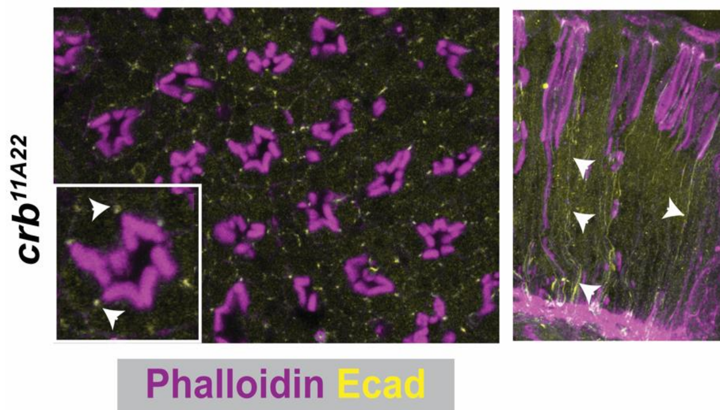

Figure II: E-cadherin localization during the growth phase in *crb*^{11A22} mutant.
Cross-sections (Left) and longitudinal projection (right) of 12µm sections of pupal eyes (at 85h APF) in *crb*^{11A22}. Sections are labelled with phalloidin (magenta) and with an antibody against E-cadherin (yellow). A magnified view of rhabdomeres is shown in the inset on left. E-cadherin, a component of the Adherens junctions, is visible in cross sections (white arrowheads). Proximally, Ecadherin is observed (white arrowheads) and rhabdomeres fail to extend.

3. Figure 2:
All of data presented here are quite new and very interesting. It is very interesting finding that a 5.9-fold increase in fa2h transcripts at pupal stages (60-80h APF) of

crb8F105 animals (Fig. 2E). Indeed, this period coincides with massive rhabdomere growth, as authors described. However, in crb mutants, rhabdomeres grow well (more than the wild type) in distal half of photoreceptors. Thus, the increasement of fa2h transcripts is hard to be explained by the failure of rhabdomere growth in crb mutant. Apical membrane extension to the retinal floor occurs around 35-45% pupal development (pd), and the photoreceptor extension along the proximo-distal axis occurs from 50%pd to adult stage.

Authors' response: Rhabdomeric growth is manifest as growth along all the three dimensions and this is explained in Figure 1 in more detail. The statement that in *crb* mutants, rhabdomeres grow well (more than the wild type) in distal half of photoreceptors" refers only rhabdomeric width, which is increased. This phenotype is already evident at 70h APF (Fig. 1D), and hence must occur earlier. All our experiments with *fa2h*, also those connected to Rh1 trafficking, are in the context of the phase of rhabdomere extension (number of microvilli) that occurs between 70h APF and adulthood.

4. Rh1Gal4 induces the expression from 70%pd and to get the effect of RNAi need the time. Thus, the strong effect of Rh1Gal4-drived Fa2h RNAi for rhabdomere elongation is amazing and surprising. Does Fa2h express in the pigment cells too?

Authors' response: From our results we cannot make any conclusion about *fa2h* expression in pigment cells. However, from data shown in Figure 4 we can conclude that *fa2h* expression in PRCs is sufficient to modulate rhabdomeric extension, since Rh1-Gal4 is specifically expressed in PRCs.

5. If Fa2h expresses mainly in the photoreceptors, please check Fa2h mRNA reduction by qPCR.

Authors' response: In Fig. 4J, we now show the effectiveness of *fa2h* overexpression and RNAi on *fa2h* mRNA expression.

6. If Fa2h expresses both in the photoreceptors and pigment cells, please check Fa2h mRNA reduction by in situ hybridization: pigment cell would give a good control.
Or please use mosaic retinas.

Authors' response: In light of the above two points, we think these experiments are not necessary as they will not affect the conclusions made in our manuscript regarding the impact of hydroxylated sphingolipids on rhabdomeric growth. The effects we see upon *fa2h* overexpression or knock-down are already evident when occurring in PRCs.

7. Figure 4:
I wonder why Rh1 does not localize in the rhabdomeres. Whole mount staining sometimes gives this penetration problem and it is sometimes hard to detect Rh1 in the rhabdomeres, however, the authors used cryo-sections. Please use better experimental conditions to show Rh1 staining in the rhabdomeres.

Authors' response: We do observe Rh1 staining in the rhabdomeres (Fig. 5C'). It should be noted that in one retinal section, we do observe Rh1 staining either "filling the rhabdomere" or localizing as a crescent pattern along the rhabdomeric membrane. This can also be seen in Fig. III below (not included in the manuscript). Localization of Rh1 in a crescent along the rhabdomeric membrane has also been extensively described in immunostaining on sections in (Satoh et al., 2005; Schopf et al., 2019)

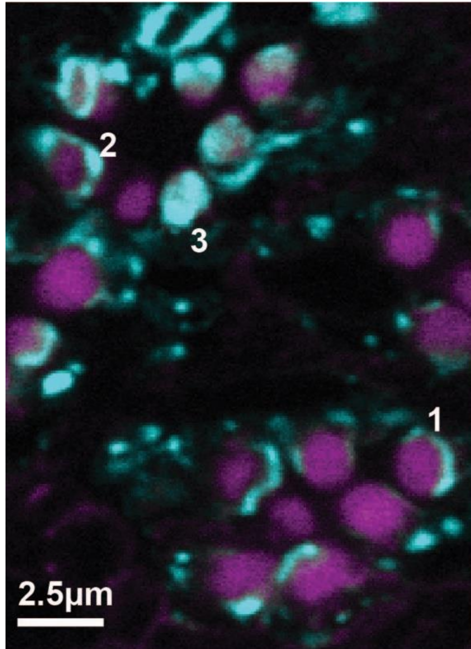

Fig. III: Rh1 immunostaining in a fly eye section.

An image of an optical slice of a 12µm thick cross section of a fly eye labelled with phalloidin (magenta) and an antibody against Rh1 (cyan). Rh1 staining is evident as a crescent pattern (1) along the rhabdomere membrane, or outlining (2), or filling the rhabdomere (3).

8. Figure 5: Again, there is no Rh1 staining within the rhabdomeres in the wild type cells at 3 hours after BLICS. It is difficult to judge whether Rh1 transport is affected or not affected by these staining data.

Authors' response: Please see the explanation above.

9. Please use better experimental conditions to show Rh1 staining in the rhabdomeres.

Authors' response: Please see above point

10. Moreover, please use mosaic eyes for BLICS experiments: because BLICS is sometimes difficult to start Rh1 transport well even in the wild type flies, probably because it is difficult to control how much all-trans-retinal the flies eat or how much 11-cis retinal is formed by blue light.
Mosaic retinas containing both the wild type cells and mutant cells (or cells expressing proteins) would help better judgement (Pocha et al., JCB 2011 is the good example).

Authors' response: For *crumbs* mutants, indeed mosaic eyes are used in Supplemental Fig. S5. However, for *fa2h* overexpression, there is no need to use mosaic eyes.

11. Which step of Rh1 transport is inhibited by overexpression of fa2h? It is not clear from the pictures in current Figures (there is no Rab11 staining).

Authors' response: We have now provided Rab11 staining images in Figure 6E, F and E", F".

12. If post-Golgi trafficking is impaired, Rh1 will be massively accumulated in the cytoplasmic vesicles (see Otsuka et al., JCS 2019). If this is the case, please show EM pictures of crb mutant photoreceptors overexpressing fa2h.

Authors' response: Otsuka et al., 2019 refers to a mutant in which Rab11 trafficking, in general, is affected. We have not stated anywhere that there are general defects in Rab11 trafficking. Our results instead indicate that Rh1 trafficking via Rab11 is affected especially under sensitized conditions of the BLICS assay. In this context, we do not observe any accumulation of cytoplasmic vesicles as compared to controls or as seen in (Otsuka et al., 2019) (See Fig. IV below; not included in the manuscript)

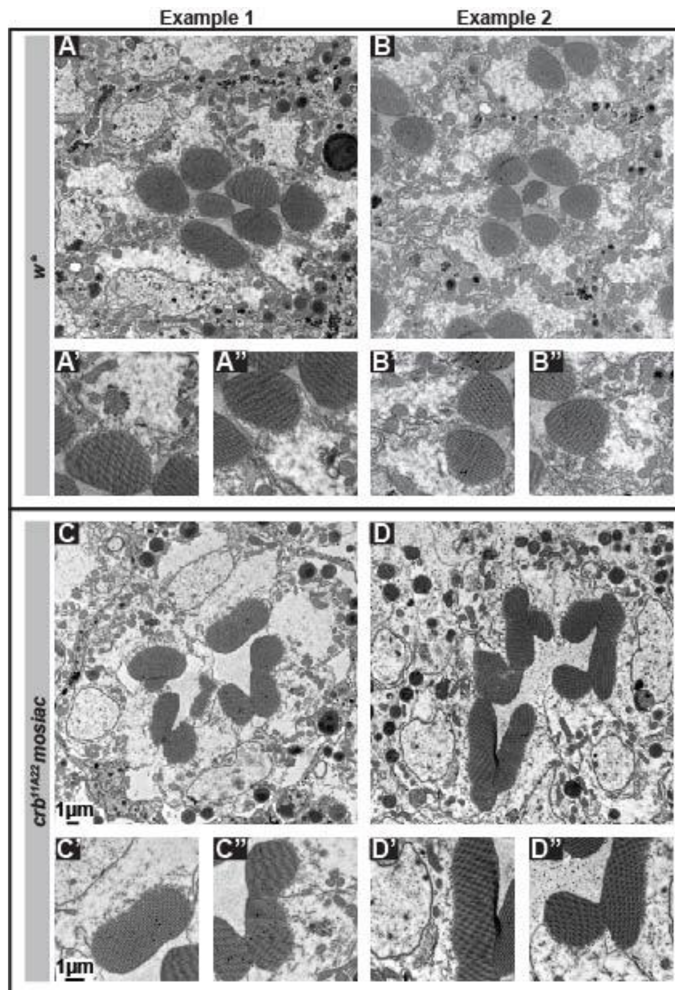

Fig. IV: No accumulation of cytoplasmic vesicles in *crb*^{11A22} mutant PRCs

A-D: Two representative examples of electron micrographs of ultrathin sections of controls (A, B; w*) and mutant adult photoreceptor cells (PRCs) (C, D; *crb*^{11A22}). Presence of nuclei indicate the distal regions of the retina.

A'-B" and C'-D": Magnified views of the PRC in controls (A'-B"; w*) and mutant PRCs (C', D"; *crb*^{11A22}). There is no abnormal accumulation of cytoplasmic vesicles in the mutants (C-D") as compared to the controls (A-B").

Scale bars are as indicated in C and C'

13. There is no figures of Rab11 staining. How we can judge the colocalization of Rab11 and Rh1 and where do they colocalize? Please show the original pictures for Rh1 and Rab11 staining.

Authors' response: We now provide Rab11, Rh1 staining in Figure 6. Original raw data can be provided/uploaded upon request.

14. Figure 6, Figure 5 might be able to include this model.

Authors' response: Figure 5 and 6 are now modified and the model has been removed.

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

Review

In this paper, the authors explore the contributions of lipidomics to the defects in rhabdomere structure known to be associated with certain alleles of crumbs (crb). Their lipid profiling revealed an increase in levels of the hydroxylated sphinglolipid ox-CerPE with only the crb mutant alleles associated with aberrant rhabdomere morphologies. They link these changes to increases in expression of the gene encoding Fatty acid-2 hydroxylase (fa2h), an enzyme in the pathway to Ox-CerPE synthesis, and show that knock down of fa2h can partially rescue the rhabdomere defects in certain crb mutants. Since the work of others suggests that fa2h expression is induced by oxidative stress and crb mutant rhabdomeres undergo oxidative stress, they examined levels and localization of the stress marker - gstD1 - in crb mutants and saw expected changes with the crb alleles associated with aberrant rhabdomere morphologies. Finally, the authors explored a potential link between the changes in lipids with trafficking of a major rhabdomere protein - Rhodopsin 1 - to the apical surface membrane, potentially explaining how changes in the lipid profile is linked to the altered rhabdomere morphologies.

Specific findings in the paper:

1. Rhabdomeres fail to fully extend their apical domains along the PD axis in three of four loss-of-function crb alleles examined. Levels of the hydroxylated sphingolipid ox-CerPE go up in the crb alleles affecting PD apical domain elongation. They show that it is the long chain fatty acid versions that are affected by loss of crb. These data are strong - although it would be helpful to know the molecular lesions associated with each of the crb alleles they examine.

Authors' response: This information is provided in Supplemental Table S1.

2. RNA levels for fa2h, which encodes fatty acid 2 hydroxylase, an enzyme in the pathway leading to ox-CerPE production, and gstD1, a marker for oxidative stress, are higher in crb alleles that affect PD elongation of rhabdomeres. A GFP reporter for gstD1 is observed in retinal cells of crb mutants with affected rhabdomeres. The GFP reporter data are not convincing.

Authors' response: The microscopy data is now in Supplemental Fig. S3 in which we also show the extracted grayscale images for the *GstD GFP* signal. We hope that this clarifies the increased intensity in PRCs (in the center of the hexagonal units). In Figure 2 we also present real time qRT-PCR data showing increased *GstD1* mRNA profiles for 3 mutant alleles.

3. Altering levels of fa2h by overexpression or RNAi can alter crb rhabdomere phenotypes with reasonable rescue of one allele with RNAi knockdown of fa2h. Quantification of phenotypes is provided in Table 1. These data are convincing.

Authors' response: No comment

4. Rhabdomere expansion is known to be linked to the amount of Rhodopsin making it to the apical surface. So the authors show that they can affect rhabdomere structure by limiting carotenoids in diet (which limits maturation and trafficking of Rhodopsin) and they show that the defects caused by this treatment are exacerbated in a *crb* mutant. These data are convincing.

Authors' response: No comment

5. The authors show that too much fa2h limits recovery of rhabdomeres following provision of the cis retinal required to make rhodopsin. These data are convincing.

Authors' response: No comment

6. The authors suggest that the amount of fa2h affects Rab11 dependent Rh1 trafficking to the rhabdomeres. We don't see this data (other than quantification) in the core figures in the paper. Thus, I am not convinced by these data.

Authors' response: We now provide Rab11, Rh1 staining in Figure 6. Original raw data can be provided/uploaded upon request.

Issues:

6. Authors have shown that in crb mutant eyes, a marker of oxidative stress goes up based on quantification of transcripts and with some less convincing immunostaining of crb mutant eye sections stained with a GFP tagged version of this marker. They have also shown that mRNA levels of the gene encoding an enzyme involved in production of ox-Cer (fa2h) go up as do levels of ox-CERPE. From this they conclude that reducing Crb increases oxidative stress, which in turn induces expression of the FA synthase gene. Although they show that two things change in crb mutants, they have not

demonstrated that one change causes the other. It could be the other way around or the changes could be independent. If they are going to push this idea, they should show that induction of oxidative stress (independent of loss of *crb*) can induce increased expression of fa2h.

Authors' response: We now provide this data in Figure 3. We altered the function of the cytoplasmic *Sod1* gene by RNAi mediated knockdown [*Sod1 IR*; (Missirlis et al., 2003)] or by using a heterozygous mutant allele (*Sod1*^{n1}/+), which is associated with a reduced function of *Sod1* (Phillips et al., 1995). We observed increased expression of *GstD1* mRNA under this condition of increased oxidative stress signalling, and increased *fa2h* mRNA and an incomplete extension of the rhabdomeres. These data support the connection between increased oxidative stress, altered *fa2h* levels, and abnormal rhabdomeric extension independent of *crumbs*.

7. The model in 6B refutes their data. If Crb normally blocks oxidative stress and oxidative stress normally blocks fa2h synthesis, then reducing Crb function would increase oxidative stress, which would further block fa2h synthesis. This is not what was observed - fa2h levels went up in the crb mutants. As suggested above, the other problem with this model is that they provide no evidence that oxidative stress is affecting fa2h levels, only that crb affects levels of both an oxidative stress marker and of fa2h.

Authors' response: We have rephrased our working model in the discussion as follows "Crb normally limits oxidative stress (Chartier et al., 2012) and the resulting oxidative status (low oxidative stress) normally limits *fa2h* expression. In *crb* mutants, however, an altered oxidative status (increased oxidative stress) causes an upregulation of *fa2h* transcription, which, in turn, results in severely reduced Rh1 delivery and improperly extended rhabdomeres. The *fa2h* dependence of rhabdomeric growth described here is only one aspect of the pleiotropic cellular response to an altered redox status of increased oxidative stress signaling due to loss of *crb*".

8. I would like to see what they were quantifying regarding vesicles with the staining of both Rab11 and Rh1 (Figure 5E). Where is the raw data or a sample image one could use to judge how easily this quantification was done?

Authors' response: We have provided Rab11, Rh1 staining in Figure 6. Original raw data can be provided/uploaded upon request.

9. Also, I am assuming that since the authors are looking at pupal and adult eyes that the alleles of crb they are examining are not lethal alleles. So, what kind of alleles are they? What is the molecular lesion in each allele and how might that be linked to the variability in rhabdomere defects?

Authors' response: Except *crb*^{13A9}, the three other alleles are in fact embryonic lethal. In these cases, we used genetic mosaics to study their function in the eye (see Materials

and Methods). The molecular lesions of alleles are now provided in Supplemental Table S1. We have also included the following sentences in the results to note the differences between alleles.
On page 8: "The magnitude of increase of *fa2h* was not as high in *crb*^{4} as compared the other two alleles (*crb*^{11A22}, *crb*^{8F105}) which reflects the trend in ox-CerPE levels (Fig. 2A)"
On page 10: "No further enhancement of the *crb*^{11A22} phenotype upon overexpression of *fa2h* (Fig. 4N) is in agreement with earlier data showing that *crb*^{11A2} is a protein null allele, while some protein is still produced in *crb*^{8F105} (Wodarz et al., 1993)."

10. The gstD-GFP staining is not so convincing given how abnormal these eyes are (Figure 2 H and I). How do the investigators know where the retinal cells are relative to the pigment cells? It would be helpful to know this. Are there other independent markers that could be used? This should be resolved in a convincing manner.

Authors' response: The microscopy data is now in Supplemental Fig. S3 in which we also show the extracted grayscale images for the *GstD GFP* signal. We hope that this clarifies the increased intensity in PRCs (in the center of the hexagonal units). In Figure 2 we now present real time qRT-PCR data showing increased *GstD1* mRNA profiles for 3 mutant alleles.

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August 31, 2020

RE: JCB Manuscript #201911100R

Dr. Sarita Hebbar
Max-Planck-Institute of Molecular Cell Biology and Genetics
Pfotenhauerstr. 108
Dresden 01307
Germany

Dear Dr. Hebbar:

Thank you for submitting your revised manuscript entitled "A role for hydroxylated sphingolipids in apical domain morphogenesis of Drosophila photoreceptors". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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We suggest slight edits of your title to:

Hydroxylated sphingolipids regulate Rab11-mediated trafficking of Rhodopsin in Drosophila photoreceptors

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11) eTOC summary: A ~40-50-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.

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

Authors have satisfied my requests from the initial review process.