Combining AFM-based functional imaging with fluorescence reporters to outline adhesion-dependent signaling

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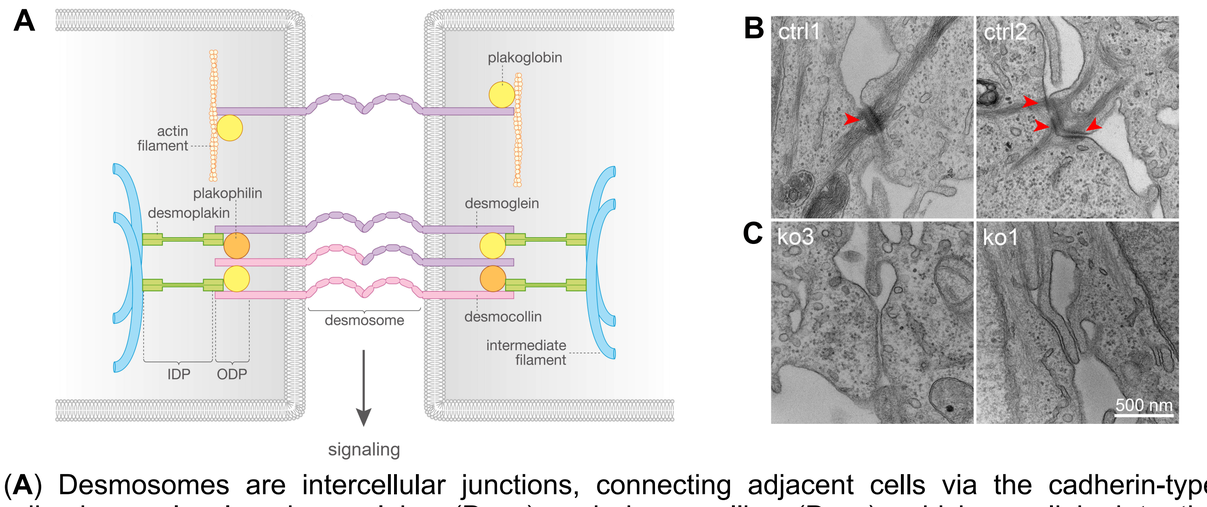
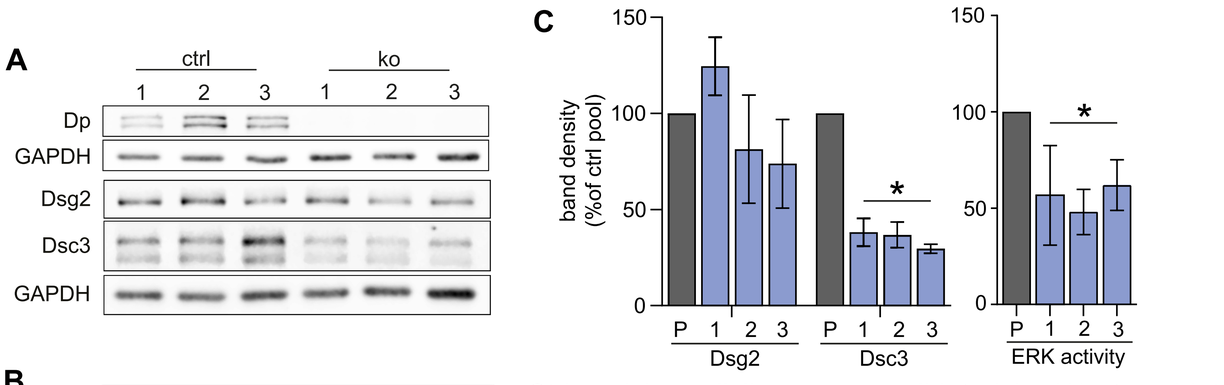
Introduction Desmosomes - adhesive structures and signaling hubs



(**A**) Desmosomes are intercellular junctions, connecting adjacent cells via the cadherin-type adhesion molecules desmogleins (Dsgs) and desmocollins (Dscs), which are linked to the intermediate filament cytoskeleton by plakophilins, plakoglobin and desmoplakin (Dp). In addition to their adhesive properties, they act as signaling hubs by yet unknown mechanisms. Electron micrographs revealed that knockout of Dp (ko) in human HaCaT keratinocytes led to complete absence of desmosomes (**C**), while desmosomal structure remained normal in control (ctrl) cells (**B**). *Schematic in (**A**) modified from Waschke and Spindler, Med. Res. Rev., 2014.*

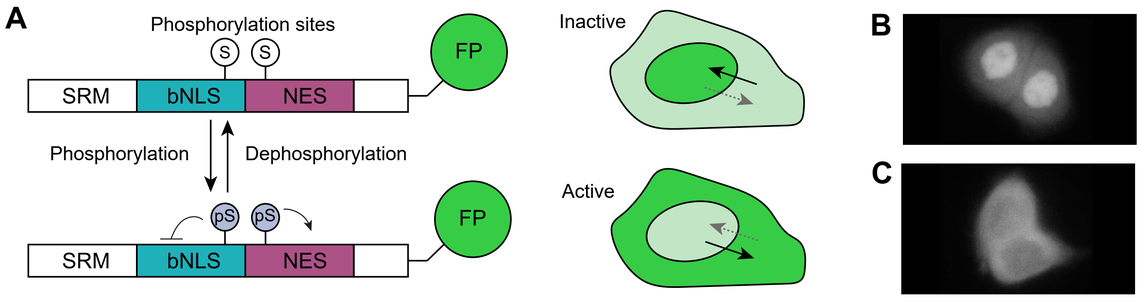
AIM: Establishing a method to investigate the modulation of ERK signaling in response to altered desmosomal adhesion using a fluorescence-based kinase translocation reporter (KTR).

Loss of Dp results in altered expression of Dsc3 and ERK



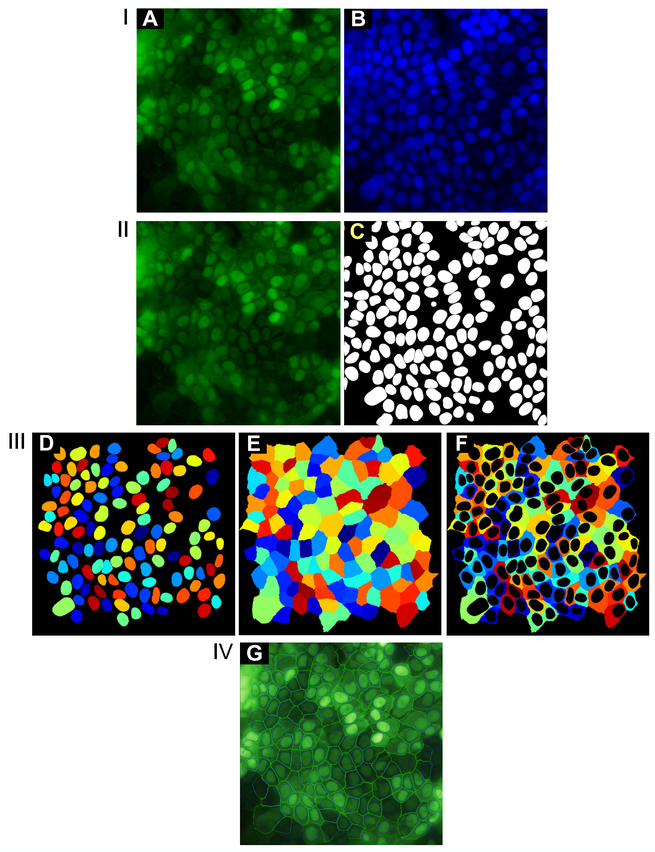
Ctrl and ko cell lines were validated by Western blot analysis. Loss of Dp led to reduced levels of Dsc3, while Dsg2 protein expression remained unchanged (**A, C**). Furthermore, ERK activity was diminished in ko cells (**B, C**). (*n=3-4, error bars represent mean ± SEM, *p<0.05)*

Kinase Translocation Reporters - a powerful tool for investigating signaling



(**A**) Kinase translocation reporters (KTRs) harbour a substrate recognition motif (SRM), phosphorylatable bipartite nuclear localization signal (bNLS) and nuclear export signal (NES) sites as well as a fluorescent protein domain. Upon low kinase activity, dephosphorylated KTRs mainly localize in the nucleus. Phosphorylation upon high kinase activity increases the nuclear export rate of KTRs by enhancing NES activity, resulting in translocation of KTRs into the cytosol. The ratio between cytosolic and nuclear fluorescence describes kinase activity. Ctrl cells expressing ERK-KTR-mClover in the (**B**) inactive or (**C**) active state. *Schematic in (**A**) modified from Kudo et al., Nat Protoc, 2018.*

Automated KTR analysis by nucleus and cytoplasm segmentation



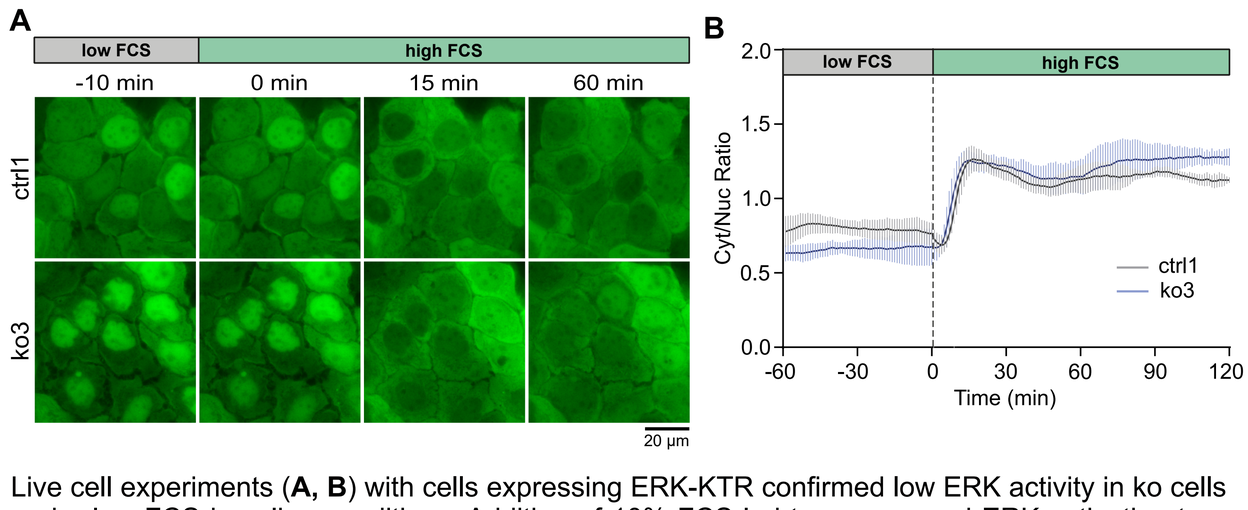
I Live cell images of ctrl cells expressing ERK-KTR (**A**). For segmentation, nuclei were stained with Hoechst33342 (**B**).

II Nuclei were detected and segmented (**C**) using the StarDist Plugin in ImageJ.

III Using CellProfiler, primary (**D**, nucleus), secondary (**E**, cell) and tertiary (**F**, cytosol) objects were identified with a seed/ propagation method.

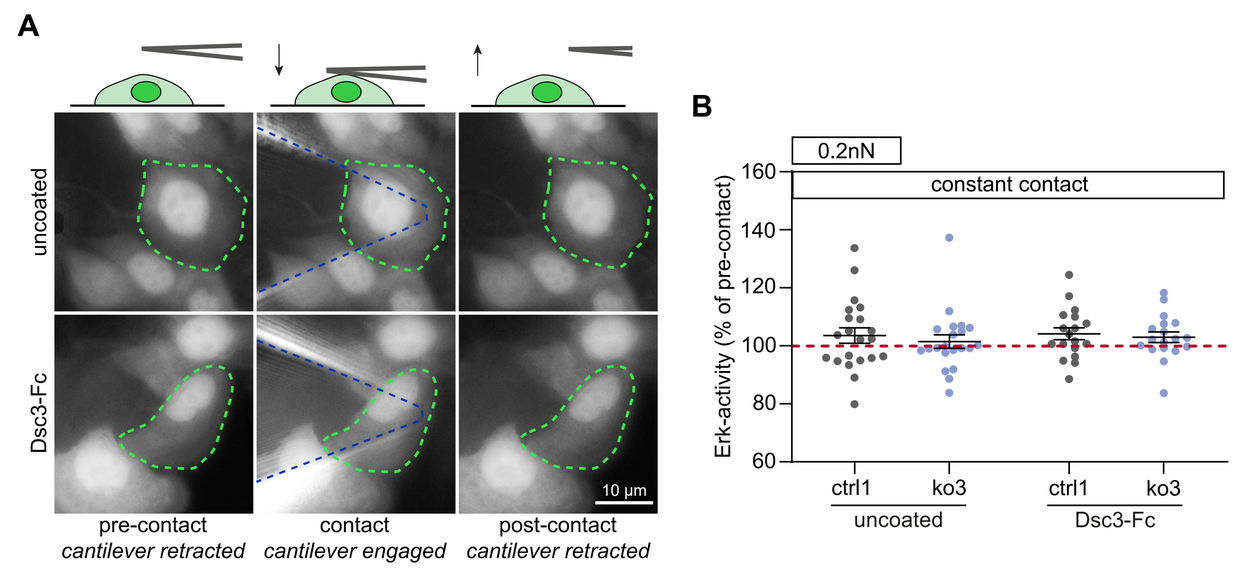
IV Mean intensity of nucleus and cytosol for each cell is analysed after completed segmentation (**G**).

ERK-KTR visualizes pronounced ERK activity upon FCS addition



Live cell experiments (**A, B**) with cells expressing ERK-KTR confirmed low ERK activity in ko cells under low FCS baseline conditions. Addition of 10% FCS led to pronounced ERK activation to a similar extent in ctrl and ko cells, visible through a translocation of ERK-KTR from the nucleus to the cytosol. (*n=3, error bars represent mean ± SD)*

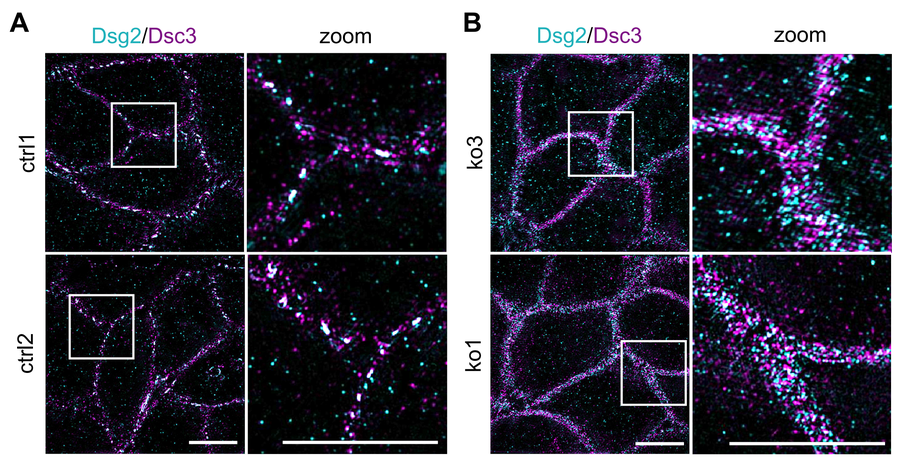
Dsc3-coated AFM cantilevers do not trigger ERK activity



AFM was utilized to mimic formation or disruption of desmosomal junctions. Dsc3-Fc- or uncoated tipless cantilevers were brought into contact with cells under constant force and retracted again after 300s (**A**).

An applied force of 0.2nN and a constant cantilever-cell contact did not trigger ERK activity in ko and ctrls cells (**B**). Uncoated cantilevers were used to apply a higher force of 0.6nN in either constant contact or tapping mode for 300s, revealing unchanged ERK activity (**C**). (*n=3. Each data point represents one cell, error bars represent mean ± SEM)*

Inhibition of ERK does not alter Dsg2 distribution in ctrl cells



Localization of Dsg2 and Dsc3 was investigated by structured illumination microscopy. While Dsg2 mainly colocalized with Dsc3 in clusters at desmosomes in ctrl cells (**A**), loss of Dp led to a more homogeneous distribution of both proteins (**B**).
(*n=3; Scale bar: 10 µm)*

To test the hypothesis that altered distribution of Dsg2 in ko cells is caused by their reduced ERK activity, ctrl cells were incubated with 10 µM of the MEK-Inhibitor U0126 for 24h and stained for Dsg2 (**C**). Inhibition of ERK did not alter the clustered appearance of Dsg2 along the membrane. (*n=3; Scale bar: 5 µm)*

SUMMARY AND CONCLUSION

- Loss of Dp leads to lack of desmosomes and diminished ERK activity which is also shown by ERK-KTR.
- ERK-KTR shows prominent activity upon stimulation with FCS, while ERK seems to be independent of mechanical manipulations with Dsc3-Fc coated cantilevers.

→ **Combining kinase activity reporters with single molecule force spectroscopy is a promising model to study the relationship between adhesion molecules and intracellular signaling.**