



Integrin $\alpha 1 \beta 1$ is a receptor for collagen XIII

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Received: 1 July 2020 / Accepted: 14 September 2020 / Published online: 11 December 2020
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Abstract

Collagen XIII is a conserved transmembrane collagen mainly expressed in mesenchymal tissues. Previously, we have shown that collagen XIII modulates tissue development and homeostasis. Integrins are a family of receptors that mediate signals from the environment into the cells and vice versa. Integrin $\alpha 1 \beta 1$ is a collagen receptor known to recognize the GFOGER (O=hydroxyproline) sequence in collagens. Interestingly, collagen XIII and integrin $\alpha 1 \beta 1$ both have a role in the regulation of bone homeostasis. To study whether $\alpha 1 \beta 1$ is a receptor for collagen XIII, we utilized C2C12 cells transfected to express $\alpha 1 \beta 1$ as their only collagen receptor. The interaction between collagen XIII and integrin $\alpha 1 \beta 1$ was also confirmed by surface plasmon resonance and pull-down assays. We discovered that integrin $\alpha 1 \beta 1$ mediates cell adhesion to two collagenous motifs, namely GPKGER and GF(S)QGEK, that were shown to act as the recognition sites for the integrin $\alpha 1$ -I domain. Furthermore, we studied the in vivo significance of the $\alpha 1 \beta 1$ -collagen XIII interaction by crossbreeding $\alpha 1$ null mice (*Itga11^{-/-}*) with mice overexpressing *Col13a1* (*Col13a1^{oe}*). When we evaluated the bone morphology by microcomputed tomography, *Col13a1^{oe}* mice had a drastic bone overgrowth followed by severe osteoporosis, whereas the double mutant mouse line showed a much milder bone phenotype. To conclude, our data identifies integrin $\alpha 1 \beta 1$ as a new collagen XIII receptor and demonstrates that this ligand-receptor pair has a role in the maintenance of bone homeostasis.

Keywords Collagen · Integrin · Cell adhesion · Bone homeostasis · ECM receptors

Introduction

The adhesion of cells to the neighboring cells and to the extracellular matrix (ECM) is critical for their survival, migration, differentiation, and signal transduction. Integrins

are an important family of molecules involved in cell-ECM adhesion process. They are heterodimeric membrane glycoproteins consisting of non-covalently associated α and β subunits (Pandolfi et al. 2017; Barczyk et al. 2010). The ligand-binding specificity of most integrins is well known (Pandolfi et al. 2017; Barczyk et al. 2010). Four collagen-binding integrins, $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, $\alpha 10 \beta 1$, and $\alpha 11 \beta 1$, are expressed in various tissues (Hemler et al. 1986; Camper et al. 1998; Gullberg et al. 1995). The collagen receptor integrin α subunits are characterized by an inserted domain named as the I domain (often called as the A domain) that mediates interaction with native collagens (Hamaia and Farndale 2014; Zeltz and Gullberg 2016).

Collagen XIII is a type II transmembrane protein composed of a short cytosolic head, a transmembrane anchor, and a long extracellular domain with three collagenous domains (COL1-3) and four non-collagenous domains (NC1-4) (Hägg et al. 1998; Heikkinen et al. 2012). Our previous in vitro studies using solid-phase assays showed that the I domain of the integrin $\alpha 1$ subunit binds to collagen XIII with similar approximated avidity as to collagen IV

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(Nykqvist et al. 2000), but the binding site in the collagen was not characterized. In another report, we suggested that collagen XIII mediates integrin $\alpha 1\beta 1$ -dependent transmigration of monocytes in renal fibrosis with a binding site different from other integrin I domain binding motifs (Dennis et al. 2010). These data prompted us to investigate the binding of collagen XIII to other collagen receptor integrins.

Collagen XIII is broadly distributed in mesenchymal tissues at low levels (Hägg et al. 2001; Sund et al. 2001; Sandberg et al. 1989; Juvonen et al. 1992; Sandberg-Lall et al. 2000). Many studies with genetically modified mice have indicated its physiological functions in musculoskeletal tissues (Ylönen et al. 2005; Härönen et al. 2017, 2019; Zainul et al. 2018; Koivunen et al. 2019) and congenital myasthenic syndrome type 19 (CMS19; OMIM #616,720) musculoskeletal phenotypes (Logan et al. 2015; Rodriguez Cruz et al. 2019).

Integrin $\alpha 1\beta 1$ is a collagen receptor (Lu et al. 2010) recognizing the GFOGER (O=hydroxyproline) sequence in interstitial collagens (W. M. Zhang et al. 2003). We consider of interest that both collagen XIII and integrin $\alpha 1\beta 1$ are induced in several tumor tissues (Väisänen et al. 2005, H. Zhang et al. 2018, Izzi et al. 2019, Mirtti et al. 2006, Zhu et al. 2007, Primac et al. 2019) and that they are involved in the regulation of bone development and homeostasis (Blumbach et al. 2012; Shen et al. 2019; Ylönen et al. 2005; Koivunen et al. 2019). Therefore, we chose to analyze the interaction between these two molecules. We found that $\alpha 1\beta 1$ mediates cell spreading on a collagen XIII-coated surface and that two collagenous motifs, GPKGER and GF(S)QGEK, in the collagen XIII ectodomain contribute in the in vitro binding to the integrin $\alpha 11$ -I domain. Additionally, we show that collagen XIII-integrin $\alpha 11$ interaction participates in the maintenance of bone homeostasis in vivo.

Materials and methods

Cells

Murine C2C12 myoblast cells from the American Type Culture Collection (ATCC) were stably transfected with a human integrin $\alpha 2$ cDNA or a human integrin $\alpha 11$ cDNA (Tiger et al. 2001), the cDNA encoding the integrin $\alpha 11$ was tagged with enhanced green fluorescent protein (EGFP) C-terminally (Erusappan et al. 2019). Mouse embryonic fibroblast cells (MEFs) were isolated from wild-type mouse embryos as described previously (Popova et al. 2004, 2007) and cultured at 37 °C in DMEM (Biochrom AG, Berlin, Germany) supplemented with 10% FCS (Euroclone, UK) and antibiotics (Cambrex, USA). Immortalized cell lines were created by induction of simian virus 40 (SV40) as described previously (Barczyk et al. 2013).

Production of integrin I domains

The recombinant I domains of the human integrins $\alpha 1$ and $\alpha 2$ were produced as described previously (Nykqvist et al. 2000). Briefly, the $\alpha 1$ -I domain cDNA was generated by PCR using a human integrin $\alpha 1$ cDNA (Briesewitz et al. 1993) as a template, cloned into the pGEX-4T-3 vector with a GST tag (GE Healthcare, USA), and the protein was expressed in *E. coli*. The $\alpha 2$ -I domain cDNA was produced by PCR using a human integrin $\alpha 2$ cDNA (Takada and Hemler 1989) as a template, cloned into the pGEX-2T vector (GE Healthcare, USA) and the GST- $\alpha 2$ I fusion protein was expressed in *E. coli*. The $\alpha 11$ -I domain cDNA was generated similarly by PCR using a human integrin $\alpha 11$ cDNA (Velling et al. 1999) as a template, and then cloned into the pAcSecG2T vector (Invitrogen, USA). The $\alpha 11$ -I domain was expressed as a GST-tagged fusion protein and secreted into cell culture media in insect High-Five cells (Invitrogen) using a Baculovirus expression system (Invitrogen). All recombinant GST-I domains were purified using glutathione-Sepharose affinity chromatography (GE Healthcare) and their purities were analyzed by SDS-PAGE.

Production of the recombinant human collagen XIII ectodomain, its collagenous peptides and triple-helical motifs

The ectodomain of human collagen XIII was produced as described previously (Tu et al. 2002). High Five insect cells (Invitrogen) were cultured in a monolayer and co-infected with a virus encoding the human collagen XIII $\alpha 1$ chain lacking the cytosolic domain (Snellman et al. 2000) and another virus encoding both the α and β subunits of human prolyl 4-hydroxylase (Lamberg et al. 1996; Nokelainen et al. 1998). The ectodomain was purified from ~ 400 ml of 48-h infected culture media sequentially using a HiTrap™ Q 5 ml column (GE Healthcare), a HiTrap™ SP 5 ml column (GE Healthcare), and a HiLoad™ Superdex™ 200 column (GE Healthcare). Its purity was analyzed by native gel electrophoresis and SDS-PAGE.

The collagenous peptides of collagen XIII were produced by enzymatic digestion of 400 μ g of the collagen XIII ectodomain with 0.4 μ g of pepsin at pH 2–3 for 30 min at room temperature and then isolated by a Superdex-200 column in 20 mM HEPES buffer, pH 7.0, containing 0.3 M NaCl.

The recombinant proteins composing the testing motifs were produced in *E. coli* or insect cells. The DNA fragment encoding (GPP)₅GFPGER(GPP)₅ was produced by a two-step PCR reaction. In the first step, the template for the reaction 1 was formed from annealing of two synthetic oligonucleotides 5'-GCCATGGCTGGACCCCCGGCCCTCTGGACCTCTGGTCCCCCTGGTCC-3' and 5'-TGGTCC

CGGTGGACCACGCTCGCCAGGGAAACCGGGAGGACCAGGGGGACC-3'. The only primer 5'-ACAAGGCCATGGCTGGACCCCC-3' was used for forward extension. The template for the reaction 2 was from annealing of another pair of oligos 5'-ACCGGATCCCGGTGGGCCAGGTGGTCCGGGTGGACCCGGTGGTCCCGGTGGACC-3' and 5'-GGTCCCCCTGGTCTCCCGGTTCCCTGGCGAGCGTGGTCCACCGGGACCA-3' and a primer 5'-GTAACCGGATCCCGGTGGGCCAGGT-3' for the reverse extension. After five cycles, the two reaction products were mixed and 25 more PCR cycles continued.

The cloning of other motifs followed the same strategy except changes in two oligonucleotides for annealing, one in italic and underlined, and another in bold and underlined.

The final PCR products were digested with NcoI and BamHI, and the purified DNA was inserted into a plasmid pHisTRX₂-Foldon (a gift kindly from J. Engel). The DNA sequence was confirmed by DNA sequencing. The DNAs encoding (GPP)₅motif(GPP)₅-foldon were further cloned by PCR using primers containing KpnI and HindIII cleavage sites (forward primer AGCTCGGTACCAGGACCCCCCGGCCCT; reverse primer CTAATTAAGCTTTTACAGGAAAGGTAGA), and the enzymatic digested PCR products were inserted into a plasmid pQE3.1 (Qiagen) for expressing the recombinant protein composing 6xHis-(GPP)₅motif(GPP)₅-foldon in an *E. coli* strain BL21(DE3). To express the fusion proteins in insect cells, the DNA fragments were cloned by PCR using a forward primer TCTGCAGCGGCCGCATGAGAGGATCTCACCAT and a reverse primer CTTCTAGAATTCTTATTACAGGAAGGTAGA and then inserted to the expression vector pVL1392 (Invitrogen) through the NotI and EcoRI cleavage. The recombinant fusion proteins were produced from the cell lysates by His-tag affinity purification using Ni-NTA resin (Qiagen). The peptide trimerization was confirmed by a gel filtration chromatography.

Cell attachment assay

The cells attached to the culture plates were monitored directly by microscopy or quantitatively analyzed using a β -hexosaminidase release assay (Landegren 1984). The ligands used for the cell attachment assays were human plasma fibronectin (Invitrogen), rat tail collagen I (Collaborative Biomedical Products, USA), collagen XIII ectodomain, and its motif-foldon fusion proteins, at a concentration of 10 μ g/ml in PBS or in 20 mM HEPES, 0.15 M NaCl, pH 7.0, 0.2% heat-inactivated BSA (crystallized, Serva, Germany) in PBS was used as a blank control. The 24-well cell culture plates (Greiner Bio-one, Germany) were coated with 300 μ l of the ligands at 4 °C overnight. The plates were rinsed 3 times in PBS, and the uncoated surface was then blocked with 0.2% heat-inactivated BSA in PBS at 37 °C for 1 h and washed with Puck's saline

(137 mM NaCl, 5 mM KCl, 4 mM NaHCO₃, 5.5 mM D-glucose, and 2 mg/L phenol red equilibrated with 5% CO₂ at 37 °C) containing 2 mM MgCl₂ and 20 μ M CaCl₂. For the attachment testing, C2C12 cells at 60–80% confluence were detached by trypsin-EDTA treatment and washed once with DMEM containing FCS to inactivate the trypsin and then 3 times with Puck's saline. The cells were added to the wells at a density of 100,000 cells/0.5 ml/well. They were allowed to attach for 1 h at 37 °C in Puck's saline, and non-attached cells were removed by washing for 3 times with Puck's saline. For the β -hexosaminidase assay, the attached cells were lysed for 2 h at 37 °C in 200 μ l 0.05 M citrate, pH 5.0, containing 0.25% Triton X-100 and 3.75 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma, USA). Fifty microliters of the cell lysates were loaded onto a 96-well microtiter plate (Nunc, Denmark) and the reaction was stopped, and color developed by adding 75 μ l of 45 mM glycine solution, pH 10.4, containing 4.5 mM EDTA. The absorbance at 405 nm was measured using a Victor microplate reader (Perkin Elmer, USA). A cell number calibration curve was made by incubating known numbers of cells under equivalent conditions. Three repeated experiments were performed in the tests. The data were normalized by taking adhesion to a fibronectin-coated surface as 100% cell attachment and that to wells coated with BSA only as 0%. In the inhibition assay, motif-fusion proteins, with the molar ratios of 1:1, 5:1, and 10:1 to the coating collagen XIII, were mixed with the cells first and then added to the collagen XIII-coated plate wells.

Immunofluorescence staining and EGFP imaging

The glass coverslips were coated and blocked in the same way as for the 24-well plates in the cell attachment assay. The cells were allowed to adhere at a density of 9000 cells/30 μ l/coverslip in DMEM containing 10% FCS or in serum-free DMEM for 1.5–2 h at 37 °C complemented with 5% CO₂. After washing the coverslips with PBS, the attached cells were fixed in acetone at – 20 °C for 8 min or in 4% PFA at room temperature for 10 min and then permeabilized in acetone at – 20 °C for 5 min. Non-specific binding sites were blocked by incubating with 10% goat serum (Zymed Laboratories, Inc., USA) diluted in PBS. The fixed cells were incubated with the antibody against integrin α 11 cytoplasmic domain (Popova et al. 2004) for 1–2 h at 37 °C or at 4 °C overnight. Cy2-, Cy3- (Jackson ImmunoResearch Laboratories, USA), Alexa Fluor 488-, and Alexa Fluor 568-conjugated secondary antibodies (Invitrogen) were used for detection. The stained cells were mounted in ImmMount mounting solution (Thermo Shandon, USA), visualized and photographed under a Leitz Aristoplan microscope (Leica Microsystems, Germany) or an Olympus BX11

(Olympus, Japan) equipped with optics for observing fluorescence. To visualize C2C12- α 11+, cells were fixed in 2% PFA + 0.1% Triton X-100 and mounted as described above.

Western blotting

To verify the expression of integrin α 11, C2C12 cells in one culture plate ($\Phi = 100$ mm) at 80–90% confluence were washed once with 10 ml of PBS and then detached with a cell scraper into 1 ml of PBS. The cells were harvested by centrifuging at $340\times g$ for 10 min and then homogenized for 1 min with 0.5 ml of 70 mM Tris/0.3 M NaCl/0.2% Triton X-100, pH 7.4, containing Protease Inhibitor cocktail (Roche Applied Science, Germany), followed by incubation on ice for 30 min. After centrifuging at $8000\times g$ for 10 min, the supernatant of the cell lysate was applied to SDS-PAGE followed by western blotting. The expression of recombinant human integrin α 11 in C2C12 cells was detected with an affinity-purified antibody against the human α 11 cytoplasmic domain (Tiger et al. 2001).

Solid-phase binding and surface plasmon resonance assays

Fifty microliters of collagen solutions, collagen I (Collaborative Biomedical Products, USA), collagen IV (BD Biosciences), collagen XIII ectodomain, its pepsin-digested fragments, and the recombinant motif-fusion proteins, were coated on the plastic surfaces of MaxiSorp™ 96-well microplates (Nunc) at 4 °C overnight. All the other steps were performed at room temperature using a buffer containing 20 mM Tris, 0.15 M NaCl, 2 mM MgCl₂, 20 μ M CaCl₂, 0.05% Tween-20, or 10 mM HEPES, 0.15 M NaCl, 2 mM MgCl₂, 20 μ M CaCl₂, pH 7.0. Three parallel samples were analyzed. The wells were blocked with 5% fat-free milk in Tris buffer or in HEPES buffer for 1 h and the samples incubated for 1.5 h with α 1-, α 2-, and α 11-I domains diluted in the buffer containing 30 μ g/ml BSA (crystallized, Serva). After thorough washing, the binding ligands were detected with a goat polyclonal anti-GST antibody (Rockland Immunochemicals, USA) and then with an anti-goat IgG secondary antibody conjugated with horseradish peroxidase (Dako, Denmark). TMB-Xtra (Kem-En-Tec Diagnostics A/S, Denmark) was used as the substrate for detection.

Surface plasmon resonance (SPR) analysis was performed at 25 °C in a Biacore® 3000 system (Biacore AB, Sweden) using a CM5 sensor chip (Biacore). Integrin α 11-I domain was immobilized onto the chip at a level of 2000 resonance units (RUs). Binding of collagen XIII was tested in 10 mM HEPES, pH 7.0, 0.15 M NaCl, 2 mM MgCl₂, 20 μ M CaCl₂ containing 0.005% P-20 surfactant (Biacore) at a flow rate of 20 μ l/min. The blank was carried out on one

of the flow cells on the sensor chip lacking the immobilized I domain protein.

Pull-down assay

Insect High-Five cells were infected with viruses encoding GST and GST- α 11-I domain respectively with MOI of 10 in TNM-FH media (Sigma) containing 10% FCS. Eighty percent of the recombinant proteins were secreted into the media due to a signaling peptide in the expression vector. Forty-eight postinfection 5 ml media from each cell culture plate were collected and dialyzed in PBS containing 0.1 mM DTT and 0.001% Triton. The media were then mixed with the glutathione-Sepharose pre-equilibrated with the dialysis buffer at 4 °C overnight. After washing out the unbound proteins, the purified collagen XIII ectodomain was incubated with the Sepharose resin in 10 mM HEPES, pH 7.0, containing 0.15 M NaCl and 2 mM MgCl₂ at room temperature for 2 h. The unbound collagen XIII was removed by extensive washing steps and the proteins absorbed on the glutathione-Sepharose were eluted by incubating with either 10 mM glutathione or one cleavage unit of thrombin at room temperature overnight. The unbound collagen XIII was incubated at room temperature overnight as a control. The elution fractions were analyzed with a western blotting using a monoclonal antibody recognizing the recombinant human collagen XIII (Tu et al. 2002).

Sequence retrieval and alignment

The full-length human integrin α 11 sequence is 1188 residues in length. We retrieved only the I domain residues ranging from 164 to 345 which comprises 182 residues in total (later renumbered from 1 to 182) from the UniProtKB (Q9UKX5). The crystal structure of the human integrin α 2-I domain bound with the collagen peptide (PDB code: 1DZI) as well as the sequence information was obtained from the PDB (Berman et al. 2000). Sequence alignment between the integrin domains of α 2 and α 11 was carried out using ClustalW2 (Larkin et al. 2007) with default settings.

Homology modeling of the human integrin α 11-I domain

The final sequence alignment obtained from the CLUSTALW2 program was submitted to the MODELLER9v1 software package (Eswar et al. 2006; Sali and Blundell 1993) for constructing the homology model of the human integrin α 11-I domain. Twenty different models were generated and the model with the lowest energy was considered as the ideal model.

Energy minimization and structure validation

Energy minimization of the human integrin $\alpha 11$ -I domain model was carried out using GROMACS version 4.0.7. (Hess et al. 2008; Van Der Spoel et al. 2005). Initially, the protein was placed in a cubic box and a SPC water model was used for solvation. After solvating, suitable ions were added to neutralize the whole system. The final system was composed of 13,073 molecules, which includes the protein (182 residues), water (12,882 molecules), and ions (9 Na⁺). The whole system was then subjected to 1000 steps of steepest descent minimization using the GROMOS96 53a6 force field until the maximum force converged to < 1000.0 kJ/mol/nm with PME (Particle Mesh Ewald) as the Coulomb type, the *r*coulomb cutoff of 1.0 and van der Waals cutoff of 1.0. For validation purposes, the minimized final model was submitted to various structure validation programs (PROCHECK, VERIFY3D and ERRAT) available through SAVeS server (<https://servicesn.mbi.ucla.edu/SAVES/>).

Collagen triple-helical peptide construction

The crystal structure of the $\alpha 2$ -I domain-collagen peptide complex was downloaded from the PDB databank, and then collagen triple-helical peptides (strands B, C, D) were manually separated from the integrin domain. Each peptide sequence comprises of 21 amino acid residues (the residues in each peptide strands were later renumbered from 1 to 21). Here, we constructed three different collagen triple-helical peptides namely, (GPP)₂GFQGEK(GPP)₃, and (GPP)₂GPKGER(GPP)₃. These collagen triple-helical peptides were constructed using COOT software package (P. Emsley et al. 2010). All these constructed triple-helical peptides were then subjected to 500 steps of steepest descent minimization using GROMACS version 4.0.7 (Hess et al. 2008; Van Der Spoel et al. 2005). The final energy minimized triple-helical peptides were then used for docking with the human integrin $\alpha 11$ -I domain.

Protein-protein docking

Docking of modeled human $\alpha 11$ -I domain and the constructed triple-helical peptides ($\alpha 11$ -(GPP)₂GFQGEK(GPP)₃ and $\alpha 11$ -(GPP)₂GPKGER(GPP)₃) were carried out using the HADDOCK program (Dominguez et al. 2003), a Web-based tool to perform protein-protein docking. The HADDOCK docking program is driven based on the Ambiguous Interaction Restraints derived from the experimental information of the intermolecular interacting residues provided by the user. We used the easy interface for all the docking calculations. However, before carrying out the docking experiments between the modeled human $\alpha 11$ -I domain and collagen XIII peptides, we first performed a control docking experiment

using the crystal structure of ligand-binding $\alpha 2$ -I domain to validate the HADDOCK docking program. Upon docking of $\alpha 2$ -I domain and its collagen triple-helical peptide (GFOGER), the program had predicted the key interacting residues as seen in the crystal structure suggesting that HADDOCK can be used for the docking calculations using the triple-helical peptides. The constructed peptides were then docked to the modeled $\alpha 11$ -I domain. The best representative docked complex was chosen based on the lowest energy cluster and HADDOCK score obtained through diverse energy terms such as van der Waals energy, electrostatic energy, desolvation energy, restraints violation energy and buried surface area. PDBsum database was used to analyze the protein-peptide interactions while the binding energy (ΔG) was calculated using the PRODIGY server (Xue et al. 2016).

Mouse line

Generation of the transgenic *Coll13a1^{oe}* mice has been described previously (Ylönen et al. 2005), and the mouse line is publicly available in the Infrafrontier EMMA repository with the strain name B6.Cg-Tg(Col13a1)2Pih/Oulu (EM:09885). Generation of *Itga11^{-/-}* mice has been described previously (Popova et al. 2007). Here, *Itga11^{-/-}* mice in C57BL/6J background were bred with *Coll13a1^{oe}* to generate a *Coll13a1^{oe};Itga11^{-/-}* double mutant mouse line, overexpressing *Coll13a1* and lacking *Itga11*. *Coll13a1^{oe}* littermates were used as controls. Permission for the maintenance of mice (license ID ESAVI/4220/04.10.07/2013) was obtained from the Finnish Animal Care and Use Committee of the State Provincial Office of Southern Finland (Hämeenlinna, Finland) and the European Community Council Directive on the protection of animals used for scientific purposes (September 22, 2010; 2010/63/EEC), national legislation and the regulations for the care and use of laboratory animals were followed.

μ CT

Tubular bone microarchitecture was assessed from left femurs of *Coll13a1^{oe};Itga11^{-/-}* mice, and they were compared to *Coll13a1^{oe}* mice. Number of mice analyzed in order of increasing age: female *Coll13a1^{oe}* (7, 4, 6, 6, 6), female *Coll13a1^{oe};Itga11^{-/-}* (3, 3, 4, 3, 2), male *Coll13a1^{oe}* (5, 4, 7, 6), male *Coll13a1^{oe};Itga11^{-/-}* (3, 3, 3, 3). The most recent guidelines for bone microstructure assessment were used (Bouxsein et al. 2010). Bones were imaged using a Skyscan 1176 scanner (Bruker microCT, Kontich, Belgium) using the following parameters: X-ray tube voltage of 50 kV, current of 500 μ A, exposure time of 4000 ms, and a 0.5 mm aluminum filter. Projection

images were collected every 0.3° over a 360° rotation. Image processing was performed with the manufacturer's software NRecon (v. 1.6.5.2), Dataviewer (v. 1.5.6.2), and CTAn (v. 1.11.10.0). Femur length was measured from reconstructed datasets from the superior top of trochanter major to the distal patellofemoral groove in mid-frontal plane. The average value of three separate measurements was used as the result for the femur length. Measurements were conducted using the manufacturer's software (Skyscan, Dataviewer v. 1.5.6.2). Besides the femur length, cortical bone morphology was assessed. Diaphyseal cortical bone was assessed using the VOI starting 0.9 mm distally from the femoral neck and extending further distally. Two different ROI lengths were used for cortical bone analysis: 3.6 mm was used for 4-week time point, whereas 5.4 mm was used for other time points. 2D structural analysis was performed to define cortical bone area (Ct.Ar) and average cortical thickness (Ct.Th). 2D volumetric analyses of datasets were conducted using CTAn software (Skyscan, v. 1.11.10.0).

Statistics

Statistical significance was determined by two-way ANOVA followed by the Benjamini, Krieger, and Yekutieli FDR post hoc test (Benjamini et al. 2006). Values of FDR-corrected p (q) < 0.05 values were considered statistically significant. No statistical methods were used to predetermine sample size.

Results

Integrin $\alpha 11\beta 1$ is localized at focal contacts in cells spreading on a collagen XIII-coated surface

To determine whether $\alpha 11\beta 1$ mediates the spreading of cells on collagen XIII, $\alpha 11$ -EGFP transfected C2C12 mouse myoblasts (C2C12- $\alpha 11+$), and mouse embryonic fibroblasts (MEFs) derived from a wild-type mouse embryo were tested. Direct fluorescence imaging of C2C12- $\alpha 11+$ cells showed that $\alpha 11$ -EGFP localized at cell-matrix adhesion structures during cell spreading on collagen XIII (Fig. 1a) and on collagen I (Fig. 1c), but not on fibronectin (Fig. 1e). The cellular localization of integrin $\alpha 11\beta 1$ on primary MEFs on collagen XIII was studied by immunofluorescence staining using an antibody against the cytoplasmic domain of the mouse integrin $\alpha 11$ subunit (Fig. 1b). Spreading on collagen I was analyzed as a control (Fig. 1d). These data indicate that both collagen XIII and collagen I, but not fibronectin, are functional ligands for integrin $\alpha 11\beta 1$.

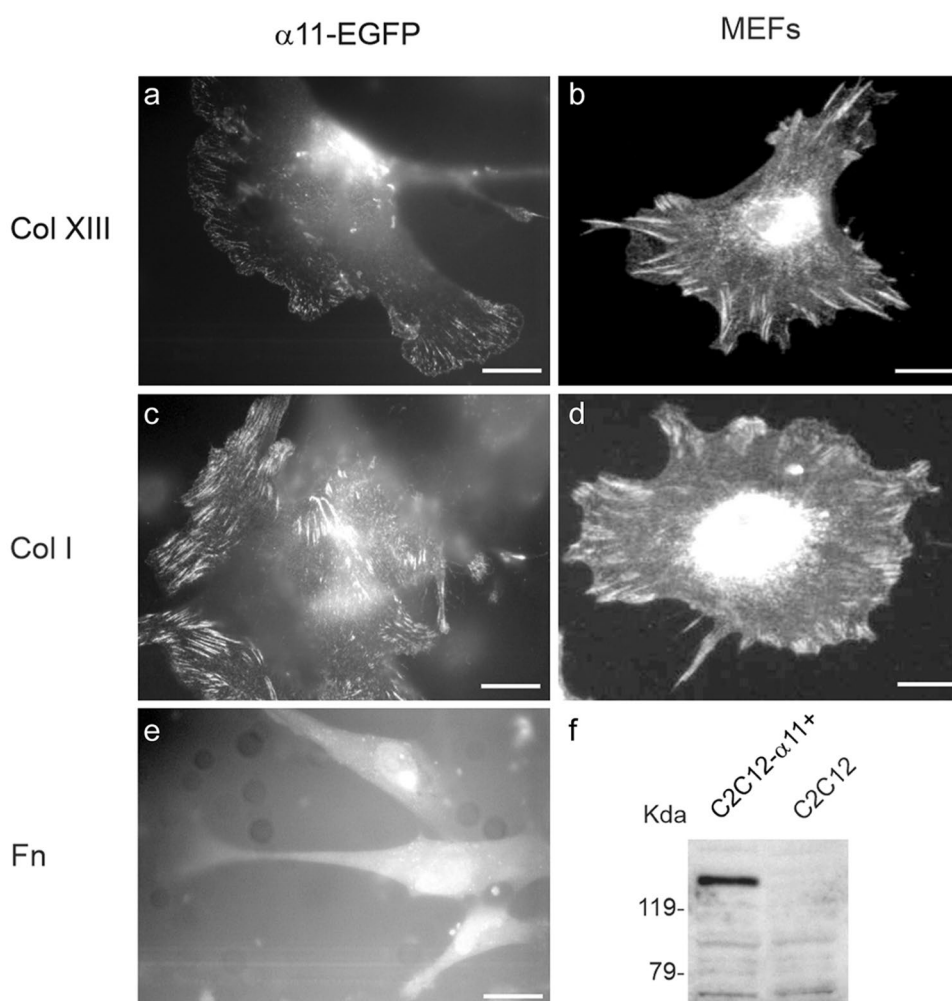
C2C12 cells expressing integrin $\alpha 11\beta 1$ adhere to collagen I and collagen XIII-coated surfaces

The interaction between collagen XIII and integrin $\alpha 11\beta 1$ was further studied by using the transfected C2C12 cells that expressed human $\alpha 11\beta 1$ as their only collagen receptor (Tiger et al. 2001). C2C12 cells transfected with the vector plasmid (MOCK) were tested in parallel. The expression of the $\alpha 11$ subunit was verified by western blotting using an antibody specific for the cytoplasmic domain of the human integrin $\alpha 11$ (Fig. 1e). C2C12 control cells did not adhere to native collagen I or to native collagen XIII, but the cells attached well to fibronectin due to the endogenous expression of fibronectin receptor integrins (Fig. 2a). Cell attachment assays utilizing C2C12 cells that were stably transfected with the full-length human integrin $\alpha 11$ cDNA (C2C12- $\alpha 11+$ cells) indicated a remarkable increase in adhesion to collagens I and XIII when compared to control C2C12 cells (Fig. 2a). The attached C2C12- $\alpha 11+$ cells started to spread on the collagen XIII-coated surfaces after 2 h (Fig. 2b). For control purposes, C2C12- $\alpha 2+$ cells were tested with similar results as in our previously published study (Fig. S1), i.e., integrin $\alpha 2$ can mediate cell spreading on collagen I but not on collagen XIII (Nykqvist et al. 2000). Integrin $\alpha 2\beta 1$ and $\alpha 11\beta 1$ have both been suggested to be the cellular receptors for fibrillar collagens (Zeltz and Gullberg 2016), but the present results demonstrate a remarkable difference in their preference for collagen XIII.

Collagen XIII binds to the I domain of integrin $\alpha 11$ subunit with high avidity in vitro

A GST- $\alpha 11$ I-fusion protein has previously been produced in *E. coli* (W. M. Zhang et al. 2003), but the protein yield and binding activity varied from batch to batch (Käpylä J. et al., unpublished). To facilitate studies on the interaction between integrin $\alpha 11\beta 1$ and collagen XIII in more detail, we set up an expression system of the $\alpha 11$ -I domain as a secreted protein in insect cells. The recombinant protein contains a GST-tag and a thrombin cleavage site between GST and the I domain. The $\alpha 11$ -I domain was purified from the medium by a one-step GST-affinity purification method. The unpurified media containing GST-tagged $\alpha 11$ -I domain or GST as a blank control was used for binding specificity test in vitro. The human collagen XIII ectodomain was expressed as a homotrimer using a baculovirus system in insect cells (Tu et al. 2002). Furthermore, collagen types I and IV were compared to collagen XIII in the binding assays. The integrin $\alpha 1$ - and $\alpha 2$ -I domains obtained from *E. coli* (Nykqvist et al. 2000) which also contained the GST-tags, were included for selected interaction studies. The binding tests were performed using an enzyme-linked immunosorbent assay (ELISA) in which collagens I, IV, and XIII were coated onto

Fig. 1 Localization of integrin $\alpha 11\beta 1$ in cells spreading on collagens XIII and I. C2C12 cells transfected with integrin $\alpha 11$ -EGFP were seeded onto coverslips coated with collagen XIII (Col XIII) ectodomain **a** and collagen I (Col I) **c** and allowed to adhere at 37 °C for 2 h. The cells were then fixed and imaged on a fluorescence microscope with direct detection of EGFP-tagged protein. **e** The C2C12- $\alpha 11+$ cells spreading on the fibronectin-coated surface. Primary wild type MEFs spread on collagen XIII **b** and on collagen I **d** under the same conditions as the C2C12- $\alpha 11+$ cells and were visualized with indirect immune fluorescence staining using an antibody against the cytoplasmic domain of mouse integrin $\alpha 11$. **e** C2C12 cells were stably transfected with integrin $\alpha 11$, and the expression of $\alpha 11$ was analyzed by western blotting using an antibody against the $\alpha 11$ cytoplasmic domain



the plastic surface as solid ligands and the I domains were used as soluble analytes. The interaction was detected using a GST antibody. The $\alpha 11$ -I domain interacted with collagen types I and XIII in a dose-dependent manner (Fig. 3a). Collagen XIII was identified to bind $\alpha 11$ -I domain with similar affinity as collagen I and stronger than collagen IV (Fig. 3b). Consistently with the previously published results (W. M. Zhang et al. 2003; Tiger et al. 2001; Nykvist et al. 2000), collagen IV showed preferential binding to the $\alpha 1$ -I domain, but no binding to the $\alpha 11$ -I domain (Fig. 3b). In contrast to collagens IV and XIII, collagen I bound well to all the tested integrin I domains (Fig. 3b).

The specific interaction between collagen XIII and integrin $\alpha 11\beta 1$ was further confirmed by surface plasmon resonance (SPR) and pull-down assays. In SPR, collagen XIII showed a typical sensorgram of binding to the immobilized fusion protein $\alpha 11$ -I domain (Fig. 3c, black curve) unlike in the blank control in which no protein was immobilized (Fig. 3c, gray curve). In a pull-down assay, collagen XIII was trapped only by the GST- $\alpha 11$ -I domain coupled to the glutathione-Sepharose but not when GST was used alone.

The protein complex could be eluted by glutathione or thrombin, indicating that the interaction site locates in the $\alpha 11$ -I domain (Fig. 3d). Due to the long incubation time at room temperature, collagen XIII eluted from the interaction complex was partly degraded, resulting in a reduced molecular mass of 60 kDa. The unbound fraction contained both this 60 kDa fragment as well as the full-length 72 kDa collagen XIII ectodomain, and after overnight incubation, mimicking the enzyme digested fractions, equal degradation of the unbound fraction was evidenced (Fig. 3d).

Integrin $\alpha 11$ -I domain binds specifically to the C-terminal collagenous domain of collagen XIII

To identify the potential integrin $\alpha 11\beta 1$ binding sites in collagen XIII, the purified collagen XIII ectodomain was digested by pepsin and the cleaved fragments were separated by gel-filtration chromatography (Tu et al. 2002). The peptide representing the COL3 domain showed similar integrin-binding capability as the intact ectodomain

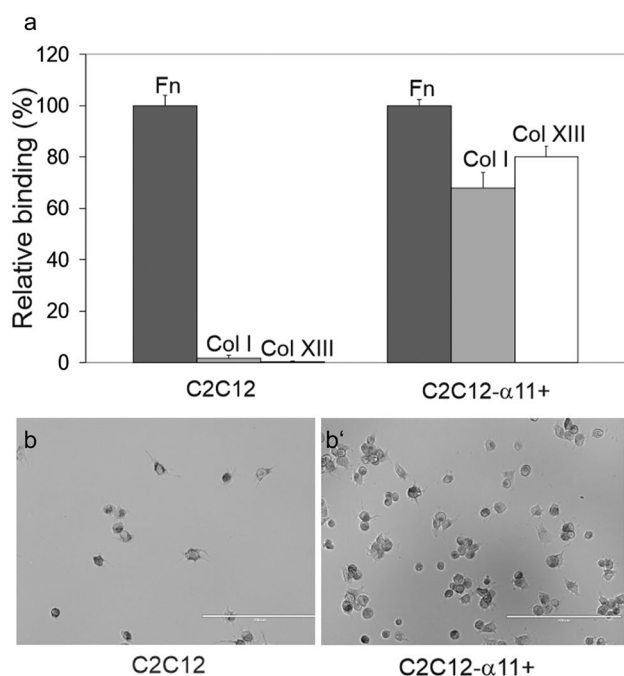


Fig. 2 Adhesion of integrin α 11-transfected C2C12 cells to fibronectin and collagens (a) C2C12 control cells and cells transfected with an integrin α 11cDNA (C2C12- α 11+) were seeded on a plastic surface coated with fibronectin, collagen I, or the collagen XIII ectodomain. The cells were analyzed quantitatively using the β -hexosaminidase release assay for the attachment to fibronectin, collagen I, and collagen XIII. Data are presented relative to fibronectin and BSA binding, where the saturated binding to fibronectin represents 100% and the background binding to a BSA-coated surface represents 0%. Values are shown as means of triplicates with standard deviation bars. (b, b') Microscopic monitoring of the attachment and morphology of C2C12 and C2C12- α 11 cells adhering on collagen XIII. Scale bars 200 μ m

(Fig. 4a). Collagen XIII does not contain the GFOGER or other previously identified integrin-binding motifs (UniProt Consortium 2019). However, the COL3 domain in collagen XIII harbors two GER and seven GEK sequences that are typically found as parts of the known integrin-binding motifs. To test these putative binding sequences, two GER motifs, namely GPKGER and GNRGER, and one GEK motif, namely GF(S)QGEK (“S” in mouse), were expressed in *E. coli* using an expressing vector encoding two (GPP) \times 5 sequences flanking the tested fragments, a foldon sequence for triple-helical formation (Frank et al. 2001), and a His-tag for the protein purification (Fig. 4c). GFPGER was chosen as a positive control and GEK-GAKGSPGLP, a peptide located on the COL2 domain and binding to integrin α 1 β 1 (Dennis et al. 2010), was produced for a negative control. Other GEK motifs were considered less interesting based on the knowledge gained from the peptide ToolKits of collagen-receptor interactions (Farndale et al. 2008). The motifs GPKGER and GF(S)QGEK showed positive binding to the integrin α 11-I

domain, albeit at a 50–60% lower approximated avidity when compared to GFPGER (Fig. 4b). GEKARGSPGLP and GNRGER showed no binding (Fig. 4b). The protein-protein interaction was further confirmed by a cell attachment assay utilizing collagen I, the ectodomain of collagen XIII, and the recombinant proteins containing the putative integrin-binding motifs. The binding of cells was markedly stronger to the GPKGER than to the GF(S)QGEK motif (Fig. 4d). Furthermore, the attachment of C2C12- α 11+ cells to the collagen XIII-coated surface could be partially inhibited by GPKGER (Fig. 4e). These data suggested that both GPKGER and GF(S)QGEK contributed to the binding of collagen XIII to the α 11-I domain. The two binding sites identified in the COL3 domain of collagen XIII lie within a highly conserved region (Fig. 4f).

Homology modeling and structure validation

To study the molecular interaction between the human α 11-I domain and the triple-helical peptides, a homology model of the human α 11-I domain was constructed using the crystal structure of the human integrin α 2-I domain (PDB code: 1DZI) (J. Emsley et al. 2000) as a template. Prior to the homology model construction, the sequence alignment of α 11 and α 2 integrin I domains were subjected to manual inspection as a quality check; this identified a shared sequence identity and similarity of 44% and 63.1%, respectively (Fig. 5), being amendable for the further modeling studies. The energy minimized model of the human α 11-I domain is shown in Fig. 6a. The root mean square deviation between the final energy minimized model of the α 11-I domain and the α 2-I domain was 0.237 (Fig. 6b), suggesting that the initial backbone structure was intact and there was not much deviation from its original conformation. Ramachandran plot analysis using PROCHECK (Laskowski et al. 1993) showed that 90.1% of the residues fall in the most favored regions and 9.3% of the residues in the additionally allowed regions (Fig. S2). Also, the assessment of the constructed model by its 3D profile using VERIFY3D (Eisenberg et al. 1997) showed that 100% of the residues are within the allowed regions (Fig. S3). Further, the reliability and the quality of the model were validated using the ERRAT program (Colovos and Yeates 1993). The comparison between the initial model (before minimization) and the final model (after minimization) showed that the quality of the modeled protein was significantly increased after minimization with the overall quality factor score of 97.093 (Fig. S4). The structure validation programs suggest that the constructed homology model of the human α 11-I domain structure is of high quality and reliable, thus, can be used for our docking studies.

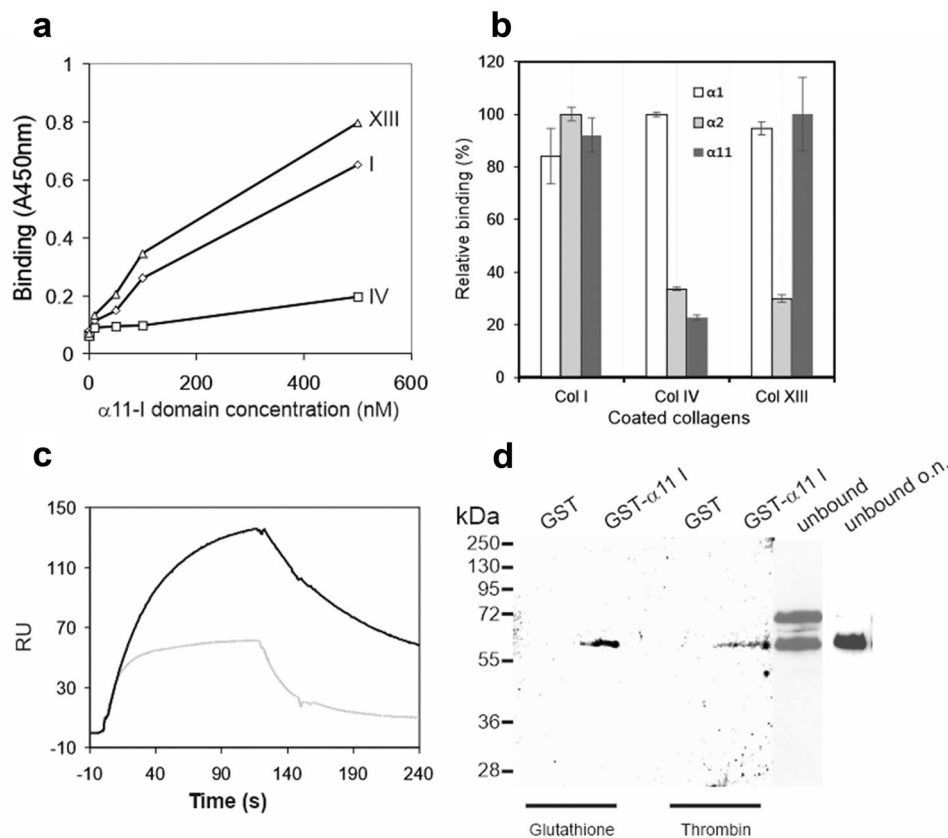


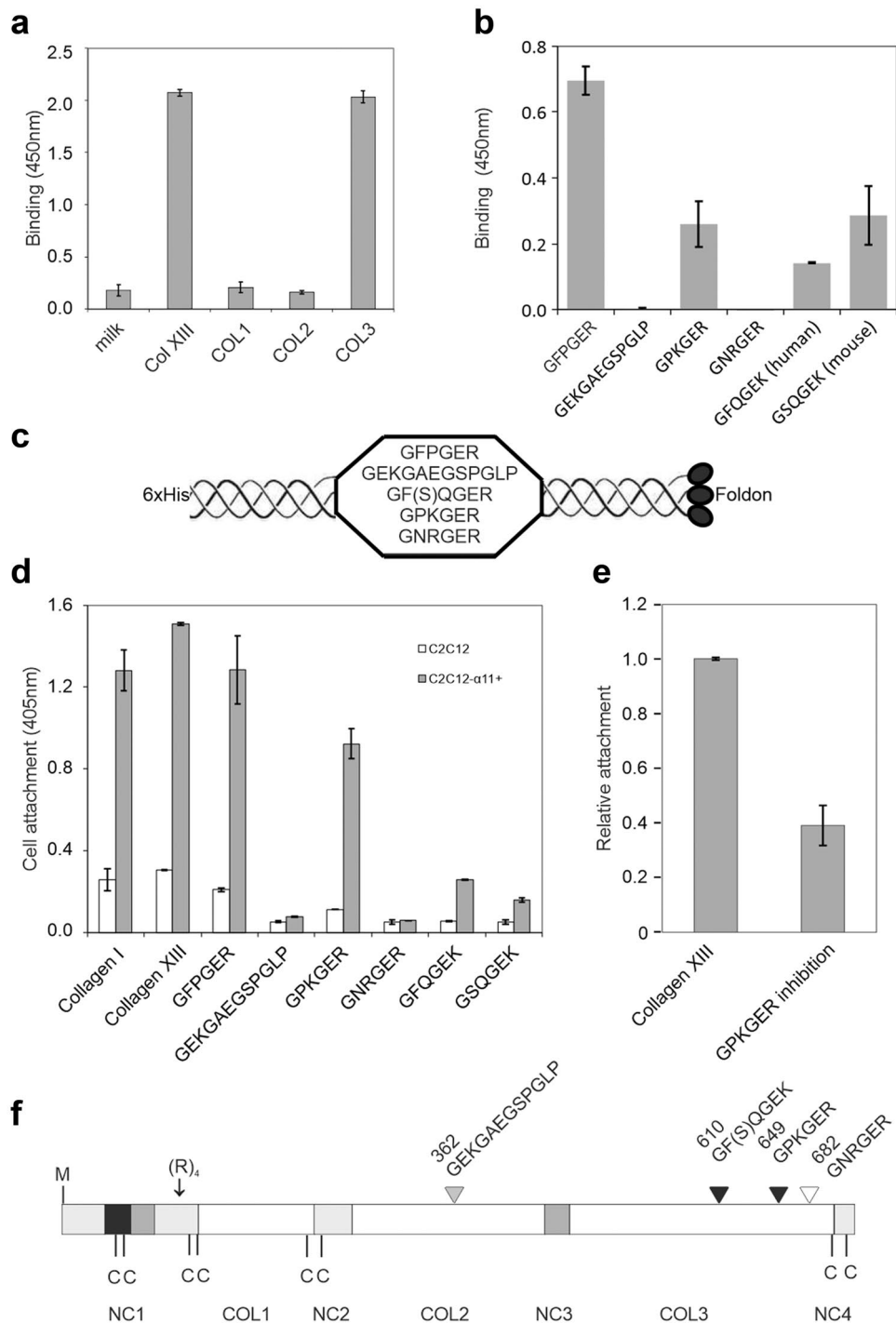
Fig. 3 Selective binding of collagen XIII to the integrin $\alpha 11$ -I domain; **a** 0.25 μ g of collagens I, IV, and XIII in 50 μ l of TBS were coated on microtiter plates. The GST- $\alpha 11$ -I domain was diluted in series in TBS containing 30 μ g/ml BSA, 2 mM $MgCl_2$, and 0.05% Tween-20 and used for binding tests employing a solid-phase assay with an antibody against GST. Five percent of fat-free milk in TBS was used for blocking the uncoated surface and for a blank control. **b** A summary of relative binding comparison of I domains to the coated collagen types I, IV, and XIII (Col I, Col IV, and Col XIII, respec-

tively). **c** SPR response of the binding of collagen XIII to the immobilized integrin $\alpha 11$ -I domain (black curve). The gray curve shows the background of the sensorgram. **d** A pull-down assay was performed by incubation of collagen XIII with the GST- $\alpha 11$ -I domain or GST protein coupled glutathione-Sepharose. The interaction complex was eluted by 10 mM of reduced glutathione or cleaved by thrombin. The eluted, unbound, and unbound incubated overnight (o.n.) collagen XIII was detected by a collagen XIII monoclonal antibody

Docking of $\alpha 11$ -I domain and triple-helical collagen peptides

To study the collagen XIII binding mode to the $\alpha 11$ -I domain, we performed docking studies using our homology model of $\alpha 11$ -I domain and the HADDOCK program (Dominguez et al. 2003). First, the interface residues that directly interact with the triple-helical collagen were identified from the crystal structure of the $\alpha 2$ -I domain. Then, the identified residues were mapped correspondingly to the $\alpha 11$ -I domain in the sequence alignment. These residues were then supplied as active residues of the $\alpha 11$ -I domain while the passive residues were set to define automatically by the HADDOCK program (Dominguez et al. 2003) to perform the docking calculations.

The HADDOCK server produced 176 different structures of the $\alpha 11$ -(GPP)₂GFQGEK(GPP)₃ complex classified in 8 clusters which represents 88.0% of the water-refined models. For the $\alpha 11$ -(GPP)₂GPKGER(GPP)₃ complex, the HADDOCK server resulted in 136 structures classified in 10 clusters representing 68% of the water-refined models. Analysis of the $\alpha 11$ -(GPP)₂GFQGEK(GPP)₃ complex revealed that the residues from all the three strands (strands B, C, and D) of the collagen peptide contributed to the interactions with the interface residues of the $\alpha 11$ -I domain (Fig. 7a). Lys12 of the strand B, Glu11 and Lys12 of the strand C, and Glu11 and Lys12 of the strand D were involved in the formation of multiple hydrogen bonds with the $\alpha 11$ -I domain. Apart from the hydrogen bonds, Glu11 and Lys12 of the strand



C, Lys12 of the strand D also participated in the salt bridge formation with the α 11-I domain residues. The energy values of the docked complexes are shown in Table 1 and detailed list of hydrogen bonds formed between the atoms of α 11-I domain and GFQGEK is shown in Table 2.

The interaction between the α 11-I domain and $(GPP)_2GPKGER(GPP)_3$ was distinct compared to the

$(GPP)_2GFQGEK(GPP)_3$ interaction with the α 11-I domain. Here, much of the interactions come from the Lys9 residue of the strand C collagen peptide (Fig. 7b). Further, Glu76 of the α 11-I domain and Lys9 and Arg12 of the collagen peptide from the strand C participated in the H-bond and salt bridge formation. Another salt bridge is formed between Glu110 of α 11-I domain and Lys9 of the

Fig. 4 Integrin $\alpha 11$ binds to the COL3 domain of collagen XIII specifically by GPKGER and GF(S)QGEK. **a** A solid-phase binding assay shows binding of the integrin $\alpha 11$ -I domain to the COL3 domain specifically. One hundred nanomolars of the collagen XIII ectodomain (Col XIII) and its pepsin-digested collagenous fragments, COL1, COL2, and COL3 in 50 μ l of HEPES buffer were coated onto a microtiter plate wells, and 700 nM of the GST- $\alpha 11$ -I domain in 50 μ l of HEPES buffer containing 2 mM $MgCl_2$ was added for binding testing. The bound I domain was detected by a GST antibody. **b** A solid-phase binding assay shows binding of the $\alpha 11$ -I domain to the recombinant proteins composed of the testing motifs flanked by the collagenous sequence 5x(GPP) and a foldon sequence at the C-termini. **c, d** A cell attachment assay indicates the binding of the specific collagen XIII motifs to the integrin $\alpha 11$. GFPGER was used as positive control. **e** Cell attachment inhibition by the GPKGER motif. **f** A schematic structure of collagen XIII compiled from previous work (Hägg et al. 1998). The black arrowheads indicate the positive binding sites and the white shows a negative binding site. The grey arrowhead points to a reported integrin $\alpha 1$ -binding site (Dennis J. et al. 2010). The numbers above the arrowheads represent the residues in the human collagen XIII sequence. The collagenous domains (COL1–3) are indicated as white boxes, the non-collagenous domains (NC1–4) as light gray boxes, coiled-coil motifs as dark gray boxes, and the transmembrane domain as a black box. The initiation methionine is marked as M, cysteine residues as C, and a furin proteolytic cleavage site consisting of four arginine residues as (R)₄

strand B. Table 3 shows the detailed list of hydrogen bonds and salt-bridges between the acceptor and donor atoms of the $\alpha 11$ -(GPP)₂GPKGER(GPP)₃ complex.

Collagen XIII-integrin $\alpha 11$ interaction regulates bone homeostasis in vivo

To study the in vivo significance of collagen XIII- $\alpha 11\beta 1$ interaction, we crossbred integrin $\alpha 11$ null mice (*Itga11*^{-/-}) with mice overexpressing *Col13a1* (*Col13a1*^{oe} (Ylönen et al. 2005, Koivunen et al. 2019)) and created a double mutant mouse line (*Col13a1*^{oe};*Itga11*^{-/-}). The *Col13a1*^{oe} mice possess a severe bone phenotype, in which especially the circumference of middiaphyseal femur is enlarged (Koivunen et al. 2019). The femoral cortical bone morphology of *Col13a1*^{oe} and *Col13a1*^{oe};*Itga11*^{-/-} mice was evaluated in females at five time points and males at four time points by micro-computed tomography (μ CT). As a result, we discovered that the *Col13a1*^{oe};*Itga11*^{-/-} double mutant mice had a significantly milder bone phenotype when compared to *Col13a1*^{oe} mice, the *Col13a1*^{oe};*Itga11*^{-/-} mice had less cortical bone overgrowth (females in Fig. 8a and males in Fig. S5a) and the bone loss (females in Fig. 8b and males in Fig. S5b) was significantly diminished when compared to *Col13a1*^{oe}. No differences were observed in the bone length between these genotypes (females in Fig. 8c and males in Fig. S5c). These results indicate that collagen XIII-integrin $\alpha 11\beta 1$ interaction has a role

in cortical bone homeostasis, and this role is evident in both genders.

Discussion

Collagens are known to be recognized by the α -I domains in collagen receptor integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$ (Gullberg and Lundgren-Akerlund 2002; Zeltz and Gullberg 2016), and it has been shown that $\alpha 1\beta 1$ and $\alpha 10\beta 1$ interact preferentially with the basement membrane collagen IV and the beaded filament forming collagen VI (Tulla et al. 2001; Nykvist et al. 2000), while $\alpha 11\beta 1$ shows similar features as $\alpha 2\beta 1$ and selects the interstitial collagens (W. M. Zhang et al. 2003). However, collagen IX, a cartilage matrix component, shows exceptionally high binding capacity to all four integrin I domains, suggesting a more complicated mechanism for cell adhesion to collagens (Käpylä et al. 2004). Here, we show the firm interaction of collagen XIII with integrin $\alpha 11\beta 1$.

The motif GFOGER in a triple-helical context has been identified as a target sequence for $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$ integrins (Knight et al. 1998, Xu et al. 2000, W. M. Zhang et al. 2003, Sipilä et al. 2014), but these integrins also bind to other ECM proteins that do not contain this particular sequence (Pandolfi et al. 2017). On the other hand, collagen IV does not bind to $\alpha 2\beta 1$ or $\alpha 11\beta 1$ as strongly as collagens I–III, although it contains the GFOGER sequence in the $\alpha 1(IV)$, $\alpha 3(IV)$, $\alpha 4(IV)$, and $\alpha 5(IV)$ chains. Additionally, collagen IX, which binds avidly to all integrin I domains, lacks the GFOGER motif. Hence, it has been proposed that GFOGER is not the only target sequence for collagen-binding integrins. Indeed, studies on the integrin-recognition motifs in collagen III have identified GROGER, GLKGEN, and GLOGEN as integrin-binding sites (Raynal et al. 2006; Kim et al. 2005). Moreover, a prokaryotic collagen sequence GLPGER has been reported to bind to integrins $\alpha 2\beta 1$ and $\alpha 11\beta 1$ (Caswell et al. 2008). A study using the collagen peptide ToolKits, which consist of sets of overlapping triple-helical peptides, identified two motifs GLOGEN and GVOGEA that are specific for $\alpha 1\beta 1$ (Hamaia et al. 2012). Additional binding motifs identified for collagen-binding integrins are GFKGER, GLQGER, GLOGER, and GASGER (Zwolaneck et al. 2014; Xu et al. 2000).

Our previous study has shown that the ectodomain of the transmembrane collagen XIII binds avidly to the $\alpha 1$ -I domain (Nykvist et al. 2000). Here, we show that C2C12 cells expressing $\alpha 11\beta 1$ spread on a collagen XIII-coated surface in the same manner as on a collagen I-coated surface. Among the integrin $\alpha 1$ -, $\alpha 2$ -, and $\alpha 11$ -I domains, the $\alpha 11$ -I domain showed strongest binding to collagen XIII, and

1DZI_ITGA2	1	ALIDVVVVCDSENSIYPWDAVKNFLEKQGLDIGPTKTQVGLIQYANNP	50
		: : : : ..:::.. . . .: . : . . : ..	
ITGA11_human	1	---DIVIVLDGSNSIYPWVEVQHFILNILKKFYIGPGQIQVGVVQYGEDV	47
1DZI_ITGA2	51	RVVFNLTNTYKTKKEEMIVATSQTSQYGGDLTNTFGAIQYARKYAYSAAASGG	100
		... : . . .: : : : :	
ITGA11_human	48	VHEFHLNDYRSVKDVVEAAASHIEQRGGTETRATFGIEFARSEAFQ--KGG	95
1DZI_ITGA2	101	RRSATKVMVVVTDGESHSDGSMKLAVIDQCMHDNILRFGIAVGLGYLNRNAL	150
		: . . : : . : : . ::	
ITGA11_human	96	RKGAKKVMIVITDGEHSDSPDLEKVIQQSERDNLNTRYAVAVLGYNRRGI	145
1DZI_ITGA2	151	DTKNLIKEIKAIASIPTERYFFNVSDEAALLEKAG--	185
		: : :: : : : : : : : : : : : ..	
ITGA11_human	146	NPETFLEIKYIASDPDDKHFFNVTDEAALKDIVDAL	182

Fig. 5 Sequence alignment. Human integrin $\alpha 2$ -I (ITGA2) and $\alpha 11$ -I (ITGA11) domains share sequence identity of 43.9% and 63.1%, respectively

the collagen XIII showed more specific interaction with the $\alpha 11$ -I domain when compared to other collagen types. These data suggest differences in the interaction of collagen XIII with $\alpha 11$ integrin compared to other α subunits.

Collagen XIII is reported to be a ligand mediating integrin $\alpha 1\beta 1$ -dependent transmigration of monocytes in Alport renal fibrosis (Dennis et al. 2010). In that study, $\alpha 1\beta 1$ -positive monocytes were shown to bind to a GEK-GAEGSPGLP sequence located in the COL2 domain of the collagen XIII. In the present study, the $\alpha 1\beta 1$ binding site in collagen XIII was localized in the COL3 domain. Some of the known integrin-binding collagenous motifs, such as GFOGER, GLOGER, and GVPGEA (see above), do not exist in collagen XIII. Furthermore, we did not consider

GXXGEN-like sequences ($X =$ any of the amino acid residues) since these motifs were not preferred in a previous study on the $\alpha 11$ -I domain utilizing the collagen peptide ToolKits (W. M. Zhang et al. 2003). In the present study, four motifs, GPKGER, GFQGEK, GSQGEK, and GNRGER from the COL3 domain, and GFPGER, used as a positive control, were chosen for the binding tests. GNRGER showed no binding to the $\alpha 11$, a finding consistent with the ToolKit data. GPKGER, GFQGEK, and GSQGEK bound to $\alpha 11$, but the interaction was more modest than that with GFPGER. Proline hydroxylation is known to be important in collagen-integrin interaction (Sipilä et al. 2018; Rappu et al. 2019). However, in this study, coexpression of prolyl 4-hydroxylase (P4H) in the insect cells did not catalyze the proline hydroxylation probably due to the short (GPP)₅ sequence, which was not sufficient for binding of the enzyme P4H to the collagen substrate. Thus, here all prolines are non-hydroxylated. While many previously reported integrin-binding motifs contain hydroxyproline (Rappu et al. 2019), there are also several exceptions, such as GLSGER and GQRGER (Siljander et al. 2004), GFKGER (Zwolaneck et al. 2014), GLQGER (Zwolaneck et al. 2014), and GASGER (Xu et al. 2000). Moreover, in the hydroxyproline containing functional domains hydroxylation of proline residues is not absolutely required for integrin binding, but it increases the avidity of the interaction. For example, the $\alpha 2$ -I domain can bind to GFPGER containing peptides, but more weakly than to GFOGER (Knight et al. 2000).

To model the interaction between the $\alpha 11$ -I domain and collagen motifs, two different collagen peptides ((GPP)₂GFQGEK(GPP)₃ and (GPP)₂GPKGER(GPP)₃) were constructed through in silico approaches. The docking analysis showed that both constructed triple-helical

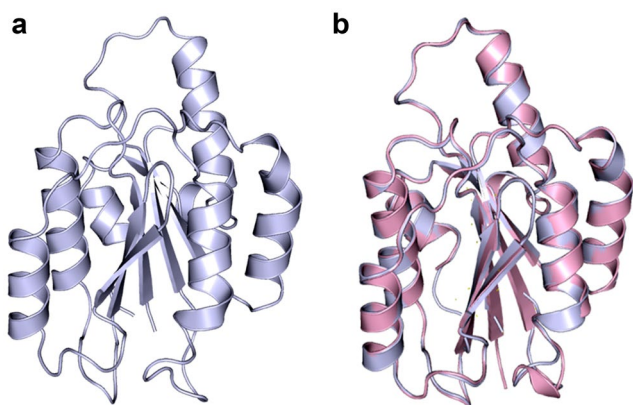


Fig. 6 Molecular model of the integrin $\alpha 11$ -I domain. **a** An energy minimized structure of human integrin $\alpha 11$ -I domain. **b** A superimposed model of the $\alpha 11$ -I domain (blue) and the crystal structure of human integrin $\alpha 2$ -I domain (pink, PDB: 1DZI) is shown. The root mean square deviation between these two structures is 0.237

Fig. 7 Docked complexes of collagen tripeptides and the $\alpha 11$ -I domain. **a** The collagen XIII tripeptide (GPP)₂GFQGEK(GPP)₃ bound to the $\alpha 11$ -I domain, sidechains of the interacting residues are labeled and shown on the right side. **b** A complex of $\alpha 11$ -I (GPP)₂GPKGER(GPP)₃ along with the interacting residues

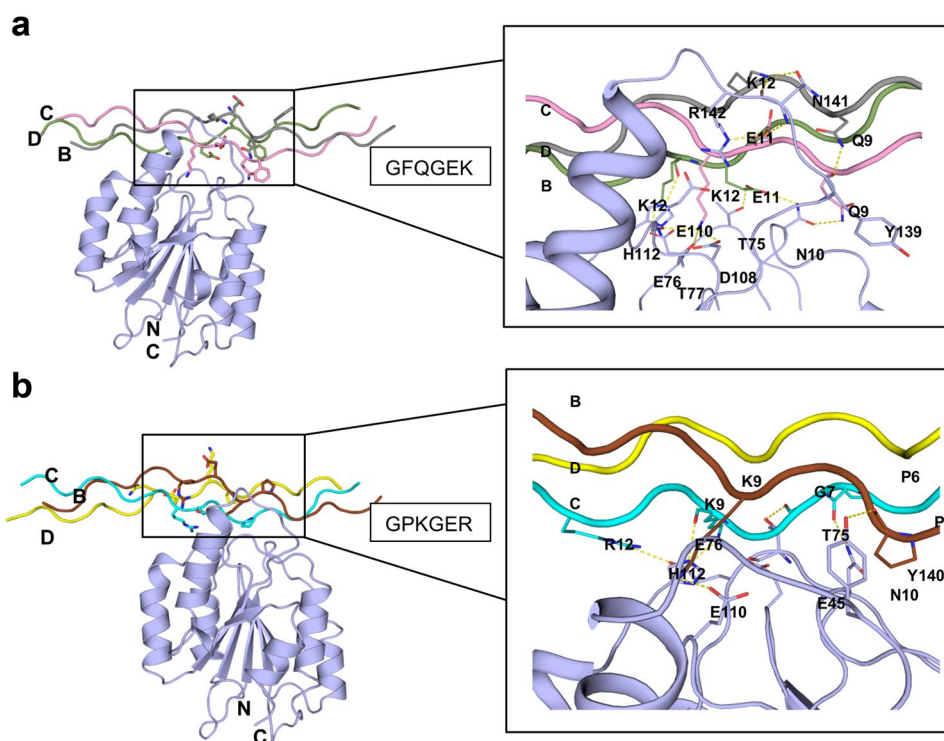


Table 1 The best representative docked complex was chosen based on the HADDOCK score and the cluster size. Binding energy (ΔG) for each of the docked complexes was calculated using the PRODIGY

server. Van der Waals, electrostatic energy, and buried surface area obtained from HADDOCK server are shown

Docked Complex	HADDOCK score	Binding energy ΔG (kcal/mol)	Van der Waals energy (kcal/mol)	Electrostatic energy (kcal/mol)	Buried Surface (\AA^2)
$\alpha 11$ -I domain-(GPP) ₂ GFQGEK(GPP) ₃	-105.99 ± 9.9	-6.7	-41.0 ± 6.1	-391.4 ± 45.7	1505.4 ± 129.6
$\alpha 11$ -I domain-(GPP) ₂ GPKGER(GPP) ₃	-60.3 ± 3.4	-7.1	-30.199 ± 4.8	-290.0 ± 37.0	1109.4 ± 109.2

Table 2 Residues contributing to hydrogen bonds between the $\alpha 11$ -I domain and triple-helical peptide (GPP)₂GFQGEK(GPP)₃ (chains B, C, and D) is shown. The atom names and the interacting distance (in Angstroms) are shown in square brackets

$\alpha 11$	(GPP) ₂ GFQGEK(GPP) ₃		
	Chain B	Chain C	Chain D
TYR139[O]	GLN9[NE2] [2.84]		
ASN141[O]	LYS12[NZ] [2.91]		
ASN141[OD1]	LYS12[NZ] [3.05]		
ASN10[OD1]		GLN9[NE2] [3.03]	
THR77[OG1]		LYS12[NZ] [2.91]	
ASP108[OD2]*		LYS12[NZ]* [2.67]	
GLU110[O]		LYS12[NZ] [2.76]	
ASN14[N]		GLU11[OE2] [3.06]	
ARG142[NE]		GLU11[OE2] [2.63]	
ARG142[NE]*		GLU11[OE1]* [2.63]	
ASN10[ND2]			GLU11[OE2] [2.78]
THR75[OG1]			GLU11[OE1] [2.62]
GLU76[OE1]*			LYS12[NZ] [2.69]*
GLU76[OE2]			LYS12[NZ] [2.69]
HIS112[NE2]			LYS12[O] [2.79]

*Involved in salt bridge formation

Table 3 Residues contributing to hydrogen bonds between the $\alpha 11$ -I domain and triple-helical peptide (GPP)₂GPKGER(GPP)₃ (chains B and C) is shown. The atom names and the interacting distance (in Angstroms) are shown in square brackets

$\alpha 11$	(GPP) ₂ GPKGER(GPP) ₃	
	Chain B	Chain C
GLU110[OE1]	LYS9[NZ] [2.66]	
GLU110[OE2]*	LYS9[NZ]* [2.66]	
TYR140[OH]	PRO6[O] [3.06]	
ASN10[ND2]		PRO6[O] [2.88]
GLU45[OE2]*		LYS9[NZ]* [2.68]
THR75[O]		LYS9[N] [2.75]
THR75[OG1]		GLY7[O] [2.77]
GLU76[OE1]*		LYS9[NZ]* [2.67]
GLU76[OE2]		ARG12[NH1] [3.34]
GLU76[OE2]*		ARG12[NH2]* [2.75]
HIS112[NE2]		LYS9[O] [2.67]

*Involved in salt bridge formation

peptides indeed bind to the interface of $\alpha 11$ -I domain. In the crystal structure of the integrin $\alpha 2$ -I domain bound to the triple-helical GFOGER peptide (J. Emsley et al. 2000), the strand C makes most of the interactions with the $\alpha 2$ -I domain whereas the strand B contributes fewer interactions and the strand D makes no interactions as it is exposed to the solvent. Consistently, this trend was observed in our docking experiments for the GPKGER peptide, where most of the hydrogen bonds came from the strand C, less hydrogen bonds from the strand B and no interaction from the strand D. It can be hypothesized that the presence of an arginine (Arg12, the 12th residue in the strand C) residue might influence the orientation of the triple-helical peptide with the integrin domain such that the strand C makes many contacts with the integrin I domain similar as seen in the template crystal structure. However, for the GFQGEK, most of the hydrogen bonds came from the strand C and multiple hydrogen bonds were observed from both the strands B and the D. It is conceivable that the change of arginine (Arg12) to lysine (Lys12) may disrupt the orientation exposing all the three strands contacting the I domain. Consequently, our experimental data supports the molecular modeling results as C2C12- $\alpha 11$ + cells attach much stronger on GPKGER than on GFQGEK (Fig. 4d). Homology modeling and docking can be considered a powerful strategy to predict protein-protein interactions if there is no crystal structure of the complexes available (Quignot et al. 2018). Accuracy of the docking depends on the quality of the homology model (Quignot et al. 2018; Rodrigues et al. 2013); thus, we have validated our model at different stages both manually as well as using various online servers to obtain a reliable

prediction of the $\alpha 11$ -I domain structure and the docked complex (see “Materials and methods”). However, despite striving for high-quality predictions in our study, it should be noted that the crystal structures of the $\alpha 11$ -I domain in an unbound and a collagen-bound state remain to be solved, allowing further confirmation of our modeling results.

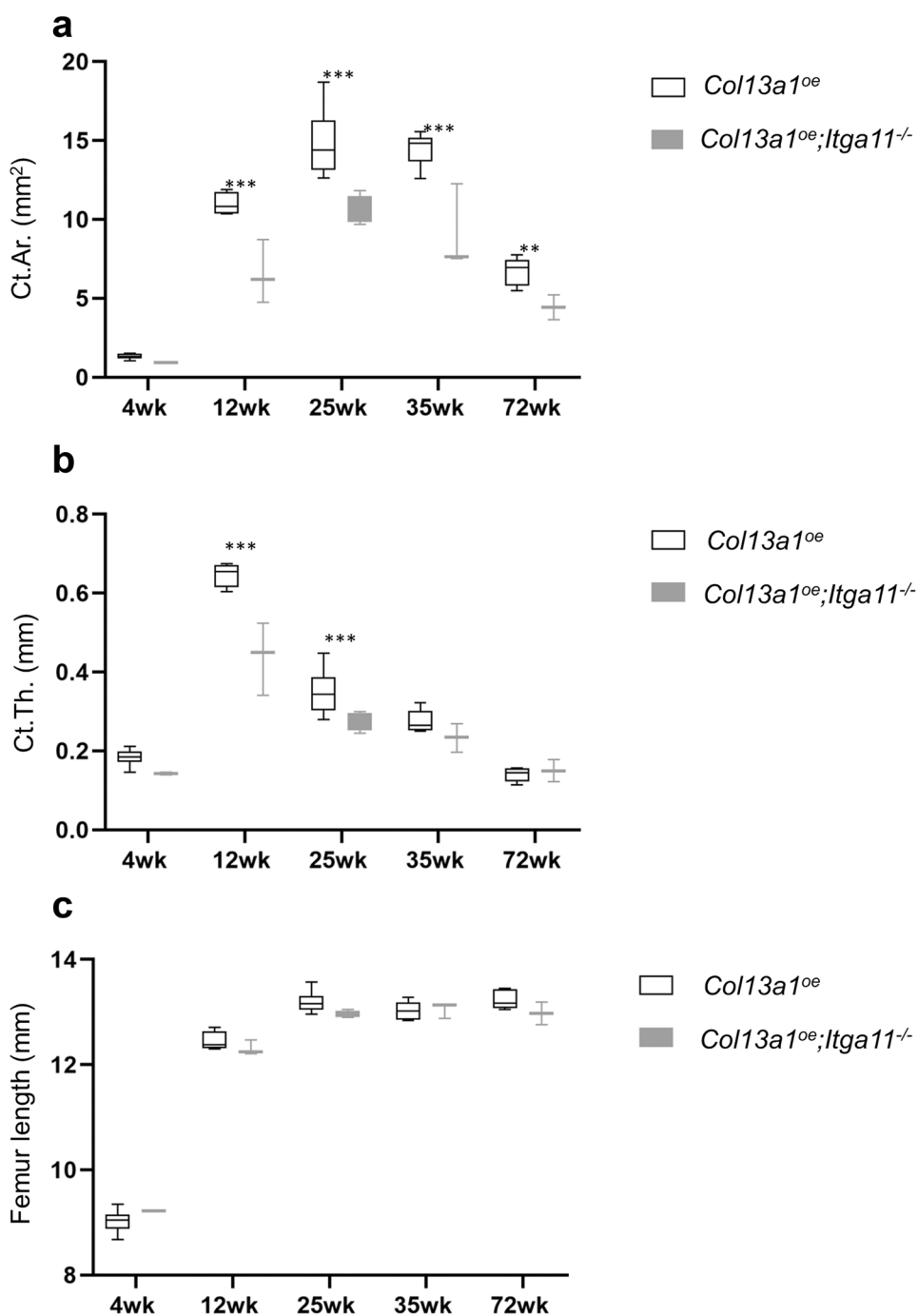
The peptide sequence GPKGER exists in 15 different collagens (collagens III, IV, V, VII, IX, XI, XIII, XIV, XV, XVII, XIX, XX, XXII, XXV, XXVII), while GFQGEK exists only in collagens XIII and IV, and GSQGER only in mouse collagen XIII and in rat XVII (UniProt Consortium 2019). It is not clear why collagen IV does not bind to $\alpha 11$ although it contains both GFOGER and GFQGEK sequences (Farndale et al. 2008, W. M. Zhang et al. 2003). Collagen IX, which binds to all the four I domains, contains the GFKGER sequence also. We propose GFKGER as a new general integrin-binding motif, albeit with modest affinity.

In the light of data obtained with cells and mutant mice (Hägg et al. 1998, 2001; Kvist et al. 2001; Sund et al. 2001; Tahkola et al. 2008; Tuomisto et al. 2008), collagen XIII appears to play a role in cell-matrix adhesion. Recent in vivo studies highlight involvement of collagen XIII in the development, differentiation, and maturation of musculoskeletal tissues and vessels and in maintaining tissue integrity (Latvanlehto et al. 2010; Härönen et al. 2017; Zainul et al. 2018; Heikkinen et al. 2019; Koivunen et al. 2019). The 150-nm flexible ectodomain of collagen XIII can potentially cross the basement membrane around myofibrils or under endothelium and can penetrate into the surrounding stroma. Thus, the in vitro properties of collagen XIII in binding to the basement membrane collagen receptor $\alpha 1\beta 1$ and to the matrix interstitial collagen receptor $\alpha 11\beta 1$ may be of physiological relevance. Moreover, COL3, the furthest collagenous domain from the plasma membrane, appeared largely responsible for the binding to the $\alpha 11$ -I domain, suggesting that collagen XIII molecules could interact with integrin $\alpha 11\beta 1$ molecules occurring in neighboring cells, but this aspect was not studied in the present study.

Under physiological conditions, integrin $\alpha 11\beta 1$ is expressed in specific subsets of fibroblasts and mesenchymal stem cells (MSCs) (Popov et al. 2011; Shen et al. 2019) and it has previously been observed to be up-regulated in carcinoma-associated fibroblasts (CAFs) in the tumor stroma (Zhu et al. 2007; Smeland et al. 2019). Collagen XIII is shown to be up-regulated by TGF- β in the reactive stromal cells of epithelial tumors and throughout mesenchymal tumors. The induction of collagen XIII expression occurs at an early stage in tumor progression in response to malignant transformation (Väisänen et al. 2005).

Integrin $\alpha 11\beta 1$ is a regulator of MSC survival on collagen I (Popov et al. 2011). Moreover, $\alpha 11\beta 1$ is found in leptin receptor positive MSCs in the bone marrow where $\alpha 11\beta 1$ has shown to

Fig. 8 Collagen XIII-integrin $\alpha 11$ interaction affects bone homeostasis. μ CT results of cortical bone area (Ct.Ar.) **a** and cortical thickness (Ct.Th.) **b**. No difference was seen in the length of the femurs **c**. Number of mice analyzed in order of increasing age: female *Col13a1^{oe}* (7, 4, 6, 6, 6), female *Col13a1^{oe};Itga11^{-/-}* (3, 3, 4, 3, 2). ** $q < 0.01$ and *** $q < 0.001$ determined by two-way ANOVA followed by FDR



be an osteolectin receptor and a regulator of osteogenesis and bone homeostasis (Shen et al. 2019). Conditional deletion of the $\alpha 11$ subunit from leptin receptor positive bone marrow cells accelerates trabecular bone loss in aged mice without alterations to body size, femoral length or cortical bone area. However, the mineral apposition rate is significantly reduced in cortical bone in these mice compared to littermate controls. We have previously shown that collagen XIII regulates bone remodeling and angiogenesis through $\beta 1$ integrins (Koivunen et al. 2019). The

novel double mutant mouse line presented here, *Col13a1^{oe};Itga11^{-/-}*, has a milder high bone mass phenotype compared to *Col13a1^{oe}* mice. More specifically, double mutants have significantly less bone overgrowth and milder osteoporosis compared to *Col13a1^{oe}* mice. A comparable phenotype was seen in both sexes up to 35 weeks of age. We were not, however, able to complete a full cohort of the male mice to the final time point on account of insufficient amount of mice available for the experiment. These data suggest that collagen XIII-triggered effects in

bone homeostasis may be mediated by $\alpha 1 \beta 1$. However, *Itga11* deficiency does not lead to full rescue of the bone phenotype indicating existence of other, unknown, mechanisms triggered by collagen XIII.

In conclusion, we identify integrin $\alpha 1 \beta 1$ as a new collagen XIII receptor and show that this ligand/receptor pair may have a role in bone homeostasis. Additionally, we propose GFKGER as a new general integrin-binding motif with moderate affinity.

Supplementary Information The online version contains supplementary material available at (<https://doi.org/10.1007/s00441-020-03300-y>).

Acknowledgments We thank Sirkka Vilmi, Aila White, and Maija Seppänen for valuable technical assistance and Veli-Pekka Ronkainen at Biocenter Oulu core facility for support with microscopy. The use of the facilities and expertise of the Biocenter Oulu Proteomics and Protein Analysis core facility, the University of Oulu Animal Facility, and the Biocenter Oulu Light Microscopy core facility are gratefully acknowledged.

Funding Open access funding provided by University of Oulu including Oulu University Hospital. This work was supported by the Finnish Centre of Excellence Programme (251314 years 2012–2017) of the Academy of Finland (294617 to JK, 308867 to TP, 268378 and 273571 to SS), the Sigrid Jusélius Foundation (TP) and by grants from Centre for Cancer Biomarkers (Centre of Excellence funded by Research council of Norway, Project No 223250 to DG), and The Norwegian Centre for International Cooperation in Education (Diku: NNA-2016/10026 to DG). European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement no. 336267 to SS. Part of research infrastructure has been supported by the European Commission Regional Development Fund (decision nr. 538/2010).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the European Community Council Directive on the protection of animals used for scientific purposes (September 22, 2010; 2010/63/EEC), national legislation and the regulations for the care and use of laboratory animals.

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References

- Barczyk MM, Carracedo S, Gullberg D (2010) Integrins. *Cell Tissue Res* 339:269–280
- Barczyk MM, Lu N, Popova SN, Bolstad AI, Gullberg D (2013) $\alpha 1 \beta 1$ integrin-mediated MMP-13-dependent collagen lattice contraction by fibroblasts: evidence for integrin-coordinated collagen proteolysis. *J Cell Physiol* 228:1108–1119
- Benjamini Y, Krieger AM, Yekutieli D (2006) Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* 93:491–507
- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000) The Protein Data Bank. *Nucleic Acids Res* 28:235–242
- Blumbach K, Niehoff A, Belgardt BF, Ehlen HW, Schmitz M, Hallinger R, Schulz JN, Bruning JC, Krieg T, Schubert M, Gullberg D, Eckes B (2012) Dwarfism in mice lacking collagen-binding integrins $\alpha 2 \beta 1$ and $\alpha 1 \beta 1$ is caused by severely diminished IGF-1 levels. *J Biol Chem* 287:6431–6440
- Bouxein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R (2010) Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res* 25:1468–1486
- Briesewitz R, Epstein MR, Marcantonio EE (1993) Expression of native and truncated forms of the human integrin $\alpha 1$ subunit. *J Biol Chem* 268:2989–2996
- Camper L, Hellman U, Lundgren-Akerlund E (1998) Isolation, cloning, and sequence analysis of the integrin subunit $\alpha 10$, a $\beta 1$ -associated collagen binding integrin expressed on chondrocytes. *J Biol Chem* 273:20383–20389
- Caswell CC, Barczyk M, Keene DR, Lukomska E, Gullberg DE, Lukowski S (2008) Identification of the first prokaryotic collagen sequence motif that mediates binding to human collagen receptors, integrins $\alpha 2 \beta 1$ and $\alpha 1 \beta 1$. *J Biol Chem* 283:36168–36175
- Colovos C, Yeates TO (1993) Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Sci* 2:1511–1519
- Dennis J, Meehan DT, Delimont D, Zalocchi M, Perry GA, O'Brien S, Tu H, Pihlajaniemi T, Cosgrove D (2010) Collagen XIII induced in vascular endothelium mediates $\alpha 1 \beta 1$ integrin-dependent transmigration of monocytes in renal fibrosis. *Am J Pathol* 177:2527–2540
- Dominguez C, Boelens R, Bonvin AM (2003) HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. *J Am Chem Soc* 125:1731–1737
- Eisenberg D, Luthy R, Bowie JU (1997) VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods Enzymol* 277:396–404
- Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC (2000) Structural basis of collagen recognition by integrin $\alpha 2 \beta 1$. *Cell* 101:47–56
- Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66:486–501
- Erusappan P, Alam J, Lu N, Zeltz C, Gullberg D (2019) Integrin $\alpha 11$ cytoplasmic tail is required for FAK activation to initiate 3D cell invasion and ERK-mediated cell proliferation. *Sci Rep* 9:15283–15286
- Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, Sali A (2006) Comparative protein structure modeling using Modeller. *Curr Protoc Bioinformatics* Chapter 5:Unit-5.6
- Farndale RW, Lisman T, Bihan D, Hamaia S, Smerling CS, Pugh N, Konitsiotis A, Leitinger B, de Groot PG, Jarvis GE, Raynal N (2008) Cell-collagen interactions: the use of peptide Toolkits to

- investigate collagen-receptor interactions. *Biochem Soc Trans* 36:241–250
- Frank S, Kammerer RA, Mechling D, Schulthess T, Landwehr R, Bann J, Guo Y, Lustig A, Bachinger HP, Engel J (2001) Stabilization of short collagen-like triple helices by protein engineering. *J Mol Biol* 308:1081–1089
- Gullberg D, Lundgren-Akerlund E (2002) Collagen-binding I domain integrins—what do they do? *Prog Histochem Cytochem* 37:3–54
- Gullberg D, Velling T, Sjöberg G, Sejersen T (1995) Up-regulation of a novel integrin alpha-chain (alpha mt) on human fetal myotubes. *Dev Dyn* 204:57–65
- Hägg P, Rehn M, Huhtala P, Väisänen T, Tamminen M, Pihlajaniemi T (1998) Type XIII collagen is identified as a plasma membrane protein. *J Biol Chem* 273:15590–15597
- Hägg P, Väisänen T, Tuomisto A, Rehn M, Tu H, Huhtala P, Eskelinen S, Pihlajaniemi T (2001) Type XIII collagen: a novel cell adhesion component present in a range of cell-matrix adhesions and in the intercalated discs between cardiac muscle cells. *Matrix Biol* 19:727–742
- Hamaia SW, Farndale RW (2014) Integrin recognition motifs in the human collagens. *Adv Exp Med Biol* 819:127–142
- Hamaia SW, Pugh N, Raynal N, Nemoz B, Stone R, Gullberg D, Bihan D, Farndale RW (2012) Mapping of potent and specific binding motifs, GLOGEN and GVOGEA, for integrin alpha1beta1 using collagen toolkits II and III. *J Biol Chem* 287:26019–26028
- Härönen H, Zainul Z, Naumenko N, Sormunen R, Miinalainen I, Shakirzyanova A, Santolero S, Kemppainen AV, Giniatullin R, Pihlajaniemi T, Heikkinen A (2019) Correct expression and localization of collagen XIII are crucial for the normal formation and function of the neuromuscular system. *Eur J Neurosci*
- Härönen H, Zainul Z, Tu H, Naumenko N, Sormunen R, Miinalainen I, Shakirzyanova A, Oikarainen T, Abdullin A, Martin P, Santolero S, Koistinaho J, Silman I, Giniatullin R, Fox MA, Heikkinen A, Pihlajaniemi T (2017) Collagen XIII secures pre- and postsynaptic integrity of the neuromuscular synapse. *Hum Mol Genet* 26:2076–2090
- Heikkinen A, Haronen H, Norman O, Pihlajaniemi T (2019) Collagen XIII and Other ECM Components in the Assembly and Disease of the Neuromuscular Junction. *Anat Rec (Hoboken)*
- Heikkinen A, Tu H, Pihlajaniemi T (2012) Collagen XIII: a type II transmembrane protein with relevance to musculoskeletal tissues, microvessels and inflammation. *Int J Biochem Cell Biol* 44:714–717
- Hemler ME, Glass D, Coblyn JS, Jacobson JG (1986) Very late activation antigens on rheumatoid synovial fluid T lymphocytes. Association with stages of T cell activation. *J Clin Invest* 78:696–702
- Hess B, Kutzner C, van der Spoel D, Lindahl E (2008) GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J Chem Theory Comput* 4:435–447
- Izzi V, Lakkala J, Devarajan R, Kääriäinen A, Koivunen J, Heljasvaara R, Pihlajaniemi T (2019) Pan-Cancer analysis of the expression and regulation of matrisome genes across 32 tumor types. *Matrix Biology Plus* 1:100004
- Juvenon M, Sandberg M, Pihlajaniemi T (1992) Patterns of expression of the six alternatively spliced exons affecting the structures of the COL1 and NC2 domains of the alpha 1(XIII) collagen chain in human tissues and cell lines. *J Biol Chem* 267:24700–24707
- Käpylä J, Jääliñoja J, Tulla M, Ylöstalo J, Nissinen L, Viitasalo T, Vehviläinen P, Marjomäki V, Nykvist P, Säämänen AM, Farndale RW, Birk DE, Ala-Kokko L, Heino J (2004) The fibril-associated collagen IX provides a novel mechanism for cell adhesion to cartilaginous matrix. *J Biol Chem* 279:51677–51687
- Kim JK, Xu Y, Xu X, Keene DR, Gurusiddappa S, Liang X, Wary KK, Hook M (2005) A novel binding site in collagen type III for integrins alpha1beta1 and alpha2beta1. *J Biol Chem* 280:32512–32520
- Knight CG, Morton LF, Onley DJ, Peachey AR, Messent AJ, Smethurst PA, Tuckwell DS, Farndale RW, Barnes MJ (1998) Identification in collagen type I of an integrin alpha2 beta1-binding site containing an essential GER sequence. *J Biol Chem* 273:33287–33294
- Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ (2000) The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* 275:35–40
- Koivunen J, Kemppainen AV, Finnilla MA, Keski-Filppula R, Haronen H, Tu H, Pellikka H, Heikkinen A, Kylmaja E, Sormunen R, Miinalainen I, Saarakkala S, Izzi V, Pihlajaniemi T (2019) Collagen XIII-derived ectodomain regulates bone angiogenesis and intracortical remodeling. *Matrix Biol* 83:6–25
- Kvist AP, Latvanlehto A, Sund M, Eklund L, Väisänen T, Hägg P, Sormunen R, Komulainen J, Fässler R, Pihlajaniemi T (2001) Lack of cytosolic and transmembrane domains of type XIII collagen results in progressive myopathy. *Am J Pathol* 159:1581–1592
- Lamberg A, Helaakoski T, Myllyharju J, Peltonen S, Notbohm H, Pihlajaniemi T, Kivirikko KI (1996) Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. *J Biol Chem* 271:11988–11995
- Landegren U (1984) Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. *J Immunol Methods* 67:379–388
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948
- Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Cryst* 26:283–291
- Latvanlehto A, Fox MA, Sormunen R, Tu H, Oikarainen T, Koski A, Naumenko N, Shakirzyanova A, Kallio M, Ilves M, Giniatullin R, Sanes JR, Pihlajaniemi T (2010) Muscle-derived collagen XIII regulates maturation of the skeletal neuromuscular junction. *J Neurosci* 30:12230–12241
- Logan CV, Cossins J, Rodriguez Cruz PM, Parry DA, Maxwell S, Martinez-Martinez P, Riepsaame J, Abdelhamed ZA, Lake AV, Moran M, Robb S, Chow G, Sewry C, Hopkins PM, Sheridan E, Jayawant S, Palace J, Johnson CA, Beeson D (2015) Congenital myasthenic syndrome type 19 is caused by mutations in COL13A1, encoding the atypical non-fibrillar collagen type XIII alpha1 chain. *Am J Hum Genet* 97:878–885
- Lu N, Carracedo S, Ranta J, Heuchel R, Soinen R, Gullberg D (2010) The human alpha1I integrin promoter drives fibroblast-restricted expression in vivo and is regulated by TGF-beta1 in a Smad- and Sp1-dependent manner. *Matrix Biol* 29:166–176
- Mirtti T, Nylund C, Lehtonen J, Hiekkanen H, Nissinen L, Kallajoki M, Alanen K, Gullberg D, Heino J (2006) Regulation of prostate cell collagen receptors by malignant transformation. *Int J Cancer* 118:889–898
- Nokelainen M, Helaakoski T, Myllyharju J, Notbohm H, Pihlajaniemi T, Fietzek PP, Kivirikko KI (1998) Expression and characterization of recombinant human type II collagens with low and high contents of hydroxylysine and its glycosylated forms. *Matrix Biol* 16:329–338
- Nykvist P, Tu H, Ivaska J, Käpylä J, Pihlajaniemi T, Heino J (2000) Distinct recognition of collagen subtypes by alpha(1)beta(1) and alpha(2)beta(1) integrins. Alpha(1)beta(1) mediates cell adhesion to type XIII collagen. *J Biol Chem* 275:8255–8261

- Pandolfi F, Franza L, Altamura S, Mandolini C, Cianci R, Ansari A, Kurnick JT (2017) Integrins: integrating the biology and therapy of cell-cell interactions. *Clin Ther* 39:2420–2436
- Popov C, Radic T, Haasters F, Prall WC, Aszodi A, Gullberg D, Schieker M, Docheva D (2011) Integrins alpha2beta1 and alpha11beta1 regulate the survival of mesenchymal stem cells on collagen I. *Cell Death Dis* 2:e186
- Popova SN, Barczyk M, Tiger CF, Beertsen W, Zigrino P, Aszodi A, Miosge N, Forsberg E, Gullberg D (2007) Alpha11 beta1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor. *Mol Cell Biol* 27:4306–4316
- Popova SN, Rodriguez-Sanchez B, Liden A, Betsholtz C, Van Den Bos T, Gullberg D (2004) The mesenchymal alpha11beta1 integrin attenuates PDGF-BB-stimulated chemotaxis of embryonic fibroblasts on collagens. *Dev Biol* 270:427–442
- Primac I, Maquoui E, Blacher S, Heljasvaara R, Van Deun J, Smeland HY, Canale A, Louis T, Stuhr L, Sounni NE, Cataldo D, Pihlajaniemi T, Pequeux C, De Wever O, Gullberg D, Noel A (2019) Stromal integrin alpha11 regulates PDGFR-beta signaling and promotes breast cancer progression. *J Clin Invest* 130:4609–4628
- Quignot C, Rey J, Yu J, Tufféry P, Guerois R, Andreani J (2018) InterEvDock2: an expanded server for protein docking using evolutionary and biological information from homology models and multimeric inputs. *Nucleic Acids Res* 46:W408–W416
- Rappu P, Salo AM, Myllyharju J, Heino J (2019) Role of prolyl hydroxylation in the molecular interactions of collagens. *Essays Biochem* 63:325–335
- Raynal N, Hamaia SW, Siljander PR, Maddox B, Peachey AR, Fernandez R, Foley LJ, Slatte DA, Jarvis GE, Farndale RW (2006) Use of synthetic peptides to locate novel integrin alpha2beta1-binding motifs in human collagen III. *J Biol Chem* 281:3821–3831
- Rodrigues J, Melquiond ASJ, Karaca E, Trellet M, Mv D, van Zundert GCP, Schmitz C, Vries SJD, Bordogna A, Bonati L, Kastrius PL, Bonvin AMJJ (2013) Defining the limits of homology modeling in information-driven protein docking. *Proteins: Struct, Funct, Bioinf* 81:2119–2128
- Rodriguez Cruz PM, Cossins J, Estephan EP, Munell F, Selby K, Hirano M, Maroofin R, Mehrjardi MYV, Chow G, Carr A, Manzur A, Robb S, Munot P, Wei Liu W, Banka S, Fraser H, De Goede C, Zanoteli E, Conti Reed U, Sage A, Gratacos M, Macaya A, Dusl M, Senderek J, Topf A, Hofer M, Knight R, Ramdas S, Jayawant S, Lochmuller H, Palace J, Beeson D (2019) The clinical spectrum of the congenital myasthenic syndrome resulting from COL13A1 mutations. *Brain* 142:1547–1560
- Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 234:779–815
- Sandberg M, Tamminen M, Hirvonen H, Vuorio E, Pihlajaniemi T (1989) Expression of mRNAs coding for the alpha 1 chain of type XIII collagen in human fetal tissues: comparison with expression of mRNAs for collagen types I, II, and III. *J Cell Biol* 109:1371–1379
- Sandberg-Lall M, Hagg PO, Wahlstrom I, Pihlajaniemi T (2000) Type XIII collagen is widely expressed in the adult and developing human eye and accentuated in the ciliary muscle, the optic nerve and the neural retina. *Exp Eye Res* 70:401–410
- Shen B, Vardy K, Hughes P, Tasdogan A, Zhao Z, Yue R, Crane GM, Morrison SJ (2019) Integrin alpha11 is an osteolectin receptor and is required for the maintenance of adult skeletal bone mass. *Elife* 8:<https://doi.org/10.7554/eLife.42274>
- Siljander PR, Hamaia S, Peachey AR, Slatte DA, Smethurst PA, Ouwehand WH, Knight CG, Farndale RW (2004) Integrin activation state determines selectivity for novel recognition sites in fibrillar collagens. *J Biol Chem* 279:47763–47772
- Sipilä KH, Drushinin K, Rappu P, Jokinen J, Salminen TA, Salo AM, Käpylä J, Myllyharju J, Heino J (2018) Proline hydroxylation in collagen supports integrin binding by two distinct mechanisms. *J Biol Chem* 293:7645–7658
- Sipilä KH, Haag S, Denessiouk K, Käpylä J, Peters EC, Denesyuk A, Hansen U, Konttinen Y, Johnson MS, Holmdahl R, Heino J (2014) Citrullination of collagen II affects integrin-mediated cell adhesion in a receptor-specific manner. *FASEB J* 28:3758–3768
- Smeland HY, Askeland C, Wik E, Knutsvik G, Molven A, Edelman RJ, Reed RK, Warren DJ, Gullberg D, Stuhr L, Akslen LA (2019) Integrin alpha11beta1 is expressed in breast cancer stroma and associates with aggressive tumor phenotypes. *J Pathol Clin Res*
- Snellman A, Tu H, Väisänen T, Kvist AP, Huhtala P, Pihlajaniemi T (2000) A short sequence in the N-terminal region is required for the trimerization of type XIII collagen and is conserved in other collagenous transmembrane proteins. *EMBO J* 19:5051–5059
- Sund M, Väisänen T, Kaukinen S, Ilves M, Tu H, Autio-Harmainen H, Rauvala H, Pihlajaniemi T (2001) Distinct expression of type XIII collagen in neuronal structures and other tissues during mouse development. *Matrix Biol* 20:215–231
- Tahkola J, Räsänen J, Sund M, Mäkilä K, Autio-Harmainen H, Pihlajaniemi T (2008) Cardiac dysfunction in transgenic mouse fetuses overexpressing shortened type XIII collagen. *Cell Tissue Res* 333:61–69
- Takada Y, Hemler ME (1989) The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J Cell Biol* 109:397–407
- Tiger CF, Fougereousse F, Grundstrom G, Velling T, Gullberg D (2001) Alpha11beta1 integrin is a receptor for interstitial collagens involved in cell migration and collagen reorganization on mesenchymal nonmuscle cells. *Dev Biol* 237:116–129
- Tu H, Sasaki T, Snellman A, Gohring W, Pirilä P, Timpl R, Pihlajaniemi T (2002) The type XIII collagen ectodomain is a 150-nm rod and capable of binding to fibronectin, nidogen-2, perlecan, and heparin. *J Biol Chem* 277:23092–23099
- Tulla M, Pentikäinen OT, Viitasalo T, Käpylä J, Impola U, Nykvist P, Nissinen L, Johnson MS, Heino J (2001) Selective binding of collagen subtypes by integrin alpha 1I, alpha 2I, and alpha 10I domains. *J Biol Chem* 276:48206–48212
- Tuomisto A, Sund M, Tahkola J, Latvanlehto A, Savolainen ER, Autio-Harmainen H, Liakka A, Sormunen R, Vuoristo J, West A, Lahesmaa R, Morse HC, Pihlajaniemi T (2008) A mutant collagen XIII alters intestinal expression of immune response genes and predisposes transgenic mice to develop B-cell lymphomas. *Cancer Res* 68:10324–10332
- UniProt Consortium (2019) UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res* 47:D506–D515
- Väisänen T, Väisänen MR, Autio-Harmainen H, Pihlajaniemi T (2005) Type XIII collagen expression is induced during malignant transformation in various epithelial and mesenchymal tumours. *J Pathol* 207:324–335
- Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ (2005) GROMACS: fast, flexible, and free. *J Comput Chem* 26:1701–1718
- Velling T, Kusche-Gullberg M, Sejersen T, Gullberg D (1999) cDNA cloning and chromosomal localization of human alpha(11) integrin. A collagen-binding, I domain-containing, beta(1)-associated integrin alpha-chain present in muscle tissues. *J Biol Chem* 274:25735–25742
- Xu Y, Gurusiddappa S, Rich RL, Owens RT, Keene DR, Mayne R, Hook A, Hook M (2000) Multiple binding sites in collagen type

- I for the integrins alpha1beta1 and alpha2beta1. *J Biol Chem* 275:38981–38989
- Xue LC, Rodrigues JP, Kastritis PL, Bonvin AM, Vangone A (2016) PRODIGY: a web server for predicting the binding affinity of protein-protein complexes. *Bioinformatics* 32:3676–3678
- Ylönen R, Kyrölähti T, Sund M, Ilves M, Lehenkari P, Tuukkanen J, Pihlajaniemi T (2005) Type XIII collagen strongly affects bone formation in transgenic mice. *J Bone Miner Res* 20:1381–1393
- Zainul Z, Heikkinen A, Koivisto H, Rautalahti I, Kallio M, Lin S, Härönen H, Norman O, Rüegg MA, Tanila H, Pihlajaniemi T (2018) Collagen XIII is required for neuromuscular synapse regeneration and functional recovery after peripheral nerve injury. *J Neurosci* 38:4243–4258
- Zeltz C, Gullberg D (2016) The integrin-collagen connection - a glue for tissue repair? *J Cell Sci* 129:1284
- Zhang H, Fredericks T, Xiong G, Qi Y, Rychahou PG, Li JD, Pihlajaniemi T, Xu W, Xu R (2018) Membrane associated collagen XIII promotes cancer metastasis and enhances anoikis resistance. *Breast Cancer Res* 20:116-y
- Zhang WM, Käpylä J, Puranen JS, Knight CG, Tiger CF, Pentikäinen OT, Johnson MS, Farndale RW, Heino J, Gullberg D (2003) alpha 11beta 1 integrin recognizes the GFOGER sequence in interstitial collagens. *J Biol Chem* 278:7270–7277
- Zhu CQ, Popova SN, Brown ER, Barsyte-Lovejoy D, Navab R, Shih W, Li M, Lu M, Jurisica I, Penn LZ, Gullberg D, Tsao MS (2007) Integrin alpha 11 regulates IGF2 expression in fibroblasts to enhance tumorigenicity of human non-small-cell lung cancer cells. *Proc Natl Acad Sci U S A* 104:11754–11759
- Zwolanek D, Veit G, Eble JA, Gullberg D, Ruggiero F, Heino J, Meier M, Stetefeld J, Koch M (2014) Collagen XXII binds to collagen-binding integrins via the novel motifs GLQGER and GFKGER. *Biochem J* 459:217–227