This is the accepted manuscript of the article, which has been published in Physiological reviews. 2019, 99(4), 1655-1699
https://doi.org/10.1152/physrev.00036.2018

Cell adhesion by integrins

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Abstract

Integrins are heterodimeric cell surface receptors ensuring the mechanical connection between cells and the extracellular matrix. In addition to the anchorage of cells to the extracellular matrix, these receptors have critical functions in intracellular signaling, but are also taking center stage in many physiological and pathological conditions. In this review we provide some historical, structural and physiological notes, so that the diverse functions of these receptors can be appreciated and put into the context of the emerging field of mechanobiology. We propose that the exciting journey of the exploration of these receptors will continue for at least another new generation of researchers.

Chapter 1: Introduction and some historical notes

It is always difficult to trace back the origin of an idea, a particular historic event or the role of its founders, that initiated a new way of thinking in a particular field of science. In the case of the cell-matrix adhesion receptors of the integrin family, we could highlight the work of Abercrombie and co-workers as well as Curtis, who explored the mechanisms allowing cells to adhere to and crawl on petri dishes, recognizing the cytoskeleton and substrate anchoring adhesion sites visible in the electron microscope or by interference reflection contrast (1, 2, 96). Cell adhesion was also a subject interesting researchers in the field of tumor biology, as a central feature of cancer cells is their ability to grow on soft agar, indicating that these cells no longer require adhesion to their tissue environment and have lost the regulatory influence of the healthy microenvironment of the tissue (273). At about that time, Richard Hynes incubated normal adhering hamster fibroblasts or their hamster sarcoma virus-transformed derivatives with an extracellular iodination solution. When analyzing the iodinated proteins by SDS-PAGE, he identified an abundant 250 kDa protein present on normal, but not on transformed cells (202). This large, external, and transformation-sensitive (LETS) glycoprotein was simultaneously found and characterized in many different laboratories and given names such as cold-insoluble globulin, cell surface protein, fibroblast surface antigen and eventually named fibronectin (296, 375, 474). Since fibronectin showed an intriguing overlap with intracellular stress fibers (204), the existence of a transmembrane link was postulated. Only a few years later it became clear that fibronectin was a major extracellular binding partner for fibroblasts and that the critical binding element in fibronectin was a short peptide Arg-Gly-Asp (RGD) (7, 334). The respective surface receptors recognizing this motif in fibronectin as well as in vitronectin were identified by Pytela and Ruoslahti (349, 350). In an alternative approach, the same fibronectin-binding surface receptors were also identified based on monoclonal antibodies that prevented cell binding to fibronectin, such as JG22, CSAT and GP135 (8, 97, 158). Shortly afterwards the integrin field enjoyed its first expansion phase, where all the different integrin receptors and the majority of their ligands were described and named, either according to biochemical or ligand-affinity data as in the case of fibronectin (α5β1) (349) and vitronectin (αvβ3) receptors (350), or by researchers working in the field of immunology according to antibody reactivity as for VLA1 to 6. Especially the latter field helped to develop the concepts of integrin-dependent adhesion during platelet activation or cytokine-mediated adhesion of leukocytes to the endothelium *via* (α4β1/VCAM-1) (110, 426) and LFA1 (αLβ2)/ICAM-1 binding (117, 383, 406). Importantly, these integrin-dependent adhesion processes were not constitutive, but could be triggered by cytokine stimulation and even β1-integrin-directed

adhesion-stimulating antibodies, proposing that the affinity of these cell surface receptors was specifically regulated (22). The analysis of integrin receptors and their ligand specificity on the vascular endothelium (85) eventually led to the idea, that the inhibition of integrin-dependent adhesions in sprouting endothelial cells could inhibit the angiogenic switch and prevent tumors from growing in the tissue (59, 138, 209), taking the research on integrin receptors to almost all domains of biomedical research.

One of us was actually in the lucky position to assist this process, as his colleagues were actively identifying, purifying and characterizing different members of the integrin family in the labs of Jürgen Engel, Mats Paulsson and Ruth and Matthias Chiquet (193, 298, 423). It was clearly the golden age, or alternative the "Sturm und Drang" period, of the integrin and extracellular matrix research, in which most of the integrin-receptor concepts were created. In this phase also the majority of the integrin knockout models were established in the labs of Richard Hynes, Reinhard Fässler, Dean Sheppard and many others (111, 132, 198, 396), leading to the quintessential integrin review published in 2002 by Richard Hynes (203).

About this time, first attempts were made to understand the structure-function relationship of integrin receptors. First, the I-domain insert of the α-subdomain of the lymphocyte integrins (αM) was crystallized in two different conformations, providing a strong argument for the association of integrin ligand binding with conformational changes in the receptor (245). While the I-domain of the α-subunit exhibited a single metal-ion-dependent ligand binding site, the revelation of the structure of the entire extracellular domain of the αvβ3 integrin receptor, identifying three differently complexed metal ions coordinating the RGD-peptide to the central Mg2+ ion, determined a breakthrough in understanding how integrin-ligand-binding was coupled to conformational changes of the integrin receptors (471, 472). The structural differences between the headpiece of the lymphocyte integrin αM and the integrin αvβ3 expressed in fibroblasts and endothelial cells allowed first considerations about the connection of integrin structure to physiological function (see Chapter 2).

It took a few additional years to understand the flexible elements of the integrins and the allosteric conditions under which the receptor was extending into a conformation that was compatible with ligand binding (470). Importantly the crystallographic studies with the αIIbβ3 integrin ectodomain were backed up by electron microscopy analysis of individual integrin heterodimers changing their conformation in the presence of Mn^{2+} ions and RGD peptides,

confirming the allosteric nature of the integrin receptor (125). With the analysis of other integrin receptors, however, the debate continues about how conformational flexibility of the integrin receptor and allosteric influence of intracellular adapters and extracellular ligands shape the function of the different integrin receptors (289, 484) (see Chapter 2).

This second phase of in-depth analysis of the integrin structure/function relation was greatly advanced by the discovery of the green fluorescent protein (GFP). The fusion of GFP to cytoskeleton proteins or integrins allowed to localize these receptors in living cells, to study their dynamic association in the plasma membrane and their cycling through the membrane systems of the cell. In migrating cells, a different behavior of β3-GFP-integrin clusters located at the front and at the rear of cells was apparent (35). Furthermore, the differences in the integrin cluster behavior between immobile, but transient clusters in the cell front, and inward sliding integrin clusters at the cell rear correlated with the dynamic exchange measured by fluorescence recovery after photobleaching (FRAP) between these different integrin-dependent adhesions. Interestingly, the dynamics of the integrin exchange depended on the regulation of the actin cytoskeleton, providing at the same time a structural and dynamic vision of the integrin receptors and their association with the actin cytoskeleton and integrin adaptor proteins such as talin and vinculin (35, 90).

However, as we are learning more and more about the different integrin receptors, their functions as well as mechanical and signaling capacities, we have entered a third and still ongoing phase of research on the integrin receptor family. This third phase involves attempts to integrate the notion of mechanosignaling with the mechanical aspects of cell linkage to the extracellular matrix. Tensional forces created between the extracellular matrix and the cytoskeleton induce changes in the extracellular visco-elastic scaffold, the integrin receptors as well as their adapter proteins, linking intracellular signaling to conformational changes in multidomain proteins (205, 450). In turn, such conformational changes can affect enzymatic reactions and lead to activation of kinases such as focal adhesion kinase (FAK) and src family kinases as well as different types of phosphatases. Thus, the large number of integrin-associated proteins, defined as the adhesome (63, 239, 387, 492), as well as their differential interaction with the plasma membrane is forming a puzzle consisting of 200 to 1000 different pieces, of which we have only limited structural and biochemical information. Under tensional stress many of these adhesome proteins will undergo conformational changes, further increasing the complexity of the adhesion site. It remains a challenging task to identify the molecular

machinery, that has constantly evolved since the moment cells started to actively explore their environment and to form multicellular organism relying on extracellular scaffolds.

Chapter 2: Structure and allosteric control of the integrin receptor

Overall integrin structure

As mentioned above, some integrins like αIIbβ3, αVβ3, and the integrins involved in immunological functions containing the β2 subunit have been studied in more detail than other members of the family, and many concepts in the field are based on these integrins. We therefore want to give a general overview about the structural organization of integrins before a more detailed discussion about structure and integrin activation based on αIIbβ3 and αVβ3 integrins. Finally, we extend the discussion to other integrins and the differences in their organization before presenting potential consequences of integrin structure for their physiological function (Chapter 3).

Ultimately, the understanding of the physiological roles of integrins requires to comprehend the link of structural organization to adhesive function. Especially crystallography, electron microscopy (EM), and conformation-specific antibodies have been pivotal to reveal different conformations of integrins and the structural organization of the α- and β-subunits (Figure 1). Both subunits are tightly bound to each other by interactions between the α-propeller and the β-I-like domain in the extracellular "head" regions of both subunits. This association occurs in the endoplasmic reticulum, and single chain integrins do not reach the cell surface (250). Probably the most drastic structural difference between integrins is the presence or absence of the ligand-binding α-I domain, inserted in the top part of the α-subunit (9 integrins have, 15 do not have an α-I domain; see Figure 1). The α-I and β-I-like domains are structurally related to the Von Willebrand factor A-domain, exhibiting both a metal ion-dependent adhesion site (463). Although showing a similar fold, the β-I-like domain in the β-subunit of integrins possesses some unique structural characteristics (see below). Integrins with an α-I domain belong to the classes of collagen-binding integrins and leukocyte specific integrins (Figure 1) and are found only in vertebrates. Functionally, the most obvious difference between integrins with and without α-I domains is the mode of ligand binding. Integrins without α-I domains bind ligands in a binding pocket formed by the α-propeller in the α-subunit headpiece and the MIDAS ion in the center of the β-I-like domain of the β-subunit (Figure 2, 3). In contrast, integrins with an α-I domain recognize ligands only with their α-I domain, which is however

structurally coupled to a β-I-like domain-binding "IEGT" peptide motif, serving as an internal integrin ligand (Figure 3D). Further analysis showed that the spatial arrangement of ligand-integrin interactions is diverse even within the respective groups of integrins with or without α-I domains (Figure 3).

Given the frequency of the RGD sequence in many extracellular matrix proteins, the group of RGD-binding integrins is considered to recognize many different ligands. Because of the importance of the RGD sequence motif, one might neglect the relevance of the structural organization around the RGD peptide and the respective specificity of the ligand binding event. While present in an exposed loop in fibronectin, the RGD-peptide is flanked by a helical motif in the latent TGF-β binding protein, which leads to the specificity in binding to αvβ6 and αvβ8 (113, 321, 484). In addition, the initial characterization of integrin-ligand binding specificity proposed the selective recruitment of the RGD ligands vitronectin and fibronectin to αvβ3 and α5β1 respectively (349, 350). More recently, we have revisited ligand specificity by creating binary choice substrates, that allow cells to simultaneously use their different integrin populations on the most relevant ECM ligand (335). In fact, when cells were given the choice between different substrates, the selection of the appropriate ligand was surprisingly specific, suggesting that cells prefer to adhere on the most fitting adhesive surface in respect to ligand density and stiffness. However, cells were also able to adhere to less-preferred ligands, indicating that flexibility in ligand recognition might explain seemingly promiscuous integrin-ligand binding. New techniques, e.g. single cell force measurements (233) and super-resolution light microscopy (292) can detect differential ligand interaction in living cells (373), and will certainly facilitate the reassessment of integrin-ligand interactions, their dynamic regulation, and their *in vivo* behavior.

The ligand-recognizing headpieces of both α- and β–subunits are sitting on top of "leg" domains (Figure 2), followed by transmembrane regions and, with the exception of β4 integrin, comparably short cytoplasmic tails. While the extracellular headpiece binds ligands, the cytoplasmic tails interacts with intracellular adapters. Especially the cytoplasmic tails of β-subunits have been analyzed in detail and are attributed to important functions in regulating integrin activity (see below) and actin linkage (Chapter 4). Functions and binding partners of the cytoplasmic tails of α-subunits are less studied and have been associated with integrin inactivation rather than activation and signaling (see Chapter 4 and (54, 344, 359)).

αIIbβ3 and αVβ3 integrin activation

Integrin activation (in terms of gaining the ability to bind ligands) is coupled to extensive structural changes in both subunits. Currently, the prevailing model for αIIbβ3 and αVβ3 integrin activation assumes a tight coupling of integrin-ligand binding with a structural change from a bent-closed to an extended-closed integrin conformation (‘switchblade model’; similar to the opening of a Swiss army knife), and a further opening of the head piece to an extended-open conformation (Figure 3E). All three conformations are present in the membrane in a dynamically regulated equilibrium that involves intracellular adapters, as well as extracellular ligands. Ligand binding affinity increases with integrin extension and head-piece opening. However, also the bent conformation is able to bind RGD ligands (472). Nevertheless, the structural rearrangement during integrin extension and subsequent head-piece opening is accompanied by several local changes in the headpiece of the β-subunit induced by the carboxyl binding of the Asp-side chain of the RGD-motif to the central MIDAS Mg^{2+} ion (Figure 3A,B): (i) the ADMIDAS site moves towards the Asp-bound Mg^{2+} ion, (ii) the α1-helix in the β-I-like domain straightens, (iii) the α7-helix makes a piston-like movement towards the hybrid domain, (iv) which swings out, thereby increasing the angle to the β-I-like domain and completing the headpiece opening (Figure 3). It seems that these discrete structural events cannot be uncoupled during the process of headpiece opening; straightening the α1-helix by mutations leads to increased overall integrin activation (495), as does constitutive hybrid domain swing-out by introducing a glycosylation site that provokes opening of the angle between the β-I-like and hybrid domain by steric interference (Figure 3A) (272). The structural integrin activation process starts with a bent state and proceeds to the extended-closed and finally to the extended-open state (347, 427, 500). In contrast to such a strict three-step process, Zhu and colleagues showed that headpiece opening of αIIbβ3 integrin is a continuous process, in which they defined eight different steps (501). They also estimated the integrin headpiece affinity for an RGD peptide in the open state to be more than 200-fold higher than in the closed conformation and thus considered the extended-open conformation to be the active, ligand-binding state. Moreover, recent electron microscopy data of different β1-integrin containing integrins, proposes that the bent-closed conformation is not typical for these integrins, but regulated essentially at the level of the integrin head-piece opening (289, 417). In addition, recent data from our group indicates at least for αVβ3 integrin, that the correlation between conformation and ligand binding is more complex: αVβ3 integrin locked in the extended-closed state was able to bind vitronectin, but not fibronectin. Only the extended-open state of αVβ3 integrin was able to bind fibronectin, a behavior that required tensional forces acting on the integrin receptor

(31). Thus, structure-function relationships differ among ligands binding the same integrin, suggesting that the extended-closed conformation might be more than just a 'not yet activated' integrin. A similar situation was demonstrated for α4β7 integrin, where two cytokines (CCL25 and CXCL10) cause different integrin conformations, binding either to MAdCAM or VCAM (452). A explanation how the same integrin can select between different ligands was offered by Cormier and colleagues (92). They argued that besides αVβ3 integrin affinity for RGD, the accessibility of the ligand to the integrin binding pocket might be a regulating factor. Figure 3 highlights some of the headpiece features influencing integrin ligand binding selectivity, carrying the analysis also to laminin-binding integrins and how ligand accessibility and binding can be enhanced by the integrin headpiece movement. More detailed research will be required to challenge the notion of RGD ligand promiscuity and to show how switching between selective and promiscuous ligand binding can be of physiological relevance *in vivo*.

Given the extensive literature about mechanosensing and mechanotransduction by integrin mediated adhesions, it is almost surprising that the experimental data about the influence of mechanical forces on the integrin structure is rather limited. Based on molecular dynamics simulations of αIIbβ3 (500) and αVβ3 integrin (347) it was hypothesized that mechanical load on the β3-subunit facilitates the headpiece opening of the integrin by increasing the hybrid domain swing-out. Therefore, one might argue that mechanical forces activate integrins, an exciting concept contributing to the emerging field of mechanobiology. So far, this idea is supported by experimental data for αLβ2 integrin (LFA1) (292, 313) and αVβ3 integrin (31, 83, 146). In line with this, β-integrin subunits are especially well suited to bear mechanical load due to a reinforcement with two polypeptide chains (between the β–I-like and hybrid domain) or a disulfide bridge in addition to a polypeptide chain between their domains (113). Domain-connections in the α-subunit miss these additional reinforcements, and the α integrin subunit may therefore unfold more easily under mechanical load.

Similarities and differences between integrins

Many cell culture studies compare αVβ3 integrin and α5β1 integrin (34, 69, 98, 369, 388). Both belong to the group of RGD integrins, bind fibronectin and are expressed in both fibroblasts and endothelial cells. Accordingly, the overall structural organization is very similar. Nevertheless, there are important structural and functional differences between αVβ3 integrin and α5β1 integrin. In a recent study, Takagi and coworkers detected αVβ3 integrin to be present in the bent, extended-closed, and extended-open conformation in the absence of ligands or

stabilizing antibodies (289). However, under identical conditions the authors failed to detect an extended-open conformation for α5β1 integrin. In contrast, the group of Timothy Springer detected all three conformations for α5β1 integrins by complexing them with conformation-specific antibodies (417). This approach also allowed them to measure affinities of specific conformations for RGD and fibronectin fragments (257). Interestingly, they detected a 4,000- to 6,000-fold increase in affinity of the extended-open compared to the extended-closed conformation for cyclic RGD (cRGD) and fibronectin fragments. This is in clear contrast to αIIbβ3 integrin, for which an only 200-fold increase was reported (501). This difference in affinity increase during headpiece opening could imply α5β1 integrin to be 'locked' to its ligand when reaching the extended-open conformation. Such a strong binding to fibronectin could have evolved to support the mechanical stretching of the ligand during fibronectin fibrillogenesis (390), which is likely to be a non-linear and visco-elastic process, in which a rapid loss of tensional load in fibronectin fibrils should not result in the immediate dissociation from the integrin receptor. On the other hand, the evolution of a synergy site in fibronectin, specifically enhancing the on-rate for α5β1 integrin binding, may help to diversify the specific features of certain integrin/ligand pairs (302). At the same time, a strong binding with a low off-rate might also set the need for precise regulation of the activity of β1 integrins by inhibitors (54) or by posttranslational modifications like phosphorylation, glycosylation, or acetylation. This example emphasizes the connection of structural differences and specific physiological tasks of α5β1 integrin in fibronectin fibrillogenesis. At the same time, it highlights the difficulties of generalizing concepts from well-studied integrins to the entire family of integrin receptors.

As mentioned above, collagen-binding integrins and leukocyte specific integrins differ from all other integrins by the presence of an αI domain in the α-subunit. Importantly, only this αI domain binds the respective ligand, in contrast to a combined ligand binding by both subunits in integrins without αI domain. This might explain why RGD-binding integrins, lacking an αI domain, evolved a bigger variety of α- and β-subunit pairings (Figure 1). Interestingly, the initial binding pocket formed by the propeller domain in the α-subunit and the β-I-like domain in the β-subunit is still present in αI domain integrins. However, it is used by the αI domain as an intramolecular 'pseudo-ligand' for recognition of the IEGT-peptide motif (Figure 3D). Additionally, αI domains have no ADMIDAS site, and their αI helix is always straightened during activation. Thus, ADMIDAS movement towards the ligand and α1-helix straightening during integrin activation might be used to fine-tune the affinity of the MIDAS site in β-I-like

domains (495, 501). Therefore, Zhang and colleagues (495) argued that αI-domain integrins, missing this fine-tuning, might be better suited for fast on/off switching than integrins without αI domain.

Another surprising mechanobiological feature of integrins are catch bonds between ligands and integrins, meaning that the lifetime of a bond increases when force is applied (82). As summarized by Cheng Zhu and colleagues (82), catch bonds are now described for α5β1 – fibronectin, αVβ3 – fibronectin, αLβ2 – ICAM-1, α4β1 – VCAM-1, and αMβ2 – ICAM-1. As these authors point out, it is more appropriate to describe these bonds as catch-slip bonds, since the bond will change from a catch bond to a slip bond when the force on the bond exceeds a certain level. Catch bonds might have evolved to stabilize cell-ECM anchorage by allowing integrin-ligand bonds to persist under mechanical load, especially when the other bonds in their surrounding break by mechanical stress. Interestingly, catch bonds are documented also for other receptor-ligand pairs than integrins (Notch-Jagged1, VWF-GPIbα, TCR-pMHC as described by Cheng Zhu and colleagues (82); E-Cadherins (356), P-Selectin-PSGL-1 (281)), as well as intracellular force-bearing connections like vinculin and actin (197). Potentially, catch bonds will emerge as the rule and not the exception whenever mechanical forces are involved in receptor ligand interactions. Still, the structural implementation of this feature within the integrin headpiece requires yet to be shown. The increasing unmasking of the positive charge of the metal ion at the MIDAS position and the consequentially tighter binding of the negatively charged Asp in the RGD peptide during integrin activation are, however, a plausible mechanism (458, 459) (Figure 2, 3). Catch bonds in αI domain integrins αLβ2 and αMβ2 have to include the αI domain, but mechanisms in the β–I-like and hybrid domain could be analogous in integrins without αI domain (82).

The *in vivo* importance of catch bonds might be best documented in the vasculature, where selectin-based catch bonds regulate leukocyte rolling in presence of shear stress caused by the blood stream (139). Additionally, recent examples of circulating tumor cells arresting in a β1-dependent manner in the blood flow might indicate the relevance of catch bonds (281).

On a first glance, the structural understanding of integrins might appear quite detailed already. However, as described here, not every integrin is studied to the same extent, and the generalization of individual integrin qualities to other integrins might be misleading. While structural features of integrins can be linked to physiological settings, it is also clear that we are

limited by techniques that allow us to test these hypotheses *in vivo*. Additionally, the examples of mechanical integrin regulation suggest that the transfer of data from experiments in the absence of force (*in vitro* studies, flow cytometry) to the *in vivo* setting is not always straightforward. Having said this, we are nevertheless convinced that the detailed understanding of even a few integrins will be useful as a framework to compare with other integrins, deducing their function based on differences and similarities.

Chapter 3: The physiological role of integrin-dependent cell adhesion explained through several examples

Integrin affinity modulation versus clustering in the plasma membrane (talin and kindlin)

When integrins recognize extracellular ligands and change from a low to a high affinity conformation, either by an outside-in or inside-out triggered mechanisms, they also start to form clusters in the membrane that are visible by light microscopy (35, 90, 336). Using super-resolution light microscopy, the initial formation of nano-clusters of 50 to 100 ligand-bound integrins can be detected (75), that will further assemble into larger integrin clusters to enable cell adhesion. The mechanistic connection between conformational activation of β3-integrins and integrin clustering is still not fully understood, but requires at least extracellular ligand-binding, talin-head/integrin interaction and talin and kindlin binding to phosphoinositol lipids in the plasma membrane (51, 90).

Although αIIbβ3 and αvβ3-integrin activation and clustering are among the best studied integrin processes, it is still not clear, why in resting platelets α2β1 integrin is in an apparently extended, ligand binding-competent, but not fully activated state (289, 312), while at the same time αIIbβ3 receptors are thought to be present in the platelet membrane in a bent-closed conformation (485). Differences between β1 and β3-integrins in the transmembrane and cytoplasmic α-domain association, also known as the inner membrane clasp (Figure 2), could account for these different integrin resting states (271). Similarly, intracellular isoform-selective integrin inhibitors could be responsible for maintaining distinct conformational pools of cell surface integrins, e.g. keeping αIIbβ3 in a bent-closed conformation and preventing it from binding plasma fibrinogen, while presenting α2β1 in an extended conformation able to bind to exposed collagen fibers at sites of vessel damage (441). Support for the model of conformational activation of αIIbβ3 integrin has come mainly from the discovery of a ligand-mimetic IgM monoclonal antibody (PAC-1) binding αIIbβ3 integrin on activated, but not

resting platelets (394). Interestingly PAC-1 exhibits an RGD-related KYD sequence in the H3 loop of the heavy chain, thought to be responsible for αIIbβ3 binding. However, a report by Tomiyama and coworkers described two different IgG antibodies with the same KYD sequence that bound equally well to resting as well as activated platelets (439). Although this discrepancy in αIIbβ3 binding by IgG and IgM antibodies can be explained by a specific conformation of the KYD-containing loop, probing αIIbβ3 integrin binding with Fab fragments of the PAC-1 antibody did not allow to discriminate between integrins on resting or talin-head activated platelets or CHO cells (62). Thus it appears possible that the large size of the PAC-1 IgM prevents it from efficiently recognizing the bent-closed αIIbβ3 integrin receptor. On the other hand it is also likely that the enhanced cell surface binding of PAC-1, e.g. observed during talin-head mediated αIIbβ3 activation (425), is due to talin-mediated (90, 380) or kindlin-induced integrin clustering (486). Such an increase in integrin clustering is particularly well detected due to the polyvalency of the PAC-1 ligand (62), therefore proposing that physiological inside-out activation of the β3-integrin receptor involves conformational changes of the integrin ectodomain as well as adapter-induced clustering of the receptors in the plasma membrane (90, 380). Kindlin appears to contribute to integrin clustering rather than to activation, co-operating with talin in this process (486).

The conformational activation of integrins has also been analyzed by a genetic screening approach based on a monovalent integrin ligand binding to the *Drosophila* αPS2βPS integrin. This study revealed mostly gain of function mutants in βPS, stressing the physiological importance of keeping integrins in a low ligand-binding affinity state. On the other hand, the mutation of the juxtamembrane **C**GF**F**N**R** sequence in αPS2 to CGF**A**N**A** enhanced ligand binding of the integrin, while the **V**GFFNR mutation led to a reduction of ligand binding (187, 220). Interestingly, the mutated cysteine residue is conserved in α3, α6, α8 and αE-integrins (Figure 1) and known to be palmitoylated in α3 and α6-integrins (480), proposing the existence of still undiscovered mechanisms to control the integrin affinity state in general (such as kindlin) or in integrin-specific situations, such as in αPS2.

The αIIbβ3 receptor on platelets

One of the best studied integrin structure-function relationship concerns the αIIbβ3 receptor expressed on platelets. Blood is coagulating through activation of platelets, that are stimulated by agonists such as ADP or thrombin, or by binding to injury-released, collagen-bound von

Willebrand factor, leading to a conformationally induced change in the affinity of αIIbβ3 integrin (also known as GPIIb/IIIa) for circulating fibrinogen in the plasma (470). Based on this physiological example, the signal-mediated conformational change of αIIbβ3 integrin and the subsequent binding of extracellular fibrinogen allowed to establish the concepts of inside-out and outside-in signaling. The activation of αIIbβ3 integrin has to be strictly regulated to avoid a fatal thrombosis, therefore it cannot be activated by the always-present ligand fibrinogen. Instead, intracellular signals are required for αIIbβ3 integrin activation, leading to fibrinogen binding and formation of a blood clot (i.e. inside-out signaling). These activating signals for αIIbβ3 integrin, on the other hand, have to originate from the outside, where a signal conveying the presence of a wound to the platelet triggers the intracellular cascade leading to αIIbβ3 integrin activation (i.e. outside-in signaling). Platelets express the collagen receptors GPVI and α2β1 integrin, both potentially sensing wound-exposed collagen, but the precise contribution of both receptors to αIIbβ3 integrin activation appears controversial (279, 309). Recent structural studies for β1 integrins in the presence and absence of ligands revealed interesting differences to β3 integrins with consequences for the structure-function relationship of both integrins. Takagi and coworkers found β1 integrins in the absence of ligands to be mostly present in the extended-closed conformation, irrespective of the ion conditions (289). The same study, but also work by the group of Timothy Springer (417), detected extended-open conformation for β1 integrins in the presence of ligands (or stabilizing antibodies). On the other side, β3 integrins conformations were strongly affected by ion conditions, revealing conformations from bent, extended-closed to extended-open. Thus, β3 integrins might be more susceptible to allosteric regulation by cytoplasmic adapters, while β1 integrins are mostly regulated by the presence of ligands.

The inside-out activation of αIIbβ3 integrin is still a matter of research, but essential features include the activation of the Rap-1 GTPase, binding the talin rod-domain to release talin autoinhibition and to induce a mechanical coupling between the actin cytoskeleton (talin rod domain) and the integrin-cytoplasmic tail (talin head domain) (64, 230, 416, 454). Since the talin-integrin connection provided an explanation of the αIIbβ3 integrin activation mechanism, critical roles for additional integrin activators were not considered at the time. However, it has become clear, that the talin-head interaction with the cytoplasmic tail of the β3-integrin receptor alone is not sufficient, and that the plasma membrane-associated adapter protein kindlin is at least equally, if not even more important than talin to induce αIIbβ3 integrin conformational

activation and fibrinogen binding, subsequently triggering platelet and cell spreading (295, 437) (Figure 2, 3).

Several publications indicated Rap1-mediated activation of integrins to include the binding of RIAM to talin, as demonstrated for αIIbβ3 integrin (179). Recent publications analyzed this process in more detail and found RIAM-mediated activation to be specific for β2 integrins, whereas within the same leukocytes α4β1 integrin is activated in a RIAM-independent manner (230, 414). Additionally, RIAM knockout mice showed no severe phenotype and unaltered β1 and β3 integrin activation (230, 414). Thus, it appears that pathways upstream of talin (and kindlin) are able to target and activate specific integrin subunits, enabling cells to react differentially to separate outside-in signals. One of these pathways may involve a direct activation of the talin-head domain by Rap1-binding, instead of an indirect, RIAM-dependent mechanisms (58, 68, 502).

The role of integrins in extracellular matrix assembly: fibronectin

So far we have mainly considered the role of integrin receptors in a cell-autonomous way, as integrins are critical for cell anchorage to the extracellular matrix, providing signals for survival and proliferation. However, integrin receptors are also used by cells to organize or remodel the extracellular matrix. For example, cultured fibroblasts synthesize extracellular matrix proteins such as fibronectin, which they incorporate into an extracellular scaffold that allows their adhesion and generates survival signaling. In the well-studied case of fibroblasts cultured on fibronectin, the αvβ3 integrin receptor assures the binding of the cell periphery to the culture substrate, while α5β1 is “spinning” or “weaving” a fibronectin network around the center of the spread cells by forming fibrillar adhesions (324). In a preformed 3D fiber network the classical distinction between focal and fibrillar adhesion is no longer maintained (95, 473). As mentioned in Chapter 1, transformed fibroblasts loose the capacity to synthesize fibronectin fibrils. In cancer tissues cancer-associated fibroblasts partially compensate this by excessive deposition of extracellular matrix in the tumor stroma (CAFs) (126, 316). Interestingly, the enhanced deposition of extracellular matrix by CAFs should be taken into consideration during the treatment of tumor patients, as the enhanced stiffness of the tumor stroma induces survival signaling in B-RAF inhibitor-treated melanoma cells (191). The mechanisms responsible for fibronectin fibril synthesis are still incompletely understood, but involve the cytoplasmic integrin adapter protein tensin1 (324). Interestingly tensin1 function is targeted also by

intracellular metabolic pathways, linking integrin-dependent fibronectin assembly to the level of glucose in the tissue and in general to the metabolic state of a cell in a tissue (157, 288). Moreover, the tracking of fluorescent β1-integrin in astrocytes has allowed to connect the assembly of fibronectin fibers in fibrillar adhesions to the simultaneous association of GFP-labeled VEGF with such newly synthesized fibronectin fibers (119). These results do not only provide a unique insight into the process of integrin-dependent fibronectin assembly, but also highlight the fact that the extracellular matrix is providing a delicately tensioned scaffold, binding and storing growth factors and releasing this pool of signaling molecules in the case of tissue injury or pathological signaling in the case of fibrosis (see Chapter 9). Rather recently, it became evident that not only tensins, but also proteins from the kank family are relevant in fibrillogenesis (420). Kank2 reduces the affinity of the talin rod for actin, thereby weakening the mechanical load on the ECM-integrin-actin axis. This process acts in parallel to the maturation of focal adhesions to fibrillar adhesions and their translocation to the cell center. It might be counterintuitive that mechanical alignment of fibronectin fibers is mediated by fibrillar adhesions under low mechanical load. Interestingly, detailed studies with atomic force microscopy revealed that the initial reorganization of fibronectin fibers already occurs in the cell periphery, where integrins are under higher mechanical load (174). Thus, we envision a model of initial fibronectin stretching in the cell periphery, including higher forces on the integrin-fibronectin link. After this opening of cryptic binding sites on fibronectin, and potentially detachment from the substrate, small fibrils are aligned and organized to form bigger fibrils. This translocation of detached fibrils might benefit from high-affinity binding even under low force, which is achieved by α5β1, but not by αVβ3 integrin (31, 388), while kank2 orchestrates the change in force level through the modulation of the talin-actin connection. Interestingly, kank2 might also be important for the effect of microtubules and focal adhesion stability (77). Kank binds simultaneously to the CLASP family of microtubules plus-end binding proteins, the R7 subdomain of talin, as well as the membrane-bound liprin/LL5 scaffold, which functionally associates focal adhesions with the vesicular transport machinery (53, 410).

The role of integrins in extracellular matrix assembly: laminin and collagen

Collagen and fibronectin are both major components of the ECM, responsible for the structural organization and mechanical integrity of the ECM. Collagen type I is a prime example for fibrillar collagens, in contrast to collagen type IV that forms networks in the basement

membrane. Four integrins, α1β1, α2β1, α10β1, and α11β1 (all containing an α-I domain; see Figure 1), are reported to bind collagens with certain preferences for either collagen I or collagen IV (222). Both collagens also have different mechanisms leading to their structural arrangement in the ECM. Collagen I is known to align with fibronectin and to gradually replace it in the ECM during wound healing (287). Interestingly, collagen I preferentially binds to relaxed fibronectin fibers (236). On the other hand, the same study (236) showed fibronectin fibers to be under increased stress in the absence of collagen I, thereby emphasizing the relevance of collagen for the mechanical state of ECM. A self-assembly of fibrillar collagen, used for surface coatings in cell culture studies, seems to be much less relevant *in vivo* (215).

The experimental observation of the basement membrane organization and its main components collagen IV, laminin, nidogen, and perlecan in epithelial cells is more complex. Collagen IV was shown to be dispensable for the initial organization of the basement membrane in the embryo (before E10 in mice), but to be essential in later developmental stages (341). Thus, like fibrillar collagen, also collagen IV is highly important for the structural integrity of the ECM. It is well accepted that in basement membranes α3β1, α6β1 and α6β4 integrins contribute to adhesion of epithelial cells by recognizing the c-terminal globular domains of the laminin α-subunit (see also Figure 3D) (483). In the absence of these integrins, the epithelia detach and blisters form (111, 112). Defects in the deposition and organization of such basement membranes have been rarely reported, but it has been recognized that laminin binding integrins are palmitoylated in either their α3, α6 **C**GFFKR sequence or β4-juxtamembrane domains (480). The absence of this reversible lipidation affects laminin-dependent adhesion and association with the palmitoylated tetraspanins in the plasma membrane (39, 479). Interestingly, the depletion of the tetraspanin CD151 causes kidney failure associated with altered glomerular basement membranes (378). Moreover, in tissue culture α3β1-integrins showed enhanced, focal adhesion-like clustering due to the absence of the tetraspanin CD151 (377), suggesting that membrane distribution and tetraspanin association of laminin-binding integrins are not only regulating the adhesion to basement membranes, but also their assembly.

Non-classical integrin mediated adhesions

Integrin-mediated adhesions were often classified according to a maturation sequence starting from nascent adhesions, leading over focal complexes and focal adhesions to fibrillar adhesions (88, 151). However, it is also clear that not all integrin adhesions follow this scheme.

Podosomes and invadopodia (now summarized as invadosomes) were already described in the 1980s (reviewed in (195, 300)), and their structural organization differs drastically from 'classical' integrin adhesions. Invadosomes have a central actin core oriented perpendicular to the substrate and surrounded by a belt of adhesome proteins like talin and vinculin. As the name indicates, invadosomes are involved in ECM degradation, thereby supporting invasion of the cell into the degraded, softened tissue. This is achieved by the delivery of matrix metalloproteases (MMPs) to sites of invadosomes, where they are secreted and digest the ECM (338). This process was shown to also occur at focal adhesions (410), but appears to be more prominent at invadosomes. For more insights about integrin recycling and endo- and exocytosis at sites of integrin adhesions we would like to refer to excellent reviews about this topic (142, 293, 328).

More recently, a new type of adhesions specific for αVβ5 integrin emerged (270). During the analysis of integrin adhesions throughout the cell cycle the authors detected an enrichment of β5 integrin to specific adhesion structures during interphase. Interestingly, αVβ5 integrin-mediated adhesions in these cells recruited no classical adhesome proteins like talin1, kindlin2 or vinculin and were not coupled to actin filaments. Additionally, their shape differed from classical adhesions; they formed a dense net of adhesive structures coined reticular adhesions. The reticular adhesions recruited adapters of clathrin-mediated endocytosis, potentially contributing to their ability to stay attached to the matrix during mitosis and to serve as a 'adhesion memory' during re-spreading after mitosis. Additional studies by other groups confirmed this dependence of αVβ5 integrin-mediated adhesions on adapters of clathrin-mediated endocytosis, in contrast to classical adhesome proteins (38, 503). Interestingly, αVβ5 integrin adhesions associated with clathrin adapters have a capacity for mechanosensing and mediate cell adhesion even in the absence of the classical adhesion machinery. β5 integrin knockout mice develop age-related retinal dysfunction due to the lack of β5 integrin-dependent phagocytosis of photoreceptors by retinal pigment epithelial cells (304). The relation of this finding to reticular adhesions in cell culture experiments remains to be shown in future experiments.

Forces in tissues

The third phase of integrin research, reconciling known features of integrins with their ability of mechanosensing and -transduction, is presumably just beginning. But can we expect that these findings have a relevance in more physiological settings, compared to cells cultured on

glass and plastic coated surfaces? We believe that recent findings strongly suggest important roles of mechanical parameters (e.g. tissue stiffness, ligand geometry, elasticity vs. visco-elasticity) in developmental and pathological settings. As we discuss in Chapters 6 and 9, integrin-mediated mechanotransduction follows a sigmoidal mechanoswitch triggered around 5 kPa substrate stiffness. It is striking that most healthy tissues have a stiffness below this point, while fibrotic tissue is stiffer than 5 kPa (see Chapter 9). At the same time, stiffness gradients observed during the development of *Xenopus* (438) and *Drosophila* (94) make clear that developing organisms consist of regions with distinct mechanical properties. Richard Harland and coauthors showed in elegant experiments that the positioning of feathers in developing chicken skin is based on mechanical signals (401). Therefore, it will be not surprising when more reports uncover the contribution of integrin mechanosensing and -transduction in development and pathologies. On a more structural level, it is interesting to note that both talin (265) and integrins (422) are aligned with the force vector of actomyosin forces. As mentioned in Chapter 2, MD simulations suggest that forces parallel to the membrane (imitating retrograde actin flow) support the extended-open conformation of αIIbβ3 integrin, while the extended-closed conformation is stabilized by forces perpendicular to the membrane (422, 500). Additionally, work in *Drosophila* indicates that integrins and talins might experience unique force vectors in different tissues (229). Combined with the findings that specific integrin conformations bind ligands selectively (see above), differential force vectors in tissues might be a mechanism to tune the physiological needs for integrin activation and signaling. This very likely includes also mechanical regulation of integrin adapter conformations (208). However, the testing of these hypotheses will require improved tools to measure forces and force vectors *in vivo*. Several studies in *Drosophila* offered interesting insights into this question and might indicate a renaissance for this model organism (166, 172, 229, 249, 431).

Chapter 4: Regulation of integrins by adapter proteins

Integrins recruit hundreds or even up to thousand different proteins, building the so-called adhesome (63, 239, 387). However, a recent meta-analysis defined a consensus adhesome of 60 proteins (194), that the authors organized in four groups: 1) ILK – PINCH - kindlin, 2) FAK - paxillin, 3) talin - vinculin and 4) α-actinin – zyxin – VASP. Most of these proteins have been mapped into functional layers with super-resolution imaging (217). The importance of these sets of proteins is reflected by their frequent discussion in reviews on integrin-mediated adhesions (199, 207, 208, 364, 371, 419).

For this review, we wanted to focus on adapters that directly bind integrins. Therefore, we curated a list of such direct integrin adapters (Table 1). Some of these proteins, like talin or FAK, are well known in the integrin field, others are less well studied and their effect on integrins might not be fully established yet. Additionally, the large diversity of the integrin family as well as their extensive functional diversity suggests that integrins are regulated in a cell- and integrin-type specific manner. For example, kindlin3 is only expressed in hematopoietic cells (49) but kindlin1 and kindlin2 show unique interactions with integrins in keratinocytes (36), indicating that they are not functionally redundant (371). Talin1 and talin2 are shown to influence mechanotransduction differently (26) and to possess altered affinity for the β1- and β3-integrin subunits (15). They also differ in their expression within tissues, with e.g. talin2 being the dominating form in striated muscle (392) and required for fibronectin assembly (345). Nevertheless, the knockout of talin1 is embryonic lethal, while talin2 knockout mice show a dystrophic phenotype (104). We assume that further detailed and isoform-specific analysis will reveal more selective integrin-adapter interactions and their evolution for specific physiological needs.

To support a conceptual understanding of integrins we want to introduce 5 functions that are mediated by integrin adapters: (i) activation, (ii) inactivation, (iii) inhibition, (iv) signaling, and (v) mechanosensing. We expect that less-studied adapters can be explained within the framework of these functions. This classification also implies that adapters can have more than one function.

Activation

Talin and kindlin activate integrins (= change the extracellular conformation) and increase their affinity for ligands in a process of inside-out activation involving interaction of these adapter proteins with the β-integrin cytoplasmic tail. Both talin and kindlin are required for integrin activation and clustering, but appear to differentially contribute to mechanosensing (talin) and signaling (kindlin) (354, 437). An important part of integrin activation are the unclasping of the α- and β-subunit at the level of the transmembrane and cytoplasmic tails as well as the physical connection to the actin cytoskeleton. The α-integrin cytoplasmic tails vary in sequence and length, but share a common GFFKR motif partially buried within the cell membrane interacting with the transmembrane domain of the β-subunit (Figure 4 and 7). The cytoplasmic tails of

β-integrins contain two conserved PTB (phosphotyrosine-binding domain) binding sequences (Figure 7): the membrane-proximal NPx(Y/F) and membrane-distal Nxx(Y/F) motifs. Talin binds both the membrane-proximal helix and the β-integrin tail up to the first, membrane-proximal NPx(Y/F) motif (16, 150, 457). Kindlin binds to the inter-NXXY-region and the membrane-distal Nxx(Y/F) motif (253). Talin and kindlin can thus bind integrin simultaneously (46). Furthermore, the binding of paxillin to kindlin has been found to promote integrin activation (149), potentially further increasing the complexity of the integrin activating intracellular adapter complex. As shown for some integrins, outside-in activation is triggered by ligand binding, and therefore also ligands can be considered as integrin activators. Additionally, mechanical load supports integrin activation (see Chapter 2) and could therefore be considered as an activator. In this context, it is important to note that the F-actin linkage to integrins is the mechanically weak point, where integrin clustering, recruitment of adapter proteins (such as vinculin) and regulation of actin (de)polymerization are likely to be involved (200, 327).

Inactivation

Integrin inactivators ensure the dynamic regulation of cell adhesion, e.g. by unbinding from areas a cell wants to avoid, allowing migration away from this location. Phosphorylation by kinases, most notably FAK and Src, increases the turnover of integrins and integrin-mediated adhesions. Src serves as an integrator of several pathways, as it was shown that local ephrin/Eph signaling influences integrin-mediated adhesions in its vicinity via a Src-FAK-paxillin cascade (84). Additionally, endocytosis allows integrin detachment from the ECM and thereby inactivates integrins: Dab2/clathrin-mediated endocytosis was shown to replace integrin activators like talin and kindlin from β3 integrin and to mediate integrin endocytosis (488). Interestingly, Dab2-mediated endocytosis was increased in the absence of mechanical load on integrins, indicating that a lack of force can participate in integrin inactivation. Thus, there might be different ways integrins can be inactivated, involving either an inside-out mechanisms, e.g. proteolytic degradation of adapter proteins (386), or phosphorylation of integrins or adapters (as review, see (148)). On the other hand, the proteolytic degradation of extracellular matrix generates protein fragments with intact integrin binding functions. Such ECM fragments, also termed matrikines, can bind to integrins in their soluble forms, maintaining the extended-open conformations of integrins without mechanical linkage to the ECM. At this point, it is not clear why such a tension-free state would enhance the exchange of talin with

other intracellular adapters, but it leads to the subsequent internalization of the complex, as observed for fibronectin-bound receptors (76, 269).

As just mentioned, an interesting aspect of integrin activation vs. inactivation is the role of force in these processes. Why are activators and inactivators needed, if increased mechanical load activates integrins and decreased mechanical load inactivates them? First of all, it is important to keep in mind that force needs a physical link to be transmitted: there is no relevant mechanical force on integrins without talin-mediated actin linkage (354). Maybe even more importantly, integrins are not purely mechanical anchors, but also measure tension, create and integrate biochemical signals that in turn will change cell adhesion, motility and proliferation. These different integrin functions should be reflected by different modes of integrin (in)activation. At the same time, the crosstalk between different modes of integrin activation would allow to integrate mechanical and biochemical signals at the level of cell adhesion.

Inhibition

Some integrins are found in the membrane in an inactive, bent conformation (see Chapter 2). Additionally, the pool of inactive integrins can be stabilized or increased by integrin inhibitors (summarized in (54)). ICAP, for example, binds to the tail of β1 integrin and prevents activation (61), while filamin A is shown to inhibit integrin activation by establishing a ternary complex with αIIb and β3 integrin subunits, preventing the separation of the integrin subunits (264) (see Figure 3E).

Signaling and Mechanosensing

Finally, some adapters of integrins are involved in signaling or mechanosensing. Signaling adapters include kinases like FAK, but also paxillin, that serves as a dynamic scaffold recruiting different GEFs and GAPs, thereby regulating Rho-GTPases signaling and the organization of the actin cytoskeleton (103). Mechanosensing adapters, on the other hand, include e.g. the Src-substrate p130Cas, that is phosphorylated upon cell stretching (385). The ability of adapters to sense and transduce mechanical signals is often coupled to force-mediated conformational changes (208). Talin is an example, having several cryptic vinculin- and hidden actin-binding sites that become accessible when the talin rod domain is put under tension (24, 353, 365). In turn, the tension-exposed vinculin binding sites will enhance the physical anchorage of the talin rod to the actin cytoskeleton via newly recruited vinculin. The examples of FAK and talin

illustrate that the same adapter can fulfill different tasks, according to the functional classification presented here.

The slanted fence model of focal adhesions

What can we say about the spatial organization of integrin adapters within focal adhesions? Super-resolution light microscopy has allowed the analysis of integrin-dependent adhesions in great details. This strategy involves on the one hand the analysis and tracking of individual integrin receptors in living cells, on the other the identification of the spatial organization of key adhesion components (217, 373). In fact, tracking of individual β3 or β1 integrins in living cells revealed the transient immobilization of these receptors within paxillin-positive focal adhesions (373, 456). Moreover, cytoplasmic adapter proteins such as talin were directly recruited from a cytoplasmic pool into focal adhesions, suggesting that the stabilization of the talin/integrin interaction seen e.g. during talin-induced integrin clustering occurs inside the adhesions themselves and not as a precursor outside of adhesions. The analysis of elongated focal adhesions by interferometric photoactivated localization microscopy (iPALM) has revealed specific membrane distances for different types of integrin adapter proteins. While the paxillin/FAK/src module is located in a membrane-proximal "signaling layer", the c-terminal F-actin binding region of talin is located distant from the membrane within the F-actin and vinculin cross-linking domain of focal adhesions. Moreover, the local tension induced on the talin-rod domain is directly reflected by the orientation of the F-actin network forming the backbone of adhesion (238). If these positional informations are integrated with the lateral F-actin/myosin tension as well as the recent interactions of paxillin and DLC within the talin R8 bundle (491), one possible orientation is that of a slanted fence, similar to mobile fence systems used in the alpine regions. These slanted fences are stabilized by slanted long poles, representing the extended talin and F-actin fibers, and held in place by vertical poles, laterally connecting the fence structure (see for example https://de.wikipedia.org/wiki/Zaun#/media/File:Schrankzaun2.JPG).

For focal adhesions, we are proposing that the flexible regions in paxillin and FAK could serve as dynamic connectors between the different layers of the focal adhesion, reacting to force changes in the lateral as well as vertical axis of the tethered talin-rod domain (Figure 6). As for the slanted fence, such a dense interaction in lateral and axial direction would increase the stability of the system against perturbations from multiple directions.

***Table 1 Integrin cytoplasmic adaptors* and their known properties.** The abbreviations of the detection methods are AC: affinity chromatography, B: biosensor assay, EA: enzyme assay, ELISA: enzyme-linked immunosorbent assay, IP: immunoprecipitation, ITC: isothermal titration calorimetry, NMR: nuclear magnetic resonance spectroscopy, PD: pulldown assay, XRAY: X-ray crystallography, Y2H: yeast two-hybrid, O: other. The PDB code refers to the available structural information in the Protein Data Bank (42). IBS1 and IBS2: integrin binding sites 1 and 2 in talin. The list is not exhaustive.

Adapter	Integrin	Approximate binding site or interaction	Detec-tion	PDB code	Refe-rence	Proposed role
12-LOX	β4	residues 661-1752	IP		(432)	fatty acid metabolism
14-3-3	β2, β1A, α4	T758-phosphorylated (by PKC) β2: KSA[pT]TTVMNP, α4: KRQYK[pS]IL	AC, IP, XRAY	2V7D, 4HKC	(48, 102, 129, 428)	integrin activator (78)
4.1B	β8	DYRVSASKKDKLILQSVCTRAVTYRREK	Y2H, IP		(283)	
4.1G	β1		IP, PD		(81)	
4.1R	β1		IP, PD		(80)	
Abl2	β1	KFEKEKMNAK; phosphorylates Y783	IP, PD, EA		(404, 453)	tyrosine kinase
ACAP1	β1	DRREFAKFEK	PD, IP		(32, 254)	integrin recycling (254)
AKT1	β3	phosphorylates T753	EA		(227)	Ser/Thr kinase
Annexin A5	β5	FQSERSRARYEMAS	O		(70)	
AP2M1	α4	QYKSILQE	XRAY	5FPI	(141)	integrin endocytosis (141)
AUP1	αIIb	KVGFFKR	Y2H, PD		(218)	recruits SYK to αIIb tail (219)
BIN1	α3	KCGFFKR	Y2H		(466)	
CD98hc	β1A, β3; not β1D or β7	β1A: NPKYEGK, β3: TNITYRGT	AC		(346, 494)	promotes integrin signaling (346)
CENP-R	β3	β3: S752P weakens the binding	Y2H, IP		(395)	
CIB1	αIIb, αV, α5, α2, α3, α4, αM, αL, α11	αIIb: LVLAMWKVGFFKRNR	Y2H, IP, ITC, O		(37, 145, 303, 400, 475, 487)	inhibits αIIbβ3 activation (490)
Csk	β3		IP		(314)	Tyr kinase
Cytohesin-1	β2	β2: WKALIHLSDLREYRRFE	Y2H, IP, PD, O		(29, 154, 232, 363)	an Arf-GEF, restrains αMβ2 activation (29), promotes α4β1 and α5β1 integrin activation (28)

Dab1	β1A, β3	membrane-distal NxxY	PD		(65)	adaptor protein
Dab2	β3, β5	membrane-distal NxxY	PD		(65)	adaptor protein
Dok1	β3, β1A, β7, β2	phosphorylated NPxY (β3, β1A, β7), phosphorylated NxxY (β3), S756-phosphorylated β2	NMR		(14, 175, 320)	adaptor protein
EED	β7, α4, αE	β7: RLSVEIYDR	Y2H, IP		(363)	polycomb protein
EIF6	β4	1st and 2nd FNIII domains and the connecting sequence	Y2H, PD		(44)	ribosome binding
EPS8	β1A, β3, β5	membrane-proximal NPxY	PD		(65)	
Erbin	β4	4th FNIII domain and C-terminal sequence (res. 1457-1752)	Y2H, PD		(133)	
Ezrin	β4		IP, PD		(451)	promotes β4 expression (451)
FAK1	β5, β3	β3: complete cytoplasmic tail β5+Y861-phosphorylated FAK1: QSERSRARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD β5+Y861-nonphosphorylated FAK1: complete cytoplasmic tail	IP, PD		(120)	Tyr kinase
FAK2	β3	β3: LYKEATSTFTNITYRGT	IP, O		(333)	Tyr kinase
FHL2	α3, α7, β1, β2, β3, β6,	α7A: AVQPSAMEAGGP, α7B: GTIQRSNWGNSQWEGS, β1A: VVNPKYEGK, α3: ARTRALYEAKRQ	Y2H, IP		(382, 465)	adaptor protein
FHL3	α3, α7, αV, β1	α7A: GTVGWDSSSGRST; α7B: DAHPILAADWHPELGP	Y2H, IP		(382)	adaptor protein
FilaminA	β1A, β1D, β3, β7, β2, β6, αIIb	β2: LFKSATTTVMN β3: PLYKEATSTFT β7: LYKSAITTTI αIIb: WKVGFFKRNRP	NMR, XRAY, PD, Y2H, B	2MTP, 2BRQ, 2JF1	(137, 211, 223, 264, 428)	actin binding; competes with talin (223)
FilaminB	β1A, β1D, β3, β6		Y2H, PD		(137)	actin binding
FilaminC	β1A		Y2H, PD		(164)	actin binding
FRMD5	β5		PD, IP		(196)	interacts with and inhibits ROCK1 (196)
Fyn	β3		IP		(18, 19, 314)	Tyr kinase
GIPC1	α3, α6, α5	α3: ERLTSDA, α6: NRNESYS	Y2H, PD		(434)	
HAX1	β6	KLLVSFHDRKEVAKFEAERSKAKWQTGT	Y2H, IP		(357)	clathrin-mediated endocytosis of αVβ6 (357)
Hck	β1, β2, β3		IP		(18)	Tyr kinase

ICAP1	β1	NxxY; KSAVTTVVNP	PD, Y2H, IP, XRAY	4DX9	(74, 267, 498)	negative regulator of β1 integrin, competes with talin (55)
ICln	αIIb	KVGFFKR	IP, PD, B, O		(241)	
ILK	β1, β3		Y2H, IP		(181)	(pseudo-) kinase, part of ILK-PINCH-Parvin complex, triggers F-actin bundling (443, 446)
Kindlin1	β1, β3, β6	β1: TTVVNPKY	AC, PD		(182, 231)	co-operates with talin to activate integrin (444)
Kindlin2	β1, β3, β5, β2	β1: VTTVVNPKYEG β3: TSTFTNITYRG	XRAY, IP	5XQ0, 5XQ1	(196, 253)	co-operates with talin to activate integrin (437)
Kindlin3	β1, β2, β3	β1: TTVVNPKY, β2: membrane-distal NPKF	PD		(294, 295)	co-operates with talin to activate integrin (248)
Lyn	β1, β2, β3		IP, PD		(18, 19)	Tyr kinase
MAPK1	β6, β3	β6: RSKAKWQTGTNPLYR, β3: EATSTFTN	IP, ELISA, PD		(6, 367)	ERK2, Ser/Thr kinase
MAPK3	αV, β3	β3: EATSTFTN	IP, PD		(260, 367)	ERK1, Ser/Thr kinase
MDGI	α1, α2, α10, α11	α1: WKIGFFKRPLKKKMEK α2: WKLGFFKRKYEKMTKNPDEIDETTELSS α10: WKLGFFAHKKIPEEEKREEKLEQ α11: WKLGFFRSARRRREPGLDPTPKVLE	Y2H, B		(308)	inhibits integrin activity (308)
Melusin	β1	KLLMIIHDRREFAKFEKEKMNAKWDT	Y2H, PD		(57)	
Moesin	β1		PD		(449)	competes with talin (449)
MYO7A	β5		IP, PD		(268)	promotes cell migration (268)
MYO10	β1, β3, β5	β1: WDTGENPIY	Y2H, IP, PD		(496)	integrin relocalization to filopodia, actin binding (496)
Neuropilin-1	α5		IP		(445)	
Nischarin	α5	IYILYKLGFFKRSL	Y2H, B		(9)	inhibits PAK kinase activity (10)

NRK2	β1A, β1D (α7β1 but not α5β1)	LIWKLLMIIHDRREFAKFEKEKMNAKW	Y2H, PD, IP		(255, 256)	nicotinamide riboside kinase 2
Numb	β3, β5	membrane-proximal NPxY	PD		(65)	clathrin-mediated endocytosis of integrin (311)
p120RasGAP	α1, α2	α2: WKLGFFKRKYEKM	IP, PD, B, O		(275)	recycling of endocytosed integrins (275)
PAK4	β5	SERSRARYE	Y2H, PD		(497)	Ser/Thr kinase
Paxillin	β1, β3, α4	α4: ENRRDSWSY, β3: LYKEATSTFTNITYRGT	AC, IP, O		(180, 266, 333)	adaptor protein
PDK1	β3	phosphorylates T753	EA		(227)	Ser/Thr kinase
PKC	β2	PKCδ, PKCβI/II, PKCα and PKCη phosphorylate β2: S745 and β2: T758 PKCα phosphorylates T760	EA		(128)	Ser/Thr kinase
PKD1	β3	EATSTFTNITYRGT	IP, PD		(467)	Ser/Thr kinase
Plastin2	β1, β2	β1: DRREFAKFEKEKMNAKWDTG β2: LSDLREYRRFEKEKLKSQWN	IP, PD, O		(162)	preferentially binds clasped αMβ2 and stabilizes its inactive state (441)
Plectin	β4	FNIII 1 and 2, N-terminal part of connector between FNIII 2 and 3	XRAY	3F7P	(332)	hemidesmosome assembly (234)
PP1C	αIIb	KVGF	IP, PD		(13, 447)	Ser/Thr phosphatase
PP2A	β1A, β1D, αIIb	dephosphorylates T788 and T789 in β1A αIIb: KVGFFKR	IP, PD, EA		(176, 225)	Ser/Thr phosphatase
PTPN1	β3		IP		(17)	Tyr phosphatase
Rab21	α1, α2, α5, α6, α11, α16	GFFKR	Y2H, IP, O		(275, 331)	small GTPase
Rab25	β1		IP, PD		(72)	small GTPase
Rab34	β3		PD		(418)	small GTPase
RACK1	β1, β2, β5, α4, αV	β2: KALIHLSDLREYRRFEKEKL	Y2H, IP		(258, 498)	adaptor protein
Radixin	β2	CWKALIHLSDLREYRRF	PD, ELISA		(433)	
RanBP9	β2, β1		Y2H, PD		(105)	Ran GTPase binding
RNF181	αIIb	KVGFFKR	PD		(60)	ubiquitin ligase

SHARPIN	α1, α2, α5, αL, αM, αD	GFFKR	PD, IP		(143, 343, 359)	inhibitor of integrin activation (359)
SHC1	αVβ3, β1, β4	DTANNPL[pY]KEATSTFTNIT[pY]RGT; β4: Y1440 binds to Shc SH2, Y1526 binds to Shc PTB	IP, NMR, O	2L1C	(99, 108, 227, 243)	adaptor outside-in signaling (93)
Shp2	β4	Y1494	IP		(43, 478)	Tyr phosphatase
Skelemin	αIIb, β3	αIIb: VGFFKRNRPP β3: KLLITIHDR	Y2H, PD, NMR, B, O		(109, 337, 360)	
SNX17	β1, β3, β5, β6, β2	membrane-distal NxxY	PD, IP		(50, 314, 411, 442)	integrin recycling from early endosomes (411)
SNX31	β5, β6, β1, β2, β3	membrane-distal NxxY	PD, IP		(442)	integrin recycling from early endosomes (442)
SRC	β3	C-terminal RGT	XRAY, IP, AC, PD	4HXJ	(18, 469)	Tyr kinase
SYK	β2		IP		(448, 476)	Tyr kinase
Syntrophin 1	β5	KFNKSYNGTVD	O		(47)	
Talin1	IBS1: β1A, β1D, β2, β3, β5, β6, β7 IBS2: β1, β2, β3, β7	IBS1: β1A: NAKWDTGENPIYKS β1D: NAKWDTQENPIYKS β3: TIHDRKEFAKFEEERARAKWDTANNPLYKEA IBS2: β1: KLLMIIHDRREFAKFEKEKMNAK β2: KALTHLTDLREYRRFEKEKLKSQ β3: KLLITIHDRKEFAKFEEERARAK β7: RLSVEIYDRREYRRFEKEQQQLN	IBS1: XRAY, NMR, PD, B IBS2: PD, ELISA, B, O	IBS1: 1MK7	IBS1: (15, 65, 150, 237, 442) IBS2: (159, 290, 370)	integrin activation (66), adaptor protein, actin binding
Talin2	IBS1: β1A, β1D, β3	β1A: NAKWDTGENPIYKS β1D: NAKWDTQENPIYKS β3: TIHDRKEFAKFEEERARAKWDTANNPLYKEA	XRAY, NMR, ITC	3G9W	(15, 16)	adaptor protein, actin binding
Tensin-1	β1A, β3	β1A: KWDTGENPIYKS β3: KWDTANNPLYKE	B		(284)	actin binding
Tensin-2	β3, β5, β7, β1A		PD		(65)	actin binding
Yes	β3 β1. β2		IP, PD		(18, 19, 314)	Tyr kinase
α-actinin	β1, β2, β3	β2: RRFEKEKLKSQ	AC, IP, O		(318, 381)	actin binding (318)

Chapter 5. Proliferation and YAP/TAZ signaling

Proliferation

Integrins are usually considered to be cell-matrix receptors. This might be mistaken as a passive "gluing" to a substrate, offering a mere structural link to the cytoskeleton. Telling the story of the paxillin discovery, Christopher Turner described this as the "dogma of the time" for integrin-mediated adhesions (103). Fittingly, integrins possess no kinase domain, potentially enhancing this belief at the beginning of integrin adhesion research. Still today, integrins and integrin-mediated adhesions are sometimes just regarded as a structural link to actin und intermediate filaments. Nevertheless, there is no doubt about the signaling capacity of integrin-containing adhesive structures. Already early on, integrin-mediated adhesions were shown to have elevated levels of tyrosine phosphorylation in v-Src transformed cells (161). On the other hand, endothelial (286) and epithelial cells (147) undergo apoptosis after detachment from the ECM (i.e. anoikis). Both examples, anoikis and increased phosphorylation after v-Src mediated immortalization, highlight the link of integrin signaling to proliferation. Anoikis is regulated by a FAK-p53 signaling axis (259), while YAP/TAZ proteins regulate substrate stiffness-dependent proliferation (see below). Especially the YAP/TAZ pathway has attracted a lot of attention in the last years, as it forms a link between mechanical input and cell proliferation. However, it is interesting to note that the MRTF/SRF signaling pathway acts in parallel to and shares certain target genes with the YAP pathway (140). Additionally, the MRTF/SRF pathway targets also genes independently of YAP, which mediate a phenotype often associated with increased YAP/TAZ signaling (140, 188).

YAP/TAZ

As integrins are increasingly recognized as mechanosensors, we want to discuss the proliferative signaling of the integrin-dependent mechanotransducers YAP/TAZ in more detail. For many years, the co-transcriptional regulators YAP and TAZ were mainly associated with the Hippo signaling cascade controlling organ size *in vivo* and contact inhibition of proliferation in cell culture (322). More recently, YAP/TAZ also emerged as important mechanotransducers, sensing a variety of mechanical inputs and integrating them into output signals controlling proliferation and stem cell differentiation (116). Stiff substrates in 2D and 3D, large adhesive areas and increased blood pressure are examples of physical parameters that induce YAP/TAZ activity by increasing their nuclear localization (323). At the same time, active YAP/TAZ increases the expression not only of proteins driving proliferation, but also of focal adhesion and actin organization (305), establishing a positive feedback ensuring persistent YAP/TAZ

activation. Mechanistically, YAP/TAZ proteins were found to be inactive as long as they reside in the cytoplasm. This is the case, when they are Ser/Thr phosphorylated, leading to the formation of a complex with phosphoSer-binding 14-3-3 adapters (116). YAP/TAZ proteins are phosphorylated by the large tumor suppressor gene 1 and 2 (LATS1/2) as part of the canonical Hippo signaling pathway. Dephosphorylated YAP/TAZ instead is enriched in the nucleus, where it binds transcription factors like TEAD1 (305). This YAP/TAZ activation is supported by a β1 integrin-Src axis and potentially explains the proliferative effects of β1 integrin expression (376). However, the precise mechanism of YAP/TAZ activation remains controversial, as some groups found also integrin-independent cell adhesion on poly-L-lysine-coated substrates to cause YAP/TAZ activation (100, 499). These reports argued that the actin network integrity is necessary for nuclear localization of YAP/TAZ. Recent publications might now be able to reconcile these different findings: Elosegui-Artola and colleagues (121) found that mechanical stretching of the nuclear membrane opens nuclear pore complexes (NPCs). An increased diameter of NPCs then allows an easier entry of YAP proteins into the nucleus. This size-dependent mechanism of YAP transport was supported by experiments showing that increasing the size of the YAP protein by attachment of one or two GFP reduces its nuclear localization. The importance of mechanical force on the nucleus for YAP/TAZ activation was also shown by Shiu, Aires and colleagues (399). Their work indicated that an actin cap, spanning over the nucleus and thereby flattening it, applied mechanical force on the nucleus, leading to YAP activation. This actin cap relied on β1 integrin localization in the perinuclear region. Thus, it is evident that both β1 integrin signaling as well as actin integrity are needed for force application on the nucleus, which then facilitates the nuclear localization of YAP/TAZ. Apart from this mechanism, also other signaling cascades for YAP/TAZ signaling have been introduced recently. Meng and colleagues (285) showed that focal adhesions on soft substrates activate RAP2, leading to a deactivation of RhoA and activation of LATS1/2. As a consequence, YAP becomes phosphorylated and remains in the cytoplasm. Thus, separate pathways might work together to balance activation and inactivation of YAP/TAZ. However, as mentioned in the beginning, it is important to better analyze these pathways in order to understand the potential crosstalk with the MRTF signaling cascade. Interestingly, both YAP/TAZ and MRTF cascades involve integrin-mediated structural linkage and signaling, rendering these pathways fascinating examples of cellular signaling, where mechanical and biochemical inputs are sensed and integrated by integrins and their downstream targets.

Chapter 6: The concept of mechanosensing: linking integrin-dependent cell adhesions to signaling

Cells are in an active crosstalk with their surrounding environment. Cells integrated in a functional tissue receive signals not only from their neighboring cells, but also sense the global forces and metabolic state of a tissue. Although chemical signaling has highly important roles in cellular homeostasis, also physical signals including mechanical cues are essential for proper tissue functions. How do cells sense mechanical signals?

Integrin-mediated adhesions have a central role in cellular mechanosensing: they are physical links between individual cells and their surrounding extracellular matrix (419). Cell-matrix adhesions can thus be considered mechanical connectors. On the intracellular side, they are linked to the actomyosin machinery *via* the cell cytoskeleton, and on the extracellular side they are coupled to extracellular scaffolds formed by proteins such as fibronectin and collagen, containing specific attachment sites for integrin receptors (177, 458, 493). Cell-matrix adhesions are considered mechanosensitive, as their size, composition and signaling capacity are known to be affected by mechanical load and substrate stiffness (123, 366).

Integrins are among the most studied mechanosensory receptors. In cellular mechanosensing, a mechanical signal is received by a mechanoreceptor, which is capable of translating the signal into a chemical cue (82). The chemical signal may then affect cellular processes such as gene expression. This process is called mechanotransduction (79).

What do we know about the details of integrin-mediated mechanosensing? First of all, integrins have several different conformations, as discussed in Chapter 2. The regulation of integrin conformation is the first level of mechanosensing in cell adhesion (Figure 8). Although integrin conformation can be modulated by chemical factors, the full activation of integrins requires mechanical signals (208). However, as indicated in Chapter 2, it is still under investigation whether mechanisms of integrin activation found in well-studied integrins can be easily transferred to others. Therefore, we should not expect the same scheme of conformational regulation to be applicable for all integrin family members.

The second level in integrin-mediated mechanosensing is the integrin-ligand binding (Figure 8). While protein-protein interactions typically have a decreased lifetime under mechanical

load, the bond between fibronectin and integrins α5β1 and αVβ3 has been found to function as a catch-bond, becoming stronger when force is applied (146, 233, 291).

The third level of integrin-mediated mechanosensing relates to the intracellular adapter proteins (Figure 3, Table 1). The cytoplasmic domain of integrin acts as a ligand for several adapter proteins, including talin (177, 228), kindlin (371), sharpin (359), tensin (156) and a-actinin (329). These adapter proteins link integrins to the cytoskeleton, but also mediate cellular signals. Importantly, the adapter proteins also act as mechanosensory elements (for review, see (153, 194, 208)). The best studied example is talin, which connects to integrin *via* its N-terminal head domain and to F-actin *via* the C-terminal rod domain (169, 228). This exposes talin to mechanical load, facilitating conformational changes. As a result, cryptic binding sites for other adhesion proteins become exposed and the particular adhesion site is reinforced (160). Proposed force-regulated talin binding partners include vinculin (135, 160, 206, 247, 353, 482), DLC1 (178, 491), RIAM (170, 244, 477), and paxillin (384), but considering the size and complexity of talin, it is likely that additional force-regulated talin interactions exist (169, 301). As mentioned above, the situation for paxillin is unique, as it is recruited to the talin-rod by short helical elements positioned in its flexible N-terminal domain, that show affinity towards FAK and parvin as well as the GIT/PIX/PAK regulatory complex. On the other hand, tension-mediated paxillin recruitment to adhesions (384) is regulated at the level of the proximal talin-binding NPXY motif of the integrin (335), as well as the integrin-tail recruited kindlin (149, 437). While the mechano-dependent recruitment of paxillin to integrin-adhesions is still not fully understood, it illustrates well how the connectivity of different intracellular adapter proteins allows different signaling outputs in responses to mechanical perturbations.

Although these signaling outputs appear to create an on/off signal, it is also important that this mechanosensing system can be used in different cellular contexts, exhibiting largely different force regimes. Our recent study focusing on the talin rod revealed the tailoring of talin properties to have significant effects on cellular mechanosensing (353). We found mechanically weakened talin to decrease cellular traction force. Even more interestingly, we noticed the recognition of extracellular matrix proteins to be altered in cells expressing mechanically weakened talin compared to cells expressing the wild-type protein. Therefore, it appears that mechanical signals are instrumental in controlling the environmental sensing *via* integrins.

The mechanically induced conformational changes in intracellular proteins contributing to cell-matrix adhesion have been discussed in our previous review (208). We are only at the beginning to understand the mechanoregulation of adhesion signaling at the molecular level. However, the mechanisms are taking shape: i) opening of binding sites due to mechanical load (example: vinculin binding sites in talin rod (115, 160, 206), ii) disappearance of binding sites due to mechanical load (DLC1 – talin, (178, 252)), iii) phosphorylation of mechano-exposed regions within proteins (p130Cas) (385), iv) and proteolytic cleavage of partially unfolded proteins (4). Figure 8 aims to summarize these mechanisms. These regulatory events, combined with the high number of components involved, makes the creation of a comprehensive model of adhesion signaling challenging. In addition, new mechanisms still emerge as e.g. the competition between kank2 and actin for talin binding, leading to a modulated force transmission to integrins (420). The motor-clutch model emerged over the years as a promising framework to explore integrin dependent mechanotransduction (73, 123). In this model, molecular clutches link F-actin to the substrate and mechanically resist myosin-driven F-actin retrograde flow (73). This model might in future incorporate additional features to model real integrin-mediated adhesions more closely.

An increasing number of reports indicate that mechanotransduction on substrates of increasing stiffness follows a sigmoidal mechanosensitivity, creating a behavioral switch at a substrate stiffness around 5 kPa. Adhesion maturation, nuclear translocation of YAP or cell spreading are all suppressed on substrates below 5 kPa and increase from there within a narrow stiffness range to reach plateau levels. Roca-Cusachs and coworkers successfully linked this behavior to the motor-clutch model (122). Importantly, this on/off mechanoswitch also implies that studies on glass substrates, with a stiffness in the MPa range, might miss important changes in mechanotransduction occurring around the physiologically relevant 5 kPa stiffness range. According to the motor-clutch model, integrin-ligand affinity is a parameter that might shift the onset of mechanotransduction from 5 kPa to softer or stiffer substrates. This also implies that seemingly redundant integrin-ligand interactions on glass substrates might cause specific, differential effects at physiological stiffness values. We recently showed $\alpha V\beta 3$ integrin to have a higher affinity for vitronectin than for fibronectin, leading to differential mechanotransduction on the respective ligands (31). We envision that the increased interest in the mechanical regulation of integrins will lead to the discovery of further force- and stiffness-dependent integrin-ligand interactions. At the same time, it is clear that also integrin adapters are an intrinsic part of the mechanosensory machinery. Talin has several cryptic binding sites, that

open under mechanical stretch, and is therefore not only of interest as integrin activator, but also as mechanosensor and mechanotransducer (see above) (170, 353, 354). Interestingly, the mechanotransduction by talin is isoform-specific, with talin2 increasing the ability of cells to spread on 1-2 kPa substrates compared to talin1 (26). This difference can be attributed to the subdomains R1-R3 in the talin rod domain (indicated as yellow rectangles in Figure 6). Interestingly, several studies found these subdomains to be relevant for mechanosensing and force-dependent structural rearrangements. While talin is involved in integrin activation, it was demonstrated that also an integrin inhibitor, Thy-1, modulates mechanosensing (see Figure 3E) (136). Therefore, mechanosensing by integrins appears to rely on the proper balance between ligand binding and unbinding.

Chapter 7: Role of integrins in viral and bacterial infections

Integrins are best known as receptors contributing to cellular attachment. However, they also act as receptors for viruses and bacteria and are otherwise involved in pathogenic processes.

There are numerous known viruses exploiting integrins for their attachment to the cell, the virus entry into the cell as well as endosome escape (reviewed in (201)). Many viruses display an RGD sequence on their surface, which enables integrin binding. Adenoviruses utilize integrin αV for virus internalization (464). Binding of the adenovirus to integrin appears to induce a conformational change of integrin into an extended conformation; simultaneously a conformational rearrangement is observed also in the virus capsid (261). Similarly, integrin αVβ3 has been identified as a cellular receptor mediating both the cell adhesion and entry of Kaposi's sarcoma-associated Herpes virus into target cells (152). Another interesting group of viruses utilizing RGD-dependent integrins are enteroviruses, being among the most common human pathogens. Within the group of enteroviruses, only a handful of virus strains appear to utilize integrins in cell recognition. One of them is the coxsackie virus A9 (CVA9), which shows preferential binding to αvβ6 with a low nanomolar K_d (393). However, in this case integrin activation might actually not promote virus internalization (393). Among other RGD-possessing viruses, HIV utilizes α4β7 *via* the RGD tripeptide in the V2 loop of gp120 to infect the cell (23), and Ebola virus appears to bind α5β1-integrin for cell entry.

Viruses do not always depend on RGD to utilize integrins in their propagation cycle. Rotavirus infection was blocked with peptides containing the α4 integrin ligand sequences Tyr–Gly–Leu and Ile–Asp–Ala. These peptides eliminated virus binding to α4 integrins and infectivity (171).

Another non-RGD integrin-dependent virus is Ross River virus, which appears to utilize the collagen-binding integrin α1β1 (263). Some integrin-interacting enteroviruses do not contain RGD-like peptides, such as echovirus 1 (41). Interestingly, this virus appears to prefer binding to closed α2β1 integrin, and the inactivating integrin mutation E336A further enhanced this integrin binding (213). Echovirus 1 makes a large contact with the I-domain, with MIDAS site not being involved in binding (226). This virus does not depend on the integrin α-subunit during the later events of virus entry – the virus can infect cells even if the α2-tail is swapped or deleted (40). Notably, the binding of echovirus 1 appears to induce cellular signaling via focal adhesion kinase (379).

The natural tendency of integrins to cluster as a response to extracellular signals is complementary to the repetitive structure found in many virus capsids. For example, enteroviruses are ~30 nm in diameter, and contain 60 copies of VP1-VP4 capsid proteins. Therefore, the RGD sequences are displayed on the virus capsid almost perfectly in line with the density of clustered integrins observed in living cells: Changede et al. (75) reported ∼100 nm clusters containing ∼50 activated β3-integrins in the early adhesions under a wide variety of conditions on RGD surfaces. Thus, the regular and dense arrangement of integrin ligands on the virus particle offers a fascinating platform for the active integrin-mediated communication between cells and viruses.

The studies of Echovirus 1 have revealed, that virus binding can lead to integrin clustering without activation (213). Further, clustering of nonactivated integrins induces transient phosphorylation of FAK and paxillin in a PKCα-dependent, but talin-independent manner (379). These findings suggest that virus-induced clustering of integrins can activate FAK without conformational integrin activation.

Coxsackievirus B3 is the most viral cause of myocarditis (for review, see (130)). Activation of Akt during coxsackievirus B3 infection has been shown to take place through a PI3K-dependent pathway (127). Inhibition of integrin-linked kinase (ILK) activity and expression significantly blocked coxsackievirus B3-triggered Akt phosphorylation on Ser473 without effect on Thr308 phosphorylation. As a consequence, ILK inhibition lead to a significant decline in coxsackievirus B3 RNA transcription, viral protein synthesis, and virus progeny release.

Integrins are involved also in bacterial infections, and the following examples provide some insights into the mechanisms. A more extensive summary of bacterial species engaging integrins within the infection cycle can be found in a review by Hauck et al. (186).

Shigella bacteria cause shigellosis, a common intestinal infection leading to diarrhea and fever. Using CHO cells expressing integrin subunits, Watarai et al. showed that integrin α5β1 promotes the entry of the *Shigella flexneri* bacteria (455). They demonstrated IpaB, IpaC and IpaD proteins to bind to α5β1. Interestingly, *Shigella* appears to utilize also other components of the cell adhesion complex during its invasion, including vinculin (326) and ILK (224).

Staphylococcus aureus is a common cause for respiratory infections. Most clinical isolates of *S. aureus* express the fibronectin-binding proteins FnBP-A and FnBP-A (330). Binding of fibronectin by FnBPs is essential for the bacterial invasion, with fibronectin functioning as a bridging molecule linking FnBP to integrin $\alpha_5\beta_1$ (reviewed in (186)). This leads to the active intake of the bacteria, which does not require other bacterial factors, since even FnBP-coated polystyrene beads are internalized by cells (405).

Invasins are a class of bacterial proteins associated with the penetration of bacteria into cells. Isberg et al. showed that α3β1, α4β1, α5β1, and α6β1 all bind the *Yersinia pseudotuberculosis* invasin protein (210). The integrin-binding domain was mapped to a 192-aa C-terminal region of invasin that does not contain any RGD sequence (251). This integrin-binding domain was found sufficient to allow bacterial entry into mammalian cells (358). Later, beads coated with a larger invasin derivative comprising the C-terminal 497 amino acids were found to be internalized more efficiently than those bearing only 197 C-terminal residues. This seemed to be explained by the homomultimerization of the immobilized invasin fragment (106), suggesting a role of integrin clustering also in bacterial invasion.

The last example of integrin-bacteria-interaction demonstrates how bacterial cells can modulate tissue structure to support bacterial colonization. In some human tissues, the turnover rate of cells can be high, and e.g. the intestinal epithelium self-renewal is completed within 2–3 days (342). Slowing down this epithelial turnover can support bacterial colonization during an infection (212). *Shigella flexneri* can reinforce host cell adherence to the basal membrane *via* ILK (224). The interaction between ILK and the effector protein OspE increases cell surface levels of β1 integrin and suppresses phosphorylation of focal adhesion kinase and paxillin. As

a result, the reduced adhesion turnover and suppressed detachment of infected cells enables *Shigella* to colonize the epithelium. In a similar fashion, some bacteria such as *N. gonorrhoeae* can bind to human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), promoting enhanced host cell adhesion *via* integrin β1 activation (297).

In summary, integrins are important mediators of viral and bacterial infections, and integrin-mediated attachment, internalization as well as control over tissue integrity are central mechanisms in pathogenic processes. One may thus ask if integrins are employed more frequently as receptors for pathogens than other cell surface proteins. This is not straightforward to address: Although integrins are widely utilized by viruses and bacteria (280, 413), also numerous other known viral receptors are known (173). Potentially the intense study of integrins has lead to their frequent identification as virus receptors. In any case, it appears remarkable, that viruses, having a highly variable structure and shape, can exploit integrins in cell recognition. One such example is the utilization of integrins by adenoviruses and enteroviruses, two different classes of viruses with different evolutionary origin, showing both five integrin binding sites located in pentagonic assembly with spacing of ~60Å (reviewed by Stewart & Nemerow (413)). We therefore speculate that integrin clustering and integrin activation are suitable cellular mechanisms for the exploitation by infectious agents, and that the integrin-pathogen interactions thus offer potential targets for development of novel drugs.

Chapter 8: Integrins and diseases

Integrins are central for the integrity of the tissues, cellular adhesion and cell-matrix interactions, and it is therefore not surprising that several diseases are associated with defects in integrins. These integrin-related diseases are an active target for drug development, and a search with the term “integrin” revealed 151 studies in ClinicalTrials.gov, reflecting the importance of this research field. A significant portion of these studies (~30%) are focusing on integrin-targeting drugs. The first integrin-targeting drug entering the market 1994 was Abciximab (ReoPro), a 47 kDa Fab fragment against αIIbβ3 based on the monoclonal antibody developed by Coller et al. 1983 (91). This antibody also binds αvβ3 (430) and αMβ2 (403); it is used to prevent blood clots during the opening of blood vessels in the heart. Eptifibatide (Integrilin) is a cyclic heptapeptide derived from a barbourin protein found in the venom of the southeastern pygmy rattlesnake. This peptide targets αIIbβ3 and is used to reduce the risk of acute cardiac ischemic events (183, 435). It was launched in Europe 1999 and in USA 1998.

Tirofiban (Aggrastat) is small molecule inhibitor for αIIbβ3. It was approved in USA 2000 and in Europe 1999 for the treatment of acute coronary syndrome. Natalizumab (Tysabri) is a humanized monoclonal antibody against α4-integrin used in the treatment of multiple sclerosis (339) and Crohn's disease (167). Natalizumab is thought to prevent immune cells from crossing blood vessel walls to reach the affected organs. The most recent integrin-targeted therapeutic antibody accepted for clinical use is Vedolizumab (Entyvio), which is a humanized monoclonal antibody specifically binding to the α4b7 integrin and blocking the interaction of α4b7 integrin with mucosal addressin cell adhesion molecule-1 (407). This leads to inhibition of the migration of memory T-lymphocytes across the endothelium into inflamed gastrointestinal tissue (372). Vedolizumab is approved for treating patients with ulcerative colitis or Crohn's disease.

The following paragraphs provide insights into the importance of integrins and integrin activation in various diseases.

Integrins and skin diseases

Integrins are important for the integrity of the skin. Experiments with genetically manipulated mice have shown that deletion of α6 or β4 leads to disappearance of hemidesmosomes and the impairment of the epidermal adhesion to the underlying basement membrane (114, 155, 307). Even modest mechanical stress causes peeling of the epidermis from its underlying tissue in these animals. In humans the equivalent disease is known as junctional epidermolysis bullosa, and the associated genetic factors are reviewed in (185).

Mice deficient in α3 integrin have only a mild skin phenotype: the epidermis of α3 integrin-deficient mice has normal morphology and other characteristics (111, 192, 235). Also the hemidesmosomes of α3 integrin-deficient mice appear intact, and most regions of the basal membrane are coherent. The only defects observed in these animals are microblisters in the limb skin regions due to ruptures within the basal membrane.

As β1 integrin is essential for mouse development (132, 412), the role of β1 in skin epithelium has been studied using conditional knockout technology (56, 352). Mice with keratinocyte-specific knockouts for β1 integrin exhibit severe skin blistering and hair defects (352). In detail, a massive failure of basement membrane organization was observed, hemidesmosomes were instable and hair follicle keratinocytes failed to remodel the basement membrane. Also, Brakebusch et al. generated mice with a keratinocyte-restricted deletion of the β1 integrin using

the cre-loxP method (56). This resulted in hair loss and hair follicle abnormalities, and the epidermis of the skin became hyperthickened. The loss of β1 also caused a reduced α6β4 expression in basal keratinocytes and a decreased number of hemidesmosomes. Moreover, disruption of the basement membrane and blister formation were observed at the dermal-epidermal junction.

Integrins have also been a target for drug development for skin diseases. Efalizumab (Raptiva) is a recombinant humanized monoclonal antibody designed to treat autoimmune diseases (462), that was originally authorized for the treatment of psoriasis by EMEA 2004 and FDA 2003 (168). Efalizumab binds to the integrin αL subunit of the αLβ2 integrin specific for leukocytes (189). However, the marketing of efalizumab was suspended 2009 due to side effects such as Guillain-Barré and Miller-Fisher syndromes, encephalitis, encephalopathy, meningitis, sepsis and opportunistic infections.

While integrin activation by intracellular proteins is discussed elsewhere in this review, in the context of skin diseases, it is relevant to pinpoint the connection between kindlin and skin diseases. The best-known disease associated with kindlins is Kindler syndrome, involving the loss of kindlin-1 expression in epidermis (402). Gene knockout of *Fermt1*, the gene encoding kindlin-1, in mice caused skin atrophy and lethal intestinal epithelial dysfunction (444).

Cancer

Integrins have a central role in cancer, as reviewed previously (107, 374), and it is not possible to give a comprehensive overview within this article. Instead, we provide here selected examples of the importance of integrin activation in cancer.

Felding-Habermann et al. (134) demonstrated activated integrin avβ3 mutant D723R to support tumor cell arrest in the circulation through the interaction with platelets. This activated αvβ3 was found to be expressed by metastatic human breast cancer cells, leading to metastatic lesions. Expression of the constitutively activated integrin mutant αvβ3-D723R promoted metastasis in a mouse model. These results support a model where breast cancer cells can exhibit a platelet-interactive and metastatic phenotype controlled by the activation of integrin αvβ3. Beside this mechanism, αvβ3 integrin might have additional and parallel modes of action supporting cancer progression and metastasis. Several studies indicated that αvβ3 integrin and

VEGF receptor signaling act synergistically to promote angiogenesis (101, 274), an important part of tumor progression. This link between αvβ3 integrin and VEGF signalling motivated clinical trials with Cilengitide, a specific inhibitor of αvβ3 and αvβ5 integrin. Also glioblastoma express αvβ3 integrin, in contrast to the healthy surrounding tissue, making αvβ3 integrin inhibition a promising strategy for the treatment of this cancer. Accordingly, orphan designation for Cilengitide (EU/3/03/184) was granted by the European Commission on 14 January 2004 to Merck KGaA, Germany, for the treatment of glioma. Unfortunately, the drug was later removed from the market, as the phase 3 study showed no positive effect in glioblastoma treatment (415). A mechanism explaining the failure of Cilengitide in this clinical trial is still missing, although it was recognized later on that a low dosing of Cilengitide activates angiogenesis in mice rather than preventing it (362). This surprising effect might be explained by systemic effects, but the partial agonism of high-affinity integrin inhibitors like Cilengitide might still challenge this therapeutic strategy. In fact, we recently showed that the correlation of the different ectodomain conformations of integrins with immobilized ligand binding is still challenging. We revealed that αvβ3 integrin binds vitronectin in the extended-closed conformation, but fibronectin only in the extended-open conformation (31). Thus, rational drug design could benefit from further research efforts focusing on integrin-ligand interactions that take into account the conformational flexibility of the integrin ectodomain. Furthermore, this research should be performed appreciating the non-redundancy of these interactions.

Chronic myeloproliferative neoplasia comprises several sub-entities, including polycythemia vera (PV), essential thrombocytosis (ET), primary myelofibrosis (PMF) and others (436). Chronic myeloproliferative neoplasia caused by the V617F mutant of Janus kinase 2 (JAK2) commonly displays abnormal integrin expression on platelets, erythrocytes and leukocytes (118). A recent study found JAK2-V617F to trigger constitutive activation of the integrin inside-out signaling molecule Rap1, resulting in translocation to the cell membrane (118). In transgenic mice expressing this JAK2-V617F protein in hematopoietic cells $JAK2^{+/VF}$ granulocytes showed increased binding of 9EG7 antibody, indicating the conformational activation of β1 integrins. Moreover, increased expression of both β1 and β2 integrins was observed (118). The researchers also demonstrated the neutralizing anti–α4 (anti-VLA-4) and anti–β_2 integrin antibodies to suppress the pathologic thrombosis observed in $JAK2^{+/VF}$ mice. Additionally, the aberrant homing of $JAK2^{+/VF}$ leukocytes to the spleen was inhibited by the neutralizing anti-β_2 antibodies or alternatively pharmacologic inhibition of Rap1. These findings suggest that JAK2-V617F, a mutation commonly associated with myeloproliferative

disorders (214), leads to integrin activation, promoting pathologic thrombosis and abnormal trafficking of leukocytes to the spleen (118).

Cancer-associated fibroblasts (CAFs) are the most abundant cells in a tumor (216). Attieh et al. found that CAFs isolated from the tumor of colon cancer patients secrete and assemble fibronectin more efficiently compared to noncancer-associated fibroblasts from the neighboring healthy tissue (25). Importantly, the amounts of secreted and assembled fibronectin correlated with the invasion index of the tumor. This study suggests mechanical signals to be important for CAF-mediated cancer cell invasion, as they induce fibronectin assembly. The authors propose that contractility of CAFs is necessary for downstream activation of the integrin-αvβ3 and assembly of fibronectin puncta. α5β1 becomes critical only at later stages of fibronectin assembly. In summary, Attieh et al. (25) revealed that fibronectin-depositing CAFs enable cancer cells to invade the matrix and this process is independent of matrix metalloproteinases.

LAD-III: Kindlin-3 is essential for proper integrin activation

A rare autosomal leukocyte adhesion deficiency syndrome called LAD-III is characterized by severe bleeding and impaired adhesion of leukocytes to inflamed endothelia. A hallmark of this recessive disease is the impaired activation of β1, β2 and β3 integrins on platelets and leukocytes (12).

Malinin et al. described a kindlin-3 point mutation causing serious bleeding, frequent infections and osteopetrosis at an early age (277). They found the symptoms to be caused by an integrin activation defect in hematopoietic cells, including platelets and leukocytes. The lymphocyte cell line established from the patient was phenotypically rescued by expression of wild-type kindlin-3, proving the association of the disease to the defective kindlin-3. The inactivation of kindlin-3 was caused by a point mutation creating a premature stop codon at the amino acid position 16. Importantly, all the clinical symptoms of the subjects were resolved by an allogenic bone marrow transplantation. Also another study by Svensson et al. (421) found a link between kindlin-3 and LAD-III, identifying two independent mutations causing decreased *KINDLIN3* messenger RNA levels and loss of protein expression.

During the last years it has become evident that integrins are not only activated by talin, but also in coordination with kindlin binding to the second NxxY motif (335). As kindlin-3 is expressed exclusively in hematopoietic cells, it is clear now that LAD-III is caused by

insufficient integrin activation due to the lacking kindlin contribution. Moser et al. 2008 (295) found kindlin-3 to interact with β1 and β3 integrin tails both in the presence and absence of talin-1, with the F3 subdomain of kindlin-3 being sufficient for this direct interaction. Later, Moser et al. 2009 (294) showed that kindlin-3 binds the cytoplasmic domain of β2 integrin. They proposed kindlin-3 to be essential for neutrophil adhesion and spreading on β2 integrin-dependent ligands such as intercellular adhesion molecule-1 and the complement C3 activation product iC3b.

Autosomal dominant polycystic kidney disease

Integrins are highly important for the kidney development, and a comprehensive overview on the phenotypes associated with various integrin mutations in mouse models has been provided by Mathew et al. (282).

Autosomal dominant polycystic kidney disease (ADPKD) is a disorder involving the development of bilateral renal cysts, accumulation of extracellular matrix and tubulointerstitial fibrosis (440). The disease is caused by the mutation of the *PKD1* or *PKD2* genes encoding polycystin-1 (PC1) or polycystin-2 (PC2), respectively (184). Interestingly, the cystic epithelia express higher levels of integrins (246).

A recent study (246) showed that depletion of PC1 in immortalized renal collecting duct cells elevated the levels of integrin-β1 and fibronectin and displayed increased integrin-mediated signaling in the presence of Mn^{2+} compared to wild-type cells. Conditional inactivation of integrin-*β*1 in collecting ducts of mice resulted in a dramatic inhibition of *Pkd1*-dependent cystogenesis with a concomitant suppression of fibrosis and preservation of normal renal function. These results suggest functional integrin-*β*1 to be required for the early events leading to renal cystogenesis in ADPKD.

Integrins and liver diseases

Chronic injury in liver is characterized by intense production of collagens and other ECM components accompanied with their decelerated degradation, leading to net matrix accumulation (reviewed in (389)). Several clinical studies have found changes in integrin expression to be associated with chronic liver diseases. Nejjari et al. (306) studied patients with chronic hepatitis C and found an increase in β1 labeling intensity in 83 out of 94 patients (88.2%). Moreover, also expression of α1, α5 and α6 integrins were pronounced (306). Popov

et al. (340) extracted total RNA from explant livers of patients undergoing liver transplantation. They found β6 integrin mRNA transcript levels to be significantly elevated in patients with chronic hepatitis B and C, primary biliary cirrhosis, primary sclerosing cholangitis and alcohol-induced liver injury. This suggests integrin overexpression to be an etiology-independent factor associated with liver fibrosis.

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease involving accumulation of lipids, cell debris and extracellular matrix proteins as well as monocyte-derived macrophages at the inflamed vascular wall. The M1/M2 macrophage ratio can be considered a determinant of plaque stability (reviewed in (325)). A study by Cho et al. revealed that M1 macrophages are exclusively found in plaques of symptomatic patients and elevated in unstable plaques (87). Aziz et al. (27) studied the role of αDβ2 integrin in atherosclerosis using both mouse and human samples. In this study the retention of macrophages was linked to a significant upregulation of integrin αDβ2 in M1 macrophages *in vitro* and in macrophages in atherosclerotic lesions. The findings suggest that αDβ2 contributes to the development of chronic inflammation *via* regulation of macrophage migration. Interestingly, our study focusing on human arterial plaques showed upregulation of integrin β2, while all other studied integrins (ITGA1, ITGAV, ITGB3, ITGB5) were downregulated (315).

Integrins and smoking

Pulmonary emphysema, largely attributable to tobacco smoke exposure, is a worldwide challenge. Morris et al. observed elevated expression of matrix metalloproteinase 12 in mice lacking the integrin αvβ6, that developed progressive spontaneous emphysema. The emphysema was prevented by transgenic β6 integrin expression, which, however, was dependent on the ability of β6 to bind and activate latent TGF-β. Importantly, the pathological characteristics of mice lacking αvβ6 integrin resemble those observed in young cigarette smokers (310).

Overbeek et al. (319) studied the effect of cigarette smoke on neutrophil migration and β_2-integrin activation. CD11b-expressing neutrophils appeared in the lungs of mice after exposure to cigarette smoke for 5 days. To interpret this finding further, they exposed freshly isolated human neutrophils to cigarette smoke extract (CSE). CSE activated αMβ2 on the neutrophils, leading to firm adhesion to fibrinogen. In response to CSE the neutrophils transmigrated

through endothelium *via* the activation of β_2-integrins, and the functional block of CD11b and CD18 decreased this transmigration.

Although e-cigarettes could be considered a "healthy" choice compared to traditional cigarettes, a recent study revealed that platelets from e-cigarette-exposed mice are hyperactive, show enhanced aggregation and granule secretion. Importantly, also these platelets showed increased activation of the αIIbβ3 integrin (351).

Chapter 9: Integrins and TGF-β activation at the onset of fibrosis

In recent years cancer cells were demonstrated to cooperate extensively with cancer-associated fibroblasts, shaping the tumor microenvironment (67). These CAFs rearrange the tumor-surrounding ECM, increase its stiffness and promote cancer cell invasion (25, 67). This pathological ECM stiffening, called fibrosis, is not restricted to cancer, but rather constitutes a pathology of its own. In a simplified way fibrosis can be described as excessive scar formation. While scars preserve the mechanical integrity of the tissue, they fail to support normal organ function, such as gas exchange in the lung or the beating of the heart. Ultimately fibrosis can lead to organ failure and death and is estimated to contribute to more than 40% of deaths in the developed world (468). The onset of fibrosis is characterized by the transformation of cells into contractile myofibroblasts, mediated by signaling molecules of the transforming growth factor β (TGF-β) family (190). Interestingly, cells do not secrete TGF-β in a soluble form, but rather as a complex with the latency associated peptide (LAP). This LAP/TGF-β complex is anchored to the ECM *via* latent TGF-β binding protein (LTBP), preventing TGF-β from binding to its receptor (190). The release of TGF-β from this complex requires the binding of integrins to an RGD sequence in LAP and mechanical load on the ECM-(LAP/TGF-β)-integrin axis (299). In fact, replacing the RGD sequence in LAP by RGE prevents integrin binding to LAP and in mice leads to a phenotype comparable to a complete TGF-β knockout (481). This highlights the importance of integrin binding in the process of TGF-β release and subsequent transformation of cells into myofibroblasts. For the same reasons, integrins are also interesting drug targets for the treatment of fibrosis, where complete TGF-β inhibition causes too many adverse side effects (240).

A survey of the literature suggests that the complete group of αV integrins, including αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8, binds to LAP. However, TGF-β activation appears to be mediated only by αVβ8 integrin and especially αVβ6 integrin (368), while a recent work indicates that also αVβ1 integrin might be relevant (361). Whether these integrins compensate each other or whether they act in a tissue- and development-specific context remains to be explored. Dong and colleagues (113) published structural data for the binding of LAP/TGF-β to αVβ6 integrin and revealed that the binding interface of αVβ6 integrin and LAP/TGF-β is highly interdigitated and larger than other integrin-ligand interfaces. This spatial arrangement of integrin and ligand might help to ensure the proper alignment of the force with the TGF-β activation axis, when mechanical load is applied to the complex. Additionally, the unusually large interface between αVβ6 integrin and LAP/TGF-β might help to increase the mechanical stability of the integrin-ligand bond. It also explains why β3 and β5 integrins cannot be receptors for the LAP/TGF-β complex. Thus, the binding of αVβ6 integrin and LAP/TGF-β offers an example of a specific structural and molecular arrangement supporting biological functions with consequences for the complete organism.

The importance of integrins in fibrosis is not limited to TGF-β activation. The interplay of physical and biochemical parameters in fibrosis increased the interest in this pathology as a promising *in vivo* example for the relevance of mechanobiology. Many healthy tissues have a stiffness (measured as Young's modulus E) below 5 kPa, but exceed this value during fibrosis. For example, lung tissue switches from 2 kPa in a healthy state up to 17 kPa in fibrosis, while liver stiffness increases from below 1 kPa and less up to 12 kPa and more (461).

As discussed in Chapter 6, mechanotransduction for many processes is regulated by an on/off-mechanoswitch that is triggered around a substrate stiffness of 5 kPa. Therefore, fibrotic tissue stiffness above 5 kPa might cause a constant on-switch of YAP/TAZ-dependent proliferation, contributing to the positive feedback loop of YAP activation and tissue stiffening (67).

Chapter 10: Outlook and perspectives

Starting with Abercrombie (1), the research of integrins and integrin-mediated adhesions has a history of 40 years. We are not aware of many other fields in biology that attracted so constant interest for such a long time. One reason might be the technical accessibility of integrin-

mediated adhesions, making it an easy target for the newest microscopic techniques or proteomic studies. At the same time, integrins and cell matrix adhesions proved repetitively that they are not passive 'gluing' structures fixing cells to the ECM. Instead, they are tightly regulated, integrating many intracellular and extracellular signals to create very diverse cellular processes ranging from adhesion, migration or ECM organization to proliferation. The multitudes of direct integrin-adapter interactions in the cell (Table 1 and Figure 4) reflect these diverse integrin functions. This complexity of integrin-mediated signaling clearly justified the ongoing interest in cell adhesion over the years. Whether the current interest in integrins and cell adhesions will last for another 40 years is hard to predict. But we feel that there are plenty of open questions remaining:

Integrin-mediated mechanosensing

We highlighted already the relevance of mechanical integrin regulation (Chapter 6) additional to the established influence of biochemical signaling. This is currently a vibrating area of research, and we expect the mechanobiology of cell adhesions to also have a profound impact on the development of integrin-targeted therapies (Chapter 8). The increasing number of examples for mechanical regulation of integrin conformation and thereby integrin activation (Chapter 2) imply, that this mechanical regulation has to be considered in the development of drugs targeting cell adhesion. For example, a drug binding to only one integrin conformation or a drug leading to allosteric head-piece opening may be more difficult to use in the clinic than a drug that acts as an integrin antagonist, blocking the conformation of integrins in the extended closed conformation upon binding (5). Alternatively, mechanical forces on integrins can affect its conformation and by this the binding affinity of the drug. Such an example has been documented for imatinib (Gleevec), which suppressed c-Kit kinase activity when stimulated with a soluble Kit-ligand, but failed to inhibit c-Kit signaling mediated by a mechanically anchored Kit-ligand (424). We assume that mechanical forces on the ligand-bound c-Kit rendered the binding pocket unavailable for imatinib, in contrast to the situation with soluble Kit-ligand, where the competitive inhibitor imatinib could bind and inhibit, c-Kit. Other examples include bacterial adhesins, giving rise to fibronectin-binding peptides that recognize only relaxed fibronectin fibrils (20). Such a load-dependent binding might be disadvantageous in a therapy, limiting the activity of the drug or antibody. At the same time, it is clear that integrins are a promising and powerful drug target, with successful therapies for thrombosis and emerging therapies for multiple sclerosis and other immune system-related diseases existing (165). In fact, immune suppression by targeting $\alpha4\beta1$ integrin (natalizumab) or $\alpha L\beta2$

integrin (efalizumab) is too effective, causing progressive multifocal leukoencephalopathy by activating the human polyomavirus JVC in some patients (276). However, conformation- and/or mechanical load-specific therapies might one day offer a less toxic, but more selective approach in therapies targeting cell adhesion.

Integrins and therapies

The safety and ultimate clinical success of integrin-targeting drugs remains still to be evaluated (Chapter 8). Nevertheless, it is indisputable that the failure of the αVβ3 integrin inhibitor cilengitide in phase III trials (including Merck giving up on further trials for this inhibitor) dampened the enthusiasm about chemotherapies targeting integrins. Are there more general, fundamental problems with drugs targeting integrins? Above, we mentioned the influence of mechanical forces on the integrin-ligand binding pocket and potential consequences for drug development. However, we also tried to raise awareness for the specificity of integrins, their ligands, and their adapters throughout this review. In other words, we might just not know enough about integrins for a faithful generalization. Structure-function relationship (Chapter 2), RIAM-dependent vs. -independent integrin activation (Chapter 3), reticular adhesions vs. focal adhesions (Chapter 3), or the relevance of αVβ1 integrin in fibrosis (Chapter 9) are examples where established findings were recently challenged. Kindlin as integrin activator is much less understood than talin, kank is still emerging as potentially important adapter, and all of them have isoforms that withstood evolutionary selection while we tend to ignore them to keep things simple. But maybe we still have to add more trees before we can clearly see the forest. In the end, integrin targeting therapies are most successful in intensively studied systems such as platelets and integrins in the immune system.

Cell adhesion and metabolism

The research of metabolism seems to expand very rapidly at the moment, and it is linked to an increasing number of topics including integrin research and the interplay between cell metabolism and cell adhesion. This is strikingly demonstrated by the effects of diabetes on ECM and integrin organization (21). Interestingly, the metabolic sensors mTOR (355) and AMPK (156) are both shown to act *via* tensins on fibrillar adhesions and endocytosis, thus organizing the ECM (Chapter 3). This link between integrin-dependent ECM organization and metabolism might also explain the correlation of fibrosis (Chapter 9) and obesity. Moreover, there are first indications that integrin and integrin adapters are targets of metabolism-

dependent posttranslational modifications like acetylation (89, 460), potentially establishing a close link between metabolism and cell adhesion.

Funding sources and acknowledgements

The authors are grateful to funding by the Swiss National Science Foundation, the Swiss Cancer League, the Swiss Society for Muscle Research to BWH, as well as grateful to the Academy of Finland which supported this research *via* grants 290506, 273192 and 136288 to VPH. We thank Dr. Steffi Goffart (University of Eastern Finland) for reading the manuscript and for her helpful comments. The authors also thank the current and previous group members, colleagues and coauthors for fruitful discussions and their significant contributions.

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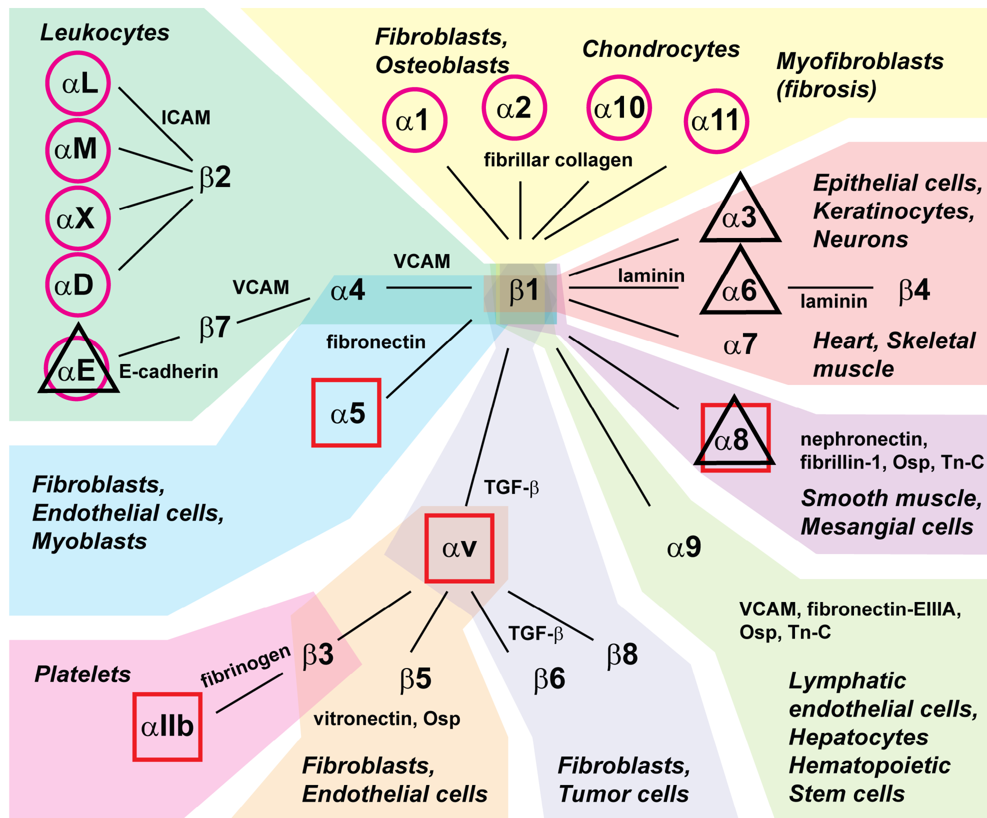
Figures and figure legends



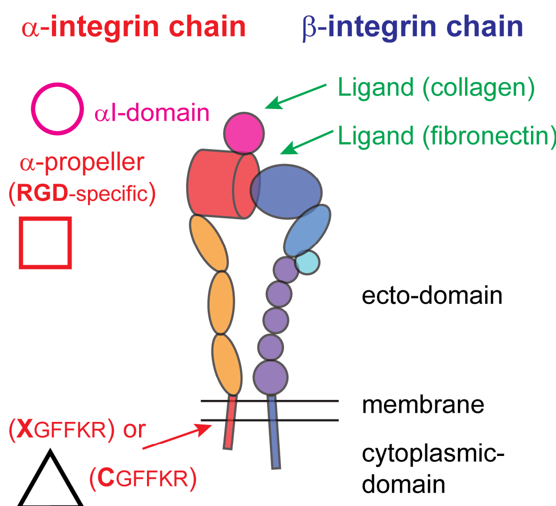


Figure 1 **Integrins, their ligands, and cellular distribution.**
As reviewed by Hynes (129), integrins are organized in 24 different heterodimers, indicated by the link between α- and β-subunits in the figure. Integrins can be classified by structural features, their ligands, and their tissue and cellular expression. Based on these criteria, we grouped integrins into nine classes indicated by the color of their background. Typical cell types expressing the respective integrins are mentioned, as well as common ligands for these integrins. We also highlighted integrins with an α-I domain (I; purple circle), those binding to RGD ligands (RGD; red square), and those with changes to the conserved GFFKR sequence in the membrane-proximal part of the α-subunit (black triangle indicates integrins with sequence

deviating from **C**GFFKR). The integrin cartoon in the lower part of the figure gives an overview about the integrin structure and is reused in the following figures. It also indicates the location of the αI domain and the GFFKR sequence in the respective integrins. Please note that αI domain integrins bind ligands (e.g. collagen) *via* this I-domain, while other integrins bind ligands (e.g. fibronectin) in binding pockets formed by both α- and β-subunits.

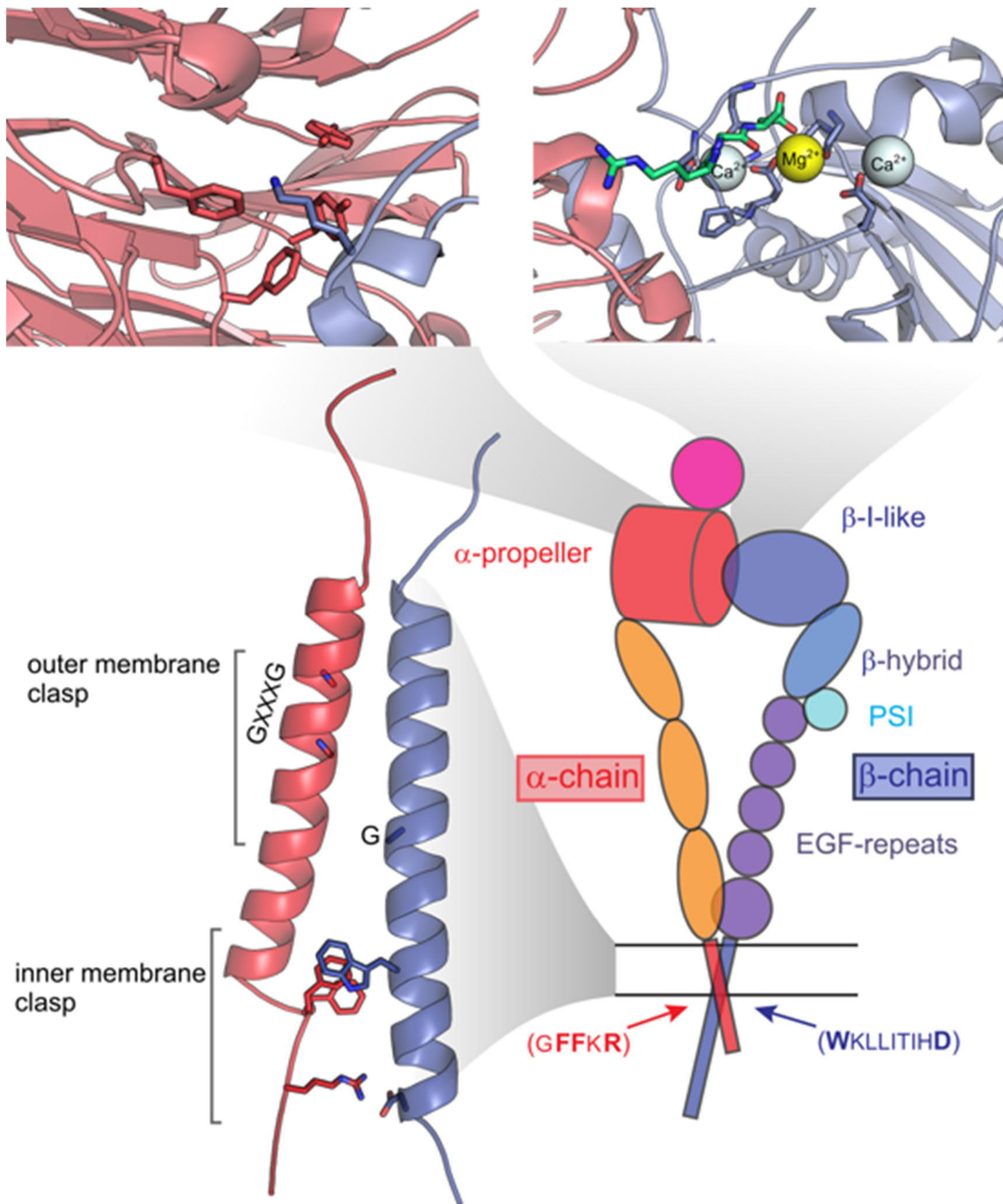


Figure 2 **Structural features of the integrin heterodimer.**

Integrins consist of α- and β-subunits, and both of the subunits are membrane anchored. The integrin subunits non-covalently associate in the extracellular domain *via* the insertion of a conserved lysine residue of the β-I-like domain (blue) into the aromatic core of the α-propeller domain (red) (upper left; respective amino acids are indicated by their structure instead of a cartoon-like representation), locking the subunits tightly together. There are also more dynamic interactions occurring at the transmembrane (outer and inner membrane clasp) and juxtamembrane regions (inner membrane clasp) of the two subunits. Conserved glycine-motifs in both transmembrane domains allow the tight association of the transmembrane domains at the outer membrane clasp, while the inner membrane clasp is stabilized by aromatic interactions. Sequence on the lower right: α-subunit: **FF**; β-subunit: **W**) and a juxtamembrane salt bridge. Sequence on the lower right: α-subunit: **R**; β-subunit: **D**); both interactions are highlighted on the lower left by showing the respective structures of the amino acids.

The cytoplasmic domains contain binding epitopes for a number of proteins, discussed further in Figure 4. The interface between the α-propeller in the integrin head piece and metal ions bound to the β-I-like domain, form a binding site for integrin ligands such as RGD (upper right zoom-in; metal ions from left to right: SyMBS (synergistic metal binding site), MIDAS (metal ion-dependent adhesion site), ADMIDAS (adjacent MIDAS)). In contrast, integrins with an α-I domain in the α-subunit bind their ligand only with the α-I domain (purple circle in the central cartoon; see also Figure 1). Ligand affinity is modulated by the coordination of the metal ions that includes amino acids of the integrin as well as the ligand (see Fig. 3C for details). Structural information regarding the lysine insertion into the α-propeller is from PDB 3VI4 (α5β1 integrin (302)), for the MIDAS site from PDB 3ZE2 (αIIbβ3 integrin (501)), and for the transmembrane domains from PDB 2K9J (αIIbβ3 integrin (242)).

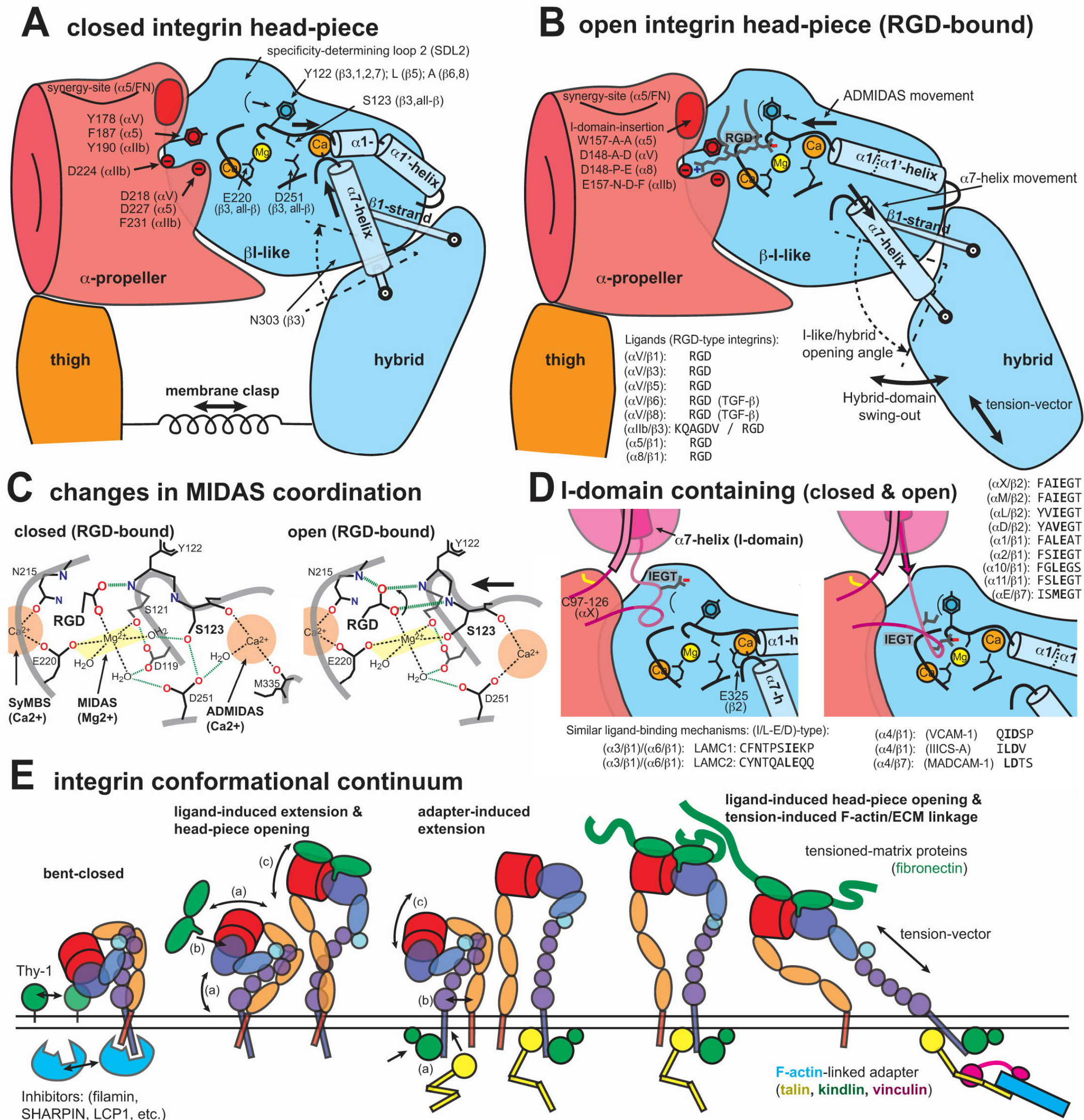


Figure 3 **Ligand-induced conformational changes and sequence specific variations in the integrin ectodomain.**

Ligne claire drawing of a prototypical integrin headpiece and mechanisms of atomic (C), small (A,B,D) and large (E) scale conformational changes. A) Closed, ligand-free state of the integrin headpiece stabilized by the ADMIDAS (adjacent to MIDAS; see also Figure 2) Ca^{2+} ion, linking the N-terminal end of the "broken" or "bent" α1/α1'-helix with the β6-α7helix loop, by coordination (large, bold arrows). In the closed conformation/state, the α7-helix (piston) is tethering the hybrid-domain to form a small βI-like/hybrid-domain angle. Experimental N-glycosylation at N303 (β3) has been used to prevent the formation of the closed integrin head-piece (90, 272). The closed head-piece conformation is further maintained by flexible associations of the lower leg domains (see E) by the membrane clasp, as indicated by a spring. Residues relevant for integrin isoform specific RGD-ligand binding of the α-propeller domain are shown by arrows (see also Figure 5). Conserved residues involved in MIDAS (metal ion depending adhesion site) coordination are labeled according to human β3 (small arrows). Ligand binding specificity in the β-chain is achieved by the

specificity-determining loop 2 (SDL2) and residue Y122 (small arrows). For example, SDL2 makes contact with the internal ligand "**I**EGT" in αI-domain containing integrins (D)(αXβ2; (391)), with MadCAM in α4β7 (489), or with the RGD**L** motif in the TGF-β binding integrins αvβ6 and αvβ8, while simultaneously avoiding steric clashes with TGF-β due to an Ala-residue at the Y122 position (113, 321)(see Fig. 5B). Integrin headpiece opening is induced by the Asp-residue binding of RGD at the MIDAS site, which leads to the movement of the ADMIDAS ion and α1-helix straightening towards the MIDAS Mg^{2+} ion (large arrow), as well as progressively stronger association of the Arg-side chain of the RGD peptide with the α-domain. ADMIDAS, S123 side-chain and backbone movement towards the MIDAS ion are linked to a rotation of the carboxyl-group of the RGD, Asp-residue and stabilization by H-bonds (thick dotted green lines in C). Due to this movement, the β6-α7 loop detaches from the ADMIDAS Ca^{2+} ion and induces the piston-like downward shift of the α7-helix, pushing the hybrid domain outwards. The hybrid domain swing-out and opening of the interdomain angle is facilitated by a flexible domain connection at the N-terminus of the β1-strand of the β-I-like domain. RGD-ligand binding and cytoplasmic adapter-mediated link to the actin cytoskeleton result in a tension vector parallel to the hybrid-domain (see also E). C) Close-up view of the SyMBS , MIDAS, and ADMIDAS coordination and their changes during integrin opening. In the closed state, the incoming RGD-ligand coordinates with the MIDAS ion, forming a single H-bond with the backbone amine of Y122. Rotation of the carboxyl side-chain establishes additional H-bonds that progressively induce the movement of S123 and backbone-associated ADMIDAS Ca^{2+} ion to directly coordinate MIDAS or D251, respectively. These changes in MIDAS and ADMIDAS coordination further stabilize RGD-ligand coordination as well as the detachment of the β6-α7-loop to induce α7-helix pistoning. D) I-domain-containing integrins show a similar mechanisms of acidic residue binding at the MIDAS (internal ligand peptide: **I**EGT), but an additional hydrophobic interaction at a preceding Ile-residue ((391), PDB: 4NEN). A similar binding mode, centered around a glutamic acid or aspartic acid residue, has been proposed for laminin-binding integrins (348, 429), as well as for α4β1-binding of the LDV-motif in the IIICS alternatively spliced domain of fibronectin, and the I/L-D-T/S consensus sequence in MAdCAM-1 and VCAM-1 binding to α4β1/7 (489). However, and in contrast to RGD-binding integrins, the binding groove of α4-binding integrins is vertical, binding parallel to the smooth, ligand-facing α-propeller surface for the latter integrins (D). E) Ligand-induced conformational changes in the integrin head-piece are proposed to be associated with the head-piece opening of the integrin. The ligand can be external (e.g. fibronectin) or, in the case of the αI-domain containing integrins, be the αI-domain serving as an internal ligand for the integrin headpiece (289). However, physiological ligands might also be recognized in cis, as proposed for Thy-1 (136) or β2 integrins and ICAM (131). Alternatively, Adair *et al.* have demonstrated FN type III9-10 binding to the bent conformation of αvβ3 (3). For α5β1, the synergy site on the α-propeller surface enhances the FN on-rate (302). A recent survey of integrin ectodomain conformations by electron microscopy demonstrated that the β1-class of integrins are more extended in the inactive state compared to αvβ3 (289). This, however, underlines the importance of the intracellular integrin adapters, as they can either prevent integrin activation, like in the case of sharpin, filamin and LCP1 (264, 359, 441), or induce leg separation and integrin extension (484), that should subsequently facilitate ligand binding, and full head-piece opening. Note that (a) to (c) indicate a temporal order. An ultimate regulation of the open head-piece conformation could be achieved by mechanical tension, changing the conformational equilibrium of the bound integrins (31, 347, 422).

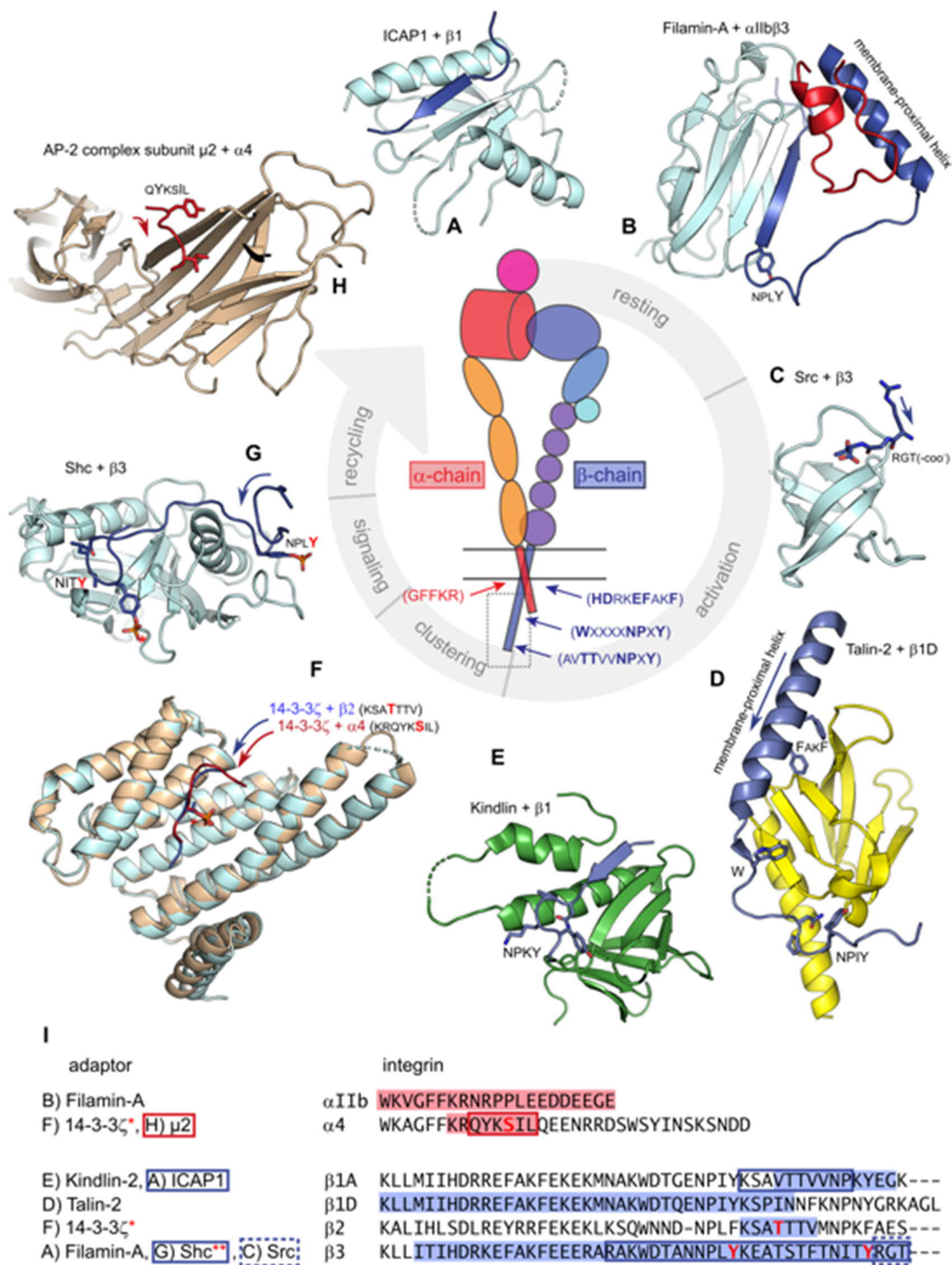


Figure 4 **Structural features of integrin ligand binding – intracellular ligands.** Cytoplasmic integrin tails comprise only a short part of the complete integrin, but interact with a wide number of adapters (or intracellular ligands; see also Table 1). Here, structures of integrins binding to cytoplasmic adapters are shown together with the amino acid sequence of integrins highlighted in red (α-subunit) or blue (β-subunit), indicating the involved amino acids in the binding of the respective adapter. Integrin inhibitors like ICAP or Filamin compete with integrin activators like kindlin or talin for binding to the same domains of the cytoplasmic integrin tail (compare structure A to structure E and structure B to structure D).

Kindlin binding arranges the membrane-distal part of the β-integrin tail, potentially priming the integrin for talin binding (30). Talin binding to the membrane-proximal part facilitates the separation of α- and β-subunit (380) and thereby integrin activation. The respective sequences in the β-subunit (indicated with blue letters) are highly conserved among integrins. Inactive integrins are stabilized by the interaction of a conserved sequence in the transmembrane part of the α-subunit (GFFKR) with the β-subunit. Integrin inactivation can occur by Tyr-phosphorylation of the NPXY-motifs, or alternatively by Ser/Thr-phosphorylation of the inter-NPXY-region, which reduce talin and kindlin association (14, 45), while allowing regulatory and recycling adapter recruitment to the integrin receptors. Alternatively, integrins can be internalized bound to their extracellular, but proteolytically fragmented ligands (see Chapter 3; (142, 293, 328)). A) ICAP1 + β1 integrin: PDB 4DX9 (267). B) Filamin-A + αIIbβ3 integrin: PDB 2MTP (264). C) Src + β3 integrin: PDB 4HXJ (469). D) Talin2 + β1D integrin: PDB 3G9W (16). E) Kindlin2 + β1A integrin: PDB 5XQ0 (253). F) 14-3-3ζ + β2 integrin (cyan and blue): PDB 2V7D (428); 14-3-3ζ + α4 integrin (light brown and red): PDB 4HKC (48). G) Shc + β3 integrin: PDB 2L1C (108). H) AP-2 complex subunit μ2 + α4 integrin: PDB 5FPI (141).

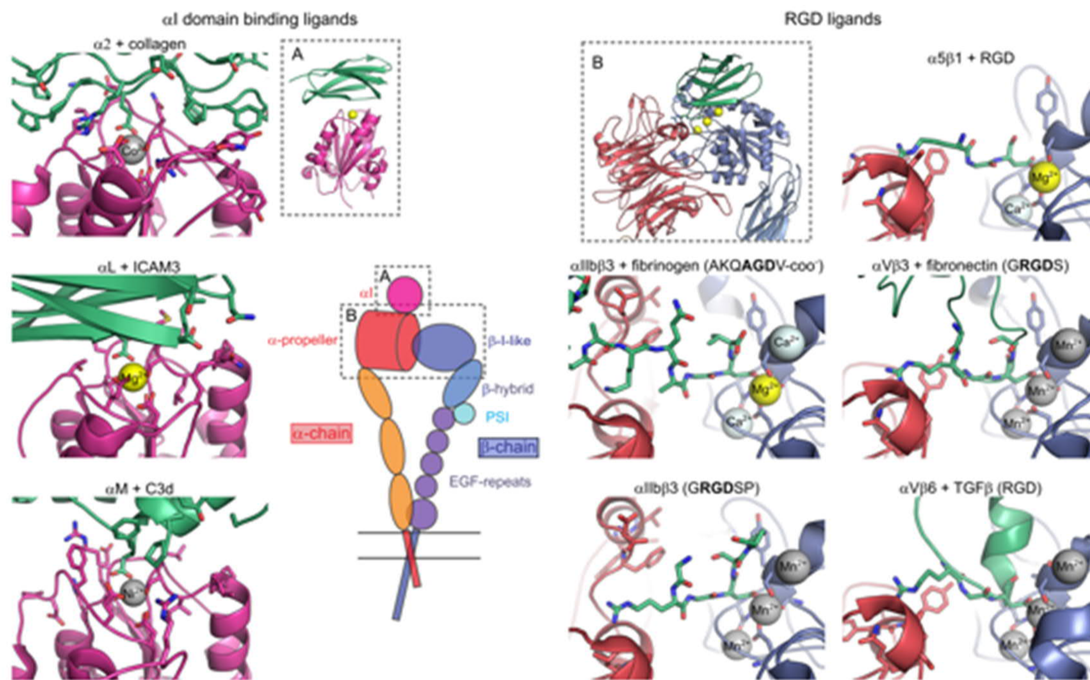


Figure 5 **Structural features of integrin ligand binding – extracellular ligands.** Integrin-ligand interactions are shown on the left for α-I domain containing integrins with the indicated ligands and on the right for integrins without the α-I domain. Please note that in the first case only the α-I domain (purple) makes contact to the ligand (green), while in the latter the heads of both the α- and the β-subunit (red and blue, respectively) contact the ligands (green). The overview of different RGD ligands with relevant integrins (right hand side) highlights the structural diversity within this group for both integrins as well as RGD ligands.
It is noteworthy that in α-I domain integrins only one metal ion site is present (MIDAS), potentially favoring a stricter on-off binding regime due to the missing fine tuning by the ADMIDAS site. The binding pocket for ligands is also more accessible compared to integrins without α-I domain (compare e.g. the orientation and peptide presentation of ICAM3 to fibrinogen binding to αIIbβ3). The accessibility might also have an impact on fibronectin binding, requiring the presentation of the RGD peptide in a short loop to integrins. High-affinity binding to α5β1 is facilitated by auxiliary binding of fibronectin to the synergy binding site on α5 (302). Lack of this auxiliary binding might contribute to conformation-specific binding of αVβ3 integrin to fibronectin. The accessibility is also expected to influence TGFβ binding; the RGD peptide in LAP/TGFβ is surrounded by a bulky α-helix. Steric hindrance with Tyr122 prevents binding to β3 integrins, in contrast to β6 and β8 (Ala) and β5 (Leu) (see also Fig. 2).
Structures shown: α2β1 + collagen: PDB 1DZI (124); αLβ2 + ICAM3: PDB 1T0P (408); αMβ2 + C3d: PDB 4M76 (33); α5β1 + RGD: PDB 3VI4 (302); αIIbβ3 + fibrinogen: PDB 2VDO (409); αVβ3 + fibronectin: PDB 4MMX (5); αIIbβ3 + RGD: PDB 3ZE2 (501); αVβ6 + LAP/TGFβ: PDB 5FFO (113).

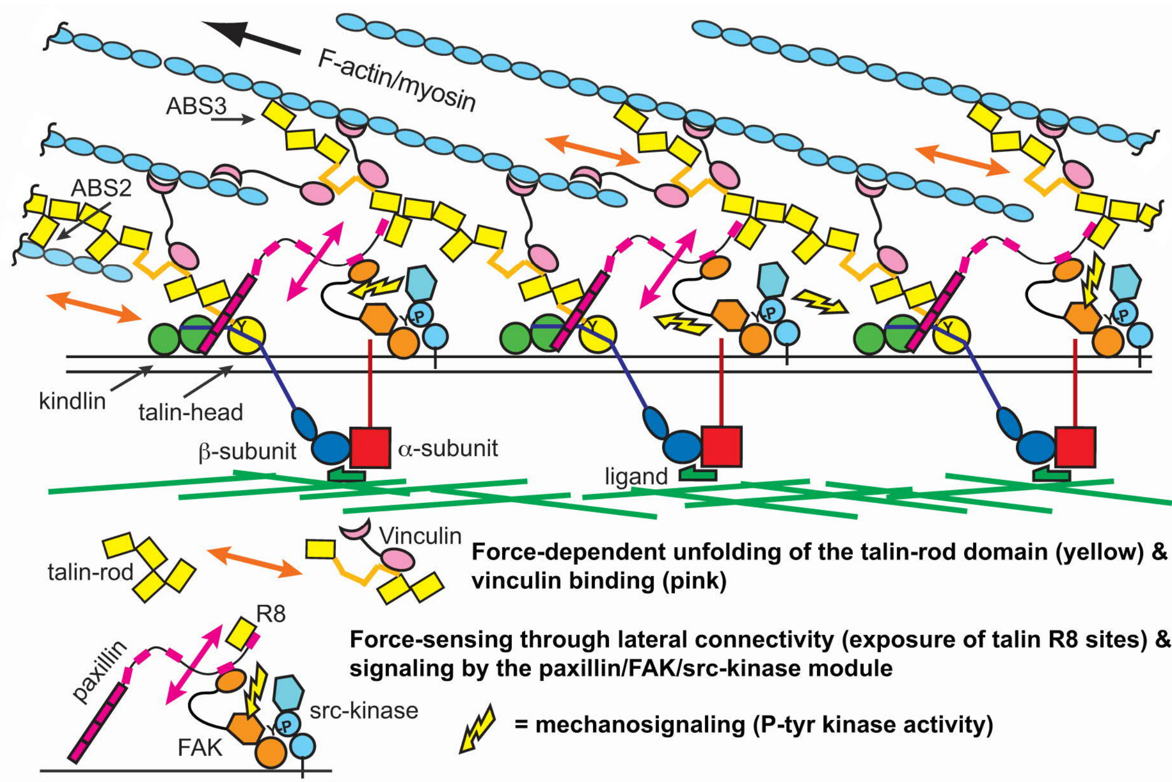


Figure 6 **Protein interplay within integrin-mediated adhesion.** This schematic figure highlights how protein interactions in cell-matrix adhesions are controlled by conformational regulation of the adhesion proteins. The integrin cytoplasmic domain acts as a binding platform for several proteins. Talin (yellow) binds with its head domain to the cytoplasmic domain of the β-subunit and with its tail connects it to the actomyosin machinery. The talin tail with its rod domains contains actin binding sites (ABS) for initial integrin-actin linkage (ABS3). Under mechanical load cryptic binding sites of talin open for one or more vinculins (light pink), as well as F-actin (ABS2) reinforcing the mechanical connection between integrin and actin (24). Vinculin (the head domain binds talin, the tail domain binds actin) can also recruit additional adapters and thereby contribute to adhesion signaling (71). Kindlin (green) co-operates with talin in integrin activation and adapter recruitment as for example paxillin (335). Paxillin (purple) is an important scaffolding protein, recruited to focal adhesions via its LIM domains and organizing Rho-GTPase signaling via Paxillin LD domains. This signaling (indicated by a yellow lightning symbol) is modulated by FAK-Src (orange, light blue)-mediated phosphorylation. FAK consists of a focal adhesion targeting domain binding to paxillin, a kinase domain and a FERM domain binding to the lipid membrane. Interactions in this web are very dynamic, with binding and unbinding events in quick succession. Accordingly, a one-to-one pairing is not to be expected. Instead, a mutual connectivity between the tension-defined ECM-integrin-talin-actin axis and perpendicular interactions based on the paxillin/FAK/talin signaling axis will reinforce and stabilize the adhesion-structure (slanted fence model; see Chapter 4).

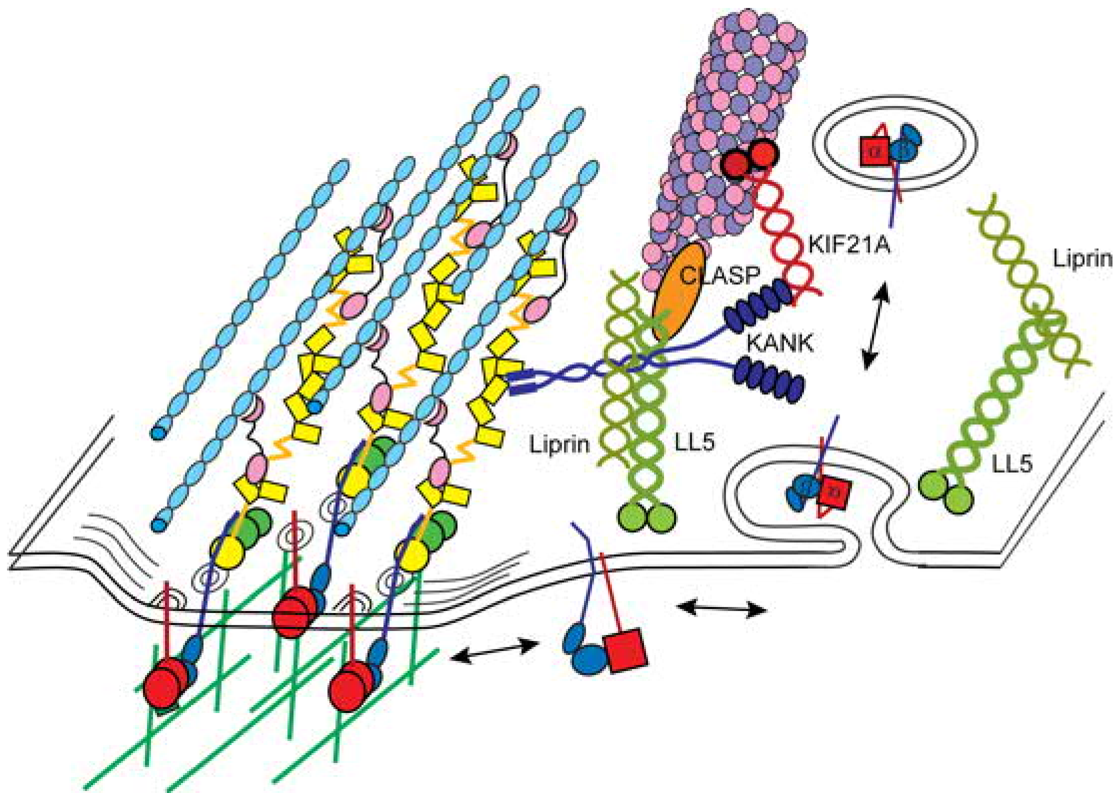


Figure 7 **Interplay between focal adhesion and microtubules.** Ligand-bound integrins are in closer proximity to the substrate compared to the surrounding membrane. The core of integrin-mediated adhesions is densely populated and might differ from the 'outer shell'. Microtubules and focal adhesions are linked *via* Kank proteins at this outer shell, which directly interacts with the talin rod domain (52). While kank can destabilize the talin association with F-actin (420), it also links talin to CLASP proteins and the liprin scaffold. CLASP is important for recruitment of microtubules to adhesion sites, which in turn controls the localized release of proteases (410). The link of kank-proteins to the liprin scaffold directs vesicular trafficking to the cell periphery, involved in directed fibronectin release at sites of adhesion to mediate fibrillogenesis (278), as well as to induce cell spreading during cancer cell migration (86).

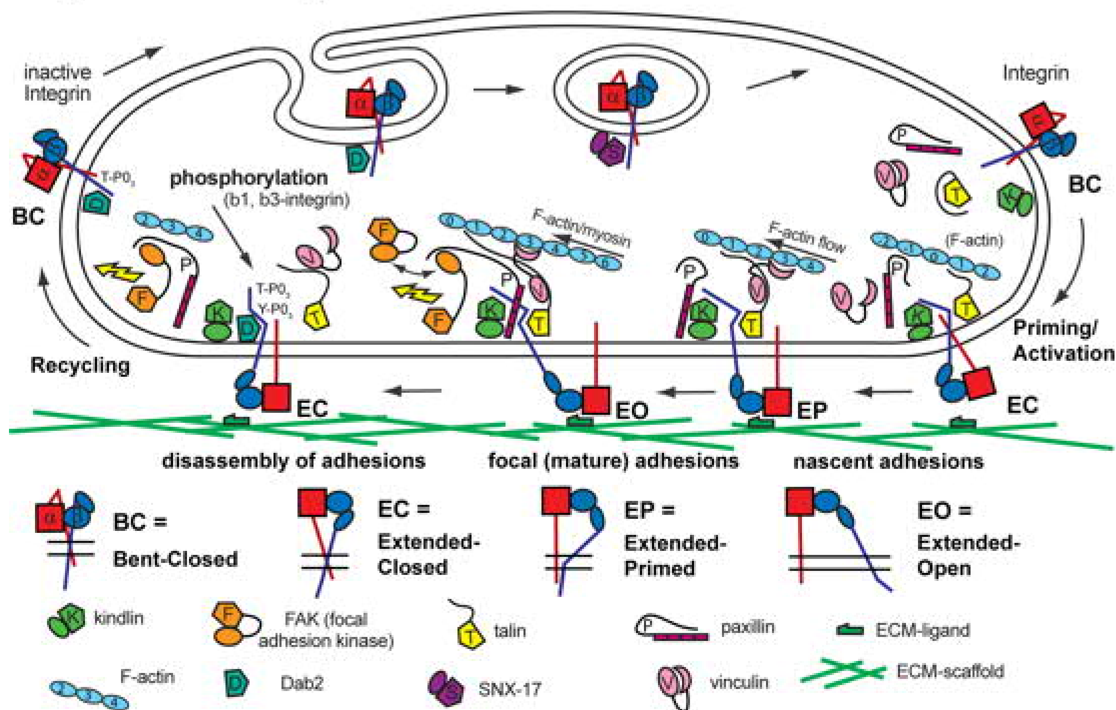


Figure 8 **Integrin activation cycle.** Integrins show the inactive, bent conformation in absence of intra- or extracellular adapters. The transmembrane domains are closely associated to support this bent structure (BC = Bent-Closed conformation). The activation process may involve both extracellular (ligand) and intracellular (talin, kindlin) triggers, leading to extension (EC = Extended-Closed) and priming (EP = Extended-Primed) of the integrin, which facilitates early adhesion events (see also Figure 3). For full activation, integrin legs are separated and arranged in the direction of the actomyosin force-vector (EO = Extended-Open conformation). The activation of integrins is accompanied by the recruitment of signaling (paxillin, FAK) and structural (talin, vinculin) adapters. Recycling of integrin-mediated adhesions can occur *via* integrin inactivation (as shown in Figure 4), with integrin phosphorylation being an important step. The release of talin allows binding to endocytosis adapters like Dab2, and subsequent recycling *via* the sorting nexin 17 (SNX-17) (35, 265). Integrins can also be internalized together with their ligands (397, 398) potentially influencing their capacity to signal from the endocytotic pathway (11) .

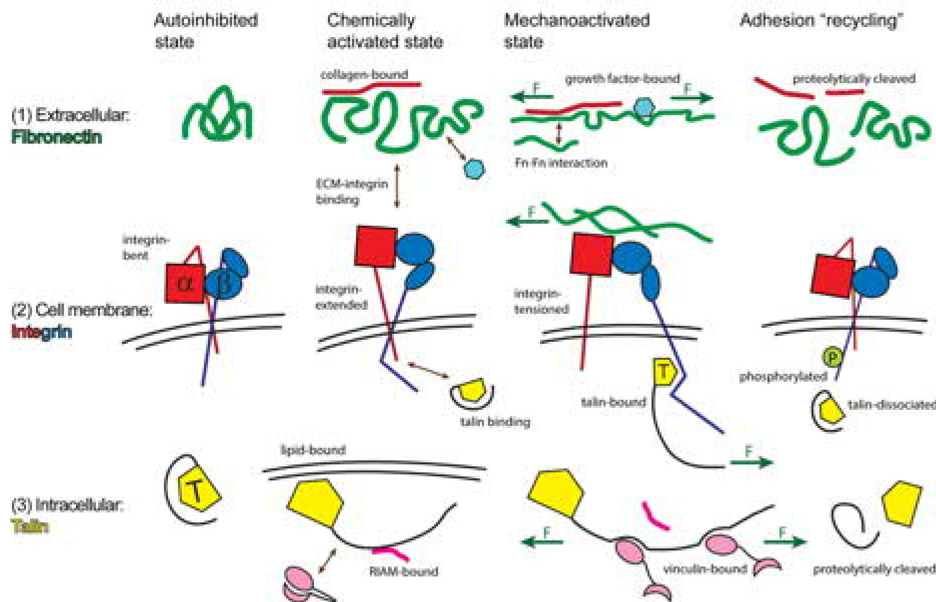


Figure 9 **Mechanoregulation of cell adhesion.** Many of the proteins participating in cell adhesion have an autoinhibited state. Shown here is fibronectin, which has a globular conformation when the protein is in the soluble state. Inactive integrin shows a closed, bent conformation. Also the intracellular protein talin exists in an autoinhibited state, where the rod domain is in contact with the head domain (163). The first step in the activation process is called "chemical activation", where conformational changes take place, leading to insoluble fiber formation and collagen binding in the case of fibronectin. Integrin activation can be triggered both by extracellular and intracellular signals, such as metal ions (289), RGD-ligand proteins (427) and talin (484). Talin may become activated by lipid-mediated triggers, such as increased PIP2 concentration (262, 380). Some of the ligands such as RIAM (170, 244) and DLC1 (491) bind only to relaxed talin. For full maturation, the adhesion complex requires mechanical signals. This leads to changes in the properties of ECM, controlling e.g. the binding of growth factors (317). In the case of integrin, mechanical load is needed for the complete activation, where leg separation takes place (500). The mechanosensory role of talin is studied widely and it involves the exposure of binding sites for other adapter proteins, including vinculin (206, 365). Additionally, mechanical tension also leads to dissociation of certain binding partners such as RIAM and DLC1 (170, 178). Finally, to recycle the adhesion, cells may utilize several molecular mechanisms. Proteolytical cleavage of ECM and intracellular proteins may be involved in the adhesion disassembly (4, 144, 221). Phosphorylation of integrin can be used to tune the binding affinity towards various intracellular partners (14).