Structural and biochemical studies of the adhesion protein talin-1

Dirk Lübbert Dedden
Oldenburg, Germany

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1. Gutachterin / 1. Gutachter: Prof. Dr. Reinhard Fässler

2. Gutachterin / 2. Gutachter: Prof. Elena Conti, PhD

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<tbody>
<tr>
<td>aa</td>
<td>aminoacid</td>
</tr>
<tr>
<td>ABS</td>
<td>actin binding site</td>
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<tr>
<td>ADMIDAS</td>
<td>metal ion-dependent adhesion site</td>
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<tr>
<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>ARP2/3</td>
<td>actin-related protein 2/3</td>
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<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>cryo-EM</td>
<td>cryo electron microscopy</td>
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<tr>
<td>CMSC</td>
<td>cortical microtubule stabilization complexes</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovaries</td>
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<tr>
<td>DAAM1</td>
<td>Disheveled-associated activator of morphogenesis</td>
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<tr>
<td>Dab2</td>
<td>disabled homolog 2</td>
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<tr>
<td>DD</td>
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<tr>
<td>DLC1</td>
<td>deleted in liver cancer 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EZH2</td>
<td>enhancer of zeste homolog 2</td>
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<tr>
<td>FA</td>
<td>Focal Adhesion</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FB</td>
<td>fibrillar adhesion</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1-erz-radin-moesin</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>IBS</td>
<td>integrin binding site</td>
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<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
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<tr>
<td>Irsp53</td>
<td>Insulin receptor substrate p53</td>
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<tr>
<td>Kank</td>
<td>KN motif and ankyrin repeat domains</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LIM</td>
<td>Lin11-Isl1-Mec3</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
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<tr>
<td>MIDAS</td>
<td>metal ion-dependent adhesion site</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NA</td>
<td>Nascent Adhesion</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>p130CAS</td>
<td>Breast cancer anti-estrogen resistance protein</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIPKιy90</td>
<td>phosphatidylinositol 4-phosphate 5-kinase Type ιy 90</td>
</tr>
<tr>
<td>pN</td>
<td>piconewton</td>
</tr>
<tr>
<td>PSI</td>
<td>plexin-semaphorin-integrin</td>
</tr>
<tr>
<td>PTB</td>
<td>phospho-tyrosine binding</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>Phosphatidylinositol-4,5-bisphosphat</td>
</tr>
<tr>
<td>RIAM</td>
<td>Rap1–GTP-interacting adapter molecule</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-Coil containing protein kinase</td>
</tr>
<tr>
<td>SAXS</td>
<td>small angle X-ray scattering</td>
</tr>
<tr>
<td>Src</td>
<td>Proto-oncogene tyrosine-protein kinase Src</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>THATCH</td>
<td>talin/HIP1R/Sl2p actin tethering C-terminal homology</td>
</tr>
<tr>
<td>TIAM</td>
<td>T-lymphoma invasion and metastasis-inducing protein</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>VBS</td>
<td>vinculin-binding site</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
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1 Summary

Integrin-mediated adhesions are responsible for the majority of cell matrix-contacts. Their regulation and correct function are vital in development, cell migration and tissue homeostasis. Integrins are class1 transmembrane receptors containing an α- and β-subunit. Their extracellular domain engages with different extracellular matrix (ECM) proteins and their cytoplasmic tail interacts with adaptor proteins, such as talin and kindlin, to establish a link to the actin-cytoskeleton. Thus, they form an anchor for cells to move and sense matrix properties.

As one of the two major integrin adaptor proteins, talin can bind to the integrin cytoplasmic β-tail and activate integrins. Talin is a 270 kDa protein that consists of a small head domain and a large rod domain. The talin head interacts with both integrin and the plasma membrane and can activate integrin’s extracellular domain, which enhances its ligand binding capabilities. The talin rod domain binds to F-actin and acts as recruitment platform for further adhesion proteins. Thus, talin functions as the linker between the actin cytoskeleton and integrin. It transmits the force generated by the actomyosin machinery, allowing cells to use the adhesion complex as an anchor for cell migration.

When both its N-terminal head an C-terminal rod domain are bound, talin adopts an elongated conformation, where the N- and C-terminus are more than 80 nm apart from each other. The mechanical tension transmitted over talin, induces the opening of some of its force dependent binding sites for vinculin and other adhesion components. This recruitment enhances the link to the actin cytoskeleton and reinforces the adhesion site.

Since regulation of cell adhesion is of critical importance for many cellular processes, integrin and its adaptor proteins also need to be tightly controlled. Talin was shown to be autoinhibited by interaction between its head and rod domains. This autoinhibited conformation was thought to be mainly present in the cytoplasm. Furthermore, several proteins and factors such as Rap1, PIPKιγ90 and RIAM were discovered to play a role in talin’s activation and recruitment to the plasma membrane.

A considerable amount of biochemical and structural research has been performed on talin’s 18 different domains; however, so far, no comprehensive model of its full length architecture and its possible autoinhibition conformation has been suggested.

In this study, talin and its interaction with some of its binding partners, such as vinculin, actin and Kank was characterized. Recombinant talin adopts different conformations
between an 80 nm robe-like shape and a 15 nm globular shape in vitro, which can be controlled and manipulated. Using cryo electron microscopy (cryo-EM) a 6.2 Å 3D-reconstruction of talin full length in its autoinhibited conformation was generated. Combining the map and the available structural information of individual domains, a model of talin was determined, revealing a general two-state inhibition and how different protein binding sites are buried within the domain arrangement. Further in vitro reconstitution showed, talin can bind to vinculin in a force independent manner depending on talin’s conformational state. Furthermore, talin acts as a scaffold of focal adhesion by binding to actin. The combination of structural and biochemical experiments gave insights into which of actin binding sites are accessible, depending on its conformational states.

Taken together, my findings suggest how talin’s closed conformation inhibits its interaction with its major binding partners actin and vinculin and suggests a mechanism for talin to be recruited and subsequently activated at the membrane.
Introduction

2 Introduction

2.1 Integrin based adhesions

The ability of cells to adhere to other cells or extracellular matrices has been an important step in the evolution of eukaryotes. Cell to cell and cell to matrix contacts are the two major adhesion classes in metazoan life. Cell to matrix contacts are not only anchors to other tissues, but a machinery for cell migration as well as signaling clusters to probe the cell’s environment. Their tight regulation and maintenance are essential in development as well as cell homeostasis. Failure of the system can cause defects in development or uncontrolled cell migration, which can result in cancer formation.

The major class of cell matrix adhesions are integrin mediated adhesions. They have two key functions: the connection of the extracellular matrix (ECM) with the cytoskeleton and the generation of signaling clusters. These clusters sense the cell’s mechanical and chemical environment and transmit signals inside the cell to adjust and regulate its behavior.

Integrins are class 1 transmembrane receptors, containing two subunits. They have a large extracellular domain which, when activated, binds to ECM proteins like fibronectin, laminin or collagen. In addition, each subunit has a single transmembrane helix as well as a short cytoplasmic tail where cellular adaptor proteins can bind and connect integrins to the cytoskeleton.

Due to their different roles and their dynamic nature, there are different types of integrin mediated adhesions: Nascent adhesions (NA) are small adhesion plaques at in the lamellopodium with a short lifetime. They are dynamic structures that locate at the leading edge of the cell. Focal adhesions (FA) are the best-studied adhesion structures. They are large protein assemblies that contain hundreds of different adhesion proteins and are connected via large actin cables with the cytoskeleton. FAs have a longer lifetime of several minutes. Fibrillar adhesions appear at locations where the most mechanical tension is exerted. They are matured adhesion complexes that create large actin stress fibers and can secrete their own fibronectin matrices.

Defects in adhesion components or their regulation are at the heart of many developmental and immunological diseases and also cause cancer formation. This makes them highly interesting therapeutic targets (Winograd-Katz et al., 2014). Therefore, the understanding of their interactions, spatial organization and regulation provides vital information for future drug design and development.
2.1.1 Molecular organization of integrin based adhesions

Integrin mediated adhesions are highly dynamic structures, containing hundreds of different proteins. Different proteomic studies each revealed thousands of proteins in various phosphorylation states, depending on the given integrin subtype (Horton et al., 2015; J. D. Humphries et al., 2009; Schiller and Fässler, 2013). A study comparing these different datasets determined a commonly found ‘core’ of 60 proteins (Horton et al., 2015). This core was determined and named the ‘consensus adhesome’. Among them are essential integrin activators like talin and kindlin, signaling proteins such as paxillin and the integrin-linked pseudokinase (ILK)-PINCH-parvin complex (IPP) and those adhesion proteins, that have the highest number of interactions with candidates that are outside of the ‘consensus adhesome’ like filamin, focal adhesion kinase (FAK) or α-actinin (Horton et al., 2016).

The architecture of FA has been studied in both x/y-direction at the plasma membrane, as well as in z-direction towards the actin cytoskeleton.

Due to recent developments in super-resolution microscopy, the architecture of FA in z-direction is quite well characterized. In vertical direction, FA organization can be divided into 3 distinct layers, with most proteins located to a specific layer (Kanchanawong et al., 2010). The first membrane proximal layer is called integrin-signaling layer and stretches approximately over 23 nm in distance away from the plasma membrane. It contains the integrin cytoplasmic tail and the talin head and is enriched in FAK, paxillin and inhibited vinculin. The second layer, named ‘force transduction layer’ reaches from 23 nm in to about 50 nm and contains the elongated talin rod and active vinculin (Case and Waterman, 2015). The last ‘actin regulatory layer’ is enriched in actin binding and crosslinks proteins like zyxin, VASP and α-actinin.

In x/y-direction the structure of focal adhesions less strictly layered. Some of the FA proteins show a polarized distribution at the membrane plane. Phosphorylated paxillin and other tyrosine phosphorylated proteins are enriched at the FA tip (Hu et al., 2015; Zaidel-Bar et al., 2007). In contrast we showed that Kank is enriched at the lateral border of the FA as a belt (Z. Sun et al., 2016). The lateral organization often depends on the highest force exerted on the FA. Using FRET tension sensors, it was observed that the highest ECM tension lies at the center of FAs. Interestingly this correlates well with paxillin localization, but not with talin and vinculin recruitment. However, these talin-tension sensor experiments also show
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that the force is directed towards the cell center, which correlates well with the tilted nature of elongated FA proteins like talin and vinculin (Kumar et al., 2018).

Some proteins require a specific orientation within FA. It was shown for talin that in its planar orientation its head is facing the cell periphery whereas its C-terminus is pointed towards the cell center (Margadant et al., 2011). In z-direction, talin’s head is located at the plasma membrane and its tail is pointing away at an angle of 15° to 25° (Paszek et al., n.d.) with an average distance of 97 nm (Liu et al., 2015).

**Figure 1: Architecture of integrin based focal adhesions in z-direction**

This model was generated from a study using interferometric photoactivated localization microscopy (IPALM) to obtain the positioning of the C- and N-termini from several adhesion proteins in z-direction. The model shows the 3 organization layers: Integrin signaling layer, the force transduction layer and the actin regulatory layer. Forces generated by actin retrograde flow and traction force through ECM are depicted as arrows in grey. Distance from the substrate in z-direction is displayed on the left side in nanometer. Legend of adhesion components located in the study is displayed on the bottom.
(Image is adopted from Case and Waterman, 2015)

It was long debated whether integrins located in the plasma membrane form clusters and whether these are always signs of integrin activation. In 2015 Changede et al. used super resolution microscopy to observe integrin clusters in FA. The study revealed integrin clusters of about 100 nm diameter containing 50 integrins on average (Changede et al., 2015). Clustering was previously associated with active integrins; however, this was challenged in 2018 by a study observing inactive integrin clusters, which were segregated in and out of FAs (Spiess et al., 2018). By the use of single molecule tracking, it was discovered that FA were permeated by freely diffusing as well as immobilized clusters of integrin; with
differences in diffusion behavior according to integrin type (Shibata et al., 2013; Rossier et al., 2012).

In general FAs can be seen as assemblies of archipel-nanoclusters that are anchored by immobile integrins to the membrane, but have components moving unhindered between them. Adhesion components exhibit various recruitment patterns to FAs. Some proteins, such as talin, α-pix and β-pix are recruited directly from the cytoplasm, whereas others followed a two-stage recruitment; first to the ventral plasma membrane and second in lateral direction towards the FA (Shibata et al., 2013).

2.1.2 Mechanobiology of integrin based adhesions

During development, cells sense their mechanical environment and the topology of their surroundings. These properties have a strong influence on where a cell migrates to, or what type of tissue is developed. Also, in later stages of multicellular life, mechanical properties can still determine cell fate. An example is the human mesenchymal stem cell system. Here, the stem cell differentiation is determined by the cell shape, which in turn is changed by the rigidity of its surrounding matrix (Engler et al., 2006). The bases for all of these processes are the cell-matrix adhesions. For all integrin mediated adhesions mechanosensitivity and mechanotransduction play an important role in maturation and disassembly.

Integrins that bind to RGD motifs on fibronectin can be activated through ligand binding. After this initial binding a ‘catch-bond’ is formed that further increases the lifetime of the integrin-ligand interaction. Catch-bonds are protein-protein interactions with increased bond-lifetime, due to additional allosteric binding properties that are induced by the application of mechanical force. In the case of α5β1 an additional binding site for fibronectin in the α-subunit is revealed, upon application of force (W. Chen et al., 2010). This catch-bond formation results in adhesion strengthening and maturation (Benito-Jardón et al., 2017; Friedland et al., 2009; Strohmeyer et al., 2017). Similar force induced activation was also recently reported for αLβ2 integrins in migrating T-cells (Moore et al., 2018; Nordenfelt et al., 2017).

Another effect of mechanical force in adhesions is the activation of force-specific binding sites on mechanosensitive adhesion proteins. Some adhesome components such as talin contain buried binding sites for interaction partners that are only available with the application of force. The application of mechanical tension can expose these cryptic sites
and lead to the recruitment of further adhesome components. It was shown for talin that under mechanical tension, additional vinculin binding sites are revealed and vinculin can be recruited (del Rio et al., 2009a). Similar behavior was observed for p130Cas, which was shown to reveal a Src specific phosphorylation site under mechanical tension (Sawada et al., 2006). FAK undergoes a conformational change under force that increases its kinase activity, leading to adhesion strengthening and maturation (Zhou et al., 2016).

In all of these cases the application of mechanical tension leads to increased recruitment and adhesion strengthening, which in turn associates more of the actomyosin machinery to the adhesion. This induces a feedback loop promoting adhesion maturation and growth.

### 2.1.3 Molecular clutch hypothesis

Cell migration is a complex process required for a multitude of cellular actions. During the cell movement, protrusions are created at the leading edge, which are made of rapidly polymerizing actin filaments. Actin is polymerized in close proximity to the leading edge with its barbed ends pointed towards the membrane. Actin is nucleated by the Arp2/3 complex, which binds to existing actin fibers and nucleates new actin filaments in a 45° angle. This creates a dynamic meshwork of polymerizing and depolymerizing actin filaments called the lamellipodium. The polymerization process of actin creates the force that drives the cell protrusion forward. However, this force can only be utilized if the protrusions are anchored to an immobile object like the ECM. When unanchored, the actin filaments get pushed back towards the cell center, a process called ‘actin retrograde flow’.

Integrin mediated adhesions function as anchors and use integrin adaptor proteins to couple actin filaments via integrin to the ECM. The ‘molecular clutch’ concept, originally proposed by Mitchison and Kirschner in 1988, explains this mechanism. It states that, like an engaged clutch, integrin adhesions coupled to actin flow can propel a cell forward, using the force that is generated by the actin polymerization at the leading edge (Mitchison and Kirschner, 1988).

Integrin has more then one binding partner, that is able to connect the transmembrane receptor to the actomyosin machinery (Brakebusch and Fässler, 2003). With binding sites for both actin and integrin, talin is thought to form initial bonds bearing a low force. Upon stronger engagement of actomyosin with the newly formed adhesion higher forces can be exerted, which may lead to partial talin domain unfolding. This allows the exposure of talin’s cryptic binding sites for vinculin, followed by vinculin recruitment and thus further
reinforcement of the clutch. It was shown that vinculin depletion results in excessive actin retrograde flow (Thievessen et al., 2013). Similarly, depletion of talin also leads to higher retrograde flow during initial cell spreading (Zhang et al., 2008).

Odde and colleagues observed, that the model predicts different behavior of the clutch system on different substrates, depending on the substrate stiffness (Chan and Odde, 2008). On hard substrates clutches would engage, but soon reach their braking point. Opposing, on soft substrates a longer loading time for talin to bind integrin would allow more clutches to engage, generating a more stable bond until breakage.

**Figure 2: Representation of Roca-Cusachs molecular clutch model**
A. actin retrograde flow is created by actin polymerization at the leading edge. Integrin adhesions anchor actin filaments to the ECM, thus acting as clutch and provide cell movement. B. Clutch model on soft substrates: Force is not sufficient to activate talin in time before the lifetime of an integrin-ECM bond runs out. Talin dis not activated and vinculin is not recruited. C. On stiff substrates, force can be generated on talin, vinculin get activated
and binds before the integrin-ECM bond slips, resulting in actin mediated adhesion reinforcement. (Swaminathan and Waterman, 2015)

This model was further evolved by the addition of the effect of talin’s force dependent vinculin recruitment and integrin’s ‘catch bond’ formation (Elosegui-Artola et al., 2016). It predicts that the integrin-substrate bond fails on low stiffness ECMS before sufficient force is transmitted to talin for clutch reinforcement through vinculin. However, on high stiffness substrates the integrin ECM bond is reinforced, force gets transmitted and vinculin recruited, thus resulting in increased traction and cell movement (Figure 2).

This bi-phasic force response due to substrate stiffness indicates that other integrin adaptors such as kindlin play a role in the integrin-actin linkage (Swaminathan and Waterman, 2017). Furthermore, it is currently discussed that the dimerization capabilities of both integrin adaptors, talin and kindlin could create a network that could prevent a system failure in case single integrin-ligand or integrin-adaptor links break (Klapholz and Brown, 2017).

2.2 Maturation of integrin based adhesions

Motility and dynamic migration are essential for development an need to strictly regulated throughout cell life; therefore integrin adhesion structures are heterogeneous in composition and can undergo drastic morphological changes depending on their mechanical and biochemical activities. Integrin adhesions start as nascent adhesions and mostly disassemble after less than two minutes. However, when engaged with force and with the required signals they can mature into focal adhesions, or even further, into fibrillar adhesions. Both are larger adhesion structures with a longer lifetime that act as more stable cell-matrix anchor (Figure 3).

The maturation of integrin adhesion structures is regulated by a multitude of factors. Biochemical processes such as phosphorylation (Choi et al., 2011), proteolysis (Franco et al., 2004) or cytosolic Ca^{2+} level play important roles. In addition, mechanical properties of the surfaces and the forces cells exert on their adhesions are essential for their maturation (Schoen et al., 2013). Upon maturation, changes in protein composition occur, through recruitment of a wide range of adhesome components, as well as reinforcement of the actin cytoskeleton with actin-crosslinker and motor proteins.
**Figure 3: Schematic view of actin based adhesion maturation in the lamellipodium.**

Lamellipodium represented as actin network (yellow) with Arp2/3 network intersections and NAs (blue). NA entering the lamellum are either disassembled, together with their actin connections and turned over, or mature force-dependent into elongated focal adhesions. FAs in the lamellum recruit actin bundling proteins such as α-actinin and later force generating myosin-II to mature and enlarge further (adopted from Vicente-Manzanares et al., 2009).

For a moving cell following biochemical signals, it is vital, that adhesions stay dynamic and that ECM anchors at the rear of cell are disassembled. Cells disassemble and internalize their adhesions at the rear end and recycle their components in order to minimize the required energy.

### 2.2.1 Nascent adhesions

To bring cells into motion, they require a dynamic cycle of assembly and disassembly of their adhesions at the lamellipodium and the rear end of the cell. At the leading edge of the cell the first visible adhesion structures are the short-lived NAs. With a diameter of less than 0.25 μm, they are the smallest visible adhesion structures triggered by integrin activation (Choi et al., 2008; Gardel et al., 2010). Their formation at the edge of the lamellipodium requires a couple of initial steps.

To initiate the NA formation, integrin needs to be activated and bound to a ligand. It is still debated how this activation is precisely achieved. It is thought that integrins can be either activated from the inside by adaptor protein binding (inside-out activation) or that they can be activated via extracellular ligand binding (outside-in signaling). Inside-out
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activation is facilitated by the integrin adaptor proteins talin and kindlin. Though it was initially thought that talin binding to the integrin cytoplasmic tail is sufficient, it was later shown that initial NA formation can occur without talin being present and that loss of kindlin is fatal for integrin activation in some cell types (Moser et al., 2009; Zhang et al., 2008; Montanez et al., 2008).

Furthermore, activated integrins need to be clustered in order to form NA. It was shown that in small adhesion structures, integrins are clustered in groups of $>$ 10 within in the plasma membrane (Wiseman et al., 2004). In addition, on the outer membrane site integrins need to be able to bind to at least three RGD nanoclusters in the ECM for a successful NA formation (Cavalcanti-Adam et al., 2007).

For further stabilization and maturation of NAs, force needs to be exerted on integrins bound to the ECM via the actin cytoskeleton. Talin is used as a mechanical linker between integrin and the actomyosin-machinery. It links integrin directly to actin, as well as indirectly via the force sensitive recruitment of vinculin. Talin acts as a clutch in transmitting force generated by the actin retrograde flow to integrin. The importance of mechanical force was shown in experiments, where either vinculin or Arp2/3 complex were knocked out, both resulting in strongly reduced NA maturation (Dang et al., 2013; Thievessen et al., 2013).

During NA formation, different adhesion signaling molecules, such as focal adhesion kinase, p130CAS or Src, are recruited to the adhesion site. Some of them, for example most LIM domain containing proteins, are recruited in a force-dependent manner. Furthermore it was shown that most of the initial adhesion components appear at the NA at about the same time. In 2014, a study by Bachir et al. showed that some of these adhesion components are recruited from and ejected to the cytoplasm in preassembled complexes. This indicates that they are reused for different adhesion complexes, which agrees with the dynamic picture of NAs (Bachir et al., 2014).

NAs can act as anchors between ECM and actin cytoskeleton or as signaling assembly to sense surrounding properties, but eventually, depending on the movement speed of the cell every NA will reach the rear end of the lamellipodium. Here, their fate is decided to either be disassembled or to mature into FAs. Thus, every NA’s lifetime and their maturation speed depends on the cell’s migration speed.

Dynamic NA assembly is vital for moving cells. On the one hand, it provides them with a fixing point to move the cells lamellipodium forward, through the cycle of actin polymerization and treadmilling. On the other hand, NAs act as mobile signaling centers to
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report the biochemical environment and ECM properties, which in turn decides over migration or resting of the cell.

2.2.2 Focal adhesions

An NA that has reached the rear edge of the lamellipodium can mature into a FA. However, for this maturation process the right signals need to be provided. During this maturation the lifetime of focal adhesions increases drastically to up to 20 minutes and its size enlarges to 1 to 5 µm. Located at the inner edge between lamellum and main cell body FAs start connecting to stress fibers and recruit more adhesome components (Figure 3). This process requires further integrin clustering, stable integrin-actin linkage and actin bundling.

One of the proteins that is suspected to have a role in some of these events is α-actinin. It was shown to directly bind integrin cytoplasmic tails and to be recruited in clusters with β1 integrin to growing FAs (Bachir et al., 2014; Roca-Cusachs et al., 2013). Further, α-actinin is able to crosslink F-actin filaments into larger actin bundles. This increases the actin amount at the FA leading to a higher local concentration of actin-bound talin, which in turn ensures more stable integrin-actin linkage (Rossier et al., 2012).

Another process of great impact for FA maturation is mechanotransduction, due to its implications in enabling the access of force-dependent binding sites in adhesome proteins and allowing the recruitment of critical components, such as vinculin recruitment to talin (del Rio et al., 2009b). Proteomic studies have shown that inhibition of myosin-II, a protein that induces mechanical force in the actin cytoskeleton, alters the adhesome protein composition, as the recruitment of some of the adhesome components depends on mechanical tension (Horton et al., 2015; Schiller and Fässler, 2013). It was observed for myosin-II inhibition that the recruitment of critical integrin adaptor proteins, such as kindlin and talin is diminished. In addition, a strong effect was observed for LIM-domain containing adhesome members, such as zyxin and paxillin (Horton et al., 2015). Interestingly, in these myosin-II deficient cells the levels of β-Pix, an adhesion maturation inhibitor was increased, which lead to increased rates of cell migration (Kuo et al., 2011).

Mechanical tension in FAs has other interesting aspects; as it facilitates for some proteins not only the additional recruitment of binding partners (for example vinculin the in case of talin), but also increased accessibility of tension-dependent phosphorylation sites, thus altering the signaling aspects in FAs. This type of effect was shown in the case of the
focal adhesion kinase and p130Cas, both gaining additional functionalities under mechanical force (Sawada et al., 2006; Zhou et al., 2016).

Another effect of tension is the formation of so-called ‘catch-bonds’. It was shown for β1-integrin that it changes its conformation under force to increase binding to synergy sites on fibronectin, thus strengthening its bond (Friedland et al., 2009). Another catch-bond formation suggested in the field is the interaction between talin and the integrin tail. In vitro they show only a weak interaction in the high micromolar range, but they are suspected to increase their bond strength and bond lifetime significantly under tension.

Overall, mechanical force on adhesion components is suggested to lead to increased phosphorylation, followed by further recruitment of adhesion components and actin, thus inducing a feedback loop to further strengthen the adhesion.

2.2.3 Adhesion disassembly

To maintain a constant movement, it is important for cells that the adhesion’s tethering to the ECM remains dynamic. Adhesions need to be assembled and reinforced at the leading edge, but also need to be disassembled at the rear end of the cell, or in regions that are not optimal for migration. Adhesion disassembly is the default fate for NAs, if maturation to FA does not occur. In FAs however, this process needs to be tightly regulated.

As FA networks are complex protein assemblies it is unlikely that their disruption follows the inverted sequence of events that are needed for their formation (Ezratty et al., 2005). It is more likely that their disassembly is orchestrated through a disruption of the weakest nodes in the network. While adhesions are diverse and consist of hundreds of different proteins, there are only two integrin adaptor proteins at the core of all adhesion architecture, namely talin and kindlin. The disruption of talin and kindlin’s binding to the cytoplasmic integrin tail eventually leads to a reduction of force in adhesions, due to the loss of the cytoskeleton-ECM linkage. Thus, the loss of force in a FA initiates adhesion disassembly, shown by experiments in the presence of blebbistatin or in cells with an inhibited ROCK pathway (Carisey et al., 2013a; Stehbens et al., 2014).

There is little known about how interactions between kindlin’s and the cytoplasmic integrin tail could be disrupted. However, several mechanisms were suggested that could destabilize the integrin-talin-actin linkage and initialize adhesion disassembly.
Talin’s sequence shows two potential cleavage sites for the calcium-induced protease calpain. Both are at critical positions within the protein; one is located in the linker region between talin’s head and rod, the other at talin’s C-terminal actin binding site, before its dimerization domain. It was shown that the inhibition of calpain hinders adhesion disassembly in cells (Bhat and Ma, 2002; Franco et al., 2004).

Upon phosphorylation through the MAP4K4 kinase, the FERM domain protein moesin can compete with talin for the interaction with the cytoplasmic integrin tail. Displacement of talin promotes adhesion disassembly (Vitorino et al., 2015). In this context, it was shown in D. melanogaster that the MAP4K4 kinase homologue missshapen is enriched at the rear end of migrating cells to promote cell migration (Lewellyn et al., 2013).

Interestingly, the histone methyl-transferase EZH2 is able to methylate talin at actin binding site 3 in the C-terminus, thus partly disrupting talin’s link to the actomyosin machinery. This leads to adhesion disassembly and was shown to promote higher levels of cell migration for neutrophils and dendritic cells (Gunawan et al., 2015).

A more global mechanism for adhesion disassembly is the internalization of integrin clusters at adhesion sites. It has been observed that microtubules, targeting adhesions structures at the front of the migrating cells tend to induce their endocytosis (Ezratty et al., 2005). Furthermore, cells can induce the clathrin-mediated endocytosis of integrins through adaptor proteins that compete with talin’s and kindlin’s binding to the NPxY and the NxxY motif on the β-integrin tail (Calderwood et al., 2003). The adaptor proteins Dab2, ARH and Numb were shown to concentrate around adhesion sites and mediate the clathrin-dependent integrin internalization (Ezratty et al., 2009).

Some proteins including FAK, ERK, paxillin and p130Cas (Webb et al., 2004) are shown to have a role in adhesion disassembly, since their absence makes adhesions more stable and increases their lifetime. In the case of p130Cas, it has recently been shown that cells make use of its degradation to enhance the stability of some selected adhesions. In those adhesions p130Cas is actively phosphorylated, which leads to the recruitment of an E3 ubiquitin ligase that induces p130Cas’s degradation (Teckchandani and Cooper, 2016). The cell’s ability to alter p130Cas levels in adhesions indicates a possible selection mechanism that allows cells to actively maintain or disrupt adhesions, depending on the current requirements.
It is necessary for multicellular organisms that their cells organize by adhering their surfaces and facilitating their cell-cell communication. During their evolution, they developed complex receptor systems, sensing their environment and are able to mediate signals to themselves as well as other cells. The receptor family responsible for interaction and communication with the ECM are integrins.

The integrin receptor family is a highly conserved group of heterodimeric transmembrane receptors, which was first described in the 1980s and named after their function of integrating the ECM with the cytoskeleton (Hynes, 1987; Tamkun et al., 1986). They mediate cell adhesion to different ECM proteins and other cell receptors such as the I-CAM and V-CAM receptor family (Xia and Kanchanawong, 2017).

Integrins are heterodimeric transmembrane receptors composed of two individual proteins the α and β subunit. There are 16 α and 8 β subunits, which form the 24 members of the integrin family. Integrin subunits are usually composed of a large extracellular domain a single transmembrane helix and a short unstructured cytoplasmic tail. Even though integrin receptors are universally expressed in metazoans each family member adopted specific roles and tasks during evolution in the organism development and tissue homeostasis, together with their specific ECM ligand (Johnson et al., 2009).

The correct function of integrin systems is vital for many types of cells. Cells in development need to travel the correct path in order to form organs correctly; leukocytes need to adhere site-specific of engage their designated targets; platelet activation needs to be tightly controlled in order to avoid catastrophic failure of the hematopoietic system. It is clear that integrin-mediated adhesions play a critical role in cancer formation, due to their role in potentially enabling cells to migrate and overcoming tissue homeostasis.

The 24 members of the integrin family are heterodimeric class 1 transmembrane receptors. They are composed of non-covalently associated α- and β-subunits, each with a large extracellular domain, a single transmembrane helix and a short cytoplasmic tail (except for β4 integrin). Cells generally express an excess of β-subunits, but present only heterodimeric integrin to the cell surface. Hence, the amount of α-subunits determines the integrin surface concentration (Santala and Heino, 1991).
The average integrin has roughly 1750 aa, where the α-subunit provides 1000 aa and the β-subunit 750 aa. The α-subunit consists of a seven-bladed β-propeller linearly connected to a thigh, a calf1- and calf2-domain followed by the transmembrane domain (TMD) and the short cytoplasmic tail (Figure 4). In 9 of the 18 α-subunits the β-propeller contains an additional αI domain between the 2nd and 3rd blade, which in those integrins directs the ligand binding (Larson et al., 1989). This αI domain assumes a Rossman fold and, together with a coordinated Mg2+ ion, forms the metal-ion-dependent adhesion site (MIDAS) (J. O. Lee et al., 1995).

The β-subunits ectodomain is composed of a plexin-sempahorin-integrin (PSI) domain followed by a hybrid domain, a βI domain and four cysteine-rich epidermal growth factors (EGF) repeats. Interestingly the βI domain also contains Mg2+ coordinating MIDAS site as well as an adjacent a Mg2+ coordinating ADMIDAS (adjacent to MIDAS) site, both within the βI domain.

Both TMD of the α or β subunit are highly conserved. They are single transmembrane helices that reach into the cytoplasm and overlap in a juxtaposed manner in the dimerized state. It was shown that the TMD have two distinct interaction points that are responsible for dimer formation. One lies within the membrane, where the dimerization is mediated via a GxxxG motif on the α-subunit’s transmembrane helix. This motif creates the binding pocket for another hydrophobic patch on the β-subunit’s transmembrane helix. The other interaction site is called the inner membrane clasp and occurs at a patch of opposing electrostatic charged residues, located at the juxtaposed region on C-terminal site of both TMDs. Through mutation studies, it was shown that in both the inner (R995D/D723R) and outer (G972L/G976L) membrane clasp that disruption of the dimerization interface results in constitutively active integrins (Hughes et al., 1995; Luo et al., 2005). Interestingly at the C-terminal end of both transmembrane helices a so called ‘snorkling lysine’ followed by a hydrophobic region can be found, which is suspected to be the reason for the tilted arrangement of the transmembrane helixes. These hydrophobic regions are responsible for both tilted and/or straight positions of the TMDs within the membrane, which is thought to play a significant role in the transmembrane signal transduction (Partridge et al., 2005).

Integrins have rather short (30-50 aa) cytoplasmic tails, except for the β4 integrin tail, which has about 1000 aa. It is widely accepted that both the α or β subunit’s cytoplasmic domains are unstructured, if not in complex with an integrin binding protein (Campbell and M. J. Humphries, 2011). It was proposed by NMR studies (LReview6) that α or β cytoplasmic tails could interact via a salt bridge between the GFFKR (α) HDR(R/)/E (β) sequences;
however, these interactions seem to be weak at best (Legate and Fässler, 2009). Of more importance are two motifs on the β integrin cytoplasmic tail, both of which function as recognition sequences for PTB domain proteins. The membrane proximal NPxY motif and the membrane distal NxxY motif act as binding sites for crucial integrin adaptor molecules such as talin and kindlin.

**Figure 4: typical domain arrangement of an αI-domain containing integrin family member.**
A.: shows the domain arrangement of both heterodimer subunits. Grey bar indicates the divalent cation-binding sites. Stars indicate the divalent cation-binding sites. Grey bar indicates the transmembrane helix and membrane dimerization sites. B.: Representation of the integrin heterodimer within the plasma membrane. Extracellular domain depicted on the top, transmembrane helixes indicated as stretched ellipsoids across the membrane and cytoplasmic tails represented by cylindric columns at the bottom. (Barczyk et al., 2010)

The ligand specificity of integrins is usually dictated by the α-subunit but several studies suggest that divalent cations binding to the MIDAS and ADMIDAS sites are of importance as well (J. O. Lee et al., 1995; Springer et al., 2008; Xiao et al., 2004). The integrin family can be classified according to their ligand binding into four subcategories. A third of the integrins (α5β1, α8β1, α11β3 and all αV integrins) recognize the RGD sequence in matrix proteins like fibronectin, vitronectin, fibrinogen and others. The αI-domain containing integrins α1β1, α2β1, α10β1 and α11β1 recognize a GFOGER motif and belong to the collagen and laminin binding integrins. Another group containing α3β1, α6β1, α7β1 and α6β4 binds to laminins, through yet unknown but specific interactions. The last subgroup contains all leukocyte-specific β2 integrins, LDV motif specific integrins (α4β1, α4β7 and α9β1) and αEβ7 integrin that interacts with the αI domains in ICAM, VCAM and E-cadherin (Figure 5).
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2.3.2 Integrin activation

The role of integrins in cells is diverse. Not only do they provide a physical link for the actin cytoskeleton to the extracellular matrix, but they can sense the extracellular environment and induce signaling cascades to alter the fate of cells. Integrins do not possess any catalytic activities themselves, but function as transmitters of signals through the plasma membrane. For this they can adopt different conformations as response to inside and outside signals. Through structural studies it was shown that that integrins have at least 3 different states (Luo et al., 2007a) (Springer and Dustin, 2012).

It was shown in a X-ray crystal structure that αVβ3 integrins ectodomain adopts a bent conformation with the N-terminal ligand binding domain facing downwards next to the crossed α- and β-subunits leg domains (Xiong et al., 2001). This ‘bent’ conformation is referred to as the inactive state, since the ligand binding domain is facing towards the plasma membrane.

**Figure 5: Integrin family overview**

Representation of the vertebrate integrin family. The 24 different heterodimers are arranged in different classes, according to their ligand binding capabilities, which is usually defined by the α-subunit (Barczyk et al., 2010)
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In 2002, an electron microscopy study on αVβ3 showed that integrin can adopt an extended open conformation upon Mn2+ addition in which both subunits legs are separated from each other and the head domain is pointing away from the membrane. In 2010 it was shown that a similar conformational change after addition of the talin head domain on αllbβ3 in lipid nanodiscs (Ye et al., 2010). In addition, it was shown that the movement of integrin’s ectodomain positions the ligand binding site ~19 nm away from the membrane, thus increasing the reach of integrin receptors in a membrane potentially surrounded by large glycosalix structures.

The third conformation named ‘extended closed’ is a transition between ‘bent’ and ‘extended open’, where the ectodomain is extended, but its leg domains and the TM domains are still closed. This conformation was suggested by negative staining electron microscopy studies (Takagi et al., 2002). It is yet unclear if there are more transition states between these three steps that remain to be uncovered.

A recent paper hinting at those dynamic short-lived states shows a cryo-EM structure of the head domain of αVβ8 integrin, where the headpiece conformation is stabilized by the β-subunits leg. The study also shows that within its extended closed conformation the integrin undergoes a dynamic ‘sunflower like’ movement, where the head domain is able to shift and rotate on the joint of the closed leg domains(Cormier et al., 2018).

It was further observed in an X-ray crystallography study, where crystals containing the integrin extracellular domain were soaked with ligand, that during the transition between the three main conformations the βI MIDAS domain is reshaped by a piston-like movement of the α7 helix. This causes the hybrid domain to pivot by 60° and finally resulting in a swingout movement of ~75 Å by the β-subunit’s leg domain away from the α-subunit (Figure 6). Since this drastic movement results in a separation the α- and β-cytosolic tail potential integrin adaptors can bind and subsequently force can be applied to the extended β-ectodomain (J. Zhu et al., 2013).

The structural information about integrin’s conformational changes is mostly derived from platelet integrins. However, it is expected that due to the high amount of sequence homology between different integrin family members this information remains true for other integrin family members.

The precise steps during the events of integrin ligand binding and head piece extension are still unclear; different models have been proposed and are highly debated.

The ‘switchblade’ model predicts that ligand binding can only occur after the integrin head piece is extended (Luo et al., 2007b). This extension is induced by ligand binding of
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talin and probably kindlin to the integrin cytoplasmic tail (Inside-out signaling). In contrast, the ‘deadbolt model’ hypothesizes that ligand binding to the extracellular domain induces the head piece extension and the leg-swingout and subsequently allows adaptor proteins to bind (Outside-In signaling) (Xiong et al., 2003).

Another model that is recently discussed is based on the kinetic energy needed to maintain the integrin in its extended open conformation. In the thermodynamic landscape the transition between bent-closed and open-extended integrin requires a high amount of activation energy. Since, the simple binding of talin and/or kindlin is probably not sufficient to provide this energy, an additional effect is needed. It is predicted that small mechanical forces of 1-3 pN are needed to keep the extended-open conformation stable (J. Li and Springer, 2017). The model predicts, that a through application of small forces the conformational equilibrium is shifted towards the extended open conformation. Hence ligand binding and small forces provided through adaptor proteins can induce and maintain the extended open conformation (Z. Sun et al., 2018).

![Figure 6: Illustration of an α-I domain integrin during activation and ligand binding.](image)

The integrin activation either by external ligand binding (A-F except E) in 5 steps, or 3 steps by internal ligand binding to β-integrins βI-domain (A,E and F). B and D are hypothetical stages, A,C,E and F were observed in electron microscopy or crystal structures. F. Forces applied by actin cytoskeleton over integrin are depicted in dashed lines. (Sen et al., 2013)
2.3.3 Integrin adaptor proteins talin and kindlin

Integrin activation can be induced in multiple ways. Whereas a ligand binding from the outside can induce the piston like movement of the β-subunit and separate the TM domains, it is believed that a similar motion can be initiated from the cytoplasm. Integrins cytoplasmic tail has many interaction partners, among them talin was first discovered to play a critical role in integrin activation. It was shown in chinese hamster ovaries (CHO) that talin can activate normally inactive integrin (Calderwood et al., 2002; Horwitz et al., 1986). Further knockout and knockdown experiments showed that talin is key in regulating integrin’s affinity and ligand binding. Talin orthologs were subsequently found in all multicellular eukaryotes and in vertebrates two talin isoforms were detected (Calderwood et al., 1999; Monkley et al., 2001a). Talin is organized in two main parts, a head and a rod domain and it was shown that the F3 subdomain of the FERM like head domain interacts with integrins membrane proximal NPxY motif. Despite the head domain being of importance for the integrin activation (Ye et al., 2010), the rod domain in needed for correct adhesion and cell spreading (Zhang et al., 2008).

In 2008 siRNA studies in mice showed that kindlin is another important integrin adaptor protein essential in integrin activation (Montanez et al., 2008; Moser et al., 2009; 2008; Ussar et al., 2008). Kindlins are structurally similar to talin head in that they are FERM domain proteins, though they have an additional pleckstrin homology (PH) domain inserted in the F2 domain. Their family has 3 members; kindlin-1 is expressed in epithelial cells, kindlin-2 is widely expressed, kindlin-3 is expressed mainly in hematopoietic cells. It was subsequently discovered that kindlin is needed for correct integrin activation (Moser et al., 2008; Theodosiou et al., 2016).

Whereas talin mainly binds to the membrane proximal NPxY motif on the integrin β-subunit tail, kindlin’s F3 domain was found to interact with the membrane distal NPxY motif (Harburger et al., 2009). It is unclear if both proteins bind the integrin tail simultaneously or if the interaction happens in tandem.

Kindlin functions as protein interaction hub and is able to bind and recruit the complex of ILK-PINCH-parvin (IPP) to the cytoplasmic integrin tail, as well as paxillin and the arp2/3 complex (Böttcher et al., 2017; Fukuda et al., 2014; Theodosiou et al., 2016).

A crystal structure of kindlin-2 without its PH domain revealed, that kindlin similar to talin is able to dimerize, though with very slow kinetics, giving rise to the hypothesis that kindlin plays a role in integrin clustering (H. Li et al., 2017).

Interestingly, it was found that kindlin can bind actin directly, thus would be able to connect
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the actin cytoskeleton directly to integrin. However, unlike talin there was so far no report on kindlin bearing any forces in vivo (Bledzka et al., 2016).

2.4 Talin

Discovered in 1983 as a protein that localizes to adhesion sites, talin was shown to be an integrin adaptor protein binding to cytoplasmic integrin tail. Shortly afterwards, it was shown that talin could also bind to F-actin. (Burridge and Connell, 1983) (Horwitz et al., 1986; Muguruma et al., 1990). Thus, talin was seen as adaptor molecule that could act as a direct link between integrin and the actin cytoskeleton. Talin’s sequence is highly conserved throughout metazoan life. Interestingly mammals acquired two isoforms of talin: talin1 and talin2, which have different tissue specificity (Gough and Goult, 2018a; Kopp et al., 2010a; Monkley et al., 2001b). Whereas talin1 is expressed in all tissues, talin2 expression is more variable as it is for example highly expressed in heart muscle cells and neurons. Both isoforms seem to complement each other as it was shown that a knockout of talin1 in mouse fibroblast could be compensated by upregulation of talin2 (Kopp et al., 2010b). However, a knockout of talin1 in mice is embryonic lethal (Monkley et al., 2000). These and other genetic studies in mice, drosophila and C. elegans show that talin is essential for flawless integrin function.

2.4.1 Talins structural organization

As one of the most important integrin activators talin is a large (~2500 aa) protein in the cytosol of the cell. It consists of two main parts, the N-terminal 4.1-ezrin-radixin-moesin (FERM) domain (~400 aa) known as the talin head and the C-terminal rod domain (~2000 aa), which are connected, via a 82 aa unstructured linker region.

FERM domains are found in many different proteins and typically consist of 3 subdomains (F1 – F3), which have membrane binding properties (Tepass, 2009). However, talin’s FERM domain is atypical as it has an additional subdomain (F0), which has a similar sequence to first subdomain (F1). Both, F0 and F1 domains share an ubiquitin like fold, whereas subdomain F3 shows a phosphotyrosine-binding (PTB) fold. It was shown that the F0 domain interacts with Rap1, which could play a role in talin’s membrane recruitment
(Bromberger et al., 2018). The F2 and F3 domains contain basic amino acid patches mostly made of lysine, acting as lipid binding sites for charged lipids. It was shown that these lipid binding sites are especially specific to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) (Chinthalapudi et al., 2018a). In addition, there is a ~40 aa loop region in the F1 domain containing several positive residues binding to negatively charged lipids. Further the F2 and F3 domain harbors actin binding site 1 (ABS1), which was recently shown to have an actin capping function while bound to the integrin tail (Ciobanasu et al., 2018).

The F3 domain also contains a conserved binding site that directly engages integrin at the membrane proximal NPxY motif on the cytoplasmic integrin tail. It was shown that both talin 1 and 2 have different specificity to different integrin tails, based on the charges of the conserved aa in the F3 domain (Gingras et al., 2010b). In addition to those binding sites it was reported that the F3 domain is able to bind different other proteins sharing the same motif. One of them is the PIPKIγ90, which is a source of PtdIns(4,5)P₂. Further interaction partners associated with talin regulation, such as RIAM, FAK, TIAM, layilin and Gα13 were reported to bind to this F3 interface as well. These multiple interaction partners make the binding interface in the F3 domain probably the most important regulation checkpoint for talin’s activity. This is supported by the crystal structure, showing an autoinhibition of talins F3 domain though binding of talins R9 domain (4F7G)(Song et al., 2012a).

The talin rod connected to the head domain via an 82 aa unstructured linker region, sensitive to calpain cleavage (Franco et al., 2004). The 2000 aa rod is constituted of 62 alpha helixes organized into 13 rod domains and a single alpha helical dimerization domain at the C-terminus. Of the 13 rod domains 9 rod domains (R1,R5,R6,R7 and R9-R13) are arranged as bundles of 5 helixes arranged one after another in a linear fashion, due to their N- and C-terminus located at opposite ends of the bundle (Figure 7). The other 4 rod domains (R2,R3,R4 and R8) are organized as 4 helix bundles with N- and C-terminus located side by side. Due to this, R2 - R4 form a distinct region at the N-terminus of the talin rod. The 4 helix bundle of R8 is inserted in between the 5 helix bundle of R7, forming a unique fold within talin (Figure 7)(Gingras et al., 2010b).

There are numerous interaction sites for different proteins along the rod (Figure 7). It was suggested that there are 11 potential vinculin binding sites in the talin rod, however all of them are buried in different helix bundles and are not directly accessible (Gingras et al., 2005; Kuo et al., 2011). Due to their N- and C-terminus location 4 helix bundle rod domains are more force sensitive and have a higher tendency to display their buried vinculin binding sites. An exception to this is the R8 rod domain. It is force resistant, due to its embedded
location within the R7 domain, this helps to retain its binding interface for its multiple interactors.

The rod further contains 2 actin binding sites (ABS2 and ABS3). ABS2 is located between the domains R4 – R8 and with the current understanding responsible for the main tension bearing actin connection (Atherton et al., 2015). ABS3 on the other side seems to act as initial actin binding point to provide force to talin and make the mechanosensitive vinculin binding site in R3 accessible.

In addition to the Integrin binding site 1 (IBS1) at the F3 domain, there is a secondary IBS in the R11 domain, though its function is not yet fully understood. It is discussed that it could play a role in integrin clustering and talin network formation at the membrane (Klapholz and Brown, 2017). The same motif in R11, as well as the rod domain R8 were shown to contain binding sites for RIAM. Rod bundle R8 acts as a binding motif for multiple other adhesion proteins, such as LIM domain containing paxillin, α-synemin and the DLC-1 receptor (H.-S. Lee et al., 2009; G. Li et al., 2011; N. Sun et al., 2008).

**Figure 7: Talin-1 domain organization and mechanical domain unfolding**

A. Domain organization and amino acid annotation of human talin-1. FERM domain indicated with its binding sites in yellow and orange, rod helixes in grey, helixes involved in actin binding (red) and helixes with VBS (purple). Further different protein interaction sites indicated on top. Lower panel shows model of the domain unfolding of a 4 and 5 helix rod domain through force (red arrow) and display of the cryptic VBS (purple). (adopted from Klapholz and Brown, 2017)

In our study, we could show that Kank is binding to talin’s R7/R8 domains via its N-terminal KN motif and that it has several functions at the adhesion site that are talin related. It is able to activate talin in vitro and subsequently enhance integrin activation. In addition,
the recruitment of Kank to actin associated talin reduces the amount of actin that is bound via talin (Z. Sun et al., 2016). It was confirmed in another study, that the interface for Kank binding on the talin rod is the R7 domain (Bouchet et al., 2016).

At the very C-terminus of the rod is the dimerization domain, consisting of a single helix, which can dimerize with its counterpart in an antiparallel manner (Gingras et al., 2008). It was suggested that talin can dimerize as a homodimer, where both rod domains are wrapping around both talin heads and form a globular structure (Goult et al., 2013a). Another EM study showed, that talin’s R13 and dimerization domain can form dimers on actin fibers (Gingras et al., 2008).

In our study we determined the structure of recombinantly purified full length talin with cryo-EM. We confidently placed 15 of the 18 domains of talin and identified it as a monomer. Via biochemical and biophysical experiments, the talin monomer was characterized in both active and inactive state. However, the position of the dimerization domain was not visible with certainty in the structure. Thus, is still an open question whether the dimerization plays a role in molecular organization of talin (unpublished data).

2.4.2 Autoinhibition and activation

Despite the most common depiction of talin as elongated chain of alpha helical bundles (Figure 7), talin also exists in an autoinhibited conformation in the cytoplasm. Since talin is required for integrin activation it is expressed in high amounts in some cell types (Calderwood et al., 1999; Tadokoro et al., 2003), which in turn makes the regulation of its activity critical. First hints for an autoinhibition function appeared from a study showing, that overexpression of talin’s head domain to have a stronger effect for integrin activation compared to the overexpression of talin full length (Goksoy et al., 2008; Song et al., 2012a).

In 1997 it was shown that talin adopts a globular structure in lower ionic strength (Winkler et al., 1997). In 2013 a cryo-EM structure showed a globular talin and fitted with SAXS models of the individual talin domains suggesting a possible architecture for the autoinhibition (Goult et al., 2013a).

Talin’s primary autoinhibition is facilitated by the interaction of the R9 with the F3 domain (Goksoy et al., 2008). This interaction surface is highly conserved and a mutation of a glutamic acid to alanine at position 1770 showed reduced wound healing in mice (Haage et al., 2018). Interestingly, it was also shown that breaking this interaction is not sufficient to
recruit all cytosolic talin to the plasma membrane (B. A et al., 2012), indicating that there are multiple layers of inhibition in talin’s architecture (Gough and Goult, 2018b).

A model in the field is the ‘push and pull’ model, which describes how the inhibition between the F3 and R9 domain could be relieved by charge; Upon binding of negatively charged lipids to the patches of basic residues on the F3 domain, acidic patches on the R9 domain would be repelled from the membrane, leading to the dissociation of the F3 – R9 link (Wang, 2012). Though, how talin is recruited to the membrane and if there are protein interactions acting in support with this mechanism is unclear. There are different candidates that play a role in talin activation.

Integrin activation studies in CHO cells suggested RIAM, the effector of the GTPase Rap1, to be a talin activator (H.-S. Lee et al., 2009). RIAM is able to bind to two regions (R8 and R11) in the talin rod domain, however it was also shown to recruit talin to the membrane and bind to a shielded region in the F3 domain of the talin head. It was suggested to then activate talin, by breaking its F3 – R9 interaction and promote integrin β3 activation (H.-S. Lee et al., 2009). Platelets however lack RIAM expression, indicating that there are different mechanisms how talin can be activated in different cell types.

In combination of in vivo (platelets) and in vitro NMR studies, it was shown that Rap1 binds to talin F0 domain and recruit talin to the plasma membrane, where talin is thought to activate platelet integrins (Bromberger et al., 2018; L. Zhu et al., 2017) (Figure 8). Recently, another study confirmed these results and proposed an additional Rap1 binding site with low affinity in the F1 domain of the talin head (Gingras et al., 2019).

**Figure 8 : Model talin recruitment via Rap1 and activation through Push and Pull**

Model shows talin head bound to the PtdIns(4,5)P₂ rich membrane, via clusters of positively charged residues (darkblue, surface representation) The F0 domain is bound to Rap1 (orange), the F1 flexible loop is shown in brown. The β-integrin tail (coral) is bound to the F3 domain and the R9 domain (cyan) is repelled from the neg. charged membrane through its patches of negatively charged residues (magenta, surface representation). Margenta arrows indicate forces binding and repelling from the membrane (Push and Pull model). (adopted from Bromberger et al., 2018)
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A study provided by Schiemer et al. in platelets found that the G-protein subunit $\text{Ga}_{\text{13}}$ binds directly to talin and is thereby able to modulate the activity of platelet integrin $\alpha\text{IIb}\beta\text{3}$. They used FRET studies to show that upon $\text{Ga}_{\text{13}}$’s binding to the F3 domain in the talin head the conformation of the full talin molecule alters (Schiemer et al., 2016).

Another possible molecule suggested to be critical for the activation of talin is the PIPK$\text{Iy90}$. This kinase is involved in the synthesis of PtdIns(4,5)$\text{P}_2$ in the plasma membrane and shows a nanomolar interaction with talin’s F3 domain. The loss of talin’s interaction with PIPK$\text{Iy90}$ leads to reduced vinculin and talin recruitment and lower ECM attachment (Anthis et al., 2009; Legate et al., 2011).

In our study, we showed that Kank has the ability in vitro to increase talin binding to integrin, thus breaking talin’s inhibition (Z. Sun et al., 2016). Kank binds at the R7 domain of talin via its KN-motif, however how precisely this resolves the inhibition is not clear.

Furthermore, we solved a cryo-EM structure of the full length talin molecule where we observed that talin’s autoinhibition is mediated via two sites. In addition to the F3 – R9 interaction we found an interaction between the R12 domain and the F2/F3 head domain. Here the R12 domain shields several lysines, responsible for PtdIns(4,5)$\text{P}_2$ binding. This interaction was observed to be unstable, allowing phospholipids to disrupt it and potentially induce talin’s activation (unpublished data).

Once talin’s primary inhibition is relieved, some of talin’s functions, such as actin binding site at the C-terminus and the lipid binding surface in the F2, F3 domain, become available. It is thought that talin can now bind to integrin and the membrane with its F2 and F3 domains and to F-actin with its C-terminal domain. This puts the rod domains under mechanical force and relieves the buried vinculin binding sites (Figure 12). Furthermore, talin’s central ABS2 gets separated from its inhibitory domains R3 and R9 to bind actin with high affinity and reinforce the stability of the adhesion (Atherton et al., 2015).

2.4.3 Mechanism of talin’s integrin binding

There are two general mechanisms of integrin activation; ligand based outside-in activation and inside-out activation. Outside-in activation is achieved by ligands of the ECM binding to integrin’s extracellular domain, whereas inside-out activation is accomplished by adaptor proteins, like talin and kindlin, binding to the cytoplasmic integrin tail. Via crystal structures and NMR studies it has been shown, that talin binds to integrin at two positions on the cytoplasmic tail with its F3 domain (Anthis et al., 2009; Lau et al., 2009).
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The first binding occurs at the NPXY motif of the integrin tail and potentially disrupts the salt bridge between integrin’s α- and β-transmembrane domain (Kalli et al., 2011). The following conformational change in integrin repositions the β-subunit’s transmembrane helix, which makes the Asp759 (β1, Asp723 in β3) available for talin binding (Lau et al., 2009). This direct interaction is supported by an interaction of a patch of basic residues on talin heads F2 and F3 domains as well as the F1-loop of the talin head, with acidic phospholipids such as PtdIns(4,5)P2 in the membrane (Figure 9) (L. Zhu et al., 2017). This lipid binding helps to orient talin head and further supports the talin-integrin interaction (Elliott et al., 2010).

![Figure 9: Model of integrin binding of talin head domain.](image)

*Figure 9: Model of integrin binding of talin head domain.*

Talin head domain binds the β3 integrin tail (PDB 2K9J) at two sites, the membrane proximal binding site and the membrane distal NPxY motif. The dotted red lines represent the cluster of basic residues of talin head that are binding to the plasma membrane. The model of talin head is represented in surface view, showing its charge distribution (red = negative, blue = positive) (PDB 3IVF). (Ye 2012 blood)

It was shown that kindlin is needed for efficient integrin activation through talin (Montanez et al., 2008; Moser et al., 2008; Theodosiou et al., 2016). However, it is not clearly understood how talin and kindlin enable integrin activation synergistically. An important question to ask is whether talin and kindlin can bind simultaneously on the β-integrin tail or whether they act in tandem. Another supporting role of kindlin might be the formation of integrin clusters via dimerization and recruitment of further adhesion proteins.
like IPP, Paxillin and even the Arp2/3 complex (Böttcher et al., 2017; Theodosiou et al., 2016).

A question of great interest is the role of talin’s catch-bond formation with integrin. It was shown in vitro that the affinity for the interaction of talin with different β-integrin tails lies in the low micromolar range (Anthis et al., 2010). The catch-bond formation through mechanical tension strengthens the lifetime of the talin-integrin bond. Furthermore, exerted forces on integrin possibly lead to its activation via mechanical force. However, no detailed mechanism for this catch-bond formation was presented so far.

It was shown that talin is of critical importance for the activation of platelets (Lefort et al., 2012) and leukocytes (J et al., 2011; Petrich et al., 2007). Interestingly, overexpression of talin head is enough to increase integrin activation in cells. In contrast, overexpression of full length talin has a less potent effect on integrin activation, indicating the presence of talin’s inhibited conformation in cells (Goksoy et al., 2008; Song et al., 2012b).

In addition to its IBS1 in the F3 domain, talin has another IBS in the R11-R12 domain. Even though the importance of this IBS is well established in flies (Ellis et al., 2011), neither its role nor its binding mechanism in mammals has been clarified so far.

### 2.4.4 Talin as mechanosensitive signaling hub

Mechanical forces are critical for the maturation of adhesion structures in cells; for example, nascent adhesions for example require forces in order to mature into focal adhesions. In recent years, research on mechanical forces at adhesion sites received more attention, and it was shown that talin functions as a mechanosensor and force-dependent binding platform for adhesion proteins. In its function as linker between integrin and actin, talin is exposed to forces generated by the retrograde flow of the actin cytoskeleton.

Talin’s chain-like domain organization makes it an ideal binding platform for other adhesion proteins. Interestingly, most of talin’s rod domains are sensitive to force and unfold once they are exposed to forces of at least 5 pN (del Rio et al., 2009a). It was shown that talin’s R3 domain, due to its threonine-rich core, especially sensitive to forces (Goult et al., 2013). It unfolds at 5 pN and allows vinculin to bind the cryptic binding sites within the domain. It was shown that the binding site of vinculin within the R3 domain is mutually exclusive to its RIAM binding site. Thus, binding of vinculin displaces RIAM, which leads to a reinforcement and maturation of small adhesion complexes, located in so called ‘sticky fingers’, into nascent adhesions (Lagarrigue et al., 2016). Similar unfolding events are
observed at higher forces for the other rod domains and their vinculin binding sites (Yao et al., 2016). The rod domain R8, however, is a unique case. In its folded state, it is a binding partner of various proteins (RIAM, DCL1, paxillin or actin). Thus, to retain its function even under higher forces, R8 is located within the 5 helix bundle R7, making it very resistant to unfolding by force.

Other interesting aspects are actin binding sites 2 and 3 of talin. It is generally accepted that, during initial assembly, ABS3 connects first with actin and thus provides an initial link, which keeps talin elongated and through which force can be transmitted over the talin rod. In the second step the provided force relieves the R3/R9 inhibition and lets ABS2 engage with actin to provide a more stable cytoskeleton linkage (Atherton et al., 2015). It is unclear whether both ABS domains can be utilized at the same time or if there are further actin-talin organization modes (Goult et al., 2018).

2.4.5 Membrane binding of talin

Talin’s FERM domain contains many different interaction sites for proteins, but also several surfaces that interact with phospholipids. Subdomain F2 and F3 both contain patches of basic residues capable of binding negatively charged lipids, specifically PtdIns(4,5)P2. In addition, F1 contains an unstructured loop, untypical for FERM domains, that was shown to enable further lipid binding (Goult et al., 2010a). Membrane binding of talin is suggested to play a role in three major processes; first, the recruitment from the cytoplasm, second it plays a role in the activation of talin and third a strengthening of talin’s weak interaction with the cytoplasmic β-integrin tail. The F3 domain is the only part of the talin head that interacts with the integrin tail; however isolated F3 domains were shown to be not very efficient in integrin activation. It was shown that rather the full talin head is needed to maintain active integrins (Bouaouina et al., 2008). The other head subdomains provide an additive effect, which is achieved by their interaction with PtdIns(4,5)P2 in the plasma membrane. Both F2 and F3 have patches of basic residues, which are directed towards the plasma membrane, when bound to integrin.

In addition, the 30 amino acid long loop in F1 contains several positively charged residues, which were shown to be essential for PtdIns(4,5)P2 interaction and thus integrin activation (Goult et al., 2010b).

In 2018, Chinthalaipudi and colleagues presented a crystal structure of talin head bound to PtdIns(4,5)P2 via a cluster of lysines (K272,K316,K324 and K343). They further showed that
disrupting the binding pocket by mutations affects integrin activation and FA formation in cells (Chinthalapudi et al., 2018b).

Furthermore, it was recently shown that talin’s F0 and F1 domain both contain an interaction site for Rap1. This interaction could function as an initial recruitment step for talin to the membrane (Bromberger et al., 2018; L. Zhu et al., 2017). Disrupting the interaction of Rap1 with talin F1 by mutating R118E was shown to reduce integrin activation in a CHO platelet model system (Gingras et al., 2019) and disrupting the binding sites on both F0 and F1 was shown to be lethal in mice (unpublished data). Recruitment of talin to the membrane via the F0/F1-Rap1 interaction could subsequently be followed by talin activation and integrin binding as predicted by the so-called ‘push and pull’ model (Figure 8).

Interestingly talin is also able to interact via its F3 domain with PIPKιγ90 with high affinity (de Pereda et al., 2005). The recruitment of PIPKιγ90 by talin stimulates PtdIns(4,5)P2 generation in FA, which induces a feedback loop that could enlarge FA (Di Paolo et al., 2002). However, despite these results indicating an important role for the PIPKιγ90-talin interaction, it was shown that a PIPKιγ90 knockout in mouse fibroblasts does not dramatically impair FA formation (Legate et al., 2011).

2.5 Other direct interactors of talin

2.5.1 Vinculin

Like talin and integrin, vinculin was discovered about 40 years ago and it was found to play a role in adhesion formation (Geiger, 1979; Geiger et al., 1980; Kuo et al., 2011). Indeed vinculin is one of the important adhesion proteins that not only takes a key role in integrin mediated cell-matrix adhesions, but also is essential for cadherin mediated cell-cell adhesions (Zemljic-Harpf et al., 2007). Vinculin itself does not have any enzymatic activity, but it acts in as reinforcement in integrin mediated adhesions, where it binds to actin and talin.

In mammals there are two vinculin isoforms, vinculin and the splice variant meta-vinculin with a 69 aa insertion. The structural organization of vinculin is quite well understood. It consists of 1066 aa, which were crystalized in full length (Bakolitsa et al., 2004) and truncations in complex with several binding partners. Vinculin is comprised of 8 anti-parallel α-helical domains, which organize in two regions. Domains D1-D3 form the larger region with 95 kDa from 116 kDa, which is called the vinculin head. They are arranged in a tri-lobar
way with a diameter of ~80 Å (Figure 10) (Bakolitsa et al., 2004). The head domain can bind to talin, α-actinin, α- and β- catenin and IpaA (Carisey and Ballestrem, 2010). The vinculin head is connected to the second region, called the vinculin tail, via a proline rich 61 aa linker, which binds different actin related proteins, such as VASP, vinexin, ponsin and the Arp2/3 complex. Finally, the vinculin tail consists of 30 kDa 5 α-helix bundle, which has a binding site for f-actin, paxillin, different phospholipids and importantly the vinculin head (Izard et al., 2004).

An important feature of vinculin is its autoinhibition, which is mediated through a sub-nanomolar interaction between the vinculin tail and head. FRET experiments showed that the inhibited form of vinculin is located in the cytoplasm, whereas the activated vinculin was found already recruited to adhesion sites (H. Chen et al., 2005). However, since the autoinhibition is strong, it is an open question how the autoinhibition of vinculin is released in the cell.

Initially, it was thought that the interaction with α-actinin is sufficient to release the inhibition, but these studies were performed with isolated domains. It was shown later, that the inhibition in the full length molecule is much stronger (Cohen et al., 2005), thus it seems unlikely that a single interaction is able to break the head to tail binding. Recent studies suggested that phosphorylation (of residues Y100, S1033, S1045 and Y1065) could activate vinculin (Golji and Mofrad, 2010; Golji et al., 2012). Another suggestion is that similar to talin, force could be the key to open vinculin’s talin and actin binding sites. This notion is supported by the observation that a loss of force in adhesions causes vinculin to inactivate (Carisey et al., 2013b; Grashoff et al., 2010). The last model suggests, that acidic phospholipids, especially PtdIns(4,5)P$_2$, might regulate vinculin’s functions. The PtdIns(4,5)P$_2$ binding at the vinculin tail could trigger a conformational change and allow talin binding via the vinculin head domain (Diez et al., 2008; Wirth et al., 2010). Interestingly it was also observed that F-actin and PtdIns(4,5)P$_2$ binding promote vinculin to form dimers and even other oligomers. Overall, the exact mode of vinculin regulation and activation needs further investigation.

In adhesions vinculin is located in the signaling layer and upon further activation migrates into the actin binding layer (Case et al., 2015). Here Vinculin plays a key role in reinforcing and rearranging adhesions. In cell migration an adhesions lifetime stretches from early focal complexes in protrusions to focal adhesion disassembly. Vinculin localizes to early adhesion complexes and promotes their maturation to NAs by recruitment of Arp2/3. Furthermore, it regulates talin by replacing talin bound RIAM and induce NA maturation to
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FA (Goult et al., 2013b). Cells deficient of vinculin lack Arp2/3 recruitment and lamellipodial protrusion (DeMali et al., 2002). In FAs vinculin stabilizes the actin-talin-integrin connection, transducing mechanical force and thus promoting integrin clustering and FA enlargement.

Most of vinculin’s functions in cell-matrix adhesions depend on its actin binding and manipulating properties. Studies showed that it can not only bind to actin filaments, but nucleate their formation, bundle multiple filaments and cap actin filaments (Jannie et al., 2015; Le Clainche et al., 2010; Shen et al., 2011; Wen et al., 2009). In addition, vinculin can recruit multiple actin modifiers, such as Arp2/3 or VASP. However, how these functions are regulated and how vinculin matures adhesion complexes will be focus of many future studies.

![Diagram of Vinculin Organization](image)

**Figure 10: Vinculins domain organization with aminoacid annotations**

Vinculin head (D1-D3 domain), linker region (D4 and unstructured) and vinculin tail (D5 domain) interaction partners are listed below the subdomains. B. Ribbon representation of the autoinhibited full-length human vinculin crystal structure with domain annotations (PDB 1TR2). C. Schematic model of vinculin in inhibited and active conformation and the probable changes in domain positioning. (Bays and DeMali, 2017)

2.5.2 Kank
The Kank protein family consists of 4 members in mammals (Kank1,2,3 and Kank4) and is evolutionary conserved up to *C. elegans* with Kank homologue VAB-19, which was initially found in 2002 (Sarkar et al., 2002). The family name Kank stands for kidney ankyrin repeat-containing protein.

The domain architecture of all family members consists of an N-terminal KN-motif followed by a coil-coil domain and a C-terminal ankyrin-repeat domain. Though the KN-motif as well as the ankyrin-repeat domain are conserved in each of the 4 family members, the coil-coil domain contains varying number of helices or is even missing in Kank4. The high similarity between the family members and the fact that both *C. elegans* and *D. melanogaster* have only one Kank homologue gave rise to the notion that gene duplication in higher mammals was responsible for its diversification (Hensley et al., 2016).

Initially, the function of *C. elegans* homologue VAP-19 was connected to adhesions. VAP-19 mutants have defects in muscle attachment and VAP-19 was described to stabilize integrin-mediated cell adhesions between uterine and vulval tissues (Matus et al., 2014). Surprisingly, it was also shown that a knockout of the only Kank homologue CG10249 in *D. melanogaster* does not lead to developmental defects, the flies suffer from defects in nephrocyte function (Gee et al., 2015). Nonetheless, through recent work in higher mammals the kank family has been suggested to be a key regulator of adhesion dynamics for two processes:

First, during fibroblast migration Kank was observed to initially locate at the tip of FAs. Later it starts to encircle and form belt around the whole adhesion structure. After completed FA encircling, Kank relocates towards the FA center, which leads to a adhesion disassembly. Kank can induce a process called adhesion sliding. This effect is frequently observed in stationary fibroblasts (Smilenov et al., 1999). Adhesion plaques can translocate towards the cell center, sliding over the substratum without getting disassembled. To slide without turnover the integrins of an adhesion need to be; loosely connected to their ligands via ‘slip bonds’, stabilized in active extended conformation and maintained in clusters.

In our study, showed that Kank2 is recruited to the FA through its N-terminal KN-motif and binds to talin’s R7/R8 rod domains. This was later confirmed by a crystal structure of Kank’s KN-motif in complex with the R7 domain of talin (Bouchet et al., 2016). Upon binding, Kank2 can activate talin and interfere with its actin binding, probably via ABS2, which is located between R4 and R8. This destabilization possibly leads to the disruption of integrin-talin catch bond and subsequently to the destabilization of the actomyosin link, resulting in adhesion sliding (Z. Sun et al., 2016).
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The second important role of Kank proteins is the guidance of microtubules to adhesion sites. Microtubules are very dynamic structures that constantly grow and shrink, but show a tendency to grow towards FA. When microtubules reach the plasma membrane, they connect with their tip to it via so-called cortical microtubule stabilization complexes (CMSCs). In close vicinity to FAs, microtubules can get linked in two ways to them: First, they can be directly linked to actin, via the microtubule-actin crosslinking factor 1 (MACF1). Second, they can be indirectly linked to talin by an interaction of Kank’s coil-coil domain with the CMSC component limprin-ß1. Subsequently, the kinesin-4 family member kif21a gets recruited through Kank’s ankyrin-repeat domain to the CMSC and further directs microtubule growth towards the lamellipodium. It was shown that cells deficient of either Kank1, Kif21a or MACF1 show reduced cell polarity due to disorganized microtubule growth (C. Li et al., 2011; van der Vaart et al., 2013).

It was further shown that Kank’s coil-coil domain is able to recruit additional F-actin regulating proteins, such as Irs5p3 and DAAM1, which is a potential RhoA activator, to the FA (Roy et al., 2009; Suzuki et al., 2017). In addition, Kank1 knockdown in NIH3T3 fibroblasts shows higher RhoA activity, indicating that Kank might play an indirect role in RhoA signaling.

![Figure 11: Model of the recruitment of microtubules to FAs in three steps:](image)

1. Recruitment of Kank1 by binding of its KN motif to the R7 domain of engaged talin in FA. 2. The initiation of a CMSC complex through Kank’s binding to liprin-b1 with its coil-coil domains. 3. The completion of the CMSC complex and subsequent association of microtubules, by clustering of ELKS, CLASP and Kif21a. (adapted from Bouchet et al., 2016)

Despite the fact that the knockout of the kank homologue in D. melanogaster does not show a distinct phenotype, genes of Kank family proteins have been connected to several rare human diseases. A large gene deletion resulting in the loss of the Kank1 gene was connected to a neurodegenerative disease called ‘type2 spastic quadriplegic cerebral palasy’
and different mutations in Kank1, 2 and 4 have been connected to human nephrotic syndromes (Gee et al., 2015).

Despite recent *in vitro* and *in vivo* studies the precise function of the Kank family members during adhesion is not fully elucidated.

### 2.5.3 Actin binding sites of talin

It was discovered that talin can bind both G-actin and F-actin *in vitro* (Goldmann and Isenberg, 1991; Schmidt, 1999). Furthermore, it was later identified that talin has at least three different ABS (Hemmings et al., 1996).

ABS1 is located in the F2 and F3 domain of talin and was found to bind actin at pH 7.0 or lower (H.-S. Lee et al., 2004). It was recently shown that that ABS1 has an actin capping function *in vitro* (Ciobanasu et al., 2018). The proposed actin binding surface is shared with the residues responsible for membrane binding of the talin head, therefore the role of ABS1 in the physiological context is unclear.

ABS2 was first determined at R7/R8 (Gingras et al., 2010a), but later discovered to work in conjunction with R4. Furthermore, it was shown in *in vitro* studies with talin fragments that the presence of the R3 or the R9 domain has an inhibiting effect on ABS2 (Atherton et al., 2015).

ABS3 is the most studied of the three ABS. It is located in R13 and the dimerization domain and called THATCH (talin/HIP1R/Sl2p actin tethering C-terminal homology) domain. The actin binding to ABS3 is dependent on a dimer formation between two dimerization domains of talin. Thus, two talin’s are bound on an actin filament in a V-shape formation (Gingras et al., 2008). It is speculated that the flexible linker between R13 and the dimerization domain allows for different talin-actin geometries within focal adhesions (Golji and Mofrad, 2014).

In addition to its three ABS, talin has the ability to connect to actin indirectly via one or more of its numerous VBS. There are 11 cryptic binding sites for vinculin distributed throughout the talin rod (Gingras et al., 2005). If unlocked and bound to vinculin, they can make an additional connection to actin via the actin binding site on the vinculin tail.

It has still been unclear, how the sequential hierarchy among the talin ABS looks like, or which role they play in enabling the recruitment of vinculin to the revealed VBS in talin.
Introduction

One model suggests that the cryptic vinculin binding sites can be accessed by force transduced via actin bound to either ABS2 or ABS3. It was shown that the deletion of ABS3 only causes a slight reduction of force across talin, still allowing vinculin recruitment. In contrast, the mutation or deletion of both ABS2 and ABS3 greatly reduces the force over talin and blocks vinculin recruitment to FA (Austen et al., 2015; Kumar et al., 2016). This highlights the role of ABS2 as major force bearing site in talin. However, this model might not be applicable to all cell types (Klapholz and Brown, 2017).

It was shown that deletion of ABS2 and ABS3 of talin2 in both mouse and fly does not block vinculin recruitment but rather leads to talin retaining most of its normal actin binding function through vinculin (Austen et al., 2015; Klapholz et al., 2015).

Another study by Atherton and colleagues showed that upon deletion of ABS3, cells either lack adhesions or have only small adhesions with low vinculin amounts. This indicates that ABS3 is important for force activation of cryptic adhesion sites, but that it also can be bypassed (Atherton et al., 2015). Deletion of ABS2 has no effect on initial adhesion formation but blocks maturation of nascent adhesions into focal adhesions. This leads to another model, which states that initial force is generated through actin binding at ABS3, opening VBS in R3 and allowing vinculin recruitment. This in turn relieves ABS2, where actin is bound, further reinforcing the adhesion and allowing maturation (Figure 12).

Though the exact role of talin’s ABS may depend on cell type and isoform, they are important for talin’s function as mechanosensor and binding hub for adhesion assembly.
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Figure 12: Schematic representation showing talin’s actin binding in two different models

Talin’s initial state: VBS in R2 and R3 are cryptic (red), ABS2 in R4-R8 is inhibited (blue). Vinculin (green) is in its inhibited state. B. Force-dependent activation: Actin binds to ABS3, generating tension on talin, stretching VBS in R2 and R3. This in turn allows vinculin binding, stabilizing R2/3 and activating ABS2. C. Vinculin-driven pathway: activated vinculin binds to VBS in R2 and R3, activating ABS2 without the need on force generated by actin binding to ABS3. D. Final engagement state of talin: ABS2 and ABS3 are bound to actin. VBS in R2 and R3 as well as further cryptic VBS along the talin rod are engaged, stabilizing the talin-actin link. (Atherton et al., 2015)
2.6 Methods of structural biology

Techniques of structural biology aim to provide information about the spatial positioning of each atom of a protein in three-dimensional space. This information can be used to understand the nature of protein interactions or mechanisms that are based on structural changes or ligand binding.

There are three prevalent methods used in structural biology: X-ray crystallography, nuclear-magnetic-resonance (NMR) spectroscopy and cryo-electron microscopy (cryo-EM). Each method has its specific strengths and limitations:

In X-ray crystallography, a monochromatic high-energy X-ray beam is shot at protein crystal to collect diffraction signals. The repetitive architecture of the crystal allows the collection of regularly spaced reflections. During the acquisition the crystal is rotated to obtain reflections of different angles. These are combined into a three-dimensional spectrum containing the amplitude information of the protein crystal. However, the phase information is missing. To solve this problem, X-ray crystallography uses different techniques, such as heavy metal soaking or molecular replacement to obtain phase information and calculate an electron density map. X-ray crystallography can be used for samples of all sizes and is able to achieve high resolution structures. However, large amounts of sample are needed and the protein needs to crystalize.

NMR spectroscopy uses a strong magnetic field to observe the local magnetic field around atomic nuclei. For protein NMR spectroscopy isotope-labeled protein samples are used to reduce the range of signals to detect. Although different specific techniques exist in NMR spectroscopy, it is limited to samples of about 35 kDa. However, it is the only structural technique that allows the observation of unstructured proteins and dynamic changes upon protein interactions.

Lastly, cryo-EM uses a high-energy electron beam that is aimed at a thin film of vitrified sample. A camera collects the scattering electron signals. Due to low signal to noise ratio thousands of images are usually collected. From these images, the scattering information is combined and processed to obtain a three-dimensional reconstruction of the protein. Cryo-EM can be used for much larger proteins and protein complexes and requires only a small amount of sample. Proteins can be observed in their native aqueous solution, but by vitrification they are fixed in their conformational state.

The spatial information that is generated by these methods is usually saved in a coordinate file and deposited in the protein data bank (PDB). Today, about 90 % of
structures deposited in the PDB are derived from X-ray crystallography and less than 8% from NMR experiments\(^1\). However, in recent years the number of entries and high-resolution structures from cryo-EM studies has increased dramatically.

2.6.1 Cryo electron microscopy

The most commonly used methods of structural biology are X-ray crystallography, NMR spectroscopy and cryo electron microscopy (cryo-EM). While X-ray crystallography is the most widespread and commonly used method, it requires high amounts of pure sample to obtain protein crystals. Since these conditions are difficult to achieve for bigger macromolecule assemblies or endogenous complexes that are usually available only in small amounts, cryo-EM has emerged as an alternative method to study those challenging projects in the recent years. In single particle cryo-EM small amounts of sample are used to obtain high resolution structures. Recent developments in hardware, as well as software improved the potential to attain structures at high resolutions, allowing even atomic modeling in electron-microscopy density maps.

To obtain high resolution structures of biological samples, a couple of obstacles have to be overcome: First, samples needed to be stable in the high vacuum of a transmission electron microscope (TEM). Second, the radiation damage on samples hit by high energy electrons needed to be reduced and third, the low contrast of biological samples in TEMs needed to be overcome. Initially, protein samples were fixated and stained with heavy metal solutions such as uranyl acetate or tungsten, but this lead to distortion of the biological samples and loss of resolution due to grain size. In the 1980’s, Dubochet and colleagues found a method to vitrify samples in their native environment by plunging them into cryogens, such as liquid ethane or propane (Dubochet et al., 1982). Though these vitrified samples suffered from low contrast, due to their biological components scattering signal being similar to the signal of the surrounding water atoms, they were natively preserved and largely protected from radiation damage.

In Cryo-EM, a TEM is used to obtain 2D projections of protein samples. These images contain hundreds of 2D projections from different directions. These can be computationally combined into a 3D reconstruction of the macromolecule that was imaged, a process that was started as far back as 1968 by De Rosier and Klug (De Rosier and Klug, 1968) but has come a long way since.

\(^1\)rcsb.org (25.05.2019)
Introduction

The low contrast of TEM images set the requirement for the high amounts of 2D particles needed to obtain a 3D model of the sample. Before the invention of direct detectors in 2012, millions of particles were often needed to obtain a sub-nanometer structure of a sample. Since the development of direct detectors, it is now possible to obtain short movie stacks with up to 10 frames per second instead of a single TEM image. This allows to correct for an effect called ‘beam-induced-motion’, in which the initial electron hits cause the sample to move in the vitrified condition. A ‘motion correction’ software aligns the single image frames and thus reduces the movement of particles within the image (Brilot et al., 2012; X. Li et al., 2013). Since over longer imaging time the sample is exposed to radiation damage, which results in a loss of high-resolution signal, a method called ‘dose weighting’ is used to extract the high-resolution information. It weights by taking into account the signal on single image frames in regard to the amount of electron radiation. The new direct detectors can detect electrons directly, which improves the signal over noise ratio dramatically, compared to the previously used charge-coupled device (CCD) cameras, which first needed to convert the electrical charges into photons before these arrived at the camera (Ruskin et al., 2013).

Altogether, these developments combined with higher image throughput, resulting from automatization of the image acquisition and processing steps, led to an increase of structures below <4 Å. These advances in the cryo-EM field allow the observation of heterogeneous complexes and different complex transition states, such as the different stages in the translation machinery (Loveland et al., 2017).

Recent years not only showed progress in hardware development, but also new reconstruction strategies and algorithms: maximum-likelihood classification, GPU based modeling or single particle CTF determination, to name some of them. In general, a typical 3D reconstruction flow is as follows: Raw frames are aligned to be combined to single images, then the contrast-transfer function is determined and then particles to be selected are determined either by using cross correlation to a template, or according to the size of the particles. The selected particles are extracted into small boxes. These boxed, low contrast images are then sorted into groups, on the basis of similar orientation, by 2D classification and combined into particle stacks (Scheres, 2014). Each particle stack then represents a view of an unknown orientation of the final 3D model (Figure 13). To determine the orientation within the final structure, the particles are compared to a crude initial model in fourier space. Since the initial model is crude, this process is used iteratively and a newly computed model from the input particles replaces the initial model at the end of each cycle.
New strategies, such as multi-body refinement (Nakane et al., 2018) make it possible to look for heterogeneities and flexibility in parts of larger macromolecular complexes. This allows insights in dynamic processes in complex formation or substrate handling of large protein machineries.

**Figure 13: Scheme of a typical cryo-EM workflow.**

Purified sample is applied to a grid and plunge-frozen in liquid ethane. Protein particles are vitrified in small μm sized holes to be observed in a TEM. Images are recorded and protein particles are picked. Particles are classified regarding to their orientation and averaged into 2D classes. The different views are aligned and they are further classified in 3D. From the best 3D class a high-resolution EM density map is generated and the structure can be refined. With sufficient resolution (<3.5 Å) an atomic model can be built into the EM density. ([Relion workflow of 20S proteasome] image adapted from Chung and Kim, 2017)


3 Aim of the thesis

Talin is an important player of the early adhesion complex, thus its regulation and interactions are of great interest. Many studies have been conducted on the structure, interactions and physical properties of talin. However, most of these studies use single domains or largely truncated constructs. So far, there is only limited information on how talin works in full length context.

In my study I aimed to recombinantly prepare full-length talin and biochemically characterize talin to gain insights into the activation mechanism of talin, which is critical for understanding of the events during early adhesion formation. I purified full length talin from two different organisms (mouse and human) and studied its interaction with the adhesion components vinculin, F-actin, as well as the integrin tail.

Talin’s regulation and inhibition is of great importance during the correct formation and disassembly of adhesions. Therefore, I opted to use structural methods to study the architecture of full length talin in its autoinhibited state. I used cryo-EM to resolve inhibited talin to a resolution of 6.2 Å and built an atomic model based on available X-ray crystallography and NMR structures of talin’s individual domains.

During autoinhibition talin adopts a compact state. Many of talin’s binding sites are not available, while others are accessible and play a role in the activation of talin. In order to understand talin’s domain-domain interplay, I used biochemical and biophysical methods to characterize full length talin, truncations and different mutation constructs as well as their interactions with some of talin’s binding partners. Based on the results obtained in this study, I proposed a model how talin can be recruited and activated on the membrane, before engaging with the actomyosin machinery and directing adhesion growth.
Aim of the thesis
4 Manuscript 1

4.1 The architecture of talin reveals an autoinhibition mechanism

Dirk Dedden, Stephanie Schumacher, Charlotte F. Kelley, Martin Zacharias, Christian Biertümpfel, Reinhard Fässler, Naoko Mizuno
(manuscript in revision)

In this study, we used recombinantly purified talin-1 and identified the molecular architecture with cryo-EM. We solved the structure of the full length protein in its autoinhibited state as a monomer to a resolution of 6.2 Å. The domain arrangement of the autoinhibited structure reveals two distinct inhibition sites. Not only could we show the importance of the inhibition site between the F3 and R9 domain in full length context, but in addition a novel inhibition site, where R12 covers the membrane binding site of the F2 and F3 domain. The structure further reveals that talin’s actin binding sites are hidden within a 15 nm globular domain arrangement of rod domains, which prevents actin and vinculin binding. We demonstrated that upon opening of the rod domains, talin unfolds into a 70 nm string-like conformation, in which its vinculin and actin binding sites are accessible. Our data shows that talin in its open conformation is able to bind vinculin in a force-independent way in 1 to 1 stoichiometry. Vinculin binding takes place in the R1 to R3 domains and can help to stabilize talin in its open conformation. Our study provides a model for how talin conformation can switch between globular and open state and suggests how talin’s activation-states could play a critical role in adhesion maturation.
The architecture of talin1 reveals an autoinhibition mechanism

Authors: Dirk Dedden1, Stephanie Schumacher1, Charlotte F. Kelley1, Martin Zacharias2, Christian Biertümpfel1, Reinhard Fässler3, Naoko Mizuno1*

Affiliations:

1 Department of Structural Cell Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany

2 Physics Department (T38), Technical University Munich, James-Franck-Str. 1, D-85748 Garching, Germany

3 Department of Molecular Medicine, Max Planck Institute of Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany

*Correspondence and lead contact: mizuno@biochem.mpg.de
Summary

Focal adhesions (FAs) are protein machineries essential for cell adhesion, migration and differentiation. Talin is an integrin-activating and tension-sensing FA component directly connecting integrins in the plasma membrane with the actomyosin cytoskeleton. However, its regulation is poorly understood. Here, we show a cryo-EM structure of full-length talin1 revealing a two-way mode of autoinhibition. The actin-binding rod domains fold into a 15-nm globular arrangement that is further interlocked by the integrin-binding FERM head. In turn, the rod domains R9 and R12 shield access of the FERM domain to integrin and the phospholipid PIP2 at the membrane. This mechanism ensures synchronous inhibition of integrin-, membrane- and cytoskeleton-binding. We also demonstrate that compacted talin1 reversibly unfolds to a ~60-nm string-like conformation, revealing interaction sites for vinculin and actin. Our data explains how fast switching between active and inactive conformations of talin could regulate FA turnover, a process critical for cell adhesion and signaling.

Keywords

Talin, Focal Adhesion, cryo-EM, actin, structure, vinculin, cytoskeleton, signaling, mechanosensor
Introduction

Focal adhesions (FA) are intracellular protein assemblies that serve as tension-sensing, anchoring points to link cells to the extracellular environment (Geiger et al., 2009; Parsons et al., 2010). FAs not only tether cells to the extracellular matrix (ECM), but also facilitate intracellular reorganisation, resulting in dynamic changes of cell functions and cell morphologies (Geiger et al., 2009; Legate et al., 2009; Parsons et al., 2010). FAs consist of hundreds of proteins in a layered arrangement that closely regulate each other (Kanchanawong et al., 2010). The first layer consists of integrin-signaling components at the plasma membrane, the second of force-transduction components, the third of actin-regulatory factors, and the fourth layer is made up of actin fibres. Several key proteins act to coordinate the individual functions of each layer, mediate crosstalk between layers, and to connect these layers with integrin receptors, the master-controller that links cytoplasmic FA complexes to the ECM (Bachir et al., 2014).

FA-mediated cellular processes are facilitated by alternating states of activation and inactivation of integrins. In migrating cells, integrin activation initiates vast FA formation, allowing cells to attach to the extracellular environment, whereas integrin inactivation, followed by disassembly of FAs, detaches cells. This cycling of on/off states allows cells to continuously change anchoring points, facilitating cellular movement. Talin is a key component in FAs, responsible for activating integrins and mediating both inside-out and outside-in signaling (Tadokoro et al., 2003) (Harburger and Calderwood, 2009; Nieswandt et al., 2007). Talin activates integrin by associating with the cytosolic tail of integrin beta-subunits. Once engaged, talin can assume an elongated conformation up to 100 nm in length (Liu et al., 2015), directly linking the beta-integrin subunit in the first layer of the FA to actin bundles in the fourth layer (Kanchanawong et al., 2010). By spanning all four layers of the FA, talin is in a unique position to act as a structural scaffold, greatly contributing to the overall composition and organization of FA complexes (Calderwood et al., 2013). In addition to its role as an integrin activator, talin also acts as a mechanosensor; it stretches like a spring and transmits tension between the ECM and the actomyosin machinery within the FA (Austen et al., 2015; Kumar et al., 2016), a process which is essential for regulating FA maturation and stability. As such, the transition between active and inactive talin likely plays a key regulatory role in FA dynamics, similar to the activation and inactivation of integrins.

Talin is a large, 270 kDa protein with 18 domains comprising a ~50 kDa globular head, a long rod made of 62 helices forming 13 helical bundle (rod) domains (R1-R13) (Calderwood et al., 2013; Goult et al., 2013), and a dimerization (DD) motif at the C-
terminus (Gingras et al., 2008). A unique conformational change of talin facilitates its spring-like behavior, through which talin can unfold into a linearly elongated 60–100 nm rod-like shape (Liu et al., 2015; Molony et al., 1987; Winkler et al., 1997). This allows it to bind to at least 11 different FA components including vinculin and actin (Goult et al., 2018). The talin head contains a 4.1-ezrin-radixin-moesin (FERM) domain with four subdomains (F0-F3), which is a common structural feature of several integrin tail-binding proteins (Elliott et al., 2010; Garcia-Alvarez et al., 2003; Goult et al., 2010; Rees et al., 1990). The FERM domain contains the integrin-binding site IBS1 (Tanentzapf and Brown, 2006; Wegener et al., 2007) and phosphatidylinositol-4,5-bisphosphate (PIP2) recognition site, allowing talin to attach to the membrane surface in a regulated way. The force-mediated stretching of talin is thought to follow (Atherton et al., 2015; Margadant et al., 2011), resulting in the exposure of up to 11 cryptic binding sites for vinculin (Fillingham et al., 2005; Gingras et al., 2005; Izard et al., 2004; Papagrigoriou et al., 2004). The binding of vinculin to talin is proposed to reinforce FA strength, as vinculin facilitates binding to actin, resulting in a cable-like configuration of F-actin and the FA (Case et al., 2015; Kanchanawong et al., 2010; Liu et al., 2015) (Carisey et al., 2013; Humphries et al., 2007) and triggering the maturation of the FA (Zaidel-Bar et al., 2003).

While the active form of talin is well characterized, the critical state of the inhibited form of talin is scarcely understood. The importance of talin inhibition is highlighted by the fact that failure in proper talin inhibition leads to morphogenetic defects during fly development (Ellis et al., 2013) and it has been implicated in the migration of metastatic cancer cells (Desinotis and Kyprianou, 2011; Fang et al., 2016; Haining et al., 2016). In its autoinhibited state, talin has a compact conformation (Goldmann et al., 1994; Goult et al., 2013; Winkler et al., 1997), with domains folded into each other, so as to occlude the interaction sites for binding partners such as integrin, membrane, vinculin and F-actin. Although individual, truncated fragments of talin have been well characterized, the regulation of full-length talin and the interplay among its domains are largely unknown on a molecular level. For example, a truncated fragment of the FERM domain (F3) was shown to interact with the R9 rod domain (Song et al., 2012); however, the overall mechanism is unclear because of the lack of information about the full-length architecture of talin.

Here, we report a cryo-EM structure of the autoinhibited form of recombinantly produced full-length talin1. The structure reveals charge-based interactions among the 13 rod domains of the talin1 monomer that entangle the protein into a compact 15 nm globular architecture, which is further secured by respective interactions between the
F2 and F3 FERM subdomains and the R12 and R9 rod domains. Interestingly, the PIP$_2$-binding surface of the FERM domain is completely covered by the rod domain R12 to occlude access to the plasma membrane. The integrin-binding site is located at the deepest part of the autoinhibition pocket. The closure of the rod domains impedes binding to vinculin and to actin via the actin-binding site 2 (ABS2), although it does not occlude the other actin-binding site, ABS3. By mimicking talin activation, we facilitated talin1 binding to F-actin and vinculin. On the basis of these results, we propose a molecular model that explains how talin activation is controlled at a PIP$_2$-enriched membrane surface and how the protein transitions from a 15 nm globular structure to a 100 nm extended structure. Our results have wide-ranging implications for the mechanistic understanding of FA and protein activation.
Results

The full-length, autoinhibited talin1 structure shows molecular closure of the rod domains by the FERM domain.

Talin is thought to adopt open and closed conformational states (Figure 1A). While open talin is amenable to further stretching by its interacting partners, the globular state is likely to take on an autoinhibited conformation that hinders access to many of talin’s binding sites, restricting interactions with other FA proteins. The autoinhibited form is thought to be the cytosolic state of talin that does not engage in FA assembly. It has been a challenge, however, to obtain a molecular view of the autoinhibited conformation. We successfully purified recombinantly expressed talin1 and pursued a full structural and biochemical characterization.

The cryo-EM analysis of full-length talin1 (talin-FL) in the autoinhibited conformation (Figure 1B, Figure S1 and S2, Table S1) revealed an intertwined architecture. Although the intrinsic flexibility of talin limited the resolution of the structural reconstruction to a global resolution of 6.2 Å (Figure S2A-C, Table S1), the clear connectivity of the rod domains within the density map facilitated robust fitting of available structures of the 13 individual rod domains and the F2 and F3 subdomains of the FERM domain (Figure 1C and S3). We performed further refinement using a flexible fitting scheme by the restraint molecular dynamics (Salomon-Ferrer et al., 2013). Our results show that the rod domains are entangled with each other, resulting in a compact organization, and the FERM domain closes the assembly via two anchoring points on rod domains R9 and R12, respectively, acting as a lid to secure the closure of the talin architecture (Figure 1D). At the first anchoring point, located in the deepest groove of the autoinhibition pocket (Figure 1E), the interacting surface between R9 and F3 agrees well with a previously reported crystal structure of isolated F3 and R9 fragments (Song et al., 2012) (Figure S4), which are necessary for talin inhibition in cells. At the second anchoring point (Figure 1E), located at the critical connecting point for the closure of talin1, K272 and K274 in F2 contacted E2288, and D2297 at the C-terminus of R12, respectively (Figure 1D).

While the F2 and F3 FERM subdomains revealed interactions necessary for talin1 autoinhibition, the densities corresponding to the F0 and F1 FERM subdomains were not visualised in our structure (Figure 1E). F0 serves as an interaction surface for the small GTPase Rap1 (Zhu et al., 2017), which acts as a recruiting factor to promote talin engagement with the plasma membrane (Goult et al., 2010; Plak et al., 2016) (Figure 1F). While these subdomains are missing from our structure, the corresponding fragments were identified by mass spectrometry (Figure S3B). This indicates that the
F0-F1 subdomains are flexibly attached to the talin core structure, presumably by a long, 30 amino-acid linker between F1 and F2 (Figure 1E). Hence, F0 and F1 are likely not part of the inhibited structure, which suggests that talin can be recruited to the membrane surface via Rap1 without a requiring that the rod domains disentangle from each other or disruption of the FERM domain interaction with R9 and R12 (Figure 1E-F).

The closed talin configuration occludes the integrin-binding site and the membrane-binding surface on the FERM domain.

The talin FERM domain was shown to interact with PIP2 (Chinthalapudi et al., 2018; Orłowski et al., 2015; Song et al., 2012). Specific residues responsible for this interaction were recently identified including K272 of F2 and K316, K324, E342 and K343 of F3 (Chinthalapudi et al., 2018). We mapped their locations to understand the geometrical relationships among them in full-length talin (Figure 1D). In the autoinhibited talin structure, the PIP2-binding surface was mostly covered by the rod domains, particularly by a region at the C-terminus of R12, ensuring that the binding site was inaccessible to PIP2 (Figure 1D and 1G). This region, containing the negatively charged amino acids E2288, E2294 and D2297 (Figure 1D), provides an acidic interface to complement the basic PIP2-binding surface. Furthermore, to understand the arrangement of integrin and the plasma membrane with respect to talin in the open and closed configurations, we superimposed the available structure of the integrin cytosolic tail in complex with F3 (Anthis et al., 2009; Wegener et al., 2007) (Figure 1G). The integrin tail was shown to locate on the surface of the truncated F3 (Song et al., 2012), where R9 also binds in our autoinhibited structure, showing that the integrin-F3 interaction and the F3-R9 autoinhibitory interaction are mutually exclusive. Moreover, the integrin transmembrane helix directly precedes the integrin tail, indicating that F2 and R12 would have to separate to enable F2 and F3 to bind the inner face of the plasma membrane (Figure 1G). We also noted that the interaction surface of F3 for R9 has been reported to be a key interface for talin-binding partners, such as riam (Yang et al., 2014) and laylin (Wegener et al., 2008), which are likely involved in talin activation (Figure S4).

The talin1 folding unit is a monomer in the autoinhibited form.

The last 50 amino acids at the C-terminus of talin1 have been crystalized as a coiled-coil in a dimeric structure (Gingras et al., 2008) (termed DD domain), suggesting the possibility that talin is capable of dimer formation. Furthermore, talin has been separately observed as a dumbbell-shaped dimer (Goldmann et al., 1994) when
interacting with actin and as entwined dimers forming a globular assembly (Goult et al., 2013), keeping the question open as to whether or not talin dimerization is necessary for its autoinhibition. Our cryo-EM structure clearly showed that talin1 monomers are capable of achieving the autoinhibited architecture.

The DD domain, which follows R13, was not clearly visible in our structure because of its high flexibility, which in turn indicates that it is not engaged in the autoinhibited conformation. Indeed, a truncated talin construct that lacked the DD domain (talin-ΔDD) (Figure S3A) was still capable of assuming the autoinhibited conformation (Figure 2A), and analysis by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS; Figure 2B) showed that both talin-FL and talin-ΔDD behave as monomers. Therefore, the DD domain appears to be inactive or not strong enough to hold talin-FL dimers together when talin is in the autoinhibited state. We further tested if we could find talin dimers as a minor component in the molecular population. Sucrose-gradient centrifugation of talin-FL in the presence of a concomitant gradient of the cross-linker glutaraldehyde (GraFix (Stark, 2010)) showed a minor population of talin that migrated differently in solution as well as on SDS-PAGE (Figure 2C), suggesting the presence of a talin-FL dimer, in agreement with the previous report (Goldmann et al., 1994). In contrast, talin-ΔDD did not display the corresponding minor band and showed only a single monomeric population, indicating that DD is the only domain in talin that is capable of facilitating dimerization.

**Weak interactions among rod domains maintain the compaction of talin.**

To explore how the molecular opening of talin is regulated, we varied the ionic environment and tested if and how the conformation was changing. When we raised the ionic environment to 500 mM salt (NaCl or KCl), the conformation of full-length talin changed from the globular, closed architecture to the open, strand-like conformation (Figure 3A and 3B) with a length of 560 Å (standard deviation [SD] = 170 Å) (Figure 3C), which fits well with the reported length of talin in a cell (Kanchanawong et al., 2010; Margadant et al., 2011). We looked for evidence of the conformational change as a function of the salt concentration using dynamic light-scattering (DLS; Figure 3D) and the results fit well with a two-state model of protein folding-unfolding. At an ionic strength of 234 mM salt, the two states were equally populated and at a physiological salt concentration of 150 mM, 81% of talin had a compact conformation. We also tested that this conformational change was reversible across fluctuating salt concentrations (Figure 3E).
Furthermore, we tested the importance of the inter-domain interactions for autoinhibition by creating point mutations as well as truncated talin constructs lacking the key domains for the interaction of F2-R12. We created a C-terminal truncation N-R11 lacking R12 and the C-terminus (Figure S3A), one of the two contact points necessary for the FERM-rod interactions, as well as N-R12 (Figure S3A). The DLS experiments showed the conformational changes of both constructs in response to the increase of salt concentration (Figure 3F). Point mutants of talin-FL altering the charge at the key interacting points on R12 (talin-FL-5K: E2288K/E2294K/D2297K/E2299K/D2300K and talin-FL-2K: E2288K/E2294K) revealed a compact conformation at 75 mM salt and underwent a conformational change similar to full-length, wild type talin upon change of the salt concentration (Figure 3I). These experiments led us to hypothesize that the rod domains themselves interact with each other to keep a compact formation.

The rod domains contain several binding sites for critical FA components. Those sites include the F-actin-binding sites ABS2 and ABS3 on R4-R8 and R13-DD, respectively (Hemmings et al., 1996). We observed additional contacts among rod domains, as shown by the mapping of neighboring domains (Figure 4A). Particularly, R4 is placed at the ‘core’ of the globular architecture and shielded by several rod domains (Figure 4B). We therefore tested the overall interactions among the rod domains by using a truncation construct that lacks the FERM domain (R1-C). DLS showed the change of the conformation in response to salt concentration (Figure 4C). Negative-stain EM images of R1-C showed a mixed population of open and not well-defined, but nevertheless compact, conformations (Figure 4D). These indicate that there is an internal interaction within the rod domains, however, it is not strong enough to keep the conformation for observation under negative-stain conditions. To visualize the nature of the interaction, we calculated the electrostatic surface potential of each of the individual rod domains surrounding R4 and estimated the interaction energies of those domains to R4 (Figure 4A and Figure S5). The calculated energy landscape for the domain-domain interaction indicated that the binding is rather weak (Figure S5B), resulting in an overall marginal stability. The rod domains may not rigidly bind to one another without cooperative, multi-module packing, as induced by the overall arrangement of the full-length protein. This explains the observation that the talin architecture is highly sensitive and how its conformation can rapidly change in response to salt.
The closed conformation of talin prevents vinculin binding, whereas the open conformation binds vinculin with 1:1 stoichiometry.

Vinculin is a major talin-binding partner that facilitates crosstalk between talin and actin through its talin-binding head domain (V-head) and its actin-binding tail domain (V-tail) (Borgon et al., 2004; Johnson and Craig, 1994). Sequence-based analysis identified 11 potential vinculin-binding sites (VBSs) in talin (Gingras et al., 2005). Experiments using combinations of talin rod fragments indicated that R3, containing two VBSs, is a potent binding site for vinculin. It has been proposed that the unfolding of the helical bundle in R3 exposes the otherwise hidden binding domain to vinculin (Izard et al., 2004; Yao et al., 2014). Our truncation experiments supported that notion, as the talin fragments R1-R8 and R1-R3 formed complexes with V-head, whereas R4-R8 did not (Figure S6). It was unclear, however, how those observations would be reflected in the context of full-length talin.

To test the binding of vinculin to talin-FL, we performed reconstitution assays using SEC (Figure 5A) at 75mM KCl, where talin shows a compact architecture. While truncated talin including R3 readily bound to vinculin (Figure S6), most of talin-FL did not form complexes with full-length vinculin, V-head, or the vinculin mutant (V-mut, N773, E775A (Cohen et al., 2005)), which exposes the talin interaction site by deregulating the autoinhibition of vinculin (Figure 5A, first row). These findings suggest that the opening of the R3 helical bundle is hindered by steric constraints in the autoinhibited form of talin. The root of R3 is connected to R4, which is buried in the core of autoinhibited talin. Therefore, we attempted to reconstitute the talin-vinculin complex with talin at 500 mM KCl where talin shows an open conformation. SEC revealed that talin and vinculin formed stable complexes with the V-mut (Figure 5A, second row). Talin formed a complex with V-head as well (Fig. 5A and 5B). SEC-MALS showed that the corresponding chromatographic peaks were monodisperse with a molecular mass of 339 kDa, corresponding to a complex comprising one talin-FL and one vinculin molecule (Figure 5C). Interestingly, the talin-FL-2K and talin-FL-5K mutants, which are designed to weaken the interactions between F2 and R12 domains, were able to form a complex with V-head at 75 mM KCl (Figure 5D and 5E). This indicates that the disruption of F2-R12 leads to a partial access of vinculin, while these mutants employ compact conformations under this condition (Figure 3I).

The compact talin can retain vinculin head as a complex but induces the dissociation of full-length vinculin.
To test the conformational change of talin in complex with vinculin, we exposed the reconstituted talin-V-head complex to low ionic strength conditions. We found that the retention volume of the SEC peak of the complex shifted from 1.27 mL (500 mM salt) to 1.31 mL (75 mM salt) (Figure 5A, Figure S7A and S7E), suggesting a possible compaction of the complex. DLS experiments of the preformed talin-FL-V-head complex indeed showed a conformational change upon variation in salt concentration, similar to talin-FL alone (Figure S7B-D). We also observed the conformational change of the complex of V-head with talin-FL-2K as well as talin-FL-5K, altogether pointing to the ability of talin to change its conformation in complex with vinculin head (Figure S7B-C).

The closed talin-V-head complex displayed a globular assembly that was similar to the closed morphology of talin alone (Figure S7F), although the talin-V-head complex had a slightly larger size (100 Å × 230 Å) than talin alone (~100 Å × 150 Å). Two-dimensional class averages of the talin-vinculin complex revealed a 60 Å protrusion from the ~100 Å globular head (Figure S7F, top panel). By combining available crystal structures of the talin VBS3 peptide bound to the vinculin N-terminal rods (N-terminal part of the vinculin head) (Izard et al., 2004), a full-length vinculin (Borgon et al., 2004), and our cryo-EM full-length talin in the autoinhibited form, we assembled a structural model of V-head binding to talin at the R3 domain with the talin helical bundle unfolded (Izard et al., 2004; Yao et al., 2014) (Figure S7G). This model matched well with the shape of the averaged talin-V-head complex (Figure S7F), indicating that the 60 Å protrusion on the talin-vinculin complex likely corresponds to vinculin binding to R3. However, when we attempted to re-close talin in complex with the V-mut including the vinculin tail, vinculin was induced to be displaced from the complex (Figure S7A). This observation suggests that the vinculin tail competes with the talin R3 domain for binding to the vinculin head.

**F-Actin binding is enhanced by the opening of talin**

Another major binding partner of talin is F-actin, which is located at the base of the FA. Acting as tension-generating machinery, actomyosin is thought to ensure the opening of talin by binding to two sites located at R4-R8 (ABS2) and R13 (ABS3; Figure 6A). Cell-based experiments with talin rod-deletion mutations suggested that ABS3 plays a role in recruiting talin to actin at the FA to activate talin, while ABS2 is critical for the maturation or growth of the FA (Atherton et al., 2015). Combining our structural findings, we hypothesized that the opening of talin is correlated with the engagement of talin to actin, and we tested the binding of various truncated talin variants to F-actin by co-sedimentation assays.
First, we tested the truncated construct N-R12 for binding to F-actin. In the tested condition at 75 mM KCl, N-R12, like talin-FL, employed an autoinhibited compact form (Figure 3F and H) that did not form a complex with vinculin (Figure 6B, Figure S7H). Reflecting the fact that N-R12 lacks ABS3, the binding of F-actin to N-R12 was reduced compared to talin-FL (Figure 6C-D). Next, we tested the C-terminal truncation construct N-R11, which lacks the R12 domain. Without R12, talin N-R11 compaction is weak enough for vinculin to associate (Figure 6B, Figure S7I). In contrast to N-R12, N-R11 bound F-actin with even higher amounts than talin-FL (Figure 6C-D), despite the fact that it lacks ABS3. Those results indicate that loosening the autoinhibitory lid (R12) of talin was directly correlated with the activation of the F-actin-binding of ABS2 as well as with vinculin binding. We also tested the truncation of the N-terminal FERM domain (R1-C) in the same way. R1-C showed binding similar to that of N-R11, although the described F-actin-binding activity of ABS1 at the FERM domain was missing from the fragment. R1-C was also capable of binding to the vinculin head (Figure 6B, Figure S7J); however, it displayed no significant enhancement of F-actin binding.

Discussion

Mechanism of talin regulation.

In cellular environments, the concentration of talin can be as high as 50 μM (Zeiler et al., 2014; Zhu et al., 2017), making talin one of the most abundant components in cells. The activation of talin within FAs is essential; however, it is also critical that the adhesion components can be properly switched off to control cell attachment and migration. Therefore, the autoinhibited state of talin is essential (Ellis et al., 2013). Information is available about interactions among individual talin fragments (Goult et al., 2013; Song et al., 2012); however, the molecular architecture and the mechanism of talin’s autoinhibition have been unknown in the regulatory context of full-length talin. We uncovered how talin can be folded to ensure its autoinhibition. A network of polar interactions keeps the 13 rod domains tangled into a compact globular architecture with the R4 domain buried in the core, thus shielding R4 and the actin-binding site ABS2 against actin binding. The compact architecture was further locked down by contacts between the F2 and F3 subdomains of the FERM head and the R12 and R9 rod domains, respectively. The PIP2 binding FERM domain was masked by the R12 domain inhibiting the interaction of talin to the inner leaflet of the plasma membrane. The densities of R11-R13 are not well defined and show flexibility in the 3D classification (Figure S1C). This suggests that the connection between R12 and FERM domain F2 is loose. The break of this interaction may be sufficient to grant an access to binding partners such as
vinculin, whose binding, however cannot open the molecule completely. The conformational change of talin can be seen as an ensemble of open and closed states, which can be shifted dynamically by the change of salt concentration in vitro. The open conformation of talin likely mimics the activated talin stabilized by the actomyosin machinery during FA initiation.

**Talin-vinculin complex formation during autoinhibition**

We observed that one vinculin-binding site is readily available when full-length talin is released from the autoinhibited state without the application of any active force. Intriguingly, talin is able to reverse back to the closed conformation while vinculin-head is still bound to R3, but only in the absence of the vinculin tail. We speculate that this may hint at a mechanism to release vinculin when talin is inactivated and no longer engaged in the FA assembly. The closure of talin and concomitant folding of R3 may weaken the interaction of vinculin head to R3. In turn, the competition of vinculin tail for the head domain becomes more effective, resulting in the inactivation of vinculin and release from the complex in the autoinhibited form. It will be interesting to structurally map the interaction surface and find out which of the talin-binding partners can bind to the autoinhibited form of talin. Such an analysis will shed light on the functional role of talin in the cytosol and help to determine if there are preformed complexes that act as precursors of FA-related complexes. Along the same line, it is possible that talin dimerization might also occur upon talin activation and elongation. Binding to actin further expands the footprint of talin and exposes the C-terminal dimerization domain, as suggested previously (Goldmann et al., 1994), while dimerization appears dispensable when talin is autoinhibited. Talin dimerization offers an additional layer of regulation and the possibility for larger complexes to be assembled from preformed FA subcomplexes. Further structural analysis of full-length talin in its active, elongated form in comparison with its autoinhibited form will provide a comprehensive view of talin function during FA initiation and maturation.

**The topological positioning of talin is a key step in FA activation.**

Our talin structure showed that the globular rod domains occlude the membrane-binding plane of the FERM domain. On the other hand, the N-terminal FERM F0-F1 subdomains and the C-terminal DD domain were not involved in maintaining the structural organization of the autoinhibited form of talin, indicating that those domains are freely accessible and not inhibited when talin is closed. In particular, F0 has been
shown to be a key binding domain for Rap1, the binding of which has been suggested as
the first step in the recruitment of talin to the cell-membrane surface (Zhu et al., 2017).

In addition, talin has a 30 amino-acid flexible linker between the F1 and F2
subdomains that separates F0-F1 from the globular autoinhibited structure (F2-DD).
This separation may allow F0-F1 to “search” for cofactors. Our findings, together with
previous results, suggest a model of how talin is recruited and correctly positioned at
the membrane surface (Figure 7, model). Autoinhibited talin is recruited to the
membrane via its freely accessible F0 subdomain in a Rap1-assisted manner. Thus, the
FERM domain orchestrates the binding of Rap1 on the one hand (Zhu et al., 2017), while
on the other it accesses the exposed, negatively charged plasma membrane via the
flexible loop inserted in the F1 subdomain (Goult et al., 2010). This recruitment process
increases the local concentration of PIP2, due to the recruitment of PIPK1y90 by the F3
subdomain (Di Paolo et al., 2002; Ling et al., 2002). PIP2 may bind to talin, opening the
R12 lid, thus shifting the conformational ensemble towards open structures. The F3-R9
interaction may then be abrogated (Figure 7, step 3), allowing the integrin tail to interact
with F3.

This rearrangement releases the rod domains from the head domain (Figure 7,
step 3), activating the interaction sites for various talin-binding proteins. The rod
domains themselves can be readily approached by vinculin and actin (as shown in
Figure 6), which could lead to the initiation of FA formation (Figure 7, step 4). Our actin
cosedimentation assays with truncated talin revealed that ABS2 is inhibited while talin
is closed. This shows that the initial pulling of talin ABS3 by the actomyosin machinery
aids the opening of talin to enable the accessibility of ABS2 as well as vinculin, as also
shown in a previous report (Atherton et al., 2015). Finally, actin binding would enable
actin retrograde force to exert tensile force on talin, enabling mechanical force to also
stabilize talin opening (Sun et al., 2019). Our structure provides a mechanistic basis to
understand the inhibition and activation of talin. Although our model is coherent,
further testing is needed to validate key events. Furthermore, other yet-unknown
activating factors may be involved to tightly regulate the inhibition and activation of
talin or to modulate individual functions of talin.

**Author Contributions**

Conceptualization, N.M.; Methodology, D.D., M.Z., C.B. and N.M.; Investigation, D.D.,
S.S. C.K. M.Z. and C.B.; Writing N.M. and D.D.; Resources N.M., R.F.; Funding Acquisition
N.M.; Supervision N.M.
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Declaration of interests

The authors declare no competing interests.
Figure 1 Structure of full-length talin.

A: Schematic of the domain organization of talin in the open form (left) and the closed, autoinhibited form (right). B: Three views of the structure of full-length talin. C: Molecular fitting of talin fragments (PDB codes: 3IVF, 1SJ8, 2L7A, 2LQG, 2L7N, 2L10, 5IC1, 2KVP, 3DYJ, 2JSW) to the EM density map. D: The molecular closure of talin achieved by F2-R12 and F3-R9 interactions and the charged amino acids surrounding the interaction interface. Bold highlighted numbers match previously published PIP2-recognition residues. E: Superimposition of the F0-F1 subdomains of the talin FERM domain structure (overlaid with grey shadow, PDB: 3IVF) onto our cryo-EM talin model. The F0 and F1 subdomains were not visualized in our structural analysis because of the flexibility of the F1-F2 linker. The small GTP Rap1-binding site on F0 is mapped in blue and labelled "Rap1". F: The NMR structure of F0-Rap1 (PDB: 6BA6). G: Comparison of the F3-R9 and F2-R12 autoinhibition sites in the closed structure with the F3-integrin tail (PDB: 2H7D) and F2-F3-PIP2 (PDB: 6MFS) as well as with the membrane-interaction sites of the FERM domain (Anthis et al., 2009), highlighting the mechanism of the autoinhibition of talin. In the inhibited form R12 forms a 'lid' covering the PIP2-binding site on F2-F3. See also Figure S1-S5.
Figure 2. The dimerization domain is not necessary for the closure of talin.

A: Negative-stain EM image of talin-ΔDD lacking the dimerization domain at 75 mM KCl, and 2D averages of the particles (right), showing a morphology similar to that of full-length talin. B: Size-exclusion chromatography coupled with multi-angle light-scattering profile of full-length talin (FL) and talin-ΔDD at 75 mM KCl, showing molecular weights corresponding to monomers. LS: light scattering, normalized, UV: absorbance. C: Centrifugation profiles of talin-ΔDD and talin-FL using the GrafFix method. Full-length talin has an extra band in the fraction with higher sucrose density (open arrowhead). Black triangles depict the gradient of sucrose concentration.
Figure 3 Two-state conformational ensemble of Talin.

A: Negative-stain EM image of full-length talin in the compact conformation at 75 mM KCl. B: Full-length talin in the open conformation in the presence of 500 mM KCl. Green arrowheads indicate examples of the open talin. C: Distribution of the lengths of the open talin molecules from B (560 ± 170 Å, mean ± S.D. N=89). D: Dynamic light-scattering (DLS) measurements of the hydrodynamic radius of full-length talin under various salt concentrations, showing the conformational change of talin as a function of salt concentration. The fitted curve indicates that talin adopts both conformations at an equal ratio at a salt concentration of 234 mM. At a physiological salt concentration of 150 mM, 81% of talin employs the compact formation. E: Reversible conformational change of talin depending on the salt concentration as determined by DLS. Left: Talin
employs a closed conformation at an ionic strength of 75 mM. Center: Talin opens and increases its size when the ionic strength is increased to 500 mM. Right: Talin closes again when the ionic strength is lowered back to 75 mM, showing that the shape change of talin is reversible. F: DLS measurements of the hydrodynamic radius of N-R11 and N-R12 under various salt concentrations, showing a conformational change comparable to talin-FL with a lower amplitude. The data point at 250 mM was removed as the measurement was outside of the dynamic range of the detector. G: Negative-stain EM images of N-R11 at 75 mM KCl, revealing mixed populations of the open (white arrowheads) and the compact (red arrowheads) form, however, it is dominated by open conformations. 2D averages of the compact entity show a globular average (right panel). H: Negative-stain EM image of N-R12 and the 2D averages of the compact entities (right panel). Magenta arrowheads indicate examples of the compact molecules. White arrowheads indicate open or deformed molecules. I: DLS measurements of point mutants of talin-FL-2K and talin-FL-5K, also showing conformational changes dependent on salt concentration.
Figure 4. Interdomain interactions of talin

A: Map of talin interdomain interactions derived from the EM structure. Binding domains relevant to this study are colored in red (actin-binding site ABS2), blue (actin-binding site ABS3), and green (vinculin-binding site). Dotted lines indicate positive (i.e. interactions are less not favorable), while solid lines depict negative interaction energies (i.e. domains are interactive) as determined by MD simulations (see STAR methods). The thickness of the lines is proportional to the calculated energies detailed in Figure S5. B: Spatial arrangement of the rod domains surrounding the R4 core in two vertical slices through the structure. C: DLS measurements of the hydrodynamic radius of R1-C under various salt concentrations in comparison to talin-FL (grey). The R1-C truncation shows a conformational change but it steadily opens up with increasing salt concentration. The size distribution could not be fit to a two-state model. D: Negative-stain EM images of talin R1-C at 75 mM KCl. Magenta arrowheads indicate examples of the compact
molecules. White arrowheads indicate open or deformed molecules. 2D averages (right) of the compact entity show globular but featureless averages. Without the FERM domain, R1-C shows mixed population of open and closed architectures.
**Figure 5. Interaction assays of talin-FL and vinculin under various conditions.**

**A:** Full-length talin and vinculin reconstitution assays using size-exclusion chromatography (SEC). An ionic strength of 75 mM was used for the reconstitution assays shown in the upper panel and 500 mM in the lower panel. SDS-PAGE profiles (talin-vinculin mixture, talin control, vinculin control) of the peaks are shown below the SEC profiles. In order to achieve complex formation, it is necessary that both talin and vinculin employ open formations (bottom-center and bottom-right). V-fl – vinculin full-length, V-mut – vinculin (N773, E775A) mutant, V-head – vinculin head. Minor shoulders at the left side of that talin peaks correspond to the degraded talin without the FERM domain. **B:** Negative-stain EM image of vinculin head bound to open talin at 500 mM KCl. **C:** SEC-multi-angle light-scattering profile of V-head bound to open talin with an calculated molecular weight of 339 kDa, indicating that the complex is made with 1:1 stoichiometry. LS: light scattering, normalized, UV: absorbance. **D:** SEC reconstitution assay of talin-FL-2K mutant and vinculin head at 75 mM salt. **E:** SEC reconstitution assay of talin-FL-5K and vinculin head. The purified proteins have never been exposed to 500 mM salt, which facilitates the opening of talin. The complex formation of these mutants and V-head is observed (indicated by red lines). See also Figure S6-S7.
Figure 6. Talin–F-actin binding assay.

A: Scheme of actin-binding domains ABS2 and ABS3 in the context of the autoinhibited talin structure. B: Table summarizing tested constructs for F-actin binding and vinculin accessibility of talin in compact conformation (75 mM KCl). C: Representative SDS-PAGE analysis of the F-actin co-sedimentation assay with various talin constructs at 75 mM salt. D: Quantification of the co-sedimented talin fragments. Data are represented as mean ± SD. N-R12 0.68 ± 0.08, N-R11 1.3 ± 0.2, N-R11 + V-head 1.1 ± 0.3, R1-C 1.1 ± 0.2 and R1-C + V-head 1.3 ± 0.4. Differences between talin-FL and N-R12 as well as N-R11 are statistically significant (marked with *), see also Figure S7.
Figure 7. Model of talin activation.

(1) Our structure of talin in the closed form reveals how inactivation is achieved through occlusion of functional sites. Rap1-mediated recruitment of talin to membranes can occur without breaking the closed formation of talin as the F0 and F1 subdomains are accessible. (2) Our structural analysis also indicated that the closed form of talin is in equilibrium with a semi-opened state due to the unstable FERM-lid connection (see Figure S1). (3) Upon binding to the PIP2-embedded membrane surface, the R12-F2 and R9-F3 interfaces are broken because of competitive interactions with PIP2 and integrin, respectively (shown in Figure 1). The binding to the integrin tail hinders the F3-rod interaction (Wegener et al., 2007) and releases the rod domains from the FERM head as well as from the plasma membrane. (4) The further opening of the rod domains may require the mechanical aid of actomyosin pulling via the ABS3 and ABS2 actin-binding sites. Vinculin may also take an active role in reinforcing the binding of talin to actin (Humphries et al., 2007).
Figure S1: Summary of cryo-EM data processing, related to Figure 1.
**Top:** Representative cryo-EM image of talin-FL at 75 mM salt with representative 2D averages. **Bottom:** Graphical scheme of image processing. 1.9 million particles were selected out of 2D classifications. Further 3D classifications and refinements facilitated the final model production (A – left). To visualize the R3 domain, the core density (R1-R10) was computationally extracted and focused 3D classification of the core density was performed. At the final step of the processing, the alignment parameters were applied to the original particles to visualize the full density (B – center). Yellow density fitted with an available structural model of R3. To assess the flexibility of the autoinhibition pocket consisting of the interface between FERM domain F2-F3 and R12-R13 (lid), extensive 3D classifications were performed, yielding a visualization of the flexible densities of R11-R13 (C – right).
Table S1: Data collection and structure refinement statistics, related to Figure 1.

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Figure S2: Quality control of cryo-EM data processing, related to Figure 1.

A: Local resolution map of the full-length talin structure, showing a resolution range between 5.5 Å and 7.9 Å. B: The Fourier shell correlation (FSC) curve of the full-length
talin structure showing a global resolution of 6.2 Å. The additional line indicates a FSC of 0.143. C: Angular distributions of the particle orientations that were incorporated into the final 3D reconstruction. D: Cryo-EM images of talin-FL in the presence of 200 mM KCl and 500 mM KCl, showing aggregations presumably due to the talin opening at 500 mM KCl. 2D classifications of talin-FL at 200 mM KCl reveal less robust averages indicating higher structural heterogeneity.
Figure S3: Talin fragments used in this study and sequencing information of full-length talin, related to Figure 1.
A: Scheme of talin fragments used in this study (top) and the structures (PDB codes) that were used as building blocks for the fitting and molecular modelling. For PDBs 3IVF, 1SJ8, 5IC1 and 3DYJ, individual domains are computationally extracted and used independently (see also STAR methods). 2K denotes charge-reversal double mutation E2288K, E2294K and 5K denotes mutations E2288K, E2294K, D2297K, E2299K, D2300K). B: Peptide detection of talin-FL by mass spectrometry. The entire sequence of talin (1-2541) is covered, showing no indication of spontaneous cleavage.
Figure S4: Talin F3-rod binding surfaces obtained from our cryo-EM map in comparison to available structures, related to Figure 1.

Top-left: Our cryo-EM model of the F3-rod binding surface. **Bottom-left:** crystal structure (PDB: 4F7G) of truncated F2-F3 and R9. **Right:** Examples of structures that bind to the talin F3-rod binding surface. From top to bottom: PDB: 4F7G superimposed to 2H7E (Integrin tail), 2MWN (Riam), and 2K00 (Layilin).
Figure S5: Talin inter-domain interactions, related to Figure 1 and 4.
A: Electrostatic surface potential of individual rod domains surrounding R4 and forming the autoinhibition core. Sites contacting each other are indicated with yellow circles. B: Interaction energies calculated by restraint molecular dynamics simulations for major interaction surfaces in the presence of 150 mM salt. Units: kCal/mol. We note that the calculated interaction energies represent enthalpic contributions and do not include the entropic penalties of restricting the large ensemble of open and flexible conformations of talin to a small ensemble of compact conformations seen in the autoinhibited state.
Figure S6: SEC reconstitution assay of various talin rod domains and the vinculin head (V-head) at 75 mM salt, related to Figure 5. The SDS-PAGE analysis corresponds to the profile of the protein mixtures (top), talin control constructs (center), and vinculin head control (bottom), respectively.
Figure S7: Conformational change of talin in complex with V-head, related to Figure 5 and 6.
A: Size-exclusion chromatography (SEC) profile of talin and vinculin constructs. Reconstitution assays of talin (open conformation) and V-head as well as V-mut were performed at 500 mM salt, and then the complex was assessed by changing the salt concentration to 75 mM. V-head stays in complex with talin (blue), while V-mut dissociates from talin (red). B-D: DLS measurements of the hydrodynamic radius of the reconstituted talin constructs (talin-FL, talin-FL-2K and talin-FL-5K) in complex with V-head under various salt concentrations. In comparison to full-length talin constructs alone (shown in grey), the increased size of the sample is observed showing complex formation. A conformational change of talin-V-head complex upon the change of salt concentration is also observed. For the case of talin-FL-5K in complex with V-head, the profile did not fit to a two-state model. E: SEC-multi-angle light-scattering profile of the talin (75mM salt, closed) -V-head complex shows a molecular weight of 362 kDa, indicating a 1:1 stoichiometry. LS: light scattering, normalized, UV: absorbance F: Negative-stain EM image of the talin-vinculin head complex in closed talin and two-dimensional averages (right), showing a long protrusion from the main globular density. G: Molecular modeling of our talin structure (white) with R3 unfolded (yellow) to accommodate the vinculin head density (green). The model was created by combining our cryo-electron microscopy full-length talin structure (white) with available structures of a part of the vinculin head (a.a. 1–258) complexed with talin peptide (a.a. 1944–1969) (PDB: 1RKC, yellow) and the vinculin head (a.a. 259–843) extracted from full-length vinculin (PDB: 1TR2, green). H-J: SEC profiles of various talin truncation constructs with V-head at 75 mM salt, showing that the complex formation of talin and V-head occurs only with N-R11 (I) and R1-C (J). N-R12 containing the R12 (lid) does not make a complex with V-head (H). In the reconstitution assay of R1-C + V-head, a Superdex S200 column was used while a Superose 6 was used for the other assays.
Table S2 List of primers and recombinant DNA used in this study, related to STAR methods.

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Recombinant DNA

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STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Naoko Mizuno (mizuno@biochem.mpg.de).

METHOD DETAILS

Protein purification

Expression constructs of full-length talin1 (talin-FL) were prepared using human talin1 DNA (a gift from Christophe Le Clainche). DNA of talin-FL was amplified using PCR and subcloned into a pCB homemade expression vector with a His tag at the C-terminus for bacterial or mammalian cells. Talin1 was expressed in E. coli BL21 (DE3) goldusing ZY auto-induction medium. Cells were lysed by sonication in 50 mM Tris-HCl pH 7.8, 500 mM NaCl, 10 mM imidazole, 1 mM DTT, 5 mM EDTA, purified by nickel-affinity chromatography (complete His-Tag purification column, Roche) and anion exchange (HiTrap Q HP, GE Healthcare). The His-tag was removed using 3C protease and proteins were further purified by size-exclusion chromatography using a Superdex 200 16/600 column (GE Healthcare) in 20 mM HEPES pH 7.8, 75 mM KCl, 0.5 mM β-Mercaptoethanol and 0.5 mM EDTA and 10% glycerol.

Talin truncation and mutation variants were obtained using mouse talin FL (pLPCXmod-Talin1-Ypet; a gift from Carsten Grashoff,) as a template and cloned into homemade expression vectors (pCB and pEC vectors). Mouse talin constructs used in this study were 1-2482 (talin-ADD), 482-2541 (R1-C) 1-2294 (N-R12), 1-2141 (N-R11), 482-1655 (R1-R8), 482-913 (R1-R3), 913-1655 (R4-R8), talin mutant E2288K/E2294K/D2297K/E2299K/D2300K (talin-FL-5K) as well as talin mutant E2288K/E2294K (talin-FL-2K) (see Table S2). The DNA fragments were amplified using PCR and subcloned into a pCB vector containing a cleavable N-terminal Venus-His8-Sumo tag or His tag. Proteins were expressed in E. coli BL21 (DE3) gold in ZY auto-induction medium. Talin truncation variants were purified using the same procedure as talin-FL. Tags were removed using 3C or Senp2 protease and proteins were further purified by size-exclusion chromatography using a Superdex 200 16/600 column (GE Healthcare) with 20 mM HEPES pH 7.5, 75 mM NaCl, 0.5 mM EDTA and 0.5 mM β-Mercaptoethanol.

Vinculin constructs were designed based on a previous report (Cohen et al., 2005) and cloned into homemade pEC vectors containing N-terminal GST-His tag and a 3C-cleavage site. V-head contains a.a. 1-851. Proteins were purified using His-affinity
purification methods and eluted with 500 mM imidazole. Protein fragments were cleaved with 3C-Protease and dialyzed over night against 20 mM HEPES at pH 7.8, 100 mM KCl, 0.5 mM β-Mercaptoethanol, concentrated and subsequently applied to size-exclusion chromatography (Superdex 200 16/600, GE Healthcare). All purified proteins were stored at −80 °C. Analytical size-exclusion chromatography experiments using talin were performed in 20 mM HEPES at pH 7.8, 0.5 mM β-Mercaptoethanol, 0.5 mM EDTA either with 75 or 500 mM KCl to test the influence of salt on the conformational change observed under negative-stain EM.

**GraFix**

The GraFix method was adapted from (Stark, 2010). Talin samples were subjected to a sucrose and glutaraldehyde gradient of 10-30% and 0-0.2%, respectively, in a buffer containing 20 mM HEPES (pH 7.5), 75 mM KCl, 0.5 mM β-Mercaptoethanol and 0.5 mM EDTA. Gradients were prepared in 2.2 ml ultracentrifuge tubes (open-top polyclear tubes, Seton) using a Gradient Station machine (model ip, Biocomp). 50 uL of talin samples at a concentration of 5 mg/ml (approximately 18.5 uM) were placed on top of the gradients and centrifuged in a TLS55 rotor (Beckman Coulter) at 50 000 rpm, for 6h at 4°C, using an Optima Max-XP ultracentrifuge (Beckman Coulter). 100 uL-Fractions were collected manually from top to bottom and quenched with 100 mM Tris (pH7.5). Fractions containing the monomeric or dimeric talin, as determined by SDS-PAGE were pooled, concentrated, and buffer-exchanged to 20 mM HEPES (pH 7.5), 75 mM KCl, 0.5 mM β-Mercaptoethanol and 0.5 mM EDTA to remove excess sucrose using Amicon-Ultra, 0.5 ml, 50 MWCO.

**Mass spectrometry**

The talin-FL sample was diluted in equal volume of buffer containing 2% sodium deoxycholate (SDC), 20 mM TCEP and 80 mM chloroacetamide in 25 mM Tris at pH 8.5 and incubated at 37°C for 20 min. The sample was then further diluted with LC-MS grade water to reduce the SDC concentration to less than 0.5% and directly digest with 1 µg of trypsin (Promega) overnight. The peptides were then acidified and purified via SDB-SCX StageTips and analysed in a Q Exactive HF mass spectrometer using a 75 min gradient. Raw data were processed using the MaxQuant platform and all identifications were filtered at 1% false discovery rate (FDR).

**Negative-stain electron microscopy**
For negative-stain EM, homemade carbon-coated grids were prepared and glow discharged before use. 5 µl of sample was applied and incubated for 1 min, blotted, washed twice in two drops of water and stained in 2% uranyl acetate for 1 min. The prepared specimens were visualized with a FEI CM200 with an operating voltage of 160 kV equipped with an Eagle CCD camera with a pixel size of 2.16 Å/pix or with a FEI Tecnai F20 at 200 kV with an Eagle CCD camera with a pixel size of 2.21 Å/pix. For 2D classifications, particles were manually or automatically picked using RELION (Zivanov et al., 2018) and extracted in 276 Å boxes. For shape assessment, 1102 (talin-ΔDD), 1749 (R1-C), 204 (N-R12), 321 (N-R11) and 5678 (talin-FL V-head complex, treated with GraFix method for stabilization) particles were selected for reference-free 2D classification using RELION, resulting in 2D class averages. The lengths of open talin were measured using bshow in BSOFT (Heymann and Belnap, 2007). Five images of negatively stained talin were filtered using median filter (n=6) as well as Gaussian filter (sigma=4) to enhance the contrast of the protein densities. Particles showing clear boundaries were manually selected and their contour length was measured using the ‘filament’ option in BSOFT, facilitating the tracing of elongated, string-like particles. The histogram was created from n=89 samples, and the distribution was fit to normal distribution (average 560 Å, S.D. 170 Å).

Cryo-EM

Samples for cryo-EM were applied to glow-discharged R1.2/1.3 Cu 200 mesh holey carbon grids at a concentration of 0.3 mg/mL in 20 mM HEPES at pH 7.8, 75 mM KCl, 0.5 mM β-Mercaptoethanol, 0.5 mM EDTA to ensure the compact formation of talin based on the DLS experiment shown in Figure 3D. Under salt concentrations of 200 mM KCl and higher, the compact formation of talin molecule was compromised in the cryo-EM environment, yielding poor 2D averages (Figure S2D). The grids were blotted at ~95% humidity at 4°C for 4 s and plunged into liquid ethane-propane using a Vitrobot Mark IV (FEI). 11007 micrographs were collected on a Titan Krios (FEI) at 300 kV equipped with a K2 Summit direct electron detector and a quantum energy filter (20 e-V)(Gatan). Micrographs were exposed for 10 s at 7.68 e-/Å²/s (total dose 76.8 e-/Å²) and 40 frames were collected in counting mode (pixel size 1.06 Å/pixel). Defocus was varied between -1 and -3 µm in steps of 0.3 µm. Movies were aligned, gain-normalized and dose-weighted using MotionCor2 (Zheng et al., 2017). Defocus values were determined using GCTF (Zhang, 2016). Particles were automatically picked with Gautomatch using the template-free mode (Gaussian blob with 220 Å diameter), imported to RELION-3 (Zivanov et al., 2018) and extracted in 224 Å-sized boxes. Extracted particles were
applied to 2D classification routine in RELION-3 to remove contaminations. 1,873,975 particles were selected and after removing low quality classes, initial 3D reconstruction was performed using cryoSPARC (Punjani et al., 2017). Using the initial model, 5 batches of 3D classification were performed, yielding several models of a closed talin structure with different completeness of densities. Particles of the most complete model were combined, further aligned and classified. 30,438 particles were selected for the final 3D refinement, yielding a final map with a global resolution of 6.2 Å with up to 5.5 Å resolution in the core of talin using the RELION post-processing routine, using the gold-standard Fourier shell correction (FCS) = 0.143 criterion. In the final map, the R3 domain was only partially resolved. To visualize the R3 domain, flexible densities were computationally subtracted from individual particles using available scheme from RELION-3, and as previously described (Bai et al., 2015), facilitating the focused 3D classification of the core density R1-R10. The particle information and the alignment parameters of the 3D class with the most prominent density of R3 were applied to the original particles that contain the flexible densities (FERM domain and R11-C) and the final map with the R3 domain was obtained. This map was used for the assignment of the R3 domain. Finally, to assess the flexibility of the autoinhibition area in the talin structure, 3D classification was performed to the 750,432 particles obtained from the initial stage of the 3D classification. It was observed that the parts corresponding to R11-R13 exhibited a weaker density compared to the core part (R1-R10) of the structure, detecting high levels of flexibility. Overall, the analysis indicated that the packing of talin-FL is sensitive to its environment, which agrees well with its conformational change observed in our biochemical analysis as well as its cellular function. The graphical summary is depicted in Figure S1.

**Molecular fitting and molecular dynamics analysis for flexible fitting**

For each of the rod fragments (R1-R13) and the FERM domains, crystal and NMR structures are available. The corresponding PDB entries are 3ivf (FERM domain), 1sj8 (rod domains R1 and R2), 2l7a (R3), 2lqg (R4), 2l10 (R6), 2l7n (R5) 5ic1 (R7, R8, R9), 2kvp (R10), 3dyj (R11, R12), 2jsw (R13). Initially, the structures containing multiple domains (3ivf, 1sj8 and 5ic1) were computationally divided into individual domains. These individual domain structures served as building blocks to build a model of the entire autoinhibited structure of talin (Figure S3).

The R1 rod domain has an asymmetric shape and only one unique well-fitted placement and orientation of this structure in the density could be identified by rigid-body fitting. The C-terminus of the fitted R1 model reaches into a neighboring helical
bundle density in the cryo-EM map, which fitted well with the R2 model. The available structure of R1-R2 complex (1sj8) was independently fitted using the colores program of the Situs package (Chacón and Wriggers, 2002) showing that they matched to each other. This fitting served as a first anchor segment. The resolution of the density was sufficient to clearly distinguish between 4-helix and 5-helix bundle segments in the density. R7 and R8 were readily identified due to the inter-winded helical bundle feature of R7 within R8. Together with the constraint on the maximum linker length to covalently connect consecutive rod-domains it was possible to define placements and also orientations of the subsequent rod domains (R3-R13) in order to fit to the density and at the same time, to allow covalent connection to the previous and subsequent rod-domains. Placements that sterically did not allow connection of consecutive fragments to form a fully connected talin chain were eliminated. Finally, remaining densities, which did not represent a helical bundle arrangement, were assessed. These remaining densities consisted of two globular entities that were identified as F2 and F3 domains due to their secondary structures features. Overall, the fitting was performed independent of any of available structural models showing the inter-domain connections. However, our placements brought the F3 segment in close binding vicinity to the R9 rod-domain. The placement coincided with the crystal structure of F2-F3 in complex with R9 (PDB 4f7g) (within an root-mean square deviation (RMSD) < 0.8 Å).

The resolution of the cyro-EM reconstruction of 6.2 Å together with the sterical constraints to covalently connect domains with smaller linker segments readily allowed the identification of the unique topology for the full chain. However, no cryo-EM densities for the FERM F0 and F1 domains and the DD domain were identified and these parts were not included during model building. Finally, a full covalently connected chain was formed by connecting N- and C-termini of consecutive rod fragments using the Leap module of the Amber16 package (Salomon-Ferrer et al., 2013) in combination with the ff14SB force field (Maier et al., 2015). The disordered connection between FERM F3 and R1 was not included and the F2 and F3 domains were treated as separate proteins not covalently connected to the rod fragments.

The emap/sander module of the Amber16 package was used to perform a flexible fitting and sterical force field optimization (based on the ff14SB force field). In order to keep the structure of each individual template structure (R1-R13 and F2 and F3) close to the corresponding experimental crystal structure distance restraints between backbone atoms within each fragment were included. These restraints kept the secondary structure and the arrangement of secondary structures in each domain to within RMSD < 1 Å from the corresponding experimental domain reference structures.
Connecting loops and side chains were nevertheless fully mobile during flexible fitting. The full start structure was first energy minimized (5000 steps) followed by a Molecular Dynamics (MD) simulation at 300 K (0.5 ns) until no further shift of the mean structure was observed followed by another round of energy minimization. The model was then subjected to manual building and correction in COOT (Emsley et al., 2010) and real-space refinement in PHENIX (Afonine et al., 2018). The final structure fitted well to the cryo-EM density with a correlation coefficient around the model (CC\text{mask}) of 0.81 without outliers in the Ramachandran plot (Table S1).

**Estimation of interaction energies between talin subunits**

The interaction energy between selected pairs of talin fragments (enthalpy) was calculated using the MMPBSA (Molecular Mechanics Poisson Boltzmann Surface Area) approach as implemented in the Amber18 package (Onufriev et al., 2000; Salomon-Ferrer et al., 2013). Pairs of talin subdomains were extracted from the compact talin autoinhibited structure in the same arrangement as observed in the full structure. For each case an ensemble of 250 conformations was generated using short MD simulation (0.1 ns, 300 K) keeping the backbone strongly restraint to the starting placement (allowing shifts < 0.1 Å). The mean domain-domain interaction energy was calculated using the MMPBSA method. It accounts for changes in electrostatic Coulomb interactions, changes in electrostatic solvation (reaction field with the aqueous environment), changes in mean van-der-Waals interactions and nonpolar (hydrophobic) contributions that depend on the buried surface area upon complex formation. In the MMPBSA approach, the change in electrostatic reaction field contributions is calculated by solving the finite-difference Poisson-Boltzmann equation for each structure. The energy of interaction was estimated by subtracting the contributions of each partner from the result of the domain-domain complex for each case. Calculations were performed at a monovalent salt concentration of 0.15 M. We note that the calculated interaction energies represent enthalpic contributions and do not include the entropic penalties of restricting the large ensemble of open and flexible conformations of talin to a small ensemble of compact conformations seen in the autoinhibited state.

**F-Actin co-sedimentation assay**

Actin was purchased from Hypermol, Germany. Actin was polymerized in 10 mM Tris pH 7.5, 50 mM KCl, 2 mM MgCl\textsubscript{2}, 0.2 mM CaCl\textsubscript{2}, 0.2 mM ATP, 0.5 mM DTT for 20 min at RT. Buffer exchange of the samples were performed using Zeba Spin desalting columns (Thermo Scientific). The buffer for all constructs was 20 mM Tris pH 7.5, 75
mM KCl. 2.5 μM talin fragments were incubated with 2.5 μM F-actin for 15 min at RT. The mixture was then ultra-centrifuged at 175 000 x g (TL-100 Ultracentrifuge, Beckmann) for 20 minutes at RT. Pellets were re-suspended to comparable volumes as supernatants with 1x SDS buffer. 6x SDS buffer was added to the supernatant and 10 μl of samples were run on SDS-page and quantified using FIJI (Schindelin et al., 2012).

**Analytical size-exclusion chromatography**

For analytical size-exclusion chromatography, the buffer exchange of the sample was performed using Zeba Spin desalting columns (Thermo Scientific) to 20 mM HEPES pH 7.5, 75 or 500 mM KCl, and 10 μM of samples were injected to either an analytical Superdex 200i 3.2/300 (for the reconstitutions of R1-C, R1-R3, R4-R8 and R4-R8 and V-head) or Superose 6i 3.2/300 (all other constructs) on an ÄKTAmicro system (GE Healthcare). Fractions were collected, mixed with 6x SDS sample buffer and 10 μl were run on SDS-PAGE.

**Dynamic Light-Scattering (DLS)**

For measurements of hydrodynamic radius changes, talin-FL, talin N-R11 and talin N-R12 were initially prepared in 20 mM HEPES pH 7.5, 75 mM KCl, 0.5 mM EDTA, 0.5 mM β-Mercaptoethanol. Further, the samples were diluted to a final concentration of 0.3 mg/ml and final KCl concentrations of 75 – 500 mM (75/100/125/150/175/200/225/250/300/350/400/450/500 mM KCl) in the same buffer in 30 μl wells in a 384-well plate. DLS measurements were performed with a Dynapro Platereader-II Dynamic Light Scattering instrument (Wyatt Technology Corporation) at 20˚C. Samples were independently prepared in triplicates and 20 measurement points per well were taken. The data was analyzed in Dynamic 7.8.1.3 (Wyatt Technology Corporation) and plotted with PRISM (GraphPad). The fitting of the curve was performed using a nonlinear regression fit with a sigmoidal curve.

**SEC-MALS**

Talin-fl and talin truncations as well as the mixtures of talin and vinculin (1:1 ratio) were prepared in buffer containing 20 mM HEPES, pH 7.5, 75 or 500 mM KCl, 0.5 mM EDTA at 3 mg/ml concentration and 20 μl were run on a Superdex 200 5/150 GL column on an ÄKTAmicro system (GE Healthcare) coupled to a Viscotek TDA302 detector (Malvern, Herrenberg, Germany) in the same buffer. Bovine serum albumin was used as a standard and the refractive index increment (dn/dc) was set to 0.180
ml/g for calculations. Data was analyzed using the OmniSEC 4.5 software (Malvern) and plotted with PRISM (GraphPad).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Quantification and statistical analysis related to EM data are integral parts of algorithms and software used.

For DLS analysis, the experiments were performed 3 times independently. The results were averaged and the error bars represent the standard deviations. For the fitting, nonlinear regression fit, sigmoidal curve fitting was applied using the software PRISM (GraphPad).

For F-actin analysis shown in Figure 6, the experiments were performed at least 3 times independently. The talin-FL band was run on the same SDS-PAGE gel as an internal control to standardize the variability of the contrast caused by staining conditions of each SDS-PAGE gels. Therefore, relative quantified values compared to the talin-FL band were used for the analysis. These values were averaged and standard deviations were obtained. Unpaired t-test was used to assess statistical significance.

**DATA AND SOFTWARE AVAILABILITY**

The cryo-EM map of human talin1 was deposited in the EMDB database with accession code EMD-4772. The flexibly fitted atomic model was deposited in the PDB with accession code 6R9T.

**Reference**


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5.1 Kank2 activates talin, reduces force transduction across integrins and induces central adhesion formation

Zhiqi Sun, Hui-Yuan Tseng, Steven Tan, Fabrice Senger, Laetitia Kurzawa, Dirk Dedden, Naoko Mizuno, Anita A. Wasik, Manuel Thery, Alexander R. Dunn and Reinhard Fässler


During my PhD study I was able to support a project of Zhiqi Sun about the role of Kank2 in focal adhesions. The study used a novel integrative adhesome analysis, which combined focal adhesion enrichment with the interactome of the integrin tail and identified the evolutionary conserved Kank family as adhesome components. The study describes that Kank2 localizes around the lateral border of FA and forms a sub-compartment, named the FA-belt in the center of sliding adhesions. There, Kank can directly interact with talin’s R7 domain via its conserved N-terminal KN motif and induce both talin’s and subsequently integrin’s activity. Furthermore Kank binding to talin has a reducing effect on talin’s actin binding capabilities, which overall weakens the link between talin and the actomyosin machinery. This leads to reduced integrin-ligand bond strength and can cause slippage between the ligand and integrin. This induces adhesion sliding and lowers the cells migration speed. This study assigns Kank2 with a role as talin activator, which has an additional function in decreasing the talin-actomyosin link to negatively regulate cell migration velocity. My major contribution to this study was the purification of proteins and protein fragments of talin and Kank2 for in vitro assays.
Kank2 activates talin, reduces force transduction across integrins and induces central adhesion formation

Zhiqi Sun1, Hui-Yuan Tseng1, Steven Tan3, Fabrice Senger3, Laetitia Kurzawa3, Dirk Dedden1, Naoko Mizuno1, Anita A. Wasik1, Manuel Thery3, Alexander R. Dunn2 and Reinhard Fässler1,4

Integrin-based adhesions play critical roles in cell migration. Talin activates integrins and flexibly connects integrins to the actomyosin cytoskeleton, thereby serving as a ‘molecular clutch’ that transmits forces to the extracellular matrix to drive cell migration. Here we identify the evolutionarily conserved Kank protein family as novel components of focal adhesions (FAs). Kank proteins accumulate at the lateral border of FAs, which we term the FA belt, and in central sliding adhesions, where they directly bind the talin rod domain through the Kank amino-terminal (KN) motif and induce talin and integrin activation. In addition, Kank proteins diminish the talin–actomyosin linkage, which curbs force transmission across integrins, leading to reduced integrin–ligand bond strength, slippage between integrin and ligand, central adhesion formation and sliding, and reduced cell migration speed. Our data identify Kank proteins as talin activators that decrease the grip between the integrin–talin complex and actomyosin to regulate cell migration velocity.

Mesenchymal cell migration on the extracellular matrix (ECM) is crucial for embryonic development, wound healing and tumour metastasis. It commences with the formation of a lamellipodium at the cell leading edge, which is stabilized by numerous small and short-lived integrin-containing nascent adhesions (NAs)1. As cells migrate, a few NAs mature into larger focal adhesions (FAs) in the lamella, which associate with the actomyosin cytoskeleton and apply traction forces against the ECM necessary to move the cell forward2.

Integrins are core components of cell–matrix adhesion sites. They are α/β heterodimers that undergo a conformational change before they bind ligands (termed integrin activation), cluster and recruit numerous proteins to their cytoplasmic domains. A hallmark of integrin-mediated adhesion is that the lifetime of integrin–ligand bonds can be flexibly increased when forces are applied to integrins3. This property, called force-induced adhesion strengthening, depends on a dynamic association between integrins and F-actin, and is essential for FAs to withstand traction forces that pull the cell forward during cell migration4. Consequently, the destabilization of the integrin–F-actin connection or a decline in myosin II activity decreases force transmission across FAs and shortens the lifetime of integrin–ligand bonds, resulting in slippage between integrin and ligand, adhesion sliding, reduced traction forces and a drop in migration speed5. The association between integrins and F-actin occurs indirectly through integrin- and/or F-actin-binding proteins such as talin and vinculin6, which serve as a ‘molecular clutch’ that couples traction and actin-driven forces in space and time7–10.

Talin consists of an amino-terminal FERM (protein 4.1, ezrin, radixin, moesin) domain, also called the talin head domain (THD), and a carboxy-terminal rod domain with 13 helical bundles (R1–R13)11. The THD binds β-integrin tails and mediates integrin activation. The rod domain contains binding sites for F-actin, vinculin and the Rap1–GTP-interacting adapter molecule (RIAM)12. Talin cycles between cytosol, where it remains in an auto-inhibited form13, and plasma membrane, where it activates integrins and links integrins to F-actin14,15. Talin activation can be induced by RIAM binding to the R8 domain16–18, phosphatidylinositol 4,5-bisphosphate synthesized by a FA-associated splice variant of phosphatidylinositol-4-phosphate 5-kinase type 1γ (PIPKIγ90)15,19 and the actin retrograde flow, probably by unleashing the talin rod from the THD20,21. The cell-type-restricted integrin activation defects in RIAM-deficient mice and normal integrin activation in PIPKIγ90-deficient mice22–24 suggest that additional talin activators probably exist.

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1Max Planck Institute of Biochemistry, 82152 Martinsried, Germany. 2Department of Chemical Engineering, Stanford University, Stanford, California 94305, USA. 3CytoMorpho Lab, Biosciences & Biotechnology Institute of Grenoble, UMR5168, CEA/INRA/CNRS/Université, 38054 Grenoble-Alpes, Grenoble, France.

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In the present paper, we identified the evolutionarily conserved Kank protein family as novel FA proteins. They consist of four members (Kank1–4) that are characterized by a Kank N-terminal motif (KN), several central coiled-coil domains and C-terminal ankyrin (Ank) repeats. The single Kank orthologue in worms, V AB19, controls epidermis–muscle attachment across activated integrins, leading to adhesion sliding and reduced cell migration.

RESULTS

Kank2 is a novel FA protein
To identify novel, β1 integrin tail-associated adhesome proteins, we compared our published FA-enriched proteome (adhesome) with the β1 integrin tail peptide interactome (Supplementary Fig. 1a). Talin, kindlin-2 and the ILK, PINCH, parvin (IPP) complex were enriched in the adhesome and the β1 tail interactome, whereas Dab-2 and SNX17, which bind integrin tails during endosomal trafficking, were not enriched in the adhesome. Interestingly, Kank2 was also enriched in the adhesome and β1 tail interactome (Supplementary Fig. 1a). Unlike kindlin-2, which preferentially bound the β1 tail, or Src and Dab-2, which preferentially bound the β3 tail, Kank2 and talin bound β1 and β3 tails equally well (Fig. 1a). Immunostaining of cells immunostained for Kank2 and talin or vinculin along depicted line scans. Scale bar, 1 μm. (d) Definition of the FA belt at the lateral FA border and the distance from the FA border to proteins in proximity to the FA (outside of FAs) along the depicted line scan. (e) Kank2-positive puncta localize to the FA border defined by ILK, kindlin-2, vinculin, talin and paxillin (mean ± s.d.; n = 8 FAs for each marker, data pooled from eight cells). Unprocessed original scans of blots are shown in Supplementary Fig. 9.
of fibronectin (FN)-seeded, immortalized mouse fibroblasts revealed that endogenous Kank2 localized to puncta at the outer border of mature kindlin-2- and talin-positive FAs behind the lamella (Fig. 1b, arrowheads, Supplementary Fig. 1c) and to thin, elongated central adhesions (Fig. 1b and Supplementary Fig. 1b), and was absent from NAs and small FAs of the lamella (Fig. 1b, open arrowheads). Blebbistatin-treated cells also lacked Kank2 in kindlin-2-positive NAs or protruding cell membranes (Fig. 1b). The recruitment of Kank2 to the FA border was pronounced in cells cultured on FN-coated crossbow-shaped micropatterns (Supplementary Fig. 1d). Line profile analysis revealed that the Kank2 puncta peaked at the outer FA border, where canonical FA proteins (talin, kindlin-2, ILK, paxillin and vinculin) showed ~50% of their plateau intensity (Fig. 1c–e). We termed this unrecognized FA compartment the ‘FA belt’.

Co-immunostaining for β1 and β3 integrins revealed that β3 integrins co-localized with paxillin in the core of mature FAs, whereas total and active (labelled with the activation epitope-reporting 9EG7 antibody) β1 accumulated in FA belts and central adhesions (Supplementary Fig. 1e–g). These data show that mature FAs are surrounded by a Kank2- and β1 integrin-enriched belt.

**Kank2 is targeted to FAs through the KN motif**

To determine how Kank2 is recruited to the FA belt, we generated Kank2 truncation/deletion mutants. (a) Domain organization of the Kank2 protein and illustration of GFP-tagged Kank2 and Kank2(1–670) together with Pxn. Scale bar, 1 µm. (b) Staining of paxillin (Pxn), F-actin and vinculin) showed 50% of their plateau intensity (Fig. 1c–e). We termed this unrecognized FA compartment the ‘FA belt’.

Co-immunostaining for β1 and β3 integrins revealed that β3 integrins co-localized with paxillin in the core of mature FAs, whereas total and active (labelled with the activation epitope-reporting 9EG7 antibody) β1 accumulated in FA belts and central adhesions (Supplementary Fig. 1e–g). These data show that mature FAs are surrounded by a Kank2- and β1 integrin-enriched belt.

**Kank2 is targeted to FAs through the KN motif**

To determine how Kank2 is recruited to the FA belt, we generated green fluorescent protein (GFP)-tagged full length (FL)- Kank2 and
Figure 3 Kank2 curbs cell migration by inducing adhesion sliding. (a) Time-lapse images of peripheral FAs in Kank2-depleted fibroblasts stably expressing FL-Kank2-GFP and paxillin-TagRFP (Pxn-TagRFP) 45 min after plating on FN. Arrowheads highlight recruitment of Kank2 to proximal borders of FAs (full arrowheads) and the developing sliding adhesions (open arrowheads). (b) Time-lapse images of cells during the migration phase 4 h after plating on FN. Arrowheads highlight recruitment of Kank2 to proximal borders of FAs (full arrowheads) and the developing sliding adhesions (open arrowheads). (c) Time-lapse images of peripheral FAs in Kank2-depleted cells stably expressing FL-Kank2-GFP and Pxn-TagRFP 45 min after plating on FN. Arrowheads highlight the proximal border of a stable FA and disassembling FA behind the lamella. Scale bars in a–d, 5μm. (e) Sliding velocities of central adhesions and FAs from indicated cells (mean ± s.d.; n=5 cells pooled from three independent experiments, >400 central adhesions and >100 peripheral adhesions analysed for each condition). (f) 2D random migration velocities on FN (dot plot and box plot with median, 95% confidence interval (CI) notch, first–third quantile box and 5th–95th percentile whiskers; n=60 and 90 cells for each cell line; data aggregated over four independent experiments). P values calculated using one-way ANOVA Tukey test in e and Kruskal–Wallis test in f. (g) Kank2-depleted cells re-expressing FL-Kank2-GFP were plated on FN-coated (10μg ml\(^{-1}\)) coverslips for 1 h or 5 h and immunostained for FN and DAPI. Scale bar, 10μm. (h) Kank2-depleted cells re-expressing FL-Kank2-GFP or GFP control were seeded on FN-coated (10μg ml\(^{-1}\)) coverslips for 12 h at confluence and immunostained for FN and DAPI. Maximal intensity projection of z-stack image series. Scale bar, 50μm.

deletion constructs of Kank2 lacking evolutionarily conserved domains (Fig. 2a): Kank2ΔKN-GFP lacking the KN motif; Kank2Δcoil-GFP lacking the liprin-β1-binding coiled-coil domain\(^{31}\); Kank2(1–670)-GFP lacking the Kif21a-binding Ank repeats\(^{31}\) and Kank2ΔKNΔcoil-GFP lacking both the KN motif and the coiled-coil domain. The Kank2 constructs were stably expressed in...
Kank2-depleted mouse fibroblasts (Supplementary Fig. 2b), which expressed mainly Kank2 (Supplementary Fig. 2a–c).

FL-Kank2-GFP also localized to the belt of paxillin-positive mature FAs and to central adhesions (Fig. 2b). In line with a previous report, we also found Kank2 adjacent to FAs in liprin-β1- and ELKS-enriched regions (Supplementary Fig. 3a–c), which capture MT plus ends and promote exocytosis. In contrast, Kank2ΔKN-GFP was absent from FA belts or central adhesions and instead accumulated in liprin-β1- and ELKS-positive regions adjacent to but clearly away from FA belts (Fig. 2b, and Supplementary Fig. 3a,b), as shown by line profile analyses (Fig. 2c,d). Although both Kank2Δcoil-GFP and Kank2(1–670)-GFP showed enrichment on FA belts (Fig. 2b–d), Kank2Δcoil-GFP also penetrated the FA core (Fig. 2e), indicating that the coiled-coil domain contributes to the exclusion of Kank2 from the FA core. Interestingly, Kank2 recruited liprin-β1 through the coiled-coil domain to FA belts, whereas ELKS always localized to the vicinity of the belt (Supplementary Fig. 3a–c). Kank2ΔKNΔcoil-GFP was diffusely distributed throughout the cytosol, whereas the GFP-tagged KN polypeptide completely overlapped with paxillin in all adhesions, including the small, peripheral NAs (Fig. 2b). These results indicate that the KN motif localizes Kank2 to FA belts and central adhesions and that additional protein regions exclude Kank2 from the FA core.

Peptide pulldowns revealed that FL-Kank2-GFP and Kank2-KN-GFP, but not Kank2ΔKN-GFP or GFP, associated with β1 integrin tails (Supplementary Fig. 3d,e). To investigate whether Kank1, Kank3 and Kank4 also localize to the belt, they were tagged with GFP and expressed in Kank2-depleted fibroblasts at similar levels as judged by GFP intensities. Kank1-GFP and Kank3-GFP localized to FA belts, whereas Kank4-GFP associated with FAs with additional cytoplasmic distribution (Supplementary Fig. 3f).

Kank2 inhibits cell migration by inducing adhesion sliding

To unravel how Kank2 is recruited to FA belts, we carried out live cell imaging of paxillin-TagRFP (red fluorescent protein) and Kank2-GFP in Kank2-depleted fibroblasts. During isotropic cell spreading on FN, Kank2-containing puncta were visible in the proximity of FAs. A few minutes after the assembly of radial FAs, Kank2 puncta appeared at their proximal tips pointing to the cell centre, from where Kank2 spread along the FA belt during the following minutes (Supplementary Video 1 and Supplementary Fig. 4a). After cell polarization (45 min after plating), Kank2 puncta continued to accumulate at the proximal border of FAs (Supplementary Video 2 and Fig. 3a), from where Kank2- and paxillin-positive, thin and elongated adhesion structures developed and moved with an average speed of ~0.3 μm min⁻¹ into the cell centre (Supplementary Video 2 and Fig. 3a). In migrating cells (4 h after plating), Kank2-GFP was recruited to the proximal tips of mature FAs and then gradually spread along the FA belt, while it remained absent from NAs in lamellipodia and FAs of the lamella (Supplementary Video 3 and Fig. 3b). The Kank2-positive FA belt formation correlated with the disassembly of the FA cores and the conversion of the FA belts into thin and motile adhesions (Supplementary Video 3 and Fig. 3b). In Kank2ΔKN-GFP-expressing cells, Kank2ΔKN-GFP-positive puncta moved to the proximity of FAs but never entered the FA belt (Supplementary Video 4 and Fig. 3c, closed arrowheads). Moreover, FAs failed to elongate and slide, and the few central adhesions that formed remained stationary (Supplementary Video 4 and Fig. 3c). The stationary FAs in Kank2ΔKN-GFP-expressing cells disassembled behind the lamella (Fig. 3d) with similar rates as in cells expressing FL-Kank2 (Supplementary Video 4 and Supplementary Fig. 4b,c), indicating that adhesion sliding is not caused by adhesion disassembly.

Our data suggest that Kank2 decorates FA belts in a KN-motif-dependent manner and induces the sliding of belts into the cell centre. To confirm that Kank2 induces adhesion motility, we recorded vinculin-mCherry co-expressed in Kank2-depleted fibroblasts with either GFP-tagged FL or mutant Kank2 (Supplementary Videos 5–10).Overlay of 10 sequential colour-coded vinculin-mCherry frames revealed that central adhesions in FL-Kank2-GFP- and Kank2(1–670)-expressing cells appeared as rainbows in overlay images due to their significant displacements, whereas central adhesions of cells expressing Kank2ΔKN-GFP, Kank2Δcoil-GFP, KN-GFP or GFP alone were stationary and appeared white in overlay images (Supplementary Fig. 4d). Single-adhesion tracking revealed that central adhesions in FL-Kank2-GFP- and Kank2(1–670)-expressing cells moved with a sliding velocity of ~0.3 μm min⁻¹, whereas sliding of central adhesions in cells expressing Kank2ΔKN-GFP, Kank2Δcoil-GFP, KN-GFP or GFP alone was slower (Fig. 3e). Furthermore, over 60% of adhesions in FL-Kank2-GFP- and Kank2(1–670)-GFP-expressing cells slid with rates higher than 0.3 μm min⁻¹, whereas the numbers of fast-sliding adhesions were significantly lower in cells expressing Kank2ΔKN-GFP, Kank2Δcoil-GFP, KN-GFP or GFP alone (Supplementary Fig. 4e). Consistent with the absence of Kank2 from adhesions in the protruding front, the sliding velocity of adhesions in protrusion was unaffected by the FL or mutant Kank2 (Fig. 3e).

Adhesion sliding correlates with reduced cell migration speed. Indeed, both Kank2-depleted cell lines showed higher migration velocities when compared with controls (Supplementary Fig. 4f). Furthermore, expression of FL-Kank2-GFP and Kank2(1–670)-GFP but not Kank2ΔKN-GFP, Kank2Δcoil-GFP, KN-GFP or GFP alone in Kank2-depleted fibroblasts decreased migration speed (Fig. 3f). Consistent with the absence of Kank2 from the cell front, cell spreading was unaffected in Kank2-depleted cells or cells expressing Kank2 constructs (Supplementary Fig. 4g).

Centrietal α5β1 integrin translocation was proposed to mediate FN fibrillogenesis. We noticed that Kank2-positive central adhesions formed within 1 h of plating on FN before FN fibrillogenesis occurred, whereas, 5 h after cell seeding, thin FN fibrils, frequently decorated with Kank2, extended from the cell periphery to the cell centre (Fig. 3g). After overnight culture, FL-Kank2-GFP-expressing cells assembled extensive networks of elongated and branched FN fibrils, compared with the few and thickened FN fibrils in GFP-expressing Kank2-depleted cells (Fig. 3h).

Kank2 induces integrin–ligand-bound slippage

Adhesion sliding can be due to ECM cleavage by metalloproteinases (MMPs) or turnover of integrin–ligand complexes. Treatment of FL-Kank2-GFP-expressing cells with the pan-MMP inhibitor GM6001 did not affect adhesion sliding (Supplementary Fig. 4h). To analyse integrin turnover, we photobleached the C-terminally GFP-tagged α5 integrin (Itgα5-GFP) in entire adhesions of Kank2-depleted cells expressing FL-Kank2-mCherry or an empty control
Figure 4 Adhesion sliding occurs at the interface between integrin and ligand. (a) Still images from representative time-lapse FRAP experiments with Itga5-GFP in Cherry-tagged FL-Kank2 or empty-vector-expressing fibroblasts. A pre-bleach image shows that Kank2-mCherry and Itga5-GFP co-localized in the region of interest (ROI, white circle). Scale bar, 2 µm (b) Fluorescence recovery curves of indicated FRAP experiments. FRAP of Itga5-GFP in central adhesions of Kank2-depleted fibroblasts transduced with either mCherry-tagged FL-Kank2 or empty plasmid. Mean optical intensities in the ROI are normalized to cytosolic background and plotted as percentage of initial intensity before bleaching (mean ± s.d.; n = 8 independent FRAPs in eight cells for each cell line). Fluorescence recovery curves are fitted to a one-phase association model. (c) Mobile Itga5-GFP fractions in bleached adhesions (mean ± s.d.; n = 8 independent FRAPs in eight cells for each cell line; P value calculated using Students t test). (d) Adhesion treadmilling (left) and integrin slippage models predict different experimental results in time-lapse images and kymographs on photobleaching of middle segments in sliding adhesions. (e–g) Time-lapse images (upper panel) and kymograph (lower panel) of Itga5-GFP (e, f) or Kank2-GFP (g) after photobleaching of the GFP signal in the middle segments of adhesion sites in Kank2-depleted fibroblasts expressing Kank2-mCherry and a low level of Itga5-GFP (e), empty plasmid (f) or Kank2-GFP (g).

plasmid, and found that FL-Kank2 increased the mobile fraction of α5 integrin from about 40% to about 70% (Fig. 4a–c). To distinguish slippage between integrin and ligand from adhesion treadmilling as the cause of adhesion sliding, we photobleached Itga5-GFP in the middle segment of an adhesion and measured GFP recovery. In the case of treadmilling defined by integrin recruitment to the proximal end and release from the distal ends the bleached middle segment should remain stationary (Fig. 4d, left panel), whereas in the case of slippage between integrin and ligand the bleached middle segment should move in the direction of the adhesion sliding, at least during the short time window before integrins are turned over (Fig. 4d, right panel). The experiment revealed...
slippage movement of integrins in Kank2-positive adhesions, but not treadmilling (Fig. 4e). Similarly, photobleaching of FL-Kank2-GFP in the middle segment of a sliding adhesion revealed the same co-sliding of bleached and unbleached areas (Fig. 4g). In contrast, photobleaching of Igα5-GFP in the middle segment of a stationary adhesion in Kank2-depleted cells revealed that neither bleached nor unbleached regions slid (Fig. 4f). These findings indicate that Kank2 destabilizes integrin–ligand complexes, leading to sliding of the Kank2–integrin complex along the ECM and the formation of FN fibrils.
Kank2 suppresses force transmission across integrins

A reduced force transmission across integrins can destabilize integrin–ligand bonds. To test this possibility, we plated fibroblasts expressing GFP-tagged FL-Kank2, Kank2ΔKN, Kank2Δcoil, Kank2(1–670), Kank2 or GFP alone on RGD peptides conjugated with a Förster resonance energy transfer (FRET)-based molecular tension sensor, which decreases FRET efficiency under tension. To visualize FRET maps recorded by total internal reflection fluorescence (TIRF) microscopy, we inverted the FRET ratios such that bright signals correspond to low FRET ratios and high traction. We found that tension had a negative correlation with FL-Kank2 binding to β1 wild-type and β1 Y783A tails using either talin-1/2 flox/flox or talin-1/2-deficient cells (talin-1/2 double knockout (DKO); mean ± s.d.; n = 4 independent pull-down experiments; P values were calculated using Student’s t test). NS, not significant. (d) Representative GST pulldown of recombinant GST-KN or GST pre-incubated with recombinant talin-1 or THD from two independent experiments. (e) Representative epifluorescence images of Venus-His-Sumo-tagged talin R4–R8 domain recruited by GST-KN but not GST control to GSH Sepharose beads from two independent experiments. Scale bar, 5 μm. Source data for c can be found in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
in FL-Kank2-expressing cells (Fig. 5h), indicating that FL-Kank2 curbs force transmission across adhesion sites. Importantly, deletion of the liprin-β1-binding coiled-coil domain (Kank2Δcoil) but not the deletion of the Kif2a-binding Ank repeats, reduced the negative correlation with the locally generated tension and failed to diminish force transmission across integrins (Fig. 5d–h).

Depletion of liprin-β1 reduced endogenous and overexpressed Kank2 levels (Supplementary Fig. 5a). Although liprin-β1 depletion slightly affected the negative correlation between FL-Kank2-GFP and tension (Supplementary Fig. 5b), force transmission remained unaffected (Fig. 5i and Supplementary Fig. 5c), indicating that the coiled-coil domain in Kank2 regulates force transmission in a liprin-β1-independent manner.

Finally, we confirmed that FL-Kank2 impairs force transmission to ECM-bound integrins with traction-force microscopy (TFM) of our cell lines on FN-coated micropatterns with a rigidity of 35 kPa (ref. 34). In line with the molecular tension sensor experiments, FL-Kank2- and Kank2(1–670)-expressing cells generated significantly

Figure 7 Kank2 induces talin and integrin activation. (a) Western blot (left) and densitometric analysis (right) of Kank2, talin and kindlin-2 binding to β1 integrin tail in control (scr shRNA) cells or Kank2-depleted (Kank2 shRNA) cells (mean ± s.d.; n = 3 independent pulldown experiments; P values were calculated using Student’s t test). (b) Western blot (left) and densitometric analysis (right) of Kank2, talin, kindlin-2 and Dab-2 binding to β1 integrin tails or scrambled peptide (β1 scr) after addition of recombinant GST-KN or GST (mean ± s.d.; n = 3 independent pulldown experiments; P values calculated using Student’s t test). (c, d) Western blot (left) and densitometric analysis (right) of recombinant talin-1 binding to β1 integrin tail after addition of recombinant GST-KN (e) or FL-Kank2 (d) (mean ± s.d.; n = 3 independent pulldown experiments; P values calculated using Student’s t test). (e) Talin-GFP co-expressed with Cherry-tagged FL-Kank2, Kank2α KN, Kank2-ΔKN in Kank2-depleted cells and stained for kindlin-2. (f) Quantification of talin-1-GFP mean optical intensity (MOI) ratio between kindlin-2-positive adhesion area and kindlin-2-negative cytosolic region in (e) (dot plot, mean ± 95% CI; n = 10 cells per cell line; P values calculated using one-way ANOVA Tukey test). (g) Binding of PAC1 antibody reporting active αIIbβ3 integrins normalized to binding of anti-total αIIbβ3 on CHO cells co-expressing talin-1-GFP with either Kank2-GFP, Kank2ΔKN-GFP or GFP, or expressing THD only (mean ± s.d.; n = 4 independent experiments; P values calculated using one-way ANOVA Tukey test). (h) Kank2-depleted fibroblasts stably transfected with GFP-tagged FL-Kank2, Kank2α KN, Kank2-ΔKN or GFP only (green), seeded on FN and immune-stained with the 9EG7 antibody reporting the exposure of a β1 integrin-specific activation epitope (using orange look-up table (LUT)). (i) Signal intensities of 9EG7 staining quantified from (h) (dot plot, mean ± 95% CI; n = 30 cells per cell line; data aggregated from three independent experiments; P values calculated using Kruskal-Wallis test). Scale bars in e, h, 10 μm. Source data for a–d, g can be found in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
To unravel how Kank2 is recruited to the FA belt and suppresses Kank2 directly binds the talin rod

less traction force than did cells expressing Kank2ΔKN, Kank2Δcoil, the KN polypeptide or the GFP control (Fig. 5j,k).

Kank2 directly binds the talin rod

To unravel how Kank2 is recruited to the FA belt and suppresses integrin-mediated force transmission, we carried out pulldown experiments with different integrin tail peptides, which revealed that the Kank2-binding profile resembled that of talin but differed from that of kindlin-2 (Fig. 6a). Furthermore, talin-binding-deficient β1 tails (β1 Y783A) pulled down neither Kank2 nor talin, but still kindlin-2 (Fig. 6a), suggesting that talin and Kank2 are co-recruited to integrin tails.
To identify Kank2-interacting proteins that mediate its recruitment to β integrin tails, we immunoprecipitated FL-Kank2-GFP in fibroblasts and determined the interacting proteins by mass spectrometry (MS). Talin-1, dynein light chain isofoms (Dynl1 and Dynl2) and liprin-β1 were identified as binding partners of Kank2 (Fig. 6b). To test whether Kank2 is recruited to β1 tails in a talin-dependent manner, we carried out β1 tail peptide pulldown in fibroblasts lacking the Talin-1 and Talin-2 genes and found that the absence of talin-1 and talin-2 expression abolished Kank2 recruitment to β1 integrin tails (Fig. 6c). Since the FA belt recruitment is KN motif dependent, we purified recombinant glutathione S-transferase (GST)-KN, full length talin-1 (FL-talin-1) and the THD (Supplementary Fig. 6a) and carried out GST pulldown experiments, which showed that the GST-KN motif pulled down FL-talin-1 but not the THD (Fig. 6d), indicating that Kank2 binds the talin-1 rod through the KN motif. Expression of different GFP-tagged domains of the talin-1 rod in HEK293 cells followed by co-immunoprecipitation of GST-KN with GFP antibodies revealed that R7R8 domains but not the R8 domain alone associated with the KN polypeptide (Supplementary Fig. 6b). Furthermore, recombinant Venus-tagged talin R4–R8 efficiently bound GST-KN-coupled glutathione beads but not control GST beads (Fig. 6e). These findings indicate that Kank2 directly binds the R7R8 domain of the talin rod through the KN motif.

Kank2 induces talin and β1 integrin activation

The R7R8 domains were shown to bind RIAM, actin and vinculin, all of which can induce activation of talin, which in turn binds and activates integrins. To investigate whether Kank2 or KN binding to talin also promotes talin–integrin interactions, we carried out a series of experiments. First, short hairpin RNA (shRNA)-mediated depletion of Kank2 in fibroblasts decreased binding of endogenous talin to β1 integrin tail peptides by about 30% (Fig. 7a), whereas doxycycline-induced expression of FL-Kank2-GFP but not Kank2ΔKN-GFP increased talin binding to β1 integrin tails in a dose-dependent manner (Supplementary Fig. 7a,b). Second, incubation of normal fibroblast lysates with GST-KN or chemically synthesized KN peptide efficiently reduced endogenous Kank2 binding to β1 integrin tail peptides and increased talin binding in a dose-dependent manner, but left kindlin-2 and Dab-2 binding unchanged (Fig. 7b and Supplementary Fig. 7c–e). Third, β1 tails pulled down about five times more recombinant FL-talin in the presence of equal molar GST-KN or recombinant FL-Kank2 (Supplementary Fig. 7f) when compared with GST only (Fig. 7c,d). Fourth, the KN-peptide-mediated activation of FL-talin depended on the THD in full length talin, as it was efficiently blocked by an excess amount of THD domain (Supplementary Fig. 7g,h). Finally, co-expression of talin-GFP and Cherry-tagged FL-Kank2 in Kank2-depleted fibroblasts increased talin-GFP signals in kindlin-2-positive central adhesions and expression of the KN motif promoted talin-GFP localization to peripheral FAs (Fig. 7e,f), whereas Kank2ΔKN- or Cherry-expressing cells had the majority of talin-1-GFP in the cytosol (Fig. 7e,f). Thus Kank2 induces talin activation and binding to integrin β tails.

Since active, integrin-tail-bound talin induces integrin activation, we next tested whether Kank2 promotes integrin activation. The expression of talin-1 together with FL-Kank2-GFP in αIIbβ3-integrin-expressing CHO cells increased binding of the αIIbβ3 integrin activation epitope-reporting PAC1 antibody when compared with cells expressing talin-1 and GFP (Fig. 7g). Importantly, expression of talin-1 and Kank2ΔKN failed to increase PAC1 binding over the talin-1/GFP control (Fig. 7g). Similarly, immunostaining with 9EG7 antibody of FN-adherent Kank2-depleted fibroblasts expressing GFP-tagged FL-Kank2, Kank2ΔKN, Kank2-KN or GFP alone revealed that FL-Kank2-GFP and Kank2-KN-GFP co-localized with active, 9EG7-positive β1 integrins and increased the number of 9EG7-positive adhesion sites, whereas Kank2ΔKN-GFP or GFP alone had no effect on 9EG7 binding (Fig. 7h,i). Altogether, these findings indicate that Kank2 activates talin and integrins in a KN-motif-dependent manner.

Kank2 interferes with F-actin binding to the ABS2 of talin

How does Kank2 binding to talin decrease force transmission across integrins? Kank2 was shown to regulate RhoA and myosin II activities through an association with RhoGDIfit in kidney podocytes. In contrast to podocytes, however, RhoGDIfit was neither identified as a binding partner of FL-Kank2-GFP in the MS-based Kank2 interactome screen nor co-localized with FL-Kank2 in fibroblasts (Supplementary Fig. 8a). Furthermore, similar GTP-bound RhoA and phospho-Ser 19-myosin light chain (pMLC) levels in cells expressing FL-Kank2, Kank2ΔKN, the KN polypeptide or GFP (Supplementary Fig. 8b,c) excluded a role for Kank2 in regulating myosin II activity.

The destabilization of the Talin–F-actin linkage can also decrease force transmission across integrins. Kank2 binds talin in the vicinity of the F-actin-binding site 2 (ABS2). To test whether Kank2 and actin binding to the talin rod is mutually regulated, we incubated recombinant His-tagged talin R4–R8-coated NiNTA beads with FL-Kank2 and actin under polymerization-permissive conditions and observed a dose-dependent inhibition of Kank2 binding to talin R4–R8 by actin (Fig. 8a,b). Consistently, recombinant FL-Kank2 but not GST-KN significantly decreased actin binding to His-tagged talin R4–R8-coated NiNTA beads (Fig. 8c,d). These results suggest that Kank2 interferes with actin binding to ABS2.

The association of talin with F-actin was shown to immobilize talin in FAs. Fluorescence recovery after photobleaching (FRAP) experiments in Kank2-depleted fibroblasts expressing FL-Kank2 or mutant versions of Kank2 showed that FL-Kank2 increased the mobile fraction of talin-GFP from 63.8 ± 9.0% to 92.4 ± 7.5% in a 5 min time window (Fig. 8e,f and Supplementary Fig. 8d), whereas neither Kank2ΔKN nor Kank2-KN affected the mobile fraction of talin. These findings confirm that Kank2 destabilizes the talin–F-actin linkage.

DISCUSSION

Kank proteins are defined by a unique KN motif. While a recent study reported that Kank proteins in HeLa cells localize to liprin-β1 and ELKS-containing clusters at the plasma membrane that capture MTs in close proximity to, but always outside FAs, we found Kank highly enriched in the β1 integrin tail interactome and the integrin-induced adhesomes, pointing to the possibility that Kank proteins also directly function inside FAs. We tested this hypothesis and found that Kank proteins are indeed present in FAs, where they play a dual role: on the one hand they control integrin activity through activating talin, and on the other hand they reduce the talin linkage with the
actomyosin, which leads to reduced force transmission across FAs, increased adhesion sliding and reduced cell migration speed.

Our analysis shows that Kank recruitment to β integrin tails is indirect and occurs through a direct interaction between the KN motif of Kank and the talin R7 domain. The consequence of the Kank–talin interaction is the activation of talin, the binding of the Kank/talin to β integrin tails, integrin activation and integrin–ligand binding. The activation of talin and integrins by Kank is KN motif dependent. In line with this finding, the KN polypeptide is sufficient to induce talin activation and recruitment to β1 integrin tails in vitro and to FAs ex vivo, and to activate εIIβ3 and β1 integrins in cells.

Our findings also demonstrate that Kank reduces the cell migration speed by converting FAs at the lamella border into thin and elongated sliding adhesions that glide into the cell centre (Fig. 7). Kank is absent from NAs and becomes recruited to the proximal tip of FAs at the lamella border either by binding to tensed, integrin-bound talin or as a de novo assembled Kank2–talin complex. From the proximal FA tips Kank gradually expands around the entire outer FA border, which is followed by the disassembly of the FA core and the gliding of the thin Kank2-positive FA border into the cell centre. We term the outer FA border, to which Kank concentrates during FA conversion, the FA belt. The exclusive localization of the KN-motif-deficient Kank2ΔKN to the previously described liprin-β1- and ELKS-containing clusters and the inability of Kank2ΔKN to induce FA sliding indicate that both FA belt recruitment and FA sliding depend on talin binding. Furthermore, the KN polypeptide also penetrates into the FA centre but fails to induce FA sliding, pointing to the functional importance of additional region(s) in Kank such as the coiled-coil domain.

We also found that Kank binding to talin destabilizes the integrin–actomyosin linkage, leading to decreased force transmission across adhesion sites, which diminishes adhesion strength and traction force, causing integrin–ligand bond slippage. FA sliding and a reduction in migration speed. The destabilization of the integrin–actomyosin linkage occurs with Kank2 binding to the talin rod. Since the KN motif is not sufficient to compete with actin for talin rod binding and for curbing force transmission across integrins, additional region(s) in Kank2 are required to execute these tasks. Such a region could be the coiled-coil domain, whose absence enabled a partial intrusion of the mutant Kank2Δcoil protein into the FA core, impaired the negative correlation with locally produced tension and failed to reduce force transmission across integrins. Mechanistically, the coiled-coil domain could hinder F-actin binding to talin-ABS2 either by oligomerizing Kank2 or by recruiting proteins such as liprin-β1. However, the localization of Kank2 to the FA belt and the normal force transduction after liprin-β1 depletion exclude liprin-β1 as potential binding partner of the coiled-coil domain for this function. Alternatively, Kank may bind as-yet-unidentified protein(s) that abrogate(s) talin–integrin tail binding on the FA belt. Such talin binding partners, however, would probably disassemble rather than convert the FA belt into a sliding central adhesion.

The reduced F-actin binding to talin by Kank2 provides a reasonable explanation for the reduced force transmitted across talin–Kank2 complexes to ligand-bound integrins, increased integrin turnover and slippage of integrin ligand bonds, which altogether leads to adhesion sliding and reduced migration speed (Fig. 8g). Consistent with our findings, cells carrying mutations in the ABS2 of talin also show reduced actomyosin coupling to integrin adhesion sites, an increase of the mobile fraction of talin and a decrease of the traction force without affecting cell adhesion or spreading.11 Kank2 was also shown to bind RhoGDIntx, which can potentially inhibit Rhoa activity at FA belts and suppress formin-dependent F-actin filament assembly and Rock-mediated myosin II activities.8,10,35,36. However, we did not detect RhoGDIntx in our MS-based Kank2 interactome or co-localized with Kank2 at FA belts. Furthermore, the normal levels of active Rhoa and pMLC in cells expressing FL-Kank2 suggest that Rhoa-dependent myosin II activity is not grossly altered, although subtle changes cannot be excluded with our assays.

Kank proteins are also found in liprin-β1- and ELKS-enriched clusters at the plasma membrane where MT plus ends are captured to allow focal exocytosis of MT1-MMP and other cargo.11,16,48 which could also contribute to adhesion sliding and cell migration velocity control. However, Kank2-induced adhesion sliding was not altered when the activities of MMPs were inhibited or MT targeting to FAs was compromised by depleting CLASP, indicating that talin–Kank2 complex on FA belts and the ELKS–liprin-β1–Kank2 complex function separately. It is conceivable, however, that Kank proteins coordinate the properties of both compartments and thereby adjust MT dynamics and ECM remodelling with adhesion strengthening.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
Z.S. and R.F. initiated the project, designed the experiments and wrote the paper; Z.S. performed the vast majority of the experiments; H.-Y.T., S.T., F.S., L.K., D.D. and A.A.W. performed experiments; Z.S., S.T., N.M., M.T., A.R.D. and R.F. analysed data; all authors read and approved the manuscript.

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36. Collectively, these findings suggest that KANK deficiency leads to podocyte dysfunction and nephrotic syndrome. J. Am. Soc. Nephrol. 25, 545–559 (2013).
METHODS

Mass spectrometry. To measure Kank1–4 levels in FA-enriched fractions and whole cell lysates, cells were serum-starved for 4 h, plated for 90 min in serum-free medium on FN-coated, BSA-blocked culture dishes, and FAs were isolated for quantitative MS analysis on an LTQ Orbitrap analyser (Thermo Electron). Data were processed using the label-free quantification (LFQ) algorithm embedded in the MaxQuant software.

For the MS analysis of anti-GFP pulldowns, the samples were run 1 cm into a 12% SDS–PAGE. The entire lane up to the front was cut into 1 mm slices and subjected to standard in gel digestions as previously described. Briefly, the gel slices were destained in ethanol followed by sequential reduction and alkylation with 10 mM dithiothreitol (DTT) and 40 mM chloroacetamide (CAA), then dried and incubated with digestion buffer containing 12.5 ng µl⁻¹ trypsin in 25 mM Tris buffer (pH 8.5) overnight. Peptides were extracted, purified in StageTips, analysed in an LTQ Orbitrap XL analyser and processed as described above. MS data were further analysed in Perseus and manually annotated for data illustration.

Antibodies. See Supplementary Table 1.

Plasmids, constructs and expression and purification of recombinant proteins. Mouse Kank1 and Kank2 complementary DNAs were cloned into pEGFP-N1 vector (Clontech) using XhoI and EcoRI sites, mouse Kank using BglII and EcoRI sites and mouse Kank4 using XhoI and AgeI sites. For retrovirus-mediated overexpression, cDNAs of FL-Kank2, Kank2ΔKN lacking amino acids 31–56, Kank2Δxolc lacking amino acids 181–240, and Kank2ΔKN lacking amino acids 31–56 and 181–240, Kank2(1–670) encoded by amino acids 1–670 and Kank2ΔKN encoded by amino acids 29–72 were inserted between XhoI and EcoRI sites of the pRetroQ-AcGFP-N1 (Clontech) vector. Expression plasmids for talin-AcGFP and talin-TAGFP were generated from pLPXcanmod-Talin1-Iperef (gift from C. Grashoff, Max Planck Institute for Biochemistry) by replacing Ypet with AcGFP or TagRFP-T. The talin rod truncations were inserted between XhoI and EcoRI of the pRetroQ-N1 vector. The expression plasmid of pLPXcanmod-Talin1-Iperef (gift from C. Grashoff, Max Planck Institute for Biochemistry) was used as previously described and to produce FL-talin-1 the pET101-talin-FL construct was used. For expression of FL-Kank2, mouse Kank2 was expressed with a cleavable N-terminal 3C protease (no 8162-1, HYPERMOL) were polymerized in PolyMix buffer (pH 8.5) overnight. Peptides were extracted, purified in StageTips, analysed in an LTQ Orbitrap XL analyser and processed as described above. MS data were further analysed in Perseus and manually annotated for data illustration.

Viral transduction and transient transfection of cell lines. SV40 large T-immortalized mouse kidney-derived fibroblasts have been previously described and used to analyse the Kank proteins. For the analysis of integrin α5-γ2 tumour cell lines, mouse kidney fibroblasts were transduced with lentivirus expressing human integrin α5-γ2, and with cells low expression were FACS-sorted. HEK293 cell lines were used for GFP pulldown of different talin rod domains. Transient transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfection of FVS-C pseudotyped retroviral vectors produced by transient transfection of HEK293T were used to generate stable cell lines as previously described. The cell lines used here were not found in the database of commonly misidentified cell lines maintained by ICLAC and NCBI BioSample. The cell lines were tested for mycoplasma contamination and were found to be negative.

Immunoprecipitation and GST pulldown. For immunoprecipitation of GFP-tagged proteins, cells were lysed in M-PER (Thermo Scientific, no. 78501) buffer and immunoprecipitated using the pMACS GFP Isolation Kit (Miltenyi Biotec) following the manufacturer’s protocols. Eluates were separated in SDS–PAGE for Western blotting or for in gel digestion and mass spectrometry analysis. For GST pulldown experiments, recombinant talin-1 and THD, respectively, were re-buffered in GST binding buffer (50 mM Tris-HCl at pH 7.5, 137 mM NaCl, 0.5% Tween-20) according to the manufacturer’s instructions and stored on ice. The percentage of polymerized integrin was estimated to be about 95% by F-Acin sedimentation assay at 100,000g for 1 h at 25°C.

In vitro F-actin competition assay. 24 µM purified alpha skeletal muscle actin (no 8162-1, HYPERMOL) and Atto665-labelled alpha skeletal muscle actin (no 8162-1, HYPERMOL) were polymerized in PolyMix buffer (100 mM KCl, 10 mM imidazole at pH 7.4, 1 mM ATP and 2 mM MgCl2) according to the manufacturer’s instructions and stored on ice. The percentage of polymerized actin was estimated to be 95% by F-Acin sedimentation assay at 100,000g for 1 h at 25°C.

To test the effects of F-actin on the interaction between the talin R4–R8 domain and FL-Kank2, 0.4 µM Venus-His-Sumago-tagged R4–R8 domain was incubated with 0.2 µM FL-Kank2 in PolyMix buffer for 1 h on ice before supplementing with 1 µM, 2 µM and 5 µM polymerized actin or empty buffer, and then incubated with Ni⁺⁺-NTA beads at 4°C for 30 min. Beads were washed with PolyMix buffer at 4°C 3 times, and bound proteins were analysed by Western blotting.

To visualize activation restores the talin R4–R8 domain, 5% BSA or 2 µM Venus-His-Sumago-tagged R4–R8 domain was incubated with 50 mM KCl, 10 mM imidazole at pH 7.1, 1 mM EDTA, 2 mM MgCl2 and incubated with Ni⁺⁺-NTA beads for 30 min. Then 10 µl buffer (20 mM HEPES at pH 7.5, 150 mM KCl, 3 mM DTT, 5% glycerol) containing 5 mM FL-Kank2, re-buffered 5 µM GST-KN motif or control buffer was added and incubated for 30 min at 4°C. 10 µl

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polymerized Atto565-labeled actin was then added and incubated for 30 min at 4 °C. Before visualization under a confocal microscope, beads were briefly washed twice with ice-cold KMEI buffer and a 20 µl mixture was then injected into a flow chamber made with glass slides and cover slips spaced by one thin layer of Parafilm.

Integrin tail peptide pull-downs. Peptide pull-downs were carried out as previously described\(^3\) with the \(\beta 1\) wild-type cytoplasmic tail peptide (HDREF\(\alpha\)KEKFKMEKN\(\alpha\)AKWD\(\alpha\)GTP\(\alpha\)K\(\alpha\)SYK\(\alpha\)S\(\alpha\)TVTP\(\alpha\)SVNP\(\alpha\)K\(\alpha\)EGK\(\alpha\))\(,\) \(\beta 1\) Y795a tail peptide (HDREF\(\alpha\)K\(\alpha\)KEKFKMEKN\(\alpha\)AKWD\(\alpha\)GTP\(\alpha\)K\(\alpha\)SYK\(\alpha\)S\(\alpha\)TVTP\(\alpha\)SVNP\(\alpha\)K\(\alpha\)EGK\(\alpha\))\(,\) \(\beta 5\) wild-type tail peptide (HDREF\(\alpha\)KEKFKMEKN\(\alpha\)AKWD\(\alpha\)GTP\(\alpha\)K\(\alpha\)SYK\(\alpha\)S\(\alpha\)TVTP\(\alpha\)SVNP\(\alpha\)K\(\alpha\)EGK\(\alpha\))\(,\) \(\beta 5\) wild-type tail peptide (HDREF\(\alpha\)KEKFKMEKN\(\alpha\)AKWD\(\alpha\)GTP\(\alpha\)K\(\alpha\)SYK\(\alpha\)S\(\alpha\)TVTP\(\alpha\)SVNP\(\alpha\)K\(\alpha\)EGK\(\alpha\)) and then incubated in a magnetic chamber (Chamlide, LCI) for TFM processing.

For FN (FF1141, Sigma) and 20 µg ml\(^{-1}\) poly-lysine (PLL)–PEG (polyethylene glycol) was added to the culture medium. 25 µM F\(8810\), Thermo Fisher) by sonication for 30 min at 4°C.

Peptide pulldowns were carried out as previously described\(^4\). The \(\alpha\)IIb\(\beta\)3 integrin activation assay. The \(\alpha\)IIb\(\beta\)3 integrin activation was assessed in CHO cells stably expressing human integrin (A5 cells) by flow cytometry using the ligand-mimetic antibody PAC1 (BD)\(^5\). CHO cells were lipofected with 1 µg of plasmids expressing eGFP-tagged Kank2 constructs and/or 3 µg TagRFP-tagged talin-1 or eGFP-tagged THD. Cells were trypsinized 24 h after transfection and incubated with the PAC1 antibody (BD) in Tyrode's buffer (pH 7.35) for 30 min on ice, washed and stained with a secondary IgM for 30 min on ice. Cells were fixed with 2% PFA–PBS for 15 min at room temperature. Image acquisition was carried out with a Zeiss LSM780 confocal laser scanning microscope equipped with a Zeiss Plan-APO ×63–numerical aperture 1.46 oil immersion objective.

Fluorescence recovery after photobleaching. FRAP and fluorescence live cell imaging were carried out on a Zeiss (Jena) LSM780 confocal laser scanning microscope equipped with a Zeiss Plan-APO ×63–numerical aperture 1.46 oil immersion objective with environmental control (5% CO\(_2\) and humidification). Cells were cultured on 10 µg ml\(^{-1}\) FN-coated glass-bottom live cell imaging chambers (bialu) for 5 h. For FRAP experiments, the ROI was bleached with full laser power at 488 nm for 30 iterations and fluorescence recovery was monitored for 5 min with 20 s intervals and 1% laser power for talin-GFP turnover, or was monitored for 5 min with 10 s intervals and 1% laser power for integrin \(\alpha 5\)-GFP turnover. No significant photobleaching was observed during the post-bleaching phase. FRAP data were recorded with ImageJ. Due to the rapid diffusion of cytosolic talin into the bleached area immediately after bleaching, the mean optical intensity outside adhesion was subtracted from the mean optical intensity in FAs to correct the background. FRAP curves were fitted to a one-phase association model to calculate the mobile fraction.

Conflonal microscopy live cell imaging. For conflonal microscopy live cell imaging, we used the same microscopic setting as for FRAP. Cells were imaged for the indicated time with 20 s or 30 s intervals with 1–3% laser power depending on the fluorophores. To block MMP activities, cells were plated for 5 h with or without 50 µM GM6001 (no sc-203979, Santa Cruz) before live cell imaging. For data illustration, fluorescent images were background-subtracted and passed through low-pass filters followed by Unsharp Mask in ImageJ.

For the tracking of sliding adhesions, a time lapse of 20 min with 2 min intervals was extracted from the full Supplementary Videos. Raw images were background-subtracted. Single adhesions were identified by thresholding and tracked with the TrackMate plugin in ImageJ. Structures with sizes below 3 pixels (0.04 µm per pixel) were excluded. Adhesion sites with lifetimes above 6 min were tracked to measure their sliding velocities. Average adhesion sliding velocities were calculated on the basis of all analysed adhesions for each cell. For the determination of sliding velocities of central adhesions, vinculin-mCherry-positive adhesions were analysed within 20 µm distance from nuclei in the first frame. For the FAs in the cell protrusions, vinculin-mCherry-positive adhesions were analysed within 10 µm behind the protruding leading edge in the first frame.

Intensity-based immunofluorescence image analysis. For line profile analysis, the fluorescence intensity along a transverse line across elongated FAs was analysed with ImageJ. The pixel position of the intensity peak for the Kank2 puncta was defined as the position of Kank2. Pixel positions corresponding to 50% of the average plateau intensity for conventional adhesion markers were manually marked on a time-lapse video.

Kindlin-2 was used to analyse the localization of talin-1-GFP at adhesion sites. GFP intensities outside kindlin-2-positive areas were considered cytosolic. Mean optical intensities of GFP within and outside kindlin-2-positive areas were manually annotated. Total kindlin-2 staining) followed by 15 min rehydration in PBS at room temperature, or with 2% PFA–PBS for 15 min at room temperature (for other immunostaining). PFA-fixed cells were permeabilized when indicated with 0.1% Triton X–100–PBS for 30 min at room temperature. Fixed cells were blocked with 5% BSA in PBS for 1 h at room temperature followed by incubation with the primary antibodies in 5% BSA in PBS overnight at 4 °C, and then with secondary antibodies for 1 h at 4 °C at room temperature. Images were collected at room temperature on a Zeiss (Jena) LSM780 confocal laser scanning microscope equipped with a Zeiss Plan-APO ×63–numerical aperture 1.46 oil immersion objective.
defined. Total intensities corresponding to 9EG7 signals and total cell areas were then measured in ImageJ.

To calculate FA disassembly rates, paxillin-TagRFP intensities during the FA disassembly phase were measured within the ROI with a fixed position that covers the initial FA area. Data were fitted into a one-phase exponential decay function and the decay rate constants were used as FA disassembly rates.

Time-lapse phase contrast video microscopy of 2D random cell migration. Cells were sparsely seeded on a six-well plate coated with 5 µg ml−1 FN in the absence of serum for 2 h. Subsequently, cell migration was recorded with a Zeiss Axiovert 200 M equipped with a ×10–numerical aperture 0.3 objective, a motorized stage (Märzhäuser), an environment chamber (EMBL Precision Engineering) and a cooled CCD (charge-coupled device) camera (Roper Scientific) at 37 °C and 5% CO₂ for 6 h with 5 min time intervals. Image acquisition and microscope control were carried out with MetaMorph software (Molecular Devices). The acquired images were analysed using the manual tracking plugin of ImageJ and the Chemotaxis and Migration Tool (ibidi).

RGD tension sensor measurements. The molecular tension sensor was adapted from the sensor previously described10, with slight modifications to the labelling chemistry. Briefly, the unnatural amino acid used to attach an ATTO647 dye was replaced with a second cysteine to attach an Alexa 647 dye for a higher labelling efficiency. This modified molecular tension sensor still presents the RGD ligand derived from FN and does not change the interactions with the cell. Perfusion chambers were attached to PEGylated coverslips as previously described40. Briefly, labelled molecular tension sensor (~100 nM) was then added to the flow cell and incubated for 30 min, followed by Pluronic F-127 (0.2% w/v) for ~1 min to prevent nonspecific cell attachment. All the above steps were preceded by a PBS wash to remove excess reagent from the previous step. The cells were then added and incubated for at least 5 h at 37 °C in DMEM high-glucose medium. Images were taken using TIRF microscopy on an inverted microscope (Nikon Ti-E) equipped with an Apo TIRF ×100–numerical aperture 1.49 oil immersion objective (Nikon).

For 2D correlation analyses, FL-Kank2-GFP and FRET images were normalized and median filtered to reduce background noise18. The channels were then manually thresholded to separate adhesions from the background signal and to create masks for each channel. The pixels from both masks were combined into a single mask, which was applied to the GFP and FRET images. We then used the resulting masked images to calculate the 2D correlation coefficient. Significance was calculated using the Wilcoxon rank sum test.

For comparison of average stress within adhesions between cell lines, the force per pixel was calculated using images of the entire cell taken on the Hamamatsu sCMOS camera. The GFP channel was normalized and processed using a moving boxcar average and thresholding. The adhesions were segmented using a watershed algorithm with a minimum adhesion size of 0.5 µm². The average force per µm² for adhesions was then calculated with an average sensor density on the basis of previous calibrations6. For the adhesion area ratio, each of the pixels from the segmented adhesions was converted to force and the ratio of the number of pixels above 1.25 pN versus below 1.25 pN was calculated. Significance was calculated using the Wilcoxon rank sum test.

RhoA GTPase activity. Indicated cells were serum-starved for 3 h and plated on FN for 1 h in the absence of serum. Active GTP-bound RhoA was affinity purified and detected with the Active Rho Pull-Down and Detection Kit (no 16116, Thermo Scientific) following manufacturer’s instructions.

Statistics and reproducibility. Statistical analysis was carried out in GraphPad Prism software (version 5.0). Statistical calculations to predetermine required sample size were not carried out. The data sets with data points above 10 were analysed with the D’Agostino and Pearson omnibus normality test. Data sets with normal distributions were analysed with either Student’s t test to compare two conditions, or with one-way ANOVA Tukey tests to compare multiple conditions. Data sets that did not follow normal distribution in the normality test or those that should not be considered as normal distribution (for example, TFM measurements) were analysed with a Kruskal–Wallis test (multiple comparison) or Wilcoxon rank sum test (two-sample comparison). In the case of TFM measurements, we carried out outlier identification and data cleaning due to apparent defects in some individual measurements. For data with replicates below 10, we assumed normal distribution on the basis of the appearance of the data and analysed with Student’s two-tailed t test. Results are depicted as mean ± s.d., mean ± s.e.m. or mean ± 95% CI as indicated in figure legends. All experiments for quantitative analysis were reproduced at least three times.

Data availability. Source data for Figs 6c, 7a–d and Supplementary Figs 7b and 8hc have been provided as Supplementary Table 2. All other data that support the conclusions are available from the authors on request.


**Supplementary Figure 1** (a) SILAC ratio plot showing specific p1wt tail interactors with high p1 wt / p1 scr and low p1 scr / p1 wt SILAC ratios in reverse SILAC labeling experiments and with high MS intensities in the FA-enriched proteome of FN-seeded fibroblasts in red. Dab-2 (green) and SNX17 (grey) show a high p1 wt / p1 scr SILAC ration but were absent from the FA-enriched proteome. (b) Split channels of Figure 1b. Mouse fibroblasts seeded on FN for 3h and immunostained for Kank2 (green), Kindlin-2 (red) and DAPI (blue). (c) Mouse fibroblasts seeded on FN for 3h and immunostained for Kank2 (green), Talin (red) and DAPI (blue). (d) Confocal image of immunostained (Kank2, green; Kindlin-2, red; DAPI, blue) wild-type fibroblasts seeded for 3h on a FN-coated micropattern. The boxed areas show Kank2 puncta around paxillin-positive FAs. (e) Mouse fibroblasts seeded on FN for 5h and immunostained for p1 integrin (Itgb1, green), p3 integrin (Itgb3, red), Paxillin (Pxn, blue) and DAPI (grey). (f,g) Mouse fibroblasts expressing Kank2-GFP seeded for 5h on FN and immunostained for p1 integrin (Itgb1, red) or active p1 integrin (9EG7, red), Paxillin (Pxn, blue) and DAPI (grey). Scale bar in b-g, 10μm.
**Supplementary Figure 2** (a) MS intensities of Kank1-4 in the FA-enriched sub-proteome and total proteome of wild-type mouse kidney fibroblasts (mean ± sd; n = 3 independent MS measurements). (b) Western blot analysis of Kank2 and β1 integrin (Itgb1) in cells expressing scrambled (shScr) and Kank2-specific shRNAs. Actin served to control protein loading. (c) Western blot analysis of Kank2 before and after shRNA-mediated knockdown using polyclonal anti-Kank2, and of indicated GFP-tagged Kank2 constructs using anti-GFP antibodies, respectively. Vinculin is used as loading control.
**SUPPLEMENTARY INFORMATION**

Supplementary Figure 3 (a,b) Immunofluorescence of GFP-tagged Kank2 constructs (green) co-stained for Liprin-β1 (a, red) or ELKS (b, red), Paxillin (Pxn; blue) and DAPI (grey). (c) Line profile analysis of GFP-tagged FL-Kank2 co-stained with Liprin-β1 and ELKS. Full arrowheads indicate the position of FA belt and open arrowheads indicate Liprin-β1- and ELKS-enriched regions outside the FA belt. Scale bar, 2 μm. (d,e) Western blot testing GFP-tagged Kank-KN (d), FL-Kank2 and Kank2ΔKN (e) binding to biotinylated β1 integrin tails. FL and mutant Kank2 proteins were detected with the anti-GFP antibody, and Kindlin-2 was used to control the β1 tail pull-down assay. (f) GFP-tagged Kank1, Kank3 and Kank4 expressed in Kank2-depleted fibroblasts and co-stained for Paxillin (Pxn; red), F-actin (blue) and DAPI (grey). Scale bars in a,b,f, 10 μm.
Supplementary Figure 4 (a) Time lapse images of cells stably expressing GFP-tagged FL-Kank2 (green) and Paxillin-TagRFP (Pm-TagRFP; red) during isotropic spreading on FN. Arrowheads indicate Kank2 recruitment to the proximal FA border. (b) Normalized Pan-TagRFP intensities during FA disassembly behind lamella in cells expressing FL-Kank2 (red) or Kank2ΔKN (blue) (mean ± sd; n = 8 cells for FL-Kank2, and n = 10 cells for Kank2ΔKN pooled from 4 independent experiments). (c) FA disassembly rates calculated from (b) as rate constants in a one-phase exponential decay (mean ± sd). (d) Ten overlaid, sequential time lapse images of Vinculin-mCherry in the cell centre temporally color-coded using spectrum look up tables (LUT). Corresponding time lapse images of GFP are shown as projection of average intensity. Scale bar, 5 μm. (e) Percentage of adhesion sites with sliding velocity above 0.3 μm/min in indicated cell lines (mean ± sd, n = 5 cells from 3 independent experiments; P values were calculated using one-way ANOVA Tukey test). (f) 2-D random migration of indicated cells on FN (box plot with median, 1st-3rd quantile box, minimal to maximal whiskers; shScr, n = 52 cells; shKank2#1, n = 58 cells; shKank2#2, n = 60 cells; data aggregated from 4 independent experiments; P values were calculated using Student t-test). (g) Cell spreading area of indicated cells at different time points (n ≥ 50 cells for each condition). (h) Percentage of adhesion sites with sliding velocity above 0.3 μm/min in Kank2-depleted fibroblasts stably expressing Paxillin-TagRFP and Kank2-GFP on FN treated with or without 50μM broad spectrum MMP inhibitor Gm6001 for 5h (mean ± sd, n = 5 cells from 3 independent experiments).
Supplementary Figure 5 (a) Western blot analysis of Kank2 and Liprin-β1 in wild-type cells expressing FL-Kank2-GFP together with scrambled (shScr) and two Liprin-β1-specific shRNAs. Vinculin served to control protein loading. (b) 2D correlation coefficient between GFP and inverted FRET signals in FL-Kank2-GFP expressing cells before and after Liprin-β1 depletion (dot plot and box plot; FL-Kank2 shScr, n = 30 cells; FL-Kank2 shLiprin-β1#1, n = 31 cells; FL-Kank2 shLiprin-β1#2, n = 33 cells; data aggregated from 3 independent experiments for each condition; P values were calculated using the Wilcoxon Rank Sum test). (c) Ratio between adhesion area with high tension (>250pN/μm²) and adhesion area with low tension (<250pN/μm²) were calculated in cells expressing indicated constructs (dot plot and box plot; FL-Kank2, n = 24 cells; Kank2ΔCol, n = 45 cells; FL-Kank2 shScr, n = 30 cells; FL-Kank2 shLiprin-β1#1, n = 31 cells; FL-Kank2 shLiprin-β1#2, n = 33 cells; data aggregated from 3 independent experiments for each condition; P values were calculated using the Wilcoxon Rank Sum test).
Supplementary Figure 6 (a) SDS-PAGE analysis of bacterially expressed and purified FL-Talin-1, Talin head domain (THD), Venus-His-Sumo-tagged Talin R4-R8 domain, GST-KN fusion protein and GST. (b) HEK293 cells expressing GFP-tagged Talin rod truncations were lysed, incubated with recombinant GST-KN, immunoprecipitated with anti-GFP antibody and analyzed with Western blot. Red asterisk marks the recombinant GST-KN protein.
Supplementary Figure 7 (a) Western blot and (b) densitometry of Talin binding to β1 integrin tails in cells treated with increasing doses of dexamethasone (Dox) to induce expression of either FL-Kank2 or Kank2ΔKN (mean ± sd; n = 3 independent experiments; P values were calculated using Student t-test). Asterisk marks additional Kank2 bands after transient induction of protein expression, which is likely due to post-translational modification. (c,d) Representative Western blot (c) and densitometry (d) of endogenous Kank2, Talin and Kindlin-2 binding to β1 integrin tails in the presence of increasing concentrations of recombinant KN-GST. (e) Western blot of endogenous Kank2, Talin and Kindlin-2 binding to β1 integrin tails in the presence of increasing concentrations of chemically synthesized KN peptide. (f) SDS-PAGE analysis of FL-Kank2 expressed and purified from HEK293 cells. (g) Western blot analysis of recombinant Talin-1 binding to β1 integrin tail in the presence of the KN peptide and increasing concentration of recombinant THD. (h) Western blot analysis of β1 integrin tail pull downs of endogenous Talin detected with an antibody recognizing THD in cells overexpressing the GFP-tagged Talin rod domain (Talin-Rod-GFP) in the presence or absence of KN peptide. Source data for panels (b) can be found in Supplementary Table 2.
Supplementary Figure 8 (a) Immunofluorescence of FL-Kank2-GFP (green) co-stained with endogenous RhoGDIα (red) and DAPI (blue). Scale bar, 10μm. (b) Western blot (left) and densitometric (right) analysis of RhoA activation in Kank2-depleted cells expressing indicated Kank2 constructs seeded on FN for 1h (mean ± sd; n = 3 independent experiments; P values were calculated using one-way ANOVA Tukey test; N.S., no significance). (c) Western blot (left) and densitometric (right) analysis of myosin light chain (MLC) phosphorylation at serine-19 (pMLC-Ser19) in Kank2-depleted cells expressing indicated Kank2 constructs seeded on FN for 1h. Actin was used to control protein loading (mean ± sd; n = 4 independent experiments; P values were calculated using one-way ANOVA Tukey test; N.S., no significance). (d) Still images from a representative time lapse FRAP experiment of Talin-1-GFP shown in rainbow LUT. Talin-1-GFP was co-expressed with Cherry-tagged FL-Kank2 or Kank2ΔKN, respectively, in Kank2-depleted fibroblasts. Co-localization of Kank2-mCherry and Talin-1-GFP in the region of interest (ROI, red circle) was confirmed in pre-bleached images. Scale bar, 2μm. Source data for panels b, c can be found in Supplementary Table 2.
Supplementary Figure 9  Original uncropped Western blots.
Supplementary Figure 9 continued
Supplementary Figure 9 continued
Supplementary Figure 9 continued
6 Outlook

Even though a structure of the autoinhibited talin has been solved in this study, a lot of open questions remain. In this regard, the two elements that could not be placed in the structure, F0/F1 and the dimerization domain, and their role in talin regulation are of high interest.

Positioning the F0/F1 domain of talin’s head into the density map resulting from our cryo-EM study was not possible. This is likely due to the unstructured linker between the F0/F1 and F2 domain. It will be interesting to figure out the role in recruitment and activation of these two domains in context with the inhibited structure. An upcoming study shows that the talin head can adopt two conformations through movement of F0/F1, one preventing membrane binding (Goul, unpublished). The mechanism of how talin is recruited to the membrane and how it is activated is not fully understood yet and future studies could help to clarify these events.

Another element that could not be clearly observed within the structure is the dimerization domain of talin. The questions, whether talin forms an inhibited dimer in the cytoplasm and how dimerization influences its inhibition are still open. Future studies of a dimerized inhibited talin might solve these issues.

This study provided evidence that the inhibition of talin is driven by two binding sites: Interactions of F3 with R9 and F2 with R12. The binding interface between F3 and R9 has been studied in vivo, by cell and mouse experiments. One study showed that mutation of E1770 to alanine leads to wound healing deficiency (Haage et al., 2018). It is intriguing to perform similar mutation studies in the R12 binding interface in vivo to fully understand its implications for talin activation.

Another interesting point to study are talin’s many binding partners, such as vinculin, actin, Kank or Rap1. This study showed that some of their interfaces are not accessible in the inhibited state of the talin molecule. To understand the function of talin as mechanosensitive scaffold in adhesions it is vital to uncover the hierarchy of binding events with its partners. It would be interesting to reconstitute a system with talin and different adhesion components in vitro to stepwise uncover the events in early adhesion formation. This system would need to include the actin cytoskeleton as well as a membrane with integrin binding sites.

Ultimately, it would be of great interest to structurally characterize an activated/engaged talin in such a reconstituted model system to understand the effects of mechanical tension in focal adhesions.
A lot of studies have been performed to understand talins activation, recruitment and function. Together with this study, it gives us some insight how talin performs its different tasks in the cell. However, future in vitro and in vivo experiments are necessary to fully uncover talin’s activation mechanism and the implications it has on cellular processes.
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