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Opening the floodgates: proteomics and the integrin adhesome

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Cell biologists studying cell adhesion have already figured out that cell–extracellular matrix connections, mediated by integrin receptors, are diverse and extremely complex structures. Dozens of adaptors — linking integrins with the cytoskeleton, and numerous enzymes and signaling proteins — regulating adhesion site dynamics, collectively referred to as the integrin adhesome, cooperate in mediating adhesion and activating specific signaling networks. Recent proteomic studies indicate that the known adhesome complexity is just the tip of the iceberg. In each existing category of molecular function the number of candidate components more than double the known components and several new categories are suggested. Proteomic analysis of different integrin heterodimers points to integrin-specific variations in composition and analysis of adhesion complexes under varying tension regimes highlights the force-dependent recruitment of different components, most notably LIM domain proteins.

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Introduction

From its inception, the field of cell–matrix adhesion has primarily been driven by advances in microscopy [1]. The fact that cells interact with the substrate at discrete sites, termed focal contacts or focal adhesions (FAs) was first observed in the early 70s by the use of electron microscopy and interference reflection microscopy [2]. The adhesion sites looked like dense plaques that associated with microfilaments at the cell's interior and with extracellular matrix fibers on the outside [3].

With the advent of immunofluorescence and cytoskeletal research at large, the molecular nature of the plaque and adhesion receptors slowly revealed itself. The first FA linkers, vinculin and talin, were discovered based on their localization at sites where microfilament bundles terminate at cell membranes [4,5]. Shortly after, a transmembrane glycoprotein named integrin, localized within FAs, was identified as the fibronectin receptor responsible for mediating cell–matrix adhesion [6,7].

Although it was clear already from interference reflection live cell imaging that FAs assemble and disassemble within minutes, the tagging of FA proteins with intrinsically fluorescent proteins enabled the study of FA dynamics, particularly in the context of cell motility, at high spatial and temporal resolution [8,9]. The microscopy-based work has shown high heterogeneity of FA, which depends on the cell type, the matrix and cellular condition. An additional level of variation was identified in different cell regions and even within individual FA sites [10–13].

The ease at which cDNA could be fused with a fluorescent protein and expressed in cells, as well as the prominence of FAs in cells cultured on glass, led to the identification of numerous proteins with a prominent FA localization. By the turn of the century the number of proteins reported to be associated with FA exceeded 50 [14]. In addition to cytoskeletal and adaptor proteins, the list of FA components contained many signaling proteins, such as tyrosine, serine/threonine, and phosphatidylinositol kinases and phosphatases and regulators of Rho GTPases, establishing the FA as a nexus for both inside-out and outside-in signaling [15,16] with important ramifications for cell behavior and fate [17].

Using known FA proteins as baits in yeast two hybrid screens and in co-immunoprecipitation assays numerous proteins potentially interacting with FAs were identified and some of them were subsequently verified and shown to localize at FAs. Thus, the inventory of FA components has steadily grown over the past decade, reaching 180 proteins in the last count [18••].

While the complexity of a cellular structure with close to two hundred parts may seem daunting, recent proteomic studies of FAs suggest the number of relevant components may in fact be substantially larger. Advances in the proteomics field combined with protocols for the isolation of integrin adhesion complexes have laid the ground for three studies utilizing proteomics technology for global analysis of FAs. In this review we examine the methodologies and results of these pioneering studies and discuss their contribution to our understanding of FA composition and regulation.

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Laying the groundwork: advances in proteomics and adhesion site isolation

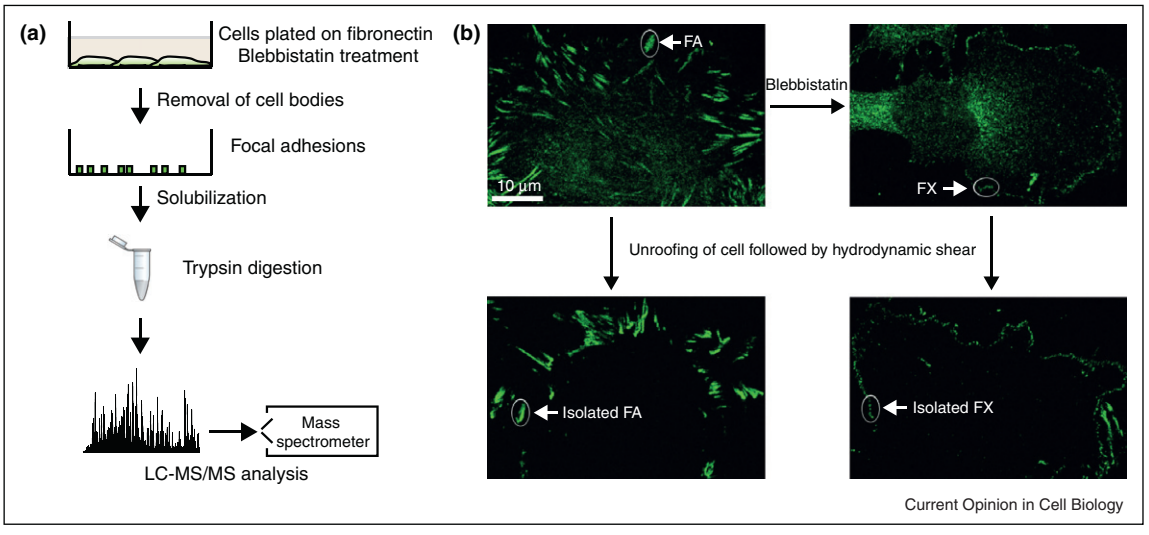
In an attempt to discover novel constituents, Kuo *et al.*, Schiller *et al.* and Humphries *et al.* isolated focal adhesions and used 'shotgun proteomics' to identify their components [19••,20••,21••]. In the 'shotgun' approach proteins from a given biological sample are first digested into peptides, and then analyzed by liquid-chromatography coupled to mass spectrometry (LC–MS) [22]. Recent developments in the proteomic field, such as the development of high-resolution mass spectrometers and developments in the computational analysis enable high accuracy in protein identification and quantification combined with coverage of large proportions of proteomes [23]. These studies aimed at broadening the view of the molecular components of FA without addressing their heterogeneity, and lay the ground for future detailed work of the localization and dynamics of these newly identified components.

The three studies discussed here differed in their proteomics methodologies, with Humphries and Kuo using low-resolution mass spectrometers combined with spectral counts for quantification, and Schiller, using high-resolution mass spectrometry and advanced label-free quantification algorithms. These differences should be considered in the interpretation of the data and the significance of the change in protein abundance between treatments. Another key determinant of proteomic data, which must be taken into consideration during interpretation, is the method used for sample preparation. As shown in Figure 1 both Kuo *et al.* and Schiller *et al.* started with cells adhering to fibronectin-coated plastic dishes and then used either osmotic pressure or a lysis buffer to unroof the cells. Application of hydrodynamic shear forces removed almost all of the cell content and basal membrane and retained integrin adhesion complexes. Humphries *et al.*, on the other hand, incubated small ligand-coated beads with cells in suspension and then used sonication and detergent extraction to isolate adhesion complexes bound to the beads. Common to both Humphries *et al.* and Schiller *et al.* was the incubation of cells with a membrane-permeable amine cross-linker prior to adhesion complex isolation. Kuo *et al.*, on the other hand, did not make use of chemical crosslinking in their sample preparation. Most importantly, each study was performed on a different cellular system, namely Kuo *et al.* isolated FA from human fibroblasts, Schiller *et al.* from mouse fibroblasts, whereas Humphries *et al.* used chronic myelogenous leukemia cells in their work. Given the experimental differences between the studies, it is interesting to examine what are the common components that were identified in all studies, and which ones are cell specific and/or method specific.

Widening the tent: potential new members of the integrin adhesome

Schiller *et al.* identified 890 proteins that showed enrichment on fibronectin compared with poly-L-lysine, and 770 proteins that were significantly different in at least one of the tested conditions; Kuo *et al.* reproducibly detected

Figure 1



A proteomic approach for the analysis of integrin adhesomes. The methodology developed and used by Schiller *et al.* [20••] and Kuo *et al.* [19••] is schematically shown in **(a)**. Adhesion complexes are gathered from fibronectin-coated plates after the removal of cell bodies. Following digestion into peptides, samples are analyzed by liquid-chromatography coupled to mass spectrometry (LC–MS). The actual isolation of integrin adhesion complexes by removal of cell bodies and application of hydrodynamic shear is shown in **(b)**. Immunolabeling of the focal adhesion protein paxillin highlights focal adhesions (FA) in control cells and focal complexes (FX) in cells treated with the myosin inhibitor blebbistatin. A cytoplasmic pool of paxillin visible in the pre-isolation images (top) is absent after isolation (bottom). Images of paxillin stained cells courtesy of Herbert Schiller and Reinhard Faessler.

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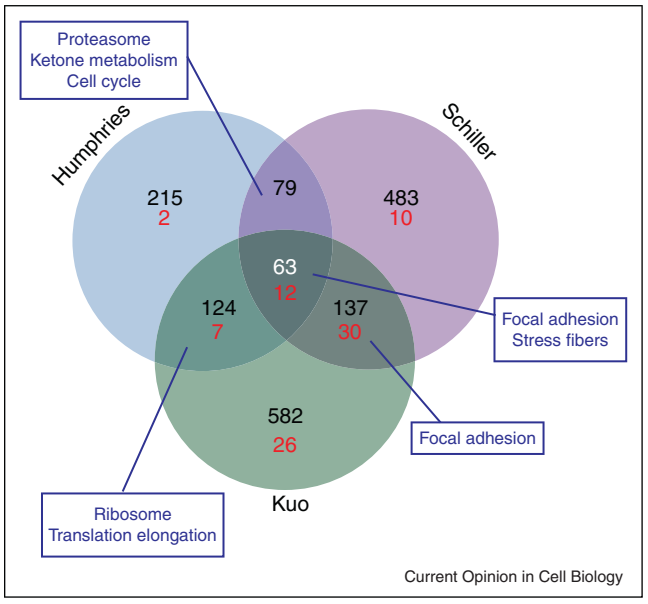
754 proteins in their isolated FAs; and Humphries *et al.* found 591 proteins to be enriched in FN or VCAM-1 samples compared to a non-integrin ligand control. In total, these studies associated 1683 proteins with integrin-mediated adhesions. The degree of overlap between these protein data sets is surprisingly low, consisting of only 63 proteins, as shown in Figure 2 (Table 1). Of the 180 known adhesome proteins only 87 are contained within this data set (Figure 2, indicated in red). The incomplete adhesome coverage is partially explained by the limited number of cell types and adhesion types analyzed in these studies as compared to the plurality of cell systems that were included in the original adhesome list. This is especially true for protein families, such as ERM, kindlins, Src, and Cas, in which different family members are expressed in different cell types. However, there are quite a few adhesome proteins that were reported in fibroblasts and were not identified in the proteomic analysis. These include some well-established components (e.g. Crk, RPTP-α, calpain-1) and other for which evidence of localization at FAs is not strong. As more proteomic studies in a variety of cell types become available it will be necessary to revisit the evidence for those adhesome components consistently absent. However, it is important to note that the majority of fibroblast adhesome components not identified by proteomics were either kinases and phosphatases or GEFs and GAPs. Such enzymes, which may only associate with FA transiently and not be bound strongly, could be lost in the proteomic sample preparation, but nevertheless be important for FA regulation.

Figure 2



Overlap and separation among three proteomic studies of integrin adhesomes. The degree of overlap between the proteomic studies of Humphries *et al.* [21••], Schiller *et al.* [20••] and Kuo *et al.* [19••] is shown in a Venn diagram. The number of proteins identified by proteomics in each region is indicated in black. The number of these proteins that are also found in the literature-based integrin adhesome of Zaidel-bar and Geiger [18••] are indicated in red. The list of 63 proteins found in all three proteomic studies is given in Table 1. Gene Ontology terms associated with the proteins in each region of the Venn diagram were assessed for enrichment versus the entire proteome. Statistically significantly enriched terms found are indicated in boxes with pointers to the relevant region.

Table 1

Candidate novel focal adhesion proteins. The list of proteins found by all three proteomic studies is given here (first row). Of the 63 proteins, 12 are known adhesome components (marked by bold), and the others should be considered candidates to being novel focal adhesion proteins. Signature molecular functions and protein domains of the literature-based integrin adhesome were used to identify proteins found in either of the proteomic studies as potential novel focal adhesion proteins, based on the fact that they share the same molecular function or domain (Figure 3). The lists of proteins belonging to a few of these categories are provided here.

Criterion	Gene/protein name
Adhesome components common to the three proteomic studies	ACTN4, ANXA1, AP2A1, AP2A2, AP2B1, AP2M1, ARF6, C1QBP, CAPZA1, CAPZB, CKAP5, CLTC, CNN2, CORO1C, CPNE1, CSNK1A1, CSNK2A1, DAB2, DDX1, EEF2, ELAVL1, EPRS, **FLNA**, FLN, FLOT1, FUS, HMGB2, HNRNPK, **ILK**, IQGAP1, **ITGA5**, **ITGB1**, **LASP1**, LOC392647, **MSN**, **MYH9**, MYL12B, NACA, NR5A1, PDAP1, PDCD10, PDLIM5, **PPP2CB**, RAB35, RAP1B, RPL10A, RPL12, RPL5, RPL8, RPLP2, SF1, SLC19A1, TFRC, **TLN1**, TTC9C, TUBB6, **VASP**, **VCL**, YBX1, YWHAE, YWHAG, YWHAQ, **ZYX**
Tyrosine kinases	**CSK**, DDR2, EGFR, EPHA2, FGFR1, FLT1, IGF2R, JAK1, KIF5B, PDGFRB, **PTK2**, PTK7, ROR2, **SRC**, TPR, YES1
Tyrosine phosphatases	**LAR**, PTP4A1, PTP4A2, PTPRK, **SHPTP2**
Ubiquitin ligases	CUL3, HECTD1, HUWE1, KCTD10, MGRN1, NEDD4, RBX1, STUB1, UBE2H, UBE2L3, UBE2M, UBE2N, UBE2R2, UBE2S, UBE2Z, UBR4
GTPases	**ARF1**, ARF4, ARF6, RND3, RHOG, ARL2, CDC42, GNA11, GNA1, GNA13, GNAI1, GNAI2, GNAQ, GNAS, GNB1, GNB2, GTPBP1, GTPBP4, **KRAS**, RAB11A, RAB11B, RAB13, RAB14, RAB18, RAB21, RAB34, RAB35, RAB3B, RAB5C, RAB7A, **RAC1**, RAC2, RALA, RALB, RAN, RAP1B, RAP2B, **RHOA**, RHOB, RHOC, RRAD, RRAS, RRAS2, SAR1A
SH2	**CSK**, **SRC**, NCK1, **PTPN11**, STAT5B, SUPT6H, **TENC1**, **TNS1**, TNS3, YES1
SH3	**ARHGEF7**, **CAS**, **CSK**, **CTTN**, DBNL, EPS8, ITSN1, ITSN2, LASP1, MPP1, MYO1E, NCK1, PACSIN2, PACSIN3, SH3BP4, SH3PXD2B, **SORBS1**, **SORBS3**, SPTAN1, **SRC**, YES1
LIM	CRIP2, **CSRP1**, CSRP2, **FBLIM1**, FHL1, **FHL2**, FHL3, **LASP1**, LIMA1, LIMCH1, LIMD1, **PINCH2**, LMO7, **LPP**, MICALL1, MICALL2, PDLIM1, PDLIM2, PDLIM4, PDLIM5, PDLIM7, **PINCH1**, **PXN**, **TES**, **TGFB1I1**, **TRIP6**, **ZYX**
FERM	EPB41, EPB41L2, **EZR**, FARP1, **KIND2**, **KIND3**, **MSN**, PLEKHH2, **PTK2**, **RDX**, **TLN1**, TLN2

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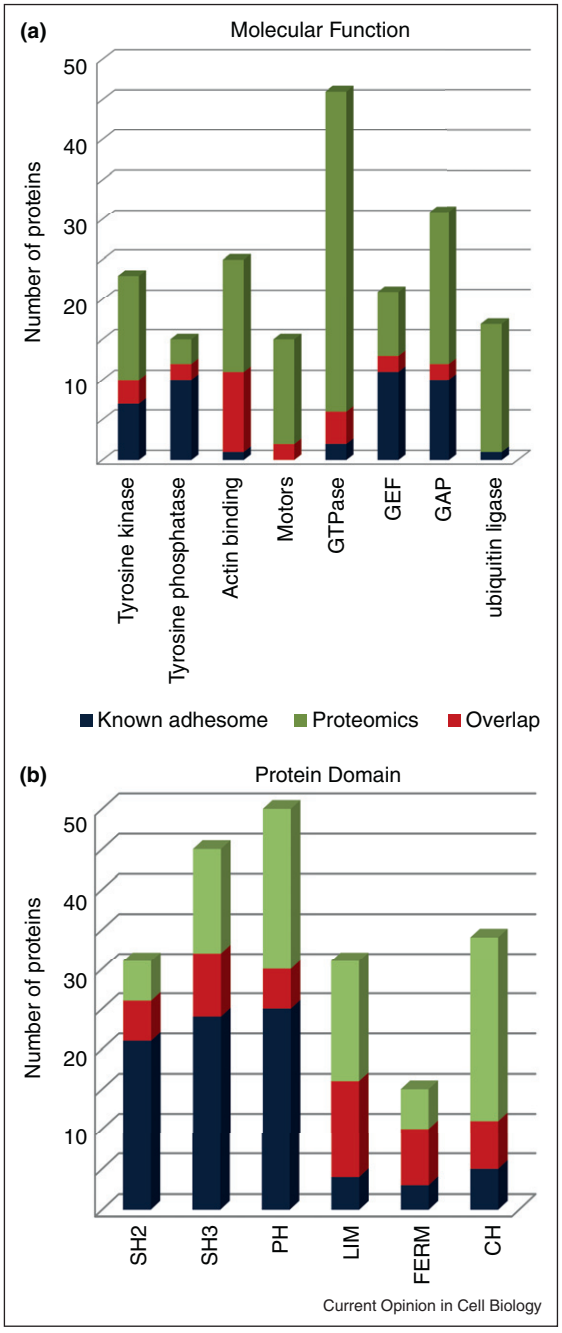
Turning our attention to the 1596 proteins identified in these studies and not appearing in the literature-based adhesome we ask how many of these are yet-to-be defined *bona-fide* components of the integrin adhesome? One way to predict which of these proteins are novel core FA constituents is based on the molecular function and domains of the known adhesome proteins. We looked for eight molecular functions representing the major structural and regulatory proteins of FA, and six protein domains characteristic of FA adapters (Figure 3). In most of these categories the proteomic experiments unearthed more than double the number of known FA proteins. For example, 16 novel tyrosine kinases were identified, 10 novel actin binders, 14 new motor proteins, and nine novel ubiquitin ligase proteins. Many of the novel tyrosine kinases, such as protein tyrosine kinase 7, discoidin domain receptor tyrosine kinase 2, receptor tyrosine kinase-like orphan receptor 2, or the ephrin type-A receptor 2, have been previously implicated in the regulation of cell adhesion or integrin signaling, however they had not been localized there. Among the F-actin binders are proteins such as eplin, adducin, α-actinin-4 and α-catenin, which are established components of cadherin-mediated adherens junctions, further substantiating the plausibility of them being true FA components. The category of GTPases exhibited a dramatic increase (40 novel proteins were identified on top of the known six), suggesting a higher involvement of this class of proteins than previously thought.

With regard to characteristic FA adaptor molecules with a variety of protein–protein interaction domains, the proteomic analysis identified 23 new proteins with calponin homology domain, 15 with LIM domains, 21 plekstrin homology, six src homology 2, 13 src homology 3 and five new proteins with FERM domains. Naturally, some proteins fall into more than one of the above categories. Altogether, this analysis showed a total of 231 new members with functions or domains that can be directly associated with FA function. But what about the remaining proteins for which a connection with FAs is not immediately clear? The accuracy of the mass-spectrometric analysis combined with appropriate statistical analysis ensures that most of the identified proteins are truly associated with FA, however their spatial distance and cellular functions are not necessarily related to cell–matrix adhesion. As an example, association of proteins through the cytoskeleton can potentially connect remote cellular compartments. To understand how these proteins found their way into the proteomic data set one must consider the cellular environment in which FAs are embedded.

It is all connected: organelles and non-adhesion complexes in the neighborhood of focal adhesions

Compartmentalization is a hallmark of eukaryotic cells. Metabolic processes are separated in space to maximize

Figure 3



Proteomics-derived candidate novel focal adhesion proteins. Signature molecular functions and protein domains of the literature-based integrin adhesome ('known adhesome', black bars) were used to identify proteins found in either of the proteomic studies as potential novel focal adhesion proteins, based on the fact that they share the same molecular function or domain ('proteomics', green bars). The number of proteins in a given group found both in the proteomic studies and in the literature-based adhesome are also marked in the chart ('overlap', red bars). The names of the proteins of a selected number of functions and domains are detailed in Table 1.

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efficiency and minimize interference. However, there is evidence in cells for numerous connections between different organelles, and FAs are no exception. Messenger RNA coding for FA proteins, translation initiation factors and ribosomes rapidly and specifically localize to FA upon integrin engagement to facilitate the local production of FA components [24,25,26•,27]. Both exocytic and endocytic pathways have been implicated in integrin traffic to/from FAs [28,29,30•], and endocytic components, such as GRAF-1, have been shown to localize at FA [31•]. Both SNARE-dependent and ESCRT-dependent membrane trafficking pathways have been shown to regulate the localization of FA proteins, such as Src and FAK [32,33]. Finally, a recent report describes an interaction between the endoplasmic reticulum and FA mediated by a kinectin–kinesin interaction [34•].

In light of this intricate connection between FA and basic cellular machineries we believe the proteomic analysis was successful in capturing the true complexity of the cellular environment intimately associated with FAs. A large number of proteins identified in these proteomic studies are associated with translation, ribosomes and membrane trafficking. If only the studies employing chemical cross-linking would have isolated these peripheral proteins we may have suspected it to be an artifact of cross-linking. However, Kuo *et al.* isolated just as many proteins with these roles. Other cellular pathways that are enriched in the proteomic list and have not yet been directly linked to FA belong to cell-cycle control, ketone metabolism and the proteasome. It will be interesting to examine the significance of these connections within cells.

The tight connection between the mediators of cell adhesion and multiple other cellular functions raises an important question: how do we define a FA? Is it a place, or a function? What is the involvement of distant interactors in the structure and activity of FA? Unlike organelles, which are defined by a membrane, FAs do not have a clear border, especially at the interface with F-actin, making it difficult to define where they end and another structure (e.g. stress-fiber) begins. In deciding whether a protein should be regarded as a FA component we favor maintaining the definition that was used to create the adhesome [35], namely that a protein must fulfill two criteria: co-localize with integrins and have a role in regulating cell adhesion. Standard cell biology methodologies — ideally in a high throughput pipeline — will have to be employed to determine which of the proteins identified by proteomics meet these criteria.

Now you see it, now you do not: the changing composition of the integrin adhesome

As soon as researches started looking at more than one FA protein in the same cell it became clear that not all adhesion sites are the same [13]. Integrin-mediated adhesion structures have been observed to vary in size, shape and dynamics [11]. Moreover, the composition of plaque proteins and their post-translational modifications (e.g. phosphorylation) can differ between cell types, at different developmental stages, and in different regions within the same cell [10,12,36].

To begin with, 24 different heterodimers of α-integrin and β-integrin have been observed in cells, each with its own set of specific ligands [37]. Humphries *et al.* compared adhesions formed by two different integrin heterodimers, namely $\alpha_5\beta_1$ integrin and $\alpha_4\beta_1$ integrin. They found the fibronectin receptor ($\alpha_5\beta_1$) recruited many more proteins compared with the VCAM-1 receptor ($\alpha_4\beta_1$). In addition, some proteins were recruited by both integrin heterodimers and others were either enriched in or exclusive to one type. The significance of this differential enrichment remains to be studied.

Force is a major player determining the structure, composition and dynamics of FAs. FAs were found to be responsive to force, either generated internally by actomyosin contractility or from an external source [38–40,41•], and the mechanosensitivity of FA is thought to enable cells to probe and respond to their mechanical environment [42–44]. The FA proteins zyxin, plectin, vinculin, FAK and filamin-A were previously shown to be recruited into FA in a tension-dependent mechanism [45•,46•,47•,48,49], but the overall changes in protein composition in response to changes in force are not yet known. In a natural course of events in cells, peripheral focal complexes, which are small actomyosin-independent integrin adhesions, mature into force-bearing FAs [36]. Inhibiting myosin II activity in cells (using the drug blebbistatin) leads to the dissolution of FAs and accumulation of focal complexes. Two of the studies reviewed here, Kuo *et al.* and Schiller *et al.*, used such treatment to compare the composition of FA with that of blebbistatin-induced focal complexes. Both studies identified proteins that were associated with FA in a tension-dependent manner, with either increased or decreased association with the structure. Thirteen proteins were found by both studies to be increased with tension. Strikingly, most of these are LIM-domain containing proteins, including zyxin, Tes, PDLIM1, Migfilin, FHL2 and FHL3, LPP, Trip6 and Hic5. Schiller *et al.* identified additional LIM-domain containing proteins and emphasized their significance in the response to tension. Kuo *et al.* identified multiple proteins that increased upon blebbistatin treatment, and emphasized the enrichment of proteins involved in Rac1-mediated lamellipodial protrusions in focal complexes. These experiments therefore show the ability of quantitative proteomics to capture the cellular dynamics of mechanosensing.

Conclusions

Integration of proteomics with focal adhesion research created an unprecedented resource of molecular components of the adhesive machinery. This unbiased

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discovery of novel FA components is an important step in understanding the mechanisms of their function and dynamics, and can serve as the starting point for multiple studies that examine the contribution of various novel components. Furthermore, the association with distinct cellular machineries raises additional hypotheses regarding regulatory mechanisms of focal adhesion. While these studies concentrated on the methodological developments, future studies will be able to address the dynamics and the variability of FAs and shed more light on the complexity of cell adhesion.

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