

# **A feedback circuitry involving $\gamma$ -actin, $\beta$ -actin and NM2A controls tight junction and apical cortex mechanics**

Corresponding Author: Professor Sandra Citi

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study by Mauperin et al., the authors investigated the roles of beta-actin and gamma-actin isoforms in regulating the organization and function of tight junctions in epithelial cells. They found that knockdown of gamma-actin increased non-muscle myosin 2A (NM2A) expression, which in turn resulted in an upregulation of beta-actin expression. Knockdown of gamma-actin also increased tight junction membrane tortuosity and altered tight junction dynamics.

This is a carefully performed and rigorous study. The manuscript is very clearly written, and the data is convincing. The literature overview and discussion are comprehensive. Some of the findings contrast with earlier studies that have examined the effects of isoform-specific actin knockdown in another cell line but the authors carefully discuss these differences. Overall, I believe the findings will be of broad interest to cell biologists and will help better understand the roles of actin isoforms in epithelial cell biology.

Some minor changes needed to data presentation are listed below:

Comments:

- 1) Line 152 – “Upregulation of NM2A was rescued by exogenous expression of gamma-actin” – this is not shown in Fig S3D, instead it shows immunoblot analysis of the expression levels of NM2A upon depletion of gamma-actin.
- 2) Lines 153-156 – a description of the panels in figure S3 that correspond to the IB and IF analysis is incorrect.

Minor comments

- 1) In Line 150, it would be “the junctional” instead of “then junctional.”

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The paper entitled “A feedback circuitry involving  $\gamma$ -actin,  $\beta$ -actin and nonmuscle myosin 2A controls membrane cortex mechanics in epithelial cells” by Maupérin et al. describes the effects of  $\gamma$ -actin knockout on  $\beta$ -actin and nonmuscle myosin 2A expression, shape of tight junctions, and tension of apical cortex using  $\gamma$ -actin knockout MDCK II cells. The paper provides an important insight into the function and regulation of individual actin isoforms. However, several points should be addressed to prove the claims by the authors.

Major points

The authors claim that the expression level of  $\beta$ -actin is upregulated in  $\gamma$ -actin via nonmuscle myosin 2A. However, the effect of RNA interference of nonmuscle myosin 2A on  $\beta$ -actin expression is examined only in MDCK II cells. Since the expression level of nonmuscle myosin 2A is not affected by the  $\beta$ -actin depletion in SKCO 15 cells (Baranwal et al., 2012), it is required to demonstrate that nonmuscle myosin 2A is involved in the regulation of  $\beta$ -actin expression in other cell lines such as EpH4 and mCCD cells.

The tortuosity of tight junctions is increased in  $\gamma$ -actin knockout MDCK cells. Since RNA interference of  $\beta$ -actin results in the increase of nonmuscle myosin 2A expression but has no effects on the tortuosity in MDCK cells, the authors claim that both  $\beta$ -actin and nonmuscle myosin 2A are required for the regulation of tortuosity of tight junctions. It is unclear which isoform(s) of actin and myosin are important for the regulation of the tortuosity of tight junctions. It is required to examine the effects of the overexpression of actin and myosin isoforms on the tortuosity of tight junctions. In addition, various other factors including Shroom3, Willin/FRMD6, Lulu, Tuba and ZO-1 are involved in the regulation of the shape of tight junctions, and ROCK is involved in the mechanism of the regulation in some cases (Hildebrand, 2005; Ishiuchi et al., 2011; Nakajima and Tanoue, 2010). The authors should examine the effects of  $\gamma$ -actin knockout on these factors to elucidate the mechanism of the regulation of tight junction tortuosity. Also, the alignment of myosin and the ultrastructure of cytoskeleton are reported to correlate to the shape of tight junctions (Fanning et al., 2012). More detailed analysis of the structure of cytoskeleton in  $\gamma$ -actin knockout cells would be helpful to understand the mechanism of the regulation of tight junction tortuosity.

The authors claim that the rescue of  $\gamma$ -actin reverted the expression of  $\beta$ -actin. The expression level of  $\gamma$ -actin in the rescue experiment is surprising low (Fig. S2). In contrast, the authors also claim that the expression of  $\beta$ -actin is also increased by the RNA interference of  $\gamma$ -actin similar to the knockout of  $\gamma$ -actin (Fig. S2). However, the expression level of  $\gamma$ -actin seems to be much higher in knockdown cells than rescue cells. The authors should explain the reason of these results.

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The terms "CGN" and "cingulin" are used in the manuscript and it is confusing. Please unify the term.

The authors discuss that the deafness by the overexpression of ZO-2 or knockout cingulin is related to the regulation of actin filament by ZO-2 or cingulin (lines 312-316). However, the barrier function of tight junction is known to be important to maintain the unique electrophysiological environment in the inner ear, which is required for the maintenance of hair cells. The authors should mention the possibility that the effect of ZO-2 or cingulin modification on the barrier function of tight junctions and electrophysiological environment in the inner ear.

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

I think my concerns have been addressed in the revised manuscript.

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Reviewer #1 (Remarks to the Author):

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- 2) Lines 153-156 – a description of the panels in figure S3 that correspond to the IB and IF analysis is incorrect.

**Response. Thank you for pointing out these errors. The text was revised: “Moreover, up-regulation of NM2A was observed upon depletion of  $\gamma$ -actin by siRNA both in MDCK cells and in additional epithelial cell lines (mCCD and Eph4), as determined by IB analysis (Fig. S3D, quantification in Fig. S3E) and by IF analysis (Fig. S3F-H, quantifications on the right).”**

Minor comments

- 1) In Line 150, it would be “the junctional” instead of “then junctional.”

**Response. Thank you for detecting the typo. The text was revised.**

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and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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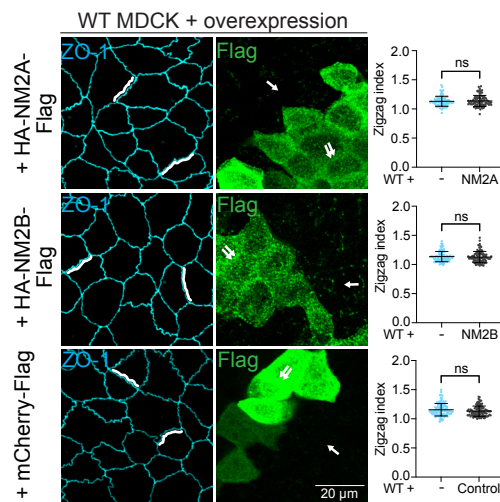
***Response. We thank this Reviewer for suggesting this experiment. The same results were obtained with additional cell lines (Eph4 and mCCD) and are now shown in revised Fig. S3 (panels K-L).***

The tortuosity of tight junctions is increased in  $\gamma$ -actin knockout MDCK cells. Since RNA interference of  $\beta$ -actin results in the increase of nonmuscle myosin 2A expression but has no effects on the tortuosity in MDCK cells, the authors claim that both  $\beta$ -actin and nonmuscle myosin 2A are required for the regulation of tortuosity of tight junctions.

***Response. The text of Discussion was revised to include “The observation that increased NM2A expression in  $\beta$ -actin-depleted cells did not result in increased tortuosity suggests that  $\gamma$ -actin cannot functionally replace  $\beta$ -actin with regards to the generation and transmission of force from the circumferential belt to the TJ membrane.”***

It is unclear which isoform(s) of actin and myosin are important for the regulation of the tortuosity of tight junctions. It is required to examine the effects of the overexpression of actin and myosin isoforms on the tortuosity of tight junctions.

***Response. We carried out overexpression of NM2A and NM2B, and of either  $\gamma$ -actin or  $\beta$ -actin, and examined the effect of this overexpression on TJ membrane tortuosity. In the case of either NM2A or NM2B, we used FLAG-tagged versions, that were correctly delivered to junctions, as well as to the cortex and cytoplasm of cells (new experiment, shown in Reviewer Fig. 1).***

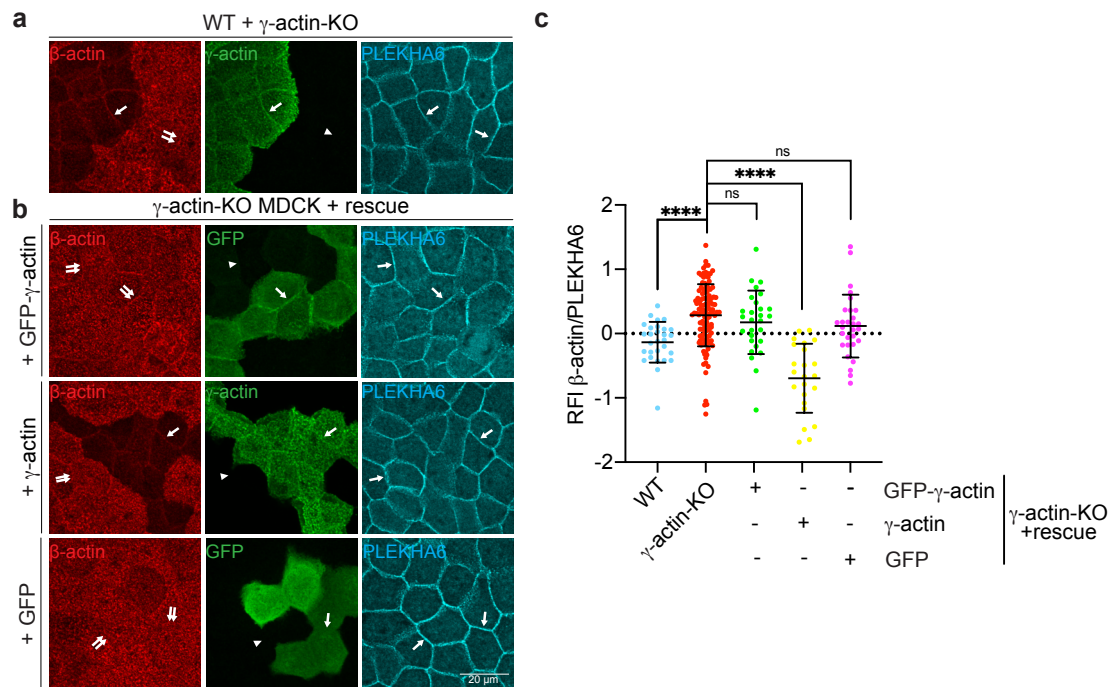


# **Reviewer figure 1\_ NM2A and NM2B overexpression in WT MDCK cells doesn't affect the TJ-membrane tortuosity.**

Immunofluorescence (IF) microscopy analysis and zig-zag index quantifications (on the right on IF panels) of endogenous ZO-1 (cyan), used as a TJ marker, in WT MDCK cells overexpressing full-length canis NM2A tagged with HA and Flag (HA-NM2A-Flag) (top panels), full-length canis NM2B tagged with HA and Flag (HA-NM2B-Flag) (middle panels) or by mCherry-Flag alone as negative control (bottom panels); distinguished via Flag antibody (green). Arrows indicate WT expression of NM2A and NM2B, double-arrows indicate overexpression of NM2A, NM2B or Control. The white line represents the TJ membrane tortuosity. Scale bar = 20  $\mu$ m. Dots shows replicates (N=3, n=97-110) and bars represent mean  $\pm$  SD. Statistical significance of quantitative data was determined by an unpaired Mann-Whitney's test (ns: not significant, \*\*\*\*p<0.0001).

*However, no effect on TJ membrane tortuosity was observed. This can be explained by the likely possibility that NM2 filaments/monomers/oligomers at steady state are already functionally saturating the system. It is also possible that no more myosin filaments/molecules can be integrated beyond a homeostatic level in a WT context, where no cytoplasmic actin isoform has been depleted.*

*In the case of cytoplasmic actins, we found that GFP-tagged  $\gamma$ -actin is not functional, although it is targeted to junctions, because it does not rescue the phenotype of increased  $\beta$ -actin expression in  $\gamma$ -actin-KO cells (new experiment, shown in Reviewer Fig. 2). This is in agreement with previous studies on cytoplasmic actins, showing that tags interfere with actin function (Rommelaere et al 2004, doi: 10.1251/bpo94, Deibler et al 2011, <https://doi.org/10.1371/journal.pone.0022941>; Nagasaki et al 2017, <https://doi.org/10.1247/csf.17016>).*

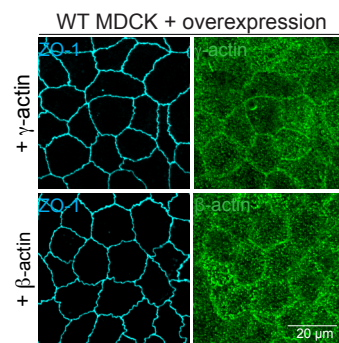


**Reviewer figure 2\_GFP-tagged  $\gamma$ -actin does not rescue the phenotype of increased  $\beta$ -actin localization in  $\gamma$ -actin-KO, unlike untagged  $\gamma$ -actin.**

(a-c) Immunofluorescence (IF) microscopy analysis (a, b) and relative fluorescence intensity (RFI) quantifications (c) of endogenous  $\beta$ -actin (red) at junctions in mixed cultures of WT and  $\gamma$ -actin-KO (a); or in  $\gamma$ -actin-KO cells rescued with full-length canis GFP-tagged  $\gamma$ -actin (GFP- $\gamma$ -actin) (top panels), full-length canis untagged  $\gamma$ -actin ( $\gamma$ -actin) (middle panel), or by GFP alone as negative control (bottom panels) (b). Arrows indicate normal labelling (as in WT cells), double-arrows indicate increased labelling for  $\beta$ -actin, arrowheads indicate loss of  $\gamma$ -actin labelling in KO cells. Quantification of RFI corresponds to the ratio between the junctional staining of  $\beta$ -actin versus the junctional marker PLEKHA6 (cyan). Scale bar = 20  $\mu$ m. Dots shows replicates and bars represent mean  $\pm$  SD. Statistical significance of quantitative data was determined by an unpaired Mann-Whitney's test (ns: not significant, \*\*\*\*p<0.0001).



*When we used un-tagged forms of either  $\gamma$ -actin or  $\beta$ -actin, we could not distinguish WT from overexpressing cells (new experiment, shown in Reviewer Fig. 3), suggesting a rheostat mechanism that prevents expressing total levels of cytoplasmic actins beyond homeostatic levels (a mechanism that is similar to what described for “auto-regulation of tubulin expression, and for actin was described in the 80s and 90s by the Bershadsky and Be Ze’ev laboratories, but has not been studied further).*



**Reviewer figure 3\_The cells overexpressing  $\gamma$ -actin or  $\beta$ -actin cannot be distinguished from the WT cells.**

Immunofluorescence (IF) microscopy analysis of endogenous ZO-1 (cyan) at junctions in WT MDCK cells overexpressing full-length canis untagged  $\gamma$ -actin ( $\gamma$ -actin) (top panels) or full-length canis untagged  $\beta$ -actin ( $\beta$ -actin) (bottom panels) (green). Scale bar = 20  $\mu$ m. N=3.

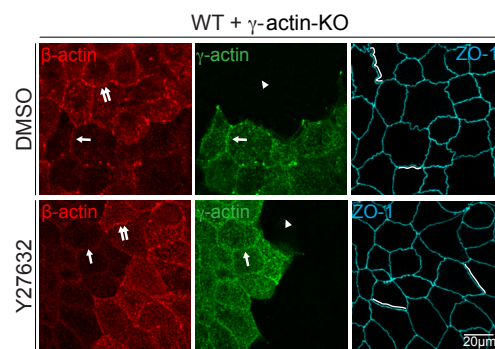
***These results confirm our longstanding experience that overexpression experiments are often either technically problematic and prone to artifacts, or/and difficult to interpret, due to the saturation of the molecular systems by the endogenous proteins and/or to constraints due to spatial architecture, existing interactors etc. In contrast, depletion, KO and rescue experiments of specific cellular components provide much clearer and more interpretable phenotypes and results. We prefer to omit Reviewer Figures 1, 2, 3 from the paper, since they provide no additional useful information.***

Comment:

In addition, various other factors including Shroom3, Willin/FRMD6, Lulu, Tuba and ZO-1 are involved in the regulation of the shape of tight junctions, and ROCK is involved in the mechanism of the regulation in some cases (Hildebrand, 2005; Ishiuchi et al., 2011; Nakajima and Tanoue, 2010). The authors should examine the effects of  $\gamma$ -actin knockout on these factors to elucidate the mechanism of the regulation of tight junction tortuosity.

***Response. We performed a new experiment (Reviewer Fig. 4), and showed that treating mixed WT and  $\gamma$ -actin-KO cells with Y27632 (which inhibits the Rho-ROCK-dependent NM2 activation) results in decreased TJ membrane tortuosity. This is consistent with the known mechanistic implication of NM2 in TJ tortuosity in WT cells (Van Itallie et al 2009 doi 10.1091/mbc.E09-04-0320, Lu et al 2021 /doi.org/10.1101/2021.05.30.446323). We prefer not to include this Figure in the revised manuscript, since it is redundant with published evidence (Van Itallie et al 2009, etc).***

***Regarding the potential implication of all of the additional proteins cited by the Reviewer in the phenotypes of  $\gamma$ -actin-KO cells, our manuscript addresses the role of  $\gamma$ -actin and we demonstrate a mechanistic implication of NM2A. Addressing this question would require to open new lines of investigation and goes beyond the scope of this paper. The text of the discussion was revised to as follows: “TJ membrane tortuosity is the result of orthogonal forces generated by the contractility of the circumferential actomyosin bundle associated with apical junctions (Tang, 2018, Citi, 2019, Citi, 2024). As such, it is regulated by different proteins directly or indirectly associated with the actomyosin cytoskeleton, including ZO-1 (Van Itallie et al, 2009; Tokuda, 2014,), Shroom (Hildebrand, 2005), Lulu (Nakajima, 2011), cingulin (Rouaud, 2023) and other factors (reviewed in (Lynn, 2020)).***



**Reviewer figure 4 ROCK inhibition by Y27632 treatment decreases the TJ-membrane tortuosity in both WT and  $\gamma$ -actin-KO cells.**

IF microscopy analysis of endogenous ZO-1 (cyan), used as a TJ marker, in mixed culture of WT and  $\gamma$ -actin-KO cells treated with DMSO (top panel) and Y27632 (bottom panel); distinguished via  $\gamma$ -actin (green). Arrows indicate normal labelling (as in WT cells), double-arrows indicate increased labelling for  $\beta$ -actin, arrowheads indicate loss of  $\gamma$ -actin labelling in KO cells. The white line represents the TJ membrane tortuosity. Scale bar = 20  $\mu$ m. N=3.

Also, the alignment of myosin and the ultrastructure of cytoskeleton are reported to correlate to the shape of tight junctions (Fanning et al., 2012). More detailed analysis of the structure of cytoskeleton in  $\gamma$ -actin knockout cells would be helpful to understand the mechanism of the regulation of tight junction tortuosity.

**Response. We carried out new experiments, and we now provide high resolution (STED) imaging, showing distinct pattern of the actin cytoskeleton, e.g. more intensely labeled contractile foci in the apical cortex of WT cells versus  $\gamma$ -actin-KO cells (new panel, Fig. 3F). The text of Results was modified accordingly.**

The authors claim that the rescue of  $\gamma$ -actin reverted the expression of  $\beta$ -actin. The expression level of  $\gamma$ -actin in the rescue experiment is surprising low (Fig. S2).

**Response. The levels are low because in this transient expression experiment only a small percentage of the cells are expressing the transgene. Fluorescent signals for actin in expressing cells are comparable to normal. The text was revised : “To confirm the specificity of the phenotype, we first rescued  $\gamma$ -actin-KO cells by re-expression of  $\gamma$ -actin, which was detected in transfected cells by IF at levels similar to WT (Fig. S2D, compare to Fig. 1A), though total protein levels were low, due to the low efficiency of transfection (IB analysis in Fig. S2E).”**

In contrast, the authors also claim that the expression of  $\beta$ -actin is also increased by the RNA interference of  $\gamma$ -actin similar to the knockout of  $\gamma$ -actin (Fig. S2). However, the expression level of  $\gamma$ -actin seems to be much higher in knockdown cells than rescue cells. The authors should explain the reason of these results.

**Response. As stated above, IB analysis shows total levels in depleted/undepleted and transfected/untransfected cells, while immunofluorescence analysis allows to detect specifically the levels of  $\gamma$ -actin and  $\beta$ -actin in depleted and rescue cells (Fig. S2 D, I-K), providing unambiguous evidence.**

Minor points

In the leaky epithelia like MDCK II cells, charge selectivity is generally more sensitive to evaluate the barrier function of tight junctions. The measurement of charge selectivity (dilution potential) would be helpful for the evaluation of  $\gamma$ -actin knockout on the barrier function of tight junction (Fig. 4).

**Response. Since the overall impact of the KO of  $\gamma$ -actin on both pore and leak pathway was not significant, and we observed no effect of  $\gamma$ -actin KO on the localization and junctional accumulation of TJ and AJ markers, we think there is no functional or molecular evidence supporting the need for a more detailed analysis of TJ barrier function.**

The terms “CGN” and “cingulin” are used in the manuscript and it is confusing. Please unify the term.

**Response. We revised the manuscript and used cingulin consistently.**

The authors discuss that the deafness by the overexpression of ZO-2 or knockout cingulin is related to the regulation of actin filament by ZO-2 or cingulin (lines 312-316). However, the barrier function of tight junction is known to be important to maintain the unique electrophysiological environment in the inner ear, which is required for the maintenance of hair cells. The authors should mention the possibility that the effect of ZO-2 or cingulin modification on the barrier function of tight junctions and electrophysiological environment in the inner ear.

***Response. To address this point, the text of the Discussion was revised to include the additional mechanisms cited by this Reviewer, as follows: “Intriguingly, either KO or mutation of cingulin in mice and humans is associated with progressive hearing loss, through increased death and apoptosis of hair cells (Zhu, Huang et al. 2023). Our results suggest that cingulin and  $\gamma$ -actin control survival of hair cells and hearing function by maintaining apical membrane stiffness and cellular integrity upon mechanical stress, through their ability to anchor  $\gamma$ -actin and NM2B to TJs (cingulin) and provide the cortex with specific biophysical stiffness properties ( $\gamma$ -actin). Other mechanisms have been described through which TJ proteins can affect hearing, such as increased apoptosis induced by overexpression of ZO-2 (Walsh, Pierce et al. 2010) and altered TJ-dependent ionic permeability resulting from loss or mutations of claudins (Ben-Yosef, Belyantseva et al. 2003, Gow, Davies et al. 2004, Nayak, Lee et al. 2013), tricellulin (Nayak, Lee et al. 2013), and occludin (Kitajiri, Katsuno et al. 2014). However, such a mechanism is unlikely for cingulin and  $\gamma$ -actin, since there is insufficient evidence for a significant effect of their KO on barrier function of epithelial cells (Guillemot, Hammar et al. 2004, Guillemot, Schneider et al. 2012, Mauperin, Sassi et al. 2023).”***