Talin: the master of integrin adhesions

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Abstract

Talin has emerged as the key cytoplasmic protein that mediates integrin adhesion to the extracellular matrix. In this Commentary, we draw on experiments performed in mammalian cells in culture and *Drosophila* to present evidence that talin is the most important component of integrin adhesion complexes. We describe how the properties of this adaptor protein enable it to orchestrate integrin adhesions. Talin forms the core of integrin adhesion complexes by linking integrins directly to actin, increasing integrin affinity for ligands (integrin activation), and recruiting numerous proteins. It regulates the strength of integrin adhesion, senses matrix rigidity, amplifies adhesions in response to force, and serves as a platform for the building of the adhesion structure. Finally, the mechano-sensitive structure of talin provides a paradigm for how proteins transduce mechanical signals to chemical signals.

Introduction

Integrins are heterodimeric transmembrane receptors composed of α and β subunits, which bind the extracellular matrix (ECM). Each heterodimer combination has selective affinity for ECM ligands, such as fibronectin, collagen or laminin, and therefore the combination of integrin subunits expressed in each cell will determine its ability to bind particular ECM substrates (Humphries et al., 2006). Almost all cells use cell-ECM adhesion during development and homeostasis, forming diverse adhesion structures that range from dynamic to permanent (Winograd-Katz et al., 2014; Maartens et al., 2015). These adhesions resist different kinds of mechanical challenges, such as shearing forces between cell layers, and pulling forces during muscle contraction.

Integrins use numerous cytoplasmic proteins to mediate their functions. The overall group of proteins involved in integrin adhesion is termed the integrin adhesome (Zaidel-Bar et al., 2007; Horton et al., 2016), and a subset assembles into integrin adhesion complexes (IACs) that link the ECM to the cytoskeleton. IACs are generally characterised by a discrete structure, as visualized by microscopy, where intracellular integrin-associated proteins

(IAPs) become highly concentrated. IACs include focal complexes, focal adhesions, fibrillar adhesions, invadopodia, and hemiadherens junctions. Not only do IACs provide important adhesive structures between cells and the ECM, they also send signals in response to adhesion and the mechanical properties of the substrate, notably stiffness, to regulate proliferation, survival and differentiation (Harburger et al., 2009; Hytonen et al., 2016).

The main 'take-home' message of this Commentary is that talin is the most important of the IAPs in mediating integrin function, and that it achieves this pre-eminence by having numerous fascinating properties. Talin was initially discovered as a protein that localises to integrin adhesions in fibroblasts (Burridge et al., 1983), and was one of the first proteins identified that binds integrin cytoplasmic tails (Horwitz et al., 1986). When it was also found to bind actin (Muguruma et al., 1990), talin emerged as a linker molecule that could directly connect integrins to the actin cytoskeleton. Numerous other IAPs that may function similarly were subsequently discovered (Calderwood et al., 2003), but the importance of talin emerged from genetics. Mutations in the single talin gene in *Drosophila* showed that talin is needed for all integrin adhesive functions (Brown et al., 2002), and once both mouse talin genes (Tln1 and Tln2) were mutated, similar strong defects were observed (Zhang et al., 2008; Conti et al., 2009; Atherton et al., 2015; Theodosiou et al., 2016). The significance of these strong phenotypes is increasing as we discover that more and more IAPs have weaker mutant phenotypes (reviewed in Bulgakova et al., 2012; Bouvard et al., 2013). Whereas talin is vital for the function of most integrins, integrins containing β subunits with divergent cytoplasmic domains, such as β4 and β8 in vertebrates and $\beta\nu$ in *Drosophila,* may not employ talin.

The authors had a talin 'epiphany' as a result of two important discoveries. Firstly, whereas integrin needs talin for all of its functions, the converse is not true; for example talin regulates cadherin gene expression (Becam et al., 2005), and causes novel eye defects (our unpublished results), independent of integrins. Secondly, the genomes of a number of organisms encode a very well conserved talin, but no integrin subunits, including protists that form multicellular fruiting bodies, such as *Dictyostelium* and *Fonticula* (our unpublished observations and Sebe-Pedros et al., 2010), indicating that talin does not need integrins to function, and that integrins may have evolved after talin. One can therefore speculate that when integrins arose, they adopted the pre-existing talin machinery, thus utilising the ability of talin to build actin-bound, mechanosensitive complexes to connect the newly evolving ECM with actin. These two findings overturned our integrin-centric view of life and elevated talin to the 'master', while reducing the integrins to one of talin's 'servants'. We will focus this Commentary on how integrins and talin work together, as little is known about integrin-independent functions. We will integrate results from both mammalian cells in culture and

Drosophila, with the aim of generating a unified model of talin function. However, we acknowledge that this assumes talin works identically in all animals, whereas it may have acquired novel functions. We recommend recent reviews for more information on talin structure (Critchley, 2009; Calderwood et al., 2013) and function in integrin activation (Shattil et al., 2010; Kim et al., 2011; Lagarrigue et al., 2016).

A simplified model of integrin adhesion: the ECM-integrin-talin-actin linkage

Before we delve into talin structure and function, we will set the context with a simple model where the core of IACs is composed of just four linked components: ECM ligand-integrin-talin-actin (Fig. 1E', and Liu et al., 2015). Talin contributes to this core in a surprisingly complex way (Fig. 1). Talin starts in a closed conformation and is activated to bind other proteins. Talin has two integrin-binding sites, three actin-binding sites, and can dimerise (see also below and Fig. 2); therefore, each talin homodimer can potentially bind four integrin heterodimers and multiple actin filaments within this core structure. The first talin molecules are recruited to ligand-bound integrins, with their orientation dependent on which integrin-binding site is used (left versus right side of Fig. 1). Talin associated with one ECM-bound integrin can expand the adhesion site by binding an adjacent, non-ligand bound integrin and stimulating its ECM-binding (see Box 1 for further details on integrin activation). The binding of talin to multiple integrins may also dictate the spacing between integrins (Fig. 1C, C'). Once integrin-bound talin binds to actin, it is stretched by mechanical forces, either in the plane of the membrane and actin cortex (left side Fig 1), or perpendicular to it (right side). The stretching of talin unfolds talin domains to expose interaction sites that recruit additional proteins, notably vinculin (Fig. 1E'). Thus, talin translates actin-mediated forces on integrins into recruitment of IAPs, which may strengthen or modify the adhesion. Finally, talin also recruits additional IAPs independent of force, either directly or indirectly, to make the full IAC (not shown in Fig. 1). With this simple framework, we can then consider all other adhesome components as modifiers of this core adhesion structure.

Talin: structure of a multifunctional adaptor molecule

Talin is a large protein of ~2,500 amino acids (aa; Fig. 2A). The N-terminal FERM domain forms the head of talin (~400 aa). FERM domains are found in numerous proteins, many of which link transmembrane proteins to the cytoskeleton (Tepass, 2009). The FERM domain of talin is atypical in that the first of the characteristic three subdomains (F1) is duplicated, adding a fourth subdomain at the N-terminus (F0) (Goult et al., 2010), a feature only shared with kindlins. The head is followed by an unstructured region (the "linker"; ~80 aa) and then the rod domain (~2,000 aa), which is formed of 13 helical bundle domains (R1-R13,

containing 62 α-helices) each consisting of four or five α-helices (Gingras et al., 2005; Goult et al., 2013a). In some non-vertebrate organisms, talin has additional sequences beyond the 62nd helix; for example, *Dictyostelium* talin B contains a villin headpiece-like domain (Tsujioka et al., 1999), and a *Drosophila* variant extends talin by ~300 aa (Senetar et al., 2005).

The solved structure of the talin head revealed an extended conformation, which can lie along the plasma membrane (Elliott et al., 2010), different from the "cloverleaf" conformation of other FERM domains. F3 binds the cytoplasmic domain of integrin β subunits (integrin-binding site 1, IBS1, Fig. 2B, Calderwood et al., 2002; Garcia-Alvarez et al., 2003). F1, F2 and F3 bind directly to membranes containing phosphatidylinositol 4,5-bisphosphate (PIP2) *in vitro*, or lipid combinations that mimic the presence of PIP2 (Anthis et al., 2009; Saltel et al., 2009; Goult et al., 2010). This direct membrane interaction is particularly relevant when considering talin function in organisms that lack integrins. In addition, the talin head binds Rap1 with F0 (Goult et al., 2010; Plak et al., 2016), actin with F2-F3 (actin-binding site 1, ABS1, Hemmings et al., 1996; Lee et al., 2004), and F3 binds many IAPs, such as focal adhesion kinase (FAK), layilin, T-cell lymphoma invasion and metastasis 1 (TIAM1) and phosphatidylinositol phosphate kinase type Iγ (PIPKIγ) (reviewed in Calderwood et al., 2013), as well as Rap1-GTP-interacting adaptor molecule (RIAM) (Yang et al., 2014).

The talin rod (R1 to R13) also contains multiple protein interaction sites. There are at least two other actin-binding sites: ABS2 is formed by R4-R8 (Hemmings et al., 1996; Atherton et al., 2015) and ABS3 is formed by R13 and adjacent α-helix 62 (McCann et al., 1997; Brett et al., 2006; Gingras et al., 2008). ABS3 is also known as the I/LWEQ domain and is found in one other protein, Hip1 and its paralogue Hip1R, which contains an ANTH domain and is involved in endocytosis (Gottfried et al., 2010). Helix 62 forms homodimers *in vitro*; it is required for actin binding and may mediate constitutive dimerisation of talin (Gingras et al., 2008). Also in the rod is a second integrin-binding site (IBS2, Rodius et al., 2008; Gingras et al., 2009), helix 50 within R11, which has remained somewhat controversial due to the difficulty of confirming direct binding. Numerous vinculin binding sites (VBSs) have been identified within the talin rod (Fig. 2A). As a VBS consists of a single α-helix, many of the 62 α-helices of talin could be VBSs; indeed, an *in vitro* analysis identified ten strong VBSs and a further nine weaker ones (Gingras et al., 2005). Binding sites have also been characterized for RIAM, paxillin, α-synemin, DLC1, Kank1 and Kank2 (Sun et al., 2008; Lee et al., 2009; Gingras et al., 2010; Li et al., 2011; Goult et al., 2013b; Bouchet et al., 2016; Sun et al., 2016; Zacharchenko et al., 2016). Furthermore, talin is

required for recruitment of all IAPs examined to date in *Drosophila* (e.g. Zervas et al., 2011), suggesting that the list of binding partners will continue to grow.

Regulation of talin interactions

How interactions between integrins and IAPs are regulated is a key problem for the field, and part of a general problem of understanding how protein complexes assemble within cells. We observe that cells assemble IACs at discrete cellular locations, but do not fully understand why interactions between components only takes place there, rather than constitutively within the cytoplasm. For example, *a priori* it is valuable for talin to bind actin only after binding integrin, because the actin concentration is much higher than integrin. We envision that newly synthesised IAC components adopt a conformation that cannot form interactions, which is then activated. This starts with integrins, where the cytoplasmic domains become available for binding following integrin binding to extracellular ligands. This could initiate a cascade, with the free integrin cytoplasmic domain binding "closed" talin, changing its conformation so that it can then bind other closed IAPs, and so on. However, there is evidence that talin and other IAPs need to be activated by other signals before joining the IAC.

Electron microscopy observations suggested that cytoplasmic talin is in a closed conformation (Molony et al., 1985), and cryo-electron microscopy generated a rolled-up dimer model, with talin heads packed at the centre of a doughnut-shaped structure, unable to bind integrins (Goult et al., 2013a). Two intramolecular interactions between the head and rod have been mapped: between R9 and F3, masking IBS1 (Goksoy et al., 2008; Goult et al., 2009; Zhang et al., 2016), and between R1-R2 and F2-F3, potentially masking membrane-binding regions of talin head (Banno et al., 2012). Disrupting the interaction between R9 and F3 enhances actin binding by talin (Banno et al., 2012), promotes integrin activation (Zhang et al., 2016) and increases focal adhesion number and assembly rate (Kopp et al., 2010). In the developing *Drosophila* embryo, this disruption increases the size of focal adhesion-like structures, retarding epithelial morphogenesis (Ellis et al., 2013). These results indicate that the amount of active talin is a limiting factor in adhesion formation, and, conversely, that too much open, active talin is detrimental.

It is not yet clear where the equilibrium between closed and open talin conformations lies, that is whether talin has to be converted into an active conformation to function, or its activity is just stimulated by factors that push the equilibrium toward an open conformation. Known activators include the phosphoinositide PIP2 (Martel et al., 2001), the Rap1 effector RIAM and the heterotrimeric G protein Gα13, both of which bind F3 and displace R9 (Yang et al., 2014; Schiemer et al., 2016), and Kank2, which binds R7 to activate talin (Sun et al.,

2016). Both RIAM and PIP2 also contribute to talin activation by bringing it to the membrane (Anthis et al., 2009; Goult et al., 2010; Chang et al., 2014)

Other interaction sites in talin are also tightly regulated. The accessibility of both actin-binding sites in the rod is negatively regulated by adjacent sequences (Fig. 3). The α-helix 57 inhibits actin binding by α-helices 58-62 of ABS3 (McCann et al., 1997; Gingras et al., 2008), and domains R2-R3 and R9 inhibit ABS2 (R4-R8, Atherton et al., 2015). Similarly, IBS2 (α-helix 50) is inhibited by α-helix 51 (Klapholz et al., 2015). VBSs are buried in the native structure, and so talin must be stretched by force for VBSs to become available for vinculin binding (Fig. 2C and 3), as discussed further below. In turn, force-dependent binding of vinculin to R2-R3 appears to alleviate the repression of ABS2 (Atherton et al., 2015), and we suspect that force also counteracts the repression of ABS3 and IBS2.

Talin is thus a classic adaptor protein, in that its only known activities are to bind to other proteins, which it does through numerous regulated binding sites, each providing a subfunction. The degree to which these individual subfunctions are required depends on the biological function of the particular integrin adhesion being examined. This variation helps explain some of the contradictory results obtained with different cell types and assays, such as whether the talin head on its own is recruited to focal adhesions or not (Nuckolls et al., 1990; Tremuth et al., 2004; Parsons et al., 2008).

Talin in integrin activation

A key activity of talin is to convert integrins into a high-affinity conformation for their ECM ligand, so-called inside-out activation (Box 1). The control of integrin activity from inside the cell is especially important in hematopoietic cells, notably platelets and lymphocytes (Lagarrigue et al., 2016). There has been some debate over whether the increase in adhesion is due to a change in the affinity of each integrin for its ligand, or an increase in the valency of the interaction brought about by integrin clustering (e.g. Lub et al., 1995). There is now strong evidence for affinity changes (reviewed in Luo et al., 2007), but clustering contributes as well.

Talin head alone can shift the integrin conformation towards an active state (Box 1), but there is increasing evidence that forces generated by actin polymerization and myosin contraction are involved in inducing or stabilizing the extended-open conformation (Comrie et al., 2015; Nordenfelt et al., 2016). This additional mechanism fits well with the discovery of other IAPs that cooperate with talin to activate integrins, such as kindlins, although the mechanism is still unclear and kindlins also cluster integrins (Moser et al., 2009; Calderwood et al., 2013; Ye et al., 2013; Rognoni et al., 2016; Georgiadou et al., 2017). Additional IAPs enhancing talin-mediated activation are RIAM (Han et al., 2006; Yang et al., 2014), zasp (Bouaouina et al., 2012) and vinculin (Lee et al., 2013), whereas other IAPs compete with

talin and reduce activation, including ICAP1 (Bouvard et al., 2003), filamin (Kiema et al., 2006) and moesin (Vitorino et al., 2015). Alternatively, SHANK proteins can sequester Rap1, reducing talin-mediated integrin activation (Lilja et al., 2017).

It has been difficult to elucidate how important talin-mediated integrin affinity changes are for integrin-ECM interactions within intact tissues. This is for three reasons. Firstly, integrins can also be activated by ligands, referred to as 'outside-in' activation (Du et al., 1991). We envision this occurs because integrins are in an equilibrium between different conformations, with the low affinity form favoured. When cells are sitting on the ECM, ligand is available for binding whenever an integrin briefly shifts to a high-affinity conformation. Cells may therefore adhere sufficiently tightly to an adjacent ECM even in the absence of inside-out integrin activation. This contrasts with the role of integrins in hematopoietic cells that have to capture soluble or fast-moving ligands, or the more dynamic adhesions involved in cell migration. Secondly, the importance of any such affinity changes may differ between organisms, preventing integration of all results into a universal model. Thirdly, as the strength of adhesion is also increased by integrin clustering, it is hard to devise experiments that distinguish affinity from clustering changes *in vivo*, especially since a single binding event can simultaneously cluster and activate an integrin, as depicted in our simple model (Fig. 1, step 2).

Experiments designed to test the importance of talin-mediated inside-out activation in the developing organism have produced mixed results (see Box 2). Our own view is that, given the ability of ECM ligands to capture transiently high-affinity integrins and the strong adhesion provided by talin-actin mediated mechanical forces and integrin clustering, it appears highly likely that inside-out activation plays a minor role in many tissues. Future identification of specific processes within tissues that require inside-out activation will clarify the physiological relevance. The role of talin in the assembly and functioning of adhesion complexes appears more crucial, as we discuss next.

Recruitment of talin to integrin adhesion sites

Once talin in the cytoplasm is 'activated' and available for interaction, it can be concentrated at sites of adhesion. However the sequence of events is unclear: does talin bind integrins and this complex binds ECM, or do integrins bind ECM first and then recruit talin? If talin is needed to activate integrins by an inside-out mechanism, it should be able to bind integrins first. A pathway fitting this idea is as follows: protein kinase C activates Rap1, which recruits its effector RIAM to the membrane; this, in turn recruits talin to the membrane where it can then activate integrins (Lee et al., 2009). RIAM is essential for inside-out activation of integrins in leucocytes (Klapproth et al., 2015), but surprisingly not in in platelets (Stritt et al.,

2015). This suggests alternative pathways, e.g. using the RIAM paralogue lamellipodin (Lagarrigue et al., 2015).

The converse model, that talin is recruited to ligand-bound integrins, fits with the behaviour of single talin molecules, which are recruited directly into focal adhesions; if talin associated with non-ligand-bound integrins before activating them, we might expect to see diffusion in the membrane, which was not observed (Rossier et al., 2012). Our favorite model is the one in Fig. 1, where talin dimers are recruited by binding to a ligand-bound integrin, and then can inside-out activate integrins that diffuse within suitable proximity. However, examination of nascent adhesions in epithelial cells revealed a third mode of initial recruitment (Bachir et al., 2014). Integrin $\alpha5\beta1$, kindlin2, talin1 and vinculin enter nascent adhesions simultaneously. However, cross-variance analysis revealed that during adhesion assembly, $\alpha5\beta1$ is bound to kindlin2, and talin bound to vinculin; however, these two subcomplexes are not bound to each other until the nascent adhesion becomes stabilised by myosin II activity (Bachir et al., 2014). Thus, talin can be retained at the adhesion without maintaining integrin binding, and interactions between IAPs can change following recruitment.

What is unequivocal is that there are multiple ways for talin to be recruited to sites of integrin adhesion, including through the integrin-binding sites in the head (IBS1) and rod (IBS2), and via R1-R4, and ABS3 (Tremuth et al., 2004; Tanentzapf et al., 2006b; Moes et al., 2007; Parsons et al., 2008; Himmel et al., 2009; Kanchanawong et al., 2010; Rossier et al., 2012). These multiple routes explain why single point mutants in either IBS1 or IBS2 did not substantially impair talin recruitment in *Drosophila*, and even the double mutant was recruited in the absence of endogenous talin, although it functioned very poorly (Ellis et al., 2011). FAK contributes to talin recruitment to nascent adhesions in mammalian cells, but its absence does not impair IAC formation in *Drosophila* (Grabbe et al., 2004; Lawson et al., 2012). To summarise, the direct binding to integrins is not essential for the association of talin with integrin adhesion sites, and there are still interactions to be discovered that contribute to talin recruitment.

The talin rod: a mechanotransducer with multiple states

Once talin is associated with ligand-bound integrin, we envision the next step to be talin binding to actin filaments through the C-terminal actin-binding site (ABS3). This is where its function as a mechanotransducer begins to come into play. Talin and vinculin play key roles in mechanotransduction, which involve two distinct, yet intertwined, aspects: the molecular clutch and stiffness sensing (reviewed in Hoffman et al., 2011; Case et al., 2015b). The molecular clutch is the mechanism required to couple rearward actin flow, produced by actin

polymerisation at the cell leading edge and actomyosin contractility, to the relatively stationary ECM adhesions, so that actin pushes the leading edge forward. Stiffness sensing refers to how cells respond to the stiffness of their substrate, and includes changes to cell morphology, the amount of traction force exerted, and gene expression. Initial modelling of the clutch predicted a peak of traction force at an optimal stiffness, with a failure due to frictional slippage as the stiffness increased further (Chan et al., 2008). However, focal adhesions do not display this behavior, but rather increase their traction force monotonically as the stiffness increases (Ghibaudo et al., 2008). This finding suggests a mechanism that reinforces the clutch so that it can operate at higher traction force, which turns out to require both talin and vinculin (Elosegui-Artola et al., 2016) (see also new computational model fromWu et al., 2017).

A variety of evidence indicates that talin is the key force-sensing molecule, and vinculin one of the key mechanoeffectors. At low traction forces, other molecules (or residual talin following knockdown) can form a clutch, but at higher forces normal levels of talin are needed (Elosegui-Artola et al., 2016). The use of force sensors has shown that the amount of force on talin increases as the substrate becomes stiffer, and the majority of force on talin is myosin II-dependent (Austen et al., 2015; Kumar et al., 2016). In contrast, the amount of force on vinculin does not change with substrate stiffness (Kumar et al., 2016), consistent with it being a mechanoeffector rather than a mechanosensor. Deletion or mutation of ABS3 causes only a slight reduction in force sensed across talin (Austen et al., 2015; Kumar et al., 2016), but blocks the normal reduction of force on talin in central fibrillar adhesions versus peripheral focal adhesions, by an unknown mechanism (Kumar et al., 2016). In contrast, mutating ABS2 or deleting both ABS2 and ABS3 substantially reduces force across talin, and eliminates its ability to recruit vinculin. Reciprocally, removing vinculin reduces force across talin-1. This gives rise to a model whereby binding of ABS2 to actin leads to vinculin recruitment, which binds to actin to increase the force exerted on talin.

The ability of talin to sense changes in force was revealed by a variety of innovative structural and biophysical studies on talin rod. The first structures of talin rod segments unexpectedly revealed that VBSs are buried within the helical bundles and thus not accessible for vinculin binding. However, this gave rise to the exciting idea that VBSs would only become available for binding once the domains become unfolded by force (Papagrigoriou et al., 2004; Fillingham et al., 2005). This was confirmed *in vitro* by using magnetic tweezers to pull open talin rod domains, demonstrating that the 13 α-helical bundles form a chain of “spring-like” structures that progressively unfold as force increases from 5 to 25pN, and binding of vinculin to talin requires some unfolding (del Rio et al., 2009; Yao et al., 2014; Yao et al., 2016). This provides a valuable paradigm for how force across a protein can lead to a chemical change, such as vinculin recruitment, and this can be

recapitulated in a cell free system (Ciobanasu et al., 2014). Bundle unfolding is reversible (Yao et al., 2016), and therefore the talin rod will respond to fluctuating force levels with cycles of recruitment and release of vinculin. In support, the length of talin substantially fluctuates (80-350nm) at adhesion sites (Margadant et al., 2011), and loss of force by inhibition of myosin II results in rapid loss of vinculin (e.g. Pasapera et al., 2010). The cyclical stretching suggests a role for talin as a 'shock absorber' between integrins and actin, buffering the impact of abrupt mechanical changes (Yao et al., 2016).

Vinculin is not needed for the amount of force across talin to increase as the substrate gets stiffer (Kumar et al., 2016), but vinculin is needed to convert the increased stretch of talin into a cellular response. Indeed, expression of a dominant-negative vinculin blocked the increase in traction force as substrate stiffness increased, as well as the stiffness-dependent translocation of the transcription factor YAP into the nucleus (Elosegui-Artola et al., 2016).

Returning to the roles of the two actin-binding sites in the rod, the force-sensor experiments highlight a major role for ABS2 in producing high levels of force on talin, in part by force-dependent vinculin recruitment, and a minor role for ABS3 in downregulation of forces in central fibrillar adhesions (Austen et al., 2015; Kumar et al., 2016). However, this model is not universal, as deletion of ABS2 and ABS3 in the second mouse talin, talin-2, did not impair vinculin recruitment (Austen et al., 2015), and a similarly deleted fly talin retains partial function through vinculin (Klapholz et al., 2015). Another study showed that ABS3 mutation of talin-1 had a variable effect, with half of the cells lacking any adhesions, and the rest having weak adhesions with little vinculin, suggesting ABS3 is needed for the initial adhesion assembly, but can be bypassed (Atherton et al., 2015). Furthermore, ABS2 mutation did not impair initial adhesions and spreading, but consistent with the force-sensor experiments, blocked maturation of nascent adhesions to focal adhesions that can exert strong forces on the substrate. This work led to an alternative model (Fig. 3): i) binding of ABS3 to actin partially stretches talin, thereby unfolding the most mechanosensitive bundles, such as R3, which then recruits vinculin; ii) vinculin-binding to R3 maintains this bundle in an unfolded state and alleviates the repression of ABS2 activity by R3; finally, iii) ABS2 binds to actin, further stretching talin and thus permitting the maturation of the adhesion. In *Drosophila,* ABS3 contributes to all talin functions and deletion of ABS2 has surprisingly mild effects (our unpublished results, Franco-Cea et al., 2010; Klapholz et al., 2015).

In contrast to vinculin, RIAM preferentially binds un-stretched, fully folded helical bundles (Fig. 2A Goult et al., 2013b; Lee et al., 2013), which makes sense if it helps recruit talin to integrins. Thus, talin rod domains can function as switches, binding one protein in a folded state, and a different protein when unfolded by force. Different forces may result in a

variety of partially unfolded talin molecules, each with certain binding interactions either switched on or off, and therefore binding to unique sets of IAPs.

To summarize, we are just beginning to get an appreciation for how the talin rod functions as a complex mechanotransducing machine, with different possible models for the sequence of events involved. Talin forms a series of spring-like switches that exchange their binding partners in response to forces generated by interaction with actin. We look forward to future work that improves our picture of IAC assembly and how the rod structure permits it to integrate multiple signals, including binding partners, post-translational modifications and forces.

Talin's role in the architecture of adhesion sites

An exciting step forward for the field was the examination of IAP distribution within focal adhesions in the Z-axis by novel developments in super-resolution microscopy. This allowed the definition of layers in the nanoarchitecture of the IAC, consisting of a membrane-proximal integrin signalling layer, containing integrins, FAK and Paxillin, which is separated by ~40nm from an actin-regulatory layer that comprises zyxin and VASP (Kanchanawong et al., 2010). Importantly, talin spans both layers, with its head close to the membrane and its C-terminus found within the actin-regulatory layer (Kanchanawong et al., 2010; Paszek et al., 2012). This orientation was also observed in muscle attachments in *Drosophila*, but not in epithelial cells (Klapholz et al., 2015). Vinculin is positioned between talin head and C-terminus and oriented in the same direction as talin, fitting with it binding to talin rod with its head domain and to the actin layer with its C-terminal actin-binding domain (Kanchanawong et al., 2010; Case et al., 2015a; Liu et al., 2015). Importantly, talin specifies the separation between the layers, as deletions within its rod domain bring the actin regulatory layer closer to the membrane (Liu et al., 2015).

As talin is essential for all integrin functions, we initially thought it had to fulfil a single, very important job that was required at all times. We were therefore surprised when structure-function analysis of talin in different developmental contexts revealed that the importance of each talin subdomain depended on the developmental event examined; for example, the talin head is essential in muscles, but only partially required in the wing epithelia, and ABS3 is essential for epithelial morphogenesis, but only partly needed in muscles or wing (Franco-Cea et al., 2010; Ellis et al., 2011; Klapholz et al., 2015). These results can be explained by a hypothesis that talin has different mechanisms of action, which involve alternative configurations and interactions (Fig. 1).

One key piece in this puzzle is IBS2, because it is difficult to see how it can bind integrins in a talin configuration where it is far away from integrins (≥25nm), such as in *Drosophila* muscles, yet a talin with a mutant IBS2 formed a defective muscle linkage (Ellis

et al., 2011; Klapholz et al., 2015). This contradiction can be resolved if IBS2 is important for making an initial talin-integrin interaction that is sufficiently strong to resist the forces resulting from the interaction between ABS3 and actin, and as forces increase, the C-terminus is pulled away from the membrane, leaving talin anchored to integrins with IBS1. Both IBS1 and ISB2 are needed for function in wing epithelia, suggesting a parallel arrangement of talin (Fig. 1, left panels), with each talin molecule bound to two integrins (and the talin dimer up to four integrins, three of which are shown in Fig. 1), which was confirmed by FRET and super-resolution microscopy (Klapholz et al., 2015). The parallel orientation of talin may sense shearing forces, or stretch between integrins (Fig. 1D,E). It will be interesting to discover if there are IAPs that regulate the association between IBS2 and integrins in different cell types, and thus the orientation of talin.

There is some evidence that IBS2-mediated interactions, and thus a parallel talin orientation, are also important in mammalian cells. A C-terminal fragment of talin from IBS2 to ABS3 rescues the proliferation defects in epithelial cells lacking talin (Wang et al., 2011), and the IBS2 region is needed for talin to form the initial clusters of ~50 integrins in nascent adhesions (Changede et al., 2015). For talin interactions alone to be sufficient to generate integrin clusters larger than four integrins, one integrin might need to be able to bind to more than one talin molecule, thus forming a network of integrins that are separated by the distance between the two IBSs in the talin molecule or dimer (Fig. 4). The position of the binding sites for IBS1 and IBS2 on the cytoplasmic domain of β-integrins suggests this could be possible, as IBS1 binds to the membrane-proximal NPxY motif and IBS2 the membrane-proximal α-helix (Wegener et al., 2007; Rodius et al., 2008; Anthis et al., 2009). This could result in the formation of a meshwork of talin molecules, generating an “intracellular matrix” (Fig. 4). This matrix may be further stabilized by intermolecular binding between talins, or crosslinking by other bifunctional IAPs. We like this concept because it helps to explain firstly, how the IAC can become a very stable structure, such as at muscle ends where a small percent of each IAP remains dynamic (Pines et al., 2012; Hakonardottir et al., 2015); secondly, how talin can serve as the essential platform for the recruitment of all other IAPs (e.g. Zervas et al., 2011); and thirdly, how integrins adopt a regular spacing within adhesions (Rossier et al., 2012). However, fragments of the head or rod domains can rescue the loss of talin in establishing nascent adhesion clusters (Changede et al., 2015), suggesting that single IBSs can function with other IAPs in generating this matrix.

A novel aspect of how talin is involved in adhesion-site architecture emerged recently with the discovery that talin recruits Kank1 and Kank2, as well as their associated 'cortical microtubule stabilization complexes' to the periphery of focal adhesions by direct binding of talin R7 to the KN motif (Bouchet et al., 2016; Sun et al., 2016). Keeping Kanks at the edge of focal adhesions requires balancing interactions with talin inside the adhesion and other

partners outside. Kank binding to talin is important both for the behaviour of focal adhesions and microtubules. In fibroblasts, Kank2 promotes the dynamic exchange of integrin and talin, sliding of adhesions, and activates talin and integrins (Sun et al., 2016). In Hela cells, Kank1 binding to talin is required for the normal reduction of microtubule growth at the cell periphery (Bouchet et al., 2016).

Taken together, it is clear that talin contributes to adhesion architecture in the Z-axis, as it controls the distance between the membrane and the actin-regulatory layer, may organise integrins into their regularly spaced arrangement in the X-Y axis, and provides linkage to associated complexes. A key architectural role fits with the exceptional conservation of talin length in *Dictyostelium*, *Drosophila* and Humans, whereas, for example, the length of filamin in these species varies several-fold.

Conclusion and Perspectives

We have described here the properties and abilities of talin that allow it to fulfil its role as the master regulator of the link between integrins and the cytoskeleton. However, it does not only directly link integrins to actin, but also has multiple, regulated interaction sites for the plasma membrane and other adhesome components. Furthermore, talin regulates the strength of integrin adhesion, both through affinity changes and clustering, and specifies the architecture of the adhesion site. Thus, it is a multifunctional tool that enables integrin adhesion in different cellular contexts, likely through different mechanisms of action, with each relying on combinations of its specific functions in a given cellular context. Given such diversity of possible functions, one wonders why additional integrin-associated proteins are needed? Do they just duplicate talin functionality and so provide robustness to the adhesion structure, or do they fulfil other unique functions, and if so, what are these? This is the subject for another review, but we anticipate that obtaining a clearer picture of talin at the heart of the adhesion structure will provide us with a framework that we can populate with the additional functions of other IAPs.

Although our understanding of the structure and function of talin has made steady progress, it has also thrown up several new questions for future research, including elucidating the different mechanisms of talin within various cellular contexts, both during development and under different physiological challenges, and what factors lead to the selection of a particular mechanism.

Answering these questions will be aided by additional work on talin function in organisms that lack integrins. So far, this type of analysis has only been performed in *Dictyostelium*, which has two talins with partially overlapping functions in cell adhesion and migration (Tsujioka et al., 2008). *Dictyostelium* does have a family of Sib transmembrane receptors with some integrin-like features and a mutant phenotype that resembles loss of

talin (Cornillon et al., 2006; Tsujioka et al., 2012), so it may be that talins always use some form of membrane receptor. However, other organisms have talin and lack both integrins and Sib receptors (our analysis), so talin may not need a transmembrane receptor to function, after all. Discovering how these adhesion structures are built and how they transduce mechanical signals may help to elucidate the original roles that accompanied the initial creation of this master adaptor protein.

Acknowledgements

We are grateful to David Calderwood, Ben Goult, Hannah Green, Jari Ylänne and the reviewers for their comments on the manuscript, and the BBSRC for funding (BB/L006669/1).

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Text boxes

Box 1. Mechanism for inside-out integrin activation by talin head.

Integrin activation involves two major conformational changes (reviewed in Luo et al., 2007). The first is the change from a bent conformation, with the ligand-binding site facing the membrane, to an extended closed conformation, with the ligand-binding site facing outward, so that it can undergo low-affinity interactions with ligands. The second is the opening of the head piece and separation of cytoplasmic domains to form the extended open conformation, which allows high-affinity binding to ligand (Fig. 1A). Integrin binding to its ligand forms a catch bond that becomes stronger when force is applied (Kong et al., 2009).

The integrin-binding region of talin was initially mapped to the rod (Horwitz et al., 1986), before effort focused on the head when it was found to both bind and activate integrins (Calderwood et al., 1999). The F3 domain in the talin head binds directly to two sites on the cytoplasmic tail of β-integrins (Fig. 2B): the most membrane-proximal of the two NPxY motifs (Calderwood et al., 1999; Garcia-Alvarez et al., 2003) and the α-helical region that lies between the membrane and this NPxY (Wegener et al., 2007; Anthis et al., 2009). Talin binding alters the orientation of the integrin β subunit transmembrane domain, separating the cytoplasmic domains of the α and β integrin subunits, thereby inducing the extended open conformation of the extracellular domains (reviewed in Shattil et al., 2010; Kim et al., 2011). Both the integrin- and membrane-binding activities are required for the talin head to 'inside-out' activate integrins in cell culture (Anthis et al., 2009; Goult et al., 2010). In an elegant experiment, the ligand affinity of single integrin heterodimers in membrane nanodiscs was shown to be increased by the talin head (Ye et al., 2010), clearly distinguishing affinity from valency. Talin has been shown to be essential for integrin inside-out activation in cultured mammalian cell assays (Calderwood et al., 1999; Tadokoro et al., 2003), as well as in platelets and leucocytes *in vivo* (Nieswandt et al., 2007; Petrich et al., 2007; Lefort et al., 2012).

Box 2. Testing the importance of inside-out integrin activation by talin in tissues.

Mutations in the *Drosophila* integrin α subunit that should constitutively activate integrins resulted in expanded and ectopic adhesion structures in the embryo (Martin-Bermudo et al., 1998), indicating that regulation of integrin activity is necessary for normal development. In contrast, constitutively active mutations in the mouse integrin β1 subunit (Vinogradova et al., 2002) did not show developmental defects (Czuchra et al., 2006). This difference may be due to effects exerted by negative regulators of integrin affinity, such as ICAP1 and sharpin

(Bouvard et al., 2013), which may counteract the effects of activating integrin mutations in the mouse, but are absent from *Drosophila*.

Drosophila embryos that completely lack talin have defects closely resembling those caused by the absence of integrins, but nonetheless integrins appear bound to the ECM (Brown et al., 2002). Similarly, myoblasts from mouse embryos lacking talin-1 and talin-2 still have activated integrin (Conti et al., 2009). Thus, either talin is not the only protein that can inside-out activate integrins, or outside-in activation is sufficient to trigger substantial adhesion. To test talin inside-out activation more specifically, three residues in talin head have been mutated: L325, R358 and W359 (Fig. 2B; equivalent *Drosophila* residues: L334, R367 and W368). Mutating any of these three residues impairs integrin activation, but only R358A and W359A impair integrin binding (Garcia-Alvarez et al., 2003; Kopp et al., 2010; Stefanini et al., 2014). Both L325R and W359A mutant mice died during embryonic development (Stefanini et al., 2014), but it was not reported whether the lethality caused by each mutant occured at the same stage, or with similar defects, as a null mutant (Monkley et al., 2000). The impairment of integrin activation by L325R was confirmed by the defects in hematopoietic cells (Stefanini et al., 2014; Yago et al., 2015). In the fly R367A caused lethality but with a much weaker phenotype than a null allele, whereas no defects were detected with L334R (Tanentzapf et al., 2006a; Ellis et al., 2014). Thus, for integrin-mediated adhesion to the ECM within intact tissues it is difficult to make a firm conclusion from current evidence regarding the importance of inside-out activation in general, and the contribution of talin in particular. This contrasts with the well-documented role of talin-mediated inside-out activation in hematopoietic cells.

Figure Legends

Fig. 1. Assembly of the ECM-integrin-talin-actin link.
For more information about talin structure and domains see Fig. 2; integrin-binding site1 (IBS1) labelled orange, other FERM subdomains yellow, IBS2 turquoise, and actin-binding sites 2 and 3 (ABS2, ABS3) red. (A) In the cytoplasm, the talin dimer has a closed conformation and has to be opened up to be able to interact with integrins. The three major conformational changes in integrin activation are also shown. Four steps (numbered on curved, dashed grey arrows) are shown for the assembly of the ECM-integrin-talin-actin link. This can be achieved via distinct orientations of talin dimers relative to the membrane: In (B, C, D and E), the talin dimer is oriented parallel to the membrane and initially recruited to integrins through its IBS2. In contrast, in (B', C', D' and E'), the talin dimer is oriented perpendicular to the membrane and its initial recruitment is mediated by IBS1. In both cases, the resulting integrin-bound talin dimers (B and B') can recruit and activate adjacent integrins (double arrows with "+"), bringing them into the adhesion site and potentially determining their spacing (double-arrows in C and C'). Talin binds to actin filaments (red rods) through its actin-binding sites (arrows in D and D'), completing the link between the ECM and actin. Force on actin and/or integrins (red arrows in E and E') is transmitted to the rod of talin, which results in unfolding of α-helical bundle domains within the rod (black double-arrows in E and E') and exposure of mechanosensitive binding sites for additional integrin-associated proteins (e.g. vinculin). The orientation of talin may switch, as indicated by the curved, dashed grey double-arrow with a question mark between E and E'.

Fig. 2. Structure and domains of talin.
(A) Domains and binding-sites of talin. The talin "Head" is an atypical FERM domain with four subdomains (F0-F3, in yellow and orange), followed by an ~80aa "Linker" that connects to the "Rod". The talin rod is composed of 61 α-helices that fold into 13 bundles (R1-R13) of 4 or 5 α-helices and one C-terminal dimerisation helix (α-helix 62, DH). Binding sites for interacting proteins are indicated: integrin-binding site 1 (IBS1) is in orange, actin-binding sites of the rod are in red, α-helices that are vinculin binding-sites (VBSs) are in purple. Vinculin-binding is indicated with purple arrows for three VBSs in R2 and R3; two dashed purple arrows indicate binding to all other VBSs in talin rod. The α-helix 50 is shown in blue and purple stripes, as it is both integrin-binding site 2 (ISB2) and a VBS. In all other figures, the IBS2-containing bundle R11 is blue. The residue numbering corresponds to mammalian talin 1. The figure has been adapted with permission from (Calderwood et al., 2013).

(B) Simplified representation of IBS1 bound to integrin. Different residues of F3 are required to bind the membrane proximal α-helix (e.g. L325 of F3) and the membrane-proximal NPxY motif (e.g. R358, W359 of F3) of the cytoplasmic region of integrin β-subunits (green). Mutating any of these three residues impairs integrin activation, but only R358A and W359A impair integrin binding. The two vertical lines indicate membrane binding.
(C) The bundles of the rod can unfold under force. Shown here are examples of four-helical (R3) and five-helical (R11) bundles unfolding under force (red arrows) and so exposing VBSs (purple) or VBS-IBS2/α-helix 50 (striped blue and purple). The unfolding of five-helical bundles may include other steps than those illustrated here, depending on the strength of the different helix-helix interactions within the bundle and the geometry of the force vector. All five-helical bundles have the same topology, with the exception of R13, which is part of ABS3.

Fig. 3. Regulation of actin binding to the talin rod.
Binding of both actin-binding sites, ABS2 and ABS3 is negatively regulated by adjacent sequences (inhibitory arrows on the left). The first step appears to be the alleviation of ABS3 inhibition, by an unknown mechanism. Once actin binds to ABS3 it exerts a pulling force on the rod of talin, which unfolds the most mechanosensitive bundle (R3) and thus exposes VBSs (centre). The unfolding of bundles may alleviate inhibition of ABS2 by R3 and R9 (blunt arrows on left diagram), enabling actin-binding to ABS2. As shown on the right, vinculin also opens up from a closed conformation to bind VBSs with its head (purple) and actin with its tail (red).

Fig. 4. Potential formation of an intracellular matrix by talin
Model for how talin dimers may generate a network of integrins. Talin can potentially connect numerous integrins, provided that both IBS1 and IBS2 can bind to the same integrin β-subunit (i.e. to the membrane-proximal NPxY with IBS1 and membrane-proximal α-helix with IBS2). Thus, talin may coordinate multiple integrins within the adhesion site. Double arrows with question marks indicate interactions that could connect rods of talin dimers in the cytoplasm, which may be direct intermolecular talin interactions or indirect through other IAPs, or actin filaments.

Figure 1
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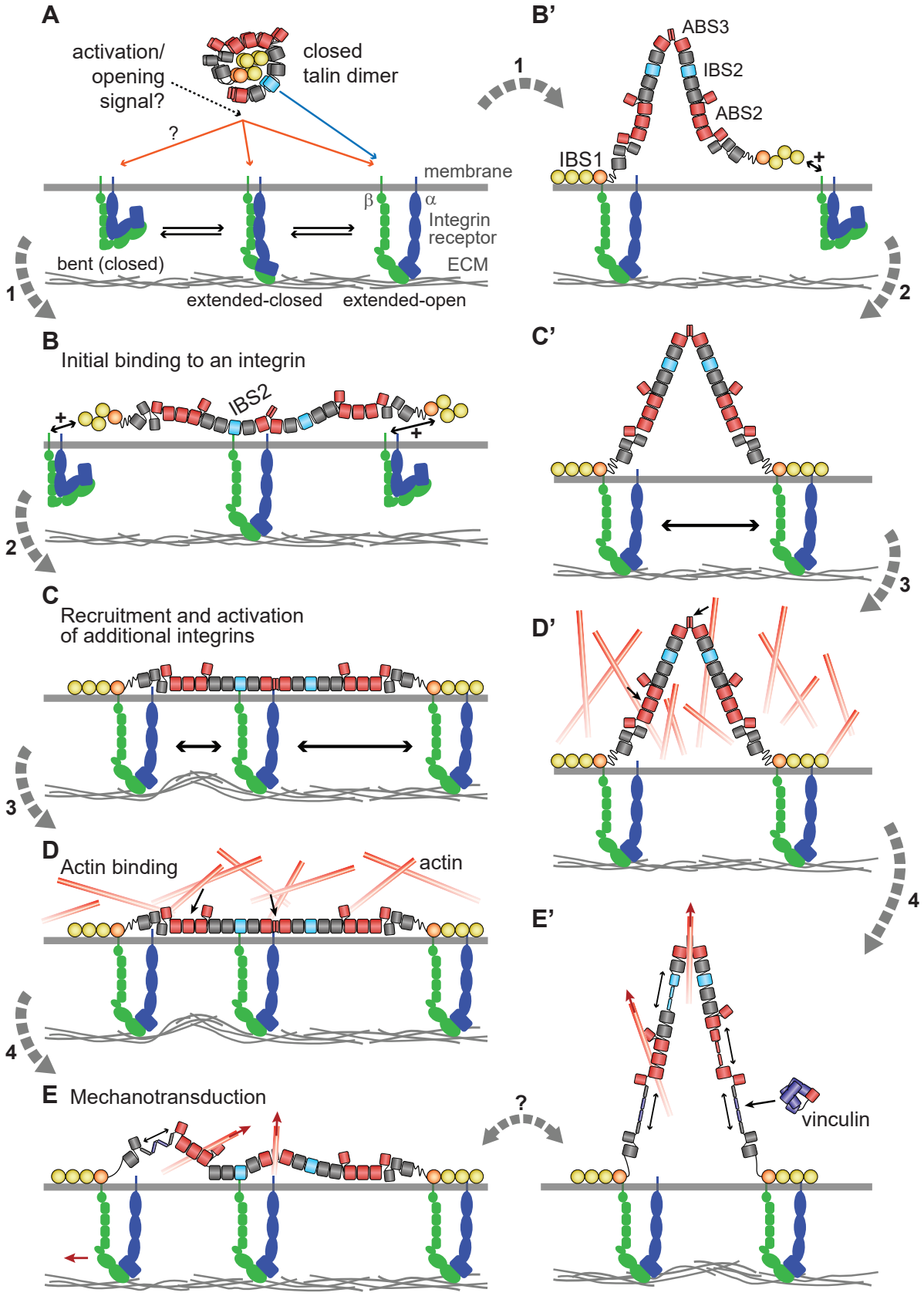

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Figure 2
Klapholz & Brown

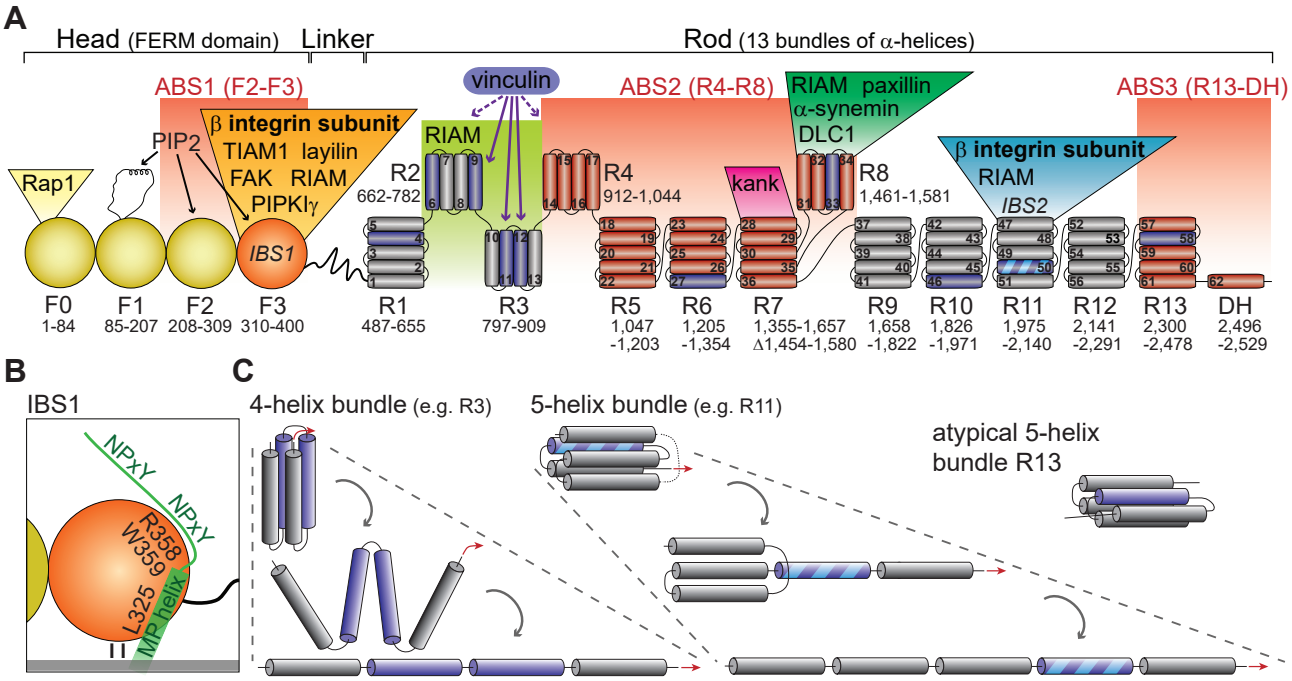

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Figure 3
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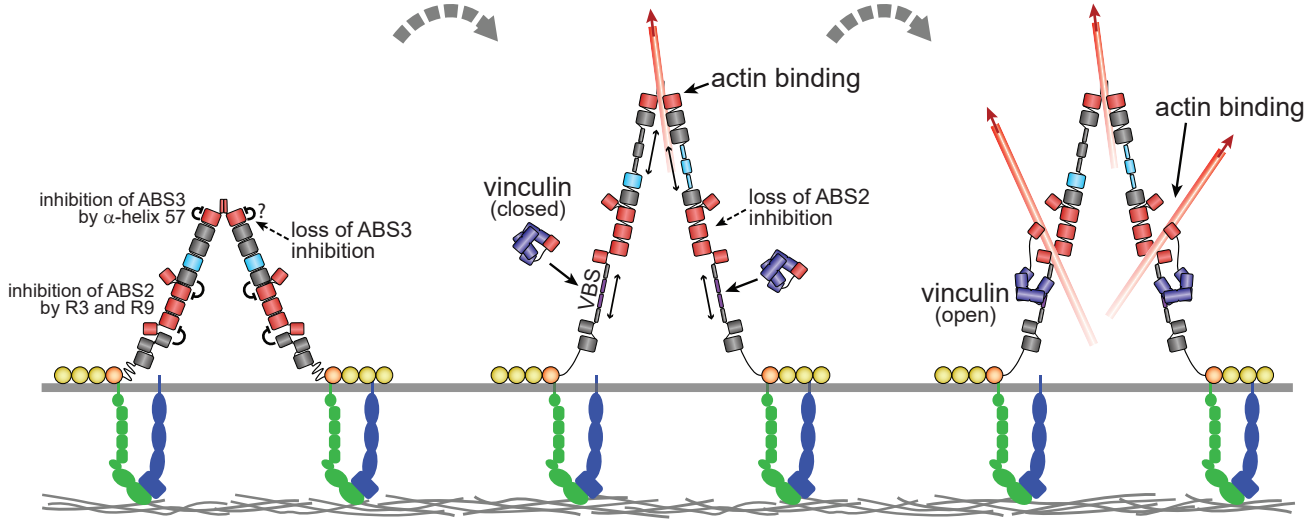

Figure 3. Regulation of actin binding to talin rod.
(Left) Binding of both actin-binding sites, ABS2 and 3 is negatively regulated by adjacent sequences (inhibitory arrows). The first step appears to be the alleviation of ABS3 inhibition, by an unknown mechanism. (Centre) Once actin binds to ABS3 it exerts a pulling force on the rod of talin, which unfolds the most mechanosensitive bundle (R3) and thus exposes VBSs. The unfolding of bundles may alleviate inhibition of ABS2 by R3 and R9 (blunt arrows on left diagram), enabling actin-binding to ABS2. (Right) Vinculin also opens up from a closed conformation to bind VBSs with its head (purple) and actin with its tail (red).

Figure 4
Klapholz & Brown

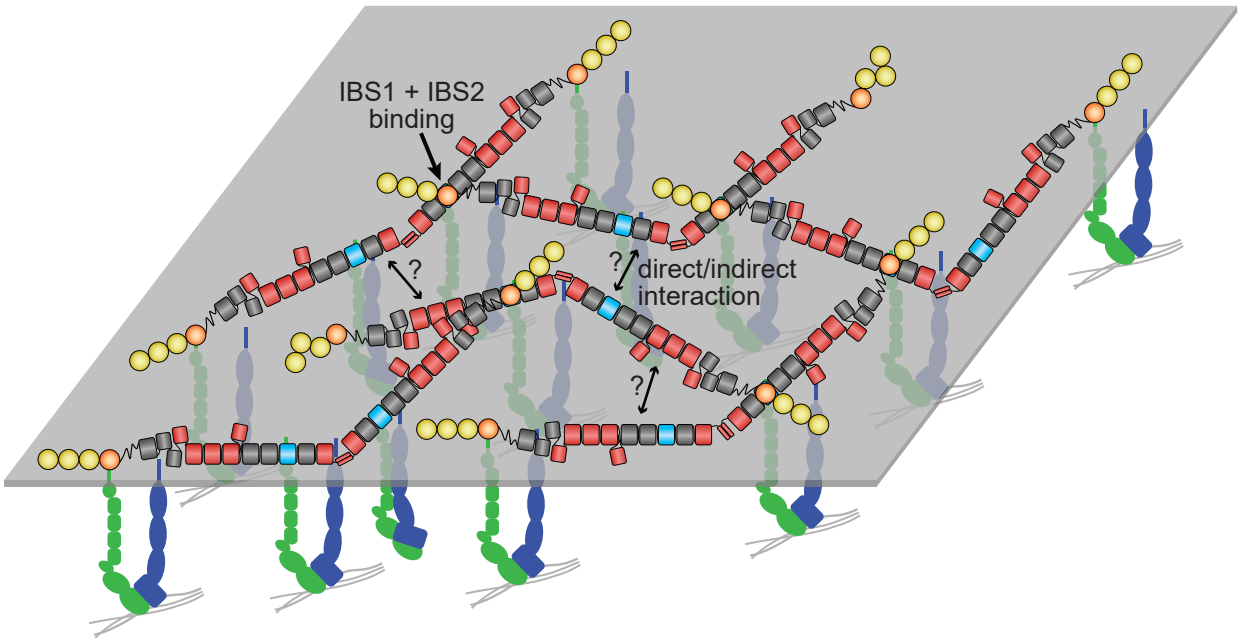

Figure 4. Does talin form an "intracellular matrix"?.
Model for how talin dimers may generate a network of integrins. Talin can potentially connect numerous integrins, provided that both IBS1 and IBS2 can bind to the same integrin β-subunit (i.e. to the membrane-proximal NPxY with IBS1 and membrane-proximal α-helix with IBS2). Thus, talin may coordinate multiple integrins within the adhesion site. Double arrows with question marks indicate interactions that could connect rods of talin dimers in the cytoplasm, which may be direct intermolecular talin interactions or indirect via other IAPs, or actin filaments.