Modeling the Kinetics of Integrin Receptor Binding to Hepatic Extracellular Matrix Proteins

Shanice V. Hudson¹,², Christine E. Dolin¹, Lauren G. Poole¹, Veronica L. Massey¹, Daniel Wilkey¹, Juliane I. Beier¹, Michael L. Merchant³, Hermann B. Frieboes¹,²,³ & Gavin E. Arteel¹,⁴

The composition of the extracellular matrix (ECM) proteins and the expression of their cognate receptors dictate cell behavior and dynamics. In particular, the interactions of ECM proteins with integrin receptors are key mediators of these cellular processes, playing a crucial role in the progression of several diseases of the liver, including inflammation, fibrosis/cirrhosis and cancer. This study establishes a modeling approach combining computation and experiments to evaluate the kinetics of integrin receptor binding to hepatic ECM proteins. ECM ligand concentration was derived from LC-MS/MS quantification of the hepatic ECM from mice exposed to chronic carbon tetrachloride (CCl₄); receptor density was derived from published literature. Mathematical models for ECM-integrin binding kinetics that were developed incorporate receptor divalence and an aggregation scheme to represent clustering. The computer simulations reproduced positive cooperativity in the receptor aggregation model when the aggregation equilibrium constant (Kₐ) was positive and greater than Keq for divalent complex formation. Importantly, the modeling projected an increase in integrin binding for several receptors for which signaling is known to be increased after CCl₄ exposure in the liver. The proposed modeling approach may be of use to elucidate the kinetics of integrin receptor binding to ECM proteins for homeostatic and diseased livers.
is partly due to an incomplete understanding of the complexity of the changes to integrin signaling under dysregulated conditions.

The kinetics of ECM-integrin interactions are highly intricate. Integrin receptor complexes are structured as non-covalently linked \( \alpha \) and \( \beta \) subunits, the various combinations of which contribute to the diversity of receptor types\(^9\) (Fig. 1). The overall rate of binding is not driven simply by ligand binding to the receptor, but also by clustering at focal adhesion points and an increase in avidity for binding additional ligand (i.e., positive cooperativity). Masson-Gandais \textit{et al.} described a two-step model wherein the \( \alpha \) subunit binds ligand first, influencing ligand recognition and determinant of association kinetics\(^10\). The \( \beta \) subunit binds second, which creates bond stabilization and determines dissociation kinetics. Ligand binding to the extracellular domain activates the receptor and initiates its conformational changes to a high-affinity state\(^11,12\). This two-step process reflects a divalent kinetics model with the \( \alpha \) subunit as the high affinity site, and the \( \beta \) subunit as the low affinity site\(^13\). In addition to binding processivity of individual receptors, ligand binding to distinct integrins favors subsequent binding by other receptors (i.e. focal adhesion clustering). Furthermore, integrin receptors bind promiscuously to various ECM ligands, creating redundancy, competition and diversity in biofunctionality\(^5,9,14\). These complex interdependent factors affect the kinetics of ECM-integrin interactions in the intact organism. Promiscuity among the repertoire of ECM ligands and integrin receptors, particularly those with RGD-binding motifs, implies a differential pattern of binding relative to the amounts of substrate available\(^15,16\).

To explore these interactions as a system, several mathematical descriptions of integrin binding have been reported with outputs related to spatial clustering and signal transduction, liver fibrosis and haptotaxis\(^17–19\). Although these models recapitulate certain aspects of ECM-integrin interactions, they typically focus on one ligand (e.g. collagen or fibronectin) as the ECM substrate. In this study, modeling of integrin receptor binding kinetics is presented that considers divalent receptor characteristics and employs a simple model of integrin clustering. The kinetic indices of each integrin for each of its ligands were initially determined to establish a single-species integrin profile. Proteomic data were compiled that assess the liver ECM under homeostatic conditions as well as experimental fibrosis. These proteomic analyses provided information on relative abundance of hepatic ECM components to calibrate substrate concentrations for the kinetic simulations. Although data from human fibrotic livers has recently been analyzed\(^20\), an animal model was chosen here as it provides a more controlled environment for initial model calibration and testing. Longer term, by testing homeostatic conditions against the experimental treatment models, how the integrin binding phenotype changes in response to injury could be determined and used to predict the ECM-integrin binding within the context of transitional tissue remodeling.

**Results**

As expected, 4 weeks of CCl\(_4\) exposure caused robust fibrotic scarring of the liver in our mouse model. The resultant phenotype of injury and fibrosis has been previously described to include degradation of basement membrane-like ECM and replacement with fibrillar collagens and other integrin ligands (Fig. 2)\(^21\). The canonical
change in ECM content during hepatic fibrosis is an increase in collagen 1 deposition. However, as has been previously described, several other proteins increase in response to CCl_{4}-induced fibrosis.

Analysis of the proteomic data (Table 1) revealed ECM protein expression profiles, and a simple conversion for relating quantitative exponentially modified protein abundance index (emPAI) values to protein mass was employed as a proteomic ruler to estimate protein concentration under homeostatic and experimental treatment conditions. Weighting the values with the concentration of extraction fractions, we estimated a relative protein concentration for ECM components. The composition of the liver ECM as quantitated via proteomic analysis has influence on integrin expression of cells that haptotactically migrate towards ECM protein gradients, and provides the pool of available ligands for subsequent binding.

Qualitatively, the majority of proteins identified were found in both the control and treatment groups; with seven proteins uniquely expressed in the CCl_{4} group and only one unique to the control group (Fig. 3). Collagens, glycoproteins and proteoglycans identified via proteomic analysis as ECM substrate were quantified and their expression profiles were previously validated to confirm relative abundance of identified proteins qualitatively and quantitatively. In particular, the amounts and distribution of collagens in the treatment group relative to the control were verified. Here, the presence of trace amounts of Col V in the CCl_{4} treatment group was validated to explore whether changes on the nanomolar scale would have pathological consequence. (Fig. 4).

Next, to provide for the capability of a system-level analysis, a computational framework was established using proteomic data for binding species to enable evaluation of integrin receptor binding kinetics (see modeling and experimental details in Methods). The model was developed taking into consideration sequential binding of integrins-ECM binding microrates have been determined for various cell types and conditions.

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The simulations were initialized using binding constants from published literature; where values were not available, parameters were estimated accordingly (see Methods). Collagen fragments for collagen I and IV were plotted together (Fig. 5) and assumed to have the same rates of binding for the purposes of these experiments. For the other fragmented protein, fibrinogen, only the gamma subunit was considered due to the binding motif located within this fragment. The binding microrates were set to recapitulate positive cooperativity in divalent receptor saturation and in receptor aggregation pairs, as stipulated in Wanant et al., wherein the aggregation equilibrium constant, K_{a}, drove cooperativity in the aggregate model (Table 3).

The simulation graphs in Fig. 5 show left-shifted curves with increased ECM ligand abundance, indicating increased affinity and avidity for ligand. This is reflected in both the curves for fully occupied divalent receptors.
From these simulation data it appears that upregulated ECMPs reached steady state values in shorter time, and that aggregation of receptors produced positive cooperativity. Considering the single divalent receptor, \( \text{Cd} \), the ECM:integrin binding pairs that had the highest steady state values include both collagen 1 and fibrinogen \( \gamma \) chain in association with the \( \alpha v \beta 3 \) integrin receptor. The combinations with the shortest time to SS were collagen 1 binding \( \alpha v \beta 3 \) or \( \alpha 1 \beta 1 \) receptors. For the aggregated receptor pairs, \( \text{Add} \), the pairs with the highest SS values include von Willebrand factor, fibrinogen \( \gamma \) chain, and collagen 1 binding \( \alpha v \beta 3 \), as well as fibronectin binding \( \alpha 5 \beta 1 \). The ECM:integrin pairings with the shortest time to SS for \( \text{Add} \) pairs were collagen 1 binding \( \alpha v \beta 3 \) and \( \alpha 1 \beta 1 \), and fibronectin binding \( \alpha 5 \beta 1 \). In nearly all cases, the \( \text{CCl}_4 \) model ECM showed faster rise to SS compared to control.

### Table 1. Quantitative Differential Protein Expression of Liver ECM following 4 Weeks of \( \text{CCl}_4 \) Exposure.

| Protein | GO Accession | MW (kDa) | Protein Abundance (µM) | Log2 FC Con CCl4 |
|---------|--------------|----------|------------------------|------------------|
| Col 1α1 | CO1A1        | 138      | 3.60                   | 6.42             |
| Col 1α2 | CO1A2        | 130      | 2.55                   | 4.25             |
| Col 3α1 | CO3A1        | 139      | 0.75                   | 1.64             |
| Col 4α1 | CO4A1        | 161      | 0.20                   | 0.23             |
| Col 4α2 | CO4A2        | 167      | 0.46                   | 0.28             |
| Col 5α1 | CO5A1        | 184      | 0                      | 0.05             |
| Col 5α3 | Q9JL2        | 172      | 0                      | 0.06             |
| Col 18α1| E9QPX3       | 182      | 0.07                   | 0                |
| Dermatopontin | DERM | 24 | 0 | 7.11 | 16.06 |
| Fibronectin | FINC | 273 | 0.18 | 0.47 | 1.40 |
| Fibrinogen \( \gamma \) chain | FIBG | 49 | 5.94 | 1.94 | −0.45 |
| Galectin-1 | LEG1 | 15 | 8.45 | 21.18 | 1.33 |
| Galectin-3 | LG3BP | 64 | 0 | 0.63 | 13.97 |
| von Willebrand factor A | VM5A | 87 | 0.33 | 0.84 | 1.33 |

The exponentially modified protein abundance index (emPAI) was used for estimation of absolute protein abundance and to approximate protein concentration of relevant integrin-binding proteins. Multidimensional protein identification technology (MudPIT) was used to artificially recombine fraction data from Mascot and SequestHT searches and produce quantitation that relates total protein signal in each treatment group. To normalize for tissue fractionation, the dimensionless emPAI score was weighted with the concentration loaded for each fraction, i.e., 0.25 µg/µL, to calculate relative protein concentration as initial parameter values for the simulations.

![Figure 3. Qualitative Venn diagram of proteomic data. Differentially expressed proteins of interest for evaluation of ECMP-integrin bindings include collagens, fibrillar proteins, glycoproteins and proteoglycans. Of seven proteins uniquely expressed in the \( \text{CCl}_4 \) experimental model, one ECMP protein, Galectin-3, was identified. One protein was unique to the control, and 90 were differentially expressed. ECMPs used for these simulations are listed in Table 1. Data for all relevant ECM proteins are given in Supplemental S1 Table.](image-url)
to the control ECM; however, fibrinogen γ chain and Col 4α2 behaved in an opposite manner, owing to the fact that CCl₄ actually downregulated these ECMPs in our dataset.

Sensitivity analysis was performed (Table 5) by evaluating the equilibrium constants listed in Table 3 for percent change in steady state after a ten-fold perturbation to the base values (Table 5). For simple integrin complex formation, i.e. when one ligand binds, increasing the $K_i$ causes a significant increase in aggregated receptors, while a ten-fold decrease causes an approximate 100% decrease in aggregate pairing. Divalent receptors are moderately decreased when $K_i$ increases, showing that decreased affinity suppresses the capacity for divalent receptor binding. Filling a single divalent receptor is negatively impacted by an increase in $K_c$, with aggregate receptor pairs decreasing ~83% for all three receptor:ligand pairings. A decrease in $K_c$ positively increases the steady state for aggregate pairs, because a lower $K_{eq}$ for the second binding event increases affinity for receptors with one bound ligand. $K_p$, the constant for filling empty paired receptors, was nominally affected by perturbation, as were perturbations to filling aggregate pairs. Finally, perturbations to the aggregation constant, $K_a$, result in a decrease in steady state for aggregated pairs when $K_a$ is increased ten-fold, and an increase in steady state when $K_a$ is decreased. This reflects the condition of a decreased equilibrium constant increasing the affinity for ligand binding, which is expected as the aggregation constant drives ligand affinity in this model.

### Discussion

Integrin binding to ECM is a vital mechanism for cell migration, invasion, proliferation, and signal transduction between cells and their microenvironment. Diseases of chronic inflammation and injury, including fibroses and cancer, involve persistent dysregulation of ECM-integrin processes and induce remodeling of the ECM. In addition to their intrinsic utility in cellular processes, association between immune cells and the ECM is regulated via the β1 & β3 integrin receptor subfamilies. Elucidating these complex cell-ECM-driven pathological conditions could lead to improved prognostics and clinical outcomes via more precise therapeutic management of the tissue microenvironment. Several mathematical models of integrin binding have been reported with outputs relating to spatial clustering and signal transduction, liver fibrosis, and cell migration. These models recapitulated

### Table 2. Binding Rate Parameters

| Integrin Species | Initial Value (nM) | Binding Kinetic Parameters ($k_{on}$ [s⁻¹·M⁻¹]; $k_{off}$ [s⁻¹]) |
|------------------|-------------------|---------------------------------------------------------------|
| Col Iβ1 β1⁴⁸,⁴⁹ | 0.0001 5.6 × 10⁴ 1.3 × 10⁻³ | 8.0 × 10⁵ $k_{on}$ 5.0 × 10⁻³ $k_{off}$ --- --- --- |
| αVβ3 | 0.05 --- --- --- --- 1.6 × 10⁸ 3.5 × 10⁻¹ 1.6 × 10⁴ 2.3 × 10⁻² |

Figure 4. Immunofluorescent staining of hepatic cryosections. OCT sections were probed with Col V primary antibody and resolved with Alexa 488-tagged secondary antibody. The CCl₄ treatment group shows a marked increase in Col V staining relative to the control.
et al. have limited clinical application. Though necessary to target multiple integrins to maximize efficacy and assumptions; indeed, even antibodies and small peptide mimetics with specificities for multiple integrins in incomplete understanding of integrin binding kinetics, which are traditionally based on single-species models.

In the current study (Table 1). Collagen I is aberrantly produced in this mouse model, and collagen V, a potent nucleator of fibrogenesis, was developed for the proposed modeling framework based on experimentally-obtained liver ECM data. During the binding pattern was established for each integrin involved in hepatic processes that are involved in fibrosis. The model from Wanant et al. was adapted to implement the basic model for divalent binding. Specifically, this model aptly describes initial integrin binding leading to a conformational switch of the receptor complex from low- to high-affinity. A model of receptor aggregation, which can describe integrin clustering upon attachment to ECM via adhesions, was also implemented. The simulations include how each integrin binds with cognate ECM ligands and incorporates the varying affinities that drive this interaction. From these calculations, the kinetic indices of each integrin for each of its binding partners were determined separately. The impact of changes to the ECM (e.g., in response to CCl4-induced fibrosis) on integrin binding was modeled by calibrating the substrate concentration based on the proteomic analyses. The extracellular matrix proteome was consistent with the known disease phenotype of the mouse model, with upregulation of specific ECMPs involved in fulminant fibrosis. The computational results show that in simulations using these ECMPs as substrate for key integrin receptors, interactions involving profibrotic integrins were predominant.

The CCl4 mouse model of liver fibrosis was chosen here due to its robustly characterized pathology and ECM/integrin phenotype (Fig. 2). This model is imperfect in its recapitulation of human liver fibrosis, but it is the current research standard and therefore has well-defined pathology and changes to the ECM. Using proteomic data from CCl4-exposed mouse livers, integrin binding can be explored within the context of fulminant fibrosis. Collagen type I, III, and type IV are excessively deposited due to activated hepatic stellate cells (HSCs) in response to myofibroblastic transformation induced by activated Kupffer cells and damaged hepatocytes. In agreement with these established phenomena, collagens I, III, and V were upregulated in the CCl4 cohort in the current study (Table 1). Collagen I is aberrantly produced in this mouse model, and collagen V, a potent nucleating effector for the co-upregulated fibronectin, exhibited a slight increase from trace levels. In contrast, collagen IV and VIII levels were decreased relative to the control. Interestingly, collagen VIII was identified at relatively minimal levels in the controls, and absent in the CCl4 treated animals (Table 1). This is contrary to an expected increase in collagen VIII following CCl4 treatment. Nevertheless, interactions simulated with this ECMP are still based on experimental proteomic analysis. Integrin receptors were not able to be resolved with this particular method of proteomic analysis, so further proteomic analysis of integrin adhesion complexes in culture is a key component of the future directions for this project.

Owing to their involvement in several critical functions that drive homeostasis and dyshomeostasis, integrins have been identified as key druggable targets in several diseases. For example, integrin inhibitors have been evaluated to suppress liver fibrogenesis, disrupt attachment and invasion of cancer cells, and to mediate immune responses. Regrettably, many of these drugs fail in early trials and rarely reach clinical use, perhaps due to an incomplete understanding of integrin binding kinetics, which are traditionally based on single-species models and assumptions; indeed, even antibodies and small peptide mimetics with specificities for multiple integrins have limited clinical application. Though necessary to target multiple integrins to maximize efficacy in vivo, perhaps the missing link is knowing which targeted doses are most effective for each anti-integrin molecule. In attempting to begin to develop a predictive tool for effective dosing, the primary goal of this work was to create

| $K_i$ (nM) | Reaction | Microrates (on/off s$^{-1}$/off s$^{-1}$) | Manipulations |
|-----------|----------|--------------------------------------|--------------|
| $K_i$     | Integrin complex formation | $k_i/k_1$ | $K_i > K_1$ increased ligand affinity after aggregation |
| $K_i$     | Filling divalent unpaired receptor | $k_i/k_1$ | $K_i = 0.01$ K decreased unpaired receptor $K_{eq}$ for binding 2nd ligand |
| $K_i$     | Empty receptor pairing with bound receptor | $k_i/k_1$ | $K_i > 0$ aggregation constant drives positive cooperativity |
| $K_i$     | Population of empty paired receptors | $k_i/k_1$ | $K_i > 0$ increased ligand affinity after aggregation |
| $K_i$     | Receptor saturation | $k_i/k_1$ | $K_i = 0.01$ K decreased aggregate receptor $K_{eq}$ for binding 2nd ligand |

Table 3. Equilibrium binding constants for receptor aggregation model. Microrate parameters are derived from published values and set to implement positive cooperativity for sequential ligand binding and receptor aggregation. The rates for integrin complex formation ($K_i$) are set to simulate an increase in ligand affinity post-aggregation. Populating an empty unpaired receptor is set with a hundredth fold decrease in $K_{eq}$ for binding the second ligand. The aggregation equilibrium constant is set at ten times the equilibrium constant for initial complex formation to allow for aggregation to drive positive cooperativity. The population of empty paired receptors dictates an increase in ligand affinity after aggregation and is set to decrease aggregate receptor $K_{eq}$ for binding second ligand for receptor saturation. These parameters are adapted from Wanant et al., and applied here to simulate positive cooperativity in receptor aggregation pairing so that the model can be initialized and implemented with proteomic data to evaluate binding profiles.
a framework to simulate simple receptor aggregation and reproduce positive cooperativity induced by aggregate pairing. The simulations were parameterized to analyze for positive cooperativity of binding in the divalent and aggregation cases. The steady state values and time to steady state for each pairing correlated to upregulation of key ECMPs in CCl4 liver injury (Fig. 5; Table 4). The integrin receptors that predominated simulations of occupancy were consistent with those known to be at play in the disease model (Fig. 2).

Table 4. Steady state Values for Simulations of Binding. Steady state Values for Simulations of Binding. Steady state (SS) values for a fully occupied single divalent receptor (C_d) and saturated aggregated receptor pairs (A_add).

| ECMP, Treatment | Integrin Receptor | Steady state [C_d] |
|-----------------|-------------------|-------------------|
| vWF, CCl4       | αvβ3             | 0.0471            |
| vWF, Control    | αvβ3             | 0.0427            |
| Fibronectin, CCl4 | αvβ3          | 0.0448            |
| Fibronectin, Control | αvβ3     | 0.0369            |
| Col 1α1, CCl4   | α1β1             | 0.0001            |
| Col 1α2, CCl4   | α1β1             | 0.0001            |
| Col 1α1, Control | α1β1           | 9.99 × 10^{-5}   |
| Col 1α2, Control | α1β1           | 9.99 × 10^{-5}   |

| ECMP, Treatment | Integrin Receptor | Steady state [A_add] |
|-----------------|-------------------|----------------------|
| vWF, CCl4       | αvβ3             | 0.0014               |
| vWF, Control    | αvβ3             | 0.0035               |
| Fibronectin, CCl4 | αvβ3           | 0.002                |
| Fibronectin, Control | αvβ3     | 0.006                |
| Col 1α1, CCl4   | α1β1             | 7.79 × 10^{-13}     |
| Col 1α2, CCl4   | α1β1             | 1.18 × 10^{-8}      |
| Col 1α1, Control | α1β1           | 1.39 × 10^{-8}      |
| Col 1α2, Control | α1β1           | 1.96 × 10^{-9}      |

Figure 5. Kinetic simulations data. Model was initialized using ligand concentrations from proteomic analysis (Table 1) and kinetic rates listed in Table 2. The ECM:integrin binding pairs fibronectin:αvβ3, von Willebrand factor: αvβ3, and collagen I:α1β1 are shown, with binding curves and percent occupancy for fully occupied single divalent receptors (Cd) and aggregated receptor pairs (Add).
This study offers a first step in which the proposed modeling framework has been initially evaluated using data from a model of fulminant fibrosis and by which other liver pathologies and how the transitional remodeling of the ECM affects ECM-integrin interactions could be explored. We acknowledge that a more comprehensive test of the model and its assumptions would require further experiments, which will be pursued in follow-up work. By testing homeostatic conditions against experimental treatment models, this platform could be broadly employed to predict or confirm changes in integrin binding (and by extension, signaling) caused by remodeling of the hepatic ECM in response to insult or injury. Longer term, a more complex stochastic model for concurrent integrin binding building upon the results of this study could be developed that considers competitive binding of multiple species. This would lay the foundation for a more detailed and nuanced analysis of ECM:integrin interactions.

**Methods**

All experiments were performed in accordance with the guidelines and regulations of the University of Louisville Office of Research Integrity and Institutional Review Board and Biosafety Committee.
Animals and treatments. Male C57BL/6J mice (4–6 w) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the University of Louisville’s Institutional Animal Care and Use Committee. Food and tap water were provided ad libitum. Mice were administered CCl4 (1 ml/kg i.p.; diluted 1:4 in olive oil; Sigma-Aldrich, St. Louis, MO) 2 ×/wk for 4 wk. Twenty-four h after the last CCl4 administration, mice were anesthetized by injection of a ketamine HCl/xylazine solution (100/15 mg/kg i.m.; Sigma-Aldrich, St. Louis, MO). Other animals received the same dose of CCl4 but only once, and were sacrificed 12–72 h after intoxication. Blood was collected from the vena cava just prior to sacrifice by exsanguination and citrated plasma was stored at −80 °C for further analysis. Portions of liver tissue were frozen immediately in liquid nitrogen, while others were fixed in 10% neutral buffered formalin or embedded in frozen specimen medium (Tissue-Tek OCT compound, Sakura Finetek, Torrance, CA) for subsequent sectioning and mounting on microscope slides.

3-step ECM extraction. Sample preparation and wash. Snap-frozen liver tissue (75–100 mg) was immediately added to ice-cold phosphate-buffered saline (pH 7.4) wash buffer containing commercially available protease and phosphatase inhibitors (Sigma Aldrich) and 25 mM EDTA to inhibit proteinase and metalloproteinase activity, respectively. While immersed in wash buffer, liver tissue was diced into small fragments and washed five times to remove contaminants. Between washes, samples were pelleted by centrifugation at 10,000 × g for 5 min and wash buffer was decanted.

NaCl extraction. Diced samples were incubated in 10 volumes of 0.5 M NaCl buffer, containing 10 mM Tris HCl (pH 7.5), protease/phosphatase inhibitors, and 25 mM EDTA. The samples were gently mixed on a plate shaker (800 rpm) overnight at room temperature. The following day, the remaining tissue pieces were pelleted by centrifugation at 10,000 × g for 10 min. The supernatant was saved and labeled as the NaCl fraction.

SDS extraction. The pellet from the NaCl extraction was subsequently incubated in 10 volumes (based on original weight) of a 1% SDS solution, containing protease/phosphatase inhibitors and 25 mM EDTA. The samples were gently mixed on a plate shaker (800 rpm) overnight at room temperature. The following day, the remaining tissue pieces were pelleted by centrifugation at 10,000 × g for 10 min. The supernatant was saved and labeled as the SDS extract.

Guanidine HCl extraction. The pellet from the SDS extraction was incubated with five volumes (based on original weight) of a denaturing guanidine buffer containing 4 M guanidine HCl (pH 5.8), 50 mM sodium acetate, 25 mM EDTA, and protease/phosphatase inhibitors. The samples were vigorously mixed on a plate shaker at 1200 rpm for 48 h at room temperature; vigorous shaking is necessary at this step to aid in the mechanical disruption of ECM components. The remaining insoluble components were pelleted by centrifugation at 10,000 × g for 10 minutes. This insoluble pellet was retained and solubilized as described below. The supernatant was saved and labeled as the GnHCl fraction.

Deglycosylation and solubilization. The supernatants from each extraction were desalted using Zeba Spin columns (Pierce) according to manufacturer's instructions. The desalted extracts were then mixed with five volumes of 100% acetone and stored at −20 °C overnight to precipitate proteins. The precipitated proteins were pelleted by centrifugation at 16,000 × g for 45 min. Acetone was evaporated by vacuum drying in a RotoVap for one hour. Dried protein pellets were resuspended in 500 µL deglycosylation buffer (150 mM NaCl, 50 mM sodium acetate, pH 6.8, 10 mM EDTA, and protease/phosphatase inhibitors) that contained chondroitinase ABC (P. vulgaris; 0.025 U/sample), endo-beta-galactosidase (B. fragilis; 0.01 U/sample) and heparitinase II (F. heparinum; 0.025 U/sample). Samples were incubated overnight at 37 °C; those containing the pellet remaining after the guanidine HCl step received 20 µL DMSO for solubilization. Protein concentrations were estimated by absorbance at 280 nm using bovine serum albumin (BSA) in deglycosylation buffer for reference standards.

LC-MS/MS analysis of samples. Sample cleanup and preparation for liquid chromatography. Pooled samples in deglycosylation buffer were thawed to room temperature and clarified by centrifugation at 5,000 × g for 5 min at 4 °C. Samples were reduced by adding 1 M DTT to 50 µL (25 µg) of each sample and then incubating at 60 °C for 30 min before addition of 8 M urea in 0.1 M Tris-HCl (pH 8.5) was added to each sample. Each reduced and diluted sample was digested with a modified Filter-Aided Sample Preparation (FASP) method. Recovered material was dried in a SpeedVac and resuspended in 200 µL of 2% v/v acetonitrile (ACN)/0.4% formic acid (FA). The samples were then trap-cleaned with a C18 PROTO™ 300 Å Ultra MicroSpin Column (The Nest Group). The sample eluates were incubated at −80 °C for 30 min, dried in a SpeedVac, and stored at −80 °C. Before liquid chromatography, dried samples were warmed to room temperature and dissolved in 2% v/v ACN/0.1% FA to a final concentration of 0.25 µg/µL. A volume of 16 µL (4 µg) of sample was injected into the Orbitrap Elite.

Liquid Chromatography. Dionex Acclaim PepMap 100, 75 µM × 2 cm nanoViper (C18, 3 µm, 100 Å) trap and Dionex Acclaim PepMap RSLC, 50 µM × 15 cm nanoViper (C18, 2 µm, 100 Å) separating column were used. An EASY n-LC (Thermo) UHPLC system was used with mobile phase buffer A (2% v/v acetonitrile/0.1% v/v formic acid), and buffer B (80% v/v acetonitrile/0.1% v/v formic acid). Following injection of the sample onto the trap, separation was accomplished with a 140 min linear gradient from 0% B to 50% B, followed by a 30 min linear gradient from 50% B to 95% B, and lastly a 10 min wash with 95% B. A 40-mm stainless-steel emitter (Thermo P/N ES542) was coupled to the outlet of the separating column. A Nanospray Flex source (Thermo) was used to
position the end of the emitter near the ion transfer capillary of the mass spectrometer. The ion transfer capillary temperature of the mass spectrometer was set at 225 °C, and the spray voltage was set at 1.6 kV.

**Mass Spectroscopy.** An Orbitrap Elite – ETD mass spectrometer (Thermo) was used to collect data from the LC eluate. An Nth Order Double Play with ETD Decision Tree method was created in Xcalibur v2.2. Scan event one of the method obtained an FTMS MS1 scan for the range 300–2000 m/z. Scan event two obtained ITMS MS2 scans on up to ten peaks that had a minimum signal threshold of 10,000 counts from scan event one. A decision tree was used to determine whether collision induced dissociation (CID) or electron transfer dissociation (ETD) activation was used. An ETD scan was triggered if any of the following held: an ion had charge state 3 and m/z less than 650, an ion had charge state 4 and m/z less than 900, an ion had charge state 5 and m/z less than 950, or an ion had charge state greater than 5; a CID scan was triggered in all other cases. The lock mass option was enabled (0% lock mass abundance) using the 371.101236 m/z polysiloxane peak as an internal calibrant.

**Proteome Data Analysis.** Proteome Discoverer v1.4.0.288 was used to analyze the data collected by the mass spectrometer. The database used in Mascot v2.4 and SequestHT searches was the 6/2/2014 version of the UniprotKB *Mus musculus* reference proteome canonical and isoform sequences. In order to estimate the false discovery rate, a Target Decoy PSM Validator node was included in the Proteome Discoverer workflow. The Proteome Discoverer analysis workflow allows for extraction of MS2 scan data from the Xcalibur RAW file, separate searches of CID and ETD MS2 scans in Mascot and Sequest, and collection of the results into a single file (.msf extension). The resulting .msf files from Proteome Discoverer were loaded into Scaffold Q+ S v4.3.2. Scaffold was used to calculate the false discovery rate using the Peptide and Protein Prophet algorithms. The results were annotated with mouse gene ontology information from the Gene Ontology Annotations Database.

**Computational Modeling.** First is considered the divalent receptor model that corresponds to ECM ligand binding of the α subunit occurring prior to the β subunit, where $k_1$ is the first-order association rate constant and $k_2$ is the dissociation constant for singly occupied receptors ($C_m$). $C_d$ indicates a fully occupied integrin receptor with two bound ECM ligands, and $k_3$ and $k_4$ define the rate constants for association and dissociation, respectively, of the doubly bound integrin receptor. Differential equations for this model are:

$$\frac{dI}{dt} = k_2 C_m - k_3 I E,$$

$$\frac{dE}{dt} = k_2 C_m - k_4 I E + k_3 C_d,$$

$$\frac{dC_m}{dt} = k_4 I E - k_2 C_m + k_5 C_d,$$

$$\frac{dC_d}{dt} = k_5 C_m E - k_4 C_d.$$

The scheme for receptor aggregation and ligand binding is shown in Fig. 6. In the model of receptor aggregation, we utilized the same scheme as Wanant et al. wherein receptors pair in a manner such that either singly- or doubly-bound receptors can aggregate only with an unbound receptor with the aggregation equilibrium constant $K_A$ (where $K_A = k_5/k_6$), and disaggregation equilibrium constant $K_A'$ ($K_A' = k_6/k_5$). Binding constants for an additional ECM ligand binding to the unbound portion of an aggregate pair are the same regardless of whether the bound portion has one or two ligands, where the equilibrium association constant is $K_A = k_5/k_6$. The equilibrium constant for adding a second ECM ligand to a singly bound receptor in any pair-configuration is $K_F = k_7/k_8$. The differential equations describing receptor aggregation are listed below:

$$\frac{dI}{dt} = k_2 C_m - k_3 I E + k_6 (A_{im} + A_{id}) - k_3 (C_m + C_d),$$

$$\frac{dE}{dt} = k_2 C_m - k_3 I E + k_4 C_d + k_6 C_{im} E + k_7 (A_{mm} + A_{md}) - k_3 E (A_{im} + A_{id}) + k_6 (A_{id} + A_{md} + A_{dd}) - k_7 E (A_{im} + A_{mm} + A_{md}),$$

$$\frac{dC_m}{dt} = k_4 I E - k_2 C_m + k_5 C_d + k_6 A_{im} - k_5 I C_m,$$

$$\frac{dC_d}{dt} = k_5 C_m E - k_4 C_d + k_6 A_{id} - k_5 I C_d.$$
where $A_{im}$ indicates an aggregate pair comprised of one unbound integrin receptor coupled with a singly-bound receptor; $A_{id}$ is the same combination, except featuring a doubly-bound receptor. A pair with two singly bound receptors is defined as $A_{mm}$ with two doubly bound receptors is $A_{dd}$ and $A_{md}$ indicates a singly bound receptor paired with a doubly bound one (Fig. 6).

The affinity of integrin receptors for ECM proteins fibronectin and laminin are generally in the micromolar range. The $K_d$ measured for ECM:integrin and, in particular, fibronectin binding, ranges between approximately $10^{-7}$–$10^{-6}$ M\(^4\); Takagi et al. report nanomolar $K_d$ values for fibronectin binding\(^4\). Mallet et al. utilized a $K_d$ of $2 \times 10^{-4}$ M for tethered RGD peptides in their model of integrin binding\(^1\).

Simulations. Computer simulations were run using Spyder for Tellurium software version 2.3.5.2; Python version 2.7\(^4\). Binding curves were plotted using SigmaPlot 13.0. The model was initialized using ligand concentrations from proteomic analysis (Table 1) and initial integrin concentrations were derived from published values. Ligand concentration was developed by collapsing the fractionated sample data using MudPIT functionality in Scaffold. Rappsilber et al. defined protein abundance index (PAI) for estimation of absolute protein abundance\(^4\), and Ishihama et al. report that the emPAI, i.e. exponentially modified PAI, is approximately proportional to protein abundance\(^2\). Using the emPAI quantitative method, proteomic output was normalized by the tissue loading concentration of 0.25 µg/µL; these values for concentration were then divided by the molecular weight of the protein to convert to molar concentration. Kinetic rates listed in Table 2 were used to calculate microrate parameters.
relative to the established binding rates from literature; where exact microrates were unavailable, rates were estimated from various published literature sources. Table 3 relates the equilibrium constants of the system relative to initial integrin complex formation, such that subsequent binding and clustering steps produce cooperativity when simulated in these proportions. Sensitivity analysis was performed by varying levels of integrin receptor concentration in 10-fold increments, to explore binding when surface membrane integrin receptor expression is upregulated or downregulated as a consequence of disease state or in response to microenvironmental fluctuations.

Data Availability. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

Conceived and organized the study (S.V.H., H.B.F., G.E.A.); directed proteomics analysis (M.L.M.); directed animal study for proteomics samples (V.L.M., J.I.B.); prepared proteomic samples (L.G.P.); performed proteomic analysis (C.E.D., D.W.); implemented modeling and simulations (S.V.H.); analyzed the results (S.V.H., H.B.F.); wrote initial manuscript (S.V.H.); revised and approved final manuscript (S.V.H., G.E.A., H.B.F.).

Additional Information

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