Author's response to reviews

Title: Small intestinal mucosa expression of putative chaperone fls485

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Author's response to reviews: see over
We thank the Editors and the Reviewers for the second thoughtful comments concerning the revised version of the manuscript. In the following lines we specify – point by point – how we revised the manuscript according to the remarks of the reviewers. All changes in the manuscript are marked by underlining.

Reviewer: Michael George
Reviewer's report:
Major Compulsory Revisions:
1. The authors have attempted to address the lack of functional analysis in their characterization of fls485 in the original manuscript by utilizing a Caco2 in vitro intestinal epithelial model system. Unfortunately, simply showing a slight increase in fls485 transcript levels between high and low density Caco2 cultures did not provide any functional insights. This set of experiments could have been designed better. The authors should assay expression of known biomarkers to demonstrate differentiation since Caco2 is a well-established model system, and attempted to determine if alterations in fls485 expression are associated with changes in Caco2 maturation/differentiation characteristics.

Please recognize, we have not simply shown a slight increase in the fls485 transcript levels between high and low density CaCo2 cultures. In order to demonstrate putative functional aspects of fls485, we have established a CaCo2 maturation model as suggested by the co-reviewer Mark Musch. An in-detail description of this model was already included in the first revised version of the manuscript, section Methods, paragraph Cell Culture and Transfection. As already stated in the revised paragraph, expression levels of the biomarker villin were used to confirm differentiation of CaCo2 cells. Villin gene expression is widely accepted as a powerful biomarker demonstrating cellular differentiation, especially the maturation of enterocytes. Following your recommendation the role of villin as a biomarker of cellular differentiation is now further substantiated by incorporation of two additional references. We do absolutely agree with you that the CaCo2 maturation model is not the best way to analyse hard functional aspects of fls485 expression. However, construction and investigation of an intestinal fls485 transgenic or knock-out mouse model were not intended with our study. We think that the in-depth functional analysis of fls485 as requested may be beyond the scope of this manuscript.

2. In situ mRNA analyses (Figure 2A/B) suggests the fls485 transcript is widespread but more concentrated in the crypt regions and, intracellularly, accumulating towards the luminal region of the plasma membrane. On the other hand, immunohistochemistry suggests fls485 protein is concentrated toward the upper regions of the villus and not in the crypts. Furthermore, the protein signal is not accumulated at the plasma membrane as the mRNA is. Because multiple products are recognized in Western blots by the antibodies used and differential staining patterns are observed, depending on the Ab clone and/or cell line used, the localization studies of tissue sections are difficult to interpret and inconclusive. The authors devote part of the Discussion to address this issue, yet in the Discussion, they also state that “immunostainings of sectioned paraffin-embedded normal human small intestinal mucosa with #7, #10 or subclones revealed an essential overlap with fls485 mRNA in situ hybridization pattern. The authors also state in the Discussion that “... colocalization of EGFP and fls485 immunosignaling was
found in EGFP-fls485158 3T3 transfectants”. However, there was no “co-localization” data presented, only Western blots confirming an EGFP-fls485158 chimeric protein was expressed. The inaccurate statements should be revised in the Discussion and the discrepancy between tissue localization of fls485 mRNA and protein really does need to be resolved. The authors’ suggestion of laser capture microdissection could be very effective in this regard. Instead of ‘essential overlap’ the phrase ‘overlap’ is used.

The tissue distribution of both fls485 mRNA and protein were addressed with mRNA in situ hybridization as well as immunohistochemistry. As stated and shown in the manuscript, the techniques visualized an overlap of mRNA and protein that was judged as essential. It is well-known from other expression studies that asymmetric distribution of mRNA and protein species along the crypt-villus axis is possible (for Ref. see Landry et al., Differentiation 1994; 56: 55-65). In this context, our statement, that an essential overlap between fls485 mRNA and protein along the crypt-villus axis exists in situ, reflects a phenomenon that is already described for several other genes. In addressing your comment, the statement is now given in a more cautious way. Moreover, a reference for asymmetric gene expression and protein synthesis along the crypt-villus axis has been included. We do absolutely agree with you that laser capture microdissection could be a powerful method to further substantiate our observation. However, this technical approach is very time-consuming. Therefore we see no chance to provide additional data for the 2nd revised version of the manuscript. Our statement that ‘colocalization of EGFP and fls485 immunsosignaling was found in transfectants’ was not illustrated in the 1st revised version of the manuscript. We think that the phrase ‘data not shown’ does not implicate that a statement is incorrect. However, in response to your criticism, additional colocalization studies with a second cell type, CaCo2 cells, were performed for anti-fls485 antibodies #7 as well as #10. The results illustrated in the revised Figure 1 give evidence for colocalization of EGFP and the fls485 truncated protein.

Minor Essential Revisions:
1. The writing is difficult to read in several places and should be edited more carefully.

To the best of our knowledge style and text edition were improved.

Discretionary revisions:
1. It would be informative perhaps to see internal localization of the EGFP-fls485158 chimeric protein and whether this may change as Caco2 cells grow to confluence and differentiate.

Following your recommendation, CaCo2 cells were used for localization studies as detailed above. We think that the proposed maturation model using the ‘EGFP-fls485158 chimeric protein’ in CaCo2 cells is not sufficient to describe functional aspects of fls485 activity, because the fls485 protein is truncated. However, the use of a full length fls485 chimeric protein could be a powerful tool in the biomarker-monitored CaCo2 maturation model. We would prefer to address this important question in further studies, because an expression construct coding the full length fls485 chimeric protein is not available at present.
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Reviewer: Mark Musch
Reviewer's report:
The addition of additional functional data on fls485 is very important and it establishes a probable role for this chaperone potentially in a number of physiological and pathophysiological states. Future work will likely establish a role for this chaperone not only in normal protein processing, but also in states of unfolded protein responses, but this awaits further work. The clarity of the manuscript was good and the revisions have made the potential roles of the chaperone even clearer and provided greater insight into its actions.

Thank you very much for your encouraging comments to continue the project.
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Reviewer: Dan Hershko
Reviewer's report:
I have carefully read the revised manuscript and the comments of the other reviewers. It seems that much effort has been put into correcting the manuscript. Nevertheless, the study still lacks in providing functional results and the biological importance of the examined protein.

Thank you very much for your comments and suggestions for improvement of the manuscript.

We do agree with you that essential parts of the study are descriptive and data concerning the fls485 bioactivity are rudimentary. The intention of our present work was in situ analysis of fls485 expression. In this setting, revealing of the fls485 function was not intended with our studies and may be behind the scope of the present manuscript. Actually, an intestinal fls485 transgenic or knock-out mouse model is not available in our lab to perform functional studies sufficiently.