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REVIEW

SUBJECT COLLECTION: ADHESION

New insights into FAK structure and function in focal adhesions

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ABSTRACT

Focal adhesion kinase (FAK; also known as PTK2) was discovered three decades ago and is now recognised as a key player in the regulation of cell–matrix adhesion and mesenchymal cell migration. Although it is essential during development, FAK also drives invasive cancer progression and metastasis. On a structural level, the basic building blocks of FAK have been described for some time. However, a picture of how FAK integrates into larger assemblies in various cellular environments, including one of its main cellular locations, the focal adhesion (FA) complex, is only beginning to emerge. Nano-resolution data from cellular studies, as well as atomic structures from reconstituted systems, have provided first insights, but also point to challenges that remain for obtaining a full structural understanding of how FAK is integrated in the FA complex and the structural changes occurring at different stages of FA maturation. In this Review, we discuss the known structural features of FAK, the interactions with its partners within the FA environment on the cell membrane and propose how its initial assembly in nascent FAs might change during FA maturation under force.

KEY WORDS: Cell adhesion, Focal adhesion kinase, FAK, Structure, Function

Introduction

Focal adhesion kinase (FAK; also known as PTK2) is a non-receptor tyrosine kinase that plays important roles in regulating cell adhesion, migration and survival. It is a multifunctional protein that can act at different sites in the cell and can be activated by various cues, including cell adhesion to the extracellular matrix (ECM) and growth factor signalling. One important role of FAK is the regulation and coordination of mesenchymal cell migration through adhesion to the ECM, and this Review will focus on this function of FAK. Other important functions of FAK at different cellular locations are summarised in Box 1.

FAK was discovered as a protein that localises to cell adhesion structures, known as focal adhesions (FAs) (Hanks et al., 1992; Schaller et al., 1992). FAs are large protein complexes that form at the cytoplasmic side of the plasma membrane and, via integrin receptors, are connected across the membrane to the ECM. This attachment to the ECM allows cells to gain traction during mesenchymal cell migration, and can be regarded as the equivalent of 'feet' touching the 'ground' when we walk. Inside the cell, the FA complex is connected to actomyosin stress fibres, which through their contraction, generate the forces for cell traction. However, as is the case for our feet on the ground, there will be no movement if the attachment is static. For productive cell migration, FAs have to be dynamic and their formation and disassembly tightly controlled (Wehrle-Haller, 2012). FAs form and make initial contact with the ECM at the cell front, where they form small and relatively unstable adhesion contacts, known as nascent FAs (Fig. 1). Attachment of nascent FAs to actomyosin stress fibres and their application of force leads to maturation into larger and stable FAs that form a tight ECM–actin linkage. As stress fibres contract, the cell body moves over the attached FAs, and once the FAs arrive at the cell rear, they need to disassemble to allow continuous cell movement (Fig. 1). In order to coordinate all these events, FAs have integrated regulatory systems that control and guide FAs through their lifecycle of formation, maturation and disassembly. To fulfil both the mechanical and the regulatory roles of FAs, they are, on the one hand, composed of structural proteins that provide a stable ECM–actin linkage, and, on the other hand, contain a signalling apparatus that is responsible for the overall coordination of the changes required during the FA lifecycle (Wehrle-Haller, 2012).

FAK is one of the main signalling components in FAs and is recruited early upon cell adhesion into nascent FAs. Its absence results in static FAs that turn over poorly, leading to greatly impaired cell migration (Ilic et al., 1995). FAK function is indeed critical in a number of processes that require controlled mesenchymal cell migration, including various events during development, tissue regeneration and wound healing. FAK-knockout embryos are not viable and have general defects in mesoderm development (Ilic et al., 1995). Studies on conditional FAK-knockout mice, as well as kinase-inactive knock-in mice, have revealed that FAK has a key role in angiogenesis and vascular development (Chen et al., 2012; Lim et al., 2010; Shen et al., 2005). In adults, FAK is required for intestinal regeneration (Ashton et al., 2010; Wang et al., 2019) and FAK activity has been shown to stimulate skeletal stem cells for new bone formation (Ransom et al., 2018). Aberrant FA-mediated cell migration also contributes in pathological settings (Winograd-Katz et al., 2014), for example when cancer cells break out of a solid tumour mass to invade neighbouring tissues, thereby initiating tumour metastasis (Nguyen et al., 2009; Sulzmaier et al., 2014). Indeed, FAK is upregulated in many tumours and correlates with a poor outcome in cancer patients (Gu and Zhou, 2018; Munguia-Calzada et al., 2019; Tang et al., 2022). The importance of FAK in cancer invasion and metastasis has further been demonstrated in a number of cancer mouse models (Lahlou et al., 2007; McLean et al., 2004; Wong et al., 2020).

FAK is a 120 kDa protein that contains a N-terminal FERM (band 4.1, ezrin, radixin, moesin homology) domain, a central kinase domain, followed by a proline-rich low-complexity region and a C-terminal FA-targeting (FAT) domain (Fig. 2A). The FERM domain is important for regulating the catalytic activity of FAK and induces an autoinhibited state by intramolecularly interacting with the kinase domain (Lietha et al., 2007). As suggested by its name, the FAT domain is responsible for recruiting FAK into the FA complex through interactions with other FA components, including paxillin. Upon FAK integration into FAs, FAK autoinhibition is

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Box 1. FAK functions at different cellular locations

Although first identified in FAs, FAK is now known to localise to a number of locations in the cell, where it performs important alternative functions (Kleinschmidt and Schlaepfer, 2017). FAK colocalises on endosomal membranes with internalised integrins and talin (Alanko et al., 2015). FAK is kept active in this complex by delivery of cholesterol and generation of $PI(4,5)P_2$ (Takahashi et al., 2021) and is shuttled in an active integrin complex to the leading edge of cells to promote cell migration (Nader et al., 2016). FAK further localises to endothelial cell–cell adherens junctions (AJs) by directly interacting with cadherin receptors (Chen et al., 2012). Endothelial AJ localisation and concomitant activation of FAK is induced by vascular endothelial growth factor (VEGF) signalling, leading to phosphorylation of β-catenin by FAK and AJ disassembly. FAK activity in this context is critical for VEGF-induced angiogenesis and vascular permeability, both important in tumour progression (Chen et al., 2012). In addition, an expanding number of functions are found for FAK in the nucleus, all affecting gene expression in various ways. Nuclear localisation of FAK is triggered by different cellular stress conditions and requires a nuclear localisation sequence situated in the KAKTLRK lipid-binding site (Lim et al., 2008). FAK has been shown to promote cell survival in the nucleus by binding to the tumour suppressor p53 and inducing its degradation through MDM2-mediated p53 ubiquitylation and proteasomal degradation (Lim et al., 2008). This does not require the catalytic activity of FAK, but other nuclear functions do. For instance, active FAK is enriched in the nucleus of squamous cell carcinoma cells, where it drives a transcriptional programme that leads to the secretion of specific chemokines that allow these tumour cells to escape anti-cancer immune responses (Serrels et al., 2015). Furthermore, mechanical stress triggers FAK activation and nuclear localisation in cardiomyocytes, where it interacts and activates the MEF2 transcription factor (Cardoso et al., 2016). A crystal structure of this complex shows a helix-swapped FAT dimer bound to two MEF2 molecules (PDB ID: 5F28). A number of other transcriptional regulations mediated by FAK have been reported, and suggest that FAK has adopted highly adaptive mechanisms to respond to cellular stresses in different contexts (Canel et al., 2017; Lim et al., 2012; Luo et al., 2009).

released, leading to efficient autophosphorylation and association with the Src kinase (Cai et al., 2008). Src then phosphorylates a number of tyrosine residues in FAK, among them two sites in the kinase activation loop, leading to full FAK activation (Lietha et al., 2007).

In this Review, we do not extensively discuss the various physiological and pathological roles FAK plays at different cellular locations (see Box 1), and we refer readers to excellent existing reviews on these topics (Alanko and Ivaska, 2016; Dawson et al., 2021; Kleinschmidt and Schlaepfer, 2017; Naser et al., 2018; Serrels and Frame, 2016; Sulzmaier et al., 2014; Urra et al., 2021; Winograd-Katz et al., 2014; Yamaguchi and Knaut, 2022). We specifically focus on fundamental structural and regulatory mechanisms of FAK in the FA complex, which are relevant across many physiological and pathological settings involving mesenchymal cell migration. We discuss the known structural features of FAK, emphasising its interactions and integration into the FA complex, and we highlight how these interactions might allow the communication between FA signalling and structural components that are responsible for force transmission.

FAK structure and regulation

High-resolution structures are available for the three globular domains of FAK, the FERM, kinase and FAT domains (Fig. 2B–F; reviewed in Tapial Martinez et al., 2020). The FERM domain displays a clover-like structure with three sub-lobes, referred to as F1, F2 and F3. The structure of the kinase domain reveals a typical kinase fold formed by a N- (N) and C-terminal (C) lobe, with the ATP-binding site buried between the two lobes. Located within the C-lobe is the regulatory activation loop containing two tyrosine residues, Y576 and Y577, which confer high catalytic activity when phosphorylated by the Src kinase. The C-terminal FAT domain exhibits a four-helix bundle and is crucial for the targeting of FAK to FAs via specific interactions with the FA proteins paxillin and talin (herein referring to talin 1) (Hoellerer et al., 2003; Lawson et al., 2012). The FAT domain harbours the Y925 phosphorylation site, which, in active FAK, is also phosphorylated by Src. Interestingly, this site is located within the first helix (H1) of the FAT domain, suggesting that partial unfolding is required for efficient phosphorylation.

In addition to structural data from the isolated FAK domains, several studies have revealed self-regulatory features that involve interdomain interactions in FAK. A crystal structure of the FERM–kinase region unveils the mode of FAK autoinhibition, with the FERM domain docked via its F2 lobe to the kinase C-lobe (Fig. 2B, right) (Lietha et al., 2007). This closed conformation is further stabilised by a stretch of the linker that connects the two domains, which is sandwiched between the FERM F1 and the kinase N-lobe. This part of the linker contains the Y397 autophosphorylation site, which in this conformation is protected from efficient phosphorylation. Similarly, the activating phosphorylation sites in the kinase activation loop, Y576 and Y577, are sequestered between the two domains (Lietha et al., 2007). Conversely, once Y576 and Y577 are phosphorylated, the activation loop adopts a conformation that prevents the FERM domain from binding and inhibiting the kinase domain (Fig. 2F), hence retaining FAK in an open and active conformation (Goñi et al., 2014; Lietha et al., 2007).

Another important regulatory feature in FAK is its oligomerisation state. In solution, FAK exists in a monomer–dimer equilibrium, and the dimer state increases the efficiency of trans-autophosphorylation (Brami-Cherrier et al., 2014). Two sites of FAK dimerisation have been identified, one in the FERM domain and the other in the FAT domain. FERM-mediated FAK dimerisation occurs via the FERM F3 lobe and involves the conserved W266 residue (Fig. 2C). These FAK dimers have been structurally characterised by small-angle X-ray scattering (SAXS), and the interface is observed as crystallographic contacts in all crystal structures containing the FAK FERM domain. The dimer is further stabilised by binding of FAT to the FERM domain, presumably in a trans conformation, providing cross-stabilisation within the dimer (Brami-Cherrier et al., 2014). A second mode of FAK dimerisation occurs via the FAT domain and involves a helix H1 swap between two FAT molecules, as observed in a FAT crystal structure (Fig. 2E; Arold et al., 2002). The propensity of the helix swap appears to stem from tension that is induced by three proline residues in the hinge linking helices H1 and H2 in FAT, which facilitates H1 release from the four-helix bundle (Kadaré et al., 2015). Modifying the rigidity of the H1–H2 linker to promote or inhibit H1 release strongly affects FAT self-association and, since Y925 is located within H1, H1 release also correlates with a high efficiency of Y925 phosphorylation by Src (Kadaré et al., 2015).

FAK integration into FAs

Super-resolution interferometric photoactivated localisation microscopy (iPALM) with fluorescently labelled FA proteins has demonstrated that FA proteins arrange into distinct bands, forming layers parallel to the membrane (Kanchanawong et al., 2010) (Fig. 3A). FAK localises to a signalling layer, placed directly on the

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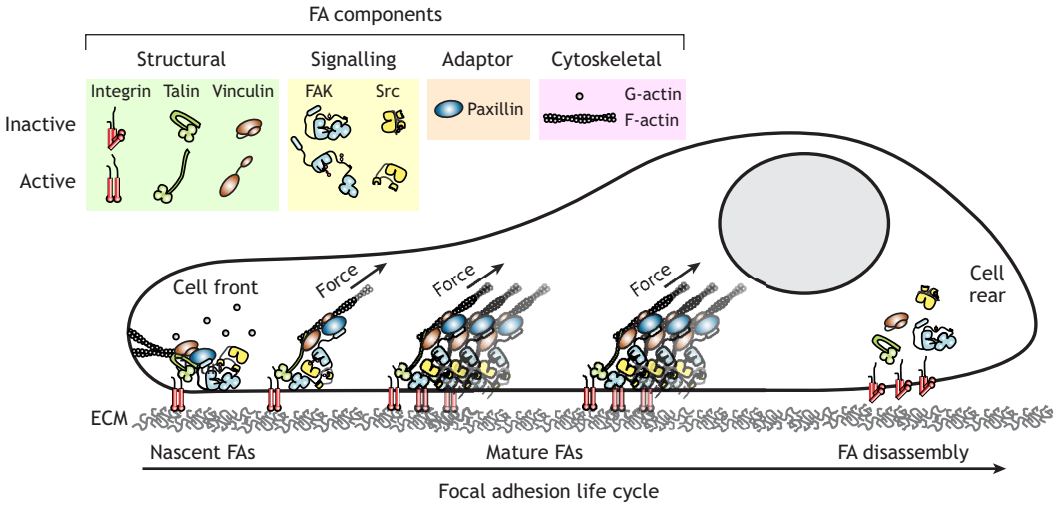

Fig. 1. Schematic illustration of the FA life cycle during mesenchymal cell migration. Nascent FAs form at the cell front (left). Force applied to FAs by contracting actomyosin fibres induces FA maturation into stable and larger FAs (centre). The generated force moves the cell body above the mature FAs. Once mature FAs arrive at the rear, FA signalling induces their disassembly (right). Inset, selection of FA proteins that can be classified into structural, signalling, adaptor and cytoskeletal components. Several of these are known to undergo conformational changes upon integration into nascent FAs and/or in response to applied forces.

membrane, with the FA adaptor paxillin right on top, slightly further away from the membrane. Actin and actin regulatory proteins are positioned some 50–60 nm above the membrane. The space in between is enriched in the structural FA proteins talin and vinculin, which connect integrins and the signalling layer to actin. The authors refer to this intermediate zone as the force-transduction layer, given that it relays the force applied by contracting actomyosin fibres to integrins and the signalling layer on the membrane (Kanchanawong et al., 2010). FAK is anchored to the FA complex at one end through interactions with the membrane, and at the other end interacts with the force-transduction layer. Below, we will discuss in some detail the structural aspects of the interactions that occur upon the incorporation of FAK into the nascent FA complex.

Interactions of FAK with the membrane occur through the lipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] (Cai et al., 2008; Goñi et al., 2014). The membrane at FA sites is enriched in $PI(4,5)P_2$ owing to the presence of the enzyme phosphatidylinositol-4-phosphate-5-kinase type Iγ, which is recruited to FAs by talin (Di Paolo et al., 2002; Ling et al., 2002). $PI(4,5)P_2$ interacts with FAK via two basic regions, one main site in the FERM F2 lobe involving a conserved K^{216}AKTLRK sequence (Cai et al., 2008), and a secondary site in the FAK kinase domain involving K621 and K627 (Hall and Schaller, 2017). Cryo-EM analysis of FAK 2D crystals bound to a $PI(4,5)P_2$ membrane, together with the dissection of biologically relevant interactions by mutagenesis studies, has revealed the structural arrangement of FAK on $PI(4,5)P_2$-containing membranes (Acebrón et al., 2020) (Fig. 3B). It shows that FAK adopts an oligomeric assembly with its FERM and kinase domains bound to the membrane. Comparison of autoinhibited and membrane-bound FAK demonstrates that membrane binding induces a large conformational change in FAK (Acebrón et al., 2020), as was previously predicted based on Förster resonance energy transfer (FRET) studies (Goñi et al., 2014), and suggests a mechanism of membrane-induced release of autoinhibitory FERM–kinase interactions (Fig. 3C). Increase in the local FAK concentration due to FA localisation and membrane binding promotes and stabilises FERM-mediated FAK dimerisation via W266, as observed by SAXS and in FERM crystals (Brami-Cherrier et al., 2014). As these FAK dimers interact via the KAKTLRK basic patch located in the FERM domain with $PI(4,5)P_2$ in the membrane, steric clashes with the membrane trigger the release of the autoinhibited kinase domain from the FERM domain. The kinase domain then rotates ∼90° around its N-terminus and interacts through its own $PI(4,5)P_2$-binding site with the membrane (Hall and Schaller, 2017). As a consequence, the Y397 autophosphorylation site, previously sandwiched between the FERM and kinase domains, is exposed, hence explaining $PI(4,5)P_2$-induced autophosphorylation as observed in biochemical experiments (Acebrón et al., 2020; Goñi et al., 2014). Furthermore, in the membrane-bound conformation of FAK, a large surface forms across the FERM and kinase domains that engages in a second FAK–FAK interface, leading to FAK oligomerisation. Intriguingly, the kinase domain bound to the membrane adopts an orientation that places the active site towards the membrane, therefore still sequestering it from efficient substrate access (Acebrón et al., 2020). This fits with biochemical data showing that membrane binding does not increase steady-state turnover activity towards exogenous substrates (Acebrón et al., 2020; Goñi et al., 2014). A tempting interpretation of this structural observation is that the initial FAK integration in nascent FAs does not activate FAK, but that owing to its dual attachment to the membrane and the force-transduction layer, FAK is primed for subsequent activation as forces are applied during FA maturation (see discussion below).

With the interactions between FAK and the membrane structurally characterised (Acebrón et al., 2020), what do we know about the interactions with the force-transduction layer? FAK is reported to interact via its C-terminal FAT domain with talin and paxillin (Hoellerer et al., 2003; Lawson et al., 2012). The interaction with talin occurs early upon cell adhesion in nascent FAs and appears to contribute to talin recruitment to nascent FAs (Lawson et al., 2012). Previous studies have indicated a direct talin–FAT interaction, but this was not reproduced in preliminary results with highly purified proteins (Naser et al., 2022 preprint), indicating that the interaction might not be direct or binary, or alternatively, that the interaction depends on a specific state, such as for example FAT dimerisation and/or Y925 phosphorylation. Previous mutagenesis experiments have defined the interaction site on FAT as involving

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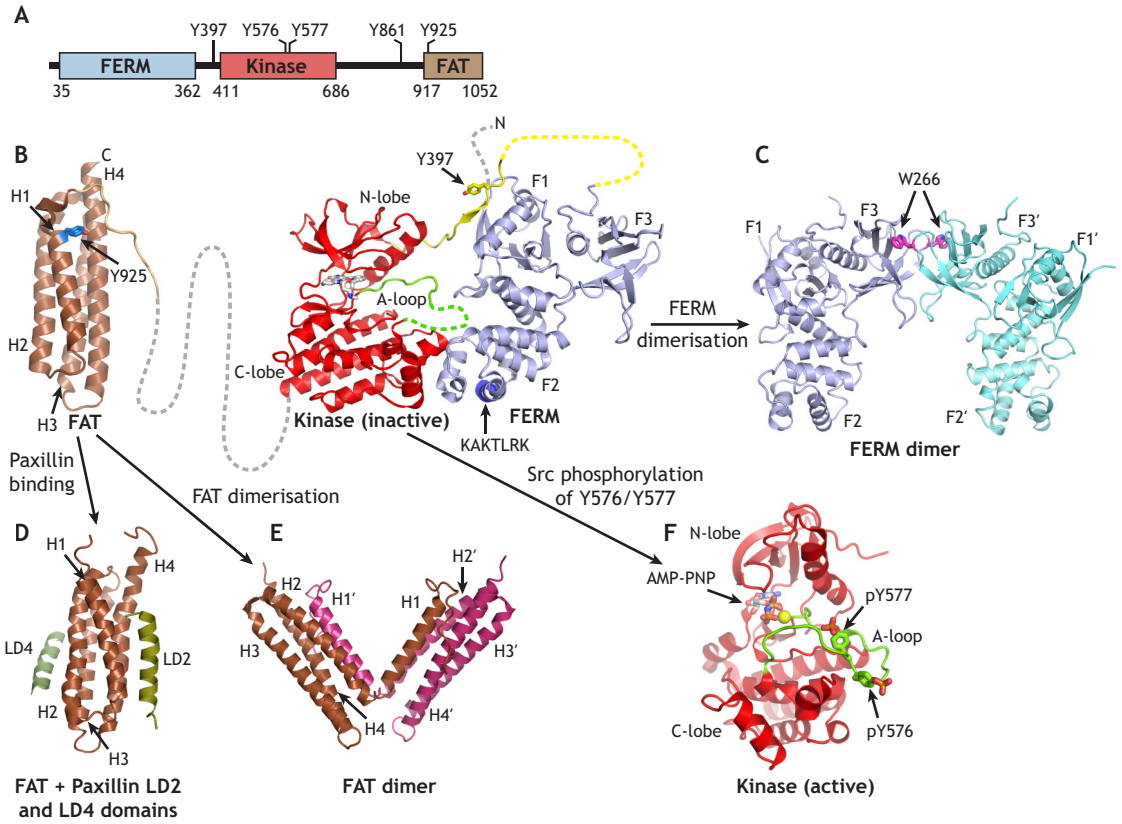

Fig. 2. Crystal structures of FAK. (A) Schematic domain structure of FAK. Domain boundaries and regulatory phosphorylation sites are indicated. (B) Structure of full-length FAK, assembled from the crystal structure of the FAK FERM–kinase region in the autoinhibited state (FAK 31–686; PDB ID: 2J0J) and the structure of the FAK FAT domain (FAK 892–1052; PDB ID: 1K05). Colouring of domains is as in A, with the FERM–kinase linker in yellow, the kinase activation loop (A-loop) in green and Y925 in the FAT domain in blue. Unstructured sections in the FERM–kinase region and portions that are not included in any structure (grey) and predicted to be unstructured are shown as dashed lines. N- and C-termini, as well as other important features are labelled. (C) FERM dimer mediated by W266 interactions in the F3 lobe as observed as crystallographic contacts in various crystal structures (shown here from PDB ID 2AEH) and by SAXS (Brami-Cherrier et al., 2014). (D) The structure of the FAK FAT domain bound to the LD2 and LD4 motifs of paxillin as defined by NMR and in crystal structures (PDB ID: 2L6F). (E) H1-swapped FAT dimer as observed in a crystal structure as a crystallographic dimer (PDB ID: 1K04). (F) Structure of the active FAK kinase domain with Y576 and Y577 in the A-loop (green) phosphorylated (PDB ID: 2J0L). A non-hydrolysable ATP analogue (AMP-PNP) and a Mg^{2+} ion (yellow sphere) are shown bound to the active site.

E1015, which is located at the beginning of helix H4 (Lawson et al., 2012). In the FAT structure, this residue is closely juxtaposed to the H1–H2 hinge; the interaction with this site could therefore be affected by H1 release and/or H1-swap-mediated FAT dimerisation. Talin has been shown to interact with FAT via its N-terminal head domain, which also interacts with cytoplasmic β-integrin receptor tails and $PI(4,5)P_2$ (Garcia-Alvarez et al., 2003; Goksoy et al., 2008). This suggests, that like the other FAK domains, the FAT domain likely also resides in close proximity to the membrane in nascent FAs, with the talin head domain potentially interacting simultaneously with the FAT domain of FAK, β-integrin tails and $PI(4,5)P_2$ in the membrane.

The FAT domain of FAK further interacts with the FA adaptor protein paxillin (Hoellerer et al., 2003). Given that paxillin also interacts with vinculin (Brown et al., 1996), the FAT–paxillin–vinculin connection establishes another important linkage between FAK and the force-transduction layer. The FAK FAT domain interacts with the two LD motifs LD2 and LD4 in paxillin simultaneously through two opposite faces in the FAT domain as observed in a crystal structure and by nuclear magnetic resonance (NMR) (Bertolucci et al., 2005; Hoellerer et al., 2003) (Fig. 2D). One binding site is formed by helices H1 and H4, and the other by helices H2 and H3 within the FAT four-helix bundle. There is some evidence suggesting that LD2 prefers binding to the H1 and H4 face and LD4 to the H2 and H3 face of the FAT domain (Bertolucci et al., 2005; Liu et al., 2002). Although for both sites, a similar affinity of ∼10 µM is reported (Liu et al., 2002; Thomas et al., 1999), mutation of the two sites indicates that only the H2–H3 site can by itself maintain a stable association between FAK and paxillin (Gao et al., 2004). The simultaneous binding of LD2 and LD4 increases the affinity ∼10-fold to sub-micromolar levels (Thomas et al., 1999). Vinculin binds to paxillin via its tail domain. The main LD motif of paxillin that interacts with vinculin is LD2, with mutations in LD2 preventing paxillin binding to vinculin (Brown et al., 1996). Although the binary interactions between paxillin and both FAK and vinculin are well characterised, it is not clear how the three proteins interact simultaneously to form the FAK–paxillin–vinculin linkage. LD2 can bind to FAK and vinculin, but given that the short LD2 motif can likely only bind to one binding partner at a time, the trimeric complex only forms if LD2 binds to vinculin and LD4 to FAK. A plausible scenario is that paxillin initially binds to FAK through an attachment of both LD2 and LD4 to the FAK FAT domain in nascent FAs, which only contain low levels of vinculin (Lawson et al., 2012). During FA maturation and increased vinculin localisation into FAs, a switch might occur, in which LD2 disengages from FAT and binds to the vinculin tail domain

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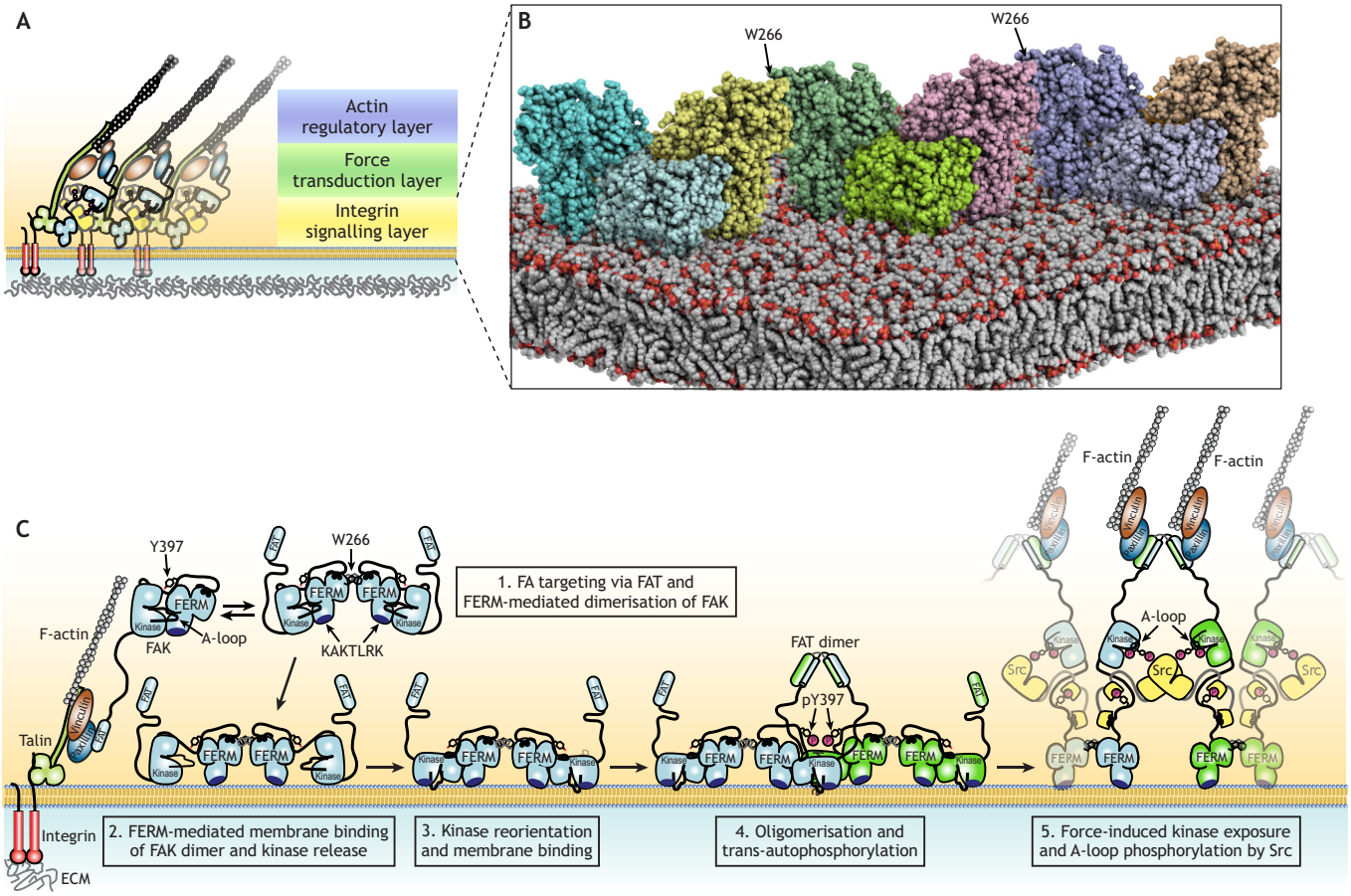

Fig. 3. FAK integration into FAs. (A) Schematic illustration of the FA architecture (left; see Fig. 1 for key of shown FA proteins) and FA layers as identified by iPALM (Kanchanawong et al., 2010) (right). (B) Oligomeric assembly of the FERM-kinase region of FAK on a PI(4,5)P_2 membrane, as observed by cryo-EM (Acebrón et al., 2020). Shown is an oligomer with six FAK molecules, each coloured differently and with FERM domains in a darker and kinase domains in a lighter tone. The kinase domains of FAK in yellow, pink and tan are mostly obscured by molecules in front. Sites of W266-mediated FERM–FERM interactions are indicated. (C) Schematic illustration of steps occurring during FAK integration into nascent FAs and proposed subsequent force-mediated activation: (1) FAT-mediated FAK localisation into FAs leads to a local increase in the concentration of FAK and promotes the formation of FERM-mediated FAK dimers. At this stage, the FAK activation loop (A-loop) is sequestered between the FERM and kinase domains in autoinhibited FAK. (2) Binding of FAK dimers via their KAKTLRK basic region in the FERM domain (dark blue) to PI(4,5)P_2-rich membranes at FAs causes the dissociation of the autoinhibitory interaction between the FERM and kinase domains. (3) The released kinase domains reorient to interact via their own basic residues with the membrane, thereby keeping the A-loop sequestered between the kinase and the membrane. (4) This aligns a large surface over the FERM and kinase domains, which engages in the formation of a second FAK–FAK interface (illustrated between blue and green FAK molecules), leading to FAK oligomerisation on the membrane. The kinase reorientation exposes the autophosphorylation site for efficient trans-autophosphorylation of Y397 in oligomeric membrane-bound FAK. The close proximity of FAK molecules might promote FAT dimerisation via helix H1 swap (shown for central two FAK molecules). (5) Src is recruited to the FAK autophosphorylation site and force application by the actin cytoskeleton in maturing FAs might release the interactions of the kinase with the FERM domain and membrane, leading to efficient phosphorylation of the FAK A-loop residues Y576 and Y577 by Src and thereby full FAK activation. The molecules shown in a faded view to the left and right indicate the formation of an oligomeric assembly extending along the membrane.

(Fig. 4A,B). What exactly could trigger this switch is unclear; it could simply occur due to differences in affinities, or LD2 release from FAT could be induced by FAT dimerisation and/or phosphorylation.

The FAK FAT domain can bind talin and paxillin simultaneously, and as mentioned earlier, PI(4,5)P_2 binding of the talin head domain will likely keep the FAK FAT domain close to the membrane in nascent FAs (Goksoy et al., 2008; Lawson et al., 2012). The vinculin tail also binds to PI(4,5)P_2 (Palmer et al., 2009), and membrane binding of talin and vinculin is thought to open up their respective autoinhibitory head–tail interactions, thereby promoting initial force-independent interactions between the vinculin head and talin tail domains (Kelley et al., 2020) (Fig. 4B). With the FAT domain and vinculin close to the membrane and both bound to talin, their proximity might further aid the handover of paxillin LD2 from the FAK FAT domain to the vinculin tail. With talin and vinculin activated and ready to engage with actin, but FAK activity still kept low by membrane interactions (see above), this assembly in nascent FAs would be perfectly primed for actin attachment and force-mediated activation of FA signalling during FA maturation (Figs 3C, 4C).

FAK under force

Nascent FAs bind to actin, but only small forces are produced as the polymerizing actin fibres push against the membrane to form membrane protrusions. Larger forces in FAs are generated through the contraction of FA-associated actomyosin fibres, and this triggers FA maturation and signalling. Both talin and vinculin experience

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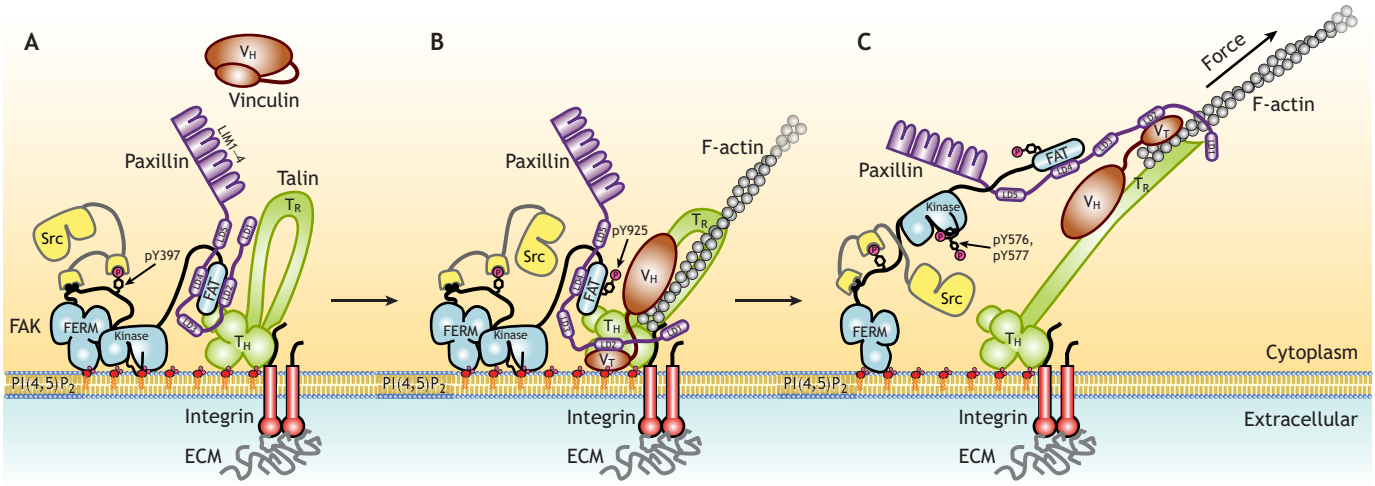

Fig. 4. Proposed schematic model of assembly of structural and signalling components in nascent FAs and force-induced changes. (A) FAK integrates into nascent FAs and adopts a conformation as observed by cryo-EM (see Fig. 3B). For clarity, only one FAK molecule of the oligomeric assembly is shown here. PI(4,5)P_2-induced autophosphorylation of Y397 (see Fig. 3C) recruits the Src kinase. The FAK FAT domain interacts with the paxillin LD2 and LD4 motifs and the talin head domain (T_H). (B) Vinculin is recruited and its tail domain (V_T) interacts with PI(4,5)P_2 in the membrane. This releases the vinculin head (V_H) and allows it to engage with the talin rod (T_R). The paxillin LD2 motif switches its binding from the FAK FAT domain to the vinculin tail. (C) Contraction of attached actomyosin fibres lifts T_R and vinculin from the membrane, pulling along paxillin and, via the LD2–LD3–LD4 linkage, the FAT domain of FAK. This, in turn, releases the FAK kinase domain from interactions with the membrane and the FERM domain, exposing the activation loop residues Y576 and Y577 for efficient phosphorylation by Src, leading to full FAK activation.

stretching forces in FAs, as demonstrated with cellular force sensors (Austen et al., 2015; Grashoff et al., 2010). Force applied to talin leads to exposure of cryptic vinculin-binding sites in the talin rod domain, resulting in a strengthening of the FA–actin linkage in mature FAs (Ciobanasu et al., 2014; del Rio et al., 2009). How force leads to activation of FA signalling is less clear, but it is likely that the connection between the force transduction and the signalling layer allows FAs to respond to forces and generate biochemical signals that control FA dynamics and directional cell migration (Wang et al., 2001). The level of FAK phosphorylation has been shown to increase when higher forces are applied externally to FAs (Torsoni et al., 2003; Wong et al., 2011; Zhou et al., 2021), as well as when cells are plated on stiffer substrates (Seong et al., 2013). The latter also leads to increased forces in FAs given that the low deformability of stiff substrates leads to a more rigid force transmission. This has important consequences in cancer; stiffening in the tumour environment leads to strongly upregulated FAK signalling and a highly invasive behaviour of tumour cells, as shown in breast cancer mouse models with increased ECM crosslinking in the tumour environment (Levental et al., 2009). Although there is currently no direct evidence, these findings point to FAK as a likely candidate to be a ‘first responder’ to force exerted on FAs by sensing the mechanical force and translating it into a biochemical signal. The notion of FAK acting as force sensor in FAs fits with the attachment points of FAK in the FA complex. With the N-terminal FERM domain attached to the PI(4,5)P_2-enriched membrane and the C-terminal FAT domain linked to the force transmission components talin and vinculin, the FAK molecule is bound to be exposed to stretching forces once force builds up in maturing FAs. The possibility of FAK activation being mediated by stretching forces was tested in single-molecule force spectroscopy experiments using atomic force microscopy (AFM) (Bauer et al., 2019). To this end, force handles were introduced into FAK at the predicted force application sites (i.e. at the membrane-binding site in the FERM domain and the C-terminus) for attachment to a functionalised AFM cantilever and surface, and these engineered FAK variants were then used in a high-throughput AFM setup. Alignment of hundreds of resulting force response curves revealed highly defined force events, which included a force peak that could be associated with the rupture of autoinhibitory FERM–kinase domain interactions, given it was not observed in the constitutively open FAK-Y180A/M183A mutant (Bauer et al., 2019). This rupture peak occurred at a force of ∼25 pN using a high cantilever retraction speed that increases forces to detectable levels. This suggests that the forces occurring in FAs in cells [for talin, on average reported at 7–10 pN (Austen et al., 2015) with peak forces of 40 pN for engaged integrins (Wang and Ha, 2013)], which are applied over significantly longer time periods [half-life for FAK in FAs is ∼10 s (Stutchbury et al., 2017)], would suffice to activate FAK by means of stretching forces.

As mentioned above, the FAT domain of FAK is connected to both talin and vinculin (the latter via paxillin), but which of these linkages could be responsible for force-mediated activation of FAK in cells? Both talin and vinculin are required for a force-related increase in FAK phosphorylation in FAs (Zhou et al., 2021). Interestingly, vinculin was found to move away from the membrane towards actin upon force application to FAs (Case et al., 2015), and the vinculin–paxillin–FAK linkage (as illustrated in Fig. 4C) could therefore pull the C-terminus of FAK along. In contrast, the talin head domain, which is connected to FAT in nascent FAs (Lawson et al., 2012), stays on the membrane in mature FAs, with only the talin C-terminus locating close to the actin regulatory layer (Kanchanawong et al., 2010). Together, this suggests that direct force application on FAK would more likely occur via the vinculin–paxillin–FAT connection, whereas talin binding to FAK might be important to form an initial assembly capable of force-mediated activation of FAK. Once under force, it is further possible that paxillin directly contributes to a stable FAT–actin linkage given that it has been demonstrated that the paxillin LIM domains share conserved features with LIM domains that specifically interact with stretched actin filaments (Winkelman et al., 2020).

Taking the structural data for FAK bound to a $PI(4,5)P_2$ membrane and its C-terminal attachments together, a structural model of FAK integration into nascent FAs and subsequent force-mediated activation is starting to emerge (Fig. 4). Upon initial FAK recruitment to nascent FAs, membrane interactions induce an oligomeric and primed form of FAK with the FERM and kinase domains attached to the membrane, as seen by cryo-EM (Acebrón et al., 2020) (Fig. 3B). In this conformation, kinase activity is still suppressed, as the active site is facing towards the membrane. Meanwhile, the C-terminal FAT domain of FAK interacts simultaneously with paxillin and talin in nascent FAs (Lawson et al., 2012), with the latter possibly dependent on other components and/or events (Naser et al., 2022 preprint). We propose a model whereby FAT initially binds to paxillin via double attachment to the LD2 and LD4 motifs in paxillin (Fig. 4A). Upon vinculin incorporation, LD2 attachment switches from FAT to the vinculin tail domain, which, in the absence of force, also interacts with $PI(4,5)P_2$ in the membrane (Fig. 4B). Interactions of talin and vinculin with membrane induce opening of their head–tail interactions, freeing the talin rod and vinculin head to interact with each other and with actin (Kelley et al., 2020). Upon contraction of attached actomyosin fibres, the talin rod and vinculin are pulled away from the membrane, as observed by iPALM (Case et al., 2015; Kanchanawong et al., 2010), pulling along paxillin and the FAT domain of FAK. Once the regions linking kinase–FAT in FAK and LD2–LD4 in paxillin are fully extended, the applied force causes detachment of the FAK kinase domain from the membrane and the FERM domain. This leads to the exposure of the kinase active site and the neighbouring phosphorylation sites in the activation loop, resulting in its efficient phosphorylation by Src and thus full catalytic activation of FAK (Fig. 4C). Although this mechanism of direct force-mediated activation of FAK seems plausible when considering structural aspects of FAK on the membrane and its anchor points in FAs, as of today it remains a hypothesis that awaits experimental confirmation, for example by using cellular force sensors, as employed for talin or vinculin (Austen et al., 2015; Grashoff et al., 2010).

Conclusions and future challenges

In this Review, we discuss the known structural aspects of FAK, what they tell us about how FAK is regulated and how this might be interpreted in the context of its integration in the FA complex. To fully understand, however, how the interactions of FAK in FAs affect FA function and cell migration, a better ultrastructural understanding of the FA complex is required at different stages of FA maturation. Currently, a rather large gap remains in our structural understanding of the entire FA complex. On the one hand, bottom-up reconstitution approaches have revealed atomic details of mostly individual FA components or small subcomplexes (for examples, see Bakolitsa et al., 2004; Chiswell et al., 2008; Dedden et al., 2019), and top-down approaches, on the other hand, have provided a low-resolution ultrastructural view of the FA architecture in cells (Kanchanawong et al., 2010; Patla et al., 2010; Stubb et al., 2019). This resolution gap severely limits the understanding of the integrated functioning of FA components and their coordinated structural changes within the FA complex, and therefore several of the models proposed here remain speculative. Despite the rapid emergence of structural details for numerous cellular megadalton complexes, aided by revolutionary advances in cryo-EM over the past decade, atomic structures that could provide insights into the FA ultrastructure are not yet available. The reason for this is likely a combination of high complexity, high dynamics under changing force conditions and an environment that requires, for its stability, attachment to the plasma membrane and transmembrane receptors on one end and to contractile actomyosin fibres at the other end – all features that do not favour high-resolution studies of reconstituted complexes.

Another feature of the highly dense FA environment that likely complicates high-resolution structural analysis is the recently described tendency of its constituents to phase-separate into liquid-like condensates (Case et al., 2022; Zhu et al., 2020). Such biomolecular condensates have been described for a number of systems, whereby mixtures of multivalent components can establish a network of weak interactions, giving rise to a type of membrane-less compartmentalisation. These condensates can selectively incorporate components that favour the interaction network and therefore provide a means of locally concentrating relevant components. The majority of interactions driving the phase separation are likely non-specific in nature; however, specific and structurally defined interactions do form within the condensates and are in fact promoted by increased local concentrations. The driving forces for biomolecular phase separation and the roles of specific versus non-specific interactions are far from being understood, but although clearly important for compartmentalisation, biomolecular phase separation does not overturn the view that specific interactions are key to regulating specific protein activities and functions (Musacchio, 2022).

Future progress to obtain a better understanding of the FA ultra-architecture and the structural changes during FA maturation will likely come from a combination of various advances. Ideally, advances in both top-down and bottom-up approaches could eventually close the resolution gap to obtain pseudo-atomic models of intact FA complexes linking integrins and the membrane with actin filaments, at different stages of the FA life cycle. There are great expectations in the structural biology community that cryo-EM tomography on thin cell slices will soon undergo a similar revolution as has occurred with single-particle cryo-EM methods (Turk and Baumeister, 2020; Wan and Briggs, 2016). In addition, improvements in shape recognition and sub-tomogram averaging, aided by new deep learning neural network algorithms, are expected to provide major advances in the near future. The critical breakthrough would be to regularly achieve a resolution in the 6–8 Å range (1 Å is 0.1 nm), in order to be able to resolve secondary structure elements of proteins and facilitate unambiguous protein identification and fitting of high-resolution structures into tomographic density maps. But important advances are also required from bottom-up reconstitution approaches. Most high-resolution structures available today are of single or binary FA components in the absence of a membrane environment. In order to better understand all the connections at atomic level, high-resolution structures of larger subcomplexes reconstituted on membranes and/or actin filaments need to be obtained in the 2–3 Å range, which for some time to come will require analysis by X-ray crystallography or single-particle cryo-EM. Combining structural studies with complementary reconstitution experiments, such as hydrogen–deuterium exchange, cross-linking and biochemical studies can greatly help in obtaining a better picture of structural changes in FAs and how they are triggered. For force-induced changes in particular, force spectroscopy experiments on reconstituted systems can provide important insights. Such complementary studies help to better understand states that are not accessible by structural methods. However, to obtain a complete atomic view of conformational transitions in FAs, all information from structural snapshots and

complementary methods will have to be fed into computational modelling algorithms (e.g. steered molecular dynamics) to obtain a full-atomic structural and dynamic view of the changes occurring during FA maturation and force application, or in other words, it will require an integrative structural biology approach (Rout and Sali, 2019).

Furthermore, it is important to point out that our view on FA structure and function is likely over-simplified as it mostly relies on reconstitution and cell culture studies, whereas FAs *in vivo* are likely to be much more diverse and adapted to specific tissue environments and cell characteristics (Yamaguchi and Knaut, 2022). FA structures *in vivo* can be highly stable, for example to provide a stable attachment of epithelial cells to basement membranes (Goodwin et al., 2016; Sidhaye and Norden, 2017), they can be dynamic and short-lived as seen during macrophage movements (Yolland et al., 2019), or cells might switch from integrin-mediated to other forms of migration (Yamada and Sixt, 2019). Nevertheless, despite the variety of FA characteristics occurring *in vivo*, the main constituents are conserved, and hence many of the basic interactions and mechanisms discussed here are also likely to be relevant in many settings *in vivo*.

FA-mediated cell migration plays an important role *in vivo* during cancer invasion and metastasis. In good agreement with findings from cells in culture (Seong et al., 2013) and proposed mechanisms of force-induced FAK activation based on force spectroscopy (Bauer et al., 2019), it has been shown that stiffening of the tumour environment (leading to higher forces in FAs) results in strong upregulation of FAK signalling and tumour invasion in a breast cancer mouse model (Levental et al., 2009). Understanding the involved mechanisms at a structural level is important and can help to identify new treatments for late-stage invasive cancers, which is highly challenging but important for types of cancers that generally are not detected at early stages, such as lung, colorectal and pancreatic cancer. A number of therapies that combine FAK inhibition with other treatments show promise and appear to prevent the occurrence of resistance to single treatments (Dawson et al., 2021). FAK-related drug resistance appears to mostly not be related to the functions of FAK in FAs, but rather to those in the nucleus, where in response to various cellular stresses (such as primary cancer treatments), FAK can act as a highly adaptive regulator of gene expression to promote cell survival (see Box 1). Of particular promise is the observation that FAK inhibition appears to sensitise otherwise unresponsive tumours to immunotherapy, for example in high-grade pancreatic and ovarian cancers (Jiang et al., 2016; Ozmadenci et al., 2022). The benefit of FAK inhibitors in cancer treatment could therefore be twofold – to inhibit invasive mesenchymal cancer cell migration, as well as blocking treatment induced cancer cell survival that causes resistance. Clearly, a detailed structural understanding of protein interfaces and mechanisms involved in cancer progression could greatly help to discover new means of blocking the invasive behaviour of cancer cells. This could lead to new generations of therapeutics that are able to inhibit FA signalling as well as affecting the FA interaction network (i.e. scaffolding), which could greatly improve the potency and likely also the specificity of such inhibitors. This notion is supported by a recently described Src inhibitor that potently inhibits Src activity but also its interaction with FAK, thereby resulting in greatly improved anti-cancer activity, while apparently being well tolerated (Temps et al., 2021).

In summary, although new insights are continuing to improve our understating of FAK structure and function, great challenges lay ahead to fully understand the detailed mechanisms of FAK functioning in its cellular environments, which are not restricted to the FA complex, to which the structural models we discuss here refer, but also include shuttling to adherens junctions, the nucleus and endosomes (Kleinschmidt and Schlaepfer, 2017).

Competing interests

The authors declare no competing or financial interests.

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