**Abstract**

p130Cas is a protein that is thought to be able to trigger signaling pathways dependent on stretch. The influence of p130Cas on focal adhesion morphology, the influence of focal adhesion morphology on force exertion, the localization of p130Cas in focal adhesions and the direct influence of p130Cas on force exertion by focal adhesions were investigated. Mouse embryonic fibroblasts re-expressing p130Cas and p130Cas knock outs were investigated on fibronectin-coated glass and micropillars. p130Cas had no influence on focal adhesion area or shape. In presence of p130Cas focal adhesion area showed a positive correlation to force exertion. Paxillin and p130Cas did not perfectly co-localize, there was a significant shift between them. p130Cas had a positive influence on force exertion by focal adhesions.
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Introduction
The mechanical properties of the cellular environment play an important role in many biological processes. The environment of a cell consists of diverse connective tissue components that form the extra cellular matrix (ECM). The physical properties of the ECM have a major influence on, for example, cell migration, which is involved in wound healing and morphogenesis, and on stem cell differentiation\(^1\). The ability of cells to probe their environment is based on the mechanosensitive nature of cell adhesions. These adhesions and especially focal adhesions are large protein complexes that are able to pull on the ECM and convert this mechanical signal into a biochemical one, such that cells adjust their morphology by rearranging their cytoskeleton\(^3\). With transmembrane adhesion receptors (integrins), the cell can pull on the ECM and pass the signal on to the intercellular part of the focal adhesion. The intercellular part can roughly be divided into a signaling layer, a force transduction layer and an actin regulatory layer (figure 1)\(^4\)\(^5\).

![Figure 1 A Model of focal adhesions and a model of the role of p130Cas in focal adhesions. (left) Schematic model of the molecular architecture of focal adhesions\(^5\). (right) Schematic model of the stretch dependent activation of p130Cas in integrin mediated signaling\(^7\).](image)

Focal adhesion complexes exist of many different proteins. A protein of particular interest is p130Cas. This protein consists of a substrate domain, flanked by an SH3 domain and Src-binding domains. These latter two domains are involved in the localization of p130Cas to focal adhesions\(^6\). The conformation of p130Cas is thought to be stretch dependent: when the protein stretches, potential phosphorylation sites in the substrate domain become accessible so that several signaling pathways are activated (figure 1)\(^7\). This mechanism could play a role in actin cytoskeleton organization, cell spreading and focal adhesion formation\(^8\).

p130cas is thought to trigger signaling pathways when it is pulled on, but its role during force exertion to probe the cellular environment for example is yet unknown. In the current study the influence of p130Cas on focal adhesion morphology and the effect of focal adhesion morphology on force exertion...
were examined. The localization of p130Cas in focal adhesions and the direct influence of p130Cas on force exertion by a cell were measured.

To get a better understanding of the localization and the importance of p130Cas during force exertion, this protein was stably expressed in p130Cas deficient mouse embryonic fibroblasts (Cas-/- MEF’s) and tagged with Venus-YFP. Cells were investigated using spinning disc confocal microscopy. Focal adhesions were examined by staining for paxillin, a known focal adhesion protein. In order to examine the force exerted by focal adhesions, cells were seeded on a PDMS micropillar array. This force sensing substrate had a stiffness comparable to muscle tissue. The micropillar array was coated with labeled fibronectin (Fn), an ECM protein, to enable the cells to attach and to make the micropillars visible. Fn-coated cover slips were used as a stiff, reference substrate.
Experimental procedures

Cells and cell culture
Cas\(^{-/}\) mouse embryonic fibroblasts (MEF’s) were kindly provided by Hisamaru Hirai (University of Tokyo). The MEF’s were grown in Dulbecco’s modified Eagle’s medium (DMEM) that contained 10% fetal calf serum (not heat activated), 100\(\mu\)g/ml pen/strep and 5m glutamax. In one cell line p130Cas Venus-YFP was re-expressed in amounts similar to normal conditions (WT). In another cell line only the Venus-YFP tag was expressed (Cas\(^{-/-}\)). Both cell lines were provided as described elsewhere\(^1\).

Fabrication of micropillar arrays and fibronectin-coated coverslips
A silicon micropillar master was provided by Bonda Tech. The actual dimensions of the pillars were 2\(\mu\)m diameter and 6.9\(\mu\)m height and 4\(\mu\)m pitch. PDMS was mixed 10:1 with cross linker, degassed and stored on the master for 20 hours at 110°C. The pillar arrays were cut out, peeled from the master and placed in a UV-ozone cleaner for 30 minutes. A PDMS stamp (10:1 cross linker) was prepared and coated with fibronectin (Fn). A Fn-solution of 50\(\mu\)g/mL Fn and 10 \(\mu\)g/mL Fn conjugated to Alexa405 in milliQ was incubated for 60 minutes. The Fn in the solution precipitated on the stamp and the rest of the solution was sucked off with a tissue. The stamp was washed with milliQ and dried under laminar flow. When dry, the stamps were applied to the pillar arrays for 15 minutes, then the pillar arrays were incubated in a 0.2% pluronic solution for 30 minutes, washed with PBS and stored in PBS at 4°C. Coverslips were cleaned by flaming with ethanol. The Fn solution as described above was used for coating.

Fixation and immunostaining
After overnight spreading on the micropillar arrays or cover slips, MEF’s were washed with warm DMEM, fixed in phosphate buffered saline (PBS) with 4% paraformaldehyde for 15 minutes, permeabilized in 0,1% TritonX in PBS for 10 minutes, and blocked with 1% bovine serum albumin (BSA) in PBS for 60 minutes. The cells were stained with Mouse anti-paxillin (BD-TL) and DyLight649 conjugated Goat-anti-Mouse IgG.

Imaging
Images were taken by a Zeiss 200 microscope with Yokogawa spinning disk confocal system. Pictures were made with a 100x objective and at 120x magnification. The lasers used were: 405 nm crystalLaser, 514nm Cobolt laser, 642nm Newport laser.

Quantitative analysis
Quantification of properties of focal adhesions was done by custom made image processing programs in Matlab. With these programs, the area of every focal adhesion was calculated as well as the area of every spot where p130Cas localized. The eccentricity of the focal adhesions and the area’s where p130Cas localized was calculated and the distance between the center of mass of paxillin and p130Cas localized in the same focal adhesion was measured. Force exertion was determined by measuring the displacement of the micropillar tips from the original position and multiplying by the spring constant of the micropillars. The dimensions of the micropillars were determined with scanning electron microscopy: \((h \times d)\) 6.9 \(\times\) 2 \(\mu\)m. The Young’s modulus of the PDMS was 2.5 MPa. These properties led to a spring constant of \(k=14.1\) nN/ \(\mu\)m.
The effective Young’s modulus for these micropillars was estimated to be in the range of 10-20kPa, which resembles the stiffness of muscle tissue\(^2\).
**Results**

In order to determine the influence of p130Cas on the focal adhesion morphology and its localization in focal adhesions, the effect of focal adhesion morphology on force exertion and the direct influence of p130Cas on force exertion, cells with and without p130Cas expression on different substrates were investigated. Figure 3 shows an overview of the phenotypes of the Cas-/- and WT cell lines seeded on pillars and glass, both coated with fibronectin.

**Focal adhesions**

Figure 3 showed, on the edges of the cells, red dots or stripes where paxillin clustered in focal adhesions. In the WT cells, the dots showed some green, caused by p130Cas also localizing in focal adhesions, and yellow, remarking where p130Cas and paxillin overlapped. In WT cells on pillars the focal adhesions formed on top of the pillars. In Cas-/- cells on pillars and both cell lines seeded on glass focal adhesions localized random. In figure 3 it was also seen that the focal adhesions in the different cell lines on the different substrates showed different morphologies.

*Figure 3 Focal adhesions of different cell lines on different substrates show different morphologies. Z-stack projection made by the spinning disk confocal microscope at 120x magnification. Of every cell line used (WT and Cas-/) on glass and on pillars, one cell is shown as an example. In every image bleu corresponded to fibronectin, red to paxillin and green to p130Cas.*
**Focal adhesion area**

Figure 3 showed that the focal adhesions in different cell types on different substrates varied in area. For example the focal adhesions in WT cells on glass were bigger than the focal adhesions in WT cells on pillars, shown in figure 4.

![Figure 4 Focal adhesions on different substrates had different areas and shapes. (left) Focal adhesions in WT cells on glass. (right) Focal adhesions in WT cells on pillars.](image)

The influence of p130Cas and the substrate on focal adhesion area and the area of p130Cas itself was examined. The area of every single focal adhesion and the area of p130Cas localizing in one focal adhesion were quantified. Paxillin was considered to cover the whole focal adhesion, therefore the areas covered by paxillin were taken as a measure for focal adhesion area.

![Figure 5 Areas covered by p130Cas were smaller than focal adhesions. Distribution of the areas of every single focal adhesion (paxillin) and areas of p130Cas localized in one focal adhesion for Cas-/ and WT cell lines on Fn-coated glass and micropillars.](image)

In cells on glass the average area of a focal adhesion in Cas-/ cells was $1.25 \pm 0.05 \mu m^2$, which was smaller than the average area of a focal adhesion in WT cells: $1.44 \pm 0.08 \mu m^2$. That the focal adhesions in WT cells on glass had a larger area was also confirmed by the distribution of the areas of focal adhesions. On pillars it was the other way around: there the focal adhesions in Cas-/ cells (mean area $1.17 \pm 0.03 \mu m^2$), were larger than the focal adhesion in WT cells (mean area $0.95 \pm 0.02 \mu m^2$). This was confirmed by the distribution of focal adhesion areas: in Cas-/ cells larger focal adhesions were seen. No clear influence of p130Cas on focal adhesion area was seen, but on glass the area focal adhesions in Cas-/ cells as well as in WT cells was larger than the focal adhesions on pillars. Also the area where p130Cas localized in one focal adhesion was larger on glass: $0.81 \pm 0.05 \mu m^2$, than on pillars: $0.73 \pm 0.02 \mu m^2$. (Figure 5, Table 1).
Table 1 On average, the largest focal adhesions were formed in WT cells on glass. Mean values and sem of the area of focal adhesions and p130Cas localization in Cas-/ and WT cells on glass coverslips and micropillars.

<table>
<thead>
<tr>
<th>Area (µm²)</th>
<th>Glass</th>
<th>Pillars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>sem</td>
</tr>
<tr>
<td>Cas-/</td>
<td>1.25</td>
<td>0.05</td>
</tr>
<tr>
<td>WT focal adhesions</td>
<td>1.44</td>
<td>0.08</td>
</tr>
<tr>
<td>p130Cas</td>
<td>0.81</td>
<td>0.05</td>
</tr>
</tbody>
</table>

With the use of pillar deflection analysis, the force applied on a pillar by a focal adhesion was calculated. This was used to examine the relation between focal adhesion and p130Cas area and force exertion. In presence of p130Cas, a larger focal adhesion area correlated positively to force exertion. Also the area covered by p130Cas in a focal adhesion showed a positive correlation to force applied by that focal adhesion. In Cas-/ cells no clear correlation was seen between focal adhesion area and force exertion (figure 5).

Figure 6 In presence of p130Cas, a larger focal adhesion area correlated to higher force exertion. The force applied by every single focal adhesion was calculated by means of pillar deflection analysis. Each data point represents a single focal adhesion. Each color indicates focal adhesions of one cell. (a) Correlation between focal adhesion area and force exertion for WT cells seeded on pillars. (b) Correlation between focal adhesion area and force exertion for Cas-/ cells on pillars. (c) Correlation of area where p130Cas localized and force exertion for WT cells on pillars.
**Focal adhesion shape**

Figures 3 and 4 showed that the focal adhesions in the different cell lines on the different substrates had different shapes. In figure 4 was seen that the focal adhesions in WT cells on pillars looked much rounder than the focal adhesions in WT cells on glass for example. To quantify the difference in focal adhesion shape and the shape of the area’s where p130Cas localized, eccentricity was used. The eccentricity is zero for a perfect circle, the eccentricity is 1 for a straight line.

![Graphs showing difference in shape between Cas-/− and WT cells on glass and pillars](image)

**Figure 7 The areas where p130Cas localized were rounder than focal adhesions.** The shape of focal adhesions and of the area’s where p130Cas is located were quantified by calculating the eccentricity. (The quantification was done on the focal adhesions of Cas-/− cells on Fn-coated glass cover slips and micropillars and the focal adhesions and areas where p130Cas localized in WT cells on Fn-coated glass cover slips and micropillars.)

The mean values of the eccentricity of focal adhesions showed that on glass the focal adhesions in Cas-/− cells (0.824±0.006) where rounder then the focal adhesions of WT cells (0.848±0.009). On pillars it was the other way around. There the focal adhesions in WT cells (0.828±0.005) were rounder than the focal adhesions in Cas-/− cells (0.855±0.005). From the distributions it was seen that on glass more focal
adhesions were elongated than in WT cells on pillars, while this difference between WT cells on pillars and Cas-/- cells on glass was not significant. The Cas-/- cells on pillars showed focal adhesions as elongated as WT cells on glass. No influence of p130Cas or the substrate was noticed. The areas where p130Cas localized were rounder than focal adhesions, but there was no significant difference in shape between p130Cas localizations on glass (eccentricity: 0.80±0.01) and p130Cas localizations on pillars (eccentricity: 0.799±0.008). (Figure 7, table 2.)

Table 2 On average, the most elongated focal adhesions were formed in Cas-/- cells on micropillars. Mean values and sem of the eccentricity of focal adhesions and p130Cas localization in Cas-/- and WT cells on Fn-coated glass coverslips and micropillars.

<table>
<thead>
<tr>
<th>Eccentricity</th>
<th>Glass mean</th>
<th>Glass sem</th>
<th>Pillars mean</th>
<th>Pillars sem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cas-/- focal adhesions</td>
<td>0.824</td>
<td>0.006</td>
<td>0.855</td>
<td>0.005</td>
</tr>
<tr>
<td>WT focal adhesions</td>
<td>0.848</td>
<td>0.009</td>
<td>0.828</td>
<td>0.005</td>
</tr>
<tr>
<td>p130Cas</td>
<td>0.80</td>
<td>0.01</td>
<td>0.799</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Like described before, the force applied on pillars by a focal adhesion was calculated. The eccentricity of focal adhesions or the area’s covered by p130Cas showed no correlation to force exertion (data not shown).

**p130Cas with respect to paxillin**

In figure 3 it was shown that p130Cas localizes mainly around the nucleus and in focal adhesions. In the focal adhesions, p130Cas did not perfectly co-localize to paxillin (figure 8). In focal adhesions the yellow spots marked where p130Cas and paxillin overlapped, but there were also red and green areas within one focal adhesion. So there was no perfect overlap.

Figure 8 p130Cas and paxillin did not perfectly co-localize. Zoom-in focal adhesions in WT cells on Fn-coated glass cover slips (left) and on Fn-coated micropillars (right).
To quantify the shift, the absolute distance between the center of mass of every area where paxillin localized and the area where p130Cas localized to the same focal adhesion was calculated.

![Graphs showing distributions of absolute distances between paxillin and p130Cas localizations.](image)

**Figure 9** There is a significant distance between p130Cas and paxillin within one focal adhesion. Distributions of the absolute distances between the centers of mass of areas where p130Cas and paxillin localized within one focal adhesion in WT cells on Fn-coated cover slips and micropillars.

The distributions and the mean values of the absolute distance between the areas covered by paxillin and p130Cas localizing in one focal adhesion showed that there was a significant distance between them. This distance was on average larger on glass (0.32±0.03 µm) than on pillars (0.23±0.01 µm). (Figure 9, table 3). Figure 10 showed that the shift between the areas covered by paxillin and p130Cas in one focal adhesion was not always in the same direction.

**Table 3** There was a significant distance between the areas covered by paxillin and p130Cas localizing in one focal adhesion. Mean values and sem of the distances between areas covered by paxillin and p130Cas localizing in one focal adhesion in WT cells on Fn-coated glass and micropillars.

<table>
<thead>
<tr>
<th>Shift (µm)</th>
<th>WT</th>
</tr>
</thead>
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<tr>
<td></td>
<td>mean</td>
</tr>
<tr>
<td>Glass</td>
<td>0.32</td>
</tr>
<tr>
<td>Pillars</td>
<td>0.23</td>
</tr>
</tbody>
</table>

**Figure 10** The shift between the areas covered by paxillin and p130Cas localized in one focal adhesion was not always in the same direction. Examples of focal adhesions in a WT cell on pillars in the yellow boxes. The shift within these focal adhesions was vertical (top) and horizontal (bottom).
Like described before, the force applied on pillars by a focal adhesion was calculated. The distance between the areas covered by paxillin and p130Cas in one focal adhesion showed no correlation to force exertion according to figure 11.

Another aspect was the orientation of these areas. Figure 12 showed that the area where p130Cas localized had, in general, the same orientation as the focal adhesion (paxillin area) it localized in. The mean value of the difference in orientation was $-1\pm2$ degrees.

**Figure 11** The distance between p130Cas and paxillin did not influence force exertion. The force applied by every single focal adhesion was calculated by means of pillar deflection analysis. Each data point represents a single focal adhesion. Each color represents the focal adhesions within one cell. Correlation between the absolute distance between the center of mass of the areas covered by paxillin and p130Cas in one focal adhesion and the force applied by that focal adhesion.

**Figure 12** The area where p130Cas localized had the same orientation as the focal adhesion it localized in. (a) Correlation between the orientation of the area covered by p130Cas and the area covered by paxillin. Each data point represents a single focal adhesion. Each color represents the focal adhesions within one cell. (b) Difference in angle between the areas covered by p130Cas and paxillin with mean $-1\pm2$ degrees.
Force exertion by focal adhesions
With the deflection of the pillars, the force applied on those pillars was calculated. Because the micropillar arrays were not perfect, from an empty array (without any cell) the deflections were measured and the pretended forces were calculated. These reference forces (shown in figure 13) showed a peak at 0.4nN (σ). The forces between 0 and 2σ were subtracted from the forces calculated from micropillar arrays in presence of a cell (figure 13) in order to display only the forces exerted by a cell. The forces applied by Cas-/− and WT cells on pillars were compared.

The mean value of the forces applied by focal adhesion in Cas-/− cells was 1.48±0.02 nN and smaller than the mean value of the forces exerted by focal adhesions in WT cells: 1.63±0.02 nN (table 4). This was confirmed by the distributions of the forces in both cell lines. It was shown that in WT cells more high forces (>3 nN) were exerted (figure 13).
Table 4 *In WT cells the focal adhesions applied, on average, the highest force on pillars.* Mean values and sem of the force applied by focal adhesions in Cas-/- and WT cells on micropillars.

<table>
<thead>
<tr>
<th>Force (nN)</th>
<th>Pillars</th>
<th>mean</th>
<th>sem</th>
</tr>
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<tbody>
<tr>
<td>Cas-/-</td>
<td>1.48</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.63</td>
<td>0.02</td>
<td></td>
</tr>
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</table>
Discussion

From figure 3 it was seen that the focal adhesions in WT cells on pillars formed right on top of the pillars. The focal adhesions in Cas-/- cells on pillars and in both cell lines on glass had a more random spreading. This could be explained by the fact that focal adhesions connect the cytoskeleton to the ECM, therefore in this case they attach to fibronectin. On glass fibronectin was available through the whole surface. There focal adhesions could attach everywhere. But on pillars the fibronectin was only on top of the pillars, that was the only place where focal adhesions could attach. Surprisingly, focal adhesions in Cas-/- cells on pillars did not only attach to the top of the pillars. A possible explanation for this phenomenon could be that the absence of p130Cas made a focal adhesion less sensitive to ECM proteins (private communication with D. M. Donato). Another explanation could be the fact that the pillar array where the Cas-/- cells were seeded on was stored in DMEM at 37 °C instead of in PBS at 4 °C. The higher temperature could have caused that the fibronectin released a bit from the pillar tips and distributed also in between the pillars. Figure 3 showed indeed a lot of background in bleu that might have been no background, but actual fibronectin in between the pillars. The pillars were also less bright then the pillars where the WT cells were seeded on.

No clear influence of p130Cas on focal adhesion area was seen in figure 5 and table 1. On glass the focal adhesions in cells expressing p130Cas had a larger area then in Cas-/- cells (mean of 1.44\pm0.08 µm² to 1.25\pm0.05 µm² respectively), while on pillars focal adhesions in cells without p130Cas expression had a larger area then in WT cells (1.17\pm0.03 µm² to 0.95\pm0.02 µm² respectively). This was remarkable because p130Cas is known to be involved in focal adhesion formation. An explanation could be that the Cas-/- cells on pillars were not seeded under the exact same conditions as the WT cells on pillars as described above. In general the focal adhesions on glass were larger than on pillars. This was as expected because glass is a much stiffer substrate than pillars and focal adhesions are known to grow bigger on stiffer substrates. Figure 6 demonstrates that in presence of p130Cas, focal adhesion area showed a positive correlation to force exertion. Also the area of p130Cas localization showed a positive correlation to force exertion. But without p130Cas expression no correlation was seen between focal adhesion area and force exertion. Which was unexpected because it was seen before that at higher force, larger focal adhesions form. A possible explanation was the lack of p130Cas. It is thought that this protein converts the signal of force exerted into a biochemical signal that leads to focal adhesion growth. In absence of p130Cas the signal does not get converted. Another explanation was the possible spreading of the fibronectin as described above. If the focal adhesions in Cas-/- cells on pillars were able to attach not only on top of the pillars, they were also able to apply forces not only on the pillars. These forces could not be measured and were not taken into account in the correlation.

Figure 7 and table 2 showed that on glass the focal adhesions in cells expressing p130Cas were more elongated than in Cas-/- cells (mean eccentricity of 0.848\pm0.009 to 0.824\pm0.006 µm² respectively), while on pillars focal adhesions in cells without p130Cas expression had a more elongated shape then in WT cells (mean eccentricity of 0.855\pm0.005 to 0.828\pm0.005 respectively). The focal adhesions in cells on pillars were expected to be more round because of the round shape of the pillars. Therefore it was
surprising that the focal adhesions in Cas-/- cells were as round as the focal adhesions in WT cells on pillars and that the focal adhesions in Cas-/- cells on pillars were as elongated as the focal adhesions in WT cells on glass. That the Cas-/- cells were not as round as expected could be explained by the possibility of fibronectin being not only attached on the pillar tips, causing the focal adhesions not attach and form to the round pillars. No influence of p130Cas expression on substrate was seen. The shape of focal adhesions also showed no correlation to force exertion.

In a focal adhesion p130Cas did not completely overlap with paxillin. Figure 9 and table 3 showed that there was a significant distance between the center of mass of areas covered by paxillin and of the areas covered by p130Cas. On glass the distance was on average 0.32±0.03 µm, on pillars the average distance was smaller: 0.023±0.01 µm. Figure 10 showed that this shift was not always in the same direction, therefore it could not be due to optical aberration. The shift might be due to the different functions of p130Cas and paxillin, therefore the might localize in different parts of the focal adhesions. But this was unlikely, because both proteins bind to the kinases Src and FAK\(^5,8\). Another explanation could be that only active, phosphorylated p130Cas localized exactly in focal adhesions, while non-activated p130Cas localized more next to the focal adhesion. In this study both activated and non-activated p130Cas was analyzed. According to figure 11 the shift had no influence on force exertion. Although p130Cas and paxillin did not completely overlap, they did have the same orientation, showed in figure 12. The mean value of the difference in orientation was -1±2 degree.

The correlation between p130Cas and force exertion in general was positive according to figure 13 and table 4. Cells expressing p130Cas showed an average force exertion of 1.63±0.02 nN, while cells without p130Cas expression had an average force exertion of 1.48±0.02 nN. This was as expected because of the model in which p130Cas converts the signal of force exertion into a biochemical signal that leads to actin re-organization, which again leads to force exertion\(^7\).
Conclusion

p130Cas had no influence on focal adhesion area, but the focal adhesion area did show a positive correlation to force exertion in presence of p130Cas. Also on focal adhesion shape p130Cas had no influence and the shape of focal adhesions showed no correlation to force exertion. P130Cas and paxillin did not completely overlap within on focal adhesion, there was a significant distance between there midpoints. This distance had no influence on force exertion. p130Cas and paxillin did have the same orientation within one focal adhesion. Force exertion by focal adhesions in general was influenced by p130Cas. Focal adhesions containing p130Cas exerted a higher force then focal adhesions without p130Cas.
References