

Final report

Title of the project:

"Regulation of cell motility by membrane-associated endosomal adaptors"

Leibniz-Institute: Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP)
Reference number: SAW-2013-FMP-5 247
Project period: 2013 – 2015
(+2 years of cost-neutral extension due to pregnancy of postdoc)
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Table of Contents

| | |
|--|-----------|
| Executive summary | 3 |
| General information | 4 |
| Publications related to SAW project | 4 |
| Press releases related to SAW project | 4 |
| Third-party funding obtained due to activities within SAW project | 4 |
| Presentations at conferences related to SAW project | 4 |
| Results subproject 1: | |
| The role of Gadkin in cell motility and immunity | 5 |
| Introduction | 5 |
| DC maturation regulates Gadkin levels and posttranslational modification | 5 |
| Loss of Gadkin alters the actin cytoskeleton of DCs | 6 |
| Loss of Gadkin impairs DC motility in vitro | 6 |
| Loss of Gadkin does not alter DC migration in vivo | 7 |
| Alternative functions for Gadkin in DCs | 7 |
| Summary | 7 |
| Results subproject 2: | |
| Function of Stonin1 at the cellular and organismic level | 8 |
| Introduction | 8 |
| Stonin1 regulates focal adhesion dynamics | 8 |
| Stonin1 as a cargo adaptor for the internalization of the oncogene NG2 | 10 |
| Stonin1 in cellular signaling | 10 |
| Stonin1 in tumorigenesis | 11 |
| Stonin1 in FA signaling and maturation | 13 |
| Regulation of Stonin1 | 14 |
| Summary | 15 |
| Statement regarding economic usability | 16 |
| Qualification of junior scientists | 16 |
| Collaborations | 16 |
| References | 17 |

Executive summary

Cell migration is crucial for development, wound healing and immune responses. Its dysregulation is involved in diseases ranging from metastatic cancer to autoimmune syndromes. Cell motility is shaped by extracellular signals cells receive via cell surface receptors which influence for example actin cytoskeleton dynamics. Cellular responses to these signals are often fine-tuned by adjusting the surface levels of the signaling receptors. Endocytosis, a process for internalizing transmembrane proteins, together with intracellular membrane transport, constitutes a powerful mechanism to regulate receptor surface levels. Therefore, defects in endocytosis have dramatic consequences ranging from impaired cell migration to oncogenic signaling. In fact, several hallmarks of cancer have been attributed to an aberrant propensity of malignant cells to endocytotically disassemble signaling and adhesion complexes. Efficient endocytic protein retrieval relies on a variety of adaptor proteins, which link e.g. cargo proteins to the endocytic machinery or vesicles to motor proteins, thereby determining what is internalized and where the cargo is transported. In addition, some adaptor proteins at the same time interact with actin regulators. However, often it is still unclear how precisely endocytic adaptors regulate the turnover of distinct cell surface receptors, affect cytoskeletal dynamics and thereby influence cell migration and tumor growth.

By employing cell biological and optical techniques in conjunction with knockout mouse models we aimed at dissecting the cellular functions of the two adaptor proteins Gadkin and Stonin1 within this project, two modulators of cell motility that act at the interface of membrane traffic and actin dynamics.

The adaptor protein Gadkin links endosomal vesicles to the motor protein KIF5 for outward transport and also binds Arp2/3, an actin nucleator which is important for migration of many cell types. The interaction with Gadkin sequesters Arp2/3 on intracellular vesicles and thereby inhibits the motility of melanoma cells as we previously showed. Directed migration is especially crucial for immune cells such as dendritic cells (DCs) which continuously sample antigens and upon pathogen encounter migrate to lymph nodes to activate T cells. To manipulate DC migration is of therapeutic interest because keeping DCs from priming autoreactive T cells is a potential avenue to treat autoimmune diseases. Since Gadkin had been suggested to affect DC-dependent inflammatory processes, we here studied its role in DCs. Our studies support the notion that Gadkin is important for DC functions. It is not only upregulated, but also phosphorylated during DC maturation. Gadkin deficient DCs have increased actin filament levels in line with reduced Arp2/3 inhibition and show impaired in vitro migration in 2D and 3D environments. Still, in vivo, these deficits do not keep the Gadkin deficient cells from reaching the lymph node suggesting a high plasticity of DC migration. However, loss of Gadkin perturbs the inflammatory responses of DCs which likely compromises immune defense.

Stonin1 has the typical structure of an endocytic adaptor protein, however, for a long time its cargo protein remained elusive. We finally identified Stonin1 as dedicated adaptor for the internalization of NG2. This proteoglycan, a co-receptor of growth factor receptors, is known to modulate cell migration. Stonin1 deficient cells display a dramatic surface accumulation of NG2, which results in increased cellular signaling and altered cellular motility. Altered signaling is a hallmark of cancer, besides, NG2 is an established oncogene, which stimulates tumor growth from within the tumor by supporting tumor cell proliferation and from within the tumor environment by promoting angiogenesis. By clearing NG2 from the plasma membrane, Stonin1 presumably influences not only cell migration, but also keeps NG2's oncogenic potential in check. Our experiments indeed show tumor suppressive effects of Stonin1 in glioma growth. In addition, we uncovered that Stonin1 influences focal adhesions, the macromolecular assemblies, which anchor cells to the substratum and have to be continuously turned over during cell migration. Stonin1's role in the maturation of focal adhesions is independent of NG2, but rather depends on its interaction with actin regulators.

General information

Project number: SAW-2013-FMP-5 247
Institution: Leibniz Forschungsinstitut für Molekulare Pharmakologie, Berlin
Applicant: PD Dr. Tanja Maritzen, Junior Group Leader
Funding Line: 5 Women in academic leadership positions
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Publications related to SAW project

* *equally contributing authors*; # *co-corresponding authors*

- Feutlinske* F, Browarski* M, Ku M, Trnka P, Waiczies S, Niendorf T, Stallcup WB, Glass R, Krause E, **Maritzen T**. *Stonin1 mediates endocytosis of the proteoglycan NG2 and regulates focal adhesion dynamics and cell motility*. **Nat Commun**. 2015; 6:8535
- Schachtner* H, Weimershaus* W, Stache V, Plewa N, Legler DF, Höpken UE, **Maritzen T**. *Loss of Gadkin affects dendritic cell migration in vitro*. **PLOS ONE**. 2015; 10(12): e0143883
- **Maritzen# T**, Schachtner H, Legler# DF. *On the move: Endocytic trafficking in cell migration*. **Cell Mol Life Sci**. 2015; 72(11):2119-34
- Mertins P, Przybylski D, Yosef N, Qiao J, Clauser K, Raychowdhury R, Eisenhaure TM, **Maritzen T**, Haucke V, Satoh T, Akira S, Carr SA, Regev A, Hacohen N, Chevrier N. *An integrative framework reveals signaling-to-transcription events in Toll-like receptor signaling*. **Cell Rep**. 2017 Jun 27;19(13):2853-2866

Press releases related to SAW project

- Press release issued by FMP: "Unterdrückung von Tumoren: Regulationsmechanismus entdeckt", published on website of FMP, Forschungsverbund Berlin e.V. and www.healthcapital.de (20.10.15)

Third-party funding obtained due to activities within SAW project

- DFG grant for the project "*Dissecting the role of Stonin1 in focal adhesion dynamics and tumor suppression*" (MA 4735/2-1; 2 PhD positions for 3 years; total volume ca.428.330 €)
- FMP "Integrated Project Program" grant for the project "*Investigating and manipulating Ena/VASP proteins in dendritic cell migration*" (1 postdoc position for 2 years; total volume ca. 176.800 €, joint project with Dr. Ronald Kühne, FMP)

Presentations at conferences related to SAW project

- 2nd International Symposium on "Membranes and Modules", Germany, 2014
- Gordon conference on "Lysosomes & Endocytosis", USA, 2014
- Gordon conference on "Directed Migration", USA, 2015
- European Cytoskeletal Forum (ECF) meeting on "Cell Adhesion and Migration", UK, 2016
- Gordon conference on "Directed Migration", USA, 2017
- European Cytoskeletal Forum (ECF) meeting on "Cytoskeleton: Mechanical Coupling from the Plasma Membrane to the Nucleus", Finland, 2017

Results Subproject 1: The role of Gadkin in cell motility and immunity

Introduction

Work within this project was based on our initial observation that the endosomal adaptor protein Gadkin does not only link AP-1 positive endosomal vesicles to the microtubule-based motor protein KIF5 for outward transport (1), but also binds Arp2/3. Arp2/3 is an actin nucleating complex which generates branched actin filaments and due to its influence on actin dynamics affects diverse processes ranging from cell migration to vesicular trafficking.

As postdoctoral researcher I showed that this interaction serves to sequester Arp2/3 on intracellular vesicles in the absence of pro-migratory signaling. This prevents unwanted Arp2/3 activation at lamellipodial protrusions and thereby inhibits cell spreading and cell migration of melanoma cells (2) under these conditions.

Cell migration is not only important for cancer cells, but also crucial for the function of numerous types of immune cells which have to quickly migrate through the organism to organize the host defense against invading pathogens. A prominent example are dendritic cells (DCs) which act as sentinels of our immune system. Stationed in peripheral tissues, immature DCs continuously sample antigens. Upon encounter of pathogens they mature and migrate to lymph nodes to activate T cells. Thus, efficient DC migration is crucial for the adaptive immune response. To understand and possibly manipulate DC migration is not only of academic interest, but likely also of therapeutic value since keeping DCs from reaching and priming T cells is a potential avenue to treat autoimmune diseases like multiple sclerosis (3).

Since the severity of a DC-dependent inflammatory phenotype in an immune compromised mouse model was reported to be modulated by a gene locus that contains among others the Gadkin gene (4), we set out to investigate the role of Gadkin in DC migration.

DC maturation regulates Gadkin levels and posttranslational modification

DCs that have not yet encountered pathogens stay within peripheral tissues and display high rates of macropinocytosis in order to continuously take up antigens. Encounter of danger signals such as lipopolysaccharides (LPS), bacterial membrane components, dramatically alters DCs. While macropinocytosis is shut down, the cells become highly migratory.

Loss of Gadkin did not affect the maturation process, however, Gadkin itself was regulated by the maturation-inducing LPS treatment of DCs. On the one hand, the protein level of Gadkin doubled within 24 h of LPS treatment (Figure 1A,B,D). On the other hand, within 30 min of the treatment, we observed the appearance of a very closely spaced additional Gadkin-specific band of lower electrophoretic mobility in immunoblots (Figure 1B,C) pointing to a post-translational modification. In fact, when the group of our collaborator Nicolas Chevrier (Harvard University, USA) performed a screen to identify proteins which are phosphorylated upon LPS stimulation of DCs, they found Gadkin among their hits (5) suggesting that the band shift we observed is due to phosphorylation of Gadkin. These findings implicate that Gadkin is involved in the processes triggered by DC maturation.

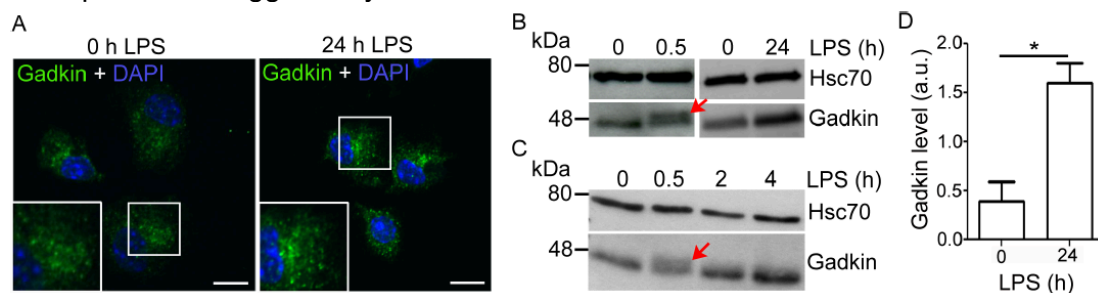


Figure 1: DC maturation increases Gadkin levels and induces its transient posttranscriptional modification

(A) Immature WT DCs were either left untreated or incubated for 24 h with LPS. Subsequently, cells were allowed to spread on fibronectin-coated cover slips for 15 min to achieve a uniform morphology prior to analysis. Cells were processed for immunofluorescence and labeled with Gadkin-specific antibodies. Nuclei were stained with DAPI. Insets show 2x enlarged perinuclear area. Scale bar: 10 μ m.

(B,C) Lysates from WT DCs treated with LPS for the indicated times were analyzed by immunoblotting using Gadkin- and Hsc70-specific antibodies. The closely spaced upper band is indicated by red arrows. (D) Quantification of immunoblot images like the one depicted in B. Gadkin levels were normalized to Hsc70 levels derived from the same blot. (N=3 independent experiments, unpaired two-tailed t-test; $*=p<0.05$; a.u., arbitrary units). Taken from (6).

Loss of Gadkin alters the actin cytoskeleton of DCs

Based on the hypothesis that Gadkin acts also in DCs as a negative regulator of Arp2/3, the amount of polymerized actin is expected to be increased in Gadkin KO DCs. In line with this, we indeed observed a higher amount of filamentous actin in DCs derived from Gadkin deficient mice (Figure 2).

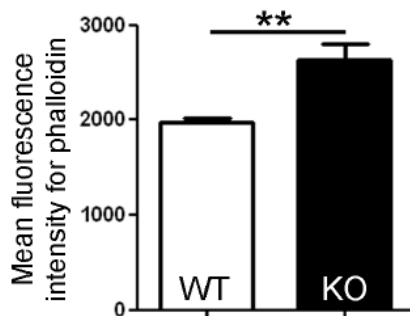


Figure 2: Gadkin KO DCs contain more F-actin

Flow cytometry-based quantification of phalloidin-stained suspension cells that had been starved for 1 h (N=7 independent experiments, unpaired t-test, $**=p<0.01$). Taken from (6).

Loss of Gadkin impairs DC motility in vitro

In B16F1 cells the loss of Gadkin led to increased cell migration due to elevated Arp2/3 activity which is important for the lamellipodia-based migration mode of these melanoma cells. Based on these findings and reports that showed the importance of Arp2/3 activators for DC migration, we assumed that loss of Gadkin might also facilitate DC migration. To determine whether loss of Gadkin indeed affects DC motility, we started off by seeding LPS-matured WT and Gadkin KO DCs into the upper reservoirs of transwells and let them migrate for 3 h towards the chemokine CCL19 or CC21 which was added to the lower reservoir. Afterwards, we assessed the number of migrated cells by flow cytometry. However, while the loss of Gadkin increased the velocity of melanoma cells, it impaired the in vitro migration of DCs. For both chemokines tested Gadkin KO cells migrated significantly less efficiently into the lower reservoir (Figure 3).

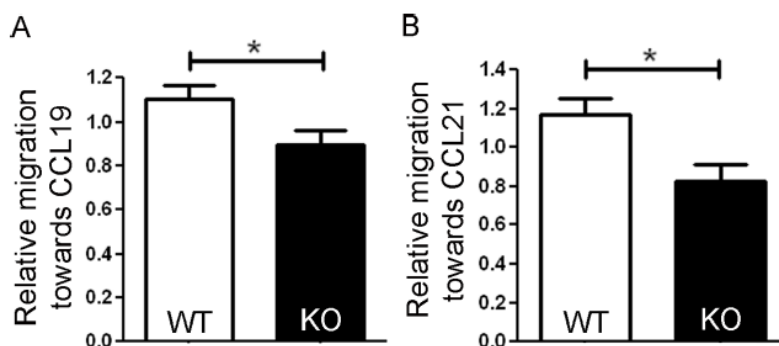


Figure 3: Loss of Gadkin impairs DC chemotaxis in 2D

(A,B) LPS-matured WT and Gadkin KO DCs were seeded into Transwell inserts and allowed to migrate for 3 h towards 250 ng/ml CCL19 (A) resp. 250 ng/ml CCL21 (B). Migration towards the chemokine was analyzed by flow cytometry. Values were subtracted for unspecific migration towards chemokine-free medium to determine the specific migration towards chemokine and then normalized to the average value of each experiment (N=6 independent experiments, unpaired two-tailed t-test, $*=p<0.05$). Taken from (6).

We obtained similar results when we turned to live cell imaging of DCs in 2D chemotaxis chambers where the cells can be tracked while they migrate along a chemokine gradient. Our analysis of the migrating cells revealed a lower velocity of Gadkin KO compared to WT cells

(6). Since ablation of genes regulating actin dynamics like Cdc42 caused especially pronounced effects in 3D DC migration, we also tracked the migration of Gadkin WT and KO DCs after embedding them in collagen type I gels for mimicking collagen-rich connective tissue. However, again we observed a decrease in migration rather than a facilitation (6).

In retrospect, the fact that we observed impaired in vitro migration of Gadkin KO DCs instead of an increase in their velocity makes sense because in 2016 a publication showed that Arp2/3-dependent actin polymerization is in fact not driving DC motility (in contrast to its function in melanoma cells), but rather diminishing the actin pool that is needed for mDia-mediated fast DC migration (7). Therefore, the decreased inhibition of Arp2/3 upon loss of Gadkin should further diminish the actin pool available for fast mDia-dependent DC migration and thus slow migration, in line with our in vitro results.

Loss of Gadkin does not alter DC migration in vivo

To test the physiological relevance of Gadkin's influence on in vitro DC migration, we turned to in vivo experiments. In our first approach we analyzed the migration of endogenous skin-resident DCs in response to skin irritation. A skin irritant was applied together with the dye FITC to the abdomen of mice. DCs attracted to the site will take up the green dye which allows to monitor their arrival at the draining lymph node. In a second approach we injected the footpads of WT mice with a mixture of WT and Gadkin KO DCs which we had differentiated and matured in vitro and finally labeled with two different dyes prior to injection to be able to distinguish between WT and KO cells.

In spite of the impairment of in vitro 2D and 3D migration of Gadkin deficient DCs, we did not observe a difference in vivo in the amount of endogenous or injected WT and Gadkin KO cells that reached the lymph node. In conclusion, loss of Gadkin does not detectably impair the efficiency of DC migration from dermal tissue to draining lymph nodes in vivo. This highlights the importance of in vivo experiments to test the physiological relevance of in vitro data and suggests a high plasticity of the migration process and/or potential compensatory changes in the constitutive Gadkin KO animals that allow overall DC migration to still work in the face of minor mechanistic defects. This is in line with previous observations on the robustness of leukocyte migration (8).

Alternative functions for Gadkin in DCs

Collaborative studies with Nicolas Chevrier (Harvard University, USA) revealed that Gadkin is important for the pro-inflammatory responses of DCs. Having been identified in a screen for LPS-induced phosphoproteins, Gadkin was considered as a candidate regulator of LPS-dependent DC responses such as the release of inflammatory cytokines. LPS triggers the activation of the Toll-like receptor 4 (TLR4) which in turn activates two distinct downstream signaling cascades involving a number of phosphorylation events which lead in the end to the transcription of antiviral and inflammatory genes. Loss-of-function experiments capitalizing on our Gadkin KO DCs confirmed that Gadkin in fact is needed for the induction of inflammatory genes (5), while the transcription of the antiviral genes was overall less affected (Figure 4). The mechanism by which Gadkin modulates the expression of inflammatory genes is currently unclear. However, TLR4-dependent signaling takes partially place at endosomes. Therefore, Gadkin's function in vesicular transport might modulate the signaling cascades upstream of inflammatory gene expression. Clearly, further investigations are needed to unravel Gadkin's exact role in immunity.

Summary

Our studies support the notion that Gadkin plays a role in mature DCs. While constitutive loss of Gadkin does not significantly affect in vivo DC migration, it appears to have strong effects on the inflammatory responses of DCs downstream of TLR4 activation.

The results presented here were published in two papers (PlosOne 2015 (6), Cell Reports 2017 (5)). Moreover, the expertise with DCs which my group gained in this project was essential for my successful application for funding by the "FMP Integrated Project Program" to study a family of actin regulatory proteins in DC migration.

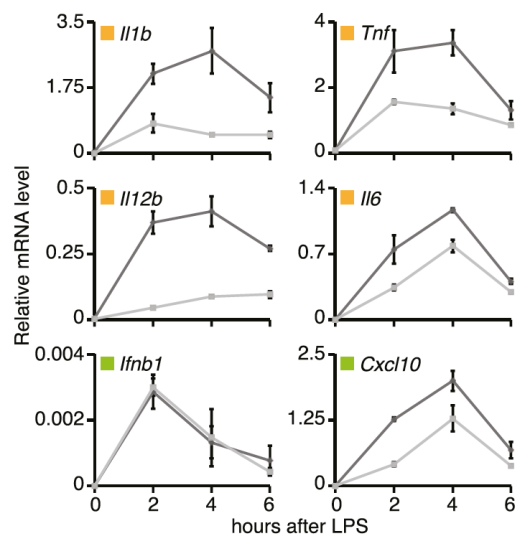


Figure 4: Reduced transcription of inflammatory cytokines upon loss of Gadkin

Gadkin WT and KO DCs were harvested at the indicated times after LPS treatment. RNA was extracted and reverse transcribed. mRNA levels of the indicated inflammatory (orange) and antiviral (green) cytokines were compared between Gadkin KO DCs (dark grey) and WT DCs (light grey) (qPCR relative to GAPDH, 3 replicates per time point, error bars represent standard deviation; Il1b, interleukin 1 β ; Tnf, tumor necrosis factor; Il12b, interleukin 12 β ; Il6, interleukin 6; lfnb1, interferon β 1; Cxcl10, C-X-C motif chemokine 10). Taken from (5).

Results Subproject 2:

Function of Stonin1 at the cellular and organismic level

Introduction

A plethora of cellular processes, among them focal adhesion (FA) dynamics and tumor growth, is orchestrated by extracellular signals cells receive via cell surface receptors. Consequently, a concise spatiotemporal regulation of receptor surface levels is essential to control cellular responses. Endocytosis is crucial for the regulation of cellular signalling by adjusting the number and localization of receptors at the cell surface. Defects in endocytosis have dramatic consequences ranging from impaired cell migration to oncogenic signaling. However, how precisely endocytosis regulates the turnover of distinct cell surface receptors remains in most cases unclear.

Generally, the internalization of many surface proteins is achieved by clathrin-mediated endocytosis. In a highly coordinated series of interactions endocytic proteins progressively bend a patch of plasma membrane into a vesicle, which is stabilized by a coat of clathrin molecules and finally pinched off by the large GTPase dynamin (9). To be sorted into the clathrin-coated pit for internalization, the cargo proteins need to have the appropriate signals. While diverse proteins contain di-leucine or tyrosine-based motifs, which are recognized directly by the general endocytic adaptor AP-2, other proteins lacking such motifs require dedicated cargo-specific endocytic adaptors that link them to the endocytic machinery (10).

Previously, we demonstrated that Stonin2 is such a cargo-specific endocytic adaptor which facilitates the internalization of the synaptic vesicle protein synaptotagmin1 (11). However, the function of the closely related protein Stonin1 remained enigmatic for a long time. The fact that combined loss of Stonin1 and Stonin2 did not aggravate the neuronal defects of Stonin2-deficient mice suggested that Stonin1 has a distinct role. Within this SAW-funded project we dissected the molecular function of Stonin1 as endocytic adaptor for the oncogenic proteoglycan NG2 (also known as CSPG4, AN2, MCSP and HMP), an FA-associated transmembrane protein serving as a co-receptor for integrins and platelet-derived growth factor receptor (PDGFR) (12) and as a promoter of cellular motility (13-19) and tumor growth (20-22). In line with this, we showed the importance of Stonin1 for FA dynamics, cellular motility (23) and tumor growth.

Stonin1 regulates focal adhesion dynamics

Our studies were based on the initial observation that Stonin 1 localizes in a polarized manner preferentially towards the leading edge of migrating cells and is often found right behind focal adhesions (FAs). In addition, Stonin1 mouse embryonic fibroblasts (MEFs) which we derived

from our Stonin1 KO mice showed alterations in the appearance of FAs (23). FAs represent complex contact sites between cells and extracellular matrix that are especially rich in cell surface receptors to mediate cellular adhesion and signaling (24). While integrins are the best-studied adhesion receptors within FAs, adhesion sites contain numerous additional cell surface proteins such as proteoglycans. Although it is clear that dynamic FA turnover is essential for cell motility and requires endocytosis, we still lack detailed knowledge about how the turnover of the various FA components is controlled. While FA disassembly was shown to rely on clathrin (25, 26) and the cargo-specific adaptors ARH and Dab2 (26) to mediate integrin uptake, the fate of other FA components remained unclear, suggesting the involvement of additional, so far unrecognized endocytic adaptor proteins.

To address a potential role of Stonin1 in FA turnover, we started off by imaging tdTomato-tagged Stonin1 together with EGFP-tagged Paxillin, an abundant FA protein. Interestingly, we found Stonin1 to preferentially accumulate at FAs during their disassembly consistent with a function in FA turnover (23). To investigate whether Stonin1 is indeed involved, we first studied the turnover of FAs by fluorescence recovery after photobleaching (FRAP). We photobleached Stonin1 WT and KO cells expressing EGFP-tagged Paxillin and monitored its reappearance. The extent of recovery was strongly reduced in the KO cells indicating an increased pool of immobile Paxillin molecules. This phenotype could be rescued by reexpressing Stonin1 in the KO cells confirming its specificity (Fig. 5).

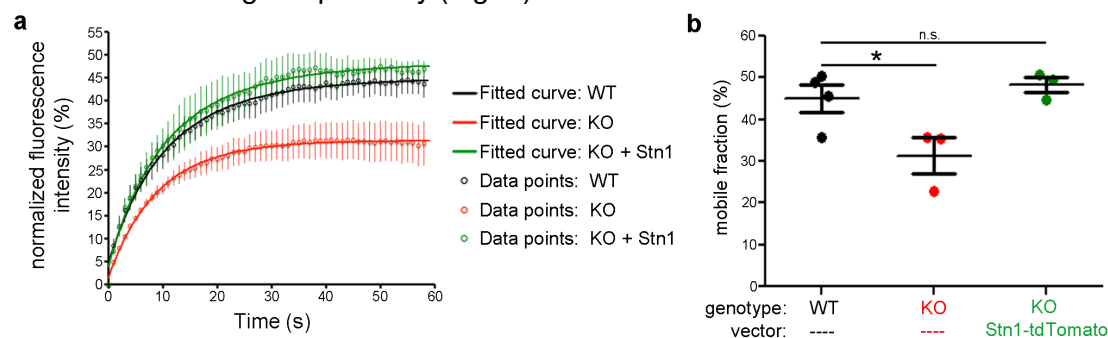


Figure 5: Slowed FA dynamics in the absence of Stonin1

(A) Curves depicting fluorescence recovery after FRAP for Paxillin-EGFP-transfected WT and Stonin1 KO MEFs. Original data points +/- SEM are shown (open circles) as well as fitted curves (solid lines). Re-expression of Stonin1-tdTomato rescued the decreased recovery in Stonin1 KO MEFs. **(B)** Quantification of the recovered i.e. mobile fraction of Paxillin-EGFP molecules based on the exponential fit of the FRAP data. Taken from (23).

To dissect in more detail whether Stonin1 affects the disassembly phase of FAs, we capitalized on the fact that microtubule regrowth after washout of the microtubule depolymerizing drug nocodazole induces synchronized FA disassembly. In line with this, WT MEFs disassemble about 50% of their FAs within 10 min of washout. However, Stonin1 KO MEFs still preserve about 90% of their FAs at this time point (Figure 6). These results identified Stonin1 as facilitator of FA disassembly. This is well in line with the altered cell motility which we had previously observed (23).

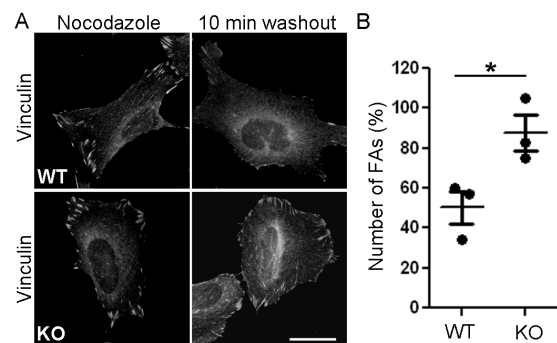


Figure 6: Stonin1 is required for FA disassembly

(A) Starved WT and Stonin1 KO MEFs were left untreated or were incubated with 10 μ M nocodazole for 4 h. After washout, to allow microtubule regrowth to trigger FA disassembly, cells were fixed and immunostained with vinculin- and tubulin-specific antibodies. Scale bar: 25 μ m. **(B)** The number of FAs in WT and Stonin1 KO cells after 10 min of nocodazole washout relative to untreated cells was quantified by thresholded particle counting of vinculin-positive FAs (mean \pm SEM, N=3, unpaired two-tailed Student's t-test, $*=p<0.05$). Taken from (23).

Stonin1 as a cargo adaptor for the internalization of the oncogene NG2

The similarity of Stonin1 to endocytic adaptors suggested that it affects FA disassembly and cell migration by facilitating the uptake of a so far un-identified cargo. Since Stonin1 does not influence the internalization of integrins (23), we had employed a proteomic approach to identify the putative cargo protein. As the cargo should accumulate on the cell surface in absence of its endocytic adaptor, we compared the surface proteome of WT and KO MEFs using SILAC-based quantitative mass spectrometry. This analysis revealed a striking accumulation of the proteoglycan NG2 on the surface of KO MEFs (fold enrichment: 34 ± 14 , $N=2$). Internalization assays confirmed that this increase in NG2 is - as presumed - a consequence of its impaired endocytosis (Figure 7A), thereby establishing Stonin1 as specific endocytic adaptor for NG2. The inefficient endocytosis of NG2 precludes its efficient delivery to the degradative pathway thus resulting also in increased total levels of NG2 (Figure 7B). NG2 is a known regulator of cellular motility (13-19), which controls, for example, the directional migration of oligodendrocyte precursor cells (13). Thus, the elevated NG2 levels in KO MEFs might underlie their altered migratory behaviour. In fact, depleting NG2 by siRNA reverted the directionality of KO cells to the WT pattern confirming that the accumulation of NG2 causes the altered cell motility of KO cells (Figure 7C).

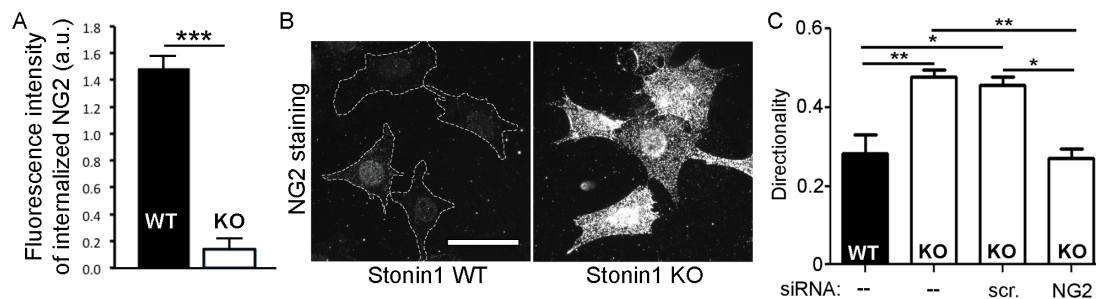


Figure 7: Stonin1 mediates the internalization of NG2 thereby affecting cell motility

(A) Impaired NG2 internalisation in Stonin1 KO MEFs. Quantification of fluorescence intensity of internalized NG2-specific antibodies after 30 min (mean \pm SEM, $N=3$, unpaired two-tailed Student's *t*-test, ***= $p<0.001$). (B) Epifluorescent images of immunostained WT and Stonin1 KO MEFs using NG2-specific antibodies. Scale bar: 50 μ m. (C) NG2 depletion rescues the directionality phenotype. Quantification of directionality (accumulated distance divided by Euclidean distance) for WT and Stonin1 KO MEFs, which were untreated or transfected with scrambled or NG2-specific siRNA (mean \pm SEM, $N=3$, one-way ANOVA + Tukey post-test, **= $p<0.01$, *= $p<0.05$). Taken from (23).

Stonin1 in cellular signaling

Since NG2 is a well known oncogene that affects cellular signal transduction cascades, we focused within this SAW project on potential alterations in the signaling of Stonin1 KO cells. NG2 being a co-receptor for the PDGFR, altered NG2 levels likely affect signaling pathways downstream of the PDGFR. In fact, we could show that Stonin1 KO cells are unable to efficiently dissolve NG2 signaling clusters resulting in increased PDGFR activation (23). Activated PDGFR is known to induce circular dorsal ruffles (CDRs) in MEFs. CDRs are actin-rich membrane structures that are involved in the bulk internalization of transmembrane proteins such as EGFR and in cell motility. In a first step we showed that NG2 colocalizes with activated PDGFR at CDRs (Figure 8A). When we induced CDR formation by incubating cells with PDGF, we found that the Stonin1 KO cells were more prone to generate CDRs than the WT cells supporting the idea that loss of Stonin1 leads to elevated PDGFR-dependent signaling (Figure 8B-C).

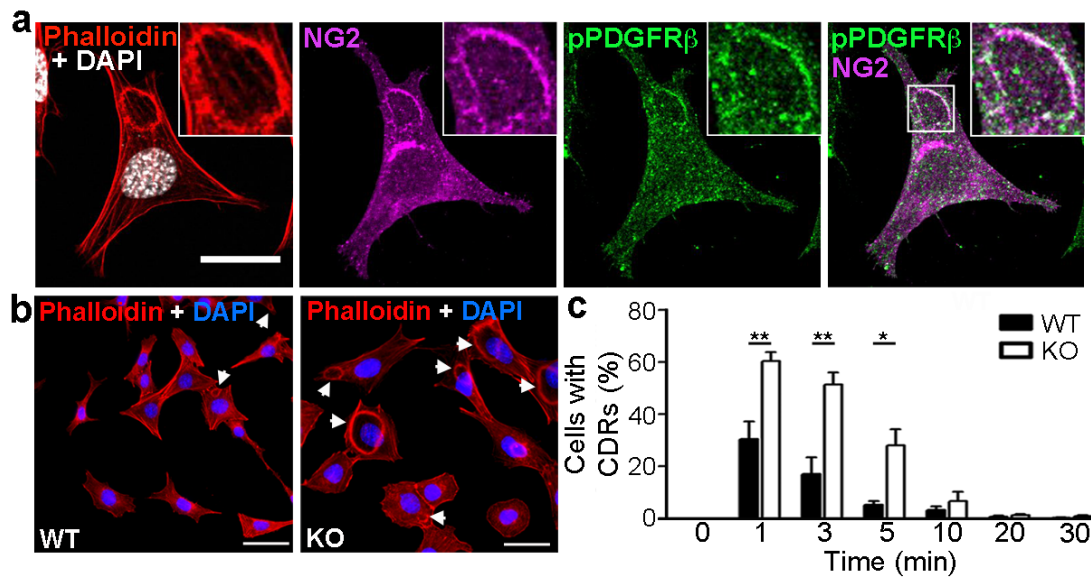


Figure 8: Increased CDR formation in Stonin1 KO MEFs

(A) NG2 localizes to CDRs. Confocal image of NG2-transfected MEF after 5 min incubation with PDGF and immunolabelled with antibodies against NG2 and phosphorylated (p) active PDGFR β . Actin was visualized by fluorescently labelled phalloidin. Nuclei were stained with DAPI. Scale bar: 25 μ m. **(B,C)** Stonin1 KO MEFs form more CDRs upon PDGF. **(B)** Example epifluorescent images of WT and Stonin1 KO MEFs treated for 3 min with PDGF and immunostained with fluorescently labelled phalloidin to visualize F-actin. Arrows point to CDRs. Scale bar: 25 μ m. **(C)** Quantification of the percentage of cells with CDRs from images as in B after different incubation times with PDGF. Taken from (23).

These results were published in 2015 in Nature Communications. However, we gained additional insights into the roles of Stonin1 during the funding period. These will be part of future manuscripts.

Stonin1 in tumorigenesis

Dysregulation of cellular signaling is a hallmark of cancer. Besides Stonin1's cargo NG2 is not only involved in cellular motility, but also a known oncogene, which is expressed by a variety of tumors such as melanomas, chondrosarcomas, myeloid leukemias and diffusely infiltrating gliomas (22) and has been targeted in preclinical models of glioblastoma multiforme and melanoma (27). In fact, several reports suggest that NG2 expression correlates with the degree of malignancy of gliomas (28-33). Glioma mouse models demonstrate an increased growth rate of NG2-positive tumors, while depleting NG2 reduces tumor growth (27). Therefore, we hypothesized that Stonin1 might restrain glioma growth via its control over NG2 surface levels. Accordingly, elevated Stonin1 expression might be beneficial for glioma patient survival. We capitalized on the glioma repository REMBRANDT to test this hypothesis. Indeed, glioma patients, whose samples displayed 3-fold elevated Stonin1 levels, had a significantly higher probability of survival (Figure 9A). In line with this, high levels of Stonin1 in patient samples correlate with low levels of NG2 (Figure 9B). If Stonin1 controls NG2 levels in the brain, both proteins should be expressed in glial cells. In fact, Stonin1 is highly expressed in cultured glial cells where it co-localizes with NG2 at steady-state (Figure 9C). To determine whether Stonin1 has an impact on NG2 levels in vivo we performed immunohistochemistry using NG2-specific antibodies on brain sections of 14 days old animals. Indeed, NG2 levels were increased in sections from Stonin1 KO brains compared to WT (Figure 9D,E).

To directly test if loss of Stonin1 promotes glioma growth, we took different approaches, since NG2 supports tumor growth in different ways: It is well established that tumor growth depends not only on properties of the tumor cells themselves, but also on properties of cells in the tumor microenvironment that support for instance tumor vascularization. In line with this, NG2 promotes tumor growth not only when present in tumor cells (20), but also by its presence in the tumor microenvironment (21). Its expression by pericytes and macrophages is crucial for

tumor vascularization (34-36), a prerequisite for tumor growth. Accordingly, early stage growth of injected intracranial tumors was dramatically reduced in pericyte- or macrophage-specific NG2 KO mice (35). In line with this, we formulated the hypothesis that loss of Stonin1 might likewise promote tumor growth either by affecting the tumor environment or by affecting the tumor cells.

To test potential effects of a Stonin1 KO tumor environment, we injected in collaboration with the group of Prof. Niendorf/Dr. Waiczies (MDC, Berlin) glioma cells into the brain of WT and Stonin1 KO mice and evaluated the tumor volume two weeks post-injection by magnetic resonance imaging (MRI). Stonin1 KO mice developed significantly larger tumors than WT mice (Fig. 9F), indicating that a Stonin1 KO tumor environment has tumor-promoting effects, presumably due to increased NG2 levels and thereby enhanced tumor vascularization.

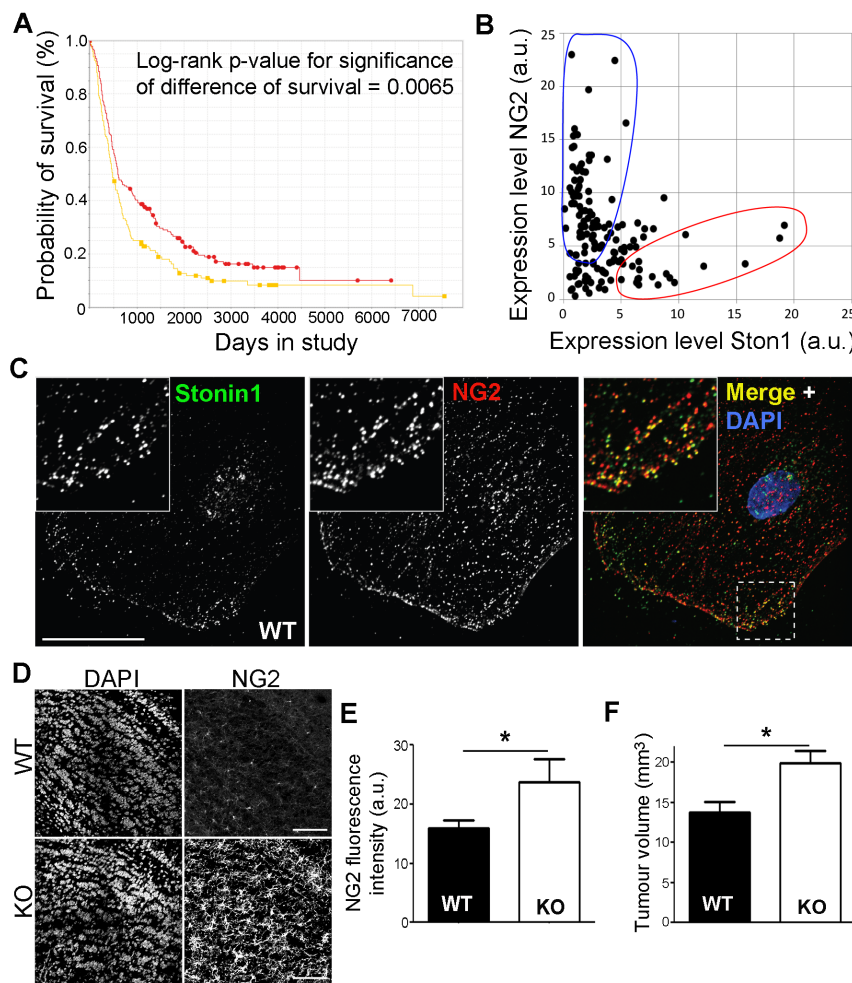


Figure 9: Stonin1, a putative tumor suppressor

(A) Elevated Stonin1 levels in gliomas correlate with significantly prolonged survival of glioma patients. Comparison between survival of patients with 3-fold higher Stonin1 expression in glioma (red; N=191) and patients with intermediate Stonin1 expression (yellow; N=150) (from REMBRANDT database). (B) Inverse correlation of Stonin1 and NG2 expression in astrocytomas (N=148). Tumors with high Stonin1 (red circle) display low NG2, while tumors with high NG2 (blue circle) display low Stonin1 (REMBRANDT). (C) Stonin1 and NG2 partially co-localize in glial cells. Confocal images of fixed glial cells immunolabelled with Stonin1- and NG2-specific antibodies. (D) Confocal images of sections of WT and Stonin1 KO olfactory bulbs of 14 days old animals immunolabelled with NG2-specific antibodies. Nuclei were stained with DAPI. Scale bar: 100 μ m. (E) Analysis of NG2 fluorescence intensity from images like in (D) (mean \pm SEM, N=5, unpaired one-tailed Student's t-test, $*=p<0.05$). (F) Increased glioma growth in Stonin1 KO mice. Glioma cells were injected into the brains of WT and Stonin1 KO mice. 14 days after injection the tumor volume was measured by MRI (mean \pm SEM, N=6, unpaired two-tailed Student's t-test, $*=p<0.05$). Unpublished work.

As NG2 also promotes tumor growth when expressed by the tumor cells themselves via enhancing their proliferation and metastatic properties, Stonin1 KO tumor cells might also give rise to larger tumors, since they are presumed to have elevated NG2-dependent signaling. To test this, we virally transformed neural precursor cells (NPCs) derived from WT or Stonin1 KO embryos with PDGF-B to render them tumorigenic and injected them into WT mice in collaboration with the group of Prof. Glass (LMU, Munich). 41 days post-injection tumor cells were histologically detected in 4/7 mice injected with WT NPCs and 4/8 mice injected with Stonin1 KO NPCs. While loss of Stonin1 did not alter the ability of transformed NPCs to induce tumors, there was a tendency to increased tumor growth in case of the KO NPCs. 3/4 animals injected with transformed Stonin1 KO NPCs had developed tumours of $>1 \text{ mm}^3$, but 0/4 animals inoculated with transformed WT NPCs (WT: $0.5 \pm 0.2 \text{ mm}^3$; KO: $3.6 \pm 1.6 \text{ mm}^3$, $n=4$). Due to the small number of mice in this experiment, which developed tumors at all, these data are not significant yet, but have to be substantiated by additional experiments for which we obtained follow-up funding by the DFG. However, our collective data suggest that Stonin1 has indeed tumor-suppressive effects in models of glioma growth. It is likely the dysregulation of NG2 in Stonin1 KO cells which promotes tumor growth. As elevated levels of Stonin1 significantly correlate with increased glioma patient survival, further insights into Stonin1's function will be important to pave the way for new approaches to glioma therapy.

Stonin1 in FA signaling and maturation

We also further investigated the role of Stonin1 in FA dynamics during the funding period. As described earlier, Stonin1 is critical for the efficient disassembly of FAs. This should lead to larger FAs at steady state as observed for other proteins that promote FA disassembly like p14 (37). However, Stonin1 KO MEFs display instead smaller FAs at steady state (23) suggesting an additional role in FA maturation. FA maturation critically involves FAK phosphorylation in response to activated integrins or growth factor receptors such as PDGFR (38). To analyze potential effects of Stonin1 loss on FA signaling and thus maturation we stimulated starved WT and Stonin1 KO cells with PDGF. After 10 min WT cells had restored FAK phosphorylation to pre-starvation levels while Stonin1 KO MEFs, even after 30 min, exhibited strongly decreased levels of pFAK (Figure 10A,B). When cells were monitored by immunofluorescence using FAKp397-specific antibodies, we observed not only a lower level of FAKp397 intensity, but also a striking difference in FAKp397 appearance (Figure 10C). In WT cells after 10 min FAKp397-positive structures were elongated and resembled well developed FAs. In contrast, Stonin1 KO cells contained dot-like and mostly peripheral FAKp397 signals resembling focal complexes. Thus, maturation of focal complexes into mature FAKp397-enriched FAs appears to be delayed in the absence of Stonin1. We confirmed that this delay is specific to the loss of Stonin1 by re-expressing Stonin1-EGFP in Stonin1 KO cells. Re-expression of Stonin1 returned the FAKp397 intensity and pattern in Stonin1 KO MEFs to WT levels, while a truncated version of Stonin1, comprising only the cargo-binding μ homology domain (μ HD), did not (Figure 10G,D). Depletion of NG2 also did not rescue the low level of FAKp397 10min after PDGF treatment (data not shown).

Interestingly, our data suggest for the first time that an endocytic protein is involved in FA maturation, as FAK activation is delayed in Stonin1 KO cells and FAs are reduced in size. FA maturation does not only require MyosinII-dependent tension, but also actin-binding proteins that mediate the formation of the lamellar actin network (39-43). Using proteomic approaches we identified actin-regulatory factors as putative Stonin1 interactors. This suggests that Stonin1 might affect FA maturation not by serving as an endocytic adaptor for NG2, but as a modulator of the actin cytoskeleton, a hypothesis that we are currently testing.

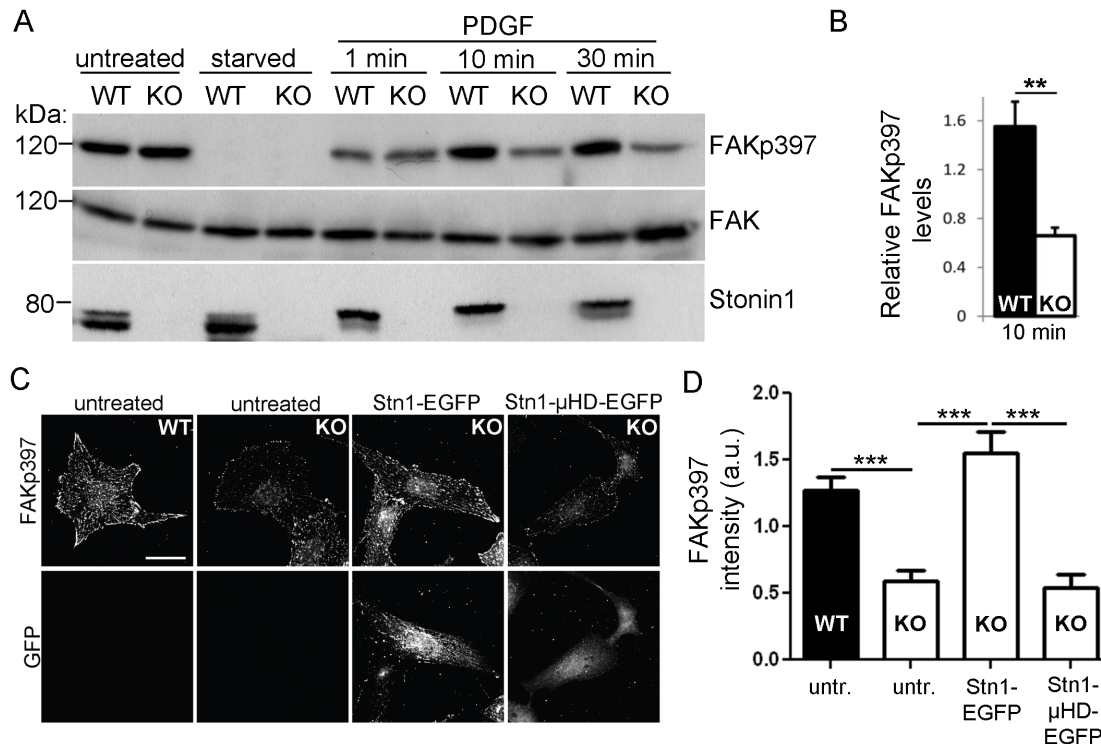


Figure 10: Delayed recovery of FAKp397 phosphorylation in Stonin1 KO MEFs

(A) Lysates from untreated, starved or starved and stimulated (50 ng/ μ l PDGF) WT and Stonin1 KO MEFs were analyzed by immunoblotting. (B) Quantification of FAKp397 levels 10 min after PDGF stimulation (mean \pm SEM, N=3, unpaired two-tailed Student's t-test, **= p <0.01). (C) FAKp397-positive structures are still small and peripheral in Stonin1 KO MEFs after 10 min PDGF stimulation, a phenotype rescued by Stonin1 full-length re-expression. Starved WT and Stonin1 KO MEFs untreated or lentivirally transduced as indicated were incubated with 50 ng/ μ l PDGF, fixed and stained with FAKp397-specific antibodies. (D) Quantification of FAKp397 fluorescence intensity (mean \pm SEM, N=3-7, one-way ANOVA + Tukey post-test, ***= p <0.001). Unpublished work.

Regulation of Stonin1

The critical functions of Stonin1 as endocytic adaptor of an oncogenic signaling protein and as modulator of FA dynamics require an elaborate regulation of its action. If Stonin1 is supposed to control the surface level of NG2 and the dynamics of FAs in a manner that is responsive to the acute cellular demands, Stonin1 itself has to be subject to acute regulation. In line with this notion, the appearance of Stonin1 as two bands of varying intensity on immunoblots depending on culture conditions suggested its dynamic posttranslational modification. Treatment with calf intestinal phosphatase revealed that Stonin1 is in fact phosphorylated (Figure 11A). Starvation as well as application of blebbistatin leads to a decrease in the phosphorylated Stonin1 variant (Figure 11B). Blebbistatin is an inhibitor of myosinII and causes FA disassembly due to loss of myosinII-dependent tensile forces, which are necessary for FA maturation and integrity (44). Its effect on Stonin1 implies that Stonin1 might be regulated coordinately with FA disassembly. Stimulation of cells with PDGF on the other hand greatly increases the phosphorylation of Stonin1 (Figure 11C).

These observations led to the question in which way phosphorylation might affect the function of Stonin1. When staining Stonin1 in PDGF-treated cells, we observed a strikingly lower signal intensity (Figure 11D-E). Since the protein amount of Stonin1 is not substantially changed by PDGF treatment according to immunoblots, this suggested that Stonin1 might be lost during the staining procedure due to a more cytosolic distribution after PDGF treatment. Thus, we formulated the hypothesis that phosphorylation might decrease the association of Stonin1 with membranes. To test this hypothesis, we performed a membrane fractionation. Indeed, only the non phosphorylated form was detectable in the membrane fraction (Figure

11F).

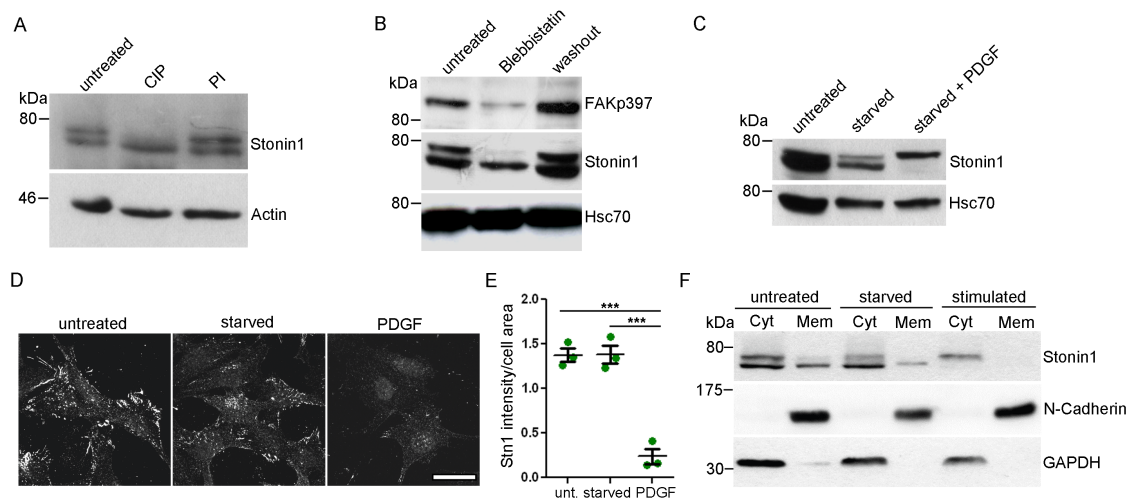


Figure 11: Stonin1 is regulated by phosphorylation

(A) Stonin1 is phosphorylated. Untreated lysates and lysates incubated for 10 min at 37°C with calf intestine phosphatase (CIP) or phosphatase inhibitors (PI) were probed by immuno-blotting. **(B)** Blebbistatin, a myosinII inhibitor, causes dephosphorylation of Stonin1. MEFs were harvested either directly or after 30 min treatment with blebbistatin (50 μ M) or after a 60 min washout. Lysates were probed by immunoblotting. **(C)** Phosphorylation is induced by PDGF. Cells were starved for 12 h in DMEM without FCS and subsequently left untreated or incubated with 50 ng/ml PDGF for 30 min. Lysates were probed by immunoblotting. **(D)** PDGF dissociates Stonin1 from membranes. Cells treated as indicated were fixed and stained with a Stonin1-specific antibody. **(E)** Quantification of Stonin1 fluorescence intensity in images as in (D) (mean \pm SEM, N=3, one-way ANOVA + Tukey post-test, ***=p<0.001). **(F)** Dephospho-Stonin1 associates with membranes. Membrane fractionation of cells treated as indicated (starvation: as above; stimulation: 10% serum for 30 min). Fractions were probed by immunoblotting. Unpublished work.

As Stonin1 is in principle a cytosolic protein without apparent lipid binding domains, it seems likely that the non phosphorylated protein is recruited to membranes via protein interactions that are abolished upon phosphorylation. To dissect the phosphorylation sites in Stonin1 and to thereby gain a detailed understanding of the phosphorylation-dependent control of Stonin1's membrane recruitment in the context of FA dynamics and endocytosis-mediated NG2 regulation will be a focus for our future work on Stonin1.

Summary

Our studies on Stonin1 illustrate how regulation by endocytic adaptors fine-tunes focal adhesion dynamics, cell migration and cellular signaling. As dysregulation of these processes is a hallmark of cancer and in light of the established function of NG2 as oncogene, it seems likely that Stonin1 might act as tumor suppressor by limiting NG2's oncogenic potential via its removal from the plasma membrane.

Our work on Stonin1 was presented at numerous conferences and has been received with great interest by the scientific community since its publication in 2015. Moreover, the data on Stonin1 which we collected during the funding period have laid the foundation for my successful application for subsequent funding of our investigations on Stonin's role in focal adhesion dynamics and tumorigenesis by the DFG (MA4735/2-1).

Statement regarding economic usability

The work performed within this project is research into basic cell biological mechanisms which promotes our understanding of the importance of endocytosis and cytoskeletal dynamics for cell signaling and cell migration and thereby for the development of cancer and immune system dysfunctions. On the long-term we expect our results to help to devise new targeted therapies for these conditions, however, there is no immediate economic usability of our findings.

Qualification of junior scientists

Habilitation: Dr. Tanja Maritzen, at Freie Universität Berlin in 2016

PhD theses: Marietta Browarski, at Freie Universität Berlin in 2017

Collaborations

- Eberhard Krause, FMP, Berlin, Germany
 - Mass spectrometrical analyses
- Uta Höpken, MDC, Berlin, Germany
 - Assistance with in vivo DC migration assays
- Thoralf Niendorf & Dr. Sonia Waiczies, MDC, Berlin, Germany
 - MRI-based tumor studies
- Rainer Glass, Ludwig-Maximilians-Universität München, Germany
 - Studies on tumors derived from Stonin1 WT and KO cells
- Nicolas Chevrier, Harvard University, Cambridge, USA
 - Studies of inflammatory responses of Gadkin deficient DCs
- William B. Stallcup, Sanford-Burnham Medical Research Institute, La Jolla, USA
 - Reagents for the study of NG2
- Daniel Legler, Biotechnologie Institut Thurgau (BITg), Kreuzlingen, Schweiz
 - Contribution to joint review

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