Stonin1 mediates endocytosis of the proteoglycan NG2 and regulates focal adhesion dynamics and cell motility

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Cellular functions, ranging from focal adhesion (FA) dynamics and cell motility to tumour growth, are orchestrated by signals cells receive from outside via cell surface receptors. Signalling is fine-tuned by the exo–endocytic cycling of these receptors to control cellular responses such as FA dynamics, which determine cell motility. How precisely endocytosis regulates turnover of the various cell surface receptors remains unclear. Here we identify Stonin1, an endocytic adaptor of unknown function, as a regulator of FA dynamics and cell motility, and demonstrate that it facilitates the internalization of the oncogenic proteoglycan NG2, a co-receptor of integrins and platelet-derived growth factor receptor. Embryonic fibroblasts obtained from Stonin1-deficient mice display a marked surface accumulation of NG2, increased cellular signalling and defective FA disassembly as well as altered cellular motility. These data establish Stonin1 as a specific adaptor for the endocytosis of NG2 and as an important factor for FA dynamics and cell migration.
Endocytosis is essential for the regulation of cellular signalling by adjusting the number and localization of receptors at the cell surface. Alterations in endocytosis affect focal adhesion (FA) dynamics and cellular migration, which crucially depend on the precise spatiotemporal regulation of the surface levels of various adhesion proteins. In fact, dysregulated endocytosis causes defects in cellular motility and is associated with cancer.

FAs represent complex contact sites between cells and extracellular matrix that are especially rich in cell surface receptors to mediate cellular adhesion and signalling. While integrins are the best-studied adhesion receptors within FAs, adhesion sites contain numerous additional cell surface proteins such as syndecans and other proteoglycans. Although it is clear that dynamic FA turnover is essential for cell motility and requires exo- and 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 and the cargo-specific adaptors ARH and Dab2 (ref. 5) to mediate integrin uptake, the fate of other FA components remains unclear.

Cargo-specific adaptors are required whenever surface proteins do not contain the necessary consensus motifs to bind to the general endocytic adapter AP-2, which recruits most cargo proteins for clathrin-mediated endocytosis. By linking their corresponding cargo to the endocytic machinery, they ensure its efficient internalization. The group of cargo-specific adaptors includes also the Stonin proteins, which interact with AP-2 and cargo proteins. While Drosophila melanogaster contains a single Stonin protein termed StonedB, homology searches in mammals revealed two Stonin orthologues termed StonedB and acts as an endocytic adaptor for the synaptic vesicle protein Synaptotagmin1 (ref. 9). In contrast to organs such as lung, fibroblasts express only Stonin1, in contrast to Stonin2, which remained enigmatic.

The fact that combined loss of Stonin1 and Stonin2 functionally similar to StonedB and acts as an endocytic adaptor for the synaptic vesicle protein Synaptotagmin1 (ref. 9). In fact, dysregulated endocytosis causes defects in cellular motility and is associated with cancer.

In this study, we deleted Stonin1 in mice and found that it acts as an important regulator of FA dynamics and cellular motility. We show that Stonin1 is crucial for the efficient internalization of the proteoglycan NG2 (also known as CSPG4, AN2, MCSP and HMP), an FA-associated transmembrane protein serving as a co-receptor for integrins and the platelet-derived growth factor receptor (PDGFR) and as a promoter of cellular motility and tumour growth. In the absence of Stonin1, NG2 accumulates at the cell surface, which alters cell migration. Thus, we establish Stonin1 as a specific endocytic adaptor for NG2 with important roles in FA dynamics and cellular motility.

**Results**

Stonin1 regulates FA dynamics. Consistent with our findings, two proteomic studies previously identified Stonin1 as a FA component. Stonin1 was one of the few proteins not detected in steady-state FAs, but found selectively when FA disassembly was triggered by blebbistatin-mediated inhibition of myosinII. MyosinII-dependent tensile forces are necessary for FA maturation and integrity. Their loss prevents FA maturation and causes the disassembly of mature FAs, resulting in an enrichment of nascent adhesions. To catch FAs in the process of disassembly, we treated cells briefly with blebbistatin. Under these conditions Stonin1 formed enlarged patches, which partially colocalized with vinculin and likely represent disassembling FAs (Fig. 2a,b), suggesting that Stonin1 preferentially localizes to disassembling FAs. To study this process in living cells, we performed dual-colour total internal reflection fluorescence (TIRF) imaging of Stonin1−/−/MEFs expressing tdTomato-Stonin1 and the FA marker enhanced green fluorescent protein (EGFP)-Paxillin. Stonin1 fluorescence accumulated precisely at the time when Paxillin fluorescence faded as FAs disassembled. Conversely, Stonin1 levels declined as new FAs formed (Fig. 2c). The fact that a fraction of FAs was stable during the time course of imaging while others progressively lost the Paxillin signal, provided us with a means to correlate FA behaviour with Stonin1 levels. Indeed, FAs with high Stonin1 levels typically underwent disassembly, whereas stable FAs usually lacked Stonin1 (Fig. 2d–f; Supplementary Movie 1).

To dissect whether Stonin1 modulates FA dynamics, we employed fluorescence recovery after photobleaching (FRAP). Previous FRAP studies demonstrated that FAs differ in their dynamic protein-exchange rate and in the density of their components, which affects FA remodelling. Paxillin-EGFP expressing wild-type (WT) and Stonin1−/−/MEFs were photobleached, and FRAP was measured. While the kinetics of recovery did not differ significantly between WT and Stonin1−/−/MEFs, the extent was strongly reduced in Stonin1−/−/MEFs (Fig. 2g,h; Supplementary Fig. 3a–c). The reduced overall recovery of Paxillin-EGFP signifies that Paxillin molecules are not efficiently exchanged, suggesting that FAs in Stonin1−/−/MEFs contain a large proportion of immobile molecules. This is similar to p14−/−/MEFs, which display enlarged and hyperstable FAs due to defective endosomal delivery of factors required for FA dynamics.
**Figure 1 | Stonin1 is an endocytic adaptor localizing close to FAs.** (a) Stonin1 and Stonin2 domain structure. SHD, Stonin homology domain; μHD, μ-homology domain. H.s., Homo sapiens; M.m., Mus musculus. (b) Stonin1 interacts with AP-2. Bead-coupled GST-AP-2-2x-ear resp. GST as control was incubated with lysate from cells overexpressing Stonin1-haemagglutinin (HA). Bound proteins were eluted from washed beads and analysed by immunoblotting with HA-specific antibodies. (c) Stonin1 associates with endocytic and FA proteins. Bead-coupled Stonin1-specific antibodies were incubated with WT and Stonin1−/− lung lysate. Bound proteins were eluted from washed beads and analysed by immunoblotting with the indicated antibodies (* indicates an unspecific band). (d) Stonin1 and Stonin2 protein expression in different tissue and cell types. Lysates from WT and Stonin1−/− cells were analysed by immunoblotting using antibodies specific for Stonin1, Stonin2 and actin. MLFs, mouse lung fibroblasts; MEFs, mouse embryonic fibroblasts. (e-j) Stonin1 co-localizes with endocytic proteins at peripheral CCPs close to FAs. (e-g) Fixed WT and Stonin1−/− MEFs were stained with antibodies specific for the indicated proteins and analysed by confocal microscopy. Insets show enlargements of boxed areas. DAPI-stained nuclei are depicted in blue. Scale bar, 25 μm. (f) Quantification of co-localization from images such as in e using Mander’s coefficients. Red: correlation of Stonin1 with endocytic proteins; green: reverse correlation (data are depicted as mean ± s.e.m., n = 9–10, unpaired two-tailed Student’s t-test for comparisons between two types of correlations for the same staining, one-way analysis of variance followed by Dunnett post-test to compare Mander’s coefficients for correlation of Numb with Stonin1 with Mander’s coefficients for correlations of other endocytic proteins with Stonin1, ***P<0.0001, **P<0.001, *P<0.01). Only the outer 2/3 of cell area were evaluated to exclude unspecific nuclear background staining. (h) Comparison of Stonin1 and AP-2 distribution across the cell based on clock scans on images such as in e (data are depicted as mean ± s.e.m., n = 14, unpaired two-tailed Student’s t-test, **P<0.01, *P<0.05). DAPI, 4,6-diamidino-2-phenylindole; NS, not significant.

We therefore addressed FA disassembly directly capitalizing on the fact that microtubule regrowth after Nocodazole washout induces FA disassembly. 10 min after Nocodazole washout, WT cells had lost about 50% of their FAs, while about 90% were still retained in Stonin1−/− MEFs (Fig. 3a,b). Only when WT cells had already started to generate new FAs about 30 min after washout, had the Stonin1−/− cells finally lost the majority of their FAs. Collectively, these data identify Stonin1 as an important facilitator of FA disassembly.

To study whether the observed changes in FA dynamics in Stonin1−/− MEFs affect FAs at steady state, we immunolabelled...
WT and Stonin1−/− cells with Paxillin-specific antibodies. Contrary to the phenotype of p14−/− MEFs, we did not observe enlarged FAs, but an increased number of small adhesions with decreased Paxillin intensity (Fig. 3c–f). This suggests that altered FA dynamics in absence of Stonin1 do not only impair FA disassembly, but likely also FA maturation.
In summary, Stonin1 is required for dynamic protein exchange within FAs and for their efficient disassembly.

Loss of Stonin1 alters cell shape and motility. FA dynamics are intimately linked to cell shape and motility. Consistent with their altered FA turnover, Stonin1

MEFs exhibited an altered shape with broader and more pronounced protrusions as evidenced by their reduced solidity (cell body outline divided by convex hull) (Fig. 4a,b). To address cellular motility, we tracked the random migration of WT and Stonin1

MEFs. WT cells showed normal persistence of leading edge protrusions and trailing ends. Persistent migration of WT and Stonin1

MEFs was, thus, presumably caused by the combined effects of greater persistence of leading edge protrusions and trailing ends.

Stonin1 interacts with NG2 facilitating its internalization. The data presented so far identify Stonin1 as an important regulator of FA dynamics and cellular motility. Loss of Stonin1 impairs FA disassembly and increases migratory directionality. The similarity of Stonin1 to endocytic adaptors suggests that it controls these processes by facilitating the internalization of a specific cargo. However, so far no cargo for Stonin1 has been identified. Endocytic adaptors previously implicated in FA disassembly promote endocytosis of integrins

and NG2 has been targeted in preclinical models of glioblastoma multiforme and melanoma

Fluorescence-activated cell sorting analysis of NG2 levels in WT and

suggesting that Stonin1 acts on a distinct cargo. To identify this cargo and to unravel the mechanism underlying Stonin1’s impact on cell motility, we employed a proteomic approach. As the cargo protein should accumulate on the cell surface in absence of its endocytic adaptor, we determined the surface proteome of WT and Stonin1

MEFs using stable isotope labelling with amino acids in cell culture-based quantitative mass spectrometry. This analysis revealed a striking accumulation of the proteoglycan NG2 on the surface of Stonin1

MEFs (fold enrichment: 34 ± 14, N = 2, data given as mean ± s.e.m., compare Supplementary Data 1). NG2 interacts with extracellular matrix components, functions as co-receptor for PDGFR and integrins and regulates cellular motility

In addition, it is a known oncogene, which has been targeted in preclinical models of glioblastoma multiforme and melanoma

Fluorescence-activated cell sorting analysis of NG2 levels in WT and
Stonin1−/− MEFs confirmed the marked increase of NG2 at the surface of Stonin1−/− cells, while β1-integrin levels were unchanged (Fig. 5b). To ascertain that elevated NG2 levels in Stonin1−/− cells are caused by defective endocytosis, we performed uptake assays with NG2-specific antibodies. These demonstrated a markedly impaired internalization of NG2 in Stonin1−/− cells (Fig. 5c), whereas uptake of β1-integrin proceeded unaltered (Fig. 5a).

So far endocytosis has not been described as a mechanism for the regulation of NG2, which is proposed to be removed from the cell surface by proteolysis. If NG2 is endocytosed, it should be detectable in endosomal vesicles. To test this, we monitored early endosomes labelled with Rab5-EGFP, together with SNAP-tagged NG2. Indeed, coincident with its disappearance from the cell surface NG2 entered Rab5-positive endosomes (Supplementary Fig. 4a). At least a fraction of internalized NG2 is presumably degraded in lysosomes, as loss of Stonin1 did not only cause NG2 surface accumulation, but also greatly increased total NG2 levels (Fig. 5d,e). Elevated NG2 levels in absence of Stonin1 were confirmed in acutely isolated mouse lung fibroblasts (MLFs) and in a second independent WT/Stonin1−/− MEF pair (Fig. 5f). In addition, we conducted rescue experiments to analyse whether the NG2 accumulation is indeed caused by Stonin1 deficiency. As expected, re-expression of EGFP-Stonin1 in Stonin1−/− cells significantly reduced NG2 levels, while EGFP expression did not (Fig. 5g).

If Stonin1 acts as an endocytic adaptor for NG2, both proteins should form a transient complex. To test this hypothesis, we incubated lung lysate as an abundant source of Stonin1 with the GST-fused cytosolic tail of NG2. Indeed, the cytosolic NG2 tail was able to precipitate endogenous full-length Stonin1 (Fig. 5h). To delineate the binding site within Stonin1, we used HEK293T cells overexpressing full-length NG2 in combination with different Stonin1 variants for co-immunoprecipitations. This revealed that NG2 binds to the Stonin1-μHD (Fig. 5i). Surprisingly, in these experiments we could not detect binding between full-length Stonin1 and NG2. This might be due to the lower expression level of ectopically expressed full-length Stonin1 as compared with the Stonin1-μHD. However, this result might also indicate that full-length Stonin1 binds less efficiently to NG2, suggesting that Stonin1 might be regulated by autoinhibition, the exact mechanism of which remains to be determined. To dissect the mode by which NG2 binds to Stonin1-μHD, we conducted affinity chromatography using progressively truncated GST fusions of the NG2 intracellular domain, which were incubated with lysates from Stonin1-μHD-expressing cells. Deleting the PDZ-binding motif QYWV at the C terminus of NG2 blocked binding of the Stonin1-μHD (Fig. 5j). We conclude from our biochemical analyses that Stonin1 associates with the C-terminal PDZ-binding motif of NG2, possibly indirectly via a PDZ domain protein, as Stonin1 lacks a PDZ domain. In addition, complex formation between NG2 and Stonin1 is also evident from their...
Figure 5 | Stonin1 facilitates NG2 internalization. (a) Normal β1-integrin internalization in Stonin1−/− MEFs. Fluorescent β1-integrin-specific antibodies internalized after 30 min were measured by flow cytometry (N = 3). (b) Increased NG2 surface levels in Stonin1−/− MEFs. Surface levels of NG2 and β1-integrin were analysed by flow cytometry (N = 3). (c) Impaired NG2 internalization in Stonin1−/− MEFs. Quantification of fluorescence intensity of internalized NG2-specific antibodies after 30 min (N = 3). (d-f) Increased total NG2 levels in Stonin1−/− MEF. (g) Lysates of WT and Stonin1−/− cells were analysed by immunoblotting with antibodies against the indicated proteins. (e) Epifluorescent images of immunostained WT and Stonin1−/− MEFs using NG2-specific antibodies. Scale bar, 50 μm. (f) Quantification of total NG2 levels from images such as in e from two independent MEF pairs and primary MLFs (N = 3, n > 100). (g) Re-expression of Stonin1 partially rescues increased NG2 levels. Quantification of NG2 immunostainings of WT and Stonin1−/−/MEFs lentivirally transduced for 72 h to re-express Stonin1-EGFP or EGFP. Scale bar, 50 μm (N = 3). (h) Stonin1 and NG2 form a complex. Left: bead-coupled GST-tagged NG2-cytosolic-tail resp. GST as control was incubated with lysates from WT and Stonin1−/−/MEFs internalized after 30 min were measured by flow cytometry (N = 3). (i) Removal of the NG2 PDZ motif significantly impairs binding of Stonin1-μHD. Bead-coupled progressively truncated GST-tagged variants of the NG2-cytosolic-tail, as well as GST were incubated with lysates from HEK293 cells overexpressing full-length (FL) haemagglutinin (HA)-tagged Stonin1 or N-resp. C-terminal truncations. After elution from washed beads, samples were analysed by immunoblotting with GST- and Gadkin-specific (= control) antibodies. Right: Coomassie gel indicating integrity of purified proteins. (j) The Stonin1-μHD interacts with NG2. Beads coupled to NG2-specific antibodies were incubated with lysates from HEK293 cells overexpressing full-length (FL) NG2-specific antibodies that move and disappear together (k) and in mobile spots (l). Scale bars, 1.5 μm. Time-lapse TIRF imaging of live Stonin1−/− MEFs transfected with Stonin1-tomato and NG2-EGFP (complete cell depicted in Supplementary Fig. 4b). All raw data are depicted as mean ± s.e.m. and compared by unpaired two-tailed Student’s t-tests (b,c,f) or one-way analysis of variance followed by Tukey’s post-test (g,j), ***P < 0.001, **P < 0.01, *P < 0.05.

Co-localization and coordinate movement in living cells (Fig. 5k,l; Supplementary Fig. 4b).

Increased NG2 clustering in Stonin1−/− MEFs. What are the consequences of impaired NG2 internalization? Clustering of NG2, for example, by antibody binding was shown to precede NG2 activation of Cdc42 (ref. 36). A comparison of the distribution of NG2 in WT and Stonin1−/− cells revealed that WT MEFs contain few NG2 clusters at steady state, while these are strikingly more prominent in Stonin1−/− cells (Fig. 6a–c). Inhibiting endocytosis with dynasore for 30 min increased the number of NG2 clusters also in WT cells, while NG2 levels remained unchanged on this short timescale. This indicates that the occurrence of NG2 clusters in Stonin1−/− cells is not a secondary result of elevated NG2 levels, but that the clusters accumulate due to lack of NG2 internalization, confirming that NG2 clusters are normally resolved by endocytosis. Intriguingly, not only inhibition of endocytosis induces NG2 clusters in WT cells, but also stimulation with PDGF (Fig. 6a–c), suggesting that NG2 clusters serve as signalling hubs under physiological conditions. The action of PDGF on NG2 is also consistent with NG2’s proposed function as a co-receptor of PDGF, which potentiates PDGF signalling.10.

If Stonin1 mediates the dissolution of NG2 clusters, it should be present in these clusters. To test this, we induced clusters in WT cells by dynasore or PDGF and performed immunostainings.
Indeed, Stonin1 localizes to NG2 clusters, which are often present at protrusions and partially co-localize with FA markers (Fig. 6d). As NG2 is a known co-receptor and potentiator of PDGFR and responds to PDGFR treatment, we speculated that PDGFR might likewise be present in NG2 clusters and might exhibit increased activation. In fact, NG2 clusters observed in WT cells upon PDGFR treatment co-clustered PDGFR, and the receptor was also present in NG2 clusters in Stonin1−/− cells (Fig. 7a). (d) Stonin1 is present in NG2 clusters. Confocal images of WT MEFs treated with PDGF or dynasore as above and immunolabelled with antibodies against the indicated proteins. Scale bar, 25 μm. DAPI, 4,6-diamidino-2-phenylindole.

Elevated circular dorsal ruffle formation in Stonin1−/− MEFs. Activated PDGFR is known to induce circular dorsal ruffles (CDRs) in MEFs, actin-rich membrane structures that are involved in the bulk internalization of transmembrane proteins such as EGFR and in cell motility.37,38 In line with enhanced activation of PDGFR in absence of Stonin1, Stonin1−/− MEFs displayed elevated CDR formation as early as 1 min after PDGFR stimulation (Fig. 7e,f). Moreover, Stonin1−/− cells required lower doses of PDGF to initiate CDRs when compared with WT MEFs (Fig. 7g). This increased PDGFR sensitivity of Stonin1−/− cells is consistent with their elevated NG2 levels, as NG2 potentiates PDGFR signalling. In line with a role of NG2 in CDR formation, NG2 and activated PDGFR colocalize at CDRs (Fig. 7h).

NG2 mediates the impact of Stonin1 on directionality. In addition to its role as potentiator of PDGFR signalling, NG2 is a known regulator of cellular motility11–17, which controls, for example, the directional migration of oligodendrocyte precursor cells11. Thus, the elevated NG2 levels in Stonin1−/− MEFs might underlie their altered migratory behaviour. To test whether the accumulation of NG2 is causally involved in the directionality increase of Stonin1−/− cells, we silenced NG2 in Stonin1−/− MEFs by short interfering RNA (siRNA) and analysed their migratory pattern. Indeed, the directionality of Stonin1−/− cells reverted to WT levels upon NG2 depletion, whereas a scrambled control siRNA did not influence the directionality of Stonin1−/− cells (Fig. 8a,b). We thus conclude that the accumulation of NG2 causes the defective cell motility of Stonin1−/− MEFs. The altered shape of Stonin1−/− cells, however, was not rescued by NG2 depletion arguing for additional NG2-independent functions of Stonin1 (Supplementary Fig. 5a,b).

Discussion

Proteoglycans such as NG2 play critical roles in cell signalling and migration and are known to promote tumourigenesis. Nevertheless, their regulation is incompletely understood. We here show that NG2 function is critically controlled by endocytosis, and identify the until now uncharacterized protein Stonin1 as a dedicated adaptor for the internalization of NG2. Stonin1−/− MEFs display markedly elevated NG2 levels, which are associated with altered FA dynamics and with an increase in cellular signalling and directional cell migration. The increased directional persistence of Stonin1−/− MEFs is directly linked to Stonin’s role as an endocytic adaptor for NG2, as NG2 depletion...
rescues this phenotype. Elevated NG2 levels likely augment directional persistence due to the interaction of NG2 with Syntenin1 (ref. 40). Cells expressing Syndecan-4 mutants with enhanced Syntenin1 binding show increased directional persistence, similar to Stonin1−/− MEFs. This is caused by Syntenin-mediated suppression of Arf6 activity, which alters the integrin composition of FAs and thereby promotes directional movement41. Thus, elevated Syntenin1 recruitment due to increased NG2 levels might contribute to the increased directionality of Stonin1−/− MEFs.

Cellular motility also depends on FA dynamics. Random migration requires highly dynamic FA turnover, whereas...
increased FA stabilization results in directionally persistent migration. Our data indicate that Ston1 is an important regulator of FA turnover. Ston1 localizes specifically to CCPs in close vicinity of FAs, accumulates at FAs during their disassembly and facilitates the disassembly process. FAs of Ston1−/− MEFs exchange their components less efficiently and disassemble more slowly in line with the impaired removal of a crucial component. Thus, greater stability of FAs in Ston1−/− MEFs likely also contributes to increased directionality. While proteomic studies of FAs reported Ston1 levels to increase upon the disassembly of mature FAs due to myosinII inhibition, NG2 levels decreased at the same time, consistent with its endocytotic removal by Ston1 during FA disassembly. Finally, persistent trailing ends of migrating cells also promote directionality. Thus, the longer and more stable trailing ends of Ston1−/− MEFs, which have also been observed upon clathrin depletion, likely contribute to elevated directionality as well.

While cells with FA disassembly defects such as p14−/− MEFs tend to have enlarged FAs, surprisingly, we observed an increased number of small adhesions in Ston1−/− MEFs at steady state. Increased levels of small adhesions have been reported upon disorganization of the lamellar stress fibre network following depletion of Septin9 (ref. 43) or ROCK inhibition. This is in line with the fact that FA maturation does not only require myosinII-dependent tension, but also actin-binding proteins that mediate the formation of the lamellar actin network. The fact that Ston1−/− cells contain decreased surface-associated levels of Septin9 (fold-reduction in Ston1−/− versus WT: 0.35 ± 0.07, N = 2, data given as mean ± s.e.m., compare Supplementary Data 1) and that Ston1 co-immunoprecipitates actin (Fig. 1c) suggests that loss of Ston1 might not only affect FAs by serving as an endocytic adaptor for NG2 but also by influencing the cytoskeleton, a hypothesis that awaits further testing. An NG2-independent function of Ston1 in regards to cytoskeletal dynamics might also underlie the observed alterations in cell shape upon loss of Ston1.

In addition to alterations in FA dynamics and cellular motility, Ston1−/− MEFs display increased PDGF-dependent signalling. This is consistent with the fact that NG2 is not only involved in cell migration but also serves as co-receptor for PDGF and promotes PDGF-mediated signalling. Our data suggest that NG2 aggregates upon PDGF stimulation into clusters that contain PDGFR and promotes PDGF activation. We show that these signalling clusters have to be resolved by Ston1-mediated endocytosis of NG2 to limit PDGF signalling. By shutting NG2 into the endosomal pathway, Ston1 limits NG2 signalling from the plasma membrane. Failure to efficiently internalize NG2 leads to elevated signalling causing for instance an increase of PDGF-induced CDRs.

On the basis of the presented data, we propose the following model: Ston1 resides at peripheral CCPs, which participate in the removal of FA components during FA disassembly and the dissolution of associated signalling clusters. During these endocytic events, Ston1 specifically binds to NG2 and facilitates its internalization. Thereby Ston1 promotes FA disassembly and limits NG2 activity, which results in decreased persistence of movement and lower levels of cellular signalling.

In summary, we identify Ston1 as an endocytic adaptor for NG2 and illustrate how endocytic regulation fine-tunes FA dynamics, cellular motility and signalling. As alterations in these processes are intimately linked to tumorigenesis and as NG2 is a well-known oncogene, Ston1 might be crucial for limiting tumour growth by keeping NG2’s oncogenic potential in check via its removal from the plasma membrane. Future studies involving tumour models will have to validate the putative tumour suppressor function of Ston1.

Methods

Reagents. For information on antibodies, and plasmids and siRNAs see Supplementary Tables 1 and 2.

Generation and genotyping of Ston1−/− mice. The mouse Ston1 gene (Ston1) is located on chromosome 17 and consists of three coding exons including Ensembl entry ENSMUST0000064035. Ston1−/− mice were generated by replacing the first coding exon, which comprises most of the protein-coding sequence, by a floxed neomycin-resistance cassette using homologous recombination in embryonic stem cells (Supplementary Fig. 1), which were injected into pronuclear stage two embryos implanted into adult foster mothers C57BL6 J mice. The resulting chimeric males were mated with Ella-Cre Deleter mice to remove the neomycin-resistance cassette. The obtained Ston1−/− mice with one disrupted Ston1 allele did not show any overt phenotypic differences to WT animals. They were backcrossed onto a C57BL6 J background and interbred to obtain WT and Ston1−/− littermates for experiments (line name: Ston1tm1.TTmar). Ston1−/− mice did not show any overt phenotypic differences. Animals were genotyped before experiments by PCR analysis of genomic DNA obtained from tail or ear biopsies. The Ston1 WT allele was detected with the forward primer TM45 (5′- CAGGACAGGAGACCTTCAGA-3′) and the reverse primer TM46 (5′- CGAGCAAGCACTCATCTGCT-3′) (product: 402 nt). The Ston1 KO allele was detected using the forward primer TM45 and the reverse primer TM49 (5′- GATGCTAGAGTTGGCCGGTC-3′) (product: 301 nt). Mice were housed in small groups in standard cages and kept on a 12 h-dark–light cycle with food and water ad libitum. All animal experiments in the present study were performed in strict accordance with ethical regulations and the animal welfare guidelines of the Landesamt für Gesundheit und Sozialen (LAGeSo) Berlin and with their permission.

Generation of MEFs. E13.5 embryos of random sex were transferred into sterile ice-cold Hanks’ balanced salt solution (HBBS). After removal of head and inner organs (which were kept for genotyping), embryonic tissue was dissected into small pieces and digested in 5 ml trypsin/EDTA for 15 min at 37°C in a shaking water bath. After brief trituration of the tissue segments, 10 ml culture medium (DMEM containing 4.5 g/l glucose, 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin) were added, and cells were centrifuged at 400 g for 5 min at room temperature, washed once with HBBS and plated in culture medium on one 10-cm culture dish per embryo and grown at 37°C and 5% CO2. After 1 day in vitro, floating cultures were washed with sterile PBS and replaced with fresh culture medium. Cells were passaged once before immortalization by Lipofectamin 2000 (Invitrogen)-mediated transfection with SV40 large T-antigen on DIV 10. Two independent MEF cell line pairs from WT and Ston1−/− embryos were established (pair #1 and #2).

Generation of primary MLFs. Lungs were dissected from 5-7-day-old sex-matched Ston1 WT and KO mice (C57BL/6) of both genders and transferred into ice-cold HBBS. Bronchi and connective tissue were removed, and the remaining tissue was dissected into small pieces, washed three times with ice-cold HBBS and digested with 37°C warm 20 mg/ml collagenase (Millipore) in DMEM containing 10 mg/ml bovine serum albumin (BSA) for 45 min at 37°C in a shaking water bath. Digestion was stopped by the addition of culture medium, and tissue pieces were washed once in culture medium. After tissue trituration, cells were washed with culture medium, plated onto one 10-cm culture dish in fresh culture medium and grown at 37°C and 5% CO2. The cultures were washed with PBS on DIV 1 and passaged 1:10 every 5 days.

Cell culture and transfections. Primary cells (MEFs and MLFs) as well as HEK293T cells (obtained from ATCC) were grown in culture medium at 37°C and 5% CO2. While HEK293T cells were transfected with plasmid DNA by calcium phosphate transfection according to standard protocols, MEFs were transfected with electroporation with the Amaxa Nucleofector IIb (Lonza) using the program A-23 designated for MEFs or by lentivirus-based transfection. For lentivirus production, the lentiviral packaging plasmid psPax2 and the VSVG envelope-expressing plasmid pMD2.G were transfected together with a Ston1-encoding pLRsinCpPT.PGK-GFP.WPRE-based plasmid into HEK293T cells. After 20 h, the transfection medium was removed and fresh culture medium was added. At 48 h post transfection, the medium was collected, debris was removed by centrifugation for 10 min at 6,000 g and the supernatant was filtered through a 0.45-μm filter before immediate use. For lentivirus-based transductions, MEFs were incubated at 60% confluency with fresh lentivirus supernatant. After 20 h, the supernatants were removed, and fresh culture medium was added. Transfection of siRNAs into MEFs was performed with RNAiMax (Invitrogen) according to the manufacturer’s instruction. On day 2, the transfection medium was exchanged for fresh culture medium. At 36 h after the medium exchange, a second round of siRNA transfection was performed for 4–6 h or overnight. Directly after removal of the transfection medium, cells were split according to the planned application.

Experiments were done on days 4–7. When seeding transfected cells on glass surfaces for microscopy, these surfaces were first coated with Matrigel.

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Preparation of lysates and immunoblot-based analysis. Cultured cells were washed briefly with PBS and scraped into lysis buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 0.1% protease inhibitor cocktail (Sigma)). After 5 min on ice, lysates were centrifuged for 5 min with 17,000g at 4°C. The protein concentration of the supernatant was determined by the Bradford assay. Tissue extracts were obtained by tissue homogenization in homogenization buffer (320 mM sucrose, 4 mM HEPES pH 7.4, 2 mM PMSF, 0.3% protease inhibitor cocktail (Sigma)) using a pestle (15 strokes at 1,000 r.p.m.). After centrifugation with 1,000 g at 4°C for 10 min, the supernatant was collected and again cleared by centrifugation for 5 min with 17,000g at 4°C to yield the final tissue extract. Tissue lysates used for binding experiments were adjusted to 1 x lysis buffer and lysed for 10 min on ice before centrifugation for 15 min with 43,500g for 4°C and again for 15 min with 265,000g at 4°C in an ultracentrifuge to obtain a clear lysate before determining the protein concentration by the Bradford assay. Before immunoblotting lysates were adjusted to 1 x Laemmli sample buffer, samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Bound primary antibodies were detected by incubation with secondary antibodies conjugated to horse radish peroxidase (Jackson Immunoresearch; dilution: 1:10,000) and a chemiluminescent substrate. Uncropped versions of all immunoblots are depicted in Supplementary Fig. 6.

MEF treatments. To assess effects of cellular stimulation, cells were either left untreated or washed twice in sterile PBS and starved 12–48 h in serum-free DMEM. Cells were then stimulated with either 10% BCS or with 50-100 ng ml⁻¹ PDGF-BB (Peprotech (termPE PDGF in the text), if not stated otherwise in the figure legend. Cultures were stimulated from 1 to 60 min before immediate lysis on the dish as described above. The dynamin superfamily Dynasore (self-synthesized) was applied at 100 µM for 30 min. The myosin-IIIa inhibitor blebbistatin (Merck–Millipore) was applied at 25 µM for 5–30 min.

Surface biotinylation and affinity purification. MEFs were cultured for 6 days in 1-lysine- and 1-arginine-free DMEM/10% FCS (Thermo Fisher Scientific Inc.) supplemented either with the ‘heavy’ amino acids ¹³C-lysine and ¹³C-¹⁵N-¹⁴N-¹⁴N-arginine (Silantes GmbH) in case of KO MEFs or normal ‘light’ amino acids in case of WT MEFs. For biotinylation of the surface, protein pool cells were placed on ice, washed twice with ice-cold PBS and incubated with 2.0 mg ml⁻¹ Sulfo-NHS-LC-Biotin (EZ-Link, Pierce/Thermo Scientific) in PBS while shaking for 30 min at 4°C. Biotinylated targets were quenched with 2-5 mM 500 mM glycine in PBS at 4°C on a shaker. Cells were harvested, and lysates were prepared as described above. After protein determination by the Bradford assay, WT and KO lysates were brought to the same concentration and mixed 1:1. Biotinylated molecules were isolated by a 1-h incubation of lysates with streptavidin beads on a rotator at 4°C. After centrifugation at 3,500g, the supernatant was transferred to a fresh tube. The beads were washed extensively and bound protein was eluted with Laemmli buffer and separated by SDS-PAGE.

Relative protein quantification by mass spectrometry. For liquid chromatography (LC)–mass spectrometry (MS)/MS analysis, coomassie-stained lanes were cut into slices and destained with trypsin digestion buffer. After digestion, the slices were washed with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate, dehydrated in acetonitrile and dried in a vacuum centrifuge. The dried gel pieces were washed with 50% (v/v) in water, mobile phase B contained 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan (with a 20 p.p.m. and 0.35 Da, respectively. Methionine oxidation and the acrylamide database (version 3.68). The mass tolerance of precursor and sequence ions was set specifically label extracellular SNAP tags. The blot was exposed to autoradiography film and scanned with a high-resolution densitometer (Fuji, Tokyo, Japan) for quantification. The integrated signal intensity of each spot was normalized to the β-actin signal intensity of each sample. The ratio of the normalized signal intensity was calculated for each treatment group.

Fluorescence recovery after photobleaching. For FRAP experiments, Paxillin-GFP transfected MEFs on Matrigel-coated 24 mm CSs were transferred to imaging solution (HBSS containing Ca²⁺, Mg²⁺, 5% FCS, 20 mM HEPES pH 7.4) at 37°C without CO₂. NG2 was engineered to contain an extracellularly located SNAP tag. SNAP surface Alexa647 (New England Biolabs) was used at 1:200–1:500 as SNAP substrate, a non-membrane-permeable probe that can be used to specifically label extracellular SNAP tags.

NG2 antibody uptake assay. Endocytosis of NG2 was assessed by quantification of internalized mouse-α/NG2.1 antibody against the extracellular domain of NG2.1, transfected MEFs were incubated with α/NG2.1 antibody (1:20 dilution) in live-cell imaging solution (HBSS containing Ca²⁺, Mg²⁺, 5% FCS, 20 mM HEPES pH 7.4) at 37°C without CO₂. NG2 was engineered to contain an extracellularly located SNAP tag. SNAP surface Alexa647 (New England Biolabs) was used at 1:200–1:500 as SNAP substrate, a non-membrane-permeable probe that can be used to specifically label extracellular SNAP tags.

Intracellular NG2 fluorescence was measured at 488 nm for Alexa488 and Alexa647 (both from Invitrogen). Intracellular NG2 was quantified by subtracting the signal from SNAP control. Data were collected during 10 min bleaching periods followed by recovery over 60 s at 1 s per frame on the spinning disc confocal microscope. FRAP data together with pre-bleach images were exported from Velocity software and further analysed in ImageJ. Fluorescence intensity plots of each frame were normalized to bleaching before averaging over Cell and corrected for background, enabling comparisons between cells. The resulting data from each experiment was fitted with a single exponential recovery curve using the software Origin to calculate the half-time of recovery (t½) and the fractional recovery. Only experimental data for which the fit resulted in an R²-value > 0.95 was included in the analysis.

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uptake was performed in the continued presence of NG2.EC in uptake solution by wash off surface-bound antibody. The acid quench control and the sample for ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9535 were blocked and permeabilized, incubated with an Alexa488 goat 2-rabbit secondary antibody and mounted as described above. Values obtained for internalized NG2 were normalized to surface amount and subtracted for acid wash background.

Flow cytometry. Flow cytometry was used to quantify surface levels of NG2 and β1-integrin and to perform an integrin uptake assay. For surface stainings, cells were removed from dishes by treatment with 100 mM EDTA in PBS for 5 min at 37 °C. EDTA was diluted by the addition of PBS, and cells were centrifuged at 4 °C for 5 min at 300g. The pellet was resuspended in PBS, and cells were fixed for 30 min on ice by adding PFA to a final concentration of 2%, and then washed once in PBS. The antibodies were applied in 3% BSA in PBS for 1 h at room temperature. Surface NG2 was stained with NG2.EC (1:100), surface integrin with a phycoerythrin (PE)-conjugated β1-integrin-specific antibody (1:50). Cells were washed once with PBS. In the case of the NG2 staining, fixed cells were incubated with a secondary mouse anti-rabbit antibody conjugated to Alexa488 diluted 1:200 in 3% BSA in PBS for 30 min at room temperature in the dark. Finally, cells were washed with PBS and dispersed by vortexing in PBS before the flow cytometric measure- ment. Samples were measured with a FACS Calibur (BD Biosciences) and CellQuest Pro and analysed with FlowJo V10.

The β1-integrin internalization assay was done in essence as described for the NG2 antibody uptake assay. Briefly, MEFs attached to CIs were surface-stained with a β1-conjugated integrin-specific antibody (1:50). Acid treatment was done as described for the NG2 antibody uptake. Cells were detached with 100 mM EDTA in PBS for 5 min at 37 °C, fixed and subjected to flow cytometric analysis as described above. Values obtained for internalized β1-integrin were normalized to surface amount and subtracted for acid wash background.

FA disassembly assay. MEFs seeded on glass CIs were serum starved for 24 h. Next, they were treated for 4–5 h with 10 μM nocodazole to completely depolymerize microtubules. During washout, nocodazole was replaced with serum-free medium for the indicated time intervals to allow microtubule regrowth. Cells were then fixed, processed for immunofluorescence as described above and imaged on an epifluorescence microscope. FA numbers were quantified by particle analysis with customized ImageJ macros (for details see Data Analyses). Mean values were normalized to the mean of the WT control before relative FA disassembly per genotype was calculated.

Migration assays. Time-lapse movies of randomly migrating cells were acquired on the Nikon microscope specified above with a 10× objective and a plan-NEO 20×/0.75 objective, using a temperature chamber at 37 °C and 5% CO2. Cells were then fixed, processed for immunofluorescence as described above and imaged on an epifluorescence microscope. FA numbers were quantified by particle analysis with customized ImageJ macros (for details see Data Analyses). Mean values were normalized to the mean of the WT control before relative FA disassembly per genotype was calculated.

Data analyses. Image analysis was performed with Fiji, an ImageJ 1.47g package. Processing was semi-automated by the use of custom-made macros in ImageJ using intensity quantifications on manually outlined cells or automatically selected particles after application of a common threshold. For three-dimensional display of reconstructed stacks, the resulting 3D images were converted using the software dedicated to this purpose. Project function. Time-lapse movies were created from single Tiff images. Its application results in a line-scan along the radius of an amorphous shape from the centre to the peripheral region of the stack for all angles and normalizes the respective radius from 0 to 1 (ROI). Hence, the result is a normalized profile from the centre outwards. An advanced mode permits the selection of specified angles to scan, which allows the division of a migrating cell in front and rear. Signal intensities can be normalized, and the distance from 0 to 1 can be binned according to the user’s needs to compute the profile of different sub-localizations of pixels between one channel and another and vice versa was judged by quantifying Mander’s coefficients.

Statistical analyses. Values are always depicted as mean ± s.e.m. Statistical significance of data was assessed by two-tailed unpaired Student’s t-tests in case of two experimental groups and one-way analysis of variance followed by either a Tukey or a Dunnett post-test in case of >2 experimental groups using GraphPad Prism software, and are indicated in the following way: ***P<0.0001; **P<0.001; *P<0.01; P<0.05. Data with arbitrary values, such as fluorescent intensities, was normalized to the mean of all samples before performing statistics. Fold increases were calculated and normalized to the respective reference, which mostly is the untreated WT sample. The number of experimental replica or animals used is given as ‘N’, the number of analysed cells is given as ‘n’. No statistical method was used to predetermine sample size. If samples were excluded, the exclusion criteria are described in the respective method section. Whenever possible, data were evaluated in a blinded manner.

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