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# ZO-2 enriched tight junction-like structures at sites of platelet-platelet contact

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## INTRODUCTION

Platelets express junction proteins that are known to regulate cell-cell adhesion, barrier formation and communication in tissue forming cells. Previous research has shown that the gap junction proteins expressed by platelets regulate platelet activation in an $\alpha_{IIb}\beta_3$-dependent manner.[1] Also, platelet activation processes are negatively regulated by endothelial cell-selective adhesion molecule (ESAM)[2] and junctional adhesion molecule A (JAM-A)[3], proteins that are part of the tight junctions (TJ) of endothelial cells. However, the role of zonula occludens 2 (ZO-2) -an intracellular component of the endothelial TJs- in the formation of inter-platelet contacts has not yet been described.

## AIM

To unravel the molecular composition, in particular ZO-2, of tight platelet-platelet contacts.

## METHOD

Using phosphoproteomics databases[4,5], ZO-2 phosphorylation in activated platelets was investigated. Isolated platelets were allowed to fully spread on a fibrinogen surface. Subsequently, samples were fixed and stained to assess F-actin polymerization in relation to ZO-2 and ESAM distribution using confocal and/or super-resolution fluorescence microscopy. Platinum replica electron microscopy (PR-EM) was applied for ultrastructural visualization of the cytoskeleton at sites of platelet-platelet interaction.

## RESULTS

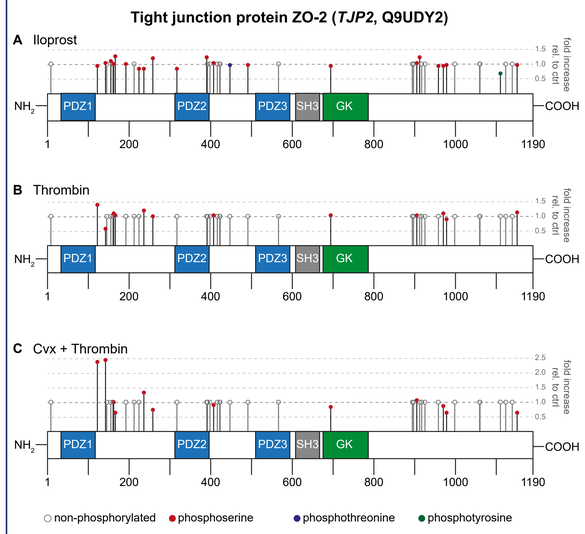


**Figure 1. Altered phosphorylation profiles of ZO-2 identified in phosphoproteome indicate differential regulation of ZO-2 upon platelet inhibition and activation.** Schematic representation of the ZO-2 protein with PDZ and guanylate cyclase (GK) domains. All phosphosites of ZO-2 as described in Uniprot are plotted. Phosphosites that are not regulated in platelets are indicated in grey, while serine, threonine and tyrosine residues that are phosphorylated upon platelet signaling are indicated in red, blue and green respectively. Fold changes in phospho-signal upon **A**) iloprost, **B**) thrombin or **C**) thrombin/convulxin (cvx) stimulation are indicated relative to that in untreated platelets (control) according to published data.[4,5]

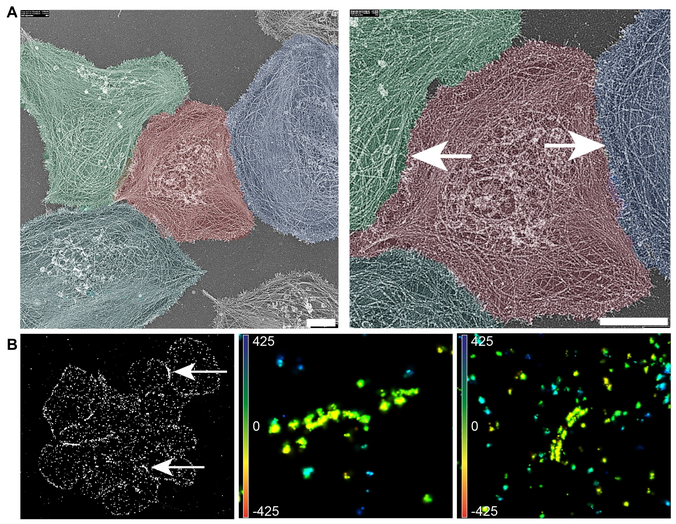


**Figure 3. Super-resolution of ZO-2 rich platelet-platelet contacts.** **A**) Platinum replica EM demonstrating compacting platelet-platelet contacts, in which circumferential actin zones of interacting platelets are merged (arrows). Bar = 1 µm. For better visualization, pseudo coloring has been applied on original PR-EM images using Adobe Photoshop. **B**) 3D *d*STORM super-resolution microscopy showing molecular clusters of ZO-2 at inter-platelet contact sites (arrows). Color code (yellow → blue) shows *z*-distance around fibrinogen surface.

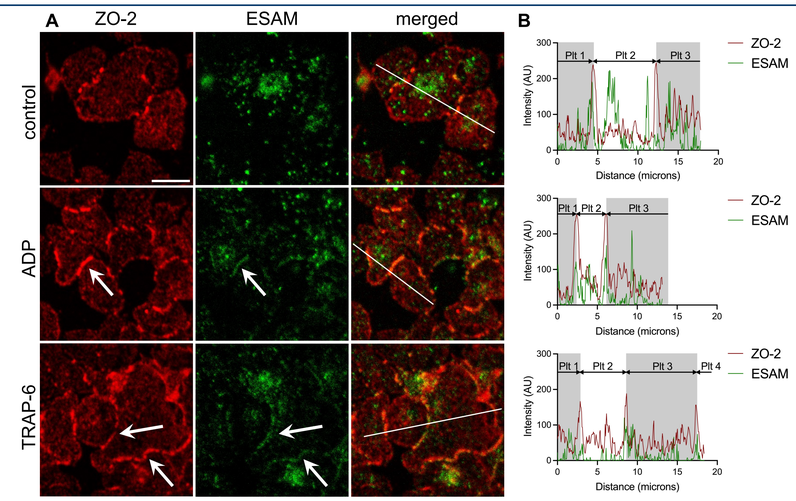


**Figure 2. ZO-2 and ESAM enriched tight junction-like structures form at sites of platelet-platelet contacts in spread platelets and become more defined upon additional platelet activation.** Platelets were spread on a fibrinogen surface in the absence or presence of ADP or TRAP-6. Samples were prepared for confocal microscopy. **A**) Representative confocal fluorescence images of ZO-2 (red) and ESAM (green). Bar = 5 µm **B**) Fluorescence intensity plots of ZO-2 and ESAM staining across multiple spread platelets (white line) indicating enrichment of ZO-2 and ESAM at sites of platelet-platelet contact.

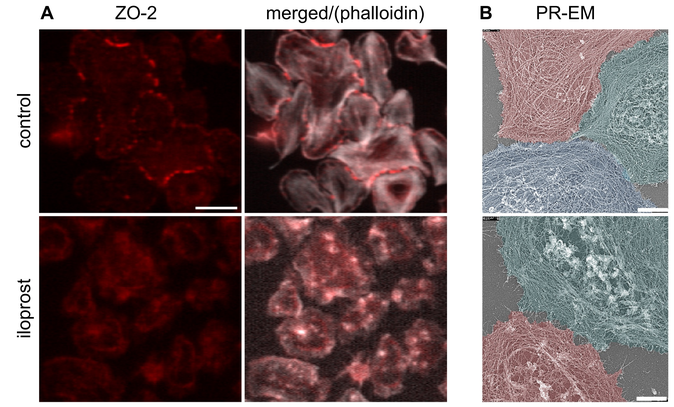


**Figure 4. Modulation of ZO-2 clusters and inter-platelet contacts by cAMP elevation.** Platelets adhered to and spread on fibrinogen were left untreated or treated with iloprost (5 nM). The spread platelets were prepared for confocal microscopy or platinum replica EM (PR-EM). **A**) Representative confocal fluorescence images, red: anti-ZO-2 AF647 mAb, gray: phalloidin, (bar = 5 µm) and **B**) platinum replica EM images (bar = 1 µm). For better visualization, pseudo coloring has been applied on original PR-EM images using Adobe Photoshop.

## REFERENCES

1. Vaiyapuri S. et al. Gap junctions and connexin hemichannels underpin hemostasis and thrombosis. *Circulation.* 2012;125(20):2479-91
2. Stalker T. et al. Endothelial cell specific adhesion molecule (ESAM) localizes to platelet-platelet contacts and regulates thrombis formation in vivo. *J Thromb Haemost.* 2009;7(11):1886-96.
3.Karshovska E. et al. Hyperreactivity of junctional adhesion molecule A-deficient platelets accelerates atherosclerosis in hyperlipidemic mice. *Circ Res.* 2015;116(4):587-99.
4. Beck F. et al. Time-resolve characterization of cAMP/PKA dependent signaling reveals that platelet inhibition is a concerted process involving multiple signaling pathways. *Blood.* 2014;123(5):e1-e10.
5.Solari F. et al. Combined quantification of the global proteome, phosphoproteome and proteolytic cleavage to characterize altered platelet functions in the human scott syndrome. *Mol Cell Proteomics.* 2016;15(10):3154-3169.

## CONCLUSIONS

Jointly, these data point to the assembly of tight junction-like structures, where intracellular components of tight junctions accumulate at sites of close platelet-platelet contact.

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