Systems-level analyses of the adhesion nexus

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Edward R. Horton
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Abstract

Cell adhesion to the extracellular matrix is mediated by the integrin family of adhesion receptors. Integrin receptor engagement initiates the formation of multimolecular protein complexes, termed integrin adhesion complexes (IACs), at the cell membrane. IACs are complex signalling hubs that are enriched in tyrosine-based phosphorylation events and form a mechanochemical connection between integrin receptors and the actin cytoskeleton. Dysregulation of individual IAC components has been reported to influence a wide range of biological processes that contribute to disease. Literature-curated and proteomic analyses of IACs have revealed an unanticipated molecular complexity of IACs in a variety of experimental contexts; however, a global consensus view of the composition of IACs, and a description of how the complex network of interactions in IACs influences global cell function, is currently lacking.

Here, multiple existing and new proteomic datasets detailing the protein composition of IACs were analysed to identify a systems-level description of IACs and to enable interrogation of IAC structure, topology and dynamics. Quantitative IAC proteomes derived from multiple cell types were integrated to generate a 2,412-protein ‘meta-adhesome’ database of proteins enriched to fibronectin-induced IACs. To investigate the putative functional adhesion landscape in an objective manner, the meta-adhesome was analysed using a combination of hierarchical clustering, gene ontology and interaction network analyses. An emergent property of the meta-adhesome was the definition of a consensus adhesome: 60 proteins commonly identified from IAC datasets that likely represent an IAC protein core composition. The consensus adhesome highlights how integrins connect to actin via multiple pathways and consists of both canonical and underappreciated IAC components.

To investigate the robustness of the IAC network, the effects of pharmacological perturbation of the key IAC kinases FAK and Src on IACs were examined. FAK activity was inhibited with the small molecule inhibitor AZ13256675, and mass spectrometry-based protein quantification revealed that IAC protein composition was unaffected upon FAK inhibition. Moreover, IAC composition was also insensitive to Src inhibition using AZD0530 and to simultaneous FAK and Src inhibition. In contrast, phosphorylation of IAC components, cell migration and cell proliferation were reduced upon FAK and/or Src inhibition. These data suggest that IAC protein composition is robust to perturbation of key kinases, while flux of signals propagated through IACs via phosphorylation is kinase dependent.

To examine IAC dynamics, the composition of IACs during IAC assembly and IAC disassembly were examined in the context of the meta-adhesome and consensus adhesome using IAC proteomic datasets. These analyses revealed the temporal dynamics of specific functional protein modules at IACs and detailed the compositional dynamics of the core cell adhesion machinery. In summary, these studies describe both a systems-level and a reductionist view of the IAC proteome, investigate the effects of kinase inhibition on IAC composition and chart IAC dynamics during their assembly and disassembly. These data demonstrate the usefulness of the meta-adhesome and consensus adhesome for future analyses of IAC proteomes.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Finally, I would like to thank my family for their support and encouragement, and Helen Frost for moving to Manchester with me and always being there.
Rationale for presenting the thesis in alternative format

The work presented in this thesis separates into three distinct sections that are all related by their experimental approach: mass spectrometry-based proteomic analysis of integrin adhesion complexes. Submission of the thesis in alternative format was beneficial for the following reasons:

- Separate introductions could be included for each results chapter, which helps to introduce the rationale and reasoning behind work presented in that chapter;
- The logical flow of the thesis as a whole is considerably improved;
- At the time of writing the thesis, manuscripts were in preparation for submission and re-writing these manuscripts for inclusion of the data in a conventional format thesis would not have been a productive use of time;
- Writing the thesis in manuscript format allowed us to visualise and determine whether the data presented formed a piece of work suitable for journal submission, and to which journal;
- The work presented includes contributions from multiple colleagues and exclusion of these data would have seriously impacted the narrative of the thesis; and
- The experience of converting experimental data into papers suitable for journal submission has been useful.

The results obtained for inclusion in the thesis form part of two manuscripts. One has been submitted (May 2015, Nature Cell Biology) and the second paper is in preparation. To aid the logical flow of the thesis as a whole and to allow supplementary figures and additional data to be included that was omitted from the submitted manuscript due to space constraints, the submitted paper has been divided between chapters 3 and 5. The student is co-first author (with A. Byron) on the submitted paper that forms chapters 3 and 5. The student is sole first author on the paper in preparation that forms chapter 4. Detailed author contributions are provided at the end of each chapter.

In addition to the work presented in this thesis, the student was involved in work to characterise the composition of IACs isolated from mesenchymal stem cells that is published online (Ajeian et al., 2015).

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>12G10</td>
<td>active integrin conformation-specific monoclonal antibody</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>4B4</td>
<td>inactive integrin conformation-specific monoclonal antibody</td>
</tr>
<tr>
<td>A.U.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>A375</td>
<td>human malignant melanoma cells</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ACTN</td>
<td>actinin</td>
</tr>
<tr>
<td>Adh</td>
<td>adherent</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride</td>
</tr>
<tr>
<td>ANXA1</td>
<td>annexin A1</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ARH</td>
<td>autosomal recessive hypercholesteremia</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 antagonist/killer</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BRIX1</td>
<td>biogenesis of ribosomes, homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CIB1</td>
<td>calcium-integrin binding protein 1</td>
</tr>
<tr>
<td>CLASP</td>
<td>cytoplasmic linker-associated protein</td>
</tr>
<tr>
<td>CO(_2)</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CRAPome</td>
<td>contaminant repository for affinity purification–MS data</td>
</tr>
<tr>
<td>CSK</td>
<td>c-src tyrosine kinase</td>
</tr>
<tr>
<td>Ctrl</td>
<td>control</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DAB2</td>
<td>disabled 2</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAVID</td>
<td>database for annotation, visualization and integrated discovery</td>
</tr>
<tr>
<td>DDX</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide</td>
</tr>
<tr>
<td>DIMT1</td>
<td>DIM1 dimethyladenosine transferase 1 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMEM-HEPES</td>
<td>DMEM supplemented with 25 mM HEPES</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dok1</td>
<td>downstream of tyrosine kinase 1</td>
</tr>
<tr>
<td>DPDPB</td>
<td>1,4-di-[3’-(2’-pyridyldithio)-propionamido]butane</td>
</tr>
<tr>
<td>DSP</td>
<td>dithiobis[succinimidyl propionate]</td>
</tr>
<tr>
<td>DTBP</td>
<td>dimethyl-3, 3’-dithiobispropionimdate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EB</td>
<td>extraction buffer</td>
</tr>
<tr>
<td>EB1</td>
<td>microtubule-associated protein 1</td>
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</table>
EB2, microtubule-associated protein 2
ECM, extracellular matrix
EDTA, ethylenediaminetetraacetic acid
EdU, 5-ethynyl-2’-deoxyuridine
ESI, electrospray ionisation
FA, formic acid
FAK [i], FAK inhibitor AZ13256675
FAK, focal adhesion kinase
FAT, focal adhesion targeting
FAU, Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed
FBLIM1, filamin binding LIM protein 1 (migfilin)
FCCS, fluorescence cross-correlation spectroscopy
FCS, foetal calf serum
FERM, 4.1, ezrin, radixin and moesin
Fig, figure
FN, fibronectin
FRAP, fluorescence recovery after photobleaching
FRNK, FAK-related non-kinase
GAD, genetic association database
GAP, GTPase-activating protein
GEF, guanine nucleotide exchange factor
GFP, green fluorescent protein
GIT, G protein-coupled receptor kinase interactor
GSK, Abl-related Gene Tyrosine Kinase
GTP, guanosine-5’-triphosphate
H1FX, H1 histone family, member X
HCl, hydrochloride
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF, human foreskin fibroblast cells
HGNC, HUGO gene nomenclature committee
IAC, integrin adhesion complex
IB, immunoblotting
IC_{50}, 50% inhibitory concentration
ICAP1, integrin cytoplasmic-associated protein 1
IF, immunofluorescence
IgG, immunoglobulin G
ILK, integrin-linked kinase
IQGAP, IQ motif containing GTPase activating protein
IQSEC, IQ motif and Sec7 domain
ITG, integrin
K562, human chronic myelogenous leukaemia cells
KEGG, Kyoto Encyclopedia of genes and genomes
LASP1, LIM and SH3 protein 1
LC, liquid chromatography
LC-MS/MS, liquid chromatography-tandem mass spectrometry
LDV, leucine-aspartate-valine
LIM, Lin-11, Isl1, and Mec-3
LIMD1, LIM domains containing 1
LPP, lipoma-preferred partner
m/z, mass to charge ratio
MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4
MAPK, mitogen-activated protein kinase
mDia1, diaphanous-related formin-1
MEF, mouse embryonic fibroblast cells
MES, 2-(N-morpholino)ethanesulfonic acid
MgCl2, magnesium chloride
MKF, mouse kidney fibroblast cells
MOC, Mander’s overlap coefficient
mRNA, messenger RNA
MRT04, mRNA turnover 4 homolog (S. cerevisiae)
MS, mass spectrometry
MS/MS, tandem MS
MS1, first m/z spectrum
MS2, second m/z spectrum
mTOR, mechanistic target of rapamycin
Na3VO4, sodium orthovanadate
NaCl, sodium chloride
NaF, sodium fluoride
NH4HCO3, ammonium bicarbonate
ns, not significant
nSC, normalised spectral count
OMIM, online mendelian inheritance in man
P4HB, prolyl 4-hydroxylase, beta polypeptide
PAGE, polyacrylamide gel electrophoresis
PBS-, CaCl2- and MgCl2-free phosphate-buffered saline
PBS+, phosphate-buffered saline containing CaCl2 and MgCl2
PLL, poly-L-lysine
PDL, poly-D-lysine
PDLIM, PDZ and LIM protein
PI3K, phosphatidylinositide 3-kinase
PINA, protein interaction network analysis
PINCH, particularly interesting new Cys-His protein
PIX, pak-interacting exchange factor
PKCα, protein kinase C α
PLCγ, phospholipase C γ
PPI, protein-protein interaction
PPIB, cyclophilin B
ppm, parts per million
PRIDE, proteomics identifications
pY, phosphotyrosine
R1, biological replicate 1
RCC2, regulation of chromosome condensation 2
RGD, arginine-glycine-aspartate
RIPA, radioimmunoprecipitation assay
RNA, ribonucleic acid
ROCK, Rho kinase
RPL23A, ribosomal protein L23a
RPMI, roswell park memorial institute
RSB, reducing sample buffer
Rsu-1, ras suppressor protein 1
RT, room temperature
s.d./SD, standard deviation
s.e.m./SEM, standard error of the mean
SDS, sodium dodecyl sulfate
SFK, Src family kinase
SH2, Src homology 2
SH3, Src homology 3
SHARPIN, shank-associated RH domain-interacting protein
SILAC, stable isotope labelling by amino acids in cell culture
SIPA1, signal-induced proliferation-associated 1
siRNA, small interfering RNA
Src [i], Src inhibitor AZD0530
Susp, suspension
TBS, tris-buffered saline
TBS-T, TBS supplemented with 0.05% (v/v) Tween-20
TCL, total cell lysate
Tf, transferrin
TGFB1I1, transforming growth factor beta 1 induced transcript 1 (hic-5)
TGM2, transglutaminase-2
TRIM, tripartite motif containing
TRIP6, thyroid hormone receptor interactor 6
U2OS, osteosarcoma cells
v, volume
VASP, vasodilator-stimulated phosphoprotein
VCAM-1(D40A), vascular cell adhesion molecule-1 mutant (Asp40→Ala)
VCAM-1, vascular cell adhesion molecule-1
w, weight
YFP, yellow fluorescent protein
Chapter 1

Introduction

1.1 Cell adhesion

A requirement for a metazoan existence is the ability of cells to form higher order structures and tissues (Johnson et al., 2009) via adhesion to each other, termed cell-cell interactions, and their extracellular matrix (ECM), termed cell-ECM interactions, thus allowing cells to detect changes and respond to cues in their immediate surroundings (Hynes, 1999). Cell adhesion is mediated by a number of cell-cell and cell-ECM cell surface adhesion receptors, such as cadherins, immunoglobulins, selectins, syndecans and integrins (Juliano, 2002; Morgan et al., 2007; Takeichi, 1990). The integrin and syndecan families of cell-ECM receptors provide direct physical connections between the ECM and the intracellular actomyosin cytoskeleton (Brakebusch and Fässler, 2003). These connections are required to allow bidirectional signalling and force transmission between the cell interior and ECM molecules across the plasma membrane (Evans and Calderwood, 2007; Hynes, 2002). The coordinated regulation of cell-ECM communication via cell-ECM interactions must be precisely controlled so that both static and migrating cells can use and respond to changes in the ECM. These responses are necessary for cells to undergo targeted relocation in developmental (Tarone et al., 2000), repair (Eckes et al., 2010) and disease processes such as osteoarthritis (Zemmyo et al., 2003), inflammatory disorders (Antonov et al., 2011) and cancer (Eke and Cordes, 2015; Seguin et al., 2015). Therefore, understanding cell adhesion biology at the molecular level is required to understand disease mechanisms and for the design of improved therapeutics. Recently, advances in network-based and quantitative approaches to study cell biology have uncovered molecular mechanisms and improved biological understanding (Ideker et al., 2003; Mast et al., 2014). In addition to candidate-based studies on individual molecules, the application of network-based approaches to study cell adhesion may provide insights into the global regulation of cell adhesion machinery.

1.2 The integrin family of adhesion receptors

Integrins, which mediate cell-ECM interactions, are large, heterodimeric membrane-spanning receptors composed of an α-subunit and a β-subunit that have large extracellular domains, a single-spanning transmembrane domain and typically short cytoplasmic domains (Campbell and Humphries, 2011; Luo and Springer, 2006). In mammals the integrin receptor family consists of 24 distinct αβ heterodimers, made up from 18 α-subunits and 8 β-subunits (Fig. 1.1a) (Hynes, 2002). The integrin heterodimers consist of four main groups depending on the mechanism of ligand binding, which are the Arg-Gly-Asp (RGD)-binding integrins, the Leu-Asp-Val (LDV)-binding integrins, the αA-domain-containing collagen-binding β1 integrins and the non-αA-domain-containing laminin-binding integrins (Fig. 1.1a) (Humphries et al., 2006). The αβ-subunit combination controls ligand-binding specificity with each integrin ligand able to bind multiple integrins and each integrin able to bind multiple ligands. For example, fibronectin (FN) binds α4β1, α4β7, α5β1, α8β1, αVβ1, αVβ3, αVβ6, αIIbβ3, vascular cell adhesion molecule-1 (VCAM-1) binds
Figure 1.1. The integrin family of adhesion receptors and their associated adhesion complexes (IACs).

(a) Diagram of integrin αβ heterodimer combinations. In mammals, 8 β and 18 α subunits form 24 distinct αβ combinations. Integrin heterodimers are classified according to their ligand binding specificities and mechanisms (RGD-binding, LDV-binding, collagen-binding and laminin-binding) and are coloured according to structural differences in the α-integrin subunit ligand binding region (αA-domain-containing integrins, green; non-αA-domain-containing integrins, yellow). A subgroup of LDV-binding integrins expressed specifically in leukocytes are indicated. (b) Schematic depicting integrins, IACs and associated adhesion-mediated signalling events. Upon integrin-ligand binding, proteins (coloured circles) recruited to integrin cytoplasmic tails form IACs, which have been shown to regulate a number of downstream signalling cascades and cell fate decisions. Data adapted from Hynes, 2002 and Humphries et al., 2006.
αβ1, αβ7, α9β1, αDβ2, laminin binds α1β1, α2β1, α3β1, α6β1, α7β1, α10β1, α6β4 and collagen binds α1β1, α2β1, α10β1, α11β1, αXβ2 (Humphries et al., 2006).

Integrin-ECM engagement regulates bidirectional signalling cascades that influence cellular processes both in normal development and disease, including cell spreading, proliferation, differentiation, survival, morphogenesis, migration and invasion (Fig. 1.1b) (Geiger and Yamada, 2011; Hynes, 2002; Winograd-Katz et al., 2014; Zamir and Geiger, 2001a). Therefore, integrin ligand binding and activation must be precisely regulated by the cell and can occur through compartmentalisation via integrin recycling (Franceschi et al., 2015), which has been shown to promote tumour cell invasion in three-dimensional (3D) environments (Jacquemet et al., 2013a); through regulation of integrin conformation (Rocco et al., 2008), which can determine the affinity of a particular integrin for specific ligands; and through integrin clustering, which leads to the formation of adhesion plaques (Paszek et al., 2009).

Integrins rely on adaptor molecules binding to their cytoplasmic domains to mediate their functions (Legate and Fässler, 2009). In ‘inside-out’ integrin signalling, binding of cytoskeletal proteins to integrin cytoplasmic domains causes conformational changes in the integrin head domains, thus regulating their affinity for extracellular ligands and ultimately cell-ECM attachment (Calderwood, 2004; Hu and Luo, 2013). In studies that examined integrin activation, integrin binding of talin (Tadokoro et al., 2003; Wegener et al., 2007) and kindlin (Harburger et al., 2009; Liao et al., 2015; Ye et al., 2013) activated integrins and increased their binding affinities for ECM molecules. Conversely, filamin regulated integrin activation by direct competition with talin-binding sites on integrins to maintain integrins in an inactivate state (Liu et al., 2015). Migfilin bound filamin with higher affinity than filamin binding to integrins, which implicated migfilin in sequestering filamin away from integrin tails to prevent filamin-mediated integrin inactivation (Ithychanda et al., 2009). Similarly to filamin, downstream of tyrosine kinase 1 (Dok1) competed with talin binding to integrins and displayed higher integrin-binding affinity than talin upon β3 integrin phosphorylation by Src family kinases (SFKs) (Oxley et al., 2008). Other negative regulators of integrin activation include calcium-integrin binding protein 1 (CIB1) and integrin cytoplasmic-associated protein 1 (ICAP1) (Millon-Frémillon et al., 2008; Yuan et al., 2006).

In ‘outside-in’ adhesion signalling, integrins are maintained in a high affinity state following initial integrin-ECM engagement (Hu and Luo, 2013), which results in integrin clustering and recruitment of cell signalling proteins to integrin cytoplasmic domains (Askari et al., 2010; Harburger and Calderwood, 2009). Recruited proteins form multiprotein complexes, termed integrin adhesion complexes (IACs) (Byron et al., 2010), which transduce intracellular signals to alter signalling pathways and downstream cellular responses (Fig. 1.1b).

1.3 Integrin adhesion complexes (IACs)

IACs are highly dynamic structures that undergo a continuous maturation process and can be characterised as nascent adhesions, focal complexes, focal adhesions, fibrillar adhesions, podosomes or invadopodia (also known as invadosomes (Linder, 2009)) depending on their size, lifetime and location (Fig. 1.2) (Cukierman et al., 2002; Geiger et al., 2001; Zaidel-Bar et al., 2004). Nascent adhesions are the smallest IAC structures (less than 0.25 μm in diameter) (Fig. 1.2), are
Figure 1.2. Characteristics of IAC structures.

Human foreskin fibroblast cells were spread on fibronectin (FN) and fixed. IACs were visualised using antibodies against vinculin (red) and α5 integrin (red) and the actin cytoskeleton was visualised by staining with fluorophore-conjugated phalloidin (green). Arrows indicate examples of IAC structures. Small nascent adhesions (green arrows) are located at the cell edge in membrane protrusions. Focal complexes (blue arrows) recruit more proteins and are larger than nascent adhesions. More stable focal adhesions (purple arrows) are mainly located at the cell periphery and are linked by stress fibres. Fibrillar adhesions (pink arrows) can be longer and are more centrally located in cells. The typical size (diameter), lifetime and cellular location of IAC structures are indicated. Scale bars, 20 μm. Data adapted from Cukierman et al., 2002, Geiger et al., 2001 and Zaidel-Bar et al., 2004.
present at the edge of the lamellipodium in membrane protrusions and have a high turnover rate, typically of less than one minute (Sun et al., 2014). Most nascent adhesions are rapidly disassembled, while a proportion of nascent adhesions recruit more proteins to form larger focal complexes (Fig. 1.2). Focal complexes are found at the leading edge of membrane protrusions and either disassemble within 5 minutes or, upon application of mechanical force from the ECM or actin cytoskeleton (Choi et al., 2008), mature into focal adhesions. Focal adhesions are 1 - 5 μm in diameter (Fig. 1.2), tend to be more stable with a lifespan of over 10 minutes and provide a robust anchorage via transcellular actomyosin-containing stress fibres (Byron et al., 2010). Focal adhesions either disassemble or elongate to form more stable fibrillar adhesions (Fig. 1.2), which tend to be more centrally located within the cell and are sites of ECM deposition and FN fibrillogenesis (Geiger and Yamada, 2011). Two other IAC structures, podosomes and invadopodia, have characteristics in common with focal adhesions but degrade the ECM at membrane protrusions in invasive cells (Block et al., 2008; Murphy and Courtneidge, 2011; Seano and Primo, 2015) and have been implicated in cancer metastasis and tumour invasion (Lohmer et al., 2014; Yamaguchi et al., 2006).

1.4 The dynamic nature of IACs

1.4.1 IAC dynamics in cell migration

IACs are highly dynamic, turning over within minutes. To investigate the dynamics of IACs, in particular focal adhesions and fibrillar adhesions, cultured fibroblast cells expressing the IAC proteins paxillin and tensin tagged with green fluorescent protein (GFP) were imaged using time-lapse microscopy (Zamir et al., 2000). This study revealed that both types of IAC are highly dynamic, with mean focal adhesion and fibrillar adhesion translocation rates of 19 and 18 μm/h, respectively (Zamir et al., 2000). The size, lifetimes and locations of all IACs in a cell (Fig. 1.2) must be precisely regulated to allow the formation of cell protrusions required for cellular sensing of the microenvironment and to permit cell retraction and polarisation during cell migration (Scales and Parsons, 2011). Efficient and effective cell migration, in particular, requires the coordinated spatiotemporal regulation of IAC assembly, stabilisation and turnover (Gardel et al., 2010). Engagement of specific integrin heterodimers contributes to the regulation of IAC stability; for example, Src-mediated syndecan-4 phosphorylation was found to control the relative abundances of membrane-localised α5β1 and αVβ3 integrins, which promoted either IAC disassembly or IAC stabilisation (Morgan et al., 2013). In a simplified model that describes cell migration, IACs assembled at the cell front in the leading edge of membrane protrusions, remained fixed to the ECM as the cell moves forward and disassembled at the cell rear to enable cell retraction (Wehrle-Haller and Imhof, 2003), which places importance on the regulation of IAC dynamics.

1.4.2 IAC assembly and maturation

The assembly of IACs has been shown to be a time-dependent hierarchical process (Zaidel-Bar et al., 2004), possibly because many IAC components are structural adaptors and are therefore required in order to allow the sequential recruitment and binding of additional IAC proteins. In support of the hierarchical recruitment of IAC components, the formation of focal complexes was visualised in cells migrating into an in vitro wound using phase-contrast and immunofluorescence
microscopy techniques (Zaidel-Bar et al., 2003). The first components recruited were αVβ3 integrin and phosphotyrosine, followed by talin and paxillin. Vinculin, α-actinin, focal adhesion kinase (FAK) and vasodilator-stimulated phosphoprotein (VASP) were found to be recruited to IACs later, which demonstrated that the molecular composition of IACs is time-dependent (Zaidel-Bar et al., 2004). Once the leading edge stopped protruding or retracted, IACs became larger and formed focal adhesions, which involved recruitment of zyxin and assembly of an actin bundle (Zaidel-Bar et al., 2004). Finally, tensin was recruited during fibrillar adhesion formation (Zaidel-Bar et al., 2004).

However, some controversy remains as to the order of proteins initially recruited to IACs, since in contrast to the studies mentioned above, it was reported that FAK was one of the earliest recruited proteins to nascent adhesions (Miyamoto et al., 1995), FAK was recruited to IACs before paxillin (Hu et al., 2014) and FAK bound paxillin in early nascent adhesions (Choi et al., 2011). Further investigation using phosphomimetic mutations that prevented paxillin phosphorylation at paxillin Y118 (Y118E) and paxillin Y31 (Y31E) revealed that paxillin inhibition led to an increased IAC maturation rate, disrupted the paxillin interaction with FAK and promoted IAC stabilisation, which suggested that both paxillin phosphorylation and paxillin-FAK binding regulate IAC formation (Choi et al., 2011). FAK has also been implicated in the recruitment of talin to IACs since a point mutation in the talin binding site on FAK prevented talin recruitment (Lawson et al., 2012), which suggested that FAK was recruited to IACs before talin. What is unknown, however, is the timing of FAK recruitment in relation to other IAC molecules since, for example, a recent study that did not assess FAK dynamics demonstrated that integrin and kindlin were the earliest proteins present in nascent adhesions (Bachir et al., 2014). In addition, IAC assembly has been shown to be dependent on the paxillin-interacting protein integrin-linked kinase (ILK), since a reduction and increase in the levels of vinculin and tensin, respectively, were observed in IACs in ILK knock-out cells (Elad et al., 2013). These studies raise the question as to what are the first molecules recruited to IACs, suggest that multiple mechanisms and heterogeneity of IAC formation may exist, and demonstrate the difficulty in assessing cause and effect from studies that inhibit IAC components.

Following their formation, a proportion of IACs are able to mature from early nascent adhesions to more stable and larger focal adhesions in a myosin II-dependent manner as a result of RhoA-stimulated cell contractility and intracellular force exerted on IACs via actin stress fibres (Chrzanowska-Wodnicka and Burridge, 1996; Geiger et al., 2009; Schwartz, 2010). Upon force generated by myosin II, it has been shown that a vinculin-talin complex associated with an integrin-kindlin complex in focal adhesions and clusters of α-actinin periodically associated with nascent adhesions throughout the IAC maturation process (Bachir et al., 2014). In other reports, an initially transient vinculin-talin interaction was shown to switch to a high-affinity binding interaction during IAC maturation that recruited paxillin (Humphries et al., 2007), and the stable vinculin-talin interaction was shown to occur in combination with vinculin binding to actin filaments (Chen et al., 2006). In addition to requiring force generated from myosin II, the maturation of nascent adhesions to focal adhesions at the back of the lamellipodium in membrane protrusions has also been shown to be mediated by α-actinin, since α-actinin depletion by RNAi prevented adhesion elongation (Choi et al., 2008).
In summary, while there is a consensus view that IAC assembly and maturation involves the sequential and hierarchical recruitment of proteins to IACs, the exact timings and order of protein recruitment of all the IAC molecules involved remains unresolved. This is possibly because relatively few IAC components have been examined in each study, IAC dynamics have been examined over different timescales between studies, and variation in IAC dynamics may exist due to cells type, ECM composition and cell exposure to different external stimuli.

1.4.3 IAC disassembly and turnover

Force is required for IAC maturation to occur and, reciprocally, the loss of force and tension exerted on IACs induces disassembly (Parsons et al., 2010). In support of this, it has been shown that the actin-crosslinking protein fascin was required to dissociate from stress fibres to allow actin to be severed by coflin, which reduced force on IACs and therefore induced IAC disassembly (Elkhatib et al., 2014). To induce IAC disassembly experimentally, in a force-dependent manner, actomyosin contractility has been inhibited in cells by treatment with the Rho kinase (ROCK) inhibitor Y-27632 or the myosin II inhibitor blebbistatin. In a study that examined IAC disassembly upon cell treatment with the ROCK inhibitor, different IAC components dissociated from IACs at different rates (Lavelin et al., 2013). The first molecules that dissociated were the cytoskeletal regulators zyxin and VASP, followed by talin, paxillin and ILK, and finally FAK, kindlin and vinculin (Lavelin et al., 2013). In other reports, zyxin and actin polymerisation were diminished at IACs in cells treated with blebbistatin but α5β1 integrin was unaffected and remained associated with the plasma membrane (Hirata et al., 2008). Further examination of additional IAC components, specifically vinculin and paxillin, has revealed that zyxin dissociated from IACs first upon blebbistatin treatment and began to reduce in abundance after 1 min (Wolfenson et al., 2011), while vinculin was found to leave IACs at a faster rate than paxillin (Wolfenson et al., 2011). These findings contradict results found upon treatment with the ROCK inhibitor that induced IAC disassembly and reported the loss of vinculin after paxillin dissociation (Lavelin et al., 2013). Further to these studies that have demonstrated the sequential loss of IAC components upon IAC disassembly, examination of IAC turnover rates by immunofluorescence imaging of IACs in membrane protrusions revealed that FAK, paxillin and zyxin dissociated from IACs with comparable kinetics (Webb et al., 2004), and therefore do not dissociate from IACs sequentially.

Despite discrepancies into the timings and order of protein dissociation from IACs, a number of IAC molecules have been implicated in the regulation of IAC disassembly. It has been reported that IAC disassembly was inhibited in FAK-null cells (Webb et al., 2004) as a consequence of reduced inhibition of Rho activity (Ren et al., 2000), and studies have shown that phosphorylation of FAK at FAKY397 induced IAC disassembly (Hamadi et al., 2005), which collectively suggested that FAK is involved in the regulation of IAC turnover. Filamin A has been reported to regulate IAC disassembly also, since filamin A depletion increased mitogen-activated protein kinase (MAPK) signalling and induced subsequent activation of the calcium-dependent protease calpain (Xu et al., 2010), which promoted IAC disassembly by cleavage of proteins from IACs and therefore regulated cell migration (Bhatt et al., 2002; Huttenlocher et al., 1997).
In addition to actin-associated IAC disassembly through loss of force at IACs, microtubule-targeting to IACs has been shown to induce IAC disruption (Kaverina et al., 1999). To study microtubule-induced IAC disassembly biochemically requires the synchronisation of IAC disruption in a cell population. To facilitate this, an assay has been developed that involves treating cells with the microtubule polymerisation inhibitor nocodazole, which binds to microtubule subunits to block their repolymerisation (Saunders and Limbird, 1997); followed by nocodazole washout, which allows microtubule polymerisation and IAC targeting to induce the coordinated disassembly of IACs across a population of cells (Ezratty et al., 2005). Using the nocodazole washout assay, several studies have revealed insights into the mechanistic regulation of microtubule-induced IAC disassembly. During disassembly, it was found that FAK phosphorylated at FAK Y397 recruited dynamin to IACs via dynamin binding to Grb2 (Ezratty et al., 2005). Upon initiation of IAC disassembly, additional studies reported that clathrin was recruited to IACs via its adaptors autosomal recessive hypercholesteremia (ARH) and disabled-2 (Dab2) and clathrin later dissociated from IACs with integrins during their endocytosis (Chao and Kunz, 2009; Ezratty et al., 2009). Arrestins have also been implicated to be involved in IAC disassembly, since clathrin displayed decreased dynamics near IACs in arrestin-deficient cells (Cleghorn et al., 2015). As well as clathrin, it has been shown that the microtubule-associated proteins CLASP1 and CLASP2 clustered around IACs via LL5β during IAC disassembly (Lansbergen et al., 2006; Stehbens et al., 2014). In addition, it was recently reported that MAP4K4 was recruited to IACs along IAC-targeting microtubules via EB2, and Arf6 was subsequently activated via IQSEC1 to enhance IAC disassembly (Yue et al., 2014). Another study reported that late endosomes carrying the p14–MP1 (LAMTOR2/3, MAPK/ERK kinase 1 partner MP1, and its endosomal adaptor protein p14) complex targeted mature IACs along microtubules and caused IQ motif containing GTPase activating protein 1 (IQGAP1) to dissociate from IACs (Schiefermeier et al., 2014), which revealed that the p14-MP1 complex regulated microtubule-associated IAC dynamics. In fact, depletion of any of the molecules implicated in microtubule-associated IAC dynamics mentioned above inhibited IAC disassembly and in some cases cell migration, whereas genetic depletion of Src or paxillin had no effect on IAC disassembly (Ezratty et al., 2005). Other molecules have been implicated in regulating IAC disassembly, such as the Abl-related Gene Tyrosine Kinase (GSK) (Peacock et al., 2007) and TRIM15 (Uchil et al., 2014). These studies suggest that multiple redundant pathways exist that regulate microtubule-dependent IAC disassembly.

In summary, the combined regulation of the actin and microtubule cytoskeletal networks contribute to efficient cell migration (Wehrle-Haller and Imhof, 2003). While many regulators of microtubule-induced IAC disassembly have been uncovered, many studies have used IAC markers such as paxillin, vinculin and zyxin to report IAC dynamics. Therefore, a global perspective on the dynamics and relative rates of loss of all IAC molecules during IAC disassembly is not known.

1.5 IAC organisation at the molecular level

Light and electron microscopy have been widely used to study cell adhesion (Humphries et al., 2015; Worth and Parsons, 2010). Early studies used electron microscopy to study IACs, which revealed that adherent cells attached to protein layers, mediated by small puncta at electron-dense attachment sites, rather than directly attaching to dishes (Abercrombie and Dunn, 1975;
Abercrombie et al., 1971; Revel and Wolken, 1973). Recent studies have been carried out using super-resolution microscopy approaches (Case et al., 2015; Hoffmann et al., 2014; Kanchanawong et al., 2010; Patla et al., 2010; Rossier et al., 2012), which have helped gain insight into the spatial organisation of IACs in molecular detail (Fig. 1.3) (Byron, 2011).

To examine the dynamics of individual proteins within IACs, specifically talin and integrins, super-resolution microscopy and single-protein tracking has been used (Fig. 1.3a) (Rossier et al., 2012). It was found that integrins transitioned through cycles of free-moving and stationary states, while talin was actively recruited to IACs (Rossier et al., 2012). In the stationary state, β3 integrins remained fixed in IACs while β1 integrins moved rearward (Rossier et al., 2012), demonstrating that integrin heterodimers display differing kinetics within IACs (Ivaska, 2012). To examine the architecture of IACs in fibroblast cells, fluorescence microscopy coupled with cryo-electron tomography has been used (Fig. 1.3b) (Patla et al., 2010). Cells that expressed yellow fluorescent protein (YFP)-tagged paxillin were imaged and paxillin-containing areas were analysed by cryo-electron tomography to build 3D density maps of IACs, which suggested that interactions between the cell membrane and the cytoskeleton were mediated through ring-shaped particles 20 - 30 nm in diameter located at the end of actin fibres on the cell membrane (Fig. 1.3b) (Patla et al., 2010). The ring-shaped particles were found to be associated with or contain vinculin, which has been suggested previously using electron microscopy (Samuelsson et al., 1993). In a further study, ILK depletion led to an increase in the number and density of these ring-shaped particles in IACs (Elad et al., 2013). However, these studies have yet to be repeated by others and it is therefore unclear whether the ring-shaped particles are authentic structures in cells. To investigate the organisation of IACs at the molecular level, super-resolution fluorescence-based imaging (Shtengel et al., 2009) of known IAC proteins in the vertical z plane was used, which determined the 3D positions of IAC components with nanometer precision (Kanchanawong et al., 2010) and revealed that the localisation of IAC proteins was stratified (Fig. 1.3c) (Byron, 2011). In this model, the cytoplasmic tails of αVβ3 integrin were located close to the inner plasma membrane, the signalling proteins FAK and paxillin were incorporated into an adjacent signalling layer, and vinculin resided further from the membrane in a force-regulatory layer (Kanchanawong et al., 2010). In agreement with previous reports that have demonstrated the involvement of zyxin and VASP in actin cytoskeletal regulation (Hirota et al., 2000; Krause et al., 2003), both proteins were located adjacent to actin in an actin regulatory layer (Kanchanawong et al., 2010). Finally, α-actinin localised proximal to actin and talin spanned IACs to form a direct link between integrins and actin (Kanchanawong et al., 2010). Others have examined IAC components using fluorescence cross-correlation spectroscopy (FCCS) and fluorescence recovery after photobleaching (FRAP), which revealed that IAC components formed pre-assembled complexes in the cytoplasm that were dynamically exchanged between the cytosol and stationary IACs (Hoffmann et al., 2014), indicating that a modular substructure may exist within IACs.

These insights have demonstrated the necessity of advanced imaging to understand the spatial organisation and dynamics of IACs at the molecular level. They also demonstrate that IACs are dynamic at different scales: IAC structures are able to translocate across the cell membrane as collective protein complexes during cell migration, for example, and individual IAC components
Figure 1.3. IAC dynamics, architecture and organisation revealed by advanced imaging.

(a) The dynamics of individual IAC components were tracked within IACs using super-resolution microscopy. As an example, the image shows the trajectories of β3 integrins overlaid on focal adhesions (grey). Trajectories are coloured to indicate mobility-type: diffusive (purple), confined (green) or immobile (red). Image is reproduced from Rossier et al., 2012. (b) The architecture of IACs was examined using cryo-electron tomography, which revealed that IACs contain ring-shaped particles. The image shows the location of ring-shaped particles (green) in a paxillin-containing IAC. Image is reproduced from Patla et al., 2010. (c) The 3D positions of IAC components in IACs were determined using super-resolution fluorescence microscopy, which are summarised in the vertical z-plane model of IACs. Schematic is reproduced from Kanchanawong et al., 2010.
within IACs are mobile at the molecular level. However, such candidate-based approaches are restricted to the analysis of known, and relatively few, IAC components.

1.6 IAC composition

In order to fully understand how proteins in IACs are involved in mechanical linkage between the ECM and the intracellular environment, and how they generate intracellular signals upon ligand binding, it is necessary to understand their molecular composition. Taking a systems approach, the large number of proteins reported to localise to IACs was collectively termed the integrin adhesome (Zaidel-Bar et al., 2007), which was based on evidence from immunofluorescence and immunoelectron microscopy, as well as biochemical, experiments (Zamir and Geiger, 2001b). The number of known adhesion-related proteins has continued to grow, and have been summarised in several updates of the literature-curated integrin adhesome (Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007), which currently consists of 232 members (Fig. 1.4) (Winograd-Katz et al., 2014). Proteins in the literature-curated adhesome have been characterised as ‘intrinsic’ molecules (148 proteins) that have been shown to colocalise with integrins and therefore localise directly to IACs, or ‘associated’ molecules (84 proteins) that activate, inhibit or bind intrinsic components (Winograd-Katz et al., 2014). The large number of proteins reported to localise to IACs, and the scale of the interactions between them (over 700 (Zaidel-Bar and Geiger, 2010)), revealed an unanticipated complexity of IAC composition.

The adhesome network divided into subnetworks of distinct functional modules based on known functions and biochemical activities of adhesome proteins (Fig. 1.4) (Geiger and Yamada, 2011; Winograd-Katz et al., 2014; Zaidel-Bar et al., 2007). The largest functional groups were the adhesion receptors, adaptors and actin regulators, which primarily consisted of structural IAC components that mediate the connections between adhesion receptors and the actin cytoskeleton. The majority of these connections were formed by at least two proteins, while relatively few molecules were reported to form a direct link between integrins and actin (eg. filamin, talin and tensin) (Zaidel-Bar et al., 2007). The other prominent functional groups contained adhesion-mediated signalling proteins such as kinases, phosphatases, GTPases, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that initiate downstream signalling cascades to regulate adhesion-dependent signalling events such as IAC assembly and turnover. Tyrosine kinases, in particular, were enriched in the adhesome compared with the entire proteome and analysis of the adhesome network substructure revealed that GEFs and GAPs were predominantly regulated by tyrosine kinases (Zaidel-Bar et al., 2007). Two of the most highly connected proteins in the network were the well-characterised non-receptor tyrosine kinases FAK and Src. FAK activation occurs upon integrin-mediated cell attachment and FAK mediates its kinase-dependent and independent functions through a C-terminal focal adhesion targeting (FAT) domain, a central catalytic domain and an N-terminal protein 4.1, ezrin, radixin and moesin (FERM) domain (Mitra et al., 2005). The FAT domain directs FAK to newly formed IACs by binding to paxillin (Schaller and Parsons, 1995), while FAK binding to talin has also been reported to be involved in this process (see section 1.4.2) (Chen et al., 1995; Lawson et al., 2012). In addition, the binding of a number of GEFs, such as p190RhoGEF (Zhai et al., 2003), to the FAT domain of FAK has been reported to regulate Rho GTPase activation, while the FERM domain of FAK has been...
Figure 1.4. Functional categories of the literature-curated integrin adhesome. The 232 members of the literature-curated adhesome (Winograd-Katz et al., 2014) were mapped onto a merged human interactome (see section 2.6.4) as described previously (Byron et al. 2015). Only proteins reported to localise directly to IACs, termed ‘intrinsic’ components, are shown in the network. Nodes (coloured shapes) represent individual proteins and edges (grey lines) represent reported protein-protein interactions. Proteins have been arranged into their assigned functional groups: cell surface receptors, green (adhesion receptors and channels); signalling proteins, yellow (GEFs, GAPs and GTPases) and orange (kinases, phosphatases and phospholipases); adaptor proteins, blue (adaptors, actin and actin regulators) and other proteins, grey (chaperones and DNA/RNA regulators) and white (cAMP phosphodiesterase, serine palmitoyltransferase and unknown). Proteins are labelled by gene name for clarity.
reported to bind at least 11 proteins (Frame et al., 2010; Walkiewicz et al., 2015). Upon recruitment to IACs in response to cell-ECM attachment, FAK has been reported to dimerise and is activated by FAK autophosphorylation at FAK$^{Y397}$ (Brami-Cherrier et al., 2014; Schaller et al., 1994). Phosphorylation at FAK$^{Y397}$ creates a binding site for a number of Src Homology 2 (SH2)-domain containing proteins including Src, phospholipase Cγ (PLCγ) and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Appeddu, 1996; Schaller et al., 1994). The sequential activation, recruitment and binding of Src results in the formation of a FAK-Src signalling complex and additional Src-dependent FAK phosphorylation at sites FAK$^{Y576}$ and FAK$^{Y577}$ maximally activates FAK (Calalb et al., 1995). Additional Src-mediated phosphorylation of FAK at FAK$^{Y925}$ recruits Grb2 to FAK (Schlaepfer et al., 1994) and activates the mitogen-activated protein kinase (MAPK) cascade (Sawai et al., 2005), which has been implicated in contributing to FAK’s role in regulating cell proliferation. FAK- and Src-dependent phosphorylation events, such as phosphorylation of residues on paxillin and p130Cas, regulate the recruitment of other proteins to IACs (Brown et al., 2005; Cary, 1998; Schaller and Parsons, 1995). For example, increased phosphorylation of p130Cas promotes Crk binding to p130Cas (Sakai et al., 1994), which increases Rac activity, cell motility and invasion (Mitra et al., 2005). Due to the high number of potential binding partners and phosphorylation substrates for FAK and Src in IACs, it is not surprising that the FAK-Src signalling complex has been proposed as a key IAC signalling hub that contributes to the regulation of multiple cellular functions and has been proposed as a potential therapeutic target in cancer (Kim et al., 2009; Sulzmaier et al., 2014).

To probe for links between other adhesome proteins and disease, a recent bioinformatics-based analysis of the adhesome investigated the roles of adhesome proteins in disease. Searching for adhesome components reported in the Online Mendelian Inheritance in Man database (OMIM, www.omim.org) revealed single adhesome components involved in inherited genetic disorders when mutated (55 proteins), and searching the Genetic Association Database (GAD) (Becker et al., 2004) identified diseases caused by mutations in multiple adhesome components (138 proteins) (Winograd-Katz et al., 2014). These analyses demonstrated that adhesome proteins are predominantly involved in cancer, as well as musculoskeletal and cardiovascular, disorders. Further understanding of adhesome function, components and signalling will help in the identification of IAC proteins as potential therapeutic targets.

As testament to its use as a resource, the integrin adhesome has been used extensively as a basis for adhesion-related research. Using the 180 components of the integrin adhesome, a computational study examined interaction dependencies in protein-protein interaction (PPI) networks using a text mining approach (Köster et al., 2012). In total, 208 sentences were extracted from a library of 59,933 articles that mentioned at least three adhesome components in their abstract, which resulted in the identification of 47 interaction dependencies and 24 allosteric regulations. Active Rac1 was found to promote binding of G protein-coupled receptor kinase interactor 1 (GIT1) to paxillin that is mutually exclusive of paxillin binding to FAK, which indicated that Rac1 activation may lead to a decrease of FAK in IACs (Köster et al., 2012). Similar literature-curation approaches have been applied to examine other cell surface adhesion receptors. Literature-curated interactomes of syndecan-1 and syndecan-4 displayed partial overlap with the integrin adhesome (Roper et al., 2012), 390 interactions between 174 components have been
reported in the evolving cadherin interactome (termed ‘cadhesome’) (Zaidel-Bar, 2013), and the
complexity of the protein machinery involved in actomyosin contractility was revealed in the
contractome (Zaidel-Bar et al., 2015). In addition, it has been proposed that the ability of cells to
invade their surroundings through ECM degradation is achieved through the transition of focal
adhesions to invadopodia, which led to the generation of a focal adhesion network based on the
integrin adhesome and an invadopodia network constructed using literature and database
searches (Hoshino et al., 2012). Two proteins, PI3K and protein kinase C α (PKCα), were found to
be highly connected in both networks (Hoshino et al., 2012), which led to the hypothesis that PI3K
and PKCα are regulators of invasive behaviour.

Literature-curated inventories detailing the molecular composition of different types of cell adhesion
complex provide useful resources in understanding the molecular details of adhesion biology.
However, there are limitations in the conclusions that can be drawn from the literature-curated
adhesome network since the adhesome is an amalgamation, based on previously published
reports of numerous cell types, receptors, ligands and experimental contexts using mainly targeted
approaches, of an IAC that may never be realised in reality and therefore doesn’t reflect a natural
complex in its entirety.

1.7 Global analyses of IACs

1.7.1 Overview

A vast body of research has been conducted to analyse IACs and IAC components that has mainly
involved candidate-based and targeted approaches to investigate the localisations, protein-protein
interactions and functional roles of individual IAC components (Horwitz, 2012; Worth and Parsons,
2010), which has been amalgamated in the creation of the literature-curated adhesome (Winograd-
Katz et al., 2014). To provide an unbiased view of IAC composition and function (Humphries et al.,
2015), two approaches have recently been used, which have characterised the global composition
of IACs using mass spectrometry (MS)-based proteomics (Bensimon et al., 2012; Sabidó et al.,
2012; Walther and Mann, 2010) and have identified adhesion-related functional regulators using
perturbation methods (Ideker and Krogan, 2012; Molinelli et al., 2013) such as small interfering
RNA (siRNA) screens.

1.7.2 Biochemical isolation and MS-based proteomic analysis of IACs

Using MS for identification of proteins in IACs has been difficult because of their instability and
inaccessibility (Byron et al., 2011). However, recent protocols have been developed that allow the
isolation and subsequent analysis of IAC protein composition (Fig. 1.5) (Jones et al., 2015; Kuo et
al., 2012). Protocols involved either incubating cells in suspension with ligand-coated beads or
plating cells onto ligand-coated dishes to induce IAC formation. IAC structures formed in attached
cells were stabilised by addition of chemical cross-linkers, followed by cell lysis using a
combination of detergents and sonication, or high-pressure water wash, to remove non-specific
IAC components (Jones et al., 2015; Kuo et al., 2012). Isolated IACs were extracted and collected
for analysis by polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting or MS for
Figure 1.5. Approaches used to isolate and analyse the molecular composition of IACs.

The schematic shows similarities and differences between workflows used by eleven studies that have isolated and analysed the protein composition of IACs by MS-based proteomics. Experimental parameters used by each protocol can be followed based on the colour of boxes and adjoining lines to give an overview of the main aspects of that experimental approach. Studies that used the same experimental parameters for IAC isolation have been grouped and are represented by the same colour. Studies that have not analysed IACs induced by the canonical ligand FN are shown in italics. FN, fibronectin; DTBP, dimethyl-3, 3'-dithiobispropionimidate; DSP, dithiobis[succinimidyl propionate]; DPDPB, 1,4-di-[3'-(2'-pyridyldithio)-propionamido]butane; SC, spectral counting; SILAC, stable isotope labelling by amino acids in cell culture; VCAM-1, vascular cell adhesion molecule-1.
identification and quantification of proteins. Such methods provide an enrichment of IAC proteins and are unlikely to provide pure IAC mixtures.

To analyse IACs by proteomics, current studies have used the ‘bottom-up’ approach of shotgun MS-based proteomics (Swanson and Washburn, 2005; Wu and MacCoss, 2002), which allows the unbiased identification of hundreds to thousands of proteins in a biological sample. Proteins are typically difficult to analyse by MS due to their large size. Therefore, trypsin is used to digest proteins into smaller peptides amenable to MS analysis (Aebersold and Mann, 2003). To reduce the complexity of the digested peptide mixtures, improve the coverage of peptides identified and prevent saturation of sample on the mass spectrometer due to protein overloading; samples are often fractionated prior to MS analysis (Aebersold and Mann, 2003). One fractionation method involves separation of the complex sample into multiple fractions by SDS-PAGE, excision of the protein gel into multiple bands, peptide elution from the gel and analysis of peptides from each band separately (Humphries et al., 2009). To further aid sample fractionation, peptides are loaded onto a reverse phase liquid chromatography (LC) column immediately prior to MS analysis, and peptides are gradually eluted onto the mass spectrometer at different times according to their physiochemical properties (Aebersold and Mann, 2003). Using this approach, eluted peptides introduced to the ion source in solution phase are ionised, typically using electrospray ionisation (ESI) (Fenn et al., 1989), and after evaporation analyte ions enter the mass spectrometer and are separated according to their mass to charge ratio (m/z). A detector records the ion intensity at different m/z values at that time point and an initial m/z spectrum is generated (MS1). In the case that tandem MS (MS/MS) is applied, a proportion of these peptides are identified for further fragmentation and a second round of MS analysis (MS2) (Aebersold and Mann, 2003). The resulting MS spectra are analysed using bioinformatics approaches and database searching to identify the proteins originally present in the sample (Sadygov et al., 2004). The use of MS/MS results in improved confidence of protein assignment of identified peptides, which has therefore enabled deeper coverage of identified proteins in complex protein mixtures (Walther and Mann, 2010) such as those collected in isolated IACs.

It is often desirable to quantify protein abundance differences of identified proteins between different experimental conditions, termed relative quantification (Elliott et al., 2009). Current studies that have analysed IAC composition by MS have used both label-free (Byron et al., 2012, 2015; Huang et al., 2014; Humphries et al., 2009; Kuo et al., 2011; Ng et al., 2014; Robertson et al., 2015; Yue et al., 2014) and labelled (Schiller et al., 2011, 2013) quantification methods (Fig. 1.5).

The use of the label-free approach for quantification of IAC proteomes involves sample preparation and MS analysis of different experimental conditions in parallel. In one approach, IAC proteins have been quantified using spectral counting (the total number of MS/MS spectra for all peptides assigned to each protein is termed its ‘spectral count’) (Fig. 1.5), which is based on the principle that proteins (and resulting peptides) in higher abundance have a higher probability of being selected for further fragmentation and MS2 analysis than lower abundance peptides, and will therefore have a higher spectral count (Lundgren et al., 2010). Ratios of spectral counts are used to calculate relative protein abundance differences between experimental conditions. A second intensity-based label-free approach uses differences in the intensities of spectral features observed...
in MS1 spectra between conditions as a relative measure of protein abundance (Lai et al., 2013; Nesvizhskii et al., 2007). The intensity-based approach has been reported to enable more accurate relative protein quantification than spectral counting (Old et al., 2005).

IAC proteomes have also been quantified using the labelled quantification approach of stable isotope labelling by amino acids in cell culture (SILAC) (Fig. 1.5) (Chen et al., 2015; Ong et al., 2002), which involves the complete incorporation of ‘heavy’ and ‘light’ amino acids into proteins from cell populations used in different experimental conditions. Heavy and light samples are collected, mixed and analysed by MS simultaneously, which can be beneficial as variability between conditions introduced from sample preparation errors and in downstream analyses is reduced, but the use of SILAC also results in increased complexity and reduced concentration of individual proteins in the protein mixture to be analysed by MS (DeSouza and Siu, 2013). In downstream bioinformatics analyses, spectra from identified peptides are assigned into their respective experimental condition based on detected m/z shifts caused by the specific amino acid isotope that was incorporated (Ong et al., 2002). Ratios of the intensities of spectral features observed in MS1 spectra between ‘heavy’ and ‘light’ conditions are used as a relative measure of protein abundance differences between experimental conditions.

### 1.7.3 Integrin-heterodimer specificity of IAC composition

The first study that isolated and reported the composition of IACs by MS examined ligand-specificity of IACs by analysing IAC proteomes induced by two integrin-ligand combinations: α5β1-FN and α4β1-VCAM-1 (Humphries et al., 2009). Using tandem MS, 620 and 418 proteins were detected in α5β1-FN and α4β1-VCAM-1 samples, respectively, and demonstrated integrin-ligand dependent specificity of IAC composition (Humphries et al., 2009). Regulator of chromosome condensation 2 (RCC2), a protein identified in the IAC proteome that linked Rac1 and Arf6 (Humphries et al., 2009), has been shown to be involved in a coronin 1C-RCC2-Rac1 pathway that regulates mesenchymal cell migration (Williamson et al., 2014) and filamin A, IQGAP1 and the IQGAP1-binding protein RacGAP1 have been identified by additional proteomic analyses of components associated with the RCC2-Rac1-Arf6 network to regulate Rac1-dependent cell migration (Jacquemet et al., 2013a, 2013b). These studies demonstrated the beneficial use of the IAC proteomic dataset and identification of novel IAC components by such an approach. In addition, α-subunit dependent IAC protein recruitment was revealed through isolation of VCAM-1-induced IACs associated with specific integrin heterodimers: α4β1, an α4β1 variant lacking the α cytoplasmic domain, and a chimeric α4β1 variant with α5 leg and cytoplasmic domains (Byron et al., 2012). IAC integrin-heterodimer specificity has also been examined in cells exposed to the same FN-rich ECM environments by isolation of IACs from mouse kidney fibroblast (MKF) cells (Schiller et al., 2013). IACs isolated from pan-integrin-knockout fibroblast cells that re-expressed either αV (αVβ3 and αVβ5), β1 (α5β1) or αV and β1 (α5β1, αVβ3 and αVβ5) integrins were analysed by MS, which identified differential recruitment of IAC molecules in a heterodimer-specific manner (Schiller et al., 2013). More recently, the composition of IACs induced by active- or inactive- β1 integrin was determined by exposing cells to integrin conformation-specific monoclonal antibodies (12G10, active; 4B4, inactive) and analysis of their induced IAC proteomes by MS, which revealed activation state-dependent IAC composition (Byron et al., 2015). Structural IAC
components and actin regulators such as talin, kindlin, vinculin and VASP were enriched to active β1 integrin-induced IACs, while the Rho GTPases RhoA, RhoC and RhoG were enriched in inactive β1 integrin-induced IACs (Byron et al., 2015). In addition, active β1 integrin-induced IACs were enriched for microtubule-associated proteins, and subsequent immunofluorescence imaging of microtubules revealed that microtubules were maintained at the cell periphery and targeted IACs when integrins were in an active conformation, in contrast to integrins in the inactive conformation (Byron et al., 2015). In summary, analyses of MS-derived IAC datasets have determined that it is not only the ECM ligand but also the integrin heterodimer-ligand combination and activation state that dictate IAC composition (Byron et al., 2012, 2015; Humphries et al., 2009; Schiller et al., 2013).

1.7.4 Effects of cytoskeletal force on IAC composition

To examine force-dependent changes in IAC composition during IAC maturation, the effects of myosin II activity on IAC composition were characterised in cells treated with the myosin II inhibitor blebbistatin (Kuo et al., 2011; Schiller et al., 2011), which revealed that the number and abundance of proteins recruited to IACs was reduced in myosin II-inhibited cells. Examination of the functional roles and structural domains of the myosin II-sensitive proteins revealed significant enrichment for proteins containing the Lin-11, Isl1, and Mec-3 (LIM) domain (Schiller and Fässler, 2013), which was in agreement with other studies that implicated the LIM domain-containing IAC proteins zyxin and paxillin in force sensing (Smith et al., 2014; Uemura et al., 2011). Conversely, a small proportion of proteins identified by MS were enriched in IACs after myosin II-inhibition, and one such protein, the Rac GEF β-PIX, was found to negatively regulate IAC maturation and cell migration (Kuo et al., 2011).

To further examine the effects of force on IACs and the roles of different integrins during IAC maturation, IACs were isolated from cells expressing αV, β1, or αV and β1 integrins that were treated with blebbistatin (Schiller et al., 2013). Expression of α5β1 integrin resulted in the recruitment of the canonical IAC proteins talin, kindlin and ILK to IACs but not the LIM domain-containing proteins upon blebbistatin treatment, which is consistent with studies that isolated IACs from blebbistatin-treated cells (Kuo et al., 2011; Schiller et al., 2011). However, in cells that did not express α5β1 integrin but uniquely expressed αV integrins, both canonical and LIM domain-containing IAC components were reduced upon blebbistatin treatment, which indicated that α5β1 integrins, but not αV integrins, are able to recruit IAC molecules in the absence of myosin II-mediated tension (Schiller et al., 2013). Subsequently, α5β1 integrin was shown to generate force via a RhoA-ROCK-myosin II pathway and αV integrins mediated structural changes when force is applied via a GEF-H1-mDia1-RhoA pathway, which suggested that integrins have distinct functional roles during IAC maturation (Schiller et al., 2013). Taken together, these analyses have revealed the compositional effects of actin cytoskeletal force on IACs and highlighted the involvement of the LIM domain in IAC maturation (Kuo et al., 2011; Schiller and Fässler, 2013; Schiller et al., 2011, 2013), although the exact functional involvement of the LIM domain in adhesion-related mechanotransduction remains unresolved (Humphries et al., 2015).
1.7.5 Other studies reporting IAC composition by MS

While integrin-heterodimer specific and tension-dependent IAC composition have been examined by MS, more recent MS-derived IAC datasets have reported IAC composition in other contexts. To identify novel regulators of microtubule-mediated IAC turnover, IACs have been isolated from cells treated with the microtubule polymerisation inhibitor nocodazole (Ng et al., 2014; Yue et al., 2014). Treatment with nocodazole disrupts the microtubule network, which increases IAC size due to increased contractility and reduced IAC turnover rates (Bershadsky et al., 1996; Ezratty et al., 2005). The abundance of the majority of IAC proteins increased in IACs isolated from cells treated with nocodazole, with the exception of α5β1 integrin that was unaffected by nocodazole treatment (Ng et al., 2014). In contrast, MAP4K4 and the microtubule-associated proteins EB1 and EB2 decreased in abundance in IACs upon nocodazole treatment and were therefore identified as novel regulators of microtubule-mediated IAC dynamics (Yue et al., 2014). Other studies have focussed on the role of GEF-H1 in regulating IAC protein composition and stem cell differentiation (Huang et al., 2014). Analysis of IACs isolated from GEF-H1-silencing mesenchymal stem cells revealed that GEF-H1 perturbation inhibited IAC formation and decreased the abundance of myosins, including myosin II, in IACs (Huang et al., 2014).

A number of post-translational modifications, specifically phosphorylation-based signalling events, have been implicated in the regulation of IACs and cell adhesion (Zaidel-Bar and Geiger, 2010). This is supported by immunofluorescence imaging of IACs that have demonstrated an enrichment of tyrosine phosphorylation events at IACs (Ballestrem et al., 2006; Geiger et al., 2001; Kirchner et al., 2003; Panetti, 2002) and that one of the most connected proteins in the literature-curated adhesome is the tyrosine-phosphorylated protein FAK (Winograd-Katz et al., 2014; Zaidel-Bar et al., 2007). To determine the extent of phosphorylation in IACs in a global manner, protocols that have been used to catalogue IAC protein composition (Jones et al., 2015; Kuo et al., 2012) have been adapted to allow phosphopeptide-enrichment of isolated IACs (Robertson et al., 2015). The combined IAC proteome and phosphoproteome from melanoma cells was reported (Robertson et al., 2015) and analysis of the phospho-adhesome revealed greater coverage of phosphorylation sites related to IAC signalling compared with previous phosphoproteomic analyses of IAC signalling molecules from whole cells (Chen et al., 2009; Schiller et al., 2013). In particular, novel phosphorylation sites and regulators of adhesion signalling were identified such as cyclin-dependent kinase 1 (Cdk1). Perturbation of Cdk1 activity using small molecule inhibitors and genetic depletion induced IAC disassembly and disrupted the actin cytoskeletal network (Robertson et al., 2015), which demonstrated the benefit of the phospho-adhesome in uncovering novel IAC regulators.

1.7.6 Non-MS and systems-based analyses of IACs

While MS-based analyses of IACs have reported the global composition of IACs in a number of contexts that have led to a number of mechanistic insights into IAC compositional variability, other non-MS-based approaches have been used to globally assess IACs in an unbiased manner and to identify novel regulators of adhesion-related functions. One such approach involved genetic ablation using siRNAs targeting a range of adhesion-related and non-adhesion-related
components, and the resultant effects on cells were analysed using mainly high-throughput microscopy approaches. Application of this approach has led to the identification of novel regulators of adhesion-related functions such as cell morphology and cell migration (Chen et al., 2009; Kiger et al., 2003; Simpson et al., 2008). Other studies have analysed the effects of genetic depletion more specifically on IACs by analysing the area and length of IACs, the intensity of paxillin staining and a number of cell morphological features using a high-throughput microscopy approach in a siRNA screen that targeted kinases, phosphatases and genes involved in migration and adhesion (Winograd-Katz et al., 2009), which identified common genes involved in regulation of adhesion formation. The most surprising observation to arise from the use of these approaches was the identification of a novel inhibitor of β1 integrin activation, the shank-associated RH domain-interacting protein (SHARPIN), which inhibited β1 integrin activation by binding to α-integrin subunits and preventing talin and kindlin binding to β1 integrin (Rantala et al., 2011). Such perturbation approaches have also been used to examine effects of genetic depletion of literature-curated adhesome components in silico (Zaidel-Bar et al., 2007) and of Caenorhabditis elegans homologs of literature-curated integrin adhesome proteins in vivo (Etheridge et al., 2015), which revealed that it is the complex network of interactions in IACs, and not major contributions from individual components, that primarily contributes to adhesive function. In summary, reducing protein expression levels and examining effects on cells using mainly microscopy, and not MS-based, approaches have led to the identification of novel regulators of adhesion and adhesion-related functions. However, effects of protein perturbation on IACs at the molecular level have not been examined.
1.8 Aims

The literature-curated adhesome has revealed the first systems-wide description of IACs at the molecular-level. However, this network view is an amalgamation, based on previously published reports of numerous cell types, receptors and ligands using mainly targeted approaches, and doesn’t reflect a natural complex in its entirety. IACs have been isolated and analysed by MS in a variety of experimental contexts to examine effects of ECM ligands, integrin heterodimer combinations and tension on the global composition of native IACs, and perturbation approaches have been used to identify novel regulators of adhesion-related functions. The MS-derived IAC datasets are context-dependent and mainly focus on the isolation of static IACs. The aims of this study are:

1. How are IACs described at the systems-level and what are the core components?

Multiple existing proteomic IAC datasets, which have been derived from multiple cell types using different IAC methodological strategies from multiple laboratories, will be analysed in combination to reveal an integrative overview of the biological roles of IAC-related molecules. The combined dataset will be analysed using a combination of hierarchical clustering, functional gene ontology-based and protein-protein interaction network analyses to examine cellular processes and molecules linked to cell adhesion. These data will lead to the generation of hypotheses describing how the complex network of interactions that form IACs influence downstream functions and global cell behaviour, and will highlight key regulatory control points in IACs.

2. How sensitive are IACs to perturbation of key kinases?

Perturbation approaches will be used to interrogate the structure and topology, and to determine key control points, of the IAC network and composition. Using pharmacological inhibitors to target key kinases located at IACs, specifically FAK and Src, the effects of kinase inhibition on IAC composition will be examined. These data will determine the contribution of FAK and Src protein activities on both protein recruitment of functional modules to IACs, and on signal propagation through the complex, to provide the link between cell-ECM adhesion and downstream cellular processes.
1.9 References


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Chapter 2

Materials and Methods

Methods are included in each results chapter. This section includes general methods in more detail.

2.1 Reagents

2.1.1 Antibodies

Table 2.1. Primary antibodies

<table>
<thead>
<tr>
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<th>Species</th>
<th>Dilution</th>
<th>Source</th>
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<td>mAb11</td>
<td>Rat</td>
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<td>IB, 1:500; IF, 1:200</td>
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</table>

BAK, Bcl-2 antagonist/killer; ILK, integrin-linked kinase; FAK, focal adhesion kinase; pY, phosphotyrosine; Rsu-1, ras suppressor protein 1; IB, immunoblotting; IF, immunofluorescence;
¹NIH, Bethesda, Maryland, USA;
²University of Münster, Germany;
³University of Manitoba, Winnipeg, MB, Canada;
⁴Uniformed Services University of the Health Sciences (USUHS), Bethesda, Maryland, USA.
### Table 2.2. Secondary antibodies

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IB, immunoblotting; IF, immunofluorescence

### 2.1.2 Other reagents

### Table 2.3. Other reagents

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IF, immunofluorescence
### 2.1.3 Buffers and solutions

#### Table 2.4. List of solutions

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<td>Extraction buffer (EB)*</td>
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<tr>
<td>Radioimmunoprecipitation assay (RIPA) buffer*</td>
<td>50 mM Tris-HCl, pH 8.0; 1% (v/v) Triton X-100; 150 mM NaCl; 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0; 2.5% (w/v) sodium dodecyl sulfate (SDS); 1% (w/v) sodium deoxycholate in H$_2$O</td>
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<td>Adhesion recovery solution</td>
<td>125 mM Tris-HCl, pH 6.8; 1% (w/v) SDS; 15% (v/v) β-mercaptoethanol</td>
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<td>Cell lysis buffer*</td>
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</tr>
<tr>
<td>5x reducing sample buffer (RSB)</td>
<td>125 mM Tris-HCl, pH 6.8; 25% (v/v) glycerol; 10% (w/v) SDS; 0.01% (w/v) bromophenol blue; 15% (v/v) β-mercaptoethanol</td>
</tr>
<tr>
<td>Tris-buffered saline supplemented with 0.05% (v/v) Tween-20 (TBS-T)</td>
<td>10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 0.05% (v/v) Tween 20</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>25 mM Tris; 190 mM glycine; 0.01% (w/v) SDS; 20% (v/v) methanol</td>
</tr>
</tbody>
</table>

*, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 10 mM sodium orthovanadate (Na$_3$VO$_4$) and 50 mM sodium fluoride (NaF) were added immediately before use, where appropriate.

### 2.1.4 Small molecule inhibitors

Inhibitors of FAK (AZ13256675, termed FAK [i]; Astra Zeneca, Macclesfield, UK) and Src (AZD0530, termed Src [i]; Astra Zeneca) were solubilised and stored in DMSO at 100 mM. For use, inhibitors were diluted to 3 mM in dimethyl sulfoxide (DMSO), and diluted further in PBS-. Where combined FAK [i] and Src [i] treatment was applied, inhibitors were diluted to 6 mM and added simultaneously to cells. For controls, a volume of DMSO equivalent to that in the highest inhibitor dose was added to the cells. Inhibitors were either added to cells pre-spread for 1 h or added to cells in suspension before plating onto the fibronectin (FN) substrate.
2.2 Mammalian cell culture

2.2.1 Cell culture

Telomerase-immortalised human foreskin fibroblast (HFF) cells and human osteosarcoma (U2OS) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, Poole, UK) supplemented with 10% (v/v) foetal calf serum (FCS; Lonza Bioscience, Wokingham, UK) and 2 mM L-glutamine and incubated at 37 °C in a humidified 5% (v/v) CO₂ atmosphere. To passage, cells were washed with PBS- and detached by incubation with Trypsin-EDTA (Sigma-Aldrich) diluted in PBS- for 10 min at 37 °C and DMEM added to quench trypsin. HFF and U2OS cells were centrifuged at 450 g and 350 g, respectively, for 4 min. The cell pellet was resuspended in complete medium and cells were added to flasks (Corning, Amsterdam, Netherlands) in fresh medium. Cells were passaged twice weekly to maintain optimal cell growth.

To freeze, cells were resuspended in cold 90% (v/v) FCS and 10% (v/v) DMEM following cell centrifugation. Cells were stored at -80 °C or in liquid nitrogen. To resurrect, cells were defrosted quickly and diluted in DMEM, centrifuged, resuspended in complete medium and added to culture flasks.

2.2.2 Coating dishes and coverslips

Prior to ligand coating, coverslips (13-mm-diameter; VWR International, Lutterworth, UK) were washed in 96% (v/v) ethanol. 10-cm-diameter culture plates (Corning), poly-D-lysine-coated glass-bottom dishes (14-mm-diameter; MatTek Co., Ashland, MA, USA) or ethanol-washed coverslips were coated with FN (Sigma Aldrich, 10 µg/mL diluted in phosphate-buffered saline containing CaCl₂ and MgCl₂ (PBS+, Sigma-Aldrich)) or transferrin (Tf, Sigma-Aldrich, 10 µg/mL – 100 µg/mL diluted in PBS- as indicated) overnight at 4 °C or 1 h at room temperature (RT). Ligand-coated surfaces were washed with PBS- to remove excess ligand. To prevent cell attachment to uncoated surfaces, dishes and coverslips were blocked with heat-denatured bovine serum albumin (BSA; Sigma-Aldrich; 10 mg/mL >99% purity BSA in PBS-, heated to 85 °C for 12 min, 0.22 µm filtered) for at least 30 min at RT. Dishes and coverslips were washed with PBS- to remove excess heat-denatured BSA prior to cell plating.

2.2.3 Plating cells for cell biological assays

Cells were washed with PBS-, detached with Trypsin-EDTA (Sigma-Aldrich) and quenched with 2.5% (w/v) BSA in DMEM supplemented with 25 mM HEPES (DMEM-HEPES, Sigma-Aldrich). Cells were washed with PBS-, resuspended in 5% (w/v) BSA in DMEM-HEPES and incubated at 37 °C in a humidified 8% (v/v) CO₂ atmosphere in suspension for 30 min to downregulate adhesion-dependent signalling events. Cells were counted using a haemocytometer and washed with PBS-. Cells were resuspended in DMEM-HEPES and plated onto ligand-coated surfaces. HFF cells from one confluent 225-cm² flask were plated onto three 10-cm-diameter dishes, which equated to approximately 1.5 x10⁶ cells per 10-cm-diameter dish. Cells were incubated at 37 °C in a humidified 8% (v/v) CO₂ atmosphere for the required times.
2.3 Biochemistry

2.3.1 Collection of total cell lysates

Following cell counting, approximately $1.5 \times 10^6$ cells kept in suspension (equivalent to the number of cells plated onto 1 10-cm-diameter dish) were centrifuged at 450 g for 4 min at 4 °C, washed in cold PBS- and centrifuged. Cells were lysed by addition of 100 µL cold cell lysis buffer (Table 2.4).

Adherent cells were washed in cold PBS-, 100 µL cold cell lysis buffer was added to each 10-cm-diameter dish and lysates were scraped and collected. One 10-cm-diameter dish was used for each experimental condition. After cell lysis, cell nuclei were separated by centrifugation at 18600 g for 4 min at 4 °C and the cytoplasmic supernatant was retained for SDS-polyacrylamide gel electrophoresis (PAGE) analysis or stored at -80 °C. Alternatively, total cell lysates were collected from 6-well plates using 2.5 x10^5 cells per well.

2.3.2 Determination of relative and absolute protein concentrations

To ensure equal protein loading between experimental conditions, relative protein concentrations were determined from total cell lysates using the Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA). For each condition, 3 µL protein sample was added to 22 µL PBS- and 225 µL working reagent (50 parts Reagent A added to 1 part Reagent B; Pierce BCA Protein Assay Kit) in a 96-well plate in technical triplicate and was incubated for 30 min at 37 °C in the dark. Absorbance intensity was read with a spectrometer at 570 nm and the average of three technical repeats was taken. After subtraction of the absorbance intensity of a blank PBS- sample, average absorbance intensity values were normalised to the smallest value across the experiment and multiplied by the maximum gel well loading size (10-well gel, 30 µL; 12-well gel, 20 µL). The calculated sample volume for loading was added to 5 µL 5x RSB (see Table 2.4) before gel loading.

In addition, the absolute concentration of total cell lysate samples was required to allow estimation of sample concentration of isolated integrin adhesion complexes (IACs, see section 2.3.3), which was obtained using a BSA calibration curve. Half-log dilutions of a BSA stock solution (2 mg/mL >99% purity BSA in PBS- diluted in cell lysis buffer (Table 2.4)) were analysed as described above using the Pierce BCA Protein Assay Kit to construct a calibration curve of protein concentration vs. absorbance intensity. The absorbance intensity of a total cell lysate sample was analysed and the BSA calibration curve was used to estimate the protein concentration of the total cell lysate.

2.3.3 Isolation of IACs from adherent cells

IACs collected from two 10-cm-diameter plates per condition were used for each SDS-PAGE gel and immunoblot. IACs collected from an additional four 10-cm-diameter plates per condition were used for each analysis by mass spectrometry (MS).

The method used to isolate IACs was adapted from protocols of similar studies that have analysed IAC composition by MS (see section 4.11) (Jones et al., 2015; Ng et al., 2014; Robertson et al., 2015). After cell incubation with DMSO or inhibitors, dimethyl-3, 3'-dithiobispropionimidate (DTBP,
Sigma-Aldrich) cross-linker, pre-equilibrated to RT, was dissolved in DMEM-HEPES and immediately added to cells to achieve concentrations and times, as required (6 mM, 3 min; 6 mM, 5 min; or 3 mM, 30 min). Cells were viewed on a bright-field microscope to check for the appearance of dark cell nuclei to confirm that cross-linking had occurred. The DTBP solution was removed from cells and further cross-linking quenched by the addition of 200 mM Tris-HCl in DMEM-HEPES (pH 8.0, 3 min). Cells were washed in cold PBS- and incubated with cold EB or RIPA buffer (3 min or 1 h, as indicated) to lyse the cells. Cell bodies were removed by sonication for 2.5 min (VibraCell VCX 500; Sonics & Materials, Newtown, CT; 20 kHz, 500 W, 22% amplitude, 2 min) or high-pressure water wash (tap water, 8-12 sec, ~150 ml/sec flow rate (Jones et al., 2015)). Plates containing isolated IACs were washed in cold PBS- and stored at 4 °C until all samples had been lysed. Plates were viewed on a microscope to check removal of cell nuclei to confirm efficient cell lysis. Isolated IACs from replicate plates were scraped into adhesion recovery buffer (100 µL) and collected.

Samples were acetone precipitated to reduce their volume by adding four times their volume of -20 °C acetone (eg. 1 mL acetone added to 250 µL sample). Samples were vortexed and incubated at -80 °C for at least 3 h, or preferentially overnight. Precipitated proteins were centrifuged at 16000 g for 15 min at 4 °C, washed twice with -20 °C acetone and centrifuged at 16000 g for 15 min at 4 °C without disturbing the protein pellet. The supernatant was removed and the precipitated proteins were dried at 37 °C for 20-30 min, resuspended in 2x RSB (diluted from 5x RSB, Table 2.4; 22.5 µL per two 10-cm-diameter plates) and heated to 70 °C at 1000 rpm for 20 min to solubilise.

Solubilised complexes were analysed by SDS-PAGE, immunoblotting and MS. As detergents in the adhesion recovery buffer prevented the use of the BCA assay, total protein in each sample was determined by loading serial dilutions of a known concentration of total cell lysate (see section 2.3.2) adjacent to isolated complex samples on an SDS-PAGE gel. This was used to create a calibration curve of lane intensity vs. protein concentration. Using this approach, it was calculated that approximately 200 µg/mL of protein was collected from FN-coated plates using the optimised protocol (Fig. 4.4a), which equated to approximately 10 µg of protein isolated per 10-cm-diameter plate. Approximately 5 µg of protein was recovered per Tf-coated 10-cm-diameter plate.

### 2.3.4 Nocodazole washout assay

For MS analysis of IAC disassembly (section 5.3.1), U2OS cells plated on FN-coated dishes were serum-starved for 16 h, treated with 10 µM nocodazole (Sigma-Aldrich) for 4 h, washed three times with DMEM and incubated for 5, 10 or 15 min after nocodazole removal at 37 °C, 5% (v/v) CO₂ (Ezratty et al., 2005).

For IF analysis of IAC disassembly (section 5.3.4), HFF cells were cultured for 16 hours in DMEM supplemented with 10% (v/v) FCS (Lonza) and 2 mM L-glutamine, then serum-starved with DMSO or 10 µM nocodazole for 4 hours. For the washout time points, cells treated with nocodazole were washed three times with DMEM and incubated for 5, 10, 15 or 30 min after nocodazole removal at 37 °C, 5% (v/v) CO₂. Cells were analysed that were fixed immediately after nocodazole removal (Noc.) or were fixed after washes with DMEM (0 time point).
2.3.5 Polyacrylamide gel electrophoresis (PAGE)

Total cell lysates were mixed with 5 µL 5x RSB (Table 2.4) and heated to 95 °C for 5 min. NuPAGE Novex Bis-Tris gels (4-12% (w/v); Invitrogen, Paisley, UK) were loaded into XCell SureLock mini-cells (Invitrogen) in NuPAGE MES SDS running buffer (Invitrogen). Protein extracts were separated by SDS-PAGE at 200V for 40 min. Precision Plus Protein All Blue standards (Bio-Rad Laboratories, Hemel Hemstead, UK) were used as molecular weight markers.

2.3.6 Gel staining

Gels were stained with Instant Blue (Expedeon Ltd, Harston, UK) for 1 h at RT to assess relative protein amount collected between each experimental condition. Stained gels were washed with Milli-Q water (Millipore Limited, Watford, UK) overnight at 4 °C and scanned using the Odyssey Infrared imaging system (700 nm, 169 μm resolution, 0.5 mm focus offset; LI-COR Biosciences, Cambridge, UK). Total lane intensity was determined using Odyssey software (LI-COR Biosciences). To calculate relative lane intensities between conditions and biological replicates, each lane intensity value was normalised to the summed intensity in each experiment.

2.3.7 Immunoblotting

Protein samples resolved by SDS-PAGE were transferred in transfer buffer (Table 2.4) onto nitrocellulose membrane (Whatman International Ltd, Maidstone, UK) using XCell II blot modules (Invitrogen) at 30 V for 90 min. Membranes were washed with PBS- and blocked with blocking buffer (Sigma-Aldrich) diluted in PBS- for 1 h at RT. Membranes were incubated with appropriate concentrations of primary antibodies (Table 2.1) diluted in blocking buffer in TBS-T (Table 2.4) overnight at 4 °C. After three 5 min washes in TBS-T, membranes were incubated with appropriate concentrations of secondary antibodies (Table 2.2) diluted in blocking buffer in TBS-T for 45 min at RT in the dark and were washed three times for 5 min in TBS-T. Membranes were scanned using the Odyssey Infrared imaging system (700 nm and 800 nm, 169 μm resolution; LI-COR Biosciences).

2.4 Mass spectrometry

2.4.1 Proteolytic digestion and peptide desalting

For comparison of IAC composition between 1 h and 4 h DMSO or FAK [i] treatment (section 4.11.3; n = 1 for each of 5 conditions), protein samples were separated by SDS-PAGE at 200 V until the dye front had migrated to the bottom of the gel. The gel was stained as described previously (section 2.3.6) and each gel lane was cut into 10 slices. For samples generated using the final IAC isolation protocol (section 4.3.3; n = 3 for each of 3 conditions), protein samples were separated by SDS-PAGE at 200 V for 2 min or until samples had migrated into the top of the gel (termed ‘gel-top’), and after staining each sample was excised into one slice. For both experiments, gel slices were cut into 1-mm³ pieces, transferred to a perforated V-bottom 96-well plate (Thermo Fisher) mounted onto a 96-well collection plate (Thermo Fisher), washed twice with 50% (v/v) acetonitrile (ACN, Sigma-Aldrich) in 12.5 mM NH₄HCO₃ and twice with ACN to dry gel pieces. Proteins were reduced by incubation in 10 mM dithiothreitol (DTT, Sigma-Aldrich) diluted in 25 mM
NH₄HCO₃ for 1 h at 56 °C and alkylated in 55 mM iodoacetamide (Sigma-Aldrich) diluted in 25 mM NH₄HCO₃ for 45 min at RT in the dark. Gel pieces were washed with 25 mM NH₄HCO₃ followed by a wash in ACN, which was repeated once more. Gel pieces were dried and incubated with 12 µg/mL trypsin (Promega, Southampton, UK) overnight at 37 °C to enable complete protein digestion (Shevchenko et al., 1996). Digested peptides were extracted by incubation with ACN in 0.2% (v/v) formic acid (FA, Sigma-Aldrich) followed by incubation with 50% (v/v) ACN in 0.1% (v/v) FA. To desalt peptides, each sample was resuspended in 5% (v/v) ACN in 0.1% (v/v) FA followed by incubation with OLIGO™ R3 beads (Applied Biosystems, Paisley, UK) in a 96-well plate with 0.2 µm PVDF membrane (Corning). Bead-bound peptides were washed twice in 0.1% (v/v) FA, eluted by two washes in 50% (v/v) ACN in 0.1% (v/v) FA, dried and resuspended in 5% (v/v) ACN in 0.1% (v/v) FA.

2.4.2 MS data acquisition

Peptides were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an UltiMate 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA, USA) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptide samples from each slice were loaded onto a pre-column (20 mm x 180 µm i.d., Waters, Hertfordshire, UK) in biological sample order rather than biological replicate order to optimise chromatograph alignment between samples in downstream analyses (Daniel Ng, personal communication). To increase the dynamic range, peptides were separated on an analytical column (250 mm x 75 µm i.d., 1.7 µm particle size, bridged ethyl hybrid C₁₈; Waters) using a gradient ranging from 8 to 33% (v/v) ACN in 0.1% (v/v) FA for 1 h per slice for samples separated over 10 slices or for 2 h for samples analysed using the gel-top approach. LC-MS/MS analyses were performed in data-dependent mode to allow automatic selection of peptides for fragmentation.

A longer LC gradient was used for the gel-top approach since it was reported that a similar number of proteins are identified using the gel-top approach and a 2 h LC gradient compared with protein separation using 10 gel slices and a 1 h LC gradient for each slice (M.C. Jones and J.A. Askari, personal communication). Therefore, the gel-top approach was used for the optimised assay to reduce errors and variability that could arise from processing large numbers of gel slices during in-gel digestion and peptide desalting, or from inaccurate band slicing for each fraction across samples.

2.4.3 MS data analysis and database searching

An intensity-based quantification approach was performed for analysis of IACs separated using the gel-top approach (section 4.3.3). Raw files were analysed and pre-processed using Progenesis software (Progenesis QI, Nonlinear Dynamics, Newcastle, UK; http://www.nonlinear.com/progenesis/qi-for-proteomics/) with automatic detection of alignment reference, which was selected as FAK [i], R2 (biological replicate 2). Raw files generated from Tf-induced complexes were not included in further analysis due to poor chromatograph alignment. Therefore, non-specific components were not removed allowing for characterisation of potentially novel IAC components (Jones et al., 2015), but comparative analyses between IACs isolated from cells treated with DMSO and FAK [i] were performed.
All MS data were searched using an in-house Mascot server (version 2.2.03, Matrix Science, London, UK) (Perkins et al., 1999) against the UniProt_Human protein sequence database. Permitted fixed and variable modifications were cysteine carbamidomethylation and methionine oxidation, respectively. Only tryptic peptides with a maximum of one missed cleavage were considered. Only doubly or triply charged monoisotopic precursor ions were considered, peptide mass tolerance was set to ±5 p.p.m and MS/MS tolerance was set to ±0.5 Da.

For analysis of samples separated using 10 gel slices per condition (section 4.11.3), protein quantification was performed using spectral counting to calculate relative quantification between conditions. Spectral counting was also performed for analysis of the IAC MEF dataset (Supplementary Table 3.2), IAC assembly dataset (Supplementary Table 5.1) and IAC disassembly dataset (Supplementary Table 5.2). Data were visualised using Scaffold (version 4.2.1; Proteome Software, Portland, OR, USA) with protein identification threshold of at least two unique peptides with 90% confidence at the peptide level and 99% confidence at the protein level. Data were exported and filtered to include only those proteins with at least four spectral counts in at least one experimental condition (Supplementary Table 4.1), the same criteria used to process individual IAC datasets used in the construction of the meta-adhesome.

For samples analysed using the gel-top approach, protein identifications were imported into Progenesis software and relative quantification was performed using protein grouping with non-conflicting (unique) peptides. Data were exported as protein measurements, and duplicate protein entries by gene name annotation were combined by addition of raw intensity values of unique peptides. Raw abundance values were normalised to the total raw abundance value in each condition. Only those proteins containing at least two peptides used for quantification were included in the dataset (Fig. 4.4–source data 1). Log_2 fold change values for each of three biological replicates were calculated between DMSO and FAK [i] conditions using normalised raw values and the average taken. In the case that a protein was not identified in all biological replicates, the average fold change was calculated using the other biological replicate values only. P-values were calculated from normalised abundance values between DMSO and FAK [i] conditions using the Holm-Sidak method for multiple comparisons (Holm, 1979). Each protein was analysed individually, without assuming a consistent standard deviation.

2.5 Immunofluorescence microscopy

2.5.1 Immunofluorescence staining

Cells were washed in PBS-, fixed with −20 °C methanol or 4% (w/v) paraformaldehyde for 7 min at RT, washed in PBS- and permeabilised with 0.5% (v/v) Triton X-100 for 10 min at RT. Permeabilised cells were washed three times with PBS- before incubation with appropriate primary antibodies diluted in 2% (w/v) BSA in PBS- for 1 h at RT. Cells were washed three times with PBS- and incubated with appropriate secondary antibodies diluted in 2% (w/v) BSA in PBS- for 30 min at RT in the dark. Stained cells were washed once in PBS-, twice in water and stored in water at 4 °C until imaging.
2.5.2 Microscopy

Images of cells plated on glass-bottom dishes were acquired on a Delta Vision RT (Applied Precision, Issaquah, WA, USA) restoration microscope using a 60×/1.42 Plan Apo objective and the Sedat filter set (Chroma 89000, Bellows Falls, VT, USA). Images were collected with a z optical spacing of 0.2 μm, five images per stack, using a Coolsnap HQ camera (Photometrics, Tucson, AZ, USA) and Softworx software (Applied Precision).

Images of cells plated on glass coverslips were acquired on a BX51 upright microscope (Olympus, Southend-on-Sea, UK) using a 60×/1.25 Plan Fln objective and specific band pass filter sets. Images were captured using a Coolsnap EZ camera (Photometrics) and MetaVue software (Molecular Devices, Sunnyvale, CA, USA).

Cells stained for Rsu-1 and caldesmon were also imaged using a spinning-disk confocal inverted microscope (Marianas, 3i) (images not shown). Images were collected with a z optical spacing of 0.2 μm, three images per stack, using a 63×/1.4 Plan Apochromat objective and SlideBook 6.0 software.

2.5.3 Image analysis

Maximum intensity projections of raw images were generated and background filtered (rolling ball, 10-pixel radius) using ImageJ (version 1.48o) (Schindelin et al., 2012). Alternatively, single slices of raw images were background filtered (rolling ball, 20-pixel radius). In each field of view, areas containing positive staining of IAC proteins (≥10 pixels) were measured and normalised to total cell area.

To quantify Rsu-1 and caldesmon colocalisation with vinculin, images were individually band-pass filtered (A trous wavelet, linear 3 × 3 filter, keeping scales 2–8) using custom software written in Python and NumPy to create a mask of vinculin-positive IAC structures. Colocalisation analysis was performed using the ImageJ plugins Coloc 2, with the mask as a region of interest to calculate Mander’s overlap coefficients (MOC) (Manders et al., 1993), and Plot_Multicolor (version 4.3) to plot line profiles.

2.6 Bioinformatics analyses

2.6.1 Processing of individual IAC datasets and meta-adhesome construction

IAC datasets (Supplementary Table 3.1) and the literature-curated adhesome were downloaded from the relevant publication (K562 (Humphries et al., 2009), A375 (Robertson et al., 2015), HFF (Ng et al., 2014), MKF1 (Schiller et al., 2011), MKF2 and MFK3 (Schiller et al., 2013), literature-curated adhesome (Winograd-Katz et al., 2014)). IAC datasets were filtered to include only proteins from cells spread on FN and the corresponding negative control in the absence of perturbation (proteins were excluded that were identified uniquely from phospho-enriched IACs (Robertson et al., 2015), vascular cell adhesion molecule-1 (VCAM-1)-induced IACs (Humphries et al., 2009), cells treated with blebbistatin (Schiller et al., 2011, 2013) or cells treated with nocodazole (Ng et al., 2014)). Two datasets were included from Schiller et al., 2013 for cells expressing αV and β1
integrins (α5β1, αVβ3 and αVβ5 integrins; cells spread for 45 min (MKF2) and 90 min (MKF3)), excluding proteins uniquely identified in cells expressing only either β1- or αV-class integrins as integrin heterodimer-specific adhesomes were not considered here. The assembled IAC datasets were reduced to include proteins satisfying at least 2 unique peptides per protein with at least four spectral counts (where spectral counts were provided, see Supplementary Table 3.1) and relative abundance satisfying log₂(FN/control) ≥ 1. In the case that a protein was detected uniquely in the FN condition, the fold enrichment value was set to 100.

The meta-adhesome database (Supplementary Table 3.3) was constructed from the seven assembled datasets containing proteins satisfying the quantitative data thresholds mentioned above (see Supplementary Table 3.1). An extra parameter was added to the database to calculate the total number of datasets in which a protein was enriched to FN-induced IACs compared with the negative control. Proteins from the literature-curated adhesome were also incorporated into the database.

2.6.2 Consensus adhesome construction and interaction network curation

Proteins enriched in at least five proteomic IAC datasets in the meta-adhesome database were incorporated into the consensus adhesome. Extracellular matrix (ECM) proteins are commonly detected in IAC datasets due to the nature of the enrichment procedure. ECM or secreted proteins (COL1A1, COL1A2, COL5A2, COL6A1, COL6A2, FGG, FN1, PCOLCE, PRSS23, SERPINE1) were excluded since, although relevant to adhesion biology, we sought to focus on intracellular components of IACs. Therefore, the remaining 60 proteins were selected on the basis of functional enrichment of adhesion-related gene ontology terms. All isoforms of literature-curated adhesome (Winograd-Katz et al., 2014) members were classified as adhesome molecules. Functional information was adapted from the HUGO Gene Nomenclature Committee (HGNC) database (Gray et al., 2015), protein domain information was assigned from InterPro (Mitchell et al., 2015) and disease annotations were extracted from the Online Mendelian Inheritance in Man database (www.omim.org).

Evidence for protein-protein interactions was manually verified and scored. Low-evidence interactions included those based on a single publication or on coprecipitation or yeast two-hybrid studies. Medium-evidence interactions were based on data from multiple sources, or a single source if there was phosphorylation or peptide binding data. High-evidence interactions were based on structural evidence of direct binding between two proteins, such as X-ray crystallography or nuclear magnetic resonance, or confirmation using a wide variety of techniques. Experimental evidence and source publications are detailed in Supplementary Table 3.9. For analysis of consensus adhesome dynamics, node colour represents hierarchical protein clusters defined during IAC assembly and IAC disassembly.

2.6.3 Hierarchical clustering and principal component analysis

Proteins or datasets were hierarchically clustered on the basis of uncentred Pearson correlation using Cluster 3.0 (C Clustering Library, version 1.50) (de Hoon et al., 2004) and visualised using Java TreeView (version 1.1.5) (Saldanha, 2004). Specific clusters were selected using Pearson
correlation thresholds as indicated in figure legends. Binary data were clustered on the basis of Jaccard distance and visualised using R (version 3.1.0). Distances between hits were computed using a complete-linkage matrix in all cases. Additional heatmaps were visualised using MultiExperiment Viewer (version 4.8.1) (Saeed et al., 2003). Principal component analysis was performed using MATLAB (version R2012a; MathWorks).

2.6.4 Protein-protein interaction network analyses

Interaction network analysis was performed using Cytoscape (version 2.8.3 and 3.0.2) (Cline et al., 2007). Proteins were mapped onto a merged human interactome consisting of physical protein-protein interactions reported in the Protein Interaction Network Analysis (PINA) database (*Homo sapiens, Mus musculus* and *Rattus norvegicus*, 28th October 2012), the ECM database MatrixDB (20th April 2012) (Launay et al., 2014) and the literature-curated integrin adhesome (Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007) as previously described (Byron et al., 2015; Humphries et al., 2009). Graph clustering was performed using the yFiles Organic algorithm implemented in Cytoscape. Topological parameters were computed from undirected graphs, excluding self-interactions, using NetworkAnalyzer (Assenov et al., 2008).

2.6.5 Functional enrichment analyses

Functional enrichment analysis was performed using DAVID (version 6.7) (Huang et al., 2009) against top-level annotations for biological process, cellular component, molecular function, KEGG pathways (Ogata et al., 1999) and protein domains from InterPro (Mitchell et al., 2015). Keywords with fold enrichment ≥ 1.5, Bonferroni-corrected $P$ value < 0.05, EASE score (modified Fisher’s exact test) < 0.05 and at least two proteins per keyword were considered significantly overrepresented. Functional enrichment analysis using DAVID was performed for analysis of the meta-adhesome (Supplementary Table 3.5), consensus adhesome (Supplementary Table 3.8), IAC assembly (Supplementary Table 5.3) and IAC disassembly (Supplementary Table 5.4) datasets.

For generation of functional enrichment networks, overrepresentation of functional terms was calculated using the Cytoscape plugin BiNGO (version 2.44) (Maere et al., 2005) for biological process, cellular component and molecular function categories and networks were visualised using Enrichment Map (version 1.2) (Merico et al., 2010) with significance settings $P < 0.001$, false discovery rate < 5% and Jaccard coefficient ≥ 0.25. Functional enrichment networks were generated for proteins identified in the meta-adhesome (Fig. 3.4; Supplementary Figs 3.2, 3.3; Supplementary Table 3.6) and consensus adhesome (Supplementary Fig. 3.5, Supplementary Table 3.9).

For generation of functional enrichment maps, overrepresentation of biological process terms was calculated using High-Throughput GoMiner (Zeeberg et al., 2005). One thousand randomisations were performed and data were thresholded for a 5% false discovery rate. Overrepresented terms with ≥5 and ≤500 assigned proteins were reported. Dataset occurrence was mapped onto proteins assigned to each overrepresented term, and the data matrix was subjected to hierarchical
clustering analysis. Functional enrichment maps were generated for proteins identified in the meta-adhesome (Supplementary Fig. 3.4) and consensus adhesome (Fig. 3.6).

2.6.6 Statistical analysis

Statistical significance was calculated as indicated in the figure legends with $P < 0.05$ considered statistically significant. All graphs were plotted using Prism (version 6.04; GraphPad Software, La Jolla, CA, USA). Figures were assembled using Illustrator (Adobe).
2.7 References


Chapter 3

Definition of a consensus adhesome by the analysis of multiple integrin adhesion complex proteomic datasets

Edward R. Horton¹,⁴, Adam Byron¹,³,⁴, Daniel H. J. Ng¹, Angélique Millon-Frémillon¹, Joseph Robertson¹, Ewa J. Koper¹, Nikki R. Paul¹, Stacey Warwood², David Knight², Jonathan D. Humphries¹ and Martin J. Humphries¹,⁵

¹Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
²Biological Mass Spectrometry Core Facility, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
³Present address: Edinburgh Cancer Research UK Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XR, UK
⁴These authors contributed equally to this work
⁵Correspondence should be addressed to M.J.H. or A.B.:

Professor Martin J. Humphries, Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
Tel.: +44 (0) 161 2755071; Fax: +44 (0) 161 2755082
E-mail: martin.humphries@manchester.ac.uk

Dr Adam Byron, Edinburgh Cancer Research UK Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XR, UK
Tel.: +44 (0) 131 6518575; Fax: +44 (0) 131 7773520
Email: adam.byron@igmm.ed.ac.uk

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3.1 Abstract

Integrin receptor activation initiates the formation of protein complexes at the cell membrane that transduce adhesion-dependent signals to control a multitude of cellular functions. Proteomic analyses of isolated integrin adhesion complexes (IACs) have revealed an unanticipated molecular complexity of IACs in a variety of experimental contexts; however, a global consensus view of the composition of IACs is currently lacking. Here, we have integrated IAC proteomes derived from multiple cell types and generated a 2,412-protein database of FN-induced IACs, which we termed the meta-adhesome. Analysis of this dataset reveals diverse functional classes of proteins recruited to IACs and establishes a consensus adhesome of 60 proteins. The consensus adhesome represents a putative core cell adhesion machinery, which includes canonical and underappreciated IAC components. The definition of this consensus view of integrin adhesome components provides a resource for the interpretation of future proteomic datasets and for the wider research community.
3.2 Introduction

Cellular adhesion to the extracellular matrix (ECM) is essential for a multicellular existence. Integrin adhesion receptors on the cell surface engage the cytoskeleton and transduce signals that control cell morphology, migration, survival and differentiation in a wide range of developmental, homeostatic and disease processes (Hynes, 2002). The interactions of integrin cytoplasmic domains with cytoskeletal, adaptor and signalling molecules are central to regulation of integrin-mediated cellular functions (Liu et al., 2000; Morse et al., 2014). The complex multimolecular structures that form the connection between integrin receptors and the actin cytoskeleton, which are termed integrin adhesion complexes (IACs), contain over 200 reported components (Winograd-Katz et al., 2014; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007). IAC proteins have been characterised as either ‘intrinsic’ components, which localise directly to IACs, or ‘associated’ components, which are effectors of intrinsic molecules (Zaidel-Bar et al., 2007). IACs have been implicated in many disease processes, and several individual components contribute to aberrant cellular signalling that can result in cancer and other pathologies in a wide variety of tissues (Winograd-Katz et al., 2014).

IACs, like other membrane receptor-associated signalling complexes, have been refractory to proteomic analysis due to their lability and inaccessibility (Byron et al., 2011). Recent approaches to isolate IACs and analyse their molecular composition using mass spectrometry (MS) have been performed in multiple cell types under various conditions (Byron et al., 2012, 2015; Huang et al., 2014; Humphries et al., 2009; Kuo et al., 2011; Ng et al., 2014; Robertson et al., 2015; Schiller et al., 2011, 2013; Yue et al., 2014). These datasets are necessarily context-dependent (e.g. cell type- or integrin heterodimer-specific) and consequently, an integrative, systems-wide description of IAC composition would be beneficial.

To enable a systems-level analysis of IACs, we computationally integrated seven IAC proteomes from multiple cell types (Humphries et al., 2009; Ng et al., 2014; Robertson et al., 2015; Schiller et al., 2011, 2013). By leveraging multiple experimental datasets, we first took a systems-wide approach to establish a global view of IAC composition using unbiased approaches and performed a functional enrichment analysis using the gene ontology, resulting in an experimentally-defined functional adhesion landscape. We next took a reductionist approach to highlight proteins reliably detected in proteomic datasets to define a robustly detected core ‘consensus’ adhesome, which consisted of well-characterised and underappreciated IAC components. These data describe the molecular composition of IACs and provide a resource for the interrogation of the global molecular connections that link integrin interactions to adhesion signalling.
3.3 Results

3.3.1 Comparative analyses of IAC proteomic datasets

To compare MS-based proteomic datasets of IACs, we assembled seven quantitative proteomic datasets detailing the composition of IACs induced by the canonical ligand fibronectin (FN) (Supplementary Table 3.1). The assembled datasets were generated from a range of cell types from different lineages: human malignant melanoma (A375) (Robertson et al., 2015), mouse embryonic fibroblast (MEF) (Supplementary Table 3.2), human foreskin fibroblast (HFF) (Ng et al., 2014), human chronic myelogenous leukaemia (K562) (Humphries et al., 2009) and mouse kidney fibroblast (MKF) (Schiller et al., 2011, 2013) cells (Supplementary Table 3.1). To distinguish and remove non-specific proteins from the data, we required the proteomic analyses to employ a negative-control ligand, which excluded several published datasets (Huang et al., 2014; Kuo et al., 2011; Yue et al., 2014), and we filtered the datasets to include only those proteins at least two-fold enriched to FN-induced IACs over the negative control.

Hierarchical clustering and principal component analyses revealed that IAC proteomes grouped based on the negative-control ligand condition used and cell type, which suggested that the choice of negative control was important and that IAC composition may be cell type-specific (Fig. 3.1a-c). Individual IAC proteomes contained hundreds of proteins (602 ± 250, mean ± s.d.; range, 314–1,023) and identified up to a third of literature-curated adhesome components (Winograd-Katz et al., 2014) (20.9 ± 7.1%, mean ± s.d.; range, 9.1–32.3%) (Fig. 3.1d). Identified literature-curated adhesome components were spread throughout different datasets (Fig. 3.1a), indicating differential dataset occurrence of adhesome proteins, while a number of high occurrence literature-curated adhesome components such as α5β1 integrin, focal adhesion kinase (FAK), integrin-linked kinase (ILK), paxillin, talin, vinculin and zyxin clustered together (Fig. 3.1a). We therefore hypothesise that the identification of a restricted number of literature-curated adhesome components was likely due to the context under which the IACs were observed such as extracellular ligand, cell type or time of IAC isolation after cell-ECM engagement (Humphries et al., 2015).

It should be noted that the IAC proteomes indicate a dramatic increase in both the scale and complexity of IAC composition. Indeed, the smallest dataset identified 314 proteins enriched to FN-complexes, which is still larger than the 232 proteins reported to localise to IACs in the current version of the integrin adhesome (Winograd-Katz et al., 2014). The datasets containing the highest level of overlap were MKF2 and MKF3 with 413 shared proteins (Fig. 3.1d), which is not surprising as the only difference between these datasets was the time point used to isolate IACs (45 min, MKF2; 90 min, MKF3). These datasets also displayed a high level of overlap with the other dataset generated using MFK cells (MKF1, Fig. 3.1), which used the same isolation approach and negative control (Fig. 3.1b, Supplementary Table 3.1). The two datasets that used the negative-control ligand transferrin (Tf) detected the highest number of IAC proteins (674, MEF; 1,023, A375) and exhibited a high level of overlap with 270 commonly enriched proteins (Fig. 3.1a,b,d). Furthermore, 533 proteins were uniquely enriched in the dataset generated using melanoma cells (A375) suggesting that these proteins may represent melanoma-specific components (Fig. 3.1a). In summary, comparative analyses identified negative control- and biochemical isolation methodology-specific variations in IAC composition and suggest that IAC composition may be cell
Figure 3.1. Comparison of FN-enriched IAC proteomes.
(a) Seven proteomic datasets of FN-enriched IACs were analysed by unsupervised hierarchical clustering. The binary heat map shows proteins at least two-fold enriched to FN over the negative control (red). Dataset occurrence is plotted for each protein (rainbow), and literature-curated adhesome (Winograd-Katz et al., 2014) components are indicated by purple bars. Details of the proteomic datasets are provided in Supplementary Table 3.1. (b) Dendogram illustrating the clustering of the FN-enriched IAC proteomes shown in a. Dataset dissimilarity is measured by Jaccard distance and the negative control used in each dataset is labelled. (c) FN-enriched proteins identified in the seven proteomic datasets were analysed by principal component analysis. A plot of the first two principal components is shown. (d) Pairwise overlaps of FN-enriched proteins identified in the seven proteomic datasets and the literature-curated adhesome were measured by Jaccard coefficient and are displayed as a hierarchically clustered heatmap (lower diagonal matrix; blue). Numbers of proteins in each overlap set are indicated (upper diagonal matrix). K562, human chronic myelogenous leukaemia cells (Humphries et al., 2009); MEF, mouse embryonic fibroblast cells (Supplementary Table 3.2); A375, human malignant melanoma cells (Robertson et al., 2015); HFF, human foreskin fibroblast cells (Ng et al., 2014); MKF 1, mouse kidney fibroblast cells (Schiller et al., 2011); MKF 2 and MKF 3, mouse kidney fibroblast cells (Schiller et al., 2013).
type-specific and time dependent. Furthermore, these analyses suggest that heterogeneity in IAC composition exists between experimental contexts even when cells are exposed to very similar extracellular microenvironments and ligands.

### 3.3.2 An experimentally defined integrin meta-adhesome

To obtain a global overview of IAC composition, we constructed a database from multiple MS-derived proteomic reports. Proteins from the seven assembled IAC datasets that were at least two-fold enriched over their respective controls (Supplementary Table 3.1; see section 3.8.8) were integrated and the resulting experimentally defined database, termed the ‘meta-adhesome’, contained 2,412 proteins observed in at least one IAC proteome (Supplementary Table 3.3).

Over half of the proteins in the meta-adhesome (1,359 proteins; 56.3%) were identified uniquely in a single dataset (Fig. 3.2a). These proteins represent low-abundance or context-specific adhesome components, or those difficult to detect by MS. The number of proteins identified in the meta-adhesome decreased exponentially as the stringency in dataset number increased (Fig. 3.2b). Four hundred and forty-eight proteins were detected in at least three datasets (Fig. 3.2b), more than the 63 proteins previously found in common between three published IAC proteomes (Geiger and Zaidel-Bar, 2012). Only 10 proteins were enriched in all seven datasets: α-actinin-4, α5 integrin, αV integrin, β1 integrin, ILK, LIM and SH3 protein 1 (LASP1), PDZ and LIM domain protein 5 (PDLIM5), transglutaminase-2, vasodilator-stimulated phosphoprotein (VASP) and vinculin (Fig. 3.2a,b). We hypothesised that a restricted set of robustly detected proteins may represent a context-independent core of IAC components (Humphries et al., 2015). Indeed, the proportion of identified proteins that were literature-curated adhesome components increased with dataset occurrence (Fig. 3.2c), suggesting that robustly detected proteins are more likely to represent canonical adhesion proteins.

To investigate the organisation of proteins identified by integrative analysis of IAC proteomes, we performed interaction network analysis (Fig. 3.2d). Proteins detected in few IAC datasets exhibited lower network connectivity in general, whereas proteins detected in all seven datasets had the highest degree and betweenness centrality measures (see methods; Supplementary Fig. 3.1, Supplementary Table 3.4), which indicates that proteins reliably detected in all seven datasets exhibit the potential to exert greater control over the interactions of other proteins in the complex. The high number of proteins identified in the meta-adhesome, together with their interconnected network of potential interactions, indicates that IACs, and the flow of information that they relay, are highly complex. Furthermore, it suggests that even the literature-curated adhesome (Winograd-Katz et al., 2014) and individual IAC proteomic datasets underestimate this complexity.
Figure 3.2. Coverage of proteins identified in the meta-adhesome.
(a) The number of proteomic datasets in which proteins in the meta-adhesome are identified (dataset occurrence) is displayed as a pie chart. Numbers of proteins identified are indicated for each segment (proportions of the meta-adhesome are shown in parentheses). (b) Line graph showing the cumulative proportion of the meta-adhesome in at least \( x \) proteomic datasets, where \( x \) is the minimum (min.) dataset occurrence category. Numbers of proteins identified are indicated for each data point. (c) Line graph showing the proportion of identified proteins that are in the literature-curated adhesome. Numbers of literature-curated adhesome proteins identified are indicated for each data point. (d) Protein-protein interaction network model of the meta-adhesome. The 2,412 meta-adhesome proteins were mapped onto a curated database of reported protein-protein interactions (see Methods). The largest connected graph component is displayed, comprising 11,430 interactions (grey lines; edges) between 2,035 proteins (circles; nodes). Node size and colour are proportional to the number of proteomic datasets in which a protein was identified. Locations of proteins identified in all seven datasets are indicated.
3.3.3 Comparative analysis of the integrin meta-adhesome with the literature-curated integrin adhesome

To visualise proteins identified in the meta-adhesome in the context of the literature-curated adhesome (Winograd-Katz et al., 2014), proteins from the meta-adhesome were mapped onto literature-curated adhesome functional categories. In total, 114 (49%) adhesome components were detected across all datasets (Fig. 3.3a), with almost half (56) detected in three or more datasets (Fig. 3.3b). The functional categories with the highest coverage in the meta-adhesome were adaptors (46; 65%), actin regulators (14; 82%) and chaperones (3; 100%) (Fig. 3.3c). Groups of proteins less well represented were GTPases, phosphatases, kinases, channels and adhesion receptors. Notably, the receptors most robustly detected were the prominent FN-binding α5β1 and αVβ3 integrins, which confirms the specificity of FN-induced IACs incorporated in the meta-adhesome. The 114 FN-specific adhesome components comprised 87 ‘intrinsic’ and 27 ‘associated’ proteins (Fig. 3.3a). These data likely reflect the ability of IAC isolation methods to stabilise and identify intrinsic structural adhesome molecules, such as adaptors and actin regulators. Associated proteins were generally enriched in fewer datasets compared with intrinsic proteins (Fig. 3.3b), with 27 intrinsic proteins but only one associated protein, ponsin (SORBS1), identified in five or more datasets, possibly due to the low stoichiometry, context-specificity or highly dynamic and labile nature of associated proteins, such as adhesion-related enzymes, within IACs.
Figure 3.3. Meta-adhesome coverage of the literature-curated adhesome.

(a) The proportion of the literature-curated adhesome identified in the meta-adhesome is plotted as a percentage bar chart. Proportions of the total literature-curated adhesome (black), intrinsic adhesome components (blue) and associated adhesome components (red) are shown. Numbers of identified proteins are indicated. (b) Line graph showing the cumulative number of literature-curated adhesome proteins identified in at least x proteomic datasets, where x is the minimum (min.) dataset occurrence category. Data for intrinsic (blue) and associated (red) adhesome components are shown. (c) Protein-protein interaction network of the literature-curated adhesome proteins identified in the meta-adhesome. Node size and colour are proportional to the number of proteomic datasets in which a protein was identified; ND, not detected (grey node). Nodes are clustered according to literature-curated adhesome functions; numbers (meta-adhesome/literature-curated adhesome total) and proportions of each functional category identified in the meta-adhesome are indicated in parentheses. Nodes are labelled with gene names for clarity (see Supplementary Table 3.3 for details).
3.3.4 Functional analyses of the integrin meta-adhesome

The meta-adhesome highlights an unanticipated complexity and increased scale in the number of identified proteins in IACs compared with the literature-curated adhesome. To examine the functional roles of these non-canonical IAC proteins, we performed functional enrichment analyses of proteins in the integrin meta-adhesome (Supplementary Table 3.5) and generated functional enrichment networks (Fig. 3.4, Supplementary Figs. 3.2, 3.3, Supplementary Table 3.6). As well as identification of proteins involved in adhesion-related functions such as cell-ECM adhesion, actin cytoskeleton organisation, angiogenesis, ECM organisation and cell polarity, proteins identified in the meta-adhesome were associated with a diverse range of non-adhesion-related cellular functions such as translation, cell cycle and metabolism, which is consistent with previous analyses of three IAC proteomic datasets (Geiger and Zaidel-Bar, 2012). In addition, proteins in the meta-adhesome contained protein domains previously linked with IACs such as actin-binding, LIM-type zinc finger and Src Homology domains, but meta-adhesome components also contained non-adhesion-related protein domains such as several RNA- and DNA-binding domains (Supplementary Table 3.5). The overrepresentation of these unexpected functional groups and protein domains in the meta-adhesome may represent non-canonical roles of known IAC components or adhesion-related functional roles of novel or underappreciated IAC components (Humphries et al., 2015). However, the most robustly detected proteins were overrepresented for numerous adhesion-related functions (Supplementary Fig. 3.4). These data represent an overview of the putative functional landscape of FN-induced IACs.
Figure 3.4. Functional enrichment network of the meta-adhesome.

In total, the meta-adhesome displayed significant overrepresentation of 317 biological process terms, which are displayed as a network. Each node represents a group of proteins enriched to a particular biological process term. Node size is proportional to the number of meta-adhesome proteins enriched to that functional category and node colour represents the level of statistical significance. Edges between nodes indicate shared proteins between two protein groups and edge thickness is proportional to the number of shared proteins between connected nodes. For clarity, clusters of functionally related terms were manually circled and assigned a cluster name and single gene annotations are not shown. All significantly overrepresented terms are listed in Supplementary Table 3.6. The network was generated using the Cytoscape plugins BiNGO (Maere et al., 2005) and Enrichment Map (Merico et al., 2010) with the following cut-off values: \( P \)-value = 0.001, false discovery rate (Q) = 5%, Jaccard coefficient = 0.25. Additional functional enrichment analyses of proteins identified in the meta-adhesome were performed using DAVID (Huang et al., 2009) (Supplementary Table 3.5).
3.3.5 Characterisation of a consensus integrin adhesome

The meta-adhesome provides a resource detailing the global composition of IACs from multiple cell types and experimental designs. To identify the core set of IAC components and thereby aid the identification of key nodes controlling adhesive functions (Humphries et al., 2015), we employed functional enrichment analysis and found that adhesion-related functions were uniquely overrepresented for proteins enriched in at least five datasets, whereas non-adhesion-related functions were overrepresented for proteins detected in at least four proteomic datasets (data not shown). This suggested the specific involvement in core adhesive functions of proteins identified in at least five datasets, which, following exclusion of ECM components to focus on intracellular components of IACs, resulted in a consensus integrin adhesome comprising 60 proteins (Supplementary Table 3.7). Hierarchical clustering and principal component analyses of reduced IAC proteomes assembled in the consensus adhesome revealed that IAC proteomes grouped together based on the biochemical isolation methodology (Fig. 3.5a-c). The group of proteins from the dataset that was generated using FN-coated beads (K562) was the most different (Fig. 3.1a-c) and the majority of literature-curated adhesome components and LIM domain-containing proteins in the consensus adhesome were identified in the six IAC datasets that were generated using FN-coated plates (Fig. 3.5a,b). Interestingly, the consensus integrin adhesome contained 29 proteins that were not members of the literature-curated adhesome (Winograd-Katz et al., 2014), which represent novel or underappreciated FN-mediated IAC components (Fig. 3.5; Supplementary Table 3.7) and suggests that the literature-curated adhesome underappreciates the complexity of even the consensus IAC adhesome. Individual IAC proteomes contained comparable coverage of consensus adhesome proteins (48 ± 11, mean ± s.d.; range, 26–59) (Fig. 3.5d), indicating that the consensus adhesome reduces the heterogeneity encountered between IAC proteomes that were assembled in the meta-adhesome and, although cell type-specific differences may still exist (Fig. 3.5b,c), the consensus adhesome consists of commonly identified IAC components.
Figure 3.5. Comparison of IAC proteomes in the consensus adhesome.

(a) Proteins identified in the consensus adhesome were analysed by unsupervised hierarchical clustering. The binary heat map shows proteins at least two-fold enriched to FN over the negative control (red). Dataset occurrence is plotted for each protein (rainbow), literature-curated adhesome (Winograd-Katz et al., 2014) components are indicated by purple bars and LIM domain-containing proteins are indicated by grey bars. Details of the proteomic datasets are provided in Supplementary Table 3.1 and details of proteins identified in the consensus adhesome are provided in Supplementary Table 3.7. (b) Dendogram illustrating the clustering of the IAC proteomes in the consensus adhesome shown in a. Dataset dissimilarity is measured by Pearson correlation and IAC isolation methodology is labelled. (c) Proteins identified in the consensus adhesome were analysed by principal component analysis. A plot of the first two principal components is shown. (d) Number of consensus adhesome proteins identified in each IAC proteome and the literature-curated adhesome are displayed. K562, human chronic myelogenous leukaemia cells (Humphries et al., 2009); MEF, mouse embryonic fibroblast cells (Supplementary Table 3.2); A375, human malignant melanoma cells (Robertson et al., 2015); HFF, human foreskin fibroblast cells (Ng et al., 2014); MKF1, mouse kidney fibroblast cells (Schiller et al., 2011); MKF2 and MKF3, mouse kidney fibroblast cells (Schiller et al., 2013).
To assess functional roles of consensus adhesome proteins, we performed functional enrichment analysis and found that pathways regulating focal adhesions, actin cytoskeleton and cell junction organisation were the most significantly enriched in the consensus IAC adhesome (Fig. 3.6, Supplementary Fig. 3.5, Supplementary Tables 3.8, 3.9). Protein domain enrichment analysis of the consensus adhesome revealed overrepresentation of the calponin-like and actinin-type actin-binding domains and, most significantly, the LIM-type zinc finger (Supplementary Tables 3.7, 3.8), which has been shown previously to be involved in force recognition at adhesion sites (Kuo et al., 2011; Schiller et al., 2011; Smith et al., 2014; Uemura et al., 2011). These data are in agreement with other studies that have reported a high number of adhesome molecules containing actin-binding and LIM domains (Geiger and Zaidel-Bar, 2012; Schiller and Fässler, 2013), highlighting the importance and prevalence of LIM and actin-binding domains in the organisation of IACs. These data confirmed the specificity of the consensus adhesome to cell-ECM interactions and links to the actin cytoskeleton.

To probe for links from the consensus integrin adhesome to inherited diseases, we searched the Online Mendelian Inheritance in Man database for genes encoding consensus adhesome proteins (Supplementary Table 3.7). Nine genes (15%) were found to cause disease, seven of which were also identified in a recent report (Winograd-Katz et al., 2014) (α-actinin-1, β3 integrin, filamin, LPP, palladin, plastin 3 and vinculin). The additional two proteins (α-actinin-4 and cyclophilin B) were related to glomerular disease and bone disorders, which have previously been linked to adhesome genes (Winograd-Katz et al., 2014). Furthermore, in support of links between the consensus adhesome and cardiovascular disease, a pathway associated with arrhythmogenic right ventricular cardiomyopathy was significantly overrepresented in the consensus adhesome (Supplementary Table 3.8).
Figure 3.6. Functional enrichment map of the consensus integrin adhesome. (a,b) Overrepresented biological process (a) and cellular component (b) terms from proteins identified in the consensus adhesome were hierarchically clustered according to proteomic dataset occurrence. This identified clusters of similarly detected proteins associated with a similar set of functional terms. Related terms are summarised (black bars). Proteins are labelled with gene names for clarity (see Supplementary Table 3.7 for details).
To validate further the consensus integrin adhesome, interactions between proteins were scored according to the level of supporting experimental evidence (Supplementary Table 3.10; see section 3.8.11). The resulting consensus adhesome interaction network contained many canonical IAC components, including all 31 identified literature-curated adhesome members (black borders, Fig. 3.7; Supplementary Table 3.7). The most highly connected proteins were literature-curated adhesome components (FAK, 15 interactions; β1 integrin, 13 interactions; paxillin, 12 interactions), which may reflect that these adhesion proteins have been studied more extensively than others (Rolland et al., 2014). In total, 11 proteins interacted directly with integrins, most of which bound to the β subunit. Proteins that directly linked integrins with actin were α-actinin, filamin, talin, tensin and, via a low-evidence α5β1 integrin interaction, FHL3. Other associated molecules may function to stabilise the connection of these integrin-actin linkers, such as PDLIM1 and PDLIM5 in facilitating the integrin-α-actinin-actin connection, or migfilin in bridging the connection between kindlin and actin via filamin. Many of the 92 connections in the network (57; 62%) were classified as low- or medium-evidence interactions and so require additional validation to confirm direct interactions. The high-evidence interactions in particular showed many potential pathways linking integrins to actin indirectly via multistep protein interactions, which can be broadly clustered into four theoretical modules (Fig. 3.7). The first module contained α-actinin and zyxin family members. The second module contained vinculin, talin and the vinculin-binding proteins vinexin and ponsin. Vinculin contained the highest number of high-evidence interactions (seven) and associated with many proteins in the third module, which centred on FAK and paxillin. The final module consisted of two submodules connected via a kindlin-ILK interaction.

Of the 29 proteins that were not literature-curated adhesome members (Fig. 3.5, Supplementary Table 3.7), six of these proteins interacted with other IAC components, such as Rsu-1 binding to PINCH and ILK, PDLIM1 and PDLIM5 binding to α-actinin, FHL3 binding to FHL2 and a transglutaminase-2-annexin A1 complex binding to β integrin subunits (grey borders, Fig. 3.7; Supplementary Table 3.10). Five underappreciated IAC proteins (caldesmon, calponin, IQGAP, PDLIM7 and plastin) were not connected to other IAC components but do bind to actin, suggesting that they were isolated as peripheral IAC components or that their connections to IAC proteins are uncharacterised. The remaining proteins in the consensus adhesome were unconnected to other proteins in the network, were not known to associate with actin, had unknown function or were involved in non-adhesion-related biological functions (Supplementary Table 3.7). The fact that these unexpected proteins were identified using the same methods used to detect many known adhesion-related proteins increases confidence in their involvement in the core adhesion machinery, but their contributions to adhesive functions remain to be elucidated and it remains possible that their association is non-specific.
Figure 3.7. Curated network model of the consensus integrin adhesome.
Protein-protein interaction network of the consensus adhesome. Interactions were manually validated and scored (high, medium, low) according to the level of experimental evidence for that interaction, shown by the thickness and saturation of the grey edges (see Supplementary Table 3.10). Thick black node border indicates literature-curated adhesome (Winograd-Katz et al., 2014) protein. Yellow node indicates actin-binding protein. The specific isoforms and subunits of proteins identified are detailed in Supplementary Table 3.7. For clarity, α-actinin is depicted as one node, even though two α-actinin isoforms (α-actinin-1 and -4) were identified. Actin is depicted for illustrative purposes but was not present in the consensus adhesome. The network comprised 41 proteins with 92 interactions, excluding actin binding. Unconnected components or components with only one low-evidence interaction are not shown in the network; proteins unconnected to the main network were ALYREF, BRIX1, DDX18, DDX27, DMT1, DNAJB1, FAU, FEN1, H1FX, HP1BP3, LIMD1, MRTO4, POLDIP3, RPL23A, SIPA1 andSYNCRI P; proteins connected to the network with a single low-evidence interaction were P4HB and PP1B.
To test whether underappreciated proteins identified in the consensus integrin adhesome localise to IACs, we visualised by immunofluorescence two consensus adhesome proteins that are not literature-curated adhesome members (Fig. 3.8). Caldesmon, an unconnected actin-binding protein (Janovick et al., 1991), localised to actin within vinculin-positive areas (Fig. 3.8a, Mander’s overlap coefficient (Manders et al., 1993) (MOC) = 0.51 ± 0.19, mean ± s.d.; n = 20 cells). Rsu-1, a protein connected to other adhesome components, which has been reported to associate with IACs in other cell types (Dougherty et al., 2008), colocalised with vinculin (Fig. 3.8b; MOC = 0.98 ± 0.03, mean ± s.d.; n = 20 cells). These data confirm IAC localisation of caldesmon and Rsu-1 and suggest that the consensus adhesome consists of IAC components that participate in regulation of the integrin-actin structural connection.
Figure 3.8. Caldesmon and Rsu-1 localisation in IACs.

(a, b) U2OS cells were spread on FN for 2 h and visualised using antibodies against caldesmon (green) (a) and Rsu-1 (green) (b). IACs were visualised by immunofluorescence staining for vinculin (red) and the actin cytoskeleton was visualised by staining with fluorophore-conjugated phalloidin (blue). Graphs show fluorescence intensity values for each channel across line segments in corresponding zoomed areas above each graph. In addition, colocalisation with vinculin-positive areas was quantified for caldesmon (MOC = 0.51 ± 0.19) and Rsu-1 (MOC = 0.98 ± 0.03). Values are mean ± s.d. (n = 20 cells, see Supplementary Table 3.11 for source data). Scale bars, 20 µm.
3.4 Discussion

Here, we performed extensive analyses of IAC proteomes, resulting in an experimentally defined meta-adhesome of 2,412 proteins. An emergent property of the meta-adhesome was the identification of a consensus adhesome comprising proteins robustly detected in IAC proteomes that likely reflect the core integrin-based adhesion machinery. The key findings from these analyses are:

1. The meta-adhesome provides a systems-wide view of IAC composition and highlights a steep reduction in the number of proteins conserved across multiple datasets, which led to the identification of a commonly identified consensus adhesome;
2. Both expected literature-curated adhesome components and underappreciated adhesion molecules are highly represented in IAC proteomes. To confirm this we validated the localisation of Rsu-1 and caldesmon to IACs;
3. A protein-protein interaction network of the consensus adhesome highlighted four major axes that form the integrin-actin structural connection; and
4. The meta-adhesome and consensus adhesome are valuable resources that can be used as reference datasets to interrogate other MS-derived IAC proteomes.

The proteomic datasets used here provide a global description of IACs in the context of FN-mediated adhesion. Analysis of the meta-adhesome overcomes the heterogeneity encountered when studying individual datasets from different laboratories and different cell types. These analyses enabled the analysis of global adhesion network properties and identified a diverse range of cellular functions associated with IACs. The heterogeneity between IAC proteomes collected from cells exposed to similar microenvironments and the increase in the number of proteins identified in the meta-adhesome compared with the literature-curated adhesome (Winograd-Katz et al., 2014) highlights an unanticipated complexity in IAC composition. One outcome of these analyses is that evidence for the localisation to IACs of 118 adhesome proteins (51%; 64 intrinsic, 54 associated) that were not detected in the meta-adhesome may be highly context-dependent or may need re-examining. Furthermore, an outstanding question is how the consensus adhesome differs for other ECM ligands such as laminin and collagen, or integrin heterodimers or cell types. That core adhesome composition differences may exist is supported by the example of ADP-ribosylation factor 1 (ARF1), which was not detected in FN-induced IAC datasets used here but was identified in VCAM-1-induced IACs (Byron et al., 2012; Humphries et al., 2009). Increasing the numbers of proteomic datasets of IACs induced by alternative ECM ligands and cell types to those previously investigated would help clarify this view.

To identify core adhesion machinery involved in adhesive function, we defined a consensus adhesome of 60 proteins robustly identified in FN-induced IAC proteomes that incorporated negative controls, which enabled us to threshold the identification of canonical and candidate IAC proteins objectively. A high proportion of actin-binding proteins were identified in the consensus adhesome, which may represent a specific subset of actin-binding proteins that localise in IACs at the ends of actin fibres, which was shown to be the case for caldesmon. Importantly, not all cellular actin-binding proteins were identified in IAC proteomes. Of the 395 actin-binding proteins...
annotated in the gene ontology. 146 (37%) and 17 (4%) were assembled in the meta-adhesome and consensus adhesome (Supplementary Table 3.12), respectively, indicating that the IAC isolation strategies allow the separation and characterisation of a functionally distinct pool of actin and its binding partners. Most (46; 90%) candidate IAC proteins common to three proteomic datasets highlighted in a recent analysis (Geiger and Zaidel-Bar, 2012) were not present in the consensus adhesome, but specific protein isoforms of known IAC proteins and additional non-canonical, unconnected consensus adhesome components were identified (specific protein isoforms, some of which are due to cell type-specific expression (Beggs et al., 1992; Bialkowski et al., 2010; Karaköse et al., 2010), were tensin-3, kindlin-2, filamin C, β-Pix, GIT2, α-actinin-1 and -4, Supplementary Table 3.7; proteins common to both studies were α-actinin-4, annexin A1, calponin 2, IQGAP1 and PDLIM5). With the exception of signal-induced proliferation-associated 1 (SIPA1) (Zhang et al., 2015) and LIM domains containing 1 (LIMD1) (Bai et al., 2011), we did not find evidence supporting the involvement of the unconnected consensus adhesome proteins to IACs or adhesive function (Fig. 3.7, legend; Supplementary Table 3.7). Some of the non-canonical consensus adhesome proteins have functional roles related to RNA processing and translation (Supplementary Table 3.7) and therefore may be involved in localised protein synthesis, which is supported by localisation of translation machinery and β-actin mRNA around IACs (Buxbaum et al., 2014; Chicurel et al., 1998; Katz et al., 2012; Willett et al., 2010, 2011). As is often the case for MS-based identification of protein complexes, some of the proteins identified may be co-purifying contaminants from the IAC isolation process, which is supported by their identification in the contaminant repository for affinity purification–MS data (CRAPome) (Mellacheruvu et al., 2013). However, the use of the CRAPome for comparative analyses of IAC proteomes is not recommended for these datasets as many canonical IAC proteins and cytoskeletal components, such as talin and β1 integrin, are also commonly identified in the CRAPome. Further investigation into the roles of specific protein isoforms and underappreciated IAC proteins in the regulation of cell-ECM adhesion is required. Conversely, some well-characterised IAC components were not enriched in all seven datasets (e.g. β3 integrin, FAK, kindlin, paxillin and talin; Fig. 3.5) or were observed in the meta-adhesive but not the consensus adhesome (e.g. p130Cas and Src family kinases). These omissions may be due to cell type-specific expression, cell type-specific IAC maturation, protein abundance at IACs, preferential use of the β1 integrin subunit or non-specific detection in negative controls. Additional studies to those that have examined the phosphoproteome (Robertson et al., 2015) and stoichiometry (Bachir et al., 2014) of IACs will provide deeper coverage of IAC composition and provide further insights into their relative functions in adhesion signalling.

The consensus adhesome contained many proteins that are evolutionarily conserved across multiple species and whose genetic depletion causes dramatic defects in integrin-mediated adhesion (Bulgakova et al., 2012; Etheridge et al., 2015; Meller et al., 2015; Sebé-Pedrós et al., 2010; Wickström et al., 2010), indicating that consensus adhesome proteins form an essential contribution to integrin function. Analysis of interactions between consensus adhesome molecules resulted in the identification of four interconnected axes that link integrins to the actin cytoskeleton. The use of proteomics is complementary to super-resolution microscopy approaches used to study IACs (Humphries et al., 2015; Worth and Parsons, 2010). Indeed, the four axes that form the
integrin-actin structural connection defined in this study support the vertical z-plane model of adhesion components (Case et al., 2015; Kanchanawong et al., 2010) where talin spans the adhesion site, FAK and paxillin are in a signalling layer proximal to integrins and α-actinin, zyxin and VASP are localised distal to integrins near actin (Byron, 2011; Kanchanawong et al., 2010). The association of α-actinin with β1 integrin (Kelly and Taylor, 2005; Otey et al., 1990) occurs in early nascent adhesions and is lost as adhesions mature (Bachir et al., 2014; Choi et al., 2008). Applied cytoskeletal force could induce α-actinin-integrin dissociation, allowing distal localisation of α-actinin as well as potentially associated actin-binding and LIM domain proteins from the membrane. Interactions with other consensus adhesome proteins may maintain the continued localisation of α-actinin and associated molecules in IACs. Important next steps will be to determine the dynamics (Rossier et al., 2012) and nanoscale localisation (Kanchanawong et al., 2010) of other consensus adhesome proteins using super-resolution microscopy and to examine the effects of force on the consensus adhesome (Schiller and Fässler, 2013).

We propose that the meta-adhesome and the consensus adhesome generated here can be used by the wider research community for removal of non-specific components from future analyses of IAC composition by MS, thus contextualising and streamlining identification of candidate adhesion molecules for follow-up studies. For example, of the 754 reproducible FN-induced IAC components identified in a published study from human fibroblasts that did not incorporate a negative control ligand condition (Kuo et al., 2011), we found 451 meta-adhesome proteins and 42 consensus adhesome proteins, which is of a similar scale to the controlled IAC proteomes analysed here (Figs. 3.1, 3.5). In summary, the data presented in this study provide a systems-wide analysis of FN-induced IAC composition and detail a comprehensive reductionist view of an experimentally defined integrin adhesome.
3.5 Acknowledgements

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3.6 Author contributions

A.B. and M.J.H. conceived the project; E.R.H., A.B., D.H.J.N., A.M.-F., J.D.H. and M.J.H. designed the experiments and interpreted the results; E.R.H., A.B., D.H.J.N., A.M.-F., J.R., E.J.K., N.R.P. and J.D.H. performed the experiments and analysed the data; S.W. and D.K. contributed to MS experimental design and data acquisition; E.R.H., A.B., J.D.H. and M.J.H. wrote the paper; all authors commented on the manuscript and approved the final version.

Further details of co-author contributions:

E.R.H. and A.B. collectively performed comparative analyses of IAC proteomes (Fig. 3.1);

E.R.H. performed analyses of the meta-adhesome (Figs. 3.2, 3.3), performed functional analyses of the meta-adhesome (Fig. 3.4; Supplementary Figs. 3.2, 3.3; Supplementary Tables 3.5, 3.6), assembled and analysed the consensus adhesome (Figs. 3.5, 3.7; Supplementary Fig. 3.5; Supplementary Tables 3.7-3.10), performed immunofluorescence experiments (Fig. 3.8) and performed analysis of actin-binding proteins (Supplementary Table 3.12);

A.B. processed MS datasets and assembled the meta-adhesome (Supplementary Tables 3.1-3.3), performed topological analyses of the meta-adhesome (Supplementary Fig. 3.1, Supplementary Table 3.4) and generated functional enrichment maps (Fig. 3.6, Supplementary Fig. 3.4);

D.H.J.N. generated the HFF dataset;
A.M-F. generated the MEF dataset (Supplementary Table 3.2);
J.R. generated the A375 dataset;
E.J.K. characterised the antibodies used against Rsu-1 and caldesmon;
N.R.P. calculated Mander’s overlap coefficients (Supplementary Table 3.11); and
J.D.H. deposited MS data in ProteomeXchange.

3.7 Competing financial interests

The authors declare no competing financial interests.
3.8 Methods

3.8.1 Reagents

FN, PDL and transferrin were from Sigma-Aldrich. Antibodies used for immunofluorescence were mouse anti-vinculin (hVIN-1, Sigma-Aldrich, V9131; 1:400), rabbit anti-caldesmon-1 (D5C8D, Cell Signaling Technology, 12503; 1:400) and rabbit anti-Rsu-1 (provided by M. L. Cutler; 1:500). Secondary antibodies were from Jackson Immunoresearch and Alexa Fluor 647-conjugated phalloidin was from Invitrogen.

3.8.2 Cell culture

Telomerase-immortalised HFF (provided by K. Clark), conditionally immortalised MEF, A375-SM (provided by I. J. Fidler) and osteosarcoma (U2OS) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS; Lonza Bioscience) and 2 mM L-glutamine. MEF cells were supplemented with interferon-γ (Sigma-Aldrich) and maintained at 37 °C in a humidified 5% (v/v) CO₂ atmosphere, except for MEF cells, which were maintained at 33 °C.

3.8.3 IAC isolation

IACs were isolated using a similar approach to the ligand affinity purification method described previously (Humphries et al., 2009; Jones et al., 2015). For isolation of IACs from MEF cells, cells were resuspended in DMEM supplemented with 25 mM HEPES (Sigma-Aldrich) and incubated in suspension for 20 min at 37 °C to down-regulate ECM adhesion signalling events. Cells were spread on tissue culture dishes coated with 10 μg/ml FN or transferrin for 120 min at 37 °C, 8% (v/v) CO₂. Cells were incubated with the membrane permeable cross-linker dimethyl-3, 3'-dithiobispropionimidate (DTBP; Sigma Aldrich; 3 mM, 30 min), washed twice with PBS, and DTBP was quenched using 1 M Tris-HCl (pH 8, 10 min), after which cells were washed twice with PBS and incubated at 4 °C. Cell bodies were removed by a combination of cell lysis in ice-cold extraction buffer [20 mM NH₄OH, 0.5% (w/v) Triton X-100] and sonication for 1 min (VibraCell VCX 500; Sonics & Materials). Protein complexes left bound to the substrate were washed five times with PBS, recovered by scraping in 100 μl recovery solution [125 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 15% (v/v) β-mercaptoethanol] and incubated at 70 °C for 10 min. A total of 1,461 proteins were identified (≥99% confidence) in IACs from MEF cells by MS, of which 674 proteins were at least two-fold enriched to FN-induced IACs over the negative control, transferrin (Tf) (Supplementary Table 3.2).

3.8.4 Immunoblotting

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Whatman). Membranes were blocked and probed as described previously (Humphries et al., 2009). Secondary antibodies used were donkey Alexa Fluor 680-conjugated anti-goat IgG or anti-mouse IgG (Life Technologies) and donkey IRDye 800-conjugated anti-mouse IgG (Rockland Immunocchemicals). Membranes were washed in the dark and scanned using the Odyssey infrared imaging system (LI-COR).
3.8.5 MS data acquisition

Following SDS-PAGE, gel lanes were sliced and subjected to in-gel digestion with trypsin (Shevchenko et al., 1996) with modifications (Humphries et al., 2009). Peptide samples were analysed by liquid chromatography (LC)-tandem MS using a nanoACQUITY UltraPerformance LC system (Waters) coupled online to an LTQ Velos mass spectrometer (Thermo Fisher Scientific). Peptides were concentrated and desalted on a Symmetry C18 preparative column (20 mm × 180 μm, 5-μm particle size; Waters) and separated on a bridged ethyl hybrid C18 analytical column (250 mm × 75 μm, 1.7-μm particle size; Waters) using a 45 min linear gradient from 1% to 25% (v/v) acetonitrile in 0.1% (v/v) formic acid at a flow rate of 200 nl/min. Peptides were selected for fragmentation automatically by data-dependent analysis.

3.8.6 MS data analysis

MS data were searched using an in-house Mascot server (version 2.2.03; Matrix Science) (Perkins et al., 1999) as described previously (Byron et al., 2015). Mass tolerances for precursor and fragment ions were 0.4 Da and 0.5 Da, respectively. Data were validated in Scaffold (version 3.00.06; Proteome Software) using a threshold of identification of at least 90% probability at the peptide level, assignment of at least two unique, validated peptides, and at least 99% probability at the protein level. These acceptance criteria resulted in an estimated protein false discovery rate of ≤0.1% for all datasets. MS data were quantified as described previously (Byron et al., 2015).

3.8.7 MS data deposition

MS data were deposited in ProteomeXchange (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) with the dataset identifier PXD000018 (DOI: 10.6019/PXD000018; MEF dataset). Details of all identified proteins are provided in Supplementary Table 3.2 for the MEF dataset.

3.8.8 Meta-adhesome construction

IAC datasets (Supplementary Table 3.1) were filtered to include only proteins from cells spread on FN and the corresponding negative control in the absence of perturbation. Proteins enriched in FN-induced IACs compared to the negative control, with relative abundance satisfying log2(FN/control) ≥ 1, were assembled into the meta-adhesome database (Supplementary Table 3.3). Two datasets were included from Schiller et al., 2013 for cells expressing αV and β1 integrins (α5β1, αVβ3 and αVβ5 integrins; cells spread for 45 min (MKF2) and 90 min (MKF3)), excluding proteins uniquely identified in cells expressing only either β1- or αV-class integrins as integrin heterodimer-specific adhesomes were not presented in this version of the meta-adhesome.

3.8.9 Consensus adhesome construction

Proteins enriched in at least five proteomic datasets in the meta-adhesome database were incorporated into the consensus adhesome. ECM or secreted proteins (COL1A1, COL1A2, COL5A2, COL6A1, COL6A2, FGG, FN1, PCOLCE, PRSS23, SERPINE1) were excluded since, although relevant to adhesion biology, we sought to focus on intracellular components of IACs. All isoforms of literature-curated adhesome (Winograd-Katz et al., 2014) members were classified as
adhesive molecules. Functional information was adapted from the HUGO Gene Nomenclature Committee (HGNC) database (Gray et al., 2015), protein domain information was assigned from InterPro (Mitchell et al., 2015) and disease annotations were extracted from the Online Mendelian Inheritance in Man database (www.omim.org).

3.8.10 Hierarchical clustering and principal component analysis

Proteins or datasets were hierarchically clustered on the basis of uncentred Pearson correlation using Cluster 3.0 (C Clustering Library, version 1.50) (de Hoon et al., 2004) and visualised using Java TreeView (version 1.1.5) (Saldanha, 2004). Individual IAC proteomes were clustered on the basis of Jaccard distance and visualised using R (version 3.1.0) (Fig. 3.1). Distances between hits were computed using a complete-linkage matrix in all cases. Additional heatmaps were visualised using MultiExperiment Viewer (version 4.8.1) (Saeed et al., 2003). Principal component analysis was performed using MATLAB (version R2012a; MathWorks).

3.8.11 Interaction network analyses

Interaction network analysis was performed using Cytoscape (version 3.0.2) (Cline et al., 2007). Enriched proteins were mapped onto a merged human interactome consisting of physical protein-protein interactions as described previously (Byron et al., 2015). Graph clustering was performed using the yFiles Organic algorithm implemented in Cytoscape. Topological parameters were computed from undirected graphs, excluding self-interactions, using NetworkAnalyzer (Assenov et al., 2008). Topological parameters assessed were node degree, which represents the number of reported protein-protein interactions for each protein and gives a measure of how connected a protein is in the network, and betweenness centrality, which is the number of shortest paths between pairwise proteins that pass through a particular protein and gives a measure of how central or key a protein is in the network (Assenov et al., 2008).

For the consensus adhesome, evidence for protein-protein interactions was manually verified and scored. Low-evidence interactions included those based on a single publication or on coprecipitation or yeast two-hybrid studies. Medium-evidence interactions were based on data from multiple sources, or a single source if there was phosphorylation or peptide binding data. High-evidence interactions were based on structural evidence of direct binding between two proteins, such as X-ray crystallography or nuclear magnetic resonance, or confirmation using a wide variety of techniques. Experimental evidence and source publications are detailed in Supplementary Table 3.10.

3.8.12 Functional enrichment analyses

Functional enrichment analysis was performed using DAVID (version 6.7) (Huang et al., 2009). Keywords with fold enrichment ≥ 1.5, Bonferroni-corrected P value < 0.05, EASE score (modified Fisher’s exact test) < 0.05 and at least two proteins per keyword were considered significantly overrepresented. All overrepresented gene ontology, KEGG pathway and InterPro domain terms are detailed in Supplementary Tables 3.5 and 3.8 for the meta-adhesome and consensus adhesomes, respectively.
For generation of functional enrichment networks, overrepresentation of gene ontology terms was calculated using the Cytoscape plugin BiNGO (version 2.44) (Maere et al., 2005) and gene ontology networks were visualised using Enrichment Map (version 1.2) (Merico et al., 2010) with significance settings $P < 0.001$, false discovery rate < 5% and Jaccard coefficient of 0.25. All overrepresented terms are detailed in Supplementary Tables 3.6 and 3.9 for the meta-adhesome and consensus adhesome, respectively.

For generation of functional enrichment maps, overrepresentation of gene ontology terms was calculated using High-Throughput GoMiner (Zeeberg et al., 2005). One thousand randomisations were performed and data were thresholded for a 5% false discovery rate. Overrepresented terms with $\geq 5$ and $\leq 500$ assigned proteins were reported. Dataset occurrence was mapped onto proteins assigned to each overrepresented term, and the data matrix was subjected to hierarchical clustering analysis as described above.

To calculate the proportion of actin-binding proteins identified in the meta-adhesome and consensus adhesome, all proteins annotated with actin-binding-related terms (actin binding, actin filament binding, actin monomer binding) were extracted from the gene ontology database (version 2.2.0, www.amigo.geneontology.org). All actin-binding proteins are detailed in Supplementary Table 3.12.

### 3.8.13 Immunofluorescence microscopy

To confirm localisation of Rsu-1 and caldesmon at IACs, U2OS cells were spread on FN-coated dishes (MatTek) for 2 h at 37 °C, 8% (v/v) CO₂. Cells were washed with PBS, fixed with $-20 \, ^\circ\text{C}$ methanol or 4% (w/v) paraformaldehyde for 7 min at room temperature (RT), permeabilised with 0.5% (v/v) Triton X-100 for 10 min, and then washed and incubated with antibodies as previously described (Byron et al., 2015). Images were acquired on a Delta Vision RT (Applied Precision) restoration microscope using a 60×/1.42 Plan Apo objective and the Sedat filter set (Chroma 89000). Images were collected with a z optical spacing of 0.2 μm, five images per stack, using a Coolsnap HQ camera (Photometrics) and Softworx software (Applied Precision). To assess colocalisation of Rsu-1 and caldesmon with vinculin, cells were also imaged using a spinning-disk confocal inverted microscope (Marianas, 3i) (images not shown). Images were collected with a z optical spacing of 0.2 μm, three images per stack, using a 63×/1.4 Plan Apochromat objective and SlideBook 6.0 software.

### 3.8.14 Image analysis and quantification

Maximum intensity projections of raw images were generated and background filtered (rolling ball, 10-pixel radius) using ImageJ (version 1.48o) (Schindelin et al., 2012). To quantify Rsu-1 and caldesmon colocalisation with vinculin, images were individually band-pass filtered (A trous wavelet, linear 3 × 3 filter, keeping scales 2–8) using custom software written in Python and NumPy to create a mask of vinculin-positive adhesion structures. Colocalisation analysis was performed using the ImageJ plugins Coloc 2, with the mask as a region of interest to calculate MOC (Manders et al., 1993), and Plot_Multicolor (version 4.3) to plot line profiles. Figures were assembled using Illustrator (Adobe).
3.9 Supplementary Figures and Tables

Supplementary Table 3.1. Details of proteomic datasets.

Supplementary Table 3.2. Proteins identified in purified mouse embryonic fibroblast adhesion complexes by mass spectrometry.

Supplementary Table 3.3. The meta-adhesome.

Supplementary Table 3.4. Topological analysis of the meta-adhesome interaction network.

Supplementary Table 3.5. Functional enrichment analysis of the meta-adhesome.

Supplementary Table 3.6. Functional enrichment networks of the meta-adhesome.

Supplementary Table 3.7. The consensus adhesome.

Supplementary Table 3.8. Functional enrichment analysis of the consensus adhesome.

Supplementary Table 3.9. Functional enrichment networks of the consensus adhesome.

Supplementary Table 3.10. Reported interactions between consensus adhesome proteins.

Supplementary Table 3.11. Source data for quantification of adhesion protein colocalisation.

Supplementary Table 3.12. Actin-binding proteins.
Supplementary Figure 3.1. Topological analysis of the meta-adhesome interaction network. (a) Clustered protein-protein interaction network model of the meta-adhesome. The largest connected graph component is displayed, comprising 11,430 interactions (black lines; edges) between 2,035 proteins (circles; nodes). Node size is proportional to degree and node colour is proportional to betweenness centrality. Black node borders indicate literature-curated adhesome (Winograd-Katz et al., 2014) components, which are labelled with gene names. (b) The number of reported protein-protein interactions (degree) for each protein is plotted according to the number of proteomic datasets in which it was identified. (c) Betweenness centrality (a measure of the control a node exerts over the interactions of other nodes in the network) for each protein is plotted according to the number of datasets in which it was identified. Box-and-whisker plots show the median (line), mean (plus sign), 25th and 75th percentiles (box) and 5th and 95th percentiles (whiskers) (n = 1,117, 518, 238, 102, 33, 25 and 10 mapped proteins identified in 1–7 datasets, respectively, with degree ≥ 1). *P < 0.05, **P < 0.01, ***P < 0.001; Kruskal–Wallis test with Dunn’s post hoc correction (see Supplementary Table 3.4 for source data).
Proteins identified in the meta-adhesome were performed using DAVID (Huang et al., 2009) discovery rate ($Q$). The enrichment map (Merico et al., 2010) with the following cut-off values: $P$-value < 0.01, false discovery rate ($Q$) = 5%. Jaccard coefficient = 0.25. Additional functional enrichment analyses of proteins identified in the meta-adhesome were performed using DAVID (Huang et al., 2009) (Supplementary Table 3.5).

Supplementary Figure 3.2. Cellular component enrichment network of the meta-adhesome.

In total, the meta-adhesome displayed significant overrepresentation of 177 cellular component terms, which are displayed as a network. Each node represents a group of proteins enriched to a particular cellular component term. Node size is proportional to the number of meta-adhesome proteins enriched to that functional category and node colour represents the level of statistical significance. Edges between nodes indicate shared proteins between two protein groups and edge thickness is proportional to the number of shared proteins between connected nodes. For clarity, clusters of functionally related terms were manually circled and assigned a cluster name and single gene annotations are not shown. The most significantly overrepresented terms, indicated by red node colour, are labelled. All significantly overrepresented terms are listed in Supplementary Table 3.6. The network was generated using the Cytoscape plugins BiNGO (Maere et al., 2005) and Enrichment Map (Merico et al., 2010) with the following cut-off values: $P$-value = 0.001, false discovery rate ($Q$) = 5%, Jaccard coefficient = 0.25. Additional functional enrichment analyses of proteins identified in the meta-adhesome were performed using DAVID (Huang et al., 2009) (Supplementary Table 3.5).
Supplementary Figure 3.3. Molecular function enrichment network of the meta-adhesome.

In total, the meta-adhesome displayed significant overrepresentation of 86 molecular function terms, which are displayed as a network. Each node represents a group of proteins enriched to a particular molecular function term. Node size is proportional to the number of meta-adhesome proteins enriched to that functional category and node colour represents the level of statistical significance. Edges between nodes indicate shared proteins between two protein groups and edge thickness is proportional to the number of shared proteins between connected nodes. For clarity, clusters of functionally related terms were manually circled and assigned a cluster name. The most significantly overrepresented terms, indicated by red node colour, are labelled. All significantly overrepresented terms are listed in Supplementary Table 3.6. The network was generated using the Cytoscape plugins BiNGO (Maere et al., 2005) and Enrichment Map (Merico et al., 2010) with the following cut-off values: \( P \)-value = 0.001, false discovery rate (Q) = 5%, Jaccard coefficient = 0.25. Additional functional enrichment analyses of proteins identified in the meta-adhesome were performed using DAVID (Huang et al., 2009) (Supplementary Table 3.5).
Supplementary Figure 3.4. Functional enrichment map of the meta-adhesome.
(a) Overrepresented biological process terms from proteins identified in the meta-adhesome were hierarchically clustered according to proteomic dataset occurrence. This identified clusters of similarly detected proteins associated with a similar set of functional terms. (b) The two clusters containing proteins detected in the most datasets (grey boxes in a; 1, 2) are shown in detail. Proteins are labelled with gene names for clarity (see Supplementary Table 3.3 for details).
Supplementary Figure 3.5. Functional enrichment networks of the consensus integrin adhesome.

(a-c) In total, proteins identified in the consensus adhesome displayed significant overrepresentation of 41 biological process (a), 82 cellular component (b) and 22 molecular function (c) terms, which are displayed as networks. Each node represents a group of proteins enriched to a particular term. Node size is proportional to the number of consensus adhesome proteins enriched to that functional category and node colour represents the level of statistical significance. Edges between nodes indicate shared proteins between two protein groups and edge thickness is proportional to the number of shared proteins between connected nodes. The most significantly overrepresented terms, indicated by red node colour, are labelled. All significantly overrepresented terms are listed in Supplementary Table 3.9. Networks were generated using the Cytoscape plugins BiNGO (Maere et al., 2005) and Enrichment Map (Merico et al., 2010) with the following cut-off values: P-value = 0.001, false discovery rate (Q) = 5%, Jaccard coefficient = 0.25. Additional functional enrichment analysis of proteins identified in the consensus adhesome was performed using DAVID (Huang et al., 2009) (Supplementary Table 3.8).
3.10 References


required to maintain muscle structure, mitochondrial ATP production, and movement forces in Caenorhabditis elegans. FASEB J. 29, 1235–1246.


Chapter 4

Inhibition of FAK and Src activity affects adhesion signalling but not composition of integrin adhesion complexes

Edward R. Horton¹, Jonathan D. Humphries¹, Guillaume Jacquemet¹,³, Simon T. Barry² and Martin J. Humphries¹,⁴

¹Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
²Oncology iMed, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire, UK
³Present address: Turku Centre for Biotechnology, University of Turku, 20520 Turku, Finland
⁴Correspondence should be addressed to M.J.H.:

Professor Martin J. Humphries, Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
Tel.: +44 (0) 161 2755071; Fax: +44 (0) 161 2755082
E-mail: martin.humphries@manchester.ac.uk

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4.1 Abstract

Integrin adhesion complexes (IACs) form a mechanochemical connection between integrin receptors and the actin cytoskeleton. IACs are also complex signalling hubs that mediate an array of phenotypic responses via post-translational modifications. Here, we investigate the robustness of the IAC network to pharmacological perturbation of the key IAC signalling components focal adhesion kinase (FAK) and Src. Inhibition of FAK using AZ13256675 reduced FAK Y397 phosphorylation but did not alter IAC composition, as reported by mass spectrometry. IAC composition was also insensitive to inhibition of Src using AZD0530 and to simultaneous treatment with both compounds. In contrast, phosphorylation of IAC proteins, cell migration and proliferation were substantially reduced by kinase inhibition. These results demonstrate that FAK and Src kinase activity regulate adhesion signalling, but not IAC composition. We conclude that the IAC is a stable structural connection, linking integrins to actin, which is nonetheless able to relay signals to functional end-points via phosphorylation.
4.2 Introduction

Cell adhesion to the extracellular matrix (ECM) is mediated by specific families of cell surface receptors such as proteoglycans and integrins (Juliano, 2002; Morgan et al., 2007). The integrin family comprises 18 α-subunits and 8 β-subunits that form 24 distinct αβ heterodimer pairs in mammals (Hynes, 2002). Upon integrin engagement with ECM ligands and integrin clustering, proteins are recruited to form multimolecular complexes (IACs) that facilitate the linkage between integrins and the actin cytoskeleton (Brakebusch and Fässler, 2003). In turn, IACs permit bidirectional signalling and transmission of mechanical force across the plasma membrane (Evans and Calderwood, 2007; Oakes et al., 2012; Hu and Luo, 2013). Over 200 components, from a diverse range of functional categories, localise to IACs as reported in the literature-curated integrin adhesome (Winograd-Katz et al., 2014; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007). Adaptors and actin regulators act as scaffolding molecules to stabilise the connection between integrins and actin, while a large number of enzymes and enzyme regulators, such as kinases, phosphatases, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and GTPases contribute to adhesion signalling events. These events influence a wide range of downstream biological functions in health and contribute to diseases such as developmental and cardiovascular disorders, inflammation and cancer (Eke and Cordes, 2015; Maartens and Brown, 2015; Mitra and Schlaepfer, 2006; Wahl et al., 1996; Winograd-Katz et al., 2014).

Phosphorylation is a post-translational modification that has been widely implicated in the regulation of adhesion signalling and dynamics (Zaidel-Bar and Geiger, 2010). Imaging cells with generic anti-phosphotyrosine antibodies or fluorescent proteins tagged to the Src Homology 2 (SH2) domain of Src has demonstrated an enrichment of tyrosine phosphorylation events at IACs (Ballestrem et al., 2006; Kirchner et al., 2003; Panetti, 2002), and numerous phosphorylation sites within IAC proteins have been identified by phosphoproteomics (Chen et al., 2009; Robertson et al., 2015; Schiller et al., 2013). Focal adhesion kinase (FAK), an extensively tyrosine-phosphorylated protein, is one of the earliest signalling molecules recruited to IACs (Kirchner et al., 2003), and one of the most connected proteins in the literature-curated adhesome (Zaidel-Bar et al., 2007). FAK is a core member of the integrin-associated adhesome, as defined by proteomic analysis of isolated IACs (Horton et al., submitted), and has a C-terminal focal adhesion targeting (FAT) domain, a central catalytic domain and an N-terminal 4.1, ezrin, radixin and moesin (FERM) domain (Mitra et al., 2005). Following cell-ECM engagement, FAK is autophosphorylated at FAKY397, which exposes an SH2 domain-binding site for Src (Schaller et al., 1994). Src recruitment results in additional Src-dependent phosphorylation of FAK at FAKY567 and FAKY577 leading to maximal adhesion-induced FAK activation (Calalb et al., 1995). The FAK-Src signalling complex, which is a potential therapeutic target in cancer (Kim et al., 2009; Sulzmaier et al., 2014), binds to and phosphorylates other IAC molecules such as pakxillin and p130Cas (Mitra and Schlaepfer, 2006; Schaller and Parsons, 1995).

To provide global insights into IAC biology, recent studies have emphasised the use of mass spectrometry (MS)-based proteomics and small interfering RNA (siRNA) screens. Biochemical isolation of IACs and subsequent analysis of molecular composition using proteomics has revealed an unanticipated complexity and heterogeneity in IAC composition in different contexts (Byron et
al., 2011, 2012, 2015; Geiger and Zaidel-Bar, 2012; Horton et al., submitted; Huang et al., 2014; Humphries et al., 2009, 2015; Jones et al., 2015; Kuo et al., 2011, 2012; Ng et al., 2014; Robertson et al., 2015; Schiller and Fässler, 2013; Schiller et al., 2011, 2013; Yue et al., 2014). Such methods have been used to examine effects of ECM ligands (Humphries et al., 2009), integrin activation states (Byron et al., 2015) and integrin heterodimer specificities (Byron et al., 2012; Schiller et al., 2013) on IAC protein composition. Perturbation approaches have been performed to examine effects of GEF-H1 on IAC composition using GEF-H1-silencing cells (Huang et al., 2014), and to determine the effects of drug perturbation on IAC composition using the microtubule polymerisation inhibitor nocodazole (Ng et al., 2014; Yue et al., 2014) and the myosin-II inhibitor blebbistatin (Kuo et al., 2011; Schiller et al., 2011, 2013). Effects of genetic ablation using siRNAs targeting mainly kinases and phosphatases have uncovered novel regulators of IAC formation (Winograd-Katz et al., 2009), cell migration (Chen et al., 2009; Simpson et al., 2008), cell morphology (Kiger et al., 2003) and β1 integrin activation (Rantala et al., 2011). An in vivo screen ablating homologues of adhesome components in nematodes revealed the importance of IACs in maintenance of muscle structure (Etheridge et al., 2015). Analysis of IAC composition upon perturbation of individual components may therefore provide insights into how the structure and topology of IAC composition is organised and controlled at the molecular level.

Here, we sought to examine the sensitivity of IACs to kinase inhibition since IACs are enriched for phosphotyrosine and kinases form adhesion signalling hubs (Zaidel-Bar et al., 2007). We targeted the signalling proteins FAK and Src, as knockout of these protein kinases in mice are embryonic lethal and cause osteoporosis, respectively, (Ilić et al., 1995; Soriano et al., 1991), and they are two of the most connected adhesome components (Zaidel-Bar et al., 2007). Rather than reducing protein expression levels to inhibit the combined contributions of scaffolding and signalling functional roles, we chose to specifically target kinase catalytic activity. Using pharmacological inhibitors and a combination of targeted and global approaches, we found that IAC protein composition is largely unaffected by inhibition of FAK and/or Src, highlighting the robustness of the IAC network. In contrast, cell migration, proliferation, tyrosine phosphorylation of IAC proteins and thus adhesion signalling, were reduced upon FAK and/or Src inhibition. These data demonstrate that kinase activity at key IAC hubs regulates adhesion signalling and information flux through IACs but is not required to maintain IAC structure or composition.
4.3 Results

4.3.1 Inhibition of FAK kinase activity using the small molecule inhibitor AZ13256675

To determine a concentration of FAK inhibitor required to inhibit FAK catalytic activity effectively, human foreskin fibroblast (HFF) cells were plated on fibronectin (FN)-coated dishes and treated with DMSO or half-log dilutions of the small molecule inhibitor AZ13256675 (FAK [i]) for 1 h. FAK activity was quantified by immunoblotting total cell lysates for the FAK autophosphorylation residue FAKY397 and, as a control, the Src-substrate site FAKY576 (Fig. 4.1a). FAKY397 and FAKY576 were normalised against total FAK in each condition (Fig. 4.1b,c). Phosphorylation of both FAKY397 and FAKY576 increased in an adhesion-dependent manner compared to cells kept in suspension (Fig. 4.1a-d). There was a dose-dependent reduction in FAKY397 and FAKY576 in FAK [i]-treated cells, with maximal inhibition obtained at 3 µM FAK [i]. The FAK autophosphorylation site FAKY397 was inhibited by 87% in cells treated with 3 µM FAK [i] (Fig. 4.1b,d), which was equivalent to FAKY397 levels from cells kept in suspension and is a similar extent of FAK inhibition reported by others (Tanjoni et al., 2010). In contrast, treating cells with 3 µM FAK [i] resulted in only 37% inhibition of the Src-substrate site FAKY576 compared to cells treated with DMSO (Fig. 4.1c), indicating the specificity of FAK [i] for FAKY397. A 50% inhibitory concentration (IC50, 0.11 µM FAK [i]) was calculated using FAKY397 values normalised to values from cells treated with DMSO (Fig. 4.1b,d). These data suggest that FAK is efficiently inhibited in cells treated with 3 µM FAK [i].

We next sought to examine the effect of FAK inhibition on the actin cytoskeleton and cell adhesion. HFF cells plated on FN-coated dishes for 1 h were treated with DMSO, 0.1 µM FAK [i] (the IC50 value) or 3 µM FAK [i] (to achieve maximal inhibition) for a further hour, and were fixed and stained for FAKY397, vinculin and actin (Fig. 4.1e). Cells treated with FAK [i] remained attached to the FN-rich matrix, and the actin cytoskeleton and morphology of cells appeared unaffected (Fig. 4.1e). Consistent with the biochemical data above (Fig. 4.1a), the cell area covered by FAKY397-containing IACs decreased in a dose-dependent manner in cells treated with FAK [i], with the highest reduction in FAKY397-area observed upon treatment with 3 µM FAK [i] (Fig. 4.1e,f). Conversely, the cell area covered by vinculin-containing IACs was unchanged in cells treated with FAK [i] at both doses (Fig. 4.1e,g). These data suggest that after 1 h FAK inhibition, a reduction in FAK activity by approximately 90% does not affect the size or number of pre-formed IAC structures.
Figure 4.1. Inhibition of FAK activity by FAK [i] in human fibroblasts.
HFF cells spread on FN for 1 h were treated with DMSO or the FAK inhibitor AZ13256675 (FAK [i]) for 1 h using half-log dilutions. Cells kept in suspension for 30 min were used to detect basal FAK activity. Untreated cells spread on FN for 2 h were used to detect maximal FAK activity. FAKY397 and FAKY576 were used to assess FAK catalytic activity. 

(a) Immunoblotting of FAK phosphorylation sites FAKY397 and FAKY576 and total FAK in total cell lysates. Molecular weight values (kDa) are displayed to the left of each blot. (b,c) Quantification of immunoblotted membranes. FAK phosphorylation values FAKY397 (b) and FAKY576 (c) were normalised to total FAK, n = 3. (d) Dose-response curve using FAKY397 as a readout for FAK activity to determine percentage inhibition relative to cells treated with DMSO. Grey lines and shading show means ± SEM for suspension and FN conditions (not used to calculate the trendline), n = 3. The 50% FAKY397 inhibitory concentration (IC50) was calculated as 0.11 µM FAK [i] using the formula y = 12.23ln(x) + 76.99, where y is percentage inhibition and x is FAK [i] concentration. 

(e) Immunofluorescence staining of HFF cells, IACs were visualised by staining for FAKY397 (green) and vinculin (red). The actin cytoskeleton was visualised by staining with fluorophore-conjugated phalloidin. Scale bars, 20 µm. 

(f,g) Quantification of the cell area covered by FAKY397- (f) and vinculin- (g) positive areas. Values are means ± SEM, n > 20 cells per condition. ***P < 0.001; ****P < 0.0001; ns, not significant; Kruskal–Wallis test with Dunn’s post hoc correction. Susp, suspension.
4.3.2 Effects of the duration and timing of FAK inhibition on IAC formation and cell adhesion

To test whether there were time-dependent changes in IACs, or defects in cell spreading or adhesion maturation, upon FAK inhibition, the effects of the duration and timing of FAK inhibition were examined. Initially, HFF cells plated on FN-coated plates were treated with DMSO or FAK [i] for one to four hours and cells were stained for FAKY397 and vinculin (Fig. 4.2) or paxillin and actin (Fig 4.2–figure supplement 1). Cells remained attached to FN-coated plates and displayed no actin cytoskeletal or cell morphological defects when treated with FAK [i] (Fig 4.2–figure supplement 1a,b). Treating cells with FAK [i] significantly reduced the area covered by FAKY397 at all time points tested (Fig. 4.2a,b). In contrast, increasing the duration of FAK inhibition made little change to the proportion of the cell area covered by vinculin- or paxillin-containing IACs (Fig. 4.2a,c; Fig. 4.2–figure supplement 1a,c).

To examine the effects of FAK inhibition on cell spreading and IAC formation, DMSO or FAK [i] were added to cells kept in suspension prior to attachment to FN (Fig 4.3, Fig. 4.3–figure supplement 1; Susp). To examine the effects of FAK inhibition on IAC maturation, cells were seeded on FN for 1 h, followed by treatment with DMSO or FAK [i] (Fig 4.3, Fig. 4.3–figure supplement 1; Adh). In both cases, cells were incubated with DMSO or FAK [i] for 2 h or 16 h total spreading times, fixed and stained for FAKY397 and vinculin (Fig. 4.3) or paxillin and actin (Fig. 4.3–figure supplement 1). Cells attached to FN-coated plates formed IACs and displayed no actin cytoskeletal, cell morphological or cell spreading defects in all conditions tested (Fig. 4.3–figure supplement 1a,b). Similar to the effects observed when extending the duration of FAK inhibition, there were small changes (not statistically significant) in the cell area covered by vinculin- and paxillin-containing IACs between cells treated with DMSO and FAK [i] (Fig. 4.3a,c; Fig. 4.3–figure supplement 1a,c). In summary, these data suggest that FAK catalytic activity is not required for cell attachment, cell spreading or the formation or maintenance of FN-induced vinculin- or paxillin-containing IACs.
Figure 4.2. Effects of the duration of FAK inhibition on cell adhesion.

HFF cells spread on FN for 1 h were treated with DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) for 1 h, 2 h, 3 h or 4 h. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for FAKY397 (green) and vinculin (red). Scale bars, 20 µm. (b,c) Quantification of the cell area covered by FAKY397- (b) and vinculin- (c) positive areas. Values are means ± SEM, n = 10 cells per condition. ****P < 0.0001; ns, not significant; two-way analysis of variance with Tukey’s post hoc correction (comparisons at each time point are shown).
Figure 4.2–figure supplement 1. Effects of the duration of FAK inhibition on paxillin and the actin cytoskeleton.

HFF cells spread on FN for 1 h were treated with DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) for 1 h, 2 h, 3 h or 4 h. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for paxillin and the actin cytoskeleton was visualised by staining with fluorophore-conjugated phalloidin. Scale bars, 20 µm. (b,c) Quantification of the cell area (b) and the cell area covered by paxillin-positive areas (c). Values are means ± SEM; n = 20 cells per condition in b and n = 10 cells per condition in c; ns, not significant; two-way analysis of variance with Tukey's post hoc correction (comparisons at each time point are shown).
Figure 4.3. Effects of FAK inhibition added to suspension or spread cells on cell adhesion.

To examine effects on cell spreading and IAC formation, DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) was added to HFF cells kept in suspension and cells were plated onto FN-coated plates (Susp). To examine effects on IAC maturation, cells kept in suspension were plated onto FN-coated plates for 1 h and treated with DMSO or FAK [i] (Adh). In both cases, cells were fixed after 2 h or 16 h total spreading times. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for FAKY397 (green) and vinculin (red). Scale bars, 20 µm. (b,c) Quantification of the cell area covered by FAKY397- (b) and vinculin- (c) positive areas. Values are means ± SEM; n = 20 cells per condition in b and n = 10 cells per condition in c; ****P < 0.0001; ns, not significant; two-way analysis of variance with Tukey’s post hoc correction (comparisons for each condition are shown).
Figure 4.3–figure supplement 1. Effects of FAK inhibition added to suspension or spread cells on paxillin and the actin cytoskeleton.

To examine effects on cell spreading and IAC formation, DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) was added to HFF cells kept in suspension and cells were plated onto FN-coated plates (Susp). To examine effects on IAC maturation, cells kept in suspension were plated onto FN-coated plates for 1 h and treated with DMSO or FAK [i] (Adh). In both cases, cells were fixed after 2 h or 16 h total spreading times. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for paxillin and the actin cytoskeleton was visualised by staining for fluorophore-conjugated phalloidin. Scale bars, 20 µm. (b,c) Quantification of the cell area (b) and the cell area covered by paxillin-positive areas (c). Values are means ± SEM; n = 20 cells per condition in b and n = 10 cells per condition in c; ns, not significant; two-way analysis of variance with Tukey’s post hoc correction (comparisons for each condition are shown).
4.3.3 Effects of FAK inhibition on IAC composition

The immunofluorescence-based candidate approach above demonstrated that FAK inhibition did not result in altered abundance of vinculin or paxillin in IACs (Fig. 4.2, Fig. 4.2–figure supplement 1). To determine global changes in IAC composition upon FAK inhibition in an unbiased manner, a proteomics workflow was used to characterise the composition of IACs isolated from cells spread on FN and treated with DMSO or FAK [i] (Fig. 4.4a, see section 4.11 for workflow optimisation). Complexes were also isolated from cells plated on the negative control ligand transferrin (Tf), which allows integrin-independent cell attachment via the transferrin receptor (Jones et al., 2015; Robertson et al., 2015). A similar level of total protein was collected between cells treated with DMSO or FAK [i] (Fig. 4.4b), which allows quantitative comparison of proteins between the two conditions. Immunoblotting of isolated complexes with antibodies directed against the canonical IAC proteins vinculin, integrin-linked kinase (ILK), paxillin and filamin A showed specific enrichment to FN-induced IACs (Fig. 4.4c). As negative controls, the transferrin receptor was enriched to Tf and the mitochondrial protein BAK was not detected in any isolated complexes (Fig. 4.4c). In agreement with immunofluorescence staining, immunoblotting revealed no differences in the abundance of vinculin, paxillin, ILK and filamin A in IACs isolated from cells treated with DMSO or FAK [i] (Fig. 4.4c). These data demonstrate the successful isolation and enrichment of IAC proteins from cells treated with FAK [i].

To allow quantitative comparison of IAC composition upon FAK perturbation in a global manner, isolated FN-induced IACs from cells treated with DMSO or FAK [i] were analysed by MS using a label-free intensity-based approach for relative protein quantification (Progenesis QI, Nonlinear Dynamics, http://www.nonlinear.com/progenesis/qi-for-proteomics/). In total, 898 proteins were identified and quantified with a minimum of two unique peptides per protein and the average log₂ fold change between DMSO and FAK [i] conditions was calculated for each protein (Fig. 4.4d, Fig. 4.4–source data 1). Many previously characterised IAC proteins were identified by MS, including talin, vinculin, ILK, paxillin, FAK and the FN-binding integrins α5β1 and αVβ3. To interrogate the dataset further, comparisons were performed with three different measures of IAC composition: the meta-adhesome (Horton et al., submitted) , which contains 2,412 proteins identified in at least one controlled FN-induced IAC MS dataset; the literature-curated adhesome (Winograd-Katz et al., 2014), which contains 232 proteins reported to localise to IACs; and the consensus adhesome (Horton et al., submitted), which contains 60 proteins that are most robustly identified in IAC MS datasets. In total, 617 meta-adhesome proteins (69% of dataset; 25% of meta-adhesome; Fig. 4.4–figure supplement 1a), 75 literature-curated adhesome proteins (8% of dataset; 31% of literature-curated adhesome; Fig. 4.4–figure supplement 1b) and 49 consensus adhesome proteins (5% of dataset; 82% of consensus adhesome; Fig 4.4–table supplement 1) were identified in IACs isolated from cells treated with DMSO and FAK [i], which is of a similar scale and coverage to other MS-derived IAC datasets (Geiger and Zaidel-Bar, 2012; Horton et al., submitted).

Surprisingly, the majority of proteins identified (863, 96%), including all proteins identified from the consensus adhesome (Fig. 4.4–table supplement 1), changed in abundance by less than two-fold upon FAK inhibition (Fig. 4.4d). In total, only 35 proteins changed in abundance by at least two-fold (20 decreased, 15 increased; Fig. 4.4d, Fig. 4.4–table supplement 2), of which 19 and 2 proteins
Figure 4.4. Effects of FAK inhibition on IAC composition.

(a) Workflow for the isolation of IACs from cells treated with DMSO or FAK [i] (see methods for details). HFF cells spread on FN for 1 h were treated with DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) for 1 h. IACs were isolated by a combination of cross-linking, cell lysis and high-pressure water wash. (b,c) Isolated IACs were analysed by SDS-PAGE (b) and immunoblotting (c). Total protein intensity values for each lane are indicated. Graph shows intensity values normalised to the summed intensities in each experiment. Values are mean ± SEM, n = 3. Complexes isolated from cells spread on transferrin (Tf) were used as a negative control to check purity of IAC isolations, and cell lysates from cells spread on FN (TCL) were used as a positive control. Molecular weight values (kDa) are displayed to the left of each blot. (d) Isolated IACs were subjected to MS-based proteomic analysis. Using an intensity-based quantification approach, 898 proteins were identified and quantified satisfying at least two unique peptides per protein. Ratios of normalised intensity values (log2(FAK [i]/DMSO)) were calculated for each protein in each replicate experiment. Graph shows mean ± SEM, n = 3. Blue shading corresponds to twofold change between conditions and the percentage of proteins within twofold change is indicated. In total, 15 and 20 proteins increased and decreased, respectively, upon FAK inhibition by at least two-fold (Fig. 4.4–table supplement 2). See Fig. 4.4–source data 1 for a full list of identified proteins.
Figure 4.4–figure supplement 1. Mass spectrometry-based proteomic analysis of IACs isolated from cells treated with DMSO or FAK [i].

HFF cells spread on FN for 1 h were treated with DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) for 1 h. IACs were isolated by a combination of cross-linking, cell lysis and high-pressure water wash and isolated IACs were analysed by MS. Ratios of normalised intensity values \( \log_2(\text{FAK [i]/DMSO}) \) were calculated for each protein in each replicate experiment. Graphs show mean ± SEM, \( n = 3 \). Blue shading corresponds to two-fold change between conditions and the percentage of proteins within two-fold change is indicated. (a) A graph of meta-adhesome (Horton et al., submitted) proteins identified by MS. Of the 617/2412 (25%) meta-adhesome proteins identified, 7 proteins increased and 12 proteins decreased upon FAK inhibition (Fig. 4.4–table supplement 2, bold). (b) A graph of literature-curated adhesome (Winograd-Katz et al., 2014) proteins identified by MS. Of the 75/232 (32%) literature-curated adhesome proteins identified, 1 protein increased and 1 protein decreased upon FAK inhibition (Fig. 4.4–table supplement 2, italics). (c,d) For each protein identified by MS, the number of unique peptides per protein used for protein quantification is plotted against the mean normalised intensity ratios \( \log_2(\text{FAK [i]/DMSO}) \). Panel d shows an enlarged area in c (red box).
Figure 4.4–table supplement 1. Consensus adhesome proteins identified in LC-MS/MS analysis of IACs isolated from HFF cells treated with DMSO or FAK [i].

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Of the 60 consensus adhesome proteins (Horton et al., submitted), 49 were identified by MS. Normalised intensity values (not shown) were used to calculate the fold change in each of three biological replicates (R1, R2, R3). P-values were calculated from normalised abundance values between DMSO and FAK [i] conditions using the Holm-Sidak method for multiple comparisons (Holm, 1979). FAK [i], FAK inhibitor AZ13256675; SD, standard deviation.
Figure 4.4–table supplement 2. Proteins identified in LC-MS/MS analysis of IACs isolated from HFF cells treated with DMSO or FAK [i] respectively. FAK [i], FAK inhibitor AZ13256675; SD, standard deviation. Literature-curated adhesome (Winograd-Katz et al., 2014) are indicated in bold and italic font, biological replicates (R1, R2, R3).

Normalised intensity values (not shown) were used to calculate the fold change in each of three biological replicates (R1, R2, R3). P-values were calculated from normalised abundance values between DMSO and FAK [i] conditions using the Holm-Sidak method for multiple comparisons (Holm, 1979). Proteins that are members of the meta-adhesome (Horton et al., submitted) or literature-curated adhesome (Winograd-Katz et al., 2014) are indicated in bold and italic font, respectively. FAK [i], FAK inhibitor AZ13256675; SD, standard deviation.

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Decreased by FAK inhibition

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were identified from the meta-adhesome and literature-curated adhesome, respectively (Fig. 4.4–figure supplement 1a,b; Fig. 4.4–table supplement 2). None of the detected changes in IAC composition upon FAK inhibition were statistically significant and most are likely to result from biological variability or inaccuracies with protein quantification, since compared with the 863 unchanged proteins, the 35 proteins that changed by at least two-fold upon FAK inhibition displayed higher variation between biological replicates (mean log₂ standard deviation, 0.57 vs. 2.28, respectively) and were often quantified using a lower number of peptides per protein (Fig 4.4–figure supplement 1c,d). These data decrease confidence in the altered abundance of these proteins and suggest that IAC composition at the protein level is largely unaffected by FAK inhibition.

4.3.4 Effects of FAK and Src inhibition on cell migration and proliferation

To ensure that the lack of change in IAC composition observed by MS upon FAK inhibition was not due to ineffective FAK inhibition upon treatment with FAK [i], we examined the effects of FAK inhibition on cell migration and proliferation, which are known outputs of FAK and Src signalling (Gilmore and Romer, 1996; Sánchez-Bailón et al., 2012; Serrels et al., 2012; Sieg et al., 1999; Westhoff et al., 2004). To also examine the role of another key kinase in IACs, we perturbed the function of Src using the small molecule Src kinase inhibitor saracatinib (AZD0530 (Hennequin et al., 2006; Plé et al., 2004), Src [i]). To determine a concentration of Src [i] required to inhibit Src catalytic activity effectively, HFF cells were plated on FN-coated dishes and treated with DMSO and half-log dilutions of Src [i] for 1 h. Src activity was assessed by immunoblotting total cell lysates for the Src substrate paxillinY118 (Serrels et al., 2006) and the Src activation site SrcY416 (Fig. 4.5a). PaxillinY118 and SrcY416 were normalised against total paxillin and Src, respectively, in each condition (Fig. 4.5b,c). PaxillinY118 increased in an adhesion-dependent manner compared to cells kept in suspension, and there was a dose-dependent reduction in paxillinY118 and SrcY416 in cells treated with Src [i], with maximal inhibition being obtained at 3 µM Src [i] (Fig. 4.5a-c). There was an 80% reduction in the level of paxillinY118 in cells treated with 3 µM Src [i] (Fig. 4.5b,d), which was similar to the level of paxillinY118 from cells kept in suspension. Cells treated with a higher dose of inhibitor (10 µM) did not show a greater reduction in paxillinY118 or SrcY416 levels (Fig. 4.5a-d). A 50% paxillinY118 inhibitory concentration (IC₅₀, 0.31 µM Src [i]) was calculated from paxillinY118 values normalised to values from cells treated with DMSO (Fig. 4.5d). These data suggest that Src activity is effectively reduced in cells treated with 3 µM Src [i], the same concentration used to inhibit FAK with FAK [i].
Figure 4.5. Inhibition of Src activity by Src [i] in human fibroblasts.

HFF cells spread on FN for 1 h were treated with DMSO or the Src inhibitor AZD0530 (Src [i]) for 1 h using half-log dilutions. Cells kept in suspension for 30 min were used to detect basal Src activity. PaxillinY118 and SrcY416 were used as readouts for Src catalytic activity. (a) Immunoblotting of paxillinY118, total paxillin, SrcY416 and total Src in total cell lysates. Molecular weight values (kDa) are displayed to the left of each blot. (b,c) Quantification of immunoblotted membranes in a. Phosphorylation values of paxillinY118 (b) and SrcY416 (c) were normalised to total protein values of paxillin and Src, respectively, to assess Src catalytic activity. n = 3. (d) Dose-response curve using paxillinY118 as a readout for Src activity to determine percentage inhibition relative to cells treated with DMSO. Dark grey lines and shading show mean ± SEM for the suspension condition (not used to calculate the trendline), n = 3. A 50% inhibitory concentration (IC50) was calculated as 0.31 µM Src [i] using the formula y=9.19ln(x)+60.81, where y is percentage inhibition and x is Src [i] concentration. Values are means ± SEM. Susp, suspension.
Studies investigating the effects of combined inhibition of kinases have been performed in melanoma cells (Ferguson et al., 2013; Hirata et al., 2015) and in numerous studies targeting the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and mechanistic target of rapamycin (mTOR) pathways (Belmont et al., 2014; El Touny et al., 2014; Hatzivassiliou et al., 2012; Mi et al., 2009). Therefore, as well as inhibiting FAK and Src independently, we chose to investigate combined FAK and Src inhibition, since these proteins form a signalling complex and have overlapping functions (Mitra and Schlaepfer, 2006).

To examine the effect of FAK and/or Src inhibition on cell migration, cells were treated with DMSO, FAK [i], Src [i] or combined FAK [i] and Src [i] and examined in scratch wound assays (Fig. 4.6a). Cells were able to migrate into wounded monolayers in all conditions tested, but migration distance and speed were reduced throughout the time course tested in inhibitor-treated cells, particularly for cells treated with Src [i] and combined FAK [i] and Src [i] (Fig. 4.6b,c). The ability of cells to migrate in a directionally-persistent manner was also impaired in cells treated with combined FAK [i] and Src [i] (Fig. 4.6d).

To investigate the effect of FAK or Src inhibition on cell proliferation, equal numbers of cells were incubated in the presence of DMSO or inhibitors for up to 4 days, fixed at various time points, and stained with crystal violet to measure total cell number. Cells were able to proliferate in all conditions tested, but proliferation was impaired in cells treated with inhibitors (Fig. 4.6e), particularly in cells treated with combined FAK [i] and Src [i]. Specifically, cells treated with DMSO showed an 8.2-fold increase in cell number from day 1 to day 4, whereas the increase in cell number was reduced in cells treated with FAK [i] (5.0-fold), Src [i] (4.9-fold) and combined FAK [i] and Src [i] (4.4-fold). To confirm the differences seen in proliferation, we investigated the effects of inhibitor treatment on shorter-term DNA synthesis as measured by EdU incorporation. Cells were plated for 16 h and EdU was added 2 h before fixation. To determine the proportion of actively dividing cells, cells were visualised with DAPI and EdU, and the proportion of EdU-positive cells was calculated (Fig. 4.6f). Serum starvation was used to induce cell cycle arrest in cells and was used as a negative control. Cells treated with DMSO showed an EdU incorporation rate of 26% (Fig. 4.6f). This was reduced to 17% in cells treated with FAK [i] and was unaffected in cells treated with Src [i], which is consistent with data from the crystal violet assay at earlier time points (Fig. 4.6e). The greatest reduction in DNA synthesis was observed in cells treated with combined FAK [i] and Src [i], with only 7% of actively dividing cells (Fig. 4.6f). These data demonstrate that cells treated with FAK [i] and Src [i] display defects in cell migration and proliferation, known functions of these kinases, and these effects are attenuated by combined FAK [i] and Src [i] inhibition. These data confirm successful inhibition of FAK and Src in cells treated with FAK [i] and Src [i].
Figure 4.6. Effects of FAK, Src and combined FAK and Src inhibition on cell migration and proliferation.

(a) Migration of HFF cells treated with DMSO, FAK inhibitor AZ13256675 (FAK [i]), Src inhibitor AZD0530 (Src [i]) or combined FAK [i] + Src [i] in scratch wound assays. Individual cells were tracked over 8 h. (b-d) Quantification of the distance (b), velocity (c) and directional persistence (d) of migrating cells in scratch wound assays. Percentage values relative to the DMSO condition are shown below bars, n = 50 cells per condition. In b, statistical significance was calculated based on the 8 h time point. (e) Cells were incubated with DMSO, FAK [i], Src [i] or FAK [i] + Src [i] for the required times, fixed and stained with 0.1% crystal violet. Additional inhibitors were added every 24 h, where appropriate, to maintain protein inhibition. Absorbance intensity was measured at 590 nm and a calibration curve (not shown) was used to estimate total cell number in each condition, n = 3. Statistical significance was calculated using values from day 4. (f) Cells were incubated with DMSO, FAK [i], Src [i] or FAK [i] + Src [i] for 16 h, EdU was added to the medium 2 h before fixation and the percentage of EdU-positive cells relative to DAPI staining in each field of view was calculated. Serum-starved cells were used as a negative control. For each condition, n = 15 imaged areas containing a total of >450 cells. Values are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Kruskal–Wallis test with Dunn’s post hoc correction.
4.3.5 Effects of FAK and Src inhibition on IAC protein composition and phosphotyrosine

The results above demonstrate that IACs are robust to perturbation of the most connected kinase within the network, FAK, and that FAK and Src are inhibited effectively upon treatment with FAK [i] and Src [i], respectively, by immunoblotting and functional analyses. To investigate further the role of adhesome kinases in IAC network structure, IACs were isolated using the previously outlined workflow (Fig. 4.4a) from cells treated with DMSO, FAK [i], Src [i] or combined FAK [i] and Src [i] and analysed by immunoblotting. Equal amounts of protein material were analysed in each condition (Fig. 4.7a), which allowed quantitative comparison of proteins between conditions. Immunoblotting of IAC proteins and the absence of non-adhesion-associated proteins (transferrin receptor and BAK) confirmed the specificity of the isolation (Fig. 4.7b). Comparisons between the four conditions demonstrated minor changes in the abundance of proteins located at IACs. Vinculin, filamin A, FAK and p130Cas were unchanged between conditions, while differences in abundances of talin, ILK, paxillin and Src were all within two-fold of the control, except for a decrease in ILK upon treatment with Src [i] (Fig. 4.7b,c). By contrast, immunoblotting of FAK and paxillin phosphorylation sites demonstrated that FAKY397 was reduced upon FAK inhibition but not Src inhibition, and paxillinY118 showed a greater reduction in phosphorylation levels upon Src inhibition than FAK inhibition (Fig. 4.7b,c), which demonstrates the relative selectivity of these compounds to FAK and Src. In addition, FAK- and Src-dependent phosphorylation sites of FAK, paxillin and p130Cas were examined in total cell lysates (Fig. 4.7–figure supplement 1). Immunoblotting confirmed changes in phosphorylation between conditions in whole cells that were similar to effects observed in isolated IACs for FAKY397 and paxillinY118 (Fig. 4.7b,c). FAKY576 was reduced to equivalent levels in cells treated with FAK [i] and Src [i] individually and this effect was further reduced with combined treatment with FAK [i] and Src [i] (Fig. 4.7–figure supplement 1a,c). This is in agreement with previous studies that have shown that Src is activated following FAK phosphorylation at FAKY397, and active Src then phosphorylates FAK at FAKY576 (Calalb et al., 1995; Schaller et al., 1994). Similar to the effects observed for paxillinY118, p130CasY249 showed a greater reduction in phosphorylation levels in cells treated with Src [i] than FAK [i], and was further reduced by combined treatment with FAK [i] and Src [i] (Fig. 4.7–figure supplement 1a,e). Taken together, these data indicate that IACs are robust at the protein level to perturbation of not only FAK but also Src and a combination of FAK and Src, and suggest that reduced phosphorylation levels of multiple IAC components do not elicit a major change in IAC composition.
Figure 4.7. Effects of FAK, Src and combined FAK and Src inhibition on IAC composition. HFF cells spread on FN for 1 h were treated with DMSO, 3 µM FAK inhibitor AZ13256675 (FAK [i]), 3 µM Src inhibitor AZD0530 (Src [i]) or combined FAK [i] + Src [i] for 1 h. IACs were isolated by a combination of cross-linking, cell lysis and high-pressure water wash. (a,b) Isolated IACs were collected and analysed by SDS-PAGE (a) and immunoblotting (b). Total protein intensity values for each lane are indicated. Graph shows intensity values normalised to the summed intensities in each experiment, n = 3. Cell lysates from cells spread on FN (TCL) were used as a positive control. Molecular weight values (kDa) are displayed to the left of each blot. (c) Quantification of immunoblotting in b. For each protein, the band intensity values from each condition were normalised to the summed intensities in each experiment. Quantification was not carried out for transferrin receptor or BAK. The fold change relative to DMSO is indicated below bars. Values are means ± SEM, n = 3.
Figure 4.7–figure supplement 1. Effects of FAK, Src and combined FAK and Src inhibition on FAK- and Src-dependent phosphorylation sites.

(a) Immunoblotting of total cell lysates collected from HFF cells spread on FN for 1 h and treated with DMSO, 3 µM FAK inhibitor AZ13256675 (FAK [i]), 3 µM Src inhibitor AZD0530 (Src [i]) or combined FAK [i] + Src [i] for 1 h. Molecular weight values (kDa) are displayed to the left of each blot. (b-e) Quantification of immunoblotted membranes in a. Phosphorylation values FAKY397 (b), FAKY576 (c), paxillinY118 (d) and p130CasY249 (e) were normalised to the corresponding total protein values, $n = 4$ in b-d and $n = 3$ in e. Values are means ± SEM.
To confirm that reducing FAK and/or Src activity did not dramatically alter IAC composition but did alter phosphorylation, we performed a targeted analysis of selected molecules and visualised their localisation in cells treated with DMSO, FAK [i], Src [i] and combined FAK [i] and Src [i]. The localisation of phosphotyrosine (pY) and α5 integrin (Fig. 4.8a), FAK<sup>Y397</sup> and vinculin (Fig. 4.8–figure supplement 1a), and paxillin<sup>Y118</sup> and paxillin (Fig. 4.8–figure supplement 2a) was visualised in cells by immunofluorescence. Similar to the profiles of a number of phosphosites observed in isolated IACs (Fig. 4.7) and whole cells (Fig. 4.7–figure supplement 1), the cell area covered by pY-positive areas was reduced upon FAK and Src inhibition individually and was almost completely depleted upon combined FAK and Src inhibition (Fig. 4.8a,b). FAK<sup>Y397</sup>- and paxillin<sup>Y118</sup>-positive areas were reduced in cells treated with FAK [i] and Src [i] (Fig. 4.8–figure supplement 1a,b; Fig. 4.8–figure supplement 2a,b), respectively, which is similar to the abundance changes of FAK<sup>Y397</sup> and paxillin<sup>Y118</sup> observed previously (Fig. 4.7b,c; Fig. 4.7–figure supplement 1a,b,d). In contrast, the area of α5 integrin, vinculin and paxillin remained unchanged upon kinase perturbation (Fig. 4.8a,c; Fig. 4.8–figure supplement 1a,c; Fig. 4.8–figure supplement 2a,c), indicating that α5 integrin, vinculin and paxillin are resistant to FAK and Src inhibition. These data demonstrate that upon perturbation of FAK, Src and combined FAK and Src, phosphorylation, and thus adhesion signalling, is affected but IAC protein levels are unchanged.

In summary, these data demonstrate that neither FAK nor Src activity are required to maintain IAC composition but are required for known functions of these protein kinases, such as cell migration and proliferation. Therefore, these findings demonstrate the ability of kinase-dependent signals to propagate through IACs without altering IAC composition, which suggests that a separation in IAC composition and phosphotyrosine-dependent signalling is possible at IACs.
Figure 4.8. Effects of FAK, Src and combined FAK and Src inhibition on phosphotyrosine and α5 integrin.

HFF cells spread on FN for 1 h were treated with DMSO, 3 µM FAK inhibitor AZ13256675 (FAK [i]), 3 µM Src inhibitor AZD0530 (Src [i]) or combined FAK [i] + Src [i] for 1 h. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for phosphotyrosine (pY, green) and α5 integrin (red). Scale bars, 20 µm. (b,c) Quantification of the cell area covered by pY- (b) and α5 integrin- (c) positive areas. Values are means ± SEM, n = 10 cells per condition. *P < 0.05; ****P < 0.0001; ns, not significant; Kruskal–Wallis test with Dunn’s post hoc correction (in c, comparisons with cells treated with DMSO are shown).
Figure 4.8–figure supplement 1. Effects of FAK, Src and combined FAK and Src inhibition on FAKY397 and vinculin.

HFF cells spread on FN for 1 h were treated with DMSO, 3 µM FAK inhibitor AZ13256675 (FAK [i]), 3 µM Src inhibitor AZD0530 (Src [i]) or combined FAK [i] + Src [i] for 1 h. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for FAKY397 (green) and vinculin (red). Scale bars, 20 µm. (b,c) Quantification of the cell area covered by FAKY397 (b) and vinculin (c) positive areas. Values are means ± SEM, n = 10 cells per condition. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant; Kruskal–Wallis test with Dunn’s post hoc correction (in c, comparisons with cells treated with DMSO are shown).
Figure 4.8–figure supplement 2. Effects of FAK, Src and combined FAK and Src inhibition on paxillinY118 and paxillin.

HFF cells spread on FN for 1 h were treated with DMSO, 3 µM FAK inhibitor AZ13256675 (FAK [i]), 3 µM Src inhibitor AZD0530 (Src [i]) or combined FAK [i] + Src [i] for 1 h. (a) Immunofluorescence staining of HFF cells. IACs were visualised by staining for paxillinY118 (green) and paxillin (red). Scale bars, 20 µm. (b,c) Quantification of the cell area covered by paxillinY118 (green) and paxillin (red) positive areas. Values are means ± SEM, n = 10 cells per condition. *P < 0.05; ***P < 0.001; ****P < 0.0001; ns, not significant; Kruskal–Wallis test with Dunn’s post hoc correction (in c, comparisons with cells treated with DMSO are shown).
4.4 Discussion

Previous studies have used MS-based proteomics to analyse the effects of nocodazole (Ng et al., 2014; Yue et al., 2014) or blebbistatin (Kuo et al., 2011; Schiller et al., 2011, 2013) on IAC composition. However, the response of IACs to perturbation of key kinases has not been examined. Here, we perturbed FAK and Src activities using small molecule inhibitors, and analysed their effects on IAC composition and adhesion signalling. Our key findings are:

1. FAK phosphorylation at FAKY397 is not required for cell spreading, formation or maintenance of IAC structures. Cell migration and proliferation, which are known outputs of FAK and Src signalling, are affected by treatment with FAK [i] and Src [i]; and

2. Analyses of IAC protein composition demonstrated that IAC composition is largely unaffected, and robust to, FAK and Src inhibition, while phosphorylation of adhesion signalling proteins and general phosphotyrosine levels are affected by perturbation of FAK and/or Src catalytic activity.

Surprisingly, IAC protein composition was robust to perturbation of FAK and/or Src. Other studies have reported small increases in IAC size upon FAK inhibition due to reduced IAC turnover (Plotnikov et al., 2012; Ren et al., 2000; Slack-Davis et al., 2007; Webb et al., 2004; Westhoff et al., 2004). The fact that IACs did not increase in size upon FAK inhibition here may be due to the averaging of effects on heterogeneous IAC structures, such as nascent adhesions, focal complexes, focal adhesions and fibrillar adhesions (Gardel et al., 2010; Zaidel-Bar et al., 2004). Kinase inhibition may affect some of these structures to a greater extent than others. Alternatively, changes in protein composition may be too small to detect by currently available MS quantification methods. Methodological advances, such as improved sensitivity of the proteomic approach and protocols to allow isolation of specific IAC structures, are necessary to detect small fluctuations in IAC composition more accurately using unbiased approaches. Nevertheless, the data presented here suggest that kinase inhibition does not cause gross changes in IAC composition.

IACs have been shown to vary in size in response to inhibitors that affect the cytoskeletal networks, such as inhibitors targeting microtubule polymerisation (Bershadsky et al., 1996; Ezratty et al., 2005; Ng et al., 2014; Yue et al., 2014), myosin-II activity (Choi et al., 2008; Kuo et al., 2012; Pasapera et al., 2010; Schiller et al., 2011, 2013), Rho-mediated contractility (Chrzanowska-Wodnicka and Burridge, 1996; Imamura et al., 2000) and more recently, cyclin-dependent kinase 1 (Robertson et al., 2015). In contrast, IACs are robust to gene knockouts of IAC proteins in silico and to prevention of FAK localisation to IACs in vitro (Gilmore and Romer, 1996; Zaidel-Bar et al., 2007). In combination with findings from these studies, the data presented here that demonstrate robustness of IAC protein composition to perturbation of FAK and Src kinase activity supports a model whereby IACs are robust cellular structures whose complex network of interactions maintains their structure upon modulation of individual components and adhesion signalling, but IACs are affected by modulation of external factors such as force or microtubule-targeting. One outstanding question is whether there are differential effects on the phospho-adesome upon modulation of intrinsic and external factors of IACs. Examination of IAC composition, and their
associated phospho-adhesomes, under different perturbation conditions will help to delineate the scaffolding and adhesion signalling contributions of IAC proteins.

Inhibition of FAK and Src individually or in combination revealed different profiles of phosphorylation of IAC proteins. Similar to studies treating cells with other FAK inhibitors (Slack-Davis et al., 2007; Stokes et al., 2011; Tanjoni et al., 2010), FAK [i] did not completely abolish FAKY397 levels, which indicates that other kinases are able to phosphorylate this phosphosite. FAK autophosphorylation at FAKY397 has been shown to occur through FAK dimerization (Brami-Cherrier et al., 2014), and inhibitors blocking FAK dimerization may be required to inhibit FAK activation entirely. FAK [i] inhibited FAKY397 to a greater extent than Src [i], and in agreement with reports showing that a reduction in FAKY397 does not affect p130Cas phosphorylation (Tanjoni et al., 2010), Src [i] inhibited the Src substrates paxillinY118 and p130CasY249 to a greater extent than FAK [i], which suggests that FAK [i] and Src [i] specifically inhibit FAK and Src, respectively. The inability of FAK [i] to cause a reduction of Src substrates suggests that a substantial decrease in FAKY397 alone does not abolish Src activity, possibly because Src has already been activated by FAK, or high enough levels of FAKY576 are maintained to enable additional Src activation, or Src activation occurs through alternative FAK-independent mechanisms. Src inhibition resulted in a decrease in FAKY576, paxillinY118 and p130CasY249; however, these phosphosites and total phosphotyrosine were all further reduced upon combined FAK and Src inhibition that also reduced FAKY397. These data suggest that both kinases must be inhibited to abolish FAK and Src kinase activities and adhesion signalling via phosphotyrosine more completely, which may be due to the close interplay between FAK and Src activation and their shared functional roles (Mitra and Schlaepfer, 2006; Sieg et al., 1998). As a number of kinases have been reported to localise to IACs and modify their components (Robertson et al., 2015; Winograd-Katz et al., 2014), it is surprising that inhibition of only two kinases in IACs, FAK and Src, almost completely abolished phosphotyrosine levels (Fig. 4.8a,b). The data presented here suggest that FAK and Src are the key adhesome kinases that regulate adhesion signalling via phosphotyrosine, which places importance on their associated regulators to control activation state.

Previous studies have shown that FAK-null cells, cells overexpressing the FAK-related non-kinase (FRNK, a dominant-negative portion of FAK that inhibits phosphorylation at FAKY397 (Richardson and Parsons, 1996)) and cells treated with the Src inhibitor PP2 display defects in cell migration (Ilić et al., 1995; Sieg et al., 1999; Slack et al., 2001). Similarly, FAK and Src have been shown to regulate cell proliferation in a number of cell types (Gilmore and Romer, 1996; Je et al., 2014; Luo et al., 2013; Sánchez-Bailón et al., 2012; Serrels et al., 2012). In agreement with these data, we found that perturbation of FAK and/or Src kinase activity resulted in defects in cell migration and proliferation, which was particularly apparent when both kinases were inhibited. However, previously reported defects during early cell spreading upon FAK inhibition and cell attachment upon FRNK overexpression (Richardson and Parsons, 1996) were not observed here. This may be because cells were able to overcome those effects at the later time points investigated here, or because it is the scaffolding role of FAK that mediates cell spreading, supported by data showing that coexpression of Src or catalytically-inactive FAK can rescue cell spreading defects (Richardson et al., 1997) and endogenous FRNK expression is increased during the early stages of
cell attachment (Nagoshi et al., 2006), indicating that FAK catalytic activity does not regulate this process.

In summary, the data presented here indicate that IAC composition is robust to perturbation of two central kinases, FAK and Src, and that adhesion signalling via phosphotyrosine is dependent on their catalytic activities to control adhesion-dependent cellular functions. Therefore, these data suggest the ability of kinase-dependent signal flux to transduce through IACs without altering IAC structural composition, which suggests that separation in IAC protein composition and phosphotyrosine-dependent signalling is possible at IACs.
4.5 Acknowledgments

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4.6 Author contributions

J.D.H. and M.J.H. conceived the project; E.R.H., J.D.H., G.J., S.T.B. and M.J.H. designed the experiments, analysed the data and interpreted the results; E.R.H. performed the experiments; E.R.H., J.D.H. and M.J.H. wrote the paper; all authors commented on the manuscript and approved the final version.

4.7 Competing financial interests

The authors declare no competing financial interests.
4.8 Methods

4.8.1 Cell culture

Telomerase-immortalised HFF cells (provided by K. Clark) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS; Lonza Bioscience) and 2 mM L-glutamine, penicillin and streptomycin and incubated at 37 °C in a humidified 5% (v/v) CO₂ atmosphere.

4.8.2 Reagents and antibodies

Bovine plasma FN, human Tf, phosphate-buffered saline containing CaCl₂ and MgCl₂ (PBS+), CaCl₂- and MgCl₂- free phosphate-buffered saline (PBS-), DMEM and DMEM containing 25 mM HEPES (DMEM-HEPES) were purchased from Sigma-Aldrich (Poole, UK). Monoclonal antibodies used were rat anti-α5 integrin (mAb11; IF, 1:200; gift from K. Yamada), mouse anti-FAK (clone 77; IB, 1:1000; BD Biosciences, Oxford, UK; 610088), rabbit anti-FAKY397 (clone 141-9; IB, 1:500; IF, 1:200; Invitrogen, Paisley, UK; 44-625G), rabbit anti-ILK (EPR1592; IB, 1:1000; Abcam, Cambridge, UK; ab76468), mouse anti-p130Cas (clone 21; IB, 1:1000; BD Biosciences; 610272), mouse anti-p130CasY249 (clone J169-757.12.2; IB, 1:1000; BD Biosciences; 558401), mouse anti-paxillin (clone 349; IB, 1:1000; IF, 1:400; BD Biosciences; 610051), mouse anti-phosphotyrosine (P-Tyr-100; IF, 1:400; Cell Signaling Technology, Danvers, MA, USA; 9411), mouse anti-transferrin receptor (H68.4; IB, 1:1000; Invitrogen; 13-6890) and mouse anti-vinculin (hVIN-1; IB, 1:400; Sigma-Aldrich; V9131). Polyclonal antibodies used were rabbit anti-BAK (IB, 1:1000; Sigma-Aldrich; B5897), rabbit anti-FAK576 (IB, 1:500; Invitrogen; 44-652G), rabbit anti-filamin A (IB, 1:1000; Bethyl Laboratories, Montgomery, TX, USA; A301-135A), rabbit anti-paxillinY118 (IB, 1:500; IF, 1:200; Invitrogen; 44-722G), rabbit anti-Src (IB, 1:1000; Cell Signaling Technology; 2108BC), rabbit anti-SrcY416 (IB, 1:500; Cell Signaling Technology; 2101BC) and goat anti-talin (clone c-20; IB, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-7534). Secondary Alexa-Fluor 680-conjugated (1:5000; Invitrogen) or Alexa-Fluor 800-conjugated (1:5000, Invitrogen) antimouse, -rabbit or -goat antibodies were used for immunoblotting. For immunofluorescence, secondary Alexa-Fluor 488-conjugated (1:200; Invitrogen) and Alexa-Fluor 594-conjugated (1:200; Invitrogen) anti-rabbit, -rat or -mouse antibodies were used. Actin filaments were visualised by Alexa-Fluor 647-conjugated phalloidin (1:200; Invitrogen). The FAK-specific inhibitor AZ13256675 (FAK [i]) and Src-specific inhibitor AZD0530 (Src [i]) were from Astra Zeneca (Macclesfield, UK).

4.8.3 Plating cells

Cells were washed with PBS-, detached with trypsin (Sigma-Aldrich) and trypsin was quenched with 2.5% (w/v) BSA in DMEM-HEPES. Cells were washed in PBS- and resuspended in 5% (w/v) BSA in DMEM-HEPES and incubated at 37 °C in a humidified 8% (v/v) CO₂ atmosphere in suspension for 30 min to downregulate adhesion-dependent signalling events. Cells were washed in PBS- and 1.5 x 10⁶ cells were plated in DMEM-HEPES on 10-cm-diameter dishes (Corning, NY, USA) coated with FN (10 μg/mL, PBS+) or Tf (50 μg/mL, PBS-). Prior to ligand coating, dishes were blocked with heat-denatured BSA (10 mg/mL >99% purity BSA, 0.22 μm filtered, 85 °C for 12
min) at room temperature (RT) for 1 h. Cells were incubated at 37 °C in a humidified 8% (v/v) CO₂ atmosphere for the required times.

4.8.4 Collection of cell lysates

For collection of cell lysates, cells were seeded at 3 x 10⁵ cells/mL for 1 h and DMSO or inhibitors against FAK (AZ13256675, Astra Zeneca) or Src (AZD0530, Astra Zeneca) were added at the required concentration. Inhibitors were added from stock solutions to give a final dilution of 1:1000 except for combined FAK and Src treatment, where the final dilution from stock solutions was 1:2000 for each inhibitor. In the case that cells were plated in the presence of inhibitors, inhibitors or DMSO were added to cells when maintained in suspension and cells were plated directly from suspension. Medium from dishes was removed and dishes were washed once in cold PBS. Lysates of adherent cells were collected by scraping in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 5 mM EDTA, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 0.5 mM 4-(2-aminooethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 10 mM Na₃VO₄, pH 7.4). For collection of suspension cells, cells in suspension were centrifuged (450 g, 4 min, 4 °C) and washed in cold PBS prior to addition of lysis buffer. After cell lysis, non-solubilized material was discarded by centrifugation (22000 g, 10 min, 4 °C). To ensure equal total protein gel loading, relative protein amounts in each sample were calculated using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA; 23225).

4.8.5 IAC isolation

IACs were isolated using a similar approach to the ligand affinity purification method described previously (Jones et al., 2015). Cells were spread on FN or Tf for 1 h, treated with inhibitors or DMSO for 1 h and incubated with dimethyl-3, 3’-dithiobispropionimidate (DTBP; Thermo Fisher Scientific; 6 mM, 5 min). DTBP was removed and quenched with 200 mM Tris-HCl (pH 8, 3 min), cells were washed in cold PBS and cell bodies were removed by incubation in cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.5 % (w/v) SDS, 1% (w/v) sodium deoxycholate; 3 min) followed by a high-pressure water wash. Protein complexes were washed and stored in cold PBS-. Protein complexes were collected by scraping in 30 µL/dish adhesion recovery solution (125 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 15% (v/v) β-mercaptoethanol) and heated to 95 °C for 10 min. Four times sample volume of -20 °C acetone was added to samples and samples were incubated at -80 °C for at least 3 h. Samples were washed three times with acetone (16000 g, 15 min, 4 °C) and samples were allowed to dry (37 °C, 20-30 min). Precipitated complexes were resuspended in 2x reducing sample buffer (RSB; 50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 4% (w/v) SDS, 0.004% (w/v) bromophenol blue, 15% (v/v) β-mercaptoethanol) and heated to 70 °C for 20 min.

4.8.6 SDS-PAGE and immunoblotting

Protein samples were separated by SDS-PAGE (4-12% (w/v) NuPAGE Novex Bis-Tris gels; Invitrogen) at 200 V for 45 min. To visualise total protein, gels were incubated in Instant Blue (Expedeon, Cambridgeshire, UK) for 1 h and washed in water overnight at 4 °C. For immunoblotting, gels were transferred onto nitrocellulose membrane (Whatman, Maidstone, UK).
and membranes were blocked with blocking buffer (Sigma-Aldrich) in PBS- for 1 h at RT. Membranes were incubated with appropriate concentrations of primary antibodies diluted in blocking buffer in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 100 mM NaCl) supplemented with 0.05% (v/v) Tween-20 (TBS-T) overnight at 4 °C. After three washes with TBS-T, membranes were incubated with appropriate secondary antibodies diluted in blocking buffer in TBS-T for 45 min at RT in the dark and were washed three times in TBS-T. Secondary antibodies used were donkey Alexa Fluor 680-conjugated anti-goat IgG, anti-mouse IgG or anti-rabbit IgG (Life Technologies) and donkey IRDye 800-conjugated anti-mouse IgG (Rockland Immunochemicals). Membranes and stained gels were scanned using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and total lane intensities were determined using Odyssey software (LI-COR).

4.8.7 In-gel digestion and peptide desalting

Protein samples were separated by SDS-PAGE at 200 V for 2 min or until all samples had migrated into the gel. The gel was stained with Instant Blue (Expedeon) and gel lanes were cut into 1-mm³ pieces, washed twice with 50% (v/v) acetonitrile (ACN) in 12.5 mM NH₄HCO₃ and twice with ACN to dry gel pieces. Proteins were reduced by incubation in 10 mM dithiothreitol (DTT) diluted in 25 mM NH₄HCO₃ for 1 h at 56 °C and alkylated in 55 mM iodoacetamide diluted in 25 mM NH₄HCO₃ for 45 min at RT in the dark. Gel pieces were washed with 25 mM NH₄HCO₃ followed by a wash in ACN, which was repeated once more. Gel pieces were dried and incubated with 12 µg/mL trypsin overnight at 37 °C to enable complete protein digestion (Shevchenko et al., 1996). Digested peptides were extracted by incubation with ACN in 0.2% (v/v) formic acid (FA) followed by incubation with 50% (v/v) ACN in 0.1% (v/v) FA. To desalt peptides, each sample was resuspended in 5% (v/v) ACN in 0.1% (v/v) FA followed by incubation with OLIGO™ R3 beads (Applied Biosystems, Paisley, UK). Bead-bound peptides were washed twice in 0.1% (v/v) FA, eluted by two washes in 50% (v/v) ACN in 0.1% (v/v) FA, dried and resuspended in 5% (v/v) ACN in 0.1% (v/v) FA.

4.8.8 LC-MS/MS data acquisition and analysis

Peptides were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an UltiMate 3000 Rapid Separation LC (RS LC, Dionex Corporation, Sunnyvale, CA, USA) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptide samples were loaded onto a pre-column (20 mm x 180 µm i.d., Waters, Hertfordshire, UK) in biological sample order rather than biological replicate order to increase likelihood of chromatograph alignment between samples in downstream analyses (Daniel Ng, unpublished data). Peptides were separated on an analytical column (250mm x 75 µm i.d., 1.7 µm particle size, bridged ethyl hybrid C₁₈; Waters) over a 2 h gradient from 8 to 33% (v/v) ACN in 0.1% (v/v) FA at a flow rate of 200 nl/min. LC-MS/MS analyses were performed in data-dependent mode to allow automatic selection of peptides for fragmentation.

Raw files were analysed and pre-processed using Progenesis software (Progenesis QI, Nonlinear Dynamics, Newcastle, UK; http://www.nonlinear.com/progenesis/qi-for-proteomics/) with automatic detection of alignment reference, which was selected as FAK [i], biological replicate 2 (R2). MS data were searched using an in-house Mascot server (version 2.2.03, Matrix Science) (Perkins et
against the UniProt_Human protein sequence database. Permitted fixed and variable modifications were cysteine carboxamidomethylation and methionine oxidation, respectively. Only tryptic peptides with a maximum of one missed cleavage were considered. Only doubly or triply charged monoisotopic precursor ions were considered, peptide mass tolerance was set to ±5 p.p.m and MS/MS tolerance was set to ±0.5 Da.

Protein identifications were imported into Progenesis software and relative quantification was performed using protein grouping with non-conflicting (unique) peptides. Data were exported as protein measurements, and duplicate protein entries by gene name annotation were combined by addition of raw intensity values of unique peptides. Raw abundance values were normalised to the total raw abundance value in each condition. The dataset was filtered to include only proteins containing at least two unique peptides that were used for protein quantification (Fig. 4.4–source data 1). Log₂ fold change values for each of three biological replicates were calculated between DMSO and FAK [i] conditions using normalised raw values and the average taken. In the case that a protein was not identified in all biological replicates, the average fold change was based on the other biological replicate values only. P-values were calculated from normalised abundance values between DMSO and FAK [i] conditions using the Holm-Sidak method for multiple comparisons (Holm, 1979). Each protein was analysed individually, without assuming a consistent standard deviation.

### 4.8.9 Immunofluorescence microscopy

Cells were plated on poly-D-lysine-coated glass-bottom dishes (14-mm-diameter; MatTek Co., Ashland, MA, USA) or ethanol-washed coverslips (13-mm-diameter; VWR International, Radnor, PA, USA) coated with FN (10 µg/mL). After appropriate inhibitor or DMSO incubation, cells were washed in PBS-, fixed in 4% (w/v) paraformaldehyde for 7 min at RT, washed in PBS- and permeabilised with 0.5% (v/v) Triton X-100 for 10 min at RT. Permeabilised cells were washed three times with PBS- before incubation with appropriate primary antibodies diluted in 2% (w/v) BSA in PBS- for 1 h at RT. Cells were washed three times with PBS- and incubated with appropriate secondary antibodies diluted in 2% (w/v) BSA in PBS- for 30 min at RT in the dark. Stained cells were washed once in PBS-, twice in water and stored in water at 4 °C until imaging. Alternatively, cells on coverslips were mounted onto glass slides (Klinipath, Duiven, Netherlands) in polyvinyl alcohol mounting medium (Sigma-Aldrich).

Images of cells plated on glass-bottom dishes were acquired on a Delta Vision (Applied Precision, Issaquah, WA, USA) microscope using a 60x/1.42 Plan Apo objective and the Sedat filter set (86000v2; Chroma, Bellows Falls, VT, USA). Images were collected with a z optical spacing of 0.2 μm using a Coolsnap HQ camera (Photometrics, Tucson, AZ, USA) and Softworx software (Applied Precision). Alternatively, images of cells plated on glass coverslips were acquired on a BX51 upright microscope (Olympus, Southend-on-Sea, UK) using a 60x/1.25 Plan Fln objective and specific band pass filter sets. Images were captured using a Coolsnap EZ camera (Photometrics) and MetaVue software (Molecular Devices, Sunnyvale, CA, USA). Exposure times for each channel were maintained when imaging cells treated with different inhibitors.
4.8.10 Image analysis

Single slices of raw images were background filtered (rolling ball, 20-pixel radius) using ImageJ (version 1.48b; National Institutes of Health, USA) (Schindelin et al., 2012). Areas containing positive staining of IAC proteins (≥10 pixels) were measured and normalised to total cell area.

4.8.11 Migration assay

For scratch wound migration assays, 2 x 10⁶ cells were seeded into 6-well plates in DMEM supplemented with 10% (v/v) FCS and 2 mM L-glutamine. After overnight incubation at 37 °C in a humidified 5% (v/v) CO₂ atmosphere, pipette tips were used to wound cell monolayers. Cells were washed in DMEM to remove detached cells, and inhibitors or DMSO were added at the required dose. Cells were maintained at 37 °C in a humidified 5% (v/v) CO₂ atmosphere and point visiting was used to allow imaging at multiple positions within the same time course. Bright field images were acquired every 5 min over 8 h and 15 min on a AS MDW live cell imaging system (Leica Microsystems, Milton Keynes, UK) using a 20x/0.5 HC Plan Fluotar objective, a Coolsnap HQ CCD camera (Photometrics) and Image Pro software (version 6.3; Media Cybernetics Ltd, Rockville, MD, USA). Cell migration was tracked manually using the ImageJ plugin MTrackJ (Meijering et al., 2012) for 5 cells per scratch wound. Quantification was performed using the Chemotaxis and Migration Tool (version 1.01; ibidi, Munich, Germany) in ImageJ (Schindelin et al., 2012) for 50 cells per condition.

4.8.12 Proliferation assays

For the crystal violet staining assay, 2 x 10⁴ cells were seeded into 12-well plates in DMEM supplemented with 10% (v/v) FCS and 2 mM L-glutamine. After 1 h incubation at 37 °C in a humidified 8% (v/v) CO₂ atmosphere, the required concentration of inhibitors and DMSO were added to each well and cells were incubated at 37 °C in a humidified 5% (v/v) CO₂ atmosphere for up to 4 days. Where applicable, additional inhibitors and DMSO were added every 24 h to maintain kinase inhibition. At required times, cells were washed in PBS- and fixed in 4% (w/v) paraformaldehyde for 15 min and stored in water at 4 °C. Once all samples were collected, cells were stained with a 0.1% (w/v) crystal violet solution for 20 min and washed three times in water to remove excess stain. Samples were dried and stained cells were extracted in 2 mL/well 10% (v/v) acetic acid for 20 min. In duplicate, 200 µL of crystal violet stained cells were loaded into a 96-well plate, absorbance was read with a spectrometer at 590 nm and the average taken to give a final absorbance value for each condition for that biological replicate (For each condition, n = 2 technical replicates and n = 3 biological replicates). Additionally, serial dilutions of a known number of cells were spread for 2 h, stained and absorbance measured as above to give a calibration curve of absorbance vs. total cell number (y = 0.0014x+0.0185, R² = 0.9821; y, absorbance intensity; x, cell number (x 10³)), which was used to infer total cell number in the DMSO- and inhibitor-treated conditions.

To calculate the proportion of actively dividing cells, the Click-iT EdU Alexa-Fluor 488 Imaging Kit (Thermo Fisher Scientific) was used according to manufacturer’s instructions. Briefly, cells were plated in DMEM supplemented with 10% (v/v) FCS and 2 mM L-glutamine for 1 h, incubated with
DMSO, FAK [i], Src [i] or combined FAK [i] and Src [i] for 16 h and EdU was added to the medium two hours before fixation. Serum-starved cells were used as a negative control. Stained cells were imaged, counted and the percentage of EdU-positive cells relative to the total number of cells was calculated for each field of view. For each condition, at least 450 cells were counted from 15 fields of view.

4.8.13 Statistical analyses

All graphs and statistical analyses were performed using Prism (version 6.04; GraphPad). Figures were assembled using Illustrator (Adobe).
4.9 Supplementary Tables

Figure 4.4–source data 1. Proteins identified in adhesion complexes from cells treated with DMSO or FAK [i].
References


4.11 Supplementary Information - Optimisation of assay to isolate IACs from cells treated with DMSO or FAK [i]

4.11.1 Overview

Methods have been developed to isolate IACs from cells in suspension bound to ligand-coated beads (Humphries et al., 2009) or from adherent cells spread on 2D substrates (Jones et al., 2015; Kuo et al., 2012). Isolation of IACs from adherent cells involves cell spreading on ligand-coated dishes, IAC stabilisation using a cell-permeable cross-linker, removal of cell bodies and collection of isolated IACs. To investigate the effects of FAK perturbation on IAC protein composition in HFF cells, IACs were isolated from cells treated with DMSO or FAK [i] and analysed by SDS-PAGE, immunoblotting and MS (Fig. 4.4). To incorporate treatment with FAK [i] a workflow previously used to isolate IACs from HFF cells (Jones et al., 2015; Ng et al., 2014) was adapted. HFF cells were plated on fibronectin (FN)-coated dishes in serum-free medium for 1 h before addition of DMSO or FAK [i] for 1 h. In the original protocol IACs were stabilised with the addition of fresh medium containing 6 mM DTBP, for 3 min. However, to maintain FAK inhibition during the cross-linker incubation period, 60 mM DTBP was added to the existing cell medium containing DMSO or FAK [i] to achieve a final concentration of 6 mM DTBP, and incubated for 3 min. DTBP was removed from dishes and quenched with 200 mM Tris-HCl in cell medium for 3 min. For removal of cell bodies, cells were incubated in RIPA buffer (Table 2.4) followed by a high-pressure water wash (Robertson et al., 2015), while other studies have incubated cells in extraction buffer (EB, Table 2.4) followed by sonication (Ng et al., 2014). Following cell lysis, IACs were collected in adhesion recovery solution (Table 2.4) and to concentrate IAC samples, protein mixtures were precipitated in cold acetone and resuspended in an appropriate volume of reducing sample buffer prior to downstream analysis (see section 2.3.3).

In the following sections, variations of the protocol used to isolate IACs from cells spread on 2D substrates were investigated, which led to the generation of an optimised workflow for the isolation of IACs from cells treated with DMSO or FAK [i] (Fig. 4.4a). In each figure the workflow used to isolate IACs is shown and particular optimisation steps under investigation are highlighted in bold text for clarity.
4.11.2 Effects of sonication and high-pressure water wash cell lysis methods on IAC recovery

To test the efficiency of cell lysis methods on IAC recovery, HFF cells were spread on FN-coated dishes (10 µg/mL) for 1 h followed by treatment with DMSO and FAK [i]. Cells were lysed using either incubation with EB followed by sonication or incubation with RIPA buffer followed by a high-pressure water wash (Supplementary Fig. 4.1a), and isolated complexes were analysed by SDS-PAGE and immunoblotting (Supplementary Fig. 4.1b-d). Additionally, complexes isolated from cells plated on Tf-coated dishes (10 µg/mL) for 1 h and treated with DMSO for 1 h were used as negative controls. Although other studies have used poly-lysine-coated dishes as a negative control ligand condition (Ng et al., 2014; Schiller et al., 2011, 2013), cells plated on Tf-coated dishes were used here as detection of the transferrin receptor can be used to check for specificity of transferrin receptor engagement (Jones et al., 2015; Robertson et al., 2015). Total cell lysates from cells spread on FN for 2 h were used as positive controls in immunoblotting. IAC samples were analysed by SDS-PAGE. Gels were stained with Instant Blue and total lane intensity values were used to compare relative total protein amounts between samples (Supplementary Fig. 4.1b). Lane intensity values were normalised to the summed lane intensities in each gel so that comparison between gels was not affected by staining efficiency or background levels of staining. Both cell lysis methods resulted in a similar amount of total protein collected from cells treated with DMSO or FAK [i] (Supplementary Fig. 4.1b). Less protein was recovered from Tf-induced complexes, which indicated reduced cell attachment to Tf compared with FN. Lane intensity values were consistently higher in samples generated using the high-pressure water wash, indicating a higher yield of total protein using this approach. Immunoblotting of isolated complexes showed enrichment of known IAC proteins to FN-induced complexes using both cell lysis methods (filamin A, vinculin, talin and paxillin; Supplementary Fig. 4.1c,d). The mitochondrial protein BAK was not identified in any isolated complexes but was detected in total cell lysates, indicating efficient removal of cell bodies using both approaches. Despite the low level detection of the transferrin receptor in IACs isolated using the high-pressure water wash, the transferrin receptor was enriched to Tf-induced complexes using both lysis methods (Supplementary Fig. 4.1c,d). These data demonstrate that IACs can be isolated from cells treated with DMSO or FAK [i], and that both sonication and high-pressure water wash cell lysis methods result in an enrichment of FN-induced IACs.

The total protein yield was higher in samples collected using a high-pressure water wash while the processing time required for cell lysis using sonication was considerably longer. We hypothesised that the longer time required to process samples using sonication for cell lysis could become problematic when increasing the scale of the experiment for MS analysis. Therefore, cell lysis by RIPA buffer incubation and a high-pressure water wash was chosen as the preferred cell lysis technique.
Supplementary Figure 4.1. Isolation of IACs from cells treated with DMSO or FAK [i] using sonication and water wash cell lysis methods.

(a) Workflow for the isolation of IACs from cells treated with DMSO or FAK [i]. HFF cells were spread on FN for 1 h followed by treatment with DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) for 1 h. IACs were isolated by a combination of cross-linking and cell lysis using extraction buffer (EB) and sonication, or RIPA buffer and a high-pressure water wash (bold). Complexes isolated from cells spread on transferrin (Tf) were used as a negative control to check purity of IAC isolations, and cell lysates from cells spread on FN (TCL) were used as a positive control. (b) Isolated IACs were analysed by SDS-PAGE. Total protein intensity values for each lane are indicated. Graph shows intensity values normalised to the summed intensities from each gel. (c) Immunoblot analysis of complexes isolated using EB and sonication for cell lysis. (d) Immunoblot analysis of complexes isolated using RIPA buffer and high-pressure water wash for cell lysis. Molecular weight values (kDa) are displayed to the left of each blot.
4.11.3 Effects of the duration of DMSO or FAK [i] treatment time on IAC composition

To determine whether the level of total protein recovered in isolated IACs was adequate for detection of known IAC proteins by MS, and to compare the effects of the duration of DMSO and FAK [i] treatment on IAC protein composition, IACs were isolated in parallel from HFF cells spread on FN for 1 h followed by treatment with DMSO or FAK [i] for 1 h and 4 h (Supplementary Fig. 4.2a). Isolated IACs were analysed by SDS-PAGE, immunoblotting and MS (Supplementary Fig. 4.2b-d). Isolated complexes from cells plated on Tf-coated dishes (10 µg/mL) for 1 h and treated with DMSO for 1 h were used as negative controls and total cell lysates from cells spread on FN for 2 h were used as positive controls in immunoblotting.

Analysis of relative total protein amounts between samples demonstrated similar levels collected from cells treated with DMSO or FAK [i] for 4 h, although there was a slight decrease in the amount of protein collected from cells treated with DMSO for 1 h compared with those treated with FAK [i] for 1 h (Supplementary Fig. 4.2b). However, immunoblotting for the β1 integrin receptor and IAC proteins (vinculin, talin and ILK) demonstrated recovery of IAC components in all isolated FN-induced IACs (Supplementary Fig. 4.2c). There was enrichment of the transferrin receptor to Tf-induced complexes and the mitochondrial protein BAK was not detected in any isolated complexes (Supplementary Fig. 4.2c). These data confirm the successful isolation of IACs from cells treated with either DMSO or FAK [i] for 1 h or 4 h.

To determine whether there was a suitable level of identification of known IAC proteins that could be quantified in future replicate experiments, isolated complexes were analysed by MS. Samples were separated by SDS-PAGE, each gel lane was cut into 10 slices and analysed by MS (see section 2.4), as described previously (Humphries et al., 2009). Proteins were identified by database searches and quantified by spectral counting (see section 2.4.3). In total, 733 and 760 proteins were identified at 1 h and 4 h time points, respectively, with 639 commonly identified proteins between both time points (Supplementary Fig. 4.2d). Comparison of the number of identified proteins between DMSO and FAK [i] conditions demonstrated a similar level of overlap at both time points (561 proteins, 1 h; 556 proteins, 4 h) and a similar number of proteins were identified uniquely in IACs isolated from cells treated with DMSO (75 proteins, 1 h; 90 proteins, 4 h) or FAK [i] (97 proteins, 1 h; 114 proteins, 4 h). These data demonstrate little difference in the number of identified proteins in isolated IACs from cells treated with either DMSO or FAK [i] for 1 h or 4 h.

To determine whether there was sufficient coverage of known IAC proteins detected by MS, the dataset was filtered using a threshold of at least four spectral counts per protein in at least one experimental condition (Supplementary Table 4.1). This was the threshold applied to the proteomic datasets used in the construction of the meta-adhesome (see section 3.8.8 and Supplementary Table 3.1) and therefore allows comparison of the MS dataset generated here to other MS-derived IAC datasets. In this reduced dataset, 583 proteins were identified at the 1 h time point (426 meta-adhesome, 38 consensus adhesome, 39 literature-curated adhesome) and 613 proteins were identified at the 4 h time point (442 meta-adhesome, 42 consensus adhesome, 42 literature-curated adhesome). The total number of proteins and the numbers of literature-curated adhesome (Winograd-Katz et al., 2014) proteins identified were similar to the numbers of proteins identified in
Supplementary Figure 4.2. Isolation of IACs from cells treated with either DMSO or FAK [i] for 1 h or 4 h.

(a) Workflow for the isolation of IACs from cells treated with DMSO or FAK [i]. HFF cells were spread on FN for 1 h followed by treatment with DMSO or 3 µM FAK inhibitor AZ13256675 (FAK [i]) for 1 h or 4 h (bold). IACs were isolated by a combination of cross-linking and cell lysis using RIPA buffer and high-pressure water wash. Complexes isolated from cells spread on transferrin for 2 h (Tf) were used as a negative control to check purity of IAC isolations.

(b) Isolated IACs were analysed by SDS-PAGE. Total protein intensity values for each lane are indicated. Graph shows intensity values normalised to the summed intensities in each experiment.

(c) Immunoblot analysis of isolated complexes. Cell lysates from cells spread on FN (TCL) were used as a positive control. Molecular weight values (kDa) are displayed to the left of each blot.

(d) Complexes in c and d were analysed by MS using 10 gel slices per condition and a 1 h liquid-chromotography gradient for each slice. Venn diagrams show the number of proteins identified in FN conditions. A list of all identified proteins is provided in Supplementary Table 4.1.
other MS-derived proteomic IAC datasets (Fig. 3.1, Supplementary Table 3.1). Using ratios of
normalised spectral count values for relative protein quantification, known IAC proteins (eg.
vinculin, kindlin 2, ILK, zyxin, α5 integrin and β1 integrin) were detected in all FN conditions but not
in Tf-induced complexes; talin was enriched to FN-induced complexes compared to Tf (≥15-fold)
and the transferrin receptor was enriched to Tf-induced complexes (≥26-fold) in all conditions
tested (Supplementary Table 4.1). These data demonstrate that there was sufficient and
comparable identification and enrichment of IAC proteins at both time points tested in MS analyses.

Isolation of Tf-induced complexes provides a useful negative control condition to reduce non-
specific proteins identified in IACs. However, as Tf-induced complexes were isolated from cells
plated for 2 h, they do not provide an ideal negative control for complexes isolated from cells
treated with DMSO or FAK [i] for 4 h. Similar to observations in other studies (Jones et al., 2015),
cells plated on Tf for 4 h formed protrusions and appeared to form IACs (data not shown), which
indicated cell-derived ECM deposition at this time point. Therefore, so Tf-induced complexes could
be continued to be used as a negative control and as there were little differences in the numbers of
proteins identified from cells treated with DMSO or FAK [i] for 1 h or 4 h, 1 h treatment was chosen
as the preferred incubation period.

4.11.4 Effects of Tf concentration on cell attachment

Consistently during the IAC isolation procedure, less total protein was recovered from isolated Tf-
induced complexes compared to FN-induced IACs, which we hypothesised was due to low
efficiency of cell attachment to plates coated with 10 µg/mL Tf. To determine whether the level of
cell attachment could be increased, HFF cells were plated on dishes coated with different
concentrations of Tf (10 µg/mL, 15 µg/mL, 20 µg/mL, 30 µg/mL, 50 µg/mL and 100 µg/mL) for 2 h
and cells were imaged by light microscopy to examine cell attachment efficiency (Supplementary
Fig. 4.3). Cells were able to attach to Tf-coated plates at all concentrations tested but were not able
to spread as indicated by small cell areas and lack of protrusions (Supplementary Fig. 4.3).
Compared with coating at lower concentrations of Tf, the level of cell attachment increased when
plates were coated with 50 µg/mL Tf but there was no further increase when plates were coated
with 100 µg/mL Tf (Supplementary Fig. 4.3). Therefore, the preferred concentration of Tf used to
cover plates was chosen as 50 µg/mL Tf.
Supplementary Figure 4.3. Effects of increased Tf concentration on cell attachment.
HFF cells were plated onto plates coated with transferrin (Tf) at 10 µg/mL, 15 µg/mL, 20 µg/mL, 30 µg/mL, 50 µg/mL or 100 µg/mL for 2 h. Cells were fixed and imaged by light microscopy. Scale bars, 400 µm.
4.11.5 Effects of RIPA buffer and PBS- incubation times on IAC recovery

During the IAC isolation procedure, it can be difficult and impractical to perform each stage of the protocol for all conditions simultaneously. We hypothesised that at certain stages in the protocol it would be possible to incubate cells in RIPA buffer or PBS- instead of directly continuing with the next step, without affecting the efficiency of protein recovery. Therefore, effects of RIPA buffer and PBS- incubation time on protein recovery were examined to identify possible insensitive breakpoints in the protocol that did not affect recovery of IAC proteins.

To investigate effects of RIPA buffer incubation time on efficiency of IAC recovery, IACs were isolated from cells spread on FN for 2 h, and then incubated in RIPA buffer for either 3 min or 1 h before applying a high-pressure water wash (Supplementary Fig. 4.4a). Collected IACs were analysed by SDS-PAGE and immunoblotting (Supplementary Fig. 4.4b,c). RIPA incubation for 3 min was tested as it was previously used by others to isolate IACs from A375 melanoma cells (Robertson et al., 2015), and incubation for 1 h was tested to assess whether prolonged RIPA incubation affects recovery of IAC components. A similar amount of total protein was recovered from cells incubated with RIPA buffer for 3 min or 1 h (Supplementary Fig. 4.4b), indicating that RIPA buffer incubation time does not affect the total protein amount recovered in isolated IACs. Known IAC proteins (talin, vinculin, ILK and FAK) were identified in FN-induced IACs but were detected in higher abundance in those isolated from cells incubated with RIPA buffer for 3 min compared with 1 h (Supplementary Fig. 4.4c). The higher abundance of these proteins was not due to insufficient cell lysis as the mitochondrial protein BAK and the transferrin receptor were not identified in either condition (Supplementary Fig. 4.4c). These data indicate improved recovery of IAC-specific proteins from cells incubated with RIPA buffer for 3 min compared with 1 h, and suggest that the protocol used to isolate IACs from HFF cells is sensitive to RIPA buffer incubation time. Therefore, the preferred RIPA buffer incubation time was 3 min.

To determine effects of PBS- incubation time on recovery of IAC components, cells were incubated in cold PBS- for 2 h at two stages in the protocol, termed positions a and b (Supplementary Fig. 4.4a). Position a refers to PBS- incubation after cross-linking with DTBP (6 mM, 3 min) and quenching with Tris-HCl but before RIPA buffer incubation. Position b refers to PBS- incubation after application of the high-pressure water wash but before collection of IACs (Supplementary Fig. 4.4a). Using the 3 min RIPA buffer incubation time, IACs were isolated from cells spread on FN for 2 h either without PBS- incubation (none), with PBS- incubation at position b, with PBS- incubation at position a or with PBS- incubation at positions a and b. Collected IACs were analysed by SDS-PAGE and immunoblotting (Supplementary Fig. 4.4d,e).

Equal total protein amounts were recovered between the four conditions, indicating that PBS- incubation at positions a and/or b does not affect total protein recovery (Supplementary Fig. 4.4d). Vinculin was detected in all four conditions, but was present in higher abundance in IACs isolated from cells not incubated with PBS- or incubated with PBS- at position b (Supplementary Fig. 4.4e). Paxillin and talin were identified in IACs from cells not incubated with PBS- or from cells incubated with PBS- at position b, but were not detected in IACs from cells incubated with PBS- at position a or positions a and b (Supplementary Fig. 4.4e). The higher abundance of these proteins from cells
Supplementary Figure 4.4. Effects of RIPA buffer and PBS- incubation times on IAC recovery.

(a) Workflow for the isolation of IACs. HFF cells were spread on FN-coated dishes for 2 h followed by cross-linking with 6 mM DTBP for 3 mins. Cell lysis was performed by incubation with cold RIPA buffer and a high-pressure water wash. IACs were collected, acetone precipitated and analysed by SDS-PAGE and immunoblotting. (b,c) To test whether RIPA buffer incubation time affects efficiency of protein recovery, IACs were isolated from cells with 3 min or 1 h RIPA buffer incubation times (bold). (d,e) To test whether PBS- incubation affects efficiency of protein recovery, IACs were isolated from cells incubated in cold PBS- for 2 h at positions a and/or b in the workflow in a. None, no PBS- incubation. Total protein intensity values for each lane are indicated. Graphs show intensity values normalised to the summed intensities in each experiment. Cell lysates from cells spread on FN (TCL) were used as positive controls. Molecular weight values (kDa) are displayed to the left of each blot.
not incubated with PBS- or from cells incubated with PBS- at position b was not due to increased cell number as similar total protein amount was collected between conditions, and was not due to insufficient cell lysis as the transferrin receptor was not identified in any IACs. These data demonstrate that the IAC isolation protocol is sensitive to PBS- incubation at position a but not at position b. Therefore, the protocol should be performed without delay until after the high-pressure water wash has been applied, at which point isolated IACs can be kept in cold PBS-, if necessary, before collection.

**4.11.6 Effects of DTBP cross-linker concentration and incubation time on IAC recovery**

We hypothesised that the loss of IAC proteins during PBS- incubation after cross-linking but before cell lysis (position a, Supplementary Fig. 4.4a) was due to incomplete cell cross-linking and the presence of active proteases. Therefore, the effects of DTBP cross-linker concentration and incubation time on IAC recovery were examined (Supplementary Fig. 4.5). IACs were isolated from HFF cells spread on FN for 2 h using incubation with 6 mM DTBP for 3 min (Ng et al., 2014), 6 mM DTBP for 5 min (Jones et al., 2015) or 3 mM DTBP for 30 min (Robertson et al., 2015) to cross-link cells (Supplementary Fig. 4.5a). Isolated IACs were analysed by SDS-PAGE and immunoblotting (Supplementary Fig. 4.5b,c).

The total protein amount collected between the three conditions varied (Supplementary Fig. 4.5b). The least amount of protein was recovered from IACs isolated from cells incubated with 6 mM DTBP for 3 min, while increasing the incubation time to 5 min led to a 1.7-fold increase in total protein recovered. Cross-linking with 3 mM DTBP for 30 min increased the amount of total protein collected by a further 2.1-fold compared with incubation with 6 mM DTBP for 5 min. The abundances of known IAC proteins (filamin A, vinculin, ILK and paxillin) showed a similar trend to the amounts of total protein collected (Supplementary Fig. 4.5c). However, the mitochondrial protein BAK and the transferrin receptor were detected in isolated IACs from cells incubated with 3 mM DTBP for 30 mins, indicating unsuccessful removal of cell bodies from these cells. Therefore, these data demonstrated both enrichment of IAC proteins and removal of non-specific IAC components from cells cross-linked with 6 mM DTBP for 5 mins, and so was the preferred cross-linking condition.
Supplementary Figure 4.5. Effects of DTBP cross-linker concentration and incubation time on IAC recovery.

(a) Workflow for the isolation of IACs. HFF cells were spread on FN-coated dishes for 2 h followed by cross-linking with 6 mM DTBP for 3 min, 6 mM DTBP for 5 min or 3 mM DTBP for 30 min (bold). Cell lysis was performed by incubation with cold RIPA buffer for 3 min and a high-pressure water wash. (b) Isolated IACs were analysed by SDS-PAGE. Total protein intensity values for each lane are indicated. Graph shows intensity values normalised to the summed intensities in each experiment. Values are mean ± SEM (n = 2). (c) Immunoblot analysis of IACs isolated using different cross-linking conditions. Cell lysates from cells spread on FN (TCL) were used as a positive control. Molecular weight values (kDa) are displayed to the left of each blot.
4.11.7 Summary

Protocols have been developed to isolate and analyse IACs by MS (Jones et al., 2015; Kuo et al., 2012). Here, an existing protocol used to isolate IACs from HFF cells (Ng et al., 2014) was adapted to allow isolation of IACs from cells treated with DMSO or FAK [i] and the effects of alterations to the protocol on IAC recovery were examined.

Complexes isolated from cells using EB followed by sonication or RIPA buffer followed by a high-pressure water wash for cell lysis both allowed enrichment of IAC proteins. IACs isolated from cells treated with DMSO or FAK [i] for 1 h or 4 h were analysed by MS, which demonstrated comparable detection of adhesome proteins to other MS-derived IAC proteomes, and demonstrated a comparable number of total proteins identified at 1 h and 4 h time points. To allow continued use of the negative control ligand condition, Tf, for identification of non-specific components, treatment with DMSO or FAK [i] for 1 h was maintained. To improve cell attachment efficiency, the concentration of Tf used to coat plates was increased to 50 µg/mL. To improve recovery of IAC components, the RIPA buffer incubation time was set to 3 min and to facilitate the IAC isolation procedure, an additional PBS- incubation step after cell lysis that did not affect efficiency of protein recovery was introduced to the protocol. Finally, the cross-linker concentration was maintained at 6 mM DTBP but the incubation time was increased to 5 min.

The final method (Fig. 4.4a) involved plating HFF cells on Tf-coated (50 µg/mL) and FN-coated (10 µg/mL) plates for 1 h and treating cells with DMSO or FAK [i] for 1 h. Cells were cross-linked with 6 mM DTBP for 5 min, and DTBP was quenched with 200 mM Tris-HCl for 3 min. After removal of Tris-HCl, cells were washed once with PBS- and immediately incubated with RIPA buffer for 3 min, followed by a high-pressure water wash. Plates were stored in PBS- until plates from all conditions had been processed. IACs were collected in adhesion recovery solution and acetone precipitated before analysis by SDS-PAGE, immunoblotting and MS. This method was also used to isolate IACs from cells treated with DMSO, FAK [i], Src [i] or combined FAK [i] and Src [i] (Fig. 4.7).

4.11.8 Supplementary Tables

Supplementary Table 4.1. Proteins identified in adhesion complexes from cells treated with either DMSO or FAK [i] for 1 h or 4 h by mass spectrometry.
4.11.9 Supplementary References


Chapter 5

Analysis of consensus adhesome dynamics during adhesion complex assembly and disassembly

Edward R. Horton\textsuperscript{1,4}, Adam Byron\textsuperscript{1,3,4,5}, Janet A. Askari\textsuperscript{1}, Daniel H. J. Ng\textsuperscript{1}, Stacey Warwood\textsuperscript{2}, David Knight\textsuperscript{2}, Jonathan D. Humphries\textsuperscript{1} and Martin J. Humphries\textsuperscript{1,5}

\textsuperscript{1}Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
\textsuperscript{2}Biological Mass Spectrometry Core Facility, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
\textsuperscript{3}Present address: Edinburgh Cancer Research UK Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XR, UK
\textsuperscript{4}These authors contributed equally to this work
\textsuperscript{5}Correspondence should be addressed to M.J.H. or A.B.:
Professor Martin J. Humphries, Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
Tel.: +44 (0) 161 2755071; Fax: +44 (0) 161 2755082
E-mail: martin.humphries@manchester.ac.uk

Dr Adam Byron, Edinburgh Cancer Research UK Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XR, UK
Tel.: +44 (0) 131 6518575; Fax: +44 (0) 131 7773520
Email: adam.byron@igmm.ed.ac.uk

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5.1 Abstract

The molecular composition and dynamics of integrin adhesion complexes (IACs), which are reported to contain hundreds of components, must be precisely regulated for efficient and effective cell migration. The composition of relatively static IACs has been characterised in multiple cell types, and we previously interrogated these data to define a consensus integrin adhesome of 60 proteins localised in IACs. Here, mass spectrometry-based proteomic analyses of IACs stabilised at multiple time points during cell attachment and IAC disruption enable us to characterise the global composition of IACs during IAC assembly and IAC disassembly. These analyses reveal distinct temporal profiles of protein recruitment relevant to specific cell adhesion processes and, using the consensus adhesome, detail the compositional dynamics of the core cell adhesion machinery.
5.2 Introduction

Cellular attachment to the extracellular matrix (ECM) is mediated by integrin receptors (Hynes, 2002) and their associated multiprotein complexes, termed integrin adhesion complexes (IACs), which have been reported to contain over 200 components in the literature-curated integrin adhesionome (Winograd-Katz et al., 2014; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007). More recently, analyses of IACs using biochemical approaches (Jones et al., 2015; Kuo et al., 2012) combined with mass spectrometry (MS)-based proteomics have reported the integrin heterodimer- and tension-dependent composition of IACs (Byron et al., 2012, 2015; Huang et al., 2014; Humphries et al., 2009; Kuo et al., 2011; Ng et al., 2014; Robertson et al., 2015; Schiller et al., 2011, 2013; Yue et al., 2014). Analyses of multiple proteomic datasets of IACs from multiple cell types has revealed an unanticipated complexity in IAC composition with a 10-fold increase in the scale of identified IAC proteins in the meta-adhesionome, which is a database of 2,412 proteins detected in IAC proteomes (see section 3.3.2), compared with the literature-curated adhesionome (Winograd-Katz et al., 2014). Despite their apparent complexity, IACs are highly dynamic, turning over on a timescale of minutes. This flexibility is important for healthy function and enables dynamic sampling of the cell microenvironment. Dynamics of individual IAC components have been studied for a handful of molecules using mainly microscopy approaches (Bachir et al., 2014; Broussard et al., 2015; Ezratty et al., 2005; Humphries et al., 2007; Lavelin et al., 2013; Rossier et al., 2012). Analysis of IAC components during IAC assembly has revealed the hierarchical recruitment of proteins to IACs (Choi et al., 2011; Zaidel-Bar et al., 2003, 2004) and the development of a biochemical assay to synchronise IAC disassembly across a cell population, which involves treating cells with the microtubule polymerisation inhibitor nocodazole followed by nocodazole removal (Ezratty et al., 2005), has led to a number of mechanistic insights into IAC disassembly (Akhtar and Streuli, 2013; Chao and Kunz, 2009; Cleghorn et al., 2015; Ezratty et al., 2005, 2009; Lansbergen et al., 2006; Peacock et al., 2007; Stehbens et al., 2014; Uchil et al., 2014; Wu et al., 2008).

An emergent property of the meta-adhesionome was the definition of a robustly identified core IAC composition, termed the consensus adhesionome, which uncovered the global core connections that link integrins with the actin cytoskeleton (see section 3.3.5). Cellular adhesion to the ECM combined with the core composition, assembly and turnover of IACs at the adhesion nexus must be spatially and temporally regulated to allow cell-ECM engagement at the leading edge and cell-ECM dissociation at the cell rear during cell migration (Garde1 et al., 2010; Scales and Parsons, 2011; Wehrle-Haller and Imhof, 2003), which has been implicated in a wide variety of pathologies such as cancer and wound repair (Schneider et al., 2010; Winograd-Katz et al., 2014). Current studies of IAC composition represent steady-state cell adhesion and therefore their proteome dynamics have not been characterised. Here, we provided the first global characterisation of IAC composition by MS-based proteomics upon initial cell-ECM attachment and IAC disruption. By filtering the acquired datasets using the meta-adhesionome and consensus adhesionome to demonstrate their value, we found that distinct groups of proteins relevant to specific cell adhesion processes displayed differing dynamics. These data describe IAC composition dynamics and provide a resource for the interrogation of the global molecular connections that link integrin interactions to adhesion signalling and cell migration.
5.3 Results and Discussion

5.3.1 Characterisation of IAC composition during IAC assembly and IAC disassembly

To identify the temporal dynamics of the adhesome, we characterised the composition of IACs by MS during their assembly and disassembly in synchronised cells (Fig. 5.1). To examine IAC assembly during the early stages of cell adhesion, complexes were isolated 3, 9 and 32 minutes after initial cell attachment to fibronectin (FN) (Fig. 5.1a); to examine IAC disassembly, complexes were isolated upon microtubule-induced disassembly and at 5, 10 and 15 minutes after nocodazole removal (Fig. 5.1b) (Ezratty et al., 2005). IACs isolated at each assembly and disassembly time point were analysed by quantitative MS (Supplementary Tables 5.1, 5.2) as previously described (Byron et al., 2012, 2015; Humphries et al., 2009; Ng et al., 2014; Robertson et al., 2015). Since the time course datasets here do not incorporate negative control ligand conditions to take into account potential non-specific protein binding during the IAC isolation process, we filtered the datasets using the meta- and consensus adhesomes (see sections 3.3.2 and 3.3.5, respectively). Across all three time points, 2,240 proteins were identified in the assembly dataset (1,266 proteins from the meta-adhesome, 39 proteins from the consensus adhesome; Fig. 5.1c). Across all four time points in the disassembly dataset, 643 proteins were identified (455 proteins from the meta-adhesome; 43 proteins from the consensus adhesome; Fig. 5.1d). These data characterise the global composition of IAC protein dynamics and will be analysed in subsequent sections to reveal the dynamic profiles of IAC components.
Figure 5.1. Isolation and mass spectrometry-based proteomic characterisation of IAC composition during IAC assembly and disassembly.

(a,b) Schematic workflows for the isolation and MS analysis of IACs during IAC assembly (a) and IAC disassembly (b). For analysis of IAC assembly, K562 cells were incubated with FN-coated beads for 3, 9 and 32 min and IACs were isolated at each time point. For analysis of IAC disassembly, adherent U2OS cells were serum starved for 16 h, treated with 10 µM nocodazole for 4 h and nocodazole washout out (Ezratty et al., 2005). IACs were isolated upon nocodazole removal and at 5, 10 and 15 min after nocodazole washout. Isolated IACs at each time point were analysed by MS.

(c,d) Area-proportional Venn diagrams showing the overlap between the meta-adhesome (proteins identified in one of seven MS datasets of IACs, see section 3.3.2) and proteins identified by MS during IAC assembly (c) or IAC disassembly (d). In each region, the total number of proteins (black text) and the number of proteins identified in the consensus adhesome (bold red text) is indicated. See Supplementary Tables 5.1, 5.2 for details of identified proteins.
5.3.2 Temporal dynamics of the integrin meta-adhesome

Hierarchical clustering analysis of meta-adhesome proteins identified in the temporal IAC profiles revealed distinct dynamics of proteins involved in specific functional processes. Twelve clusters were detected in the assembly dataset (SA1-12, Supplementary Fig. 5.1), and 17 clusters in the disassembly dataset (SD1-17, Supplementary Fig. 5.2). Protein clusters were analysed by functional enrichment analysis to determine whether IAC proteins may be involved in the dynamic regulation of specific functional processes during IAC formation and disruption (Supplementary Figs. 5.1, 5.2; Supplementary Tables 5.3, 5.4).

Proteins involved in membrane organisation and protein transport, which may localise to the plasma membrane to coordinate morphological changes during the initial phases of cell spreading, increased during IAC assembly (SA7, SA9; Supplementary Fig. 5.1). Proteins involved in actin cytoskeletal or adhesive functions were generally more abundant at the later time point of IAC assembly (SA5, Supplementary Fig. 5.1) and decreased during IAC disassembly with minor differences in kinetics between different cluster profiles (SD2-5; Supplementary Fig. 5.2). Analysis of protein domains overrepresented in IACs has revealed the importance and prevalence of the LIM-type zinc finger and actin-binding domains in IACs (see section 3.3.5) (Geiger and Zaidel-Bar, 2012; Kuo et al., 2011; Schiller et al., 2011). Proteins containing actin-binding domains were in high abundance late during IAC assembly (SA5, SA9; Supplementary Fig. 5.1), which suggests that the integrin-actin connection is formed and stabilised late during IAC assembly by these proteins. During IAC disassembly, proteins containing actin-binding and LIM domains decreased in abundance (SD2, SD3; Supplementary Fig. 5.2). Specifically, LIM domain-containing proteins were lost from IACs completely after 15 min, whereas proteins containing the actinin-type actin-binding domain decreased but were still present after 15 minutes IAC disassembly (SD2, SD3; Supplementary Fig. 5.2). In contrast, proteins involved in RNA processing and translation peaked early during IAC assembly followed by a decrease in abundance during IAC maturation (SA1, Supplementary Fig. 5.1) and proteins containing RNA-binding domains were in high abundance early during IAC assembly (SA1, SA8; Supplementary Fig. 5.1), which suggests recruitment of translation machinery to IACs at early time points. Proteins involved in RNA processing and translation increased during IAC disassembly (SD10, Supplementary Fig. 5.2). Proteins involved in RNA processing and translation may be involved in localised protein synthesis at IACs (Chicurel et al., 1998; Willett et al., 2010, 2011), or they may be contaminants isolated during the IAC enrichment procedure. In summary, these data suggest that RNA processing and translation proteins display reciprocal temporal profiles to cytoskeleton and adhesion proteins during IAC assembly and disassembly.

5.3.3 Temporal dynamics of the consensus integrin adhesome

To examine the dynamics of the core adhesion machinery we performed hierarchical clustering analysis of consensus adhesome proteins detected in the temporal IAC profiles. These analyses revealed that different components display distinct dynamics. Six protein groups were identified in the assembly dataset (A1-6, Fig. 5.2), and four proteins groups were determined in the disassembly dataset (D1-4, Fig. 5.3), revealing complexity in the dynamics of the abundance of consensus adhesome proteins during IAC assembly and disassembly.
Figure 5.2. Temporal profiling of the consensus adhesome during IAC assembly.

IACs isolated from K562 cells after 3, 9 and 32 min incubation with FN-coated beads were analysed by MS (Supplementary Table 5.1). (a) Throughout IAC assembly, 39 of the 60 consensus adhesome proteins were identified and were analysed by unsupervised hierarchical clustering, revealing distinct temporal profiles of protein recruitment to IACs. Six clusters, labelled A1-6, were chosen on the basis of a Pearson correlation threshold greater than 0.9 and are indicated by coloured bars. Clusters are shown alongside corresponding profile plots, with the mean temporal profile for each cluster indicated by a red line. Quantitative heat map displays mean spectral counts as a proportion of the maximum spectral count for each given protein. Proteins are labelled with gene names for clarity. Proteins also identified during IAC disassembly (Fig. 5.3, Supplementary Table 5.2) are indicated by an asterisk. Literature-curated adhesome (Winograd-Katz et al., 2014) proteins and their isoforms are in bold. Proteins able to bind actin or integrin are indicated by black bars. (b) Network model of the consensus adhesome (see Fig. 3.6). Node colour represents clusters in a (white, not detected - except ZYX/zyxin). For clarity, the two α-actinin isoforms identified are depicted as one node (α-actinin-1, A4; α-actinin-4, A3).
Figure 5.3. Temporal profiling of the consensus adhesome during IAC disassembly.

IACs were isolated from U2OS cells upon nocodazole removal and 5, 10 and 15 min after nocodazole washout to examine changes in IAC composition throughout IAC disruption (Ezratty et al., 2005) and analysed by MS (Supplementary Table 5.2). (a) Throughout IAC disassembly, 43 of the 60 consensus adhesome proteins were identified and were analysed by unsupervised hierarchical clustering, revealing distinct temporal profiles of protein dissociation from IACs. Four clusters, labelled D1–4, were chosen on the basis of a Pearson correlation threshold greater than 0.9 and are indicated by coloured bars. Clusters are shown alongside corresponding profile plots, with the mean temporal profile for each cluster indicated by a red line. Quantitative heat map displays mean spectral counts as a proportion of the maximum spectral count for each given protein. Proteins are labelled with gene names for clarity. Proteins also identified during IAC assembly (Fig. 5.2, Supplementary Table 5.1) are indicated by an asterisk. Literature-curated adhesome (Winograd-Katz et al., 2014) proteins and their isoforms are in bold. Proteins able to bind actin or integrin are indicated by black bars. (b) Network model of the consensus adhesome (see Fig. 3.6). Node colour represents clusters in a (white, not detected - except SORBS1/ponsin).
β1, α5 and αV integrins increased in abundance as IACs matured (A5, A6; Fig. 5.2), reaching maximum abundance by 30 minutes in this system. More surprisingly, however, the integrin abundance levels were stable throughout IAC disassembly (D3, Fig. 5.3), which was also the case for annexin A1 and transglutaminase-2. These data suggest that plasma membrane-associated proteins remain localised to ventral membrane structures during IAC disassembly, which is inconsistent with previous reports that have shown endocytosis of cell surface α5 integrin during IAC disassembly while total α5 integrin levels remained unchanged (Ezratty et al., 2009). Integrins localised to the plasma membrane may remain bound to FN during IAC disassembly, which has been proposed previously (Palecek et al., 1996), or FN-bound proteins may be released into the extracellular environment during IAC disassembly. As well as being reported to bind integrins (Akimov et al., 2000), transglutaminase-2 is also able to bind and crosslink FN (Jeong et al., 1995; Radek et al., 1993) and so may have been isolated in this way.

During the maturation and disassembly of IACs, an intuitive hypothesis would be that the majority of proteins would increase linearly throughout IAC maturation, and similarly decrease in abundance as IACs disassemble. Temporal analysis of IAC dynamics by MS supported this hypothesis for some proteins. For example, talin and filamin gradually increased in abundance during IAC assembly (A1, Fig. 5.2) and decreased in abundance during IAC disassembly (D4, Fig. 5.3). The unconnected consensus adhesome proteins grouped together: BRIX1, DDX18, DDX27, DMT1, FAU, MRTO4 and PPIB during IAC assembly (A2, Fig. 5.2); and FAU, H1FX, P4HB, PPIB and RPL23A during IAC disassembly (D3, Fig. 5.3). This suggests that these proteins may perform similar roles during adhesion dynamics, or could indicate that the association and identification of these proteins in IACs is non-specific.

Most connected consensus components were generally detected in high abundance at the later time point of IAC assembly, although rather than residing in the same cluster in the assembly dataset they were distributed throughout different cluster profiles (Fig. 5.2), indicating distinct dynamics of protein recruitment. α-actinin was most highly abundant at earliest times of IAC assembly (A3, A4; Fig. 5.2), which is in agreement with studies that have reported an association between α-actinin and β1 integrin in early nascent adhesions that is lost as adhesions mature (Bachir et al., 2014; Choi et al., 2008; Otey et al., 1990). Conversely, α-actinin-associated proteins in the consensus adhesome that contain actin-binding and LIM domains, such as LIM and SH3 protein 1 (LASP1), PDZ and LIM domain protein 1 (PDLIM1), PDLIM5, vasodilator-stimulated phosphoprotein (VASP), vinculin and zyxin; were most highly abundant at the later time point of IAC assembly (A6, Fig. 5.2), which is in support of studies showing hierarchical adhesion site formation (Bachir et al., 2014; Miyamoto et al., 1995; Zaidel-Bar et al., 2003, 2004). Surprisingly, the majority of proteins in the focal adhesion kinase (FAK)-paxillin module of the consensus adhesome were not identified during IAC assembly (Fig. 5.2b), which may indicate that the FAK-paxillin module is recruited to larger, more stable IACs at later time points to those examined here or that only a subset of IAC proteins are recruited to IACs formed on FN-coated beads.

During IAC disassembly, protein abundance of the two largest clusters of consensus adhesome proteins decreased during the time course but with different kinetics (D1, D4; Fig. 5.3) and, with the exception of transglutaminase-2, the integrin-binding proteins were split between these two
clusters. Proteins almost completely absent from IACs after 15 minutes in the consensus adhesome included most of the adaptor proteins from the FAK-paxillin module (FAK, FHL2, FHL3, Hic-5/TGFB1I1, paxillin and TRIP6) and the α-actinin module (LPP and zyxin) (D1, Fig. 5.3b). Proteins that decreased in abundance but were still present after 15 minutes included 13 of the 17 actin-binding proteins, four of which were integrin-binding (filamin, talin, tensin and α-actinin; D4, Fig. 5.3). These data suggest that during IAC disassembly, adaptor proteins located between actin and integrins are lost earlier and at a faster rate than actin-binding proteins and that the integrin-actin linkage is disrupted late in the adhesion disassembly process. Moreover, these data suggest that different modules of consensus components display different dynamic temporal profiles during IAC assembly and disassembly.

5.3.4 Temporal dynamics of adhesion modules during IAC disruption

To confirm the temporal differences in IAC components revealed by MS upon microtubule-induced IAC disassembly (Ezratty et al., 2005), IAC proteins were visualised during nocodazole washout (Fig. 5.4, Supplementary Fig. 5.3). Vinculin, zyxin and α5 integrin were analysed, as each protein displayed different IAC disassembly dynamics and were present in different clusters during disassembly (Fig. 5.3). In addition, phospho-paxillinY118, paxillin, phospho-FAKY397 and β1 integrin were visualised to test whether proteins within the same cluster displayed similar disassembly dynamics (Supplementary Fig. 5.3). Consistent with previous studies (Ng et al., 2014), α5 integrin did not significantly increase upon nocodazole treatment (Fig. 5.4). Upon nocodazole washout, the area of the ventral cell surface covered by α5 integrin did not change (Fig. 5.4), and similar results were obtained for β1 integrin (Supplementary Fig. 5.3). In support of the different rates of loss, the decrease in vinculin (30 min; Fig. 5.4) was delayed compared with the loss of zyxin (10 min; Fig. 5.4) and other adhesion molecules (phospho-FAKY397, 10 min; paxillin and phospho-paxillinY118, 15 min; Supplementary Fig. 5.3). Although the exact timings of loss were variable between the MS and immunofluorescence experiments, which may be because microtubule-induced IAC disassembly occurs at different rates in different cell types or because MS analysis averages over many cells compared with single-cell immunofluorescence analyses; these data validate the findings obtained by MS that indicate different adhesion molecules display distinct temporal profiles during IAC disassembly.
Figure 5.4. Changes in consensus adhesome components during IAC disassembly.
(a) HFF cells treated with DMSO, 10 µM nocodazole or after nocodazole removal at different times were stained for vinculin, zyxin and α5 integrin. Scale bars, 20 µm. (b–d) Quantification of images in a. Vinculin, zyxin and α5 integrin levels were quantified as a proportion of total cell area. Box-and-whisker plots show the median (line), mean (plus sign), 25th and 75th percentiles (box) and 5th and 95th percentiles (whiskers) (n = 10 cells). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; Kruskal–Wallis test with Dunn’s post hoc correction (comparisons with the nocodazole treatment group are shown; see Supplementary Table 5.5 for statistics source data).
In summary, the data presented in this study catalogue the first global characterisation of IAC dynamics during the initial phases of their assembly and disassembly, the coordinated regulation of which is required to permit efficient cell migration. We generated time-course IAC datasets during IAC assembly and disassembly and, by filtering the acquired datasets using the meta-adhesome and the consensus adhesome, the main findings from this study are:

1. Proteins involved in cytoskeletal and adhesive functions display reciprocal temporal profiles to proteins involved in RNA processing and translation during assembly and disassembly of IACs;

2. Consensus adhesome molecules are recruited to, and disassembled from, IACs with distinct kinetics, suggesting that these processes are differentially regulated and are not simply reciprocal events. Therefore it is important to examine multiple adhesion molecules when reporting IAC dynamics;

3. Adaptor and LIM domain-containing proteins are lost from IACs earlier than actin-binding proteins, which we validated by immunofluorescence for consensus adhesome molecules during IAC disassembly, suggesting that the integrin-actin structural connection is disrupted late in the IAC disassembly process and that adaptor proteins may be primary targets for disassembly.

These data not only provide a resource for the research community that detail the global proteome changes in IAC composition during IAC assembly and disassembly, but demonstrate an example of how the meta-adhesome and consensus adhesome can be used by future studies of IACs to interrogate the molecular composition of cell-ECM interactions.
5.4 Acknowledgments

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5.5 Author contributions

A.B. and M.J.H. conceived the project; E.R.H., A.B., J.A.A., D.H.J.N., J.D.H. and M.J.H. designed the experiments and interpreted the results; E.R.H., A.B., J.A.A., D.H.J.N. and J.D.H. performed the experiments and analysed the data; S.W. and D.K. contributed to MS experimental design and data acquisition; E.R.H., A.B., J.D.H. and M.J.H. wrote the paper; all authors commented on the manuscript and approved the final version.

Further details of co-author contributions:

E.R.H. performed hierarchical clustering and functional enrichment analyses of meta-adhesome dynamics (Fig. 5.1, Supplementary Figs. 5.1, 5.2; Supplementary Tables 5.3, 5.4), analysed consensus adhesome dynamics (Figs. 5.2, 5.3) and performed immunofluorescence experiments (Fig. 5.4, Supplementary Fig. 5.3, Supplementary Table 5.5);

A.B. processed MS datasets (Supplementary Tables 5.1, 5.2);
J.A.A. generated the IAC assembly dataset (Fig. 5.1a, Supplementary Table 5.1);
D.H.J.N. generated the IAC disassembly dataset (Fig. 5.1b, Supplementary Table 5.2); and
J.D.H. deposited MS data in ProteomeXchange.

5.6 Competing financial interests

The authors declare no competing financial interests.
5.7 Methods

5.7.1 Reagents

FN and nocodazole were from Sigma-Aldrich. Antibodies used for immunofluorescence were mouse anti-vinculin (hVIN-1, Sigma-Aldrich, V9131; 1:400), rabbit anti-phospho-FAK\(^{\text{Y397}}\) (141-9, Invitrogen, 44-625G; 1:200), rabbit anti-phospho-paxillin\(^{\text{Y118}}\) (Invitrogen, 44-722G; 1:200), mouse anti-paxillin (349/Paxillin, BD Biosciences, 610052; 1:400), mouse anti-zyxin (ZZ001, Thermo Fisher Scientific, 39-6000; 1:400), rat anti-α5 integrin (mAb11, provided by K. M. Yamada; 1:200) and rat anti-β1 integrin (9EG7, provided by D. Vestweber; 1:200). Secondary antibodies were from Jackson Immunoresearch.

5.7.2 Cell culture

Human chronic myelogenous leukaemia (K562) cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (FCS; Lonza Bioscience) and 2 mM L-glutamine. Telomerase-immortalised human foreskin fibroblast (HFF, provided by K. Clark) and osteosarcoma (U2OS) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FCS and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified 5% (v/v) CO\(_2\) atmosphere.

5.7.3 IAC isolation

IACs were isolated using a similar approach to the ligand affinity purification method described previously (Humphries et al., 2009; Jones et al., 2015). For analysis of IAC assembly, paramagnetic beads (4.5 μm-diameter; M-450 Dynabeads; Life Technologies) coated with FN were incubated with K562 cells in DMEM supplemented with 0.2% (w/v) BSA and 25 mM HEPES at 70 rpm for either 1, 7 or 30 min at 37 °C. Bead-bound cells were incubated with the membrane permeable cross-linker dimethyl-3,3'-dithiobispropionimidate (DTBP; Sigma Aldrich; 10 mM, 2 min) and lysed, and isolated protein complexes were washed and eluted as described previously (Humphries et al., 2009).

For analysis of IAC disassembly, U2OS cells plated on FN-coated dishes were serum-starved for 16 h, treated with 10 μM nocodazole for 4 h, washed three times with DMEM and incubated for appropriate times after nocodazole removal at 37 °C, 5% (v/v) CO\(_2\) (Ezratty et al., 2005). Cells were incubated with DTBP (6 mM, 3 min), and DTBP was quenched with 1 M Tris-HCl (pH 8.5), followed by a combination of cell lysis in ice-cold extraction buffer [20 mM NH\(_4\)OH, 0.5% (w/v) Triton X-100] and sonication for 2.5 min (VibraCell VCX 500; Sonics & Materials) to lyse cells. Protein complexes left bound to the substrate were washed five times with PBS, recovered by scraping in 100 μl recovery solution [125 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 15% (v/v) β-mercaptoethanol] and incubated at 70 °C for 10 min.

5.7.4 Immunoblotting

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Whatman). Membranes were blocked and probed as described previously (Humphries et al., 2009). Secondary
antibodies used were donkey Alexa Fluor 680-conjugated anti-goat IgG or anti-mouse IgG (Life Techno-
logies) and donkey IRDye 800-conjugated anti-mouse IgG (Rockland Immunochemicals). Membranes
were washed in the dark and scanned using the Odyssey infrared imaging system (LI-COR).

5.7.5 MS data acquisition

Following SDS-PAGE, gel lanes were sliced and subjected to in-gel digestion with trypsin (Shevchenko et al., 1996) with modifications (Humphries et al., 2009). Peptide samples were analysed by liquid chromatography (LC)-tandem MS using a nanoACQUITY UltraPerformance LC system (Waters) coupled online to an LTQ Velos mass spectrometer for analysis of IAC assembly (Thermo Fisher Scientific) or using an UltiMate 3000 Rapid Separation LC system (Thermo Fisher Scientific) coupled online to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) for analysis of IAC disassembly. Peptides were concentrated and desalted on a Symmetry C_{18} preparative column (20 mm × 180 μm, 5-μm particle size; Waters) and separated on a bridged ethyl hybrid C_{18} analytical column (250 mm × 75 μm, 1.7-μm particle size; Waters) using a 45 min linear gradient from 1% to 25% or 8% to 33% (v/v) acetonitrile in 0.1% (v/v) formic acid for IAC assembly or IAC disassembly samples, respectively, at a flow rate of 200 nl/min. Peptides were selected for fragmentation automatically by data-dependent analysis.

5.7.6 MS data analysis

MS data were searched using an in-house Mascot server (version 2.2.03; Matrix Science) (Perkins et al., 1999) as described previously (Byron et al., 2015). Mass tolerances for precursor and fragment ions were 0.4 Da and 0.5 Da, respectively, for LTQ Velos data (assembly dataset) or 5 ppm and 0.5 Da, respectively, for Orbitrap Elite data (disassembly dataset). Data were validated in Scaffold (version 3.00.06; Proteome Software) using a threshold of identification of at least 90% probability at the peptide level, assignment of at least two unique, validated peptides, and at least 99% probability at the protein level. These acceptance criteria resulted in an estimated protein false discovery rate of ≤0.1% for both datasets. MS data were quantified as follows: raw normalised spectral counts were normalised to the total number of spectra identified in the corresponding sample, the mean normalised spectral counts were calculated across biological replicates (n = 2 and n = 3 for IAC assembly and IAC disassembly datasets, respectively) as described previously (Byron et al., 2015). Mean normalised spectral counts were displayed as a percentage of the maximum mean normalised spectral count for each protein across the time course to allow comparison between proteins.

5.7.7 MS data deposition

MS data were deposited in ProteomeXchange (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) with the dataset identifiers PXD002159 (IAC assembly dataset) and PXD002129 (DOI: 10.6019/PXD002129; IAC disassembly dataset). Details of all identified proteins are provided in Supplementary Tables 5.1 and 5.2 for the IAC assembly and IAC disassembly datasets, respectively.
5.7.8 Hierarchical clustering analyses

Meta-adhesome and consensus adhesome proteins identified during IAC assembly and IAC disassembly were hierarchically clustered on the basis of uncentred Pearson correlation using Cluster 3.0 (C Clustering Library, version 1.50) (de Hoon et al., 2004) and visualised using Java TreeView (version 1.1.5) (Saldanha, 2004). Distances between hits were computed using a complete-linkage matrix in all cases.

5.7.9 Interaction network analyses

The consensus adhesome network was constructed as described previously (see sections 3.8.9 and 3.8.11). Briefly, proteins enriched in at least five proteomic datasets in the meta-adhesome database were incorporated into the consensus adhesome, excluding ECM or secreted proteins, and evidence for protein-protein interactions was manually verified and scored (see Supplementary Tables 3.6-3.9). Node colour represents hierarchical protein clusters defined during IAC assembly and IAC disassembly. Zyxin and ponsin were detected in the IAC assembly and disassembly datasets, respectively, but were not assigned into protein cluster profiles and were therefore depicted as white nodes.

5.7.10 Functional enrichment analyses

Functional enrichment analysis was performed using DAVID (version 6.7) (Huang et al., 2009). Keywords with fold enrichment ≥ 1.5, Bonferroni-corrected P value < 0.05, EASE score (modified Fisher’s exact test) < 0.05 and at least two proteins per keyword were considered significantly overrepresented.

5.7.11 Immunofluorescence microscopy

To validate MS data of IAC disassembly, HFF cells were treated with nocodazole and nocodazole was washed out as described previously (Ezratty et al., 2005). Cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde for 7 min at room temperature (RT), permeabilised with 0.5% (v/v) Triton X-100 for 10 min, and then washed and incubated with antibodies as described previously (Byron et al., 2015). Images were acquired on a Delta Vision RT (Applied Precision) restoration microscope using a 60×/1.42 Plan Apo objective and the Sedat filter set (Chroma 89000). Images were collected with a z optical spacing of 0.2 μm, five images per stack, using a Coolsnap HQ camera (Photometrics) and Softworx software (Applied Precision).

5.7.12 Image analysis and quantification

Maximum intensity projections of raw images were generated and background filtered (rolling ball, 10-pixel radius) using ImageJ (version 1.48o) (Schindelin et al., 2012). Areas containing positive staining of IAC proteins were measured and normalised to total cell area. Box-and-whisker plots were generated using Prism (version 6.04; GraphPad). Figures were assembled using Illustrator (Adobe).
5.8 Supplementary Figures and Tables

Supplementary Table 5.1. Proteins identified during integrin adhesion complex assembly by mass spectrometry.

Supplementary Table 5.2. Proteins identified during integrin adhesion complex disassembly by mass spectrometry.

Supplementary Table 5.3. Functional enrichment analysis of meta-adhesome proteins co-clustered during integrin adhesion complex assembly.

Supplementary Table 5.4. Functional enrichment analysis of meta-adhesome proteins co-clustered during integrin adhesion complex disassembly.

Supplementary Table 5.5. Statistics source data for quantification of cell adhesion area during nocodazole washout.
Supplementary Figure 5.1. Hierarchical clustering analysis of meta-adhesome proteins identified during IAC assembly.

IACs were isolated from K562 cells after 3, 9 and 32 min incubation with FN-coated beads and analysed by MS. Throughout IAC maturation, 1,266 of the 2,412 meta-adhesome proteins were identified (Fig. 5.1c, Supplementary Table 5.1) and were analysed by unsupervised hierarchical clustering, revealing distinct temporal profiles of protein recruitment to IACs. Quantitative heat map displays mean spectral counts as a proportion of the maximum spectral count for each given protein. Twelve clusters were chosen on the basis of a Pearson correlation threshold greater than 0.8, labelled SA1–12, and are indicated by blue and green bars. Literature-curated adhesome (Winograd-Katz et al., 2014) and consensus adhesome proteins identified in each cluster are indicated by gene name (bold, literature-curated and consensus adhesome; regular, consensus adhesome; italic, literature-curated adhesome). Clusters are shown alongside corresponding profile plots, with the mean temporal profile for each cluster indicated by a red line. The most significantly overrepresented functional annotations for selected clusters are listed. Full details of enriched functional terms are provided in Supplementary Table 5.3.
Supplementary Figure 5.2. Hierarchical clustering analysis of meta-adhesome proteins identified during IAC disassembly.

IACs were isolated from adherent U2OS cells upon nocodazole removal and at 5, 10 and 15 min after nocodazole washout to examine changes in IAC composition throughout IAC distruption (Ezratty et al., 2005). Isolated IACs at each time point were analysed by MS. Throughout IAC disassembly, 455 of the 2,412 meta-adhesome proteins were identified (Fig 5.1d, Supplementary Table 5.2) and were analysed by unsupervised hierarchical clustering, revealing distinct temporal profiles of protein dissociation from IACs. Quantitative heat map displays mean spectral counts as a proportion of the maximum spectral count for each given protein. Seventeen clusters were chosen on the basis of a Pearson correlation threshold greater than 0.8, labelled SD1–17, and are indicated by blue and green bars. Literature-curated adhesome (Winograd-Katz et al., 2014) and consensus adhesome proteins identified in each cluster are indicated by gene name (bold, literature-curated adhesome and consensus adhesome; regular, consensus adhesome; italic, literature-curated adhesome). Clusters are shown alongside corresponding profile plots, with the mean temporal profile for each cluster indicated by a red line. The most significantly overrepresented functional annotations for selected clusters are listed. Full details of enriched functional terms are provided in Supplementary Table 5.4.
Supplementary Figure 5.3. Changes in additional consensus adhesome components during IAC disassembly.

(a) To examine IAC dynamics during microtubule-induced IAC disassembly (Ezraty et al., 2005), HFF cells treated with DMSO, 10 µM nocodazole or after nocodazole removal at different times were stained for phospho-paxillinY118, paxillin, phospho-FAKY397 and β1 integrin. Scale bars, 20 µm.

(b–e) Quantification of images in a. Phospho-paxillinY118, paxillin, phospho-FAKY397 and β1 integrin levels were quantified as a proportion of total cell area. Box-and-whisker plots show median (line), mean (plus sign), 25th and 75th percentiles (box) and 5th and 95th percentiles (whiskers) (n = 10 cells). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; Kruskal–Wallis test with Dunn’s post hoc correction (see Supplementary Table 5.5 for statistics source data).
5.9 References


Chapter 6

Discussion

6.1 Overview

Multiple studies have determined the composition of IACs in a variety of contexts. The first systems-level analysis of IACs led to the creation of the literature-curated adhesome (Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007), which currently consists of 232 members (Winograd-Katz et al., 2014). The literature-curated adhesome consists of 20 functional protein groups that are involved in cell-ECM interactions and has revealed an unanticipated complexity in IAC composition (Zaidel-Bar et al., 2007). More recently, several studies have isolated IACs using a variety of approaches (Jones et al., 2015; Kuo et al., 2012) and analysed their composition by mass spectrometry (MS), which has revealed novel insights into effects of integrin heterodimers (Byron et al., 2012, 2015; Humphries et al., 2009; Schiller et al., 2013), tension (Kuo et al., 2011; Schiller et al., 2011, 2013), microtubules (Ng et al., 2014; Yue et al., 2014) and GEF-H1-silencing (Huang et al., 2014) on IAC protein composition. In addition, the phosphorylation profile of IACs has been reported (Robertson et al., 2015). Here, current and new proteomic datasets of IACs were integrated and analysed to permit a systems-level interrogation of the global composition, structure and topology of IACs at the molecular-level.

While several IAC proteomic datasets have been generated, a comprehensive analysis of these datasets in combination, which has currently not been performed, could lead to hypotheses describing how IACs influence global cell behaviour. Initially, all published and several unpublished quantitative proteomic datasets of FN-induced IACs that were generated from multiple cell types using multiple experimental approaches in different laboratories were analysed and compared (chapter 3), which revealed heterogeneity in IAC composition between different datasets. The proteomic datasets were combined to create the meta-adhesome database, which demonstrated a large increase in the scale and complexity of IAC composition compared to analyses of IAC datasets individually or the literature-curated adhesome. However, comparison of the meta-adhesome with the literature-curated adhesome indicated that the meta-adhesome likely represents a snapshot of the FN-specific IAC composition and that IAC isolation methodologies are suited to the identification of particular adhesome functional groups, such as adaptors and actin regulators, than others. The increased scale in the number of proteins identified in IAC proteomes raises the question what are the additional identified proteins and what are their functional roles in IACs? Functional analyses of proteins in the meta-adhesome revealed that the meta-adhesome consists of proteins involved in a wide range of cellular processes that impact on global cell function and cell fate. Furthermore, an emergent property of the meta-adhesome was the definition of a robustly identified IAC core of 60 proteins, termed the consensus adhesome. The consensus adhesome represents commonly identified IAC proteins and consists of both canonical and non-canonical IAC components that form the structural connection between integrins and actin.
While the consensus adhesome represents proteins commonly identified in IAC proteomes, the modular nature of the adhesome has not been fully investigated. While it has been suggested that IACs may contain a modular substructure (Byron et al., 2010; Zaidel-Bar et al., 2007), which is supported by a study that demonstrated that IAC components assemble in the cytoplasm and are recruited to IACs as pre-formed complexes (Hoffmann et al., 2014), it is unknown whether inhibition of key control points in the network causes particular protein modules to dissociate from IACs. To address these issues and to investigate the robustness of the IAC network to perturbation, effects of pharmacological inhibition of the IAC components FAK and Src on IACs were examined (chapter 4). Inhibition of FAK kinase activity upon treatment with AZ13256675 was confirmed by immunoblotting and immunofluorescence analyses. A workflow was adapted that allowed the isolation of IACs upon FAK inhibition and analyses of this MS-based proteomics dataset revealed that IACs are largely robust to FAK inhibition at the protein-level. By immunoblotting, IAC composition was also found to be insensitive to Src inhibition (using AZD0530) and upon combined inhibition of both FAK and Src. However, phosphorylation at IACs and biologically-relevant readouts of adhesion signalling, such as cell migration and cell proliferation, were sensitive to kinase inhibition, which suggests that signalling propagated through IACs, but not the protein composition of IACs, is dependent on kinase activity. These data challenge the view of how IACs relay chemical signals while maintaining a mechanical connection between integrins and actin.

Finally, to illustrate the usefulness of the meta-adhesome and consensus adhesome as resources to filter non-controlled IAC proteomic datasets, IAC dynamics were examined (chapter 5). To demonstrate this, two datasets detailing the composition of IACs during the initial phases of IAC assembly and IAC disassembly were interrogated in the context of the meta-adhesome and consensus adhesome. Analyses of these data suggested that IAC assembly and disassembly are not reciprocal events, and demonstrated that different IAC proteins display different dynamics. Moreover, analyses of IAC disassembly revealed that adaptor proteins were lost from IACs earlier than a group of actin-binding proteins, indicating that adaptor proteins located between integrins and actin may be primary targets for disassembly. In summary, the data presented here demonstrate several examples of how MS-derived IAC datasets can be interrogated to reveal novel insights and hypotheses into adhesion biology.

6.2 Comparison of IAC proteomes and construction of the meta-adhesome

One of the most surprising outcomes to arise from comparative analyses of IAC proteomes was the relatively low level of overlap and similarity in identified proteins observed between IAC datasets (section 3.3.1). To reduce heterogeneity between datasets as much as possible, the seven IAC datasets that were compared and incorporated into the meta-adhesome were generated from IACs isolated from cells exposed to similar FN-rich cellular microenvironments and from unperturbed experimental conditions. However, the datasets used different negative controls, cross-linkers and cell lysis approaches and IACs were isolated from different cell types that had been allowed to spread for different times. Analyses suggested that the choice of negative control appeared to be important, since datasets co-clustered according to the choice of negative control ligand. To
increase confidence in the identification of potentially novel IAC proteins, complexes isolated from negative control conditions that allow integrin-independent cell attachment are necessary to reduce the number of non-specific IAC components identified in downstream analyses (Jones et al., 2015). Negative control ligands that have been used include a VCAM-1 mutant (D40A) (Byron et al., 2012; Humphries et al., 2009), which does not bind integrins due to a mutation in the integrin binding site of VCAM-1; transferrin (Robertson et al., 2015), which is the ligand for the transferrin receptor; and poly lysine (Ng et al., 2014; Schiller et al., 2011, 2013), which allows cell attachment through electrostatic interactions (Schöttelndreier et al., 1999) (Fig. 1.5). Therefore, in future studies that analyse IAC composition by MS, it may be beneficial to incorporate multiple negative control ligand conditions to remove bias introduced from the use of one particular negative control. An alternative approach would be to compare the IAC proteome with the total cellular proteome, which would allow IAC-specific proteins to be identified more easily. One approach to reduce heterogeneity introduced from methodological differences between future datasets would be to streamline the methods used to isolate IACs and apply a uniform method to the isolation of IACs from multiple cell types, which would allow cell type-specific variations in IAC composition to be examined. Additional studies that use similar methods from multiple cell types, if technically possible, will help to determine whether the variation observed in IAC proteomes is predominantly due to cell-type, methodological or time-dependent effects. This is problematic, however, since methodology-specific variations in protocols used to isolate IACs such as cell culture/ligand binding, use of cross-linkers and cell lysis approach are typically cell type-dependent (Jones et al., 2015). In addition, the type and number of IACs in cells will vary hugely depending on the cell culture conditions, such as cell passage number and medium conditions. Therefore, it is almost impossible to standardise experimental conditions across multiple cell types to obtain a definitive comparison between datasets. In contrast, negative control ligand conditions are not necessarily required for comparisons of known IAC protein abundances between different experimental conditions (Byron et al., 2015; Huang et al., 2014; Kuo et al., 2011; Yue et al., 2014) (Fig. 1.5). This places importance on the biological question being addressed: is the aim of the study to identify candidate novel IAC components or to detect compositional changes in IACs between experimental conditions?

Despite variations in their experimental designs, current studies that have performed MS-based proteomic analyses of IACs have revealed context-dependent inventories of proteins located at IACs. An interesting observation was that many components of the literature-curated adhesome were not identified in any IAC datasets (section 3.3.3). One interpretation of this could be that these proteins may be cell type-specific components that were not identified due to the choice of cell types incorporated in the meta-adhesome. Many of the adhesion receptors were not identified, which is because datasets incorporated in the meta-adhesome were from cells exposed to similar FN-rich microenvironments that would exclude the identification of non-FN-binding integrins. Another explanation is that the evidence for the inclusion of some of the unidentified literature-curated adhesome components may need re-examining. Alternatively, some of the unidentified literature-curated adhesome components may be in low abundance and are therefore difficult to detect by MS, or are highly context-specific as the literature-curated adhesome is based on data generated from different cells, ligands and experimental conditions (Winograd-Katz et al., 2014). To
investigate these issues further, an additional analysis has been performed (Fig. 6.1). The baseline
expression profile of one of the cells lines used, A375 melanoma cells, was examined using the
Expression Atlas website (http://www.ebi.ac.uk/gxa/home). Many IAC proteins from the literature-
curated adhesome with high expression were not identified in any of the IAC datasets analysed
here. Conversely, many proteins with low expression profiles were identified by these proteomic
approaches (e.g. migfilin, filamin C, ponsin, palladin, GIT2 and ILK). Therefore, this analysis
indicates that the proteomic analysis of IAC components is able to identify low abundance IAC
components and does not simply identify the most highly expressed proteins from these cells, and
indicates that there is little correlation between proteins identified in the consensus adhesome and
expression level.

In contrast, protein groups that were well represented in the meta-adhesome were adaptors and
actin regulators, and the meta-adhesome identified a higher proportion of intrinsic components that
localise directly to IACs than associated members. Intrinsic components are likely to be more
stable in IACs and are therefore isolated more easily, while associated components interact
transiently and are more likely to be lost upon IAC isolation. One possible method that could be
used in future studies to identify more peripherally associated components would be to use
proximity biotinylation labelling (Roux et al., 2012). Using this approach, a bacterial biotin ligase is
fused to the protein of interest and neighbouring proteins are biotinylated in the presence of biotin.
Biotinylated proteins are extracted using streptavidin affinity chromatography and are analysed by
MS (Roux et al., 2012). Proximity biotinylation labelling was recently used to examine the
composition of E-cadherin cell-cell based junctions (Guo et al., 2014) and could be applied to the
analysis of IACs. In summary, IAC proteomes incorporated in the meta-adhesome largely
represent structural members of the adhesome. However, known IAC components not identified by
IAC proteomes may be highly cell type- or context-specific, or difficult to isolate and detect by MS,
and therefore their exclusion from IAC proteomes does not indicate that they are functionally
redundant.

One of the most striking outcomes to arise from proteomic analyses is the increased scale in the
number of proteins identified in IACs compared with the number of proteins reported in the
literature-curated adhesome and from candidate-based studies (Geiger and Zaidel-Bar, 2012;
Winograd-Katz et al., 2014), and the scale of the meta-adhesome extends this complexity further
with a 10-fold increase in the number of proteins identified (section 3.3.2). Functional analyses of
the meta-adhesome (section 3.3.4) revealed that some proteins present in IAC proteomes are
involved in adhesion-related functions, which confirms the successful isolation and enrichment of
IACs using these biochemical approaches. Other functional roles of meta-adhesome proteins were
more unexpected, such as proteins involved in RNA processing and translation, for example.
These proteins may localise to IACs to allow localised protein synthesis, which is supported by
several studies that have demonstrated localisation of translation machinery (Chicurel et al., 1998;
Willett et al., 2010, 2011), β-actin mRNA (Buxbaum et al., 2014; Katz et al., 2012) and the
endoplasmic reticulum (Zhang et al., 2010) in the vicinity of IACs. This raises an important issue
regarding the definition of IACs. Many studies use canonical IAC components such as vinculin or
paxillin to define IAC areas, and proteins were characterised as literature-curated adhesome
components if they colocalise with integrins and/or are functionally involved in adhesion (Geiger
Figure 6.1. Baseline expression profile of adhesome components in A375 cells. The baseline expression profile of one of the cell lines used in the construction of the meta-adhesome, A375 melanoma cells, was examined using the Expression Atlas website (http://www.ebi.ac.uk/gxa/home). RNA-seq data generated by Genentech was extracted for this cell line and expression levels of proteins from the consensus adhesome and literature-curated adhesome were analysed. Proteins with high expression are labelled. Box-and-whisker plot shows the median (line), 25th and 75th percentiles (box) and 5th and 95th percentiles (whiskers) ($n = 60$ consensus adhesome proteins, 85 literature-curated adhesome proteins identified in 1–4 IAC proteomic datasets and 115 unidentified literature-curated adhesome proteins). ****$P < 0.0001$; Kruskal–Wallis test with Dunn’s post hoc correction. FPKM, fragments per kilobase of transcript per million mapped reads.
and Zaidel-Bar, 2012; Zaidel-Bar et al., 2007). Unlike other organelles, IACs do not have a clear boundary and are highly dynamic so it is difficult to define exactly where an IAC begins and ends (Geiger and Zaidel-Bar, 2012), especially concerning the overlapping area between IACs and actin (see section 3.4 and Supplementary Table 3.12). It is likely that the IAC isolation methods isolate patches of membrane close to IACs and short struts of actomyosin that are linked to IACs. Therefore, it is the vicinity of the IAC that is isolated and analysed by MS. In addition, another explanation for the identification of some functional groups in IACs is that these proteins may have multiple independent roles in cells that are consequently enriched in the functional analyses of the meta-adhesome. For example, IAC components are present in cadherin-based cell-cell junctions (Guo et al., 2014; Zaidel-Bar, 2013) and have been implicated in growth factor signalling (Eberwein et al., 2015), which may explain why these processes were enriched in the meta-adhesome. Alternatively, it may be that links between adhesion and unexpected functional processes have currently not been characterised, or some of these proteins may be non-specific components. However, one intriguing and outstanding question is why are these proteins enriched to FN-induced IACs compared with complexes isolated from cells attached to negative control substrates? These proteins may be present in high abundance in cells or may be located near to IACs and are therefore cross-linked in the IAC isolation process and are isolated non-specifically, but have no functional role in adhesion. Further work is required to investigate the functional contributions of the non-canonical IAC components to cell-ECM adhesion.

6.3 The consensus adhesome

An emergent property of the meta-adhesome was the identification of 60 proteins commonly enriched in FN-induced IAC proteomes, termed the consensus adhesome (section 3.3.5). Network analyses of the consensus adhesome revealed that 41 proteins form a protein-protein interaction network that was constructed from evidence based upon interactions reported in the literature. The resulting consensus adhesome network suggested four interconnected axes that link integrins to actin, which were compiled based on reported functional protein groups described in the literature. The interactions in the network used to define these axes represent known interactions; however, additional interactions may occur that are currently undefined (Huttlin et al., 2015; Rolland et al., 2014). The number of reported protein-protein interactions is constantly evolving and several large-scale proteomics-based studies are currently aiming to characterise the global protein-protein interactome in cells (Huttlin et al., 2015; Rolland et al., 2014). Additional interactions between consensus adhesome components observed from such large-scale studies could be incorporated into the consensus adhesome network in the future. One intriguing observation in the current network is the low number of interactions between the group of proteins containing the ILK-PINCH-Parvin (IPP) complex (Wickström et al., 2010) compared with the rest of the network. As suggested by others, the IPP complex may be recruited to IACs as a pre-assembled complex (Hoffmann et al., 2014), which could then be anchored to IACs through interactions with paxillin (Lorenz et al., 2008; Nikolopoulos and Turner, 2001, 2002; Wang et al., 2008) and kindlin (Fukuda et al., 2014). Further studies are required to characterise the complete interactions between all consensus components, which could be achieved using proximity biotinylation labelling (Roux et al., 2012) (see section 6.2).
Out of the 60 proteins incorporated in the consensus adhesome, we found no evidence that 18 components are able to interact with any other consensus components, or to actin. Whether these unconnected components in the consensus adhesome are able to interact with the rest of the network remains unresolved, and the interactions between the unconnected components and canonical IAC proteins may be uncharacterised. The question remains as to whether the unconnected components are functionally important in adhesion, whether they truly localise to IACs or whether they interact with IAC components. For example, the unconnected consensus adhesome components signal-induced proliferation-associated 1 (SIPA1) and LIM domains containing 1 (LIMD1) have been shown to be involved in adhesion-related functions (Bai et al., 2011; Zhang et al., 2015). Another explanation is that these components were isolated non-specifically and are contaminants from the IAC isolation process. In support of this, the unconnected IAC components generally co-clustered during IAC assembly and IAC disassembly (section 5.3.3). In addition, of the 42 consensus adhesome proteins also identified in an additional IAC proteomics dataset (Kuo et al., 2011), only three of the unconnected consensus proteins (ALYREF, PPIB and P4HB) were identified. To confirm the IAC localisation of consensus adhesome components, the IAC localisation of two underappreciated consensus components, Rsu-1 and caldesmon, was analysed by immunofluorescence using antibodies, which revealed that both components localise to vinculin-positive IAC structures (section 3.3.5). Other underappreciated components could be analysed by immunofluorescence microscopy using antibodies or overexpression of consensus components tagged with fluorescent probes, if reagents are available. To identify additional connections in the consensus adhesome, the unconnected components could be affinity purified from cells and the collected complexes analysed by MS to identify possible binding partners. Further studies involving genetic depletion of consensus components in model organisms such as mice and flies, or using siRNA in cells, and analysis of their effects on adhesion-related functions such as cell attachment, spreading and migration will help to delineate proteins involved in adhesion-related functional processes from non-specific IAC consensus components. For example, a recent study analysed the effects of depleting literature-curated adhesome homologues in nematodes using a siRNA-based screen (Etheridge et al., 2015), which could be repeated by targeting consensus adhesome proteins. To summarise it is apparent that additional work is required to either confirm or exclude the role of the full complement of consensus adhesome components identified here in cell-ECM adhesion.

The consensus adhesome represents commonly identified IAC components in FN-induced IACs. However, only 10 components were enriched in all seven datasets, indicating that heterogeneity in IACs still exists between datasets within the consensus adhesome. In particular, some well characterised IAC components were not enriched in all seven datasets, some of which are due to cell-type specific expression; for example, K562 cells do not express kindlin-2 or β3 integrin (Geiger et al., 2012). Paxillin, FAK and kindlin-2 were enriched in all six datasets using adherent cells and in the IAC disassembly dataset, but they were not enriched in the dataset generated using bead-bound K562 cells or the IAC assembly dataset, which was also generated using bead-bound K652 cells. These cells attach to FN-coated beads in solution rather than spreading on FN-coated plates and may not, therefore, form mature focal adhesions required for the substantive recruitment of some adhesion molecules. Additionally, β3 integrin was not enriched in one dataset.
generated from mouse kidney fibroblast (MKF) cells (MKF1), which suggests that these cells preferentially use β1 integrins. Talin was enriched in all datasets except for one dataset that also used MKF cells (MKF3), in which it was 1.92-fold enriched to FN-induced IACs compared with complexes isolated from the negative control, which excluded this protein from that particular dataset as only those proteins enriched at least two-fold over the control were included in each dataset to reduce the identification of non-specific IAC components.

IACs are highly dynamic structures that can be characterised as nascent adhesions, focal complexes, focal adhesions, fibrillar adhesions, invadopodia and podosomes depending on their size, localisation and maturation state (Cukierman et al., 2002; Geiger et al., 2001; Linder, 2009; Zaidel-Bar et al., 2004). Current approaches to isolate IACs and analyse their composition by MS-based proteomics result in the combined analysis of these heterogeneous IAC structures from a cell population to give a compositional snapshot at a particular time point. Several studies have sought to investigate the effects of IAC maturation state and tension on IAC composition by treating cells with the myosin II inhibitor blebbistatin (Kuo et al., 2011; Schiller et al., 2011, 2013), which causes IACs to disassemble and therefore IACs isolated from blebbistatin-treated cells represent a more homogeneous population of smaller nascent adhesions and focal complexes. These studies have gained valuable insight into the identification of mechanosensitive proteins in IACs such as the class of LIM domain-containing proteins that are present in more mature IACs but are reduced upon blebbistatin treatment (Schiller and Fässler, 2013). A high proportion of consensus adhesome proteins are LIM domain-containing proteins (15/60 proteins), which may be lost in smaller nascent adhesions and focal complexes. In addition, the lowest number of consensus components was identified in the dataset generated from K562 cells using FN-coated beads, with a high proportion of the absent proteins being force-sensitive LIM domain-containing proteins. This suggests that this dataset likely represents immature IAC structures, such as focal complexes and nascent adhesions that form before the application of myosin-generated cytoskeletal forces. Therefore, these data suggest that the consensus adhesome contains commonly identified IAC molecules from both nascent and mature IACs as defined by proteomics, and that immature IACs formed in the absence of cytoskeletal force share many compositional similarities to mature IACs that are under tension. Techniques that allow the isolation of specific-IAC structures are required to assess the global composition of native heterogeneous IAC structures more completely, which in the future could be achieved using imaging MS (Römpp and Spengler, 2013; Steinhauser and Lechene, 2013), which allows MS analysis of specific selected cell areas. However, improved spatial resolution would be required to apply this technique to analysis of IACs. In summary, the consensus adhesome represents a network-based view of commonly identified proteins in IACs that defines the structural connections between integrins and actin in the context of FN-mediated cell adhesion.

6.4 Robustness of the IAC network to kinase inhibition

The consensus adhesome represents the structural composition of IACs that links integrins to the actin cytoskeleton. One outstanding question is how robust are IACs to inhibition of key control points in the network. One hypothesis would be that protein perturbation induces IAC disassembly and the integrity of the network architecture is compromised. It may be that specific modules dissociate from IACs upon protein inhibition, which would suggest that key proteins regulate the
recruitment and maintenance of particular sub-complexes in IACs. While numerous studies have examined effects of small molecule inhibition or knockdown of individual IAC components on adhesion using targeted methods such as immunofluorescence microscopy (Carisey et al., 2013; Ezratty et al., 2005; Plotnikov et al., 2012; Webb et al., 2004) and it has been shown that IACs are sensitive to force inhibition (Kuo et al., 2011; Schiller et al., 2011, 2013), the effects of perturbation of individual IAC kinases on IACs at the systems-level have not been characterised. To investigate the robustness of the IAC network, effects of inhibiting the activity of the key adhesion signalling hub FAK were examined, followed by analysis of IACs upon Src inhibition (chapter 4). To analyse effects of FAK inhibition on IACs at the systems-level, IACs were isolated and analysed by MS upon FAK inhibition (section 4.3.3). These data, in combination with analysis upon Src inhibition (section 4.3.5), demonstrated that IACs are robust at the protein-level to the absence of FAK/Src activity and tyrosine-based phosphorylation events at IACs. This result was surprising since FAK and Src are two of the most connected kinases in IACs (Winograd-Katz et al., 2014; Zaidel-Bar et al., 2007) and IACs are enriched for tyrosine-based phosphorylation (Ballestrem et al., 2006; Robertson et al., 2015). Other studies have demonstrated that inhibition of the kinase mechanistic target of rapamycin (mTOR) results in unperturbed IAC composition (Caroline Roberts, unpublished data), while in contrast inhibition of cyclin dependent kinase 1 (Cdk1) resulted in IAC disassembly (Robertson et al., 2015). Inhibition of Cdk1 activity, however, resulted in disruption of the actin cytoskeletal network and may have caused IACs to disassemble indirectly through loss of actomyosin-generated contractility exerted on IACs. These findings collectively suggest that IAC protein composition regulates the mechanical connection between integrins and actin, while kinases and phosphorylation contribute to adhesion signalling and relay of chemical signals. In summary, we conclude that FAK and Src kinase activity are not required to maintain IAC protein composition, but are required for phosphorylation-based adhesion signalling. This suggests that FAK and Src kinase activities do not regulate the recruitment and maintenance of protein modules to IACs as one might expect, but do contribute to the propagation of signals through IACs. In the future, analysis of the phosphorylation profile of IACs during their assembly and disassembly at multiple time points by phosphoproteomics will help to identify specific phosphorylation switches and pathways activated in response to adhesion.

6.5 Use of the consensus adhesome to assess IAC dynamics

To reduce the identification of non-specific components identified in IAC proteomes, several studies have incorporated the use of complexes isolated from cells attached to a negative control ligand for removal of non-specific components (Fig. 1.5). However, it is not always appropriate to generate negative control isolations in parallel to the analyses of IACs. Some experimental conditions require cells to be in long term culture; for example, the IAC disassembly dataset was generated from cells cultured in serum-free conditions for 16 h before nocodazole treatment (see chapter 5). Cells plated on negative control ligands would begin to modulate their environment in this time, and may begin to produce their own ECM. Another issue for some cell types is their inability to attach well to negative control ligands. Therefore, the meta-adhesome and consensus adhesome can be used to filter other MS-derived IAC datasets, thus streamlining downstream analyses and reducing complexity in the number of non-specific proteins under investigation. The
IAC assembly and disassembly datasets presented here did not incorporate negative control ligand conditions to allow identification of non-specific IAC components identified by MS. Therefore, these datasets were filtered using the meta-adhesome (section 5.3.2) and consensus adhesome (section 5.3.3), which allowed analyses of previously identified proteins that may be relevant in these processes. These analyses revealed the dynamics of diverse functional protein groups in IACs and reported the IAC dynamics of the core integrin-actin structural connection. These data represent an example of how future datasets can be analysed using the meta-adhesome and consensus adhesome. This approach is analogous to other targeted proteomics approaches (Doerr, 2012), but does not allow the identification of potentially novel IAC components. Other IAC proteins such as Src family kinases and p130Cas that were not incorporated in the consensus adhesome are functionally important in IACs (Schlaepfer et al., 1997; Vuori et al., 1996). Also, additional non-consensus proteins may be recruited to IACs upon experimental variations from which IACs are isolated, such as when cells are exposed to different ECM substrates and different integrin heterodimers are engaged. Therefore, analysis of the meta-adhesome and consensus adhesome is one approach that should be used in combination with other analyses, such as identification of other known IAC components, analysis of IAC components that are in high abundance or those displaying the largest changes between experimental conditions.

6.6 Future work

The work presented in this thesis has examined IAC proteomes generated from FN-induced IACs from cells in culture. One outstanding question is how does IAC composition change for cells exposed to different ECM ligands? Additional studies that analyse IAC composition from cells exposed to different ECM microenvironments will help to determine ECM-dependent changes in IAC composition. The fact that the consensus composition would be affected is demonstrated through comparative analysis of the FN-induced consensus adhesome with two VCAM-1-induced IAC datasets (Byron et al., 2012; Humphries et al., 2009), since only 10 out of 60 consensus adhesome proteins were identified in these additional datasets (proteins common to all these datasets were α1-actinin-4, annexin A1, filamin C, IQGAP1, β1 integrin, SYNGR1, talin, VASP, vinculin and zyxin). Other MS-derived proteomic datasets of IACs induced from alternate ECM substrates such as laminin or collagen would help to clarify this view.

To investigate effects of integrin attachment to a particular ECM substrate on IAC composition, cell spreading time must be long enough to allow IAC formation but cells may begin to modulate their environment and produce their own cell-derived ECM if cells are seeded for too long, which would reduce the integrin-ligand specificity of IACs formed. Therefore, to control the integrin heterodimer-ligand combination used by cells, most studies have analysed IAC composition at early cell spreading times, typically of less than four hours. Another unanswered question is what is the composition of IACs from cells in longer term cell culture? These IAC structures may be more similar to IACs from cells in vivo as cells are exposed to cell-derived ECM and not one ECM substrate. Future studies that investigate the composition of IACs in vivo and from cells in 3D environments will be useful in determining the functional contribution of IACs in their native environments. In summary, to obtain a true understanding of how IACs are able to relay chemical and mechanical cues between the ECM and the actin cytoskeleton will require the combined
understanding of IAC kinetics, stoichiometry, protein composition and post-translational modification profile, such as phosphorylation and ubiquitination.

6.7 Summary

In conclusion, the data presented here have distilled the available IAC proteomes and enabled the definition of a core consensus adhesome that consists of canonical and underappreciated molecules that form the integrin-actin structural connection. Using two small molecule inhibitors targeting the IAC kinases FAK and Src, IAC composition was shown to be robust to kinase inhibition during abrogation of phosphorylation-based adhesion signalling. Thus these data suggest a possible separation in the regulation of the structural and signalling contributions of IAC components in adhesive function. Analyses of two datasets that reported the compositional changes in IACs during their assembly and disassembly revealed protein modules that are recruited to and disassembled from IACs with distinct kinetics. These data demonstrate how interrogation of IAC proteomic datasets at the systems-level can be used to reveal insights into the molecular workings of IACs.
6.8 References


Appendix – CD containing supplementary files