Activation of Discoidin Domain Receptors by collagen VI and collagen I fibrils

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Declaration of originality

I herewith declare that I am the sole author of this thesis, that all data presented is my work and that any contributions from third parties are appropriately acknowledged.

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Abstract

Discoidin domain receptors (DDRs) are members of the Receptor Tyrosine Kinase (RTK) family. There are two DDRs, DDR1 and DDR2. In embryo development, DDR1 function is vital for mammary gland development, whereas DDR2 is important in long bone growth. The DDRs also have key roles in several cellular functions, such as cell migration, adhesion, proliferation and matrix remodeling. The DDRs are unique RTK members due to the fact that they are activated by an extracellular matrix component, collagen. It is well known that the DDRs are activated by fibrillar collagens, such as collagen I, when present as single triple helices. However, it is less clear whether collagen I fibrils or fibers, the form in which collagen I is present in tissues, can act as functional DDR ligands and induce DDR phosphorylation. In this thesis, I show that DDR1 activation is induced by collagen I fibrils generated in vitro from different sources, and furthermore by extracellular matrix secreted from fibroblastic cells. I also show that DDR2 is activated to a much lower extent in comparison to DDR1. Another collagen type that is present in many tissues is collagen VI. Collagen VI has not been characterized as a DDR ligand. Collagen VI is secreted in the form of tetramers to extracellular matrices, where it associates to form microfibrils. In this thesis, I show that collagen VI tetramers and microfibrils bind strongly to both DDRs. However, they can induce only DDR1 phosphorylation, not DDR2 phosphorylation. Our preliminary data further suggest that the triple-helical region in collagen VI is the functional region that promotes DDR1 activation. Furthermore, I show that collagen VI binds to DDR2 by occupying another binding site than the fibrillar binding site. Finally, preliminary experiments suggest that collagen VI microfibrils can inhibit collagen I-induced DDR2 autophosphorylation. These data suggest that collagen VI binding to DDR2 could serve as a limiting factor for DDR2 activation in pathological conditions, such as osteoarthritis.
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Abbreviations

ADAM — A Disintegrin and Metalloproteinase
ADAMTs — A Disintegrin And Metalloproteinase with Thrombospondin motifs
APS — Ammonium Persulphate
BSA — Bovine Serum Albumin
CDM — Cell-derived Matrix
COPII — coat protein complex II
Cos 7 — CV-1 origin SV40
DDR — Discoidin Domain Receptor
DMEM/F12 — Dulbecco’s modified Eagle medium/F12
DS — Discoidin
ECM — Extracellular Matrix
EDTA — Ethylenediaminetetraacetic Acid
EGFR — Epidermal Growth Factor Receptor
EJM — Extracellular Juxtamembrane
EMT — Epithelial-to-Mesenchymal Transition
ER — Endoplasmic Reticulum
ERK — Extracellular Signal Regulated
FBS — Fetal Bovine Serum
FGFR — fibroblast growth factor receptor
GAGs — Glycosaminoglycans
GPCs — Golgi to Plasma carriers
GPVI — Glycoprotein VI
HEK-293 — Human Embryonic Kidney cells 293
HEPES — 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP — Horseradish protein
IGFR — Insulin-like Growth Receptor
IJM — Intracellular Juxtamembrane
Abbreviations

KD—Kinase Domain
LAIR—Leukocyte-associated Immunoglobulin-like Receptor
MMP—Metalloproteinase
MT-MMP—Membrane-type Matrix Metalloproteinase
NCS—Neonatal Calf Serum
OA—Osteoarthritis
OD—Optical Density
OPD—O-phenylenediamine dihydrochloride
OptiMEM—Eagle’s Minimum Essential Medium
PBS—Phosphate-buffered Saline
PCR—Polymerase Chain Reaction
PFA—Paraformaldehyde
PMSF—Phenylmethylsulfonyl Fluoride
P/S—Penicillin/streptomycin
RMPI—Roswell Park Memorial Institute Medium
RTK—Receptor Tyrosine Kinase
SDS—Sodium Dodecyl Sulphate
SDS—PAGE—Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHG—Second Harmonic Generation
SLRPs—Small Leucine Rich Proteoglycans
SMED-SL—Spondylo-meta-epiphyseal Dysplasia with Short Limbs
Stat—Activators of transcription
TEMED—Tetraethylmethylenediamine
VO3—Orthovanadate
vWF—von Willebrand Factor
Chapter 1: General Introduction

1.1 Extracellular matrix composition

The extracellular matrix (ECM) constitutes an essential part of all tissues. It constitutes a three-dimensional non-cellular network that surrounds the cells present in each tissue and consists mainly of fibrous proteins, glycoproteins and proteoglycans (Mongiat et al. 2016; Frantz et al. 2010; Bonnans et al. 2014; Mouw et al. 2014). ECM displays biochemical and biomechanical characteristics that regulate significant cellular processes such as differentiation, homeostasis and migration (Rozario & DeSimone 2010; Theocharis et al. 2016). Their overall architectures are tissue-dependent and they are very dynamic due to the function of proteolytical enzymes. Any severe alteration of the ECM composition can lead to a variety of diseases such as Marshall syndrome, Stickler syndrome type II and type III, corneal endothelial dystrophy and Crohn’s disease (Frantz et al. 2010; Jarvelainen et al. 2009; Pozzi et al. 2017; Bonnans et al. 2014).

The ECM macromolecules include primarily proteoglycans (e.g. aggrecan) and fibrous proteins, such as fibronectin, laminin, elastins and collagens (Frantz et al. 2010; Mouw et al. 2014; Humphrey et al. 2014). Collagens, which are ubiquitous components of all ECMs providing tensile strength to a tissue, (Ricard-Blum 2011; Kadler et al. 2007) are described later on in detail. Proteoglycans, another important ECM component, represent a group of biological molecules which are known to play an architectural role in ECMs as they are responsible for hydrogel formation of the ECM and they have been linked with various diseases such as tumor-related diseases (Schaefer & Iozzo 2008; Frantz et al. 2010; Schaefer 2014). Apart from their mechanical role in tissues, proteoglycans participate in many cellular processes such as cell adhesion, migration, proliferation and apoptosis. They also participate in cell signaling via interactions with transforming growth factor, type 1 insulin-like growth factor receptor and epidermal growth factor receptor (EGFR) (Schaefer & Iozzo 2008;
In addition to growth factors, they also interact with several components of the ECM such as chemokines, cytokines and ECM constituents like collagen and cell-surface receptors (Theocharis et al. 2016; Iozzo & Schaefer 2015). Proteoglycans consist of a core protein covalently linked to one or more negatively charged glycosaminoglycan (GAG) chains. GAG chains, which are heteropolysaccharides, belong in one of the following groups: keratan sulfate, dermatan sulfate, chondroitin sulfate, heparan sulfate or hyaluronan (Couchman & Pataki 2012; Mouw et al. 2014). GAG chains present variation in their molecular size which is tissue-dependent (Theocharis et al. 2016). Proteoglycans are classified into two main groups, hyaluronan and the rest of the proteoglycans. Hyaluronan, which does not include a protein core, participates in several cellular processes and it is an abundant component of the pericellular matrix. It acquires a structural role in the ECM but also can also participate in signaling by activating pathways such as the PI3K/Akt pathway (Schaefer & Schaefer 2009; Schaefer 2014; Iozzo & Schaefer 2015). Proteoglycans can also be classified into four groups based on their location and core protein: extracellular, pericellular, transmembrane and intracellular proteoglycans. Extracellular proteoglycans include hyalectans and small leucine-rich proteoglycans (SLRPs) (Schaefer 2014; Theocharis et al. 2016). Hyalectans further include aggrecan, versican, neurocan and brevican. SLRPs are further divided into five classes. The most abundant SLRPs are decorin and biglycan, members of class I SLRPs (Schaefer 2014; Theocharis et al. 2016). Pericellular proteoglycans, such as perlecan, are components of the basement membranes and consist of heparan sulfate chains. Cell-surface proteoglycans include syndecans and glypicans and they also mainly consist of heparan sulfate chains. Finally, intracellular proteoglycans include serglycin and they are present in secretory compartments (Schaefer 2014; Theocharis et al. 2016).

Fibronectin is a key glycoprotein that is found in interstitial extracellular matrices (Bonnans et al. 2014). The tissues’ form of fibronectin is the fibrillar state (Theocharis et al. 2016). Molecular fibronectin, is a disulfide-linked dimer of approximately 500kDa, with each subunit consisting of many fibronectin type I, II and III repeats (Theocharis et al. 2016; Maurer et al. 2016). Even though it is coded
by a single gene, different fibronectin isoforms occur due to alternative splicing (Theocharis et al. 2016; Singh et al. 2010). Fibronectin fibers vary in diameter, ranging from 10nm to a few micrometers, and length, as they can reach up to many micrometers (Theocharis et al. 2016). Fibronectin is responsible for orchestrating the structural arrangement of interstitial ECM (Frantz et al. 2010; Humphrey et al. 2014; Singh et al. 2010). At the cellular level, fibronectin plays vital roles in cell adhesion and migration. Finally, fibronectin has been closely linked to various types of cancer and cardiovascular diseases (Tsang et al. 2010; Rozario & DeSimone 2010; Frantz et al. 2010). Fibronectin has various ligands, both soluble and transmembrane: it binds to heparan, collagen as well as integrins (Pankov & Yamada 2002).

Laminin constitutes a basic component of all basement membranes. It is a heterotrimer consisting of an α-, β- and γ-chain (Aumailley et al. 2005). To date, five α-, three β- and three γ-chains have been identified (Theocharis et al. 2016). There are only 16 laminin isoforms in mammalians since not all laminin subunits can participate in the formation of a laminin molecule (Pozzi et al. 2017). The three chains incorporate characteristic cross-shaped, Y-shaped or rod-shaped conformations. The α-chain is much longer than β- and γ- chains, including a globular region located in the C-terminal region. All three chains include a coiled-coil domain (Mouw et al. 2014). Laminin serves a structural role via its interactions with itself as well as other ECM components, particularly perlecan, fibulin-1, agrin, collagen IV and entactins, assisting in the formation of basement membranes (Theocharis et al. 2016; Durbeej 2010; Mouw et al. 2014). It also associates with cellular receptors such as integrins and syndecans. Published literature has shown that laminin has vital functions both at the tissue- and cellular-level. In tissues, it is vital in organogenesis, angiogenesis and early embryonic stages (Durbeej 2010) whereas its cellular functions include cell differentiation, adhesion and migration (Iorio et al. 2015). Mutations in laminin subunits result in various diseases. Junctional epidermolysis is caused by mutations in any subunit of laminin isoform 332. Such patients suffer from skin fragility and blistering (Kivirikko et al. 1996; Pulkkinen et al. 1994; Muhle et al. 2005). Pierson syndrome is caused by mutations in β2 chain whereas congenital muscular dystrophy 1A is a result of mutations in subunit α2
Finally, laminin has been associated with tumor growth, particularly with cell invasion and survival (Marinkovich 2007; Tran et al. 2008).

Elastin, a key molecule in many tissues such as skin, lung and blood vessels, is secreted in the ECM in the form of tropoelastin (Mouw et al. 2014). Tropoelastin molecules are cross-linked, a reaction catalyzed by members of the lysyl-oxidase family (Frantz et al. 2010; Lucero & Kagan 2006). Tropoelastin molecules associate head-to-tail forming mature elastin networks, which serve as ligands for microfibrils (Wagenseil & Mecham 2007). The mature elastic fiber consists of elastin networks, which are cross-linked tropoelastin molecules, bound on their surface with microfibrils (Mouw et al. 2014; Theocharis et al. 2016). Microfibrils consist mainly of fibrillins and microfibril-associated glycoproteins with characteristic elevated cysteine levels (Wagenseil & Mecham 2007). Both fibrillins and microfibril-associated glycoproteins perform structural roles: fibrillins assist in the assembly of microfibril molecules whereas microfibril-associated glycoproteins assist in the formation of elastic fibers (Wagenseil & Mecham 2007; Theocharis et al. 2016). Elastin provides elasticity to the tissue, since it allows the matrix to stretch (Frantz et al. 2010). More specifically, hydrophobic residues present in the elastin molecule are responsible for the elastin’s ability to resist tensile stress (Muiznieks et al. 2010). Mutations in elastin, particularly in fibrillin-1, have been associated with Marfan syndrome (Sengle & Sakai 2015; Muiznieks et al. 2010; Theocharis et al. 2016).

Collagens are ubiquitous components of ECMs. There are 28 collagen types present in human tissues; they account for the characteristic tissue stiffness and they provide tensile strength to the tissue they are found in (Myllyharju & Kivirikko 2004; Ricard-Blum 2011; Kadler et al. 2007). Apart from their biomechanical role in tissues, collagens directly interact with cells and furthermore participate in processes such as cell migration, adhesion and angiogenesis (Kadler et al. 2007; Theocharis et al. 2016). Collagens have also been linked to various pathological conditions in several tissues (Jarvelainen et al. 2009). Collagens form particular supramolecular assemblies in tissues and can be categorized according to the type of assembly into different
families, such as network forming, fibril-associated or fibrillar collagens, with fibrillar collagens being the most abundant collagens found in tissues (Ricard-Blum 2011).
Table 1.1: List of most important collagens, the tissue they are present and the functions they participate in.

<table>
<thead>
<tr>
<th>Collagen Type</th>
<th>Distribution</th>
<th>Supramolecular assembly</th>
<th>Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>Tendons, bone, cartilage, skin, cornea</td>
<td>Fibrillar collagen</td>
<td>Stiffness, tensile strength</td>
<td>(Kadler et al. 2007; Theocharis et al. 2016)</td>
</tr>
<tr>
<td>Collagen type II</td>
<td>Articular cartilage, vitreous humour, nucleus pulposus</td>
<td>Fibrillar collagen</td>
<td>Stiffness, tensile strength</td>
<td>(Kadler et al. 2007; Theocharis et al. 2016)</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>Liver, skin, lungs, spleen, cornea</td>
<td>Fibrillar collagen</td>
<td>Fibrillogenesis, cardiac function</td>
<td>(Ivanova &amp; Krivchenko 2012; Hulmes 2008; Liu et al. 1997)</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>Basement membranes</td>
<td>Network forming collagen</td>
<td>Molecular filtration</td>
<td>(Kadler et al. 2007; Theocharis et al. 2016)</td>
</tr>
<tr>
<td>Collagen type V</td>
<td>Co-distributes with collagen I</td>
<td>Fibrillar collagen</td>
<td>Structural role, interacts with several ECM components</td>
<td>(Theocharis et al. 2016; Mak et al. 2016)</td>
</tr>
<tr>
<td>Collagen Type</td>
<td>Distribution</td>
<td>Supramolecular assembly</td>
<td>Functions</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>------------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Collagen type VI</td>
<td>Skeletal muscles, adipose tissue, nervous system, cornea articular cartilage, skin</td>
<td>Beaded-microfilaments</td>
<td>architectural role, links cells with the ECM</td>
<td>(Cescon et al. 2015)</td>
</tr>
<tr>
<td>Collagen type IX</td>
<td>Articular cartilage, vitreous humour</td>
<td>Fibril-associated collagen with interrupted triple-helices</td>
<td>Coats collagen surface, participates in collagen-proteoglycan interactions</td>
<td>(Kadler et al. 2007; Theocharis et al. 2016; Cremer et al. 1998)</td>
</tr>
<tr>
<td>Collagen type X</td>
<td>Articular cartilage</td>
<td>Network forming collagen</td>
<td>Bone formation</td>
<td>(Kadler et al. 2007; Luo et al. 2017; Theocharis et al. 2016)</td>
</tr>
<tr>
<td>Collagen type XI</td>
<td>Articular cartilage, vitreous humour</td>
<td>Fibrillar collagen</td>
<td>Fibril growth, skeletal morphogenesis</td>
<td>(Li et al. 1995; Kadler et al. 2007; Theocharis et al. 2016)</td>
</tr>
</tbody>
</table>

The hallmark of the collagen structure is the triple-helix (Shoulders & Raines 2009). All collagen triple-helices consist of three polypeptide chains called α-chains, that wind around one another very tightly, with a pitch of 9.5 Å (Knupp & Squire 2005) (Figure 1.1). Each α-chain consists of Gly-X-Y triplets, where Gly is glycine and X and Y are often proline and hydroxyproline respectively (Myllyharju & Kivirikko 2004; Viguet-Carrin et al. 2006; Kadler et al. 2007). Collagens can be homotrimers, when
they consist of three identical α-chains, or heterotrimers, when they consist of different α–chains. Collagen I, the most abundant collagen, is a heterotrimer consisting of two α1–chains and one α2-chain (Hulmes 2008). Each α-chain coils into a left-handed helix and the three α-chains wrap around one another to form a right-handed coiled conformation, the triple helix, which associates very tightly due to the presence of glycine molecules in the inner part of the triple-helix (Myllyharju & Kivirikko 2004; Kadler et al. 2007).

Figure 1.1: Structure of the triple-helix. The three α-chains (represented in green, blue, orange), which are coiled in a left-handed manner, wrap around one another in order to form a right-handed coiled conformation, the triple-helix. Each α-chain of the triple-helix, consists of Gly-X-Y triplets, where X and Y are usually proline and hydroxyproline. Glycine is located deep within the triple-helix, allowing the collagen molecule to pack tightly. Figure 1 depicts only a small section of the triple-helical molecule. Image obtained from (Leitinger 2011) with permission.

1.2 Synthesis and post-translational modifications of triple-helical collagen I

Most data on collagen synthesis, post-translational modifications, packing and secretion have been acquired for collagen I, a member of the fibrillar family. Individual collagen I α-chains are synthesized by ribosomes present on the membrane of the rough endoplasmic reticulum (ER), enter the lumen of the rough ER and are subjected to post-translational modifications prior to assembly of the triple-helix (Walter & Johnson 1994). First, prolines present in the Y of the Gly-X-Y triplet are hydroxylated by the enzyme prolyl-4-hydroxylase. Hydroxyprolines contribute to the stability of the α-chain, particularly due to their participation in hydrogen bond formation and thus of the triple-helix molecule (Torre-Blanco et al. 1992; Myllyharju 2005). Proline hydroxylation also assists collagen molecules to become more resistant to heat-denaturation (Viguet-Carrin et al. 2006; Canty &
Kadler 2005). Although less frequently, proline hydroxylation may also occur when proline is present in the X position, by the enzyme prolyl-3-hydroxylase. Apart from proline residues, lysines are also hydroxylated by lysine hydroxylases (Hulmes 2008). Another post-translation modification is O-glycosylation of the hydroxyl group of hydroxylysine residues. This is catalyzed by two glycosyltransferases, galactosyltransferase and galactosylhydroxylsyl-glucosyltransferase (see Figure 1.2) (Viguett-Carrin et al. 2006). Hydroxylysine glycosylation varies and is cell- and tissue-dependent (Viguett-Carrin et al. 2006).

The next step is the initiation of collagen trimerization, which takes place with a C- to N- direction (Hulmes 2008). Published literature has identified that the C-propeptide region, the non-collagenous domain located at the C-terminus of the collagen molecule, is extremely conserved between different collagens with the exception of 15 amino acids and contributes to the selection of the appropriate α-chains and subsequently to the correct α-chain stoichiometry (Hulmes 2008). Next, inter- and intra-chain disulphide bonding occurs, a reaction catalyzed by protein disulfide isomerase. Protein disulfide isomerase is also responsible for obstruction of collagen α-chain aggregation, acting as a chaperone, and behaves as the b subunit of the porlyl-4-hydroxylase enzyme (Myllyharju & Kivirikko 2004; Myllyharju 2003). The peptid bonds prolines participate in are in cis conformation. However, trans conformation is energetically favored so the peptide bonds need to acquire the trans state prior to the trimerization being complete (Galat & Metcalfe 1995). This function is catalyzed by a family of enzymes called cis-trans isomerases and includes peptidylproline cis-trans isomerases and immunophilins (Canty & Kadler 2005; Barik 2006).

At this stage, the procollagen I (another term for immature triple-helical collagen) molecule, which is approximately 300nm long, has properly assembled (Sweeney et al. 2008; Shoulders & Raines 2009). It consists of the triple-helical region, flanked by the N- and C-terminal non-collagenous propeptides, located at the N- and C-terminus of the procollagen molecule respectively (see Figure 1.2) (Shoulders & Raines 2009). The heat shock protein 47 transfers the procollagen to the cis-Golgi
apparatus, where it dissociates from the triple-helix (Canty & Kadler 2005). Published literature has shown that both folded and unfolded procollagen molecules can act as HSP47 ligands (Satoh et al. 1996; Sauk et al. 1994). HSP47 can also serve as a regulator of collagen aggregation (Smith et al. 1995; Tasab et al. 2002). Studies showed that heat shock protein 47 knockout mice died only a few days after birth, demonstrating that heat shock protein 47 is vital for survival (Nagai et al. 2000). Another protein that has been reported to possibly function as a collagen chaperone is SPARC (Martinek et al. 2007). C-propeptide regions have also been associated with proper triple-helical folding (McAlinden et al. 2003). BiP/Grp78 and Grp94 have also been related to quality control performed to procollagen molecules (Koide & Nagata 2005) prior to secretion to cis-Golgi by HSP47 chaperone (Ricard-Blum 2011).

1.3 Trafficking of triple-helical collagen I via Golgi and secretion to the ECM

Procollagen proteins are transferred from ER to cis-Golgi using vesicles called Endoplasmic Reticulum to Golgi Intermediate Compartments or Vesicular Tubular Clusters, which appear as protrusions from the membrane of the ER (Mironov et al. 2003). The transition from ER to Golgi is regulated by coat protein complex II (COPII) that includes vesicles that coat the membrane of the saccular Vesicular Tubular Clusters (Canty & Kadler 2005; Hulmes 2008). Currently, it is thought that procollagen I is loaded to COP-II vesicles by a protein called TANGO1. Then, COP-II vesicles increase in size as a result of ubiquitination of protein SEC31 (Malhotra & Erlmann 2015). Procollagen is then transferred through the Golgi apparatus, via vesicles that form from Golgi distensions. Those vesicles occur due to the lateral aggregation of procollagen molecules (Banos et al. 2008). Finally, procollagen molecules are secreted to ECM using Golgi to Plasma membrane carriers (GPCs)(Banos et al. 2008). The range of their diameter is 0.3-1.7μm and their shape varies (Polishchuk et al. 2000).
1.4 Formation of mature collagen I molecule and its assembly into higher-order aggregates, fibrils and fibers

Procollagen I is secreted to the ECM where it is subjected to further modifications. As mentioned earlier, procollagen I consists of the triple-helical region flanked by N- and C-propeptides. These regions serve as substrates for specific zinc-dependent metalloproteinases (Hulmes 2008; Kadler et al. 2007). More specifically, N-propeptide is cleaved by members of the A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTs) family that function as N-proteinases (Porter et al. 2005), whereas C-propeptide is cleaved by members of the tolloid family, particularly Bone Morphogenetic Protein-1, that function as C-proteinases (Unsold et al. 2002; Gopalakrishnan et al. 2004) (see Figure 1.2). ADAMTs are collagen-type specific enzymes: procollagen I serves as substrate for ADAMTs-2, -3 and -14 (Colige et al. 2002; CM & BV 1993; Le Goff et al. 2006), procollagen II is cleaved by ADAMTs-3 (Fernandes et al. 2001) whereas procollagen III is cleaved by ADAMTs-2 (Wang et al. 2003). They are also tissue-specific as ADAMTs-2 is present in skin and lung tissue whereas ADAMTs-3 is present in bone and cartilage (Le Goff et al. 2006). N- and C-propeptide regions of collagen V are recognized by different N and C-proteinases: N-propeptide is cleaved by Bone Morphogenetic Protein-1 (Le Goff et al. 2006) and C-propeptide is cleaved by furin (Seidah & Prat 2002). After cleavage, the mature collagen (also called tropocollagen) consists of the triple-helix flanked by the N- and C-telopeptides respectively (see Figure 1.2). N- and C-telopeptides, are non-collagenous regions located at the N- and C-terminus of the mature collagen molecule respectively that participate in the formation of higher-ordered semicrystalline tropocollagen aggregates, which are known as fibrils and fibers (see Section 1.4) (Orgel et al. 2011; Ghazanfari et al. 2016).
Figure 1.2: Assembly of collagen I fibers. α-chains are synthesized by ribosomes present on the membrane of the rough ER. In the ER, α-chains are subjected to proline and lysine hydroxylation and also to glycosylation. Then, trimerization of the α-chains, disulphide formation and cis-trans isomerization take place. The newly synthesized collagen I molecule consisting of the three α-chains flanked by N- and C-propeptides (procollagen) is then trafficked to the ECM and subjected to cleavage of N- and C-propeptides by N- and C-proteinases respectively. The mature collagen molecules (tropocollagen) then aggregate spontaneously and form collagen microfibrils. The collagen microfibrils are then stabilized by covalent cross-links catalyzed by lysyl oxidase. Many collagen microfibrils aggregate to form the final collagen structures present in ECMs, fibrils and fibers. Image adjusted from (Shoulders & Raines 2009) with permission.

There are two different proposed models regarding the location of the cleavage of the N- and C-propeptides (Figure 1.3). According to the Birk model, cleavage of N- and C-propeptides takes place extracellularly, particularly in structures formed on the cell-surface protruding inside the cell membrane (Banos et al. 2008). The Birk model is based on data obtained by transmission electron microscopy of embryonic chick embryos. On the other hand, the Kadler model supports that initial cleavage of N- and C-propeptides takes place inside the cell, particularly in the GPCs (Canty et al.
According to this model, after procollagen cleavage, GPCs fuse with the plasma membrane to form the so-called “fibropositors”, which are projections present on the cell surface (Canty et al. 2004; Canty et al. 2006). The Kadler model is based on pulse chase experiments conducted with tendons from chick embryos. Additionally, the authors performed transmission electron microscopy using sections from embryonic chick tendons and mouse tails. However, there are a few issues arguing against the Kadler model, the most important being that the optimal pH of the proteolytic enzymes appears to be different to the pH optimum in the interior of the GPCs (Banos et al. 2008).
Figure 1.3: Summary of the two models showing formation of collagen I fibrils. Newly synthesized collagen α-chains enter the rough ER, they are then transferred to the Golgi apparatus via Vesicular Tubular Clusters and then using GPCs they are secreted to the ECM. According to the Birk model, cleavage of the N- and C-propeptides takes place extracellularly, particularly in deep channels protruding inside the surface of cells. According to the Kadler model, cleavage of the N- and C-propeptides takes place inside the cell, particularly in the GPCs. GPCs then fuse with the cell-membrane, form the so-called “fibropositors”, which secrete the collagen molecules to the ECM. Image taken from (Banos et al. 2008) with permission.

N- and C-propeptides display specific structural roles. The C-propeptide, apart from participating in the correct α-chain composition, hinders initiation of the fibrillogenesis process, which has been shown to be an entropy driven process (Kadler et al. 1987; Kadler et al. 2008). Consequently, cleavage of the C-propeptide results in the spontaneous self-assembly of tropocollagen molecules into higher-ordered structures, fibrils (Canty & Kadler 2005; Agarwal 2016) (see Figure 1.2). On the other hand, N-telopeptides participate in the regulation of the final fibril size (Viguet-Carrin et al. 2006). N- and C- telopeptides also regulate the assembly of collagen monomers (a term often used to describe collagen when present as single triple helices, not when present in fibrillar state) into higher-order structures, fibrils
N- and C-telopeptides participate in the formation of covalent cross-links with residues of the triple-helical region (Prockop 1998). This process is catalyzed by members of the lysyl oxidase family (Hulmes 2008; Maki 2009). Lysyl oxidase converts lysine or hydroxylysine residues present in the N- or C-telopeptides to peptidyl aldehydes (Hulmes 2008). Peptidyl aldehydes then react with lysines, hydroxylysines or aldehydes present in telopeptides to form covalent cross-links (Hulmes 2008) (see Figure 1.2). Covalent cross-links form either at intramolecular or intermolecular level (Ricard-Blum 2011). A study by Kuznetsova et al. (1999) supports that triple-helical collagens with no telopeptides present can also form fibrils, however such structures lack the strength and stability of typical fibrils where covalent cross-links are present (Kuznetsova & Leikin 1999). Thus, it is evident that telopeptides and covalent cross-linking are not necessary for fibrillogenesis to take place. They rather accelerate fibrillogenesis and provide stable and robust fibrils, which greatly contribute to the biomechanical characteristics of collagens and thus, of the tissue they are present in (Shoulders & Raines 2009; Ricard-Blum 2011).

1.5 Collagen I fibrils

1.5.1 Models of tropocollagen molecular packing

To date, there are two models describing the structural symmetry of tropocollagen I molecule, which forms, as mentioned earlier a right-handed triple-helical conformation. The Rich and Crick/Fraser model, which suggests that the “true repeat” of the triple-helical conformation is 8.58nm, consisting of 10 Gly-X-Y triplets, forming three turns, with the pitch of the triple-helical conformation being 2.86nm (Okuyama et al. 2006; Orgel et al. 2011; Orgel et al. 2014). The second model is the Cohen and Bear/Okuyama model, which suggests that the “true repeat” of the triple-helical conformation is 6nm, consisting of 7 Gly-X-Y triplets, forming two turns with the pitch of the triple-helical conformation being 2nm (Okuyama et al. 2006; Orgel et al. 2011; Orgel et al. 2014). When triplets contain low imino acid levels, then the packing of tropocollagen resembles more the Rich and Crick/Fraser model, whereas in triplets with high levels of imino acids tropocollagen packing resembles more the
1.5.2 Structure of collagen I fibrils

The Hodge-Petruska model dictates that each microfibril molecule consists of five tropocollagen molecules. Each tropocollagen molecule is also called “segment” (see Figure 1.4B) (Orgel et al. 2011). Using crystal structures obtained from studies with collagen peptides, along with lower resolution data, Orgel et al. (2001, 2006) proposed a model suggesting that each microfibril molecule consists of five tropocollagen segments which are organized in a quasi-hexagonal arrangement (Orgel et al. 2006; Orgel et al. 2001; Orgel et al. 2011; Brodsky & Persikov 2005), where each segment abstains 1.3nm from each neighboring one (Perumal et al. 2008; Orgel et al. 2011) (Figure 1.4C). These data describing the three-dimensional collagen packing further supported the Hodge-Petruska model, which also suggested that each collagen microfibril consists of five segments (Orgel et al. 2011). Each segment appears in the microfibril structure with a D-period of 67nm and 64nm in hydrated and dehydrated fibrils respectively (Wess & Orgel 2000). The D-period, which refers to the periodicity of the collagen segments within the microfibril molecule, is divided into the overlap region (0.46D), where all five segments are present and the gap region (0.54D), where only four segments are present (Orgel et al. 2011; Orgel et al. 2000). Finally, the molecular arrangement of fibrils was further verified when collagen I fibrils were imaged using transmission electron microscopy after heavy metal staining. This staining induces the collagen fibril to appear with a characteristic dark/light pattern, where dark staining represents the gap region whereas light staining represents the overlap region (Orgel et al. 2011), verifying the quasi-hexagonal collagen arrangement model.

Fibrillar size and diameter is tissue-dependent and varies based on the stage of development (Starborg et al. 2009). In tendons, fibril diameter varies from 30-300nm (Banos et al. 2008). Similar to fibrillar size, the final fibrillar structure is also tissue-
dependent. In tendons, monomers assemble to fibrils, fibrils assemble to fibers (bundles of fibrils), fibers assemble to fascicles (bundles of fibers) and fascicles form tertiary fiber bundles (Banos et al. 2008; Orgel et al. 2011). Previous literature reports that fibrils are present in tissues in unipolar or bipolar orientation (Kadler et al. 1996). Bipolar fibrils appear to have only the N-terminal telopeptides exposed, since after eight D-periods the fibril molecule flips around the fibril axis through 180°. Unipolar fibrils sustain the same orientation throughout the fibril (Starborg et al. 2009; Kadler 2004). However, there are conflicting publications regarding which fibrillar telopeptide is exposed: the Perumal et al. (2008) model supports that the C-telopeptide is exposed whereas the Herr and Farndale (2009) model supports that the N-telopeptide is exposed (Perumal et al. 2008; Herr & Farndale 2009).

1.5.3 Regulation of fibrillogenesis and fibril final size

An important ECM component that affects the final structure of collagen I fibrils is fibronectin. As mentioned in Section 1.1, fibronectin is a glycoprotein, present in the ECM where it forms fibrillar networks. In order for fibronectin to polymerize, interactions with integrin molecules are vital (Kadler et al. 2008; Singh et al. 2010). Studies using fibronectin-null cells have shown that fibronectin absence is linked to the absence of collagen I fibrils (Sottile & Hocking 2002; Dallas et al. 2005). In two independent studies where fibronectin-deficient vascular smooth muscle cells or mouse embryonic fibroblasts were incubated with an anti-α2β1 antibody, fibril formation was hindered (Velling et al. 2002; Li et al. 2003). On the other hand, when the antibody was removed from vascular muscle cells, collagen I fibrils were again visible (Velling et al. 2002; Li et al. 2003). This suggests an indirect and direct role for fibronectin and integrin respectively as regulators of collagen I fibril assembly (Li et al. 2003; Singh et al. 2010). Studies by Jokinen et al. (2004) using in vitro generated collagen I fibrils also suggests that integrins could promote collagen I fibrillogenesis (Jokinen et al. 2004).
Another collagen present in ECM, collagen V, has been shown to co-polymerize with collagen I fibrils, affecting the final collagen I fibril size (Kadler et al. 2008). Data obtained using rotary-shadow electron microscopy showed that collagen I fibrils co-localize with collagen V in chick corneal stroma. These data were acquired after partial dissociation of the fibrillar molecule, showing that collagen V is buried within the collagen I fibril (Birk et al. 1990; Kadler et al. 2008). Studies by Wenstrup et al. (2004a) showed that in dermal fibroblastic cultures isolated from patients with Ehlers-Danlos syndrome, data obtained by transmission electron microscopy and fibril analysis supported that the collagen I fibrils assembled in the ECM of those patients were bigger in diameter compared to those produced from control cell-lines, but surprisingly they had weaker biomechanical characteristics, particularly lower stiffness and tensile strength (Wenstrup et al. 2004a). Another study conducted from the same group showed that mice with collagen V α1-chain

**Figure 1.4: Structure of collagen I fibril.** (A) Tropocollagen I molecules consist of three polypeptide α-chains, where D-period is 6.8-6.6nm. (B) Tropocollagen molecules aggregate spontaneously to form microfibrils. Each microfibril consists of five tropocollagen molecules, each of which appears with a D-period of 67nm. (C) Microfibril molecules are organized in a quasi-hexagonal arrangement where each tropocollagen abstains 1.3nm from its neighboring tropocollagen. Image adapted from (Orgel et al. 2014) with permission.
deficiency did not survive. They also showed that the collagen V deficiency was inversely proportional to the biomechanical characteristics of the dermis and the diameter of the fibrils assembled (Wenstrup et al. 2004b). Those studies argue for a crucial role of collagen V as a regulator of collagen I fibril diameter and thus of the fibrillar biomechanical properties (Kadler et al. 2008).

Another ECM component that has been extensively studied in vitro for its ability to affect the final size of collagen I fibrils are proteoglycans. As mentioned above, proteoglycans consist of a core protein and a number of GAG chains. Decorin and biglycan, which are highly expressed in tendons and have dermatan sulfate chains, may compete for binding to collagen I fibrils and have been shown to restrain their diameter (Banos et al. 2008; Lechner et al. 2006; Rada et al. 1993). Lumican and fibromodulin, which have keratan sulfate protein core and are also present in tendons, have been shown to decrease fibrillogenesis rate (Ezura et al. 2000; Neame et al. 2000; Kalamajski & Oldberg 2010).

**1.5.4 Degradation of collagen I fibrils**

Collagen I, both in triple-helical and fibrillar form, serves as substrate for zinc-dependent metalloproteinases (MMPs), serine or cysteine proteinases (Kadler et al. 2007; Ricard-Blum 2011; Van Doren 2015; Bonnans et al. 2014; Itoh 2015). MMP-1, MMP-8 and MMP-13 (collagenases) cleave collagen I fibrils (Visse & Nagase 2003; Lu & Stultz 2013), recognizing a specific cleavage site, a specific bond between Gly-Ile/Leu producing fragments with size three- and one-quarter of the fibril molecule (Perumal et al. 2008; Visse & Nagase 2003; Lu & Stultz 2013). MMP-2 and MMP-9 (gelatinase A and B respectively) cleave specifically gelatin or the fragments produced by the previous MMPs (Visse & Nagase 2003; Bigg et al. 2007). Apart from soluble MMPs, membrane type I MMP-14 (MT1-MMP) also cleaves collagen I, along with collagen II and collagen III (Visse & Nagase 2003; Ohuchi et al. 1997; Fields 2013; Nagase et al. 2006; Itoh 2015). Extensive studies have shown that ECM remodeling by MMPs and MT-MMPs is essential for the development and tissue
repair of the respective tissue (Ricard-Blum 2011; Bonnans et al. 2014). A study by Hombeck et al. (1999) indicates the significance of MT1-MMP collagenolytic activity, demonstrating that mice with MT1-MMP knockdown presented skeletal abnormalities (Holmbeck et al. 1999). Previous studies have shown that collagen I-mediated degradation by collagenases plays an important role in keratinocyte migration (Pilcher et al. 1997) and osteoclast function (Holliday et al. 1997). However, apart from maintaining healthy tissues, MMPs have also been related with several pathological conditions, such as cancer, fibrosis and arthritis (Bonnans et al. 2014; Nagase et al. 2006; Itoh & Nagase 2002). Apart from MMPs, cysteine proteases such as cathepsin K, have the ability to promote dissociation of glycosaminoglycans from the fibril surface, expose the triple-helix and finally cause its degradation (Panwar et al. 2013). Similarly, serine proteases degrade the triple-helical region of collagen I fibrils after they promote fibril disassembly (Ran et al. 2013).

1.5.5 Collagen I-related diseases

Osteogenesis Imperfecta is one of the most common collagen I-related diseases (Marini et al. 2007). Clinical symptoms include fragile or fractured bones (Marini et al. 2007). The basis of the disease is genetic: mutations in α1- and α2-chains, mainly amino acid substitutions of glycine and exon skipping respectively (Marini et al. 2007). Such mutations cause a variety of effects, the most important being the inability of the secreted collagen I to interact with other components of the ECM and thus form proper fibrils (Marini et al. 2007). Another disease that was reported to be associated with collagen I mutations, more specifically with substitution of arginine to glycine in α1-chain, is classical Ehlers-Danlos syndrome (Nuytinck et al. 2000). Clinical symptoms include hypermobility and skin hyper elasticity (Nuytinck et al. 2000). Other types of collagen I-related diseases include atherosclerosis (Katsuda et al. 1992) and hypertensive heart disease related to over-accumulation of collagen I in the respective ECM (Diez et al. 2005).
1.6 Collagen VI microfibrils

1.6.1 Structure of collagen VI molecule

Collagen VI is present in all connective tissues (Kielty & Grant 2002), particularly in articular cartilage, skin, nervous system and muscle tissues (Cescon et al. 2015). It plays an important architectural role, as it participates in the correct assembly of the ECM, especially in cartilage and skeletal muscles (Cescon et al. 2015). At the cellular level, it has been shown to play a cytoprotective role, particularly in the nervous system (Vitale et al. 2001; Cheng et al. 2011). Collagen VI is a heterotrimer molecule, consisting of three different collagen VI α-chains. Earlier studies identified three genes coding for the three different collagen VI α-chains, α1 (VI), α2(VI) and α3 (VI) respectively (Fitzgerald et al. 2013). Genes coding for α1- and α2-chains are located in tandem in chromosome 21 locus 22.3 whereas the gene coding for α3-chain is located in chromosome 2 locus 37 (Cescon et al. 2015). More recently, three more genes were discovered, coding for α4(VI), α5(VI) and α6(VI) chain respectively. The genes for those chains are present in tandem, located in chromosome 3, locus 21 (Gara et al. 2008; Fitzgerald et al. 2008). Compared with α3-α6 chains, α1(VI) and α2(VI) chains have lower molecular weight, approximately 130-150kDa, whereas chains α3-α6 can reach more than 300kDa. Collagen VI monomers (again, a term used to describe collagen VI when present in triple-helical form, not in higher order forms such as tetramers or microfibrils) reach approximately 500kDa (Cescon et al. 2015). Collagen VI monomers consist of a short triple-helical region (approximately 336 amino acids long) flanked by large non-collagenous N- and C-globular domains (Baldock et al. 2003). As shown in Figure 1.5A, the triple-helix of each chain incorporates a cysteine residue, that has a stabilizing effect on the collagen VI higher-ordered structure (Fitzgerald et al. 2013). This cysteine is present 50 amino acids away from the N-terminal end of the triple-helix in chains α3-α6 (Fitzgerald et al. 2008; Gara et al. 2008) but it is further away, 89 amino acids away from the N-terminus, in α1- and α2-chains (Fitzgerald et al. 2013). The N-terminal end of the different α-chains consists of a number of von Willebrand Factor (vWF) type A domains (Godwin et al. 2016). Little is known regarding the N-terminal region’s
biological function. Studies by Fitzgerald et al. (2001) and Beecher et al. (2011) support that the N-terminal region of the α3-chain serves as a ligand for ECM components whereas the N5 domain of the same chain, which is located within the N-terminal region, serves an important structural role as it regulates the final collagen VI conformation (Beecher et al. 2011; Fitzgerald et al. 2001). Studies have shown that the C-terminal region of the respective collagen VI α-chain participates in the α-chain assembly, like in the case of fibrillar collagens, and the formation of the final collagen VI higher-order structure (Ball et al. 2001; Tooley et al. 2010; Lamandé et al. 2006). While the C-terminal regions of α1- and α2- chains appear to be similar to each other, α3-α6 chains have distinct differences. More specifically, while α1 and α2-chains consist only of vWF type A factor domains in their non-collagenous regions, α3-6 chains include one or more collagen VI-specific domains and furthermore a full (α3 chain) or a partial (α4 chain) Kunitz domain (see Figure 1.5A) (Fitzgerald et al. 2013). However, the overall structural pattern of α3-α6 chains suggests that α4-α6 chains could serve as substitutes for the α3-chain (Fitzgerald et al. 2008; Gara et al. 2008).

Triple-helical collagen VI assembles in a C- to N-terminal direction, like in the case of collagen I. As shown in Figure 1.5B, collagen VI monomers associate in an antiparallel direction to form a dimer, a supercoiled left-handed structure that is 75nm long (Baldock et al. 2003). Subsequently, dimers interact with each other with their C-terminal domains aligned, forming tetramers (Knupp & Squire 2005; Hulmes 2008; Fitzgerald et al. 2013). Dimeric and tetrameric assemblies are stabilized by disulphide bonds occurring between the triple-helical regions with the contribution of the C-terminal domains of the collagen VI molecules (Sato et al. 2002; Baldock et al. 2003). Only collagen VI tetramers are secreted from cells. In the ECM, collagen VI tetramers end-to-end assemble with each other, resulting in the final supramolecular collagen VI structure, beaded microfilaments (Baldock et al. 2003; Fitzgerald et al. 2013), (Figure 1.5) with a D-period of 110nm (Hulmes 2008). Collagen VI microfibrils are stabilized by non-covalent bonds that occur among the globular domains of the respective tetramers (Baldock et al. 2003).
Figure 1.5: Structure of the α1-α6-chains and assembly of collagen VI monomers to form the final collagen VI supramolecular assembly, microfibrils. (A) Architecture of collagen VI α1-α6 chains (α1(VI)-α6(VI)). Each chain consists of a non-collagenous N-terminal region, consisting of vWF type A domains (N1-N7), the triple helix and a non-collagenous C-terminal region, again comprising two or more vWF type A domains (C1, C2, C4). (B) Collagen VI monomers bind antiparallel to form dimers. Dimers then interact with one another using their C-terminal domains in alignment, resulting in tetramers. Only collagen VI tetramers are secreted from cells where they assemble end-to-end resulting in the assembly of collagen VI beaded microfilaments. Image adapted from (Godwin et al. 2016) with permission.

1.6.2 Collagen VI distribution and functions

Collagen VI is an abundant component of all connective tissues (Kielty & Grant 2002; Gara et al. 2011). It is particularly present in the pericellular matrix of articular
cartilage, surrounding its sole cellular component, chondrocytes (Poole et al. 1988; Youn et al. 2006). Collagen VI tethers any biomechanical changes from the ECM to chondrocytes and was shown to affect their proliferation rate (Cescon et al. 2015). It can also affect the structural properties of chondrocytes and of the rest of the collagens present in the ECM (Wilusz et al. 2014; Cescon et al. 2015). Increased collagen VI levels are also present in skin, particularly in hypodermis and in hair follicles (Keene et al. 1988; Gara et al. 2011). Schwann cells in the peripheral nervous system also produce collagen VI, in addition to the early-established collagen VI synthesis by meningeal cells (Cescon et al. 2015). Studies by Cheng et al. (2011) and Vitale et al. (2001) have demonstrated that collagen VI can inhibit neuron death and promote differentiation of Schwann cells respectively (Vitale et al. 2001; Cheng et al. 2011). Collagen VI is also present in skeletal and heart tissues, where it is synthesized by the respective interstitial fibroblasts. It is also located in lungs and in tendons (Cescon et al. 2015). Finally a study by Fitzgerald et al (2008) identified that collagen VI mRNA is present in lung, kidney, liver, spleen, thymus, testis, and colon (Fitzgerald et al. 2008).

1.6.3 Collagen VI ligands

Several of the collagen VI functions are attributed to its interactions with other ECM proteins. Published literature has shown that collagen VI interacts with collagen I (Bonaldo et al. 1990), collagen II (Bidanset et al. 1992) or collagen XIV (Brown et al. 1994). It has also been shown to interact with proteoglycans such as decorin, biglycan (Wiberg et al. 2001; Bidanset et al. 1992), lumican (Takahashi et al. 1993), heparan and hyaluronan (Specks et al. 1992). Furthermore, it associates with WARP (Hansen et al. 2012), matrilin-1 (Wiberg et al. 2003), fibulin-2 (Sasaki et al. 1995) and microfibril-associated MAGP1 glycoprotein (Finnis & Gibson 1997). A study by Sabatelli et al. (2001) demonstrated that collagen VI is vital for the formation of fibronectin fibrils secreted from cultured fibroblasts (Sabatelli et al. 2001). Apart from its ability to bind to other ECM components, studies have demonstrated that
collagen VI can also bind to transmembrane receptors, particularly $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_{10}\beta_1$ integrins (Doane et al. 1998; Tulla et al. 2001).

### 1.6.4 Collagen VI-related diseases

Mice with knockdown of collagen VI $\alpha_1$-chain have been shown to develop osteoarthritis (OA), low levels of mineral density as well as ossification and furthermore abnormal structure of knee trabecular bone (Alexopoulos et al. 2009; Christensen et al. 2012). Collagen VI has been associated with many skin-related diseases such as atopic dermatitis (Söderhäll et al. 2007), follicular keratosis and striae rubrae (Lettmann et al. 2014). It has been also linked with diseases of the nervous system, such as Alzheimer’s diseases (Cheng et al. 2009) and progressive myoclonus epilepsy, which was a result of a mutation in the $\alpha_2$(VI) chain (Karkheiran et al. 2013). Mutations in collagen VI $\alpha$-chains can also result in muscular dystrophies. A recent study by Merlini et al. 2008 identified an autosomal myosclerosis myopathy caused by deletion of exon 27 in collagen VI $\alpha_2$-chain. This mutation results in deletion of the collagen VI C1 domain, leaving C2 domain unharmed (Merlini et al. 2008). Bethlem myopathy and Ulrich congenital muscular dystrophy have been also linked with mutations in $\alpha_1$-$\alpha_3$ chains and $\alpha_2$- or $\alpha_3$-chains respectively (Lampe & Bushby 2005). Finally, elevated collagen VI levels have been linked to several cancers, particularly pancreatic cancer and melanomas (Burchardt et al. 2003; Kang et al. 2014). It has been demonstrated that collagen VI has certain tumor-related functions: it participates in epithelial to mesenchymal transition (EMT), inflammation and angiogenesis (Park & Scherer 2012; Chen et al. 2013).

### 1.7 Cell membrane-located collagen receptors

Integrins are well-established cell adhesion ECM receptors (Leitinger 2011; Srichai & Zent 2010; Campbell & Humphries 2011). All integrins are heterodimers, consisting of two subunits that are non-covalently linked. Each subunit consists of an extracellular region, a transmembrane helix and a short cytoplasmic region. The two
subunits associate extracellularly, forming a structure consisting of a β-propeller located at their apex, the I-like domain and the I domain, where the ligand-binding site is accommodated (Leitinger & Hohenester 2007; Leitinger 2011; Barczyk et al. 2010; Campbell & Humphries 2011). They can mediate signals either from the intracellular environment to the ECM or the opposite (Leitinger & Hohenester 2007; Heino 2014; Barczyk et al. 2010; Campbell & Humphries 2011). Their activation is accompanied by a conformational change in their extracellular region. A subfamily of β1 integrins, α1β1 α2β1 α10β1 and α11β1 have been shown to function as collagen receptors (Popova et al. 2007; Srichai & Zent 2010; Heino 2014; Leitinger & Hohenester 2007; Leitinger 2011). They have collagen-related preferences: α1β1 and α11β1 integrins bind to collagen IV with higher-affinity (Tiger et al. 2001; Gardner et al. 1996) whereas α2β1 and α10β1 integrins bind preferentially to fibrillar collagens (Tulla et al. 2001; Jokinen et al. 2004). Moreover, a study by Jokinen et al. (2004) demonstrated that α1β1 integrins bind preferentially to soluble collagen I whereas α2β1 bind also to collagen I fibrils (Jokinen et al. 2004). The expression of collagen-binding integrins is tissue-specific, as α1β1 is present in mesenchymal cells, and α2β1 is present on the surface of epithelial cells and platelets (Leitinger 2016; Leitinger 2011) whereas α10β1 is present in chondrocytes (Gigout et al. 2008) and α11β1 in interstitial fibroblasts (Tiger et al. 2001). Several studies used synthetic triple-helical peptides encompassing amino acids sequences of the collagen II or III triple-helix, to identify specific amino acid motifs that interact with members of the β1 integrin family. More specifically, the GFOGER motif, which is present in collagens I-III serves as binding site for α1β1, α2β1 and α11β1 integrins (Knight et al. 1998; Knight et al. 2000; Zhang et al. 2003). The GLOGER motif, present in collagens II and III, serves as binding motif for α1β1 and α2β1 integrins (Xu et al. 2000) whereas the GROGER motif, present in collagens II and III, serves as binding site for α2β1 integrins (Raynal et al. 2006). Integrins are the major transmembrane receptors responsible for cell adhesion. They also participate in cell migration and cell spreading (Gullberg & Lundgren-Åkerlund 2002). However, they have been shown to participate in tumor progression, angiogenesis, fibrosis, OA and wound healing (Leitinger 2011).
Another well-established collagen receptor is glycoprotein VI (GPVI). GPVI is present on the surface of platelets and participates in platelet activation and aggregation (Leitinger 2011; Stegner et al. 2014; Ozaki et al. 2013). GPVI consists of an ectodomain region, a mucin-like stalk region, a transmembrane region and a short cytoplasmic tail. The ectodomain region consists of two immunoglobulin-like domains (Moroi & Jung 2004). Upon collagen binding, GPVI associates with the Fc receptor γ-chain, and this interaction is stabilized by a salt bridge formed between their transmembrane domains (Moroi & Jung 2004). Collagens serve as functional GPVI ligands only in fibrillar conformation, not in the form of single triple-helices (Miura et al. 2002). Experiments using synthetic triple-helical peptides consisting of (GPO)10 triplets showed that these peptides were able to bind to GPVI, but they could not induce GPVI signaling (Kehrel et al. 1998; Morton et al. 1995). Clustering of GPVI, which is induced by its multivalent ligands, fibrillar collagens, results in activation of several signaling molecules such as PI3K and Rac 1 and finally to increased calcium signals (Watson et al. 2010). However, platelet aggregation by GPVI dictates its co-operation with integrins, particularly α2β1 integrins. Studies have indicated that co-operation of GPVI and integrins is vital for thrombus formation (Auger et al. 2005; Mazzucato et al. 2009; Kuijpers et al. 2003). Results so far suggest that the GPVI role appears to be related to aggregation rather than adhesion of platelets (Leitinger 2011; Pugh et al. 2010; Ozaki et al. 2013).

Another collagen receptor is leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) (Leitinger 2011; Kang et al. 2016). The LAIR family consists of two members, LAIR-1 and LAIR-2 (Leitinger 2011). LAIR-1 is expressed on the surface of cells of the immune system. It consists of an extracellular immunoglobulin domain, a stalk region, a short transmembrane region and a cytoplasmic domain. The cytoplasmic domain includes two immunoreceptor tyrosine-based inhibitory motifs (Leitinger & Hohenester 2007; Leitinger 2011; Kang et al. 2016). Ligand binding results in the phosphorylation of tyrosines present in the cytoplasmic region, which serve as docking sites for signaling adaptors such as SHP-1 and SHP-2 (Meyaard 2008). Lebbink et al. 2006 identified collagens I-IV and XVII as LAIR-1 ligands using in vitro assays (Lebbink et al. 2006). Ligand-binding results in inhibition of processes such as
target cell lysis and cytotoxic activity catalyzed by NK and T cells respectively. Finally, Tomlinson et al. (2007) demonstrated that when LAIR-1 and glycoprotein VI co-exist on the cell surface, LAIR-1 activation can block glycoprotein VI I signaling (Tomlinson et al. 2007). LAIR-2 is a soluble protein, as it consists only of an extracellular region that is homologous to LAIR-1. Published literature has shown that it can also bind to collagens in vitro and suggested that LAIR-2 could inhibit the ability of LAIR-1 to serve as functional collagen ligand in vivo (Lebbink et al. 2008). The last group of transmembrane collagen receptors is discoidin domain receptors which will be discussed in detail in Section 1.8.

1.8 Discoidin domain receptors

1.8.1 The Receptor tyrosine kinase family

Collagens also serve as ligands for discoidin domain receptors (DDRs) (Leitinger 2014). The DDRs are members of the Receptor Tyrosine Kinase (RTK) family. RTKs are glycoproteins consisting of 58 members in humans (Lemmon & Schlessinger 2010; Kennedy et al. 2016). The RTK family includes several subfamilies such as the EGFR family, that regulates epithelial cell growth and proliferation (Lemmon et al. 2014), the vascular growth factor receptor family, that plays key roles in angiogenesis and several types of cancer, the platelet-derived growth factor receptor family, insulin-like growth receptor (IGFR) family, the fibroblast growth factor receptor (FGFR) family (Goel & Mercurio 2013; Karpov et al. 2015) and the hepatocyte growth factor receptor family (Pathi et al. 2016). All RTKs consist of an extracellular region comprising the ligand binding site, which presents structural diversities due to the different ligands for each RTK family. More specifically, it can consist of cadherin-like, discoidin-like, fibronectin type III-like, kringle-like or leucine-rich like domains. This high diversity among the RTK ectodomain is attributed to the ligands of the respective RTK member (Karpov et al 2015; Ségaliny et al. 2015). Furthermore, each RTK member comprises a transmembrane region, an intracellular juxtamembrane region, a tyrosine kinase domain and a C-terminal tail (Hubbard 2004; Lemmon & Schlessinger 2010; Karpov et al 2015). The tyrosine kinase domain is enzymatically
active and includes the activation loop and the ATP binding site. Kinase structures typically are bilobal with characteristic C- and N-lobes located at the C- and N-terminal region of the tyrosine kinase domain respectively. The C-lobe, which consists of α helices, participates in the ATP chelation whereas the N-lobe, which consists of β-sheets, regulates the ATP stabilization and orientation (Ségaliny et al. 2015; Karpov et al. 2015). The tyrosine kinase domain is relatively conserved among the RTKs (Karpov et al 2015).

It is believed that RTKs are present mainly as monomers and become homodimers upon ligand binding (Lemmon & Schlessinger 2010). Most RTKs are activated by small diffusible proteins such as growth factors upon a few seconds after ligand binding (Lemmon & Schlessinger 2010). Prior to ligand binding, RTKs are auto-inhibited in various ways. Upon ligand binding, dimerization of the receptor takes place, which brings the kinase domains into close proximity and may result in rotation of its N- and C-lobe, resulting in the C-terminal region of the “donor” interacting with the N-terminal region of the “receiver” kinase within a dimer. This results in trans-phosphorylation of the tyrosine residues present in the activation loop of the dimerized receptor and stabilization of the dimer conformation (see Figure 1.6) (Karpov et al 2015; Maruyama 2014). Phosphorylation of those residues results in stabilization of the activated receptor conformation, hydrolysis of the ATP and further phosphorylation of the receptor’s tyrosine residues present in the intracellular juxtamembrane region, the kinase domain and the C-terminal tail (Sun & Bernards 2014; Lemmon & Schlessinger 2010).
Figure 1.6: Structural change of the kinase domain upon RTK activation. Activation of the kinase domain is inhibited by the juxtamembrane region, the activation loop and the C-terminal chain. Upon ligand binding, ATP binding occurs and phosphorylation takes place, since the juxtamembrane region, the activation loop and C-terminal chain change their alignment within the RTK molecule. Image obtained from (Karpov et al. 2015) with permission.

Phosphorylated tyrosine residues then act as docking sites for signaling proteins containing Src homology 2 domains or phosphotyrosine binding domains (Pawson 2007; Pathi et al. 2016). Those signaling and adaptor molecules activate signaling pathways and thus RTKs regulate several fundamental cellular processes such as migration, differentiation and proliferation (Lemmon et al. 2014). More specifically, RTKs usually activate the MAPK pathway, which regulates cell proliferation, migration, differentiation, apoptosis and angiogenesis; the PI3K/Akt/mTOR pathway, which also participates in cell apoptosis and cell cycle progression; the PLCγ or the JAK/STAT pathway (Ségaliny et al. 2015). Published literature has shown that RTKs are associated with disease progression of a number of diseases, such as tumors, diabetes, atherosclerosis and bone-related diseases (Malek & Davis 2016; Shiuam & Chen 2016; Karpov et al. 2015). More specifically, several RTKs have been related to tumorigenic states, such as EGFR to tumors in lung or skin (Morgensztern et al. 2015), FGFR to tumors in the large intestine (Mathur et al. 2014) and platelet-derived growth factor receptor to stomach and colorectal cancers (Saplacan et al. 2017; Nordby et al. 2017). Low VEGFR levels have been related to hypercholesterolemia, atherosclerosis and diabetes (Karpov et al. 2015). FGFR knockdown mice have been shown to present mesoderm defects (Su et al. 2014).
FGFR has also been related to osteoglophonic dysplasia (White et al. 2005; Farrow et al. 2006; Su et al. 2014). To date, several inhibitors using RTKs as therapeutic targets have been developed for diseases such as several types of cancer (Fauvel & Yasri 2014; Hempel et al. 2017; Yadav et al. 2017). For example, chemical compounds such as the 3-chlorobenzylamine derivative 5b and the 3-methoxybenzylamine compound 5a have been developed against EGFR and IGF-1R, which have been shown to participate in cancer progression (Hempel et al. 2017). Another inhibitor was developed against human epidermal growth factor receptor 2, which has been shown to participate in gastro-oesophageal junction cancer (Bang et al. 2010). Finally, chemical compounds against EGFR have also been developed due to the fact that it has been shown to participate in brain metastases of non-small cell lung cancer cells (Lee et al. 2012).

### 1.8.2 Discoidin Domain Receptors

As mentioned earlier, the DDRs are members of the RTK family. The DDR subfamily comprises two members, DDR1 and DDR2. DDR1 and DDR2 have different functions during embryo development, they are very broadly distributed in adult organisms, and found in many different tissues (see Section 1.8.3). At the cellular level, they play roles in cell migration, differentiation, adhesion and matrix remodeling (Leitinger 2011; Leitinger 2014). They are unique among RTKs in the sense that they are activated by a matrix component, collagen, whereas most other RTKs are activated by small diffusible proteins such as growth factors, as mentioned earlier (Karpov et al. 2015; Fu et al. 2013a). Both DDRs are activated by collagens of the fibrillar family, but they also present preferences regarding the non-fibrillar collagens they can bind to (see Section 1.8.6) (Vogel et al. 1997; Shrivastava et al. 1997; Leitinger & Kwan 2006; Hou et al. 2001). Another unique DDR characteristic is that DDR activation, which is manifested by autophosphorylation of tyrosine residues present in their intracellular region, is slow and sustained (Vogel et al. 1997; Shrivastava et al. 1997). This is not the case though for the rest of the RTKs, which are activated within seconds after ligand binding. The DDRs have been shown to play important roles in
several diseases such as organ fibrosis, OA, kidney diseases and several types of cancer (see Section 1.8.10) (Valiathan et al. 2012; Carafoli & Hohenester 2013; Borza & Pozi 2014; Rammal et al. 2016; Multhaupt et al. 2016). It is thus believed that the DDRs could serve as therapeutic targets for the diseases they participate in (Valiathan et al. 2012; Kothiwale et al. 2015; Rammal et al. 2016; Multhaupt et al. 2016).

1.8.3 DDR expression in tissues and functions

DDR1 is present on the surface of epithelial cells, in skin, kidney, placenta, brain and lungs (Alves et al. 1995; Leitinger 2014). DDRs have also been found on immune cells (Kamohara et al. 2001; Chetoui et al. 2011). DDR function is essential during development, however their role in adult tissues has not been clarified yet. Mice with DDR1 knockdown present abnormalities related to mammary gland development (Vogel et al. 2001), kidney function and inner ear structure (Leitinger 2014; Torban & Goodyer 2009). More specifically, DDR1 knockout mice are infertile, unable to secrete milk and they present increased matrix deposition (Vogel et al. 2001). Abnormalities of the inner ear structure result in deficiency of the auditory function (Meyer zum Gottesberge et al. 2008). Finally, kidney abnormalities include increased matrix deposition and altered glomerular basement membrane architecture (Gross et al. 2004). DDR2 is highly expressed in the heart, muscle and connective tissues, on the surface of mesenchymal cells (Alves et al. 1995; Karn et al. 1993; Lai & Lemke 1994). Similarly to DDR1, DDR2 has also been located on the surface of immune cells (Afonso et al. 2013). DDR2 function is closely related to long bone growth. Mice with DDR2 overexpression have elevated bone growth whereas mice with DDR2 knockout expressed dwarfism due to a defect in chondrocyte proliferation (Labrador et al. 2001; Kawai et al. 2014). Mice with a spontaneous autosomal-recessive mutation, called slie mice, which was shown to cause deletion of expression of the DDR2 gene, also expressed dwarfism along with infertility of both males (inability of spermatogenesis) and females (mice were anovulatory) (Kano et al. 2008). A rare human genetic disorder, spondylo-meta-epiphyseal
dysplasia with short limbs (SMED-SL) is caused by a number of DDR2 mutations (Borochowitz et al. 1993; Bargal et al. 2009; Langer et al. 1993; Mansouri et al. 2016). Such mutations result either in the inability of DDR2 to traffick to the cell-surface or to interact with collagen (Bargal et al. 2009; Ali et al. 2010; Al-Kindi et al. 2014). SMED-SL patients suffer from short stature with disproportionately short limbs, bone abnormalities and premature calcifications (Borochowitz et al. 1993). Published literature has demonstrated that DDR2 serves a regulating role in ossification, endochondrial and intramembranous, and furthermore it can regulate secretion of lysyl-oxidase (Khosravi et al. 2014; Zhang et al. 2011; Lin et al. 2010). Finally, a recent publication showed that mice with a DDR2 germline gene deletion show functional and structural abnormalities in the heart (Cowling et al. 2014). More specifically, these mice have smaller size heart and the cardiac fibroblasts isolated from these mice deposited very low amounts of collagen (Cowling et al. 2014).

**1.8.4 DDR Domain structure**

A main structural DDR characteristic is their discoidin (DS) homology domain located at the N-terminal region of the extracellular region. DS domains are named like that due to their high level of similarities with a protein secreted from *Dictyostelium discoideum*, the protein Discoidin I (Kiedzierska et al. 2007). The DDRs also have a DS-like domain, an extracellular juxtamembrane (EJM) region, a transmembrane region, an intracellular juxtamembrane region (IJM) and a kinase domain (KD) with a C-terminal tail (Fu et al. 2013a) (see Figure 1.7). They are single-span type I glycoproteins (Leitinger 2011; Fu et al. 2013a; Carafoli & Hohenester 2013).
Figure 1.7: Structural organization of DDR1 isoforms and DDR2. DDR1a, DDR1b and DDR2 consist of the DS domain, the DS-like domain, the transmembrane (TM) region, the kinase domain and the juxtamembrane region. N- and O-glycosylation sites (green and purple shapes) are present in the DS-like and the EJM region. Image obtained from (Leitinger 2014) with permission.

The DS domain consists of approximately 160 amino acids and presents a 59% identity at the amino acid sequence, between DDR1 and DDR2 (Carafoli & Hohenester 2013; Leitinger 2014). The DS domain is where the collagen binding site is accommodated (Leitinger 2003; Abdulhussein et al. 2004). The structure of the DDR2 DS domain, unbound and bound to a collagen-mimetic triple-helical peptide was defined at atomic level detail using NMR and X-Ray crystallography respectively (Ichikawa et al. 2007; Carafoli et al. 2009). Carafoli et al. (2009) demonstrated that two apolar residues, Methionine and Phenylalanine located on the leading and middle chain of the triple-helical collagen-mimetic peptide respectively, interact with the DDR amphiphilic pocket (Carafoli et al. 2009). Published literature has shown that residues present in loops 1, 2 and 4 of the DS domain are vital for the DDR-fibrillar collagen interaction (Ichikawa et al. 2007; Carafoli et al. 2009). More specifically, the amino acid residues located on the DDR2 DS domain, Trp52, Thr56, Asp69, Arg105, Glu113, and the Cys73-Cys177 disulfide bridge, which are conserved in DDR1, were shown to interact with the collagen-mimetic peptide (see Figure 1.8).
Furthermore, the structure of the composite of the DDR1 ectodomain bound to an anti-DDR1 antibody was obtained using X-Ray crystallography (Carafoli et al. 2012). The DS domain consists of eight β-strands formed in two anti-parallel sheets (Ichikawa et al. 2007; Carafoli et al. 2009; Carafoli et al. 2012). It adopts a β-barrel structure, which is stabilized by two disulfide bridges that link the N- and C-terminus of the DS domain (Carafoli et al. 2009; Ichikawa et al. 2007). The collagen binding site is shaped by five protruding loops on the surface of the DS domain (Leitinger 2003; Abdulhussein et al. 2004; Ichikawa et al. 2007; Carafoli et al. 2009; Carafoli et al. 2012). Furthermore, the amino acids responsible for the DDR-collagen interactions were found to be strictly conserved between DDR1 and DDR2 (Carafoli et al. 2009; Xu et al. 2011a). A conserved patch on the surface of the DS domain but located away from the collagen binding site was also shown to serve an important role during DDR activation (Carafoli et al. 2012). However, its exact function has not been identified yet: it has been hypothesized that it could serve as a low-affinity binding site or regulate receptor dimerization (Carafoli et al. 2012). Amino acids in the DDR1 DS domain that are located at the periphery of the fibrillar collagen binding pocket have been shown to participate in binding to collagen IV (Xu et al. 2011a).
Figure 1.8: X-ray crystallographic structure of the DDR2 DS domain-collagen mimetic triple-helical peptide interaction. (A) Binding of the collagen mimetic triple-helical peptide, containing the leading (yellow) middle (orange) and trailing (red) chain, to the DDR2 DS domain (cyan). X represents norleucine. The DDR2 DS domain consists of β loops (β1-β8). Disulphide bonds present in the DDR2 DS domain are in green. (B) Another view of the DDR2-mimetic collagen interaction. Selected residues are labeled. DDR2 and collagen chains are represented in the same colours as in (A). Hydrogen bonds are represented with dashed lines. Image adapted from (Carafoli et al. 2009) with permission.

The DS-like domain consists of approximately 180 amino acids and presents a 51% identity at the amino acid sequence, between DDR1 and DDR2 (Carafoli et al. 2012; Leitinger 2014). It has similar structure to the DS domain. It also adopts a β-barrel conformation with two anti-parallel β-sheets consisting of eight β strands, but with five additional strands present between β1 and β2 strands. Furthermore, the DS-like domain contains a calcium-binding site and two glycosylated asparagines at residues 260 and 211, which are highly conserved in DDR1 and DDR2 (Carafoli et al. 2012). The DS-like domain and the EJM region in both DDRs contain N- and O-glycosylation sites (Fu et al. 2013a) (see Figure 1.6).

The DDR1 EJM region, which is unusually flexible (Xu et al. 2014), contains 50 residues, whereas the DDR2 EJM region contains 32 residues (Carafoli & Hohenester 2013). The transmembrane region consists of approximately 20 residues and is
critical for receptor dimerization (Noordeen et al. 2006). Noordeen et al (2006) demonstrated that the isolated DDR1 transmembrane region forms a very strong-self association, which was attributed to the presence of a leucine-based motif. Later on, Finger et al (2009) further verified the ability of the DDR transmembrane regions to form a very strong helix interaction motif, which was identified among the strongest of all such motifs tested in RTKs (Finger et al. 2009). The DDRs IJM regions are unusually long, consisting of 170, 143 and 142 residues in DDR1b, DDR1a and DDR2 respectively (see Section 1.8.4) (Carafoli & Hohenester 2013). The DDR KD consists of approximately 300 residues and, in contrast to most RTKs, does not have a C-terminal tail (Leitinger 2014). The KD is very similar among RTKs, as mentioned earlier (see Section 1.8.1). Detailed DDR1 KD structure, which consists of an N- and a C-lobe and also includes the activation loop, was obtained along with a type II inhibitor from two independent studies (Kim et al. 2013; Johnson et al. 1996). The KD includes several tyrosines which become phosphorylated upon ligand binding. Three tyrosines present in the DDR1 activation loop get phosphorylated, tyrosines 792, 796 and 797 (Kim et al. 2013). Apart from the tyrosines present in the activation loop, another 5 tyrosines are present in the DDR1b kinase domain and totally 7 in the IJM region of DDR1b (Lemeer et al. 2012). The DDR2 activation loop also contains 3 tyrosines, tyrosines 736, 740 and 741 (Yang et al. 2005). Another 7 tyrosines are present in the kinase domain and 4 in the IJM region (Valiathan et al. 2012).

1.8.5 DDR Genomic structure

The genes encoding for DDR1 and DDR2 are located in chromosome 6(6p21.3) (Perez et al. 1994; Edelhoff et al. 1995) and 1(1q23.3) respectively (Karn et al. 1993). The DDR1 gene consists of 17 exons whereas the DDR2 gene consists of 19 exons (Karn et al. 1993; Playford et al. 1996). The DDR1 proteins have five isoforms (DDR1a-e) which form due to alternative splicing (Playford et al. 1996) whereas DDR2 has only one single isoform (see Figure 1.6). DDR1a-c are functional proteins, whereas DDR1d and DDR1e are truncated, non-functional proteins (Alves et al. 2001; Playford et al. 1996). DDR1d lacks the entire kinase domain whereas parts of the IJM region are not present in DDR1e (half of exon 10 and entire exons 11 and 12 are missing), as well as
the ATP binding site (Alves et al. 2001) (see Figure 1.6). DDR1b has 37 extra residues present in the IJM region compared to DDR1a (Alves et al. 1995). DDR1a and DDR1b are the most common isoforms present in tissues (Borza & Pozzi 2014; Leitinger 2014).

1.8.6 DDR ligands and binding motifs

Collagens serve as functional ligands for both DDRs. Collagens of the fibrillar family bind and activate both DDRs, but collagen II induces stronger DDR2 autophosphorylation signal than DDR1 (Vogel et al. 1997; Shrivastava et al. 1997; Leitinger et al. 2004). Prerequisite for DDR activation is for collagens to be present in the triple-helical conformation, as the DDRs do not bind to heat-denatured collagens (e.g. gelatin) (Vogel et al. 1997; Shrivastava et al. 1997; Leitinger 2003). Agarwal et al. (2002) first attempted to identify the collagen I binding motif for DDR2 using atomic force microscopy (Agarwal et al. 2002). Leitinger (2003) using recombinant DDR constructs found that the collagen binding site is accommodated in the DDR DS domain (Leitinger 2003). Furthermore, Leitinger (2003) and Abdulhussein et al. (2004) identified three adjacent loops on the surface of the DS domain as the collagen I binding site (Leitinger 2003; Abdulhussein et al. 2004). In order to define DDR binding motifs on collagens, Leitinger et al. (2004) used solid phase binding assays and recombinant collagen II variants. They found that the DDR2 binding site was located on the second quarter of collagen II triple-helical region (Leitinger et al. 2004). Later on, studies using the so-called “Toolkit peptides”, identified one high-affinity binding motif for both DDR1 and DDR2, present on collagens II and III, the GVMGFO motif, where O is hydroxyproline (Konitsiotis et al. 2008; Xu et al. 2011a). Furthermore, two more DDR2 binding sites on collagen II and three on collagen III were identified with the same method, however their specific amino acid sequence was not determined (Konitsiotis et al. 2008; Xu et al. 2011a). Carafoli et al. (2009) further constructed a crystal structure of DDR2 DS domain bound to a synthetic triple-helical peptide encompassing the GVMGFO motif, showing that the triple-helical peptide binds to an amphiphilic pocket on the surface of the DS domain.
Those DDR2 residues interacting with the peptide were found to be strictly conserved between DDR1 and DDR2. Furthermore, a crystallographic structure of the DDR1 ectodomain bound to the Fab fragment of an anti-DDR1 antibody showed very similar conformation of the DDR1 DS domain (Carafoli et al. 2009). Moreover, this structure revealed that a second region present in the DS domain, a highly conserved patch, far from the collagen binding pocket could serve as a secondary, low affinity collagen binding site (Carafoli et al. 2012). The DDRs can also bind to non-fibrillar collagens, more specifically DDR1 binds to the network forming collagen IV found in all basement membranes (Vogel et al. 1997; Shrivastava et al. 1997) and to collagen VIII (Hou et al. 2001) whereas DDR2 binds to collagen X (Leitinger & Kwan 2006). Since the DDRs also have non-fibrillar ligands, it is conceivable that DDRs bind to non-fibrillar collagens using another motif rather than the GVMGFO motif, since the GVMGFO motif is not present in non-fibrillar collagens such as collagen IV (Xu et al. 2011a).

1.8.7 Mechanism of DDR activation

As mentioned earlier, typical RTKs are activated by small-diffusible proteins such as growth factors and activation takes place within a few second after ligand binding (Lemmon & Schlessinger 2010). However, this is not the case for the DDRs. The only functional DDR ligands identified so far are collagens. Moreover, DDR activation is slow and sustained and, depending on the cell type, may be present even after 18 hours upon ligand binding (Vogel et al. 1997; Shrivastava et al. 1997). Unlike other RTKs, which are thought to exist as monomers on the surface of cells, the DDRs form stable non-covalently linked dimers, which are likely to form during biosynthesis (Noordeen et al. 2006; Mihai et al. 2009; Xu et al. 2014). While we understand at atomic level detail how collagen binds to the DDRs, the details of how collagen binding results in activation of the DDR kinase domain are still unknown. However, the fact that the DDRs are present on the cell-surface as ligand-independent dimers suggests that ligand-induced dimerization, like in the case of typical RTKs, (Lemmon & Schlessinger 2010) does not apply as an activation mechanism or the DDRs.
Furthermore, the lack of conformational changes detected in the crystal structure of the unbound DDR1 DS domain and the DDR2 DS domain bound to a triple-helical peptide encompassing the GVMGFO motif suggests that no significant conformational changes occur upon ligand binding (Carafoli et al. 2009; Carafoli et al. 2012; Ichikawa et al. 2007). A lack of conformational changes within a DDR dimer was further suggested by Xu et al. (2014), who showed that cysteine mutations in the EJM region and insertions or deletions in the EJM region did not hinder collagen-induced DDR1 autophosphorylation (Xu et al. 2014). However, Noordeen et al. (2006) using a TOXCAT reporter assay demonstrated that a leucine-zipper motif present in the transmembrane region was vital for DDR1 activation, as mutations in it did not allow DDR1 to autophosphorylate (Noordeen et al. 2006). A plausible theory is that collagen binding induces DDR clustering (oligomerization, rather than dimerization) on the cell-surface (see Section 1.8.8). In terms of ligand requirements, the question of whether collagens can serve as DDR ligands when present in fibrillar form has not been addressed in depth. Early studies with soluble collagen showed that this form of collagen could activate the DDRs (Shrivastava et al. 1997; Vogel et al. 1997; Leitinger 2003). However, due to the fact that when incubated with cells, collagens may assemble into higher-ordered aggregates during the incubation phase at 37°C, it was not certain whether collagens function as DDR ligands in the form of single triple helices or in fibrillar form. Later studies have showed that collagen in the form of single triple-helices is sufficient to induce DDR activation, as using triple-helical peptides encompassing the GVMGFO motif showed that these peptides, which cannot assemble into fibrillar structures, could induce DDR activation with the same kinetics as collagen I (Konitsiotis et al. 2008; Xu et al. 2011a). Finally, a study by L’Hote et al. (2002) suggested that when cells were in suspension, DDR1 was activated quicker in comparison to when it was present on adherent cells. The authors furthermore hypothesized that the slow DDR1 phosphorylation kinetics could be attributed to binding with an inhibitory protein, perhaps a phosphatase, keeping DDR1 in an inactive state (L’hote et al. 2002). However, no further publication supported these findings.
1.8.8 Endocytosis and shedding as mechanisms of regulation of DDR signaling

After ligand-induced activation, many RTKs are subjected to endocytosis along with their ligand (Karpov et al. 2015). The receptor-ligand complex is transferred to endosomes and then the receptor can be either degraded or trafficked back to the cell membrane (Karpov et al. 2015). There are two studies supporting DDR endocytosis. Mihai et al (2009) used DDR1b tagged with yellow fluorescent protein suggesting that upon ligand binding DDR1 is aggregated, endocytosed, transferred to endosomes and then recycled back to the cell membrane. They also suggested that DDR1 is activated while transferred back to the cell membrane, inside cytoplasmic vesicles (Mihai et al. 2009). The same group published a second study on DDR1 endocytosis. Again, using full-length DDR1 tagged with yellow fluorescent protein, they imaged DDR1 endocytosis by confocal microscopy using live-cell imaging (Yeung et al. 2013). They demonstrated DDR1 oligomerization using both full-length DDR1 protein and DDR1 consisting only of the DDR1 ectodomain. The authors also showed that when cells were incubated with an inhibitor of clathrin-mediated endocytosis, dynasore, DDR1 endocytosis was inhibited (Yeung et al. 2013). The question of the stoichiometry of the DDR receptor upon activation remains unanswered, but it has been suggested that DDR1 forms, upon ligand collagen binding, tetramers, hexamers or octamers (Yeung et al. 2013). Finally, a recent publication by Fu et al (2014) again demonstrated that DDR1 receptor could be endocytosed and that endocytosis was regulated by a specific N-glycosylated residue located in the DS-like domain, asparagine 211 (Fu et al. 2014). Furthermore, they showed that asparagine 211 plays a key role in the regulation of DDR1 activation, as mutations in this residue resulted in constitutive DDR1 activation (Fu et al. 2014). It is well established that a tyrosine-based motif, the NPXY motif, is responsible for the clathrin-mediated endocytosis of many receptors (Bonifacino & Traub 2003). DDR1b and DDR1c contain the NPXY motif in their IJM region but, DDR1a and DDR2 do not contain this motif. This differentiation in the expression of the NPXY motif could mean that different DDR1
isoforms could be endocytosed using different mechanisms or could lack endocytosis as a mechanism of regulation of DDR signaling (e.g. DDR2) (Leitinger 2014).

Regulation of RTK signaling is also achieved via release of the receptor ectodomain, the so-called shedding, due to function of several proteolytic enzymes (Adrain & Freeman 2014). Early evidence suggests that MMPS can induce DDR1 shedding. Vogel et al. (2002) demonstrated that upon ligand binding, DDR1 ectodomain can be subjected to cleavage by zinc MMPs. Furthermore, they detected the C-terminal region of the DDR1 ectodomain in the conditioned medium of the cultured cells (Vogel 2002). Later on, Slack et al. (2006) detected the N-terminal region of the cleaved DDR1 protein (Slack et al. 2006). Fu et al (2013b) showed that only MT-MMPs could cleave the DDR1 ectodomain, not soluble metalloproteinases (Fu et al. 2013b). Furthermore, they identified two MMP cleavage sites, located in the DDR1 EJM region. More specifically, the first one (Pro407–Val408) was located 19 amino acids away from the transmembrane region, whereas the second one (Ser397–Leu398) was located 9 amino acids away (Fu et al. 2013b). Apart from ligand-induced DDR1 shedding, DDR1 can also get cleaved in a ligand-independent manner. Shitomi et al. (2015) using two DDR1 constructs that cannot autophosphorylate, a kinase-dead and a construct with a deletion in the IJM region, identified that DDR1 shedding, which was induced by collagen binding to the receptor, can occur independently of DDR1 activation by a disintegrin and metalloproteinase 10 (ADAM 10). DDR1 shedding, which resulted in a soluble C-terminal DDR1 fragment with 60kDa molecular weight, was also verified when cells were pre-incubated with dasatinib, a tyrosine kinase inhibitor (Shitomi et al. 2015). Thus, DDR1 shedding may result in termination of receptor autophosphorylation and signaling, decrease the number of available DDR1 molecules on the cell-surface or behave as “decoy receptor”. In the latter model, collagens could bind to the soluble DDR1, not the membrane-anchored one, and thus regulate the matrix scaffold (Leitinger 2014; Fu 2013b). To date, proteolytic enzymes have not been shown to induce DDR2 shedding (Fu 2013a).
1.8.9 DDR-induced signaling

DDR activation is manifested by autophosphorylation of tyrosines present in the IJM region and the tyrosine kinase domain, as mentioned earlier. Phosphorylated tyrosines can then serve as docking sites for different molecular adaptors and signaling proteins, triggering different signaling pathways and thus different cellular responses. DDR1 has been shown to promote cell migration, adhesion and EMT whereas DDR2 can promote EMT and matrix remodeling (Multhaupt et al. 2016; Leitinger 2014). There are 13 possible phosphorylation sites in DDR1a, 15 in DDR1b and DDR1c and 14 in DDR2. Only a few direct DDR signaling partners have been identified. Phosphorylation of tyrosine 513 in DDR1b protein has been shown to recruit the molecular adaptors non-catalytic region of tyrosine kinase adaptor protein 2 (Nck2) (Koo et al. 2006) or ShcA (Vogel et al. 1997). Candidate downstream DDR1 signaling molecules include SHP-1 (Abbonante et al. 2013) and SHP-2 phosphatases (Koo et al. 2006; Wang et al. 2006), PI3 kinase and signal transducers (Dejmek et al. 2003; Suh & Han 2011; L’hote et al. 2002), activators of transcription (Stat) members (Faraci-Orf et al. 2006; Wang et al. 2006) Src kinases and containing inositol polyphosphate 5-phosphatase 1/2 (Lemeer et al. 2012). Finally, studies by two independent groups have demonstrated that DDR1 activation is dependent on Src kinase phosphorylation (Dejmek et al. 2003; Lu et al. 2011). As mentioned earlier, DDR1 participates in several signaling pathways, in a cell-and a ligand-dependent manner. In adipose stromal cells and pancreatic cancer cells, DDR1 activates the JNK pathway (Shintani et al. 2008; Ghosh et al. 2013). Also, it can both promote and inhibit ERK1/2 activation in megakaryocytes and in mesangial cells respectively (Curat 2002; Abbonante et al. 2013). In pancreatic cancer cells, DDR1 incubation with collagen I promotes Pyk2 phosphorylation (Shintani et al. 2008) whereas DDR1 incubation with collagen XV suppresses it (Clementz et al. 2013). Furthermore, DDR1 has been shown to co-operate or antagonize with β1 integrins. DDR1 and β1 integrins do not recruit the same signaling molecules, but following an independent signaling pathway, they can end up promoting or suppressing each other’s function. In MDCK cells, DDR1 has been shown to inhibit cell migration and cell spreading.
promoted by α2β1 integrins (Wang et al. 2006; Yeh et al. 2009). On the other hand, in pancreatic cancer cells, DDR1 and β1 integrins have been shown to act synergistically promoting cell scattering (Shintani et al. 2008). They have also been shown to co-operate in order to promote cell adhesion in many cell lines such as MDCK cells and smooth muscle cells (Wang et al. 2006; Hou et al. 2001). DDR1 is capable of enhancing the function of β1 integrins either by increasing the cell-surface available receptors or by influencing their affinity (Xu et al. 2012; Staudinger et al. 2013).

There is limited information regarding the signaling molecules activated by DDR2 autophosphorylation. ShcA has been shown to act as a DDR2 adaptor molecule, (Ikeda et al. 2002) whereas SHP-2, phospholipase C-like 2, Nck1 and phosphatidylinositol-4-phosphate 3-kinase have been identified as DDR2-activated signaling molecules (Iwai et al. 2013). Similar to DDR1, it has been demonstrated that DDR2 phosphorylation is highly associated with Src kinase activity (Ikeda et al. 2002; Yang et al. 2005). DDR2 signaling is also cell-type dependent: it promotes ERK2 activation in breast cancer cells (Zhang et al. 2013) and in hepatocellular carcinoma cells (Xie et al. 2015), in chondrocytes it promotes ERK1/2 and p38 activation (Xu et al. 2005; Xu et al. 2007) whereas in transfected embryonic kidney cells it promotes p38 and JNK pathway (Poudel et al. 2013). Finally DDR2 has been shown to participate in osteoblastic differentiation, however it is not clear yet whether it promotes Runx2 via the p38 or the ERK1/2 (Zhang et al. 2011; Lin et al. 2010).

1.8.10 DDR-related diseases

Fibrosis
Data provided by several studies suggest that the DDRs participate in lung fibrosis progression. A study using DDR1 knockout mice has shown that DDR1 regulates not only fibrosis but also inflammation in bleomycin-induced lung injury, a mouse model for human idiopathic pulmonary fibrosis (Avivi-Green et al. 2006). DDR1 knockdown resulted in decreased collagen deposition, lower mRNA levels of tenascin-C and
attenuation of fibrosis (Avivi-Green et al. 2006). Yang et al. (2013) also identified that DDR2 participates in lung fibrosis using mice carrying a targeted deletion of collagen I gene in lung epithelial cells. More specifically, they identified that fibrosis was attenuated in mice with deletion of collagen I gene in lung epithelial cells compared to their normal littermates. Furthermore, they suggested that lung epithelial cells synthesize collagen I, which results in activation of DDR2 receptor located on fibroblasts, promoting inflammation and sustained fibrosis (Yang et al. 2013). In addition to lung fibrosis DDR2 is also thought to participate in liver fibrosis. Depending on the stage of the disease, DDR2 can have both a pathogenic and a protective role. In alcoholic liver fibrosis, DDR2 knockdown attenuated the progression of liver fibrosis, whereas DDR2 up-regulation resulted in collagen accumulation, MMP-2 secretion and thus matrix remodeling (Zhang et al. 2010; Luo et al. 2013). Zhang et al. (2010) also identified increased levels of DDR2 mRNA in alcoholic liver fibrosis rats using real time Polymerase Chain Reaction (PCR) (Zhang et al. 2010). However, DDR2 knockdown in chronic human liver disease, resulted in increased levels of inflammation and fibrosis, due to accumulation of hepatic stellate cells, up-regulation of MMP-2 and MMP-13 and increased collagen I levels. Furthermore, DDR2 knockdown resulted in dysregulation of the interactions between hepatic stellate cells and macrophages (Olaso et al. 2011). Finally, a recent study by George et al (2016) identified that angiotensin-II stimulated rat cardiac fibroblasts, express higher DDR2 levels, both in mRNA and protein levels in a Nuclear Factor-κB dependent way (George et al. 2016). Additionally, they showed that in angiotensin-II treated cells, DDR2 promotes collagen II expression, suggesting that DDR2 is implicated in cardiac fibrogenesis (George et al. 2016). It is evident that DDRs, via regulation of collagen deposition, participate in the progression of organ fibrosis, particularly lung fibrosis in the case of DDR1 and liver fibrosis in the case of DDR2.

**Osteoarthritis and Rheumatoid Arthritis**

DDR2 is expressed on the surface of chondrocytes, the only cell type present in articular cartilage. Xu et al. (2005, 2007, 2010) showed that DDR2 over-expression is linked to OA progression. Using mouse models and human tissue samples they found
that DDR2 up-regulation leads to MMP-13 overexpression. MMP-13 degrades the collagen II network leading in further DDR2 up-regulation (Xu et al. 2005; Xu et al. 2007; Xu et al. 2010). Preliminary data by the same group also suggest that pericellular matrix acts as a physical limit, inhibiting DDR2 from coming in contact with collagen II fibrils, which are located in the territorial and interterritorial matrix (Xu et al. 2010; Xu et al. 2011b). The DDR2-MMP-13-OA link has been verified by another two independent groups: Vonk et al. (2011) using chondrons, which are chondrocytes surrounded by intact pericellular matrix, and chondrocytes with no pericellular matrix, showed that after incubation with collagen I or collagen II monomers, DDR2 was activated only in chondrocytes with no pericellular matrix present but not in chondrons (Vonk et al. 2011). Hou et al. (2012) using another OA mouse model, also found DDR2 and MMP-13 overexpression. Thus, DDR2 could be used as a therapeutic target for OA treatment (Richters et al. 2014; Murray et al. 2015; Salazar et al. 2014). DDR2 has also been linked to rheumatoid arthritis (Hou et al. 2012; Majkowska et al. 2017). A very recent study by Majkowska et al (2017) using human rheumatoid synovial or dermal fibroblasts demonstrated that DDR2 induces activation of MT1-MMP, which then promotes proMMP-2 activation. They also showed that MT1-MMP up-regulation, which has been shown to play a key role in rheumatoid arthritis progression (Sabeh et al. 2010; Miller et al. 2009; Kaneko et al. 2016), was dependent on collagen-induced DDR2 signaling (Majkowska et al. 2017). Another study by Su et al. (2009) showed that DDR2, which is highly expressed in synovial fibroblasts, promotes MMP-13 overexpression (Su et al. 2009). In conclusion, it is suggested that DDR2, apart from OA progression, is also associated with rheumatoid arthritis progression.

**Atherosclerosis**

DDR1 is thought to participate in atherosclerosis progression. Hou et al. (2001) demonstrated that knockdown of DDR1 in smooth muscle cells isolated from mouse aortas resulted in decreased collagen deposition and matrix remodeling and further showed that DDR1 has a pathogenic role in intimal thickening (Hou et al. 2001). Another study conducted by Ferri et al. (2004) also showed that DDR1 in smooth muscles participated in MMP secretion in vitro suggesting a possible implication in
atherosclerosis. This was not the case for DDR2 though, even though DDR2 was present in smooth muscle cells (Ferri et al. 2004). This was also verified by Hou et al. (2012), who showed that DDR2 knockdown in smooth muscle cells did not alter their ability to migrate or proliferate (Hou et al. 2012). Hou et al. (2002) using the same mouse model, further demonstrated that DDR1 regulates smooth muscle cell migration in atherosclerosis (Hou et al. 2002). The hypothesis that DDR1 has a pathogenic role in atherosclerosis was further demonstrated by Franco et al. (2008, 2009) who showed that there was increase in fibrillar collagen and collagen IV deposition and in MMP secretion resulting in containment of the atherosclerotic lesion in DDR1 knockdown mice (Franco et al. 2008; Franco et al. 2009).

Cancer
The DDRs have been shown to play a pathogenic role in the progression of numerous cancer types. To this end, there is increasing interest in targeting DDRs as a potential therapeutic target in relation to cancer (Kim et al. 2013; Valiathan et al. 2012; Rammal et al. 2016; Kothiwale et al. 2015). DDR1 has been associated with prostate, breast, colon, pancreatic, liver, brain and lung cancer. DDR2 has been linked to breast, melanoma, lung, prostate and thyroid cancer (Valiathan et al. 2012; Rammal et al. 2016). Studies using several cancer cell lines have identified that both DDRs participate in tumor progression both as positive and negative regulators of various cellular functions. For example, the DDRs can promote EMT, cell proliferation, migration, survival and invasion (Leitinger 2014). DDR1 can also negatively affect cell proliferation, EMT and migration whereas DDR2 has been reported to affect negatively only cell migration and proliferation (Rammal et al. 2016; Multhaupt et al. 2016). DDR1 has been closely related to lung cancer progression. Many of those studies were conducted using non-small cell lung carcinoma cell-lines. DDR1, which was one of the most highly phosphorylated kinases in lung cancer (Rikova et al. 2007), was shown to promote EMT, migration and invasion (Walsh et al. 2011; Miao et al. 2013; Yang et al. 2010). DDR1 participates in pro-survival pathway signaling, activation of the Ras/Raf/ERK, PI3K/Akt or NFkB pathways when incubated under genotoxic conditions (Ongusaha et al. 2003; Das et al. 2006). It is evident that DDR2 also plays an important role in both lung cancer progression and metastasis.
A study by Hammerman et al. (2011) has shown that 4% of lung cancer samples acquired DDR2 mutations, located across the whole DDR2 structure. Application of dasatinib, a non-selective kinase inhibitor, resulted in decreased proliferation of squamous lung cancer cells expressing the mutated DDR2 receptor. The same effect resulted from knockdown of the mutated DDR2 gene in those cells (Hammerman et al. 2011). DDR2 is also related to invasive breast cancer and high levels of metastasis (Ren et al. 2013; Zhang et al. 2013). Zhang et al. (2013) identified that in breast cancer, collagen I-induced DDR2 autophosphorylation resulted in activation of the ERK2 signaling pathway, in vitro migration and invasion of cancer cells via stabilization of SNAIL1 (Zhang et al. 2013). A recent study revealed the key role of DDR1 in metastatic reactivation of breast cancer (Gao et al. 2016). More specifically, Gao et al. (2016), using genetic screening showed that a tetraspanin called TM4SF1 is strongly activated in metastatic breast cancer. Soluble collagen I-induced activation of TM4SF1 then activates DDR1, which interacts with PKCα, and then promotes activation of signaling molecules JAK2 and STAT3, resulting in re-activation of lung, brain and bone metastatic cancer cells. The authors furthermore showed that DDR1 participates in breast cancer cell metastasis in a kinase-independent way. Their results were obtained both from in vivo and in vitro experiments (Gao et al. 2016). Another recent study, using human and mouse samples, supports the hypothesis that DDR2 plays a key role in metastasis of breast cancer cells (Gonzalez et al. 2017). More specifically, it mediates an interaction between breast cancer cells and breast cancer-associated mesenchymal stromal cells, promoting increased deposition of collagen I and subsequently DDR2 overexpression. This results in increased migration and invasion of breast cancer cells (Gonzalez et al. 2017). Based on the above, it is evident that DDRs, via their interactions with collagens, can regulate several cellular processes and play key roles in cancer progression.
Project hypothesis and aims

So far, DDR activation has mainly been promoted using collagen in the form of single triple helices. However, collagens are present in tissues in the form of fibrils or fibers (Myllyharju & Kivirikko 2004), and it is this form of collagen that would be the native ligand for the DDRs. Several studies have used polymerized collagens as DDR ligands. Two independent studies by Wall et al. (2005) and Bhadriraju et al. (2009) have shown DDR2 activation using collagen in the form of a fibrillar gel and thin collagen film respectively (Wall et al. 2005; Bhadriraju et al. 2009). Very recent evidence published by Coehlo et al. (2017) suggests that DDR1 is activated by collagen I fibrils, using again fibrillar collagen gels (Coelho et al. 2017). Another study by et al Assent et al (2015) using breast cancer cells MCF-7 showed that endogenous DDR1 was phosphorylated after 24h incubation with 3D collagen gel (Assent et al. 2015). A study by Juin et al. (2014) also indicates that DDR1 autophosphorylation can be promoted by collagen I fibrils. In this case, DDR1 activation was again induced using polymerized collagen gels (Juin et al. 2014). However, it is not clear whether these collagen gels include any soluble collagen I, which could account for the observed DDR activation. As mentioned earlier, collagen does not need to be in the fibrillar conformation to induce DDR activation, as experiments conducted with triple-helical collagen mimetic peptides promoted DDR activation as efficiently as full-length collagens (Konitsiotis et al. 2008; Xu et al. 2011a). Therefore, the question whether fibrils can induce DDR activation still remains unanswered.

As mentioned above, both DDRs interact with a number of different collagen types. However, collagen VI, a widely distributed collagen type, has not been tested for its interactions with the DDRs. As mentioned in the Section 1.6.1, collagen VI is present in the ECM in the form of microfibrils. There are several tissues where DDRs and collagen VI co-exist (Cescon et al. 2015), and we therefore hypothesized that collagen VI is a functional ligand for the DDRs.
Based on the above, first sub-hypothesis of this PhD was formatted: “**DDRs become phosphorylated by binding to collagen I fibrils, in the absence of soluble collagen I**”. To test this hypothesis, I used collagen I fibrils from three different collagen sources: rat-tail collagen I, applying a protocol to induce *in vitro* fibrillogenesis; ECM, secreted from NIH-3T3 mouse fibroblastic cells and native equine fibrils. As mentioned earlier, based on the fact that collagen VI and DDRs co-exist in several tissues (Cescon et al. 2015), the second sub-hypothesis of this PhD was formatted: “**collagen VI tetramers, the form by which collagen VI is secreted from cells, and collagen VI microfibrils, the final collagen VI assembly in tissues, can bind to the DDRs and induce DDR autophosphorylation**”. To this end, we tested the interactions of chick cornea collagen VI tetramers and microfibrils to DDRs. Furthermore, we tested whether collagen VI microfibrils bind to the DDRs using the same DDR binding site as fibrillar collagens.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Mammalian cells and cell culture reagents

CV-1 origin SV40 (Cos-7) cells were purchased from American type culture collection (Teddington, UK). Human embryonic kidney 293 (HEK-293) cells were purchased from the American tissue culture collection, Manassas, VA, Canada, and HEK293-EBNA cells were purchased from Invitrogen. T47D human breast cancer cells were obtained from the Imperial Cancer Research Fund Cell Production laboratory, London. Trypsin/Ethylenediaminetetraacetic acid (EDTA), Roswell Park Memorial Institute Medium (RPMI), Eagle’s Minimum Essential Medium (OptiMEM), 100x penicillin/streptomycin (P/S), glutamine, Foetal Bovine Serum (FBS), Neonatal Calf Serum (NCS) and Dulbecco’s modified Eagle medium/F12 (DMEM/F12) were purchased from Gibco, Life technologies. 6-, 12- and 24-well plates, 25cm² tissue culture (T-25) flasks and 75cm² tissue culture (T-75) flasks were purchased from Triple Red Ltd. Hyperflask Cell Culture Vessels were purchased from Corning®. FuGENE 6 was purchased from Promega, USA. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from AppliChem, PanreaC, TW Companies.

2.1.2 Chemicals

Gelatin, glutaraldehyde, ethanolamine, ammonium hydroxide (NH₄OH), aprotonin, ampicillin, orthovanadate (VO₃), Triton X-100, paraformaldehyde (PFA), casein, urea, Phenylmethylsulfonyl Fluoride (PMSF), ascorbic acid, o-phenylenediamine dihydrochloride (OPD), calcium chloride (CaCl₂) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Gillingham, UK). Acetic acid and methanol were purchased from Fisher Chemicals.
Tetraethylmethylenediamine (TEMED) was purchased from Fluka Biochemika (Buchs, Switzerland). Ammonium persulphate (APS), sodium dodecyl sulphate (SDS), Ponceau-S, β-mercaptoethanol, Tween-20 were purchased from Sigma-Aldrich (Gillingham, UK). Pre-stained protein marker (broad range 11-190kDa) was purchased from New England Biolabs Inc. (Hitchin, UK).

### 2.1.3 ECM proteins

Acid-soluble rat-tail collagen I (C-7661) was either purchased from Sigma (denoted as Sigma collagen in this thesis) or kindly provided by Dr. Uwe Hansen, University of Muenster (denoted as Hansen collagen in this thesis). Briefly, Dr. Uwe Hansen extracted rat tail tendons using 0.1% acetic acid solution and centrifuged them at 17,000 rpm for 1 h at 4°C. The pellet produced was subjected to further extraction and the two supernatant solutions produced were afterwards centrifuged for 15 min at 5,000 rpm. Then, the two supernatant solutions were combined and dialyzed against storage buffer. The solution produced was centrifuged at 5,000 rpm for 15 min and the protein concentration was determined using a Bicinchoninic Acid protein assay. Collagen I fibrils from equine (HORM) were kindly provided by Prof. Richard Farndale, Cambridge University. Collagen II was purchased from Chondrex Inc. Collagen IV, acid-soluble from human placenta (C-5533) was purchased from Sigma-Aldrich (Gillingham, UK). Collagen VI in the form of tetramers (10-100μg/ml) and microfibrils (10-800μg/ml), triple-helical or pepsinized, was purified from bovine cornea (Hansen et al. 2012) and kindly provided by Dr. Uwe Hansen. Mouse laminin (#354232) was purchased from Corning.
### 2.1.4 List of primary and secondary antibodies used

**Table 2.1:** List of primary antibodies used. IF; Immunofluorescence, WB; Western Blotting

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<th>Applied in</th>
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<td>produced in our lab(Carafoli et al. 2009)</td>
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Table 2.2: List of secondary antibodies used. SP; solid phase binding assay, IF; Immunofluorescence, WB; Western Blotting

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<tr>
<td>HRP anti-goat IgG</td>
<td>Rabbit</td>
<td>1:500</td>
<td>ZYMED, Laboratories Inc. San Francisco, CA, USA</td>
<td>WB</td>
</tr>
<tr>
<td>HRP anti-rabbit IgG</td>
<td>Goat</td>
<td>1:500</td>
<td>Dako, Ely, UK</td>
<td>WB</td>
</tr>
<tr>
<td>HRP anti-mouse IgG</td>
<td>Sheep</td>
<td>1:500</td>
<td>GE Healthcare</td>
<td>WB</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Bacterial transformation

For the transformation of DH5α competent Escherichia coli (Invitrogen, Life technologies) cells, DNA containing the DDR1 or DDR2 vector was diluted to 10ng/μl using distilled water. Bacteria were thawed on ice and then 1μl of DNA (10ng/μl) was added to 50μl of bacteria. Bacteria were incubated for 30min on ice, heat-shocked for 2min at 42°C and then incubated for another minute on ice. Finally, the transformed bacteria were plated onto Lb agar plates supplemented with 100μg/ml ampicillin and incubated overnight at 37°C. Colonies resistant to ampicillin were grown overnight at 37°C (Innova 4000, New Brunswick incubator). The next day, a colony was selected and transferred into a universal tube with 5ml of Lysogeny Broth supplemented with ampicillin (100μg/ml). Universals were incubated in a shaker (R100/TW, Luckam) at 37°C at 200rpm for a few hours until the culture was cloudy. These cultures were then transferred into autoclaved conical flasks in a total volume of 100ml Lysogeny Broth supplemented with ampicillin (100μg/ml) and left to grow overnight at 37°C, with shaking at 200rpm.

2.2.2 Isolation and determination of DNA concentration

The bacterial cultures were used for plasmid isolation. Bacteria were subjected to alkaline lysis in order to release the plasmid DNA, which was isolated according to the manufacturer’s instructions using a QIAspin Plasmid Maxiprep kit (Qiagen, Crawley, UK). Purified DNA was re-suspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0). A NanoDrop ND-1000 UV spectrophotometer (Thermo Scientific) at 260nm wavelength and a NanoDrop ND-1000 version 3.2.1 software were used to estimate the concentration of the DNA isolated.
2.2.3 Restriction digests

DNA samples were incubated with relevant restriction enzymes (Promega, UK or New England Biolabs) according to the various plasmid DNAs and expected band pattern. This mixture included 0.1μg/ml Bovine Serum Albumin (BSA) (Fisher Scientific), 10U restriction enzyme, the respective reaction buffer (Promega, UK or New England Biolabs) and approximately 500ng of DNA. The mixture was incubated for approximately 1h at 37°C and the resulting DNA bands were visualised using agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis

Samples containing the DNA incubated with restriction enzymes were mixed with 1x loading dye (20% ficoll, 0.25% orange G, 60mM EDTA, pH 8.0) and loaded in a 0.8% (w/v) agarose gel next to a 1kb DNA ladder, range 0.5-10kb (New England Biolabs Inc.). 30ml of gel were prepared using 1x Tris-acetate EDTA (TAE, 40mM Tris, 1mM EDTA, 20mM acetic acid, pH 8.0) buffer supplemented with 3μl of GelRed™ (Biotinum). Samples were run in an agarose gel running system Model 200/2.0 (BioRad) and visualized at 365nm with a Gel Doc XR+ System (BioRad) using a DNA gel documentation system and PDQuest version 7.4.0 software (BioRad).

2.2.5 Purification of DNA from agarose gels

Agarose gels that contained the DNA band of interest were visualized using a low intensity hand held UV light, and the band was cut from the gel using a sterile razor blade. The isolated gel piece containing the DNA of interest was subjected to purification using a QIA quick gel extraction kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions.
2.2.6 DNA ligations

Next, the isolated DNA was ligated into a specific vector in a molar ratio 3:1 (insert:vector). More specifically, DNA ligations were performed using the following components: 1x ligase buffer (Life Technologies), 30ng of vector, 0.8μl ligase T4 (Life Technologies), and the appropriate ng of insert (after calculation). The mixtures were incubated at room temperature for one hour. Ligated DNA was then used for bacterial transformation (see Section 2.2.1) and isolation of produced DNA (see Section 2.2.2). DNA produced was then digested with the appropriate enzymes to test for the expected DNA band pattern (see Section 2.2.3 and 2.2.4). After verification, the ligated expression vector DNA was used for cell transfection (see Section 2.2.8).

2.2.7 Mammalian cell culture

HEK-293 and Cos-7 cells were cultured in T-25 flasks using DMEM/F12 supplemented with 10% FBS. NIH-3T3 cells were cultured in T-25 or T-75 flasks using DMEM/F12 supplemented with 10% NCS. T47D cells were cultured in T-25 flasks using RMPI supplemented with 10% FBS. DMEM/F12 medium containing 10% FBS or 10% NCS was supplemented with 2mM glutamine and 100U/ml penicillin-100μg/ml streptomycin (complete medium). Cells were cultured in a 5% CO₂ incubator at 37°C (Galaxy 170 S, New Brunswick). When confluent, cells were rinsed with 1x Phosphate Buffered Saline (1x (PBS), 137mM NaCl, 18mM KH₂PO₄, 2.7mM KCl, 80mM Na₂HPO₄, pH 7.4) and detached by incubation with 0.5ml trypsin/EDTA for 1-3min at 37°C. After trypsinization, 4.5ml complete medium was added to de-activate trypsin’s function. Cells were either split into a new T-25 flask, for maintenance, or seeded onto cell-culture plates according to their confluency and requirement of the experiments.
2.2.8 Cell transfection

2.2.8.1 Transfection using Calcium-Phosphate precipitation

HEK-293 cells, which do not express any endogenous DDR proteins, were trypsinized from T25 flasks (see Section 2.2.7) and detached cells were re-suspended in a universal tube with complete medium at ratio 1ml cells:2.5ml total medium, seeded into 6-well plates (1ml/well) where also 1ml/well complete medium was added. When seeded on 24-well plates, HEK-293 cells were re-suspended with complete medium at ratio 1ml cells: 4ml total medium and seeded 0.5ml/well. Cells were approximately 60% confluent prior to transfection. Medium was replaced with fresh complete medium 1h prior to transfection. For transfection of cells in 6-wells, the following solution was prepared (per well): 5μg DNA was diluted in a total volume of 175μl dH₂O, then mixed with 25μl of 2M CaCl₂. In a separate tube, 200μl 2xHepes-buffered Saline (2xHBS, 290mM NaCl, 50mM HEPES buffer, 1.5mM Na₂HPO₄, pH 7.1)/well was prepared. The solution containing CaCl₂ and DNA was added drop-wise into the 2xHBS buffer while vortexing (Vortex-2 Genie, Scientific Industries). The solution used for the transfection of 24-well plates consisted of the following (per well): 1.25μg DNA in 43.7μl dH₂O, mixed with 6.3μl 2M CaCl₂. In this case, 50μl of 2xHBS, per well, was prepared in a separate tube. Again, the DNA/CaCl₂ solution was added to the 2xHBS solution while vortexing. The transfection mixture was incubated for 30min at room temperature prior to cell transfection. Cells on 6-well plates were transfected with 200μl/well whereas cells on 24-well plates were transfected with 50μl/well of transfection mixture. After 16h of incubation at 37°C, the transfection solution was replaced with serum-free medium.

2.2.8.2 Transfection using FuGENE6 reagent

Cos-7 cells were trypsinized from T25 flasks (see Section 2.2.7) and detached cells were re-suspended in a universal tube with complete medium at ratio 1ml cells:4.5ml total medium, seeded on 6-well plates (1ml/well) where also 1ml/well complete medium was added. When seeded on 24-well plates, Cos-7 cells were re-
suspended with complete medium at ratio 1ml cells:8ml total volume and seeded 0.5ml/well. Cells were approximately 50% confluent prior to transfection. Medium was replaced with fresh complete medium just before transfection. The transfection solution used for cells seeded on 6-well plates consisted of the following: 200μl/well 1xOptiMEM, 1.2μg/well DNA and 3μl/well FuGENE. The transfection solution used for cells seeded on 24-well plates consisted of the following: 50μl/well 1xOptiMEM, 0.3μg/well DNA and 0.75μl/well FuGENE. The transfection solution was incubated for 15min at room temperature and then added to the cells drop-wise on 6-well or 24-well plates, 200μl and 50μl per well, respectively. Medium was replaced 4h after transfection with fresh complete medium, and cells were incubated at 37°C in a 5% CO₂ incubator. FuGENE and 1xOptiMEM were equilibrated at room temperature and 37°C respectively prior to transfection.

Table 2.3: List of plasmids used in transient or stable cell transfections.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Vector</th>
<th>cDNA encoded</th>
<th>Cloned by</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRKDDR1a</td>
<td>pRK5</td>
<td>Full length DDR1a</td>
<td>SUGEN</td>
</tr>
<tr>
<td>pRKDDR1b</td>
<td>pRK5</td>
<td>Full length DDR1b</td>
<td>SUGEN</td>
</tr>
<tr>
<td>pcDDR2</td>
<td>pcDNA3.1/Zeo</td>
<td>Full length DDR2</td>
<td>B.Leitinger(Leitinger 2003)</td>
</tr>
<tr>
<td>pRKDDR2</td>
<td>pRK5</td>
<td>Full length DDR2</td>
<td>D.Gavriilidou</td>
</tr>
<tr>
<td>pc-DDR2-FLAG</td>
<td>pcDNA3.1/Zeo</td>
<td>Full length DDR2 fused to FLAG tag in the C-terminal</td>
<td>N.Noordeen(Noordeen 1989)</td>
</tr>
</tbody>
</table>
2.2.9 Coverslip or well preparation prior to addition of cells

Prior to coating with ECM substrates, coverslips (VWR International LTD) were first microwaved for 5min at maximum power and then rinsed with 1xPBS. Coverslips or wells were coated with 700μl/well soluble collagen I (Sigma or Hansen) or laminin at 10μg/ml using coating buffer (50mM Tris, 100mM NaCl pH 8.8) and incubated overnight at 4°C.

2.2.10 Fibrillogenesis in vitro

2.2.10.1 Collagen I fibrillogenesis in vitro

Collagen I (both Sigma and Hansen) concentration was varied in the optimization phase. Sigma collagen and Hansen collagen were stored in 0.1M acetic acid at 1mg/ml and 1.2mg/ml respectively. Collagen I, which is insoluble in physiological pH, was diluted to various concentrations (10-250μg/ml) using a fibrillogenesis buffer (437mM NaCl, 18mM KH₂PO₄, 2.7mM KCl, 80mM Na₂HPO₄, pH 7.4) in Eppendorf tubes and incubated for 24h at 37°C, according to protocols from the literature (Harris et al. 2013; Birk et al. 1990). Tubes were then centrifuged for 30min at 4°C at 13,000rpm (AccuSpin™ MicroR, Fisher Scientific) and pellets containing the collagen I fibrils were re-suspended in 2x sample buffer supplemented with urea, diluted from a 10x sample buffer (5% w/v SDS, 25%w/v glycerol, 0.0025% w/v bromophenol blue, 8M urea, 50mM Tris, pH 8.8). Pellets were boiled for 5min at 100°C and stored at -20°C until future use. Supernatants containing the soluble collagen I were incubated at -80°C overnight in Eppendorf tubes with 3 volumes of ethanol (100%) relative to the final volume of collagen I, in order to produce pellets containing the soluble collagen I. Next day Eppendorf tubes were centrifuged for 30min at 4°C at 13,000rpm, supernatants were decanted and pellets were re-suspended in 15μl 2x sample buffer supplemented with urea, boiled for 5min at 100°C and used immediately or stored at -20°C until future use. Samples were analysed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% gels (see Section 2.2.17). After gel running, bands were stained with 15ml of Instant Blue.
(Expedeon) for 1h and de-stained with distilled water overnight on a shaker at room temperature. Gels were rinsed with distilled water and dried using the DryEase® Mini-Gel Drying System (Invitrogen). More specifically, the gel was incubated on a plate shaker with 10ml of Gel-Dry™ Drying Solution (Invitrogen, composition proprietary) for 20min at room temperature, put in between two DryEase® Mini Cellophanes, which were stabilized using two specific DryEase frames and left overnight to dry. The next day an image of the dried gel was taken using a commercial scanner.

2.2.10.2 Fibrillogenesis in vitro for immunostaining or activation assays

Collagen I fibrillogenesis was optimized for immunostaining or activation assays. More specifically, collagen I (Sigma or Hansen) was diluted to several concentrations (50-250μg/ml) using a fibrillogenesis buffer (see Section 2.2.10.1) and coated onto 19mm microwaved coverslips (see Section 2.2.9). Coverslips were incubated for 24h at 37°C, centrifuged at 3000rpm for 20min at 4°C in a plate centrifuge (Eppendorf 5810R, Fisher Scientific) and the supernatant was decanted. Coverslips were rinsed with 1xPBS and used for activation assays or fixed with 4% PFA/PBS and used for immunostaining of fibrils.

2.2.11 Coverslip coating with equine fibrils

Equine fibrils (HORM) were kindly provided by Prof. Farndale, Cambridge University. 19mm coverslips were microwaved at maximum strength and rinsed once with 1M HCl:50% ethanol, twice with 300mM NaCl and finally once with 1xPBS (1ml/well for every rinsing step). Coverslips were then air-dried and coated with 10μg/ml equine fibrils diluted in 0.01M acetic acid. Coverslips were incubated with fibrils overnight at 4°C in a 6cm petri dish, filled in with tissue soaked in 0.01M acetic acid, and the next day coverslips were used for immunostaining (see Section 2.2.14).
2.2.12 Production of cell-derived matrix

In order to produce cell-derived matrix (CDM), NIH-3T3 cells were seeded on 19mm glass coverslips and grown for several days. Before cell seeding, coverslips were pre-treated to help the matrix to remain attached. Dry coverslips were first sterilized by microwaving for 5min using maximum power, then transferred into 12-well plates and rinsed with 1xPBS. PBS was aspirated and coverslips were coated with 700μl/well autoclaved and filtered 0.2% gelatin and incubated for 1h at 37°C. Gelatin was aspirated and coverslips were cross-linked with 500μl/well 1% filtered glutaraldehyde for 30min at room temperature and finally with 500μl/well 1M filtered ethanolamine for 30min at room temperature. Coverslips were rinsed 3 times with 1xPBS (500μl/well) on a shaker for 5min after each incubation step. Finally, coverslips were incubated for 30min at 37°C with 500μl complete medium per well prior to cell seeding. If medium remained red, there was no ethanolamine residues, whereas if medium turned to purple, it was aspirated and coverslips were rinsed again with 1xPBS to remove any ethanolamine left and incubated again with complete medium for 30min prior to cell seeding.

NIH-3T3 cells, prior to cell seeding, were cultured in T-25 flasks until they reached passage 20. At passage 20, cells were expanded into T-75 flasks in order to produce higher number of cells. When confluent, cells were rinsed with 1xPBS and incubated with 3ml trypsin/EDTA for 3min at 37°C to induce cell detachment. Trypsin function was de-activated using 12 ml of complete medium and then cells were counted using a haemocytometer (Copens Scientific, Assistant). A total number of 1x10^5 cells were seeded per coverslip in a total volume of 700μl complete medium. Cells were allowed to attach overnight at 37°C in a 5% CO₂ incubator and the next day the medium was replaced with fresh one containing 50μg/ml filtered ascorbic acid. Ascorbic acid was pre-diluted in 1xPBS at 1mg/ml concentration, protected from air and light. Preparation of medium containing ascorbic acid and cell feeding were performed under very low light levels. Medium was replaced every two days for a total of 9 days and then cells were lysed using pre-warmed fibroblast lysis buffer
(0.1% Triton X-100, 20mM NH₄OH, diluted in 1xPBS). Wells were rinsed with 1xPBS and then incubated with 1ml lysis buffer/coverslip for 10min at 37°C once or twice until no cells were visible under the light microscope. Cell lysate was decanted, wells were rinsed with 1xPBS and stored with 1ml/well 1xPBS supplemented with 20U/ml penicillin-20μg/ml streptomycin at 4°C until used for experiments.

2.2.13 DDR activation assays

2.2.13.1 Activation assays using cells seeded on wells or coverslips

HEK-293 cells were seeded onto 24 well plates (for detection of DDR phosphorylation by Western blotting) and Cos-7 cells were seeded on 13mm microwaved coverslips in 24-well plates (for immunofluorescence), transfected with DDR1 or DDR2 expression constructs (see Section 2.2.8) and incubated with 0.5ml serum-free medium at 37°C. After 16h the medium was aspirated and the cells were stimulated with the appropriate collagen solution for 90min unless stated otherwise, at 37°C in a 5% CO₂ incubator. Collagen I was diluted to 10μg/ml from 1mg/ml stock and used as a positive control. Acetic acid at 1mM was used as a negative control. Collagen VI was diluted to various concentrations ranging from 2μg/ml to 100μg/ml. All collagens were diluted in an incubation medium that consisted of serum-free medium/10mM HEPES, pH 7.2 or 1xOptiMEM. Stimulated cells were either lysed (see Section 2.2.15) or fixed with 4% PFA/PBS (see Section 2.2.14).

2.2.13.2 Activation assays using cells in suspension

Cos-7 cells were seeded on 6-well plates (for detection of DDR phosphorylation by immunofluorescence), transfected with DDR1 or DDR2 constructs (see Section 2.2.8) and incubated with 2ml/well serum-free medium prior to stimulation. T47D cells were seeded on 6-well plates and the next day they were incubated with serum-free medium. After 16h of incubation at 37°C, serum-free medium was aspirated and cells were rinsed with 1xPBS. PBS was aspirated and cells were incubated for 3min at 37°C with 1ml/well pre-warmed non-enzymatic cell dissociation solution (Sigma
Aldrich). Cells were then re-suspended by gentle pipetting and incubated at 37°C with complete medium (5ml/well). After 1h of incubation at 37°C, medium containing the cells was centrifuged for 5min at 1000rpm (AccuSpin 1, Fischer Scientific), cells were re-suspended in serum-free medium (2ml/well) and added either to collagen-coated coverslips or wells (see Section 2.2.9-2.2.12) for immunofluorescence analysis (see Section 2.2.14) or analysis by Western blotting respectively (see Section 2.2.18).

A similar protocol was applied for testing DDR autophosphorylation by Western blotting using HEK-293 cells. In this case though, cells were re-suspended by pipetting using fresh serum-free medium and were added straight to collagen-coated tissue culture plate wells (See Sections 2.2.10-2.2.12). Prior to stimulation, wells were blocked for 1h with 500μl/well 1% BSA, diluted in 1xPBS. Finally, BSA was aspirated, wells were rinsed with 1xPBS (500μl/well) and 700μl of cells were added and incubated at 37°C at 5% CO₂ for 90min. For time-course experiments, cell stimulation varied from 15-90min. Stimulated cells were lysed (see Section 2.2.15) and used for analysis by Western blotting (see Section 2.2.18).

2.2.14 Immunostaining
2.2.14.1 Immunostaining of permeabilized cells

The stimulation medium was aspirated and coverslips were rinsed with 1xPBS and fixed with 4% PFA/PBS. All steps were performed in room temperature. After 15min of fixing, coverslips were rinsed 3 times with 1xPBS and permeabilized with 0.1% Triton X-100/PBS, for 5min at room temperature. Triton X-100/PBS was then aspirated and coverslips were blocked for 1h with filtered 5% BSA/PBS (blocking buffer). Coverslips were rinsed 3 times with 1xPBS and then incubated for 1h with primary antibody diluted in 5% BSA/PBS (30μl/coverslip). Coverslips were rinsed three times to get rid of any unbound antibody and then incubated for 30min with secondary antibody, diluted in 5% BSA/PBS (50μl/ coverslip), under dark conditions. The coverslips were rinsed 3 times with 1xPBS and 1 time with distilled water, then
mounted with Prolong anti-fade mounting medium (Life Technologies); 10μl and 15μl of mounting medium for 13mm and 19mm coverslips, respectively. Coverslips were left overnight at room temperature in order for the mounting medium to polymerase and the next day they were imaged using wide-field or confocal microscopy or stored at 4°C in the dark for future use.

2.2.14.2 Immunostaining of non-permeabilized cells

After 90min of stimulation, cells were rinsed once with ice-cold 1xPBS and then incubated with primary anti-DDR1 antibody diluted in 5% BSA/PBS (30μl/coverslip) for 1h on ice. Coverslips were then rinsed three times with 1xPBS at room temperature and fixed with 4% PFA/PBS for 15min at room temperature. The rest of the procedure was the same as described in Section 2.2.14.1.

2.2.14.3 Immunostaining of collagen I fibrils

After 24h of incubation, coverslips with immobilized collagen I fibrils or CDM (see Sections 2.2.10-2.2.12) were rinsed with 1xPBS and fixed with 4% PFA/PBS for 15min at room temperature. The rest of the procedure was the same as described in Section 2.2.14.1. Coverslips were imaged the next day using wide-field or confocal microscopy or stored at 4°C for future use.

2.2.14.4 Image analysis and quantification

Images were acquired using either wide-field or confocal microscopy. Wide-field images were acquired using a BX51 microscope (Olympus) and Simple-PCI acquisition software. Confocal images were taken by Leica SP5 MP/FLIM microscope using oil lenses at 63x/1.4 magnification. Confocal images were prepared as maximum intensity images with z stacks=0.71μm. Images were processed using Fiji software and imaging quantification was performed using an ImageJ macro, optimized by David Corcoran, another member in the Leitinger lab.
2.2.15 Cell lysis

After 90min of collagen stimulation (see Section 2.2.13) cells were placed on ice. The supernatant was transferred into Eppendorf tubes, wells were rinsed twice with cold 1xPBS (500μl/well) and the washes were transferred in the respective tubes. The tubes were centrifuged for 2min at 13,000rpm at 4°C (AccuSpin™ MicroR, Fisher Scientific) and the supernatant was decanted. Cells on 24- or 12-well plates were incubated with 50μl or 80μl lysis buffer respectively (150mM NaCl, 1mM EDTA, 1% v/v NP-40, 0.05mg/ml aprotonin, 1mM PMSF, 5mM NaF, 1mM VO₃, 50mM Tris, pH 7.4) for 30min on ice. Cells were then scraped off, and the lysates were transferred into the respective Eppendorf tubes and incubated for another 30min on ice. Finally, Eppendorf tubes were centrifuged for 10min at 13,000rpm at 4°C and the supernatant containing the cell lysate was transferred into new tubes. Lysates were used immediately for Western blotting (see Section 2.2.18) or immunoprecipitation (see Section 2.2.16) or stored at -20°C for future use.

2.2.16 Immunoprecipitation

After cell lysis (see Section 2.2.15), 10μl of lysate from each sample was stored at -20°C whereas the rest was used for immunoprecipitation. 1μg of anti-DDR1 antibody and lysis buffer were added to each sample in a final volume of 500μl. Samples were mixed overnight by rotation at 4°C and the next day protein G or protein A beads (GE Healthcare) were added to each sample. Protein G or protein A beads (approximately 40μl washed beads/sample) were first centrifuged for 1min at 13,000rpm at 4°C in order to get rid of any ethanol. Beads were rinsed with cold 1xPBS (1ml/sample), centrifuged for 1min at 4°C, and the supernatant was aspirated. Rinsing was repeated three times and then ice-cold 1xPBS was added to the slurry in equal volume to the packed beads. 80μl of total bead slurry were added per sample and samples were incubated for 90min on a roller at 4°C (SRT1, Stuart). Finally, samples were centrifuged for 1min at 4°C at 13,000rpm, the supernatant was decanted and pellets were boiled with 15μl of 2x sample buffer (125mM Tris, 2%
SDS, 50% glycerol, 0.1% bromophenol blue, 2% β-mercaptoethanol, pH 6.8) for 5 min at 100°C. The boiled samples were used immediately for analysis by SDS-PAGE and Western blotting.

2.2.17 SDS-PAGE

Samples containing 10μl of cell lysate and 2.5μl of 5x sample buffer (300mM Tris pH 6.8, 10% SDS, 50% glycerol, 0.1% bromophenol blue, 10%, β-mercaptoethanol) were mixed in Eppendorf tubes and boiled for 5 min at 100°C. Samples were loaded onto 7.5% polyacrylamide gels and separated by SDS-PAGE using a Mini Protean 3 Cell Tank (BioRad) and a running buffer composed of 0.2M Tris, 0.4M glycine, 1% SDS, pH 8.3.

2.2.18 Western Blotting

After SDS-PAGE, gels were used for protein transferring. Proteins were transferred onto nitrocellulose membrane (GE Healthcare) for 1 h on ice at 200mA using a wet transfer Mini-Trans blot apparatus (Invitrogen, Life Technologies) and a blotting buffer composed of 0.2M Tris, 0.4M glycine, 20% v/v methanol, 0.1% SDS, pH 7.5. After the transfer, membranes were stained using Ponceau-S for total protein levels and then de-stained using 1xPBS supplemented with 0.5% (v/v) Tween-20 (PBST, rinsing buffer). De-stained membranes were blocked for 1 h at room temperature on a shaker or overnight at 4°C with 5% dried skimmed milk (Marvel) diluted in PBST. After 1h of incubation, membranes were rinsed thoroughly until there was no milk left and incubated for 1 h at room temperature or overnight at 4°C with the appropriate primary antibody diluted in PBST, on a roller. Membranes were rinsed 3 times for 10min with PBST and incubated for 1h with the respective secondary HRP conjugated antibody, diluted in PBST. Membranes were rinsed 6 times with PBST and then incubated for 5min with ECL 2 solution (GE Healthcare) which creates chemiluminescence and chemifluorescence. The blots were imaged using the Ettan DIGE scanner (GE Healthcare Biosciences) which detects chemifluorescence or
myECL Imager (Fisher Scientific) which detects chemiluminescence. For the detection of phospho-specific signals, membranes were blocked with 5% BSA/PBS and incubated with primary antibodies diluted in 5% BSA/PBS. In order to re-probe blots with anti-DDR1, anti-ERK or anti-Akt antibodies, blots were incubated for 10min on a roller with antibody stripping solution, followed by blocking with milk/PBS as above. Blots were again probed with primary and secondary antibodies and imaged using either the Ettan DIGE imager or the myECL imager. Bands produced by Western blotting were quantified using ImageJ software.

2.2.19 Statistical analysis

Statistical analysis was performed after quantification of immunostaining (see Section 2.2.14) or Western blotting data (see Section 2.2.18). Statistical analysis was performed in SPSS software using un-paired t-test. Statistical significance was characterized as any result where p was lower than 0.1.

2.2.20 Protein production

Production of DDR2-Fc protein has been previously optimized in our lab (Xu et al. 2011a). Episomally transfected HEK293-EBNA cells expressing the DDR2-Fc vector were grown in a T-25 flask using complete DMEM/F12 supplemented with 1μg/ml puromycin. DDR2-Fc protein encodes for the extracellular region of DDR2 up to Threonine<sup>398</sup>, fused to the Fc fragment of human IgG2, cloned in the pcDNA3.1/Zeo vector (H. Xu et al. 2011). When 100% confluent, cells were trypsinized (see Section 2.2.7), transferred into T-75 flasks until confluent and finally expanded into several T-175 flasks. When cells were 100% confluent, they were transferred into a HYPERFlask Cell Culture Vessel (Corning) and grown until confluent. Once confluent, the cells were incubated with 500ml serum-free medium supplemented with 1μg/ml puromycin. After 1 week of incubation, conditioned medium was aliquoted into 50ml Falcon tubes and centrifuged for 10min at 3000rpm (AccuSpin 1, Fisher Scientific) to get rid of any detached cells. The supernatant from all tubes was combined and
transferred into a flask and 80μg/ml PMSF and 1mM EDTA protease inhibitors were added. The conditioned medium was stored at -80°C until further use. Cells were incubated with 500ml fresh serum-free medium and the previous procedure was repeated another three times, until four rounds of conditioned media were produced.

2.2.21 Protein purification

We purified DDR2-Fc from conditioned media produced from 3 rounds on a protein A column on an ÄKTA™ Purifier. The combined conditioned medium was loaded onto the column at 1mg/ml. The protein was eluted using gentle Ag/Ab elution buffer (Perbio) and dialyzed against 1xTris Buffered Saline (50mM Tris, 150mM NaCl pH 7.5). The final yield of DDR2-Fc protein was 37mg. In order to concentrate the isolated protein, we used ultrafiltration (Vivaspin, 30kDa molecular weight cut off).

DDR1-Fc, which was used in solid phase binding assays (see Section 2.2.22) was previously produced and purified (Xu et al. 2011a). It contains the DDR1 extracellular region up to Threonine416, fused to the Fc fragment of human IgG2 sequence, encoded in the pcDNA3.1/Zeo vector (Xu et al. 2011a).

2.2.22 Solid phase binding assay

Solid phase binding protocol has been previously optimized in our lab (Leitinger 2003). Collagens were coated at concentrations ranging from 10-100μg/ml using a coating buffer (50mM Tris, 100mM NaCl pH8.8) onto 96-well plates (Nunc Maxisorp; 50μl/well) and incubated overnight at 4°C. The next day, wells were rinsed using 1xPBS to get rid of any unbound collagen and then blocked for 1h with 1mg/ml BSA diluted in 1xPBST (when binding to DDR2-Fc was tested) or 40μg/ml casein diluted in 10mM Tris pH 7.4 (when binding to DDR1-Fc was tested). Wells were rinsed using 150μl/well rinsing buffer (0.5mg/ml BSA in 1xPBST or 40μg/ml casein in 10mM Tris pH 7.4.) and incubated for 3h at room temperature with serial dilutions of a DDR
construct containing the extracellular region of DDR1 or DDR2 fused to the Fc region of the human IgG1 sequence (Fawcett et al. 1992) (50μl/well, diluted in rinsing buffer). Wells were rinsed 6 times with rinsing buffer and then incubated for 1h at room temperature with HRP conjugated anti-human Fc antibody, diluted in rinsing buffer. Wells were then rinsed 3 times with rinsing buffer (150μl/well), 3 times with 1xPBS (150μl/well) and finally incubated with 75μl/well substrate solution (0.5mg/ml OPD, hydrogen peroxide 30%v/v, 50mM citrate-phosphate buffer, pH 5.0) for 6min. Stopping solution (3M H₂SO₄, 50μl/well) was added to stop the reaction and absorbance was measured at 492nm using a plate reader (Sunrise Tecan).
Chapter 3: DDR activation by collagen I fibrils

Introduction

Collagens are major components of all extracellular matrices (Myllyharju & Kivirikko 2004; Leitinger 2011; Bonnans et al. 2014; Theocharis et al. 2016). They provide tensile strength and stiffness in the respective tissue and they participate in many cellular processes, such as cell adhesion and matrix remodeling. Collagens form a variety of supramolecular assemblies such as fibrillar assemblies. Fibrillar collagens include collagens I, II, III, V and XI and constitute one of the most abundant collagen families present in ECM (Ricard-Blum 2011; Kadler et al. 1996). They display broad ligand specificity which includes other ECM proteins, such as fibronectin, as well as cell-surface receptors such as integrins and DDRs. The DDRs, as mentioned in Chapter 1, are unique members of the RTK family that are activated strictly only by collagen, rather than by small diffusible proteins like most other RTKs (Leitinger 2011). Early studies showed that DDR activation is manifested by autophosphorylation of tyrosines present in the DDR kinase domain and the IJM region (Vogel et al. 1997; Shrivastava et al. 1997; Leitinger 2003). Those tyrosines serve as docking sites for several molecular adaptors which orchestrate downstream signaling (Leitinger 2014).

Two independent studies showed that collagens, when presented in triple-helical form, are responsible for DDR activation (Vogel et al. 1997; Shrivastava et al. 1997). Previous work showed that fibrillar collagens can activate both DDRs, but DDR1 and DDR2 have distinct preferences for non fibrillar collagens: collagen IV activates only DDR1 (Vogel et al. 1997; Leitinger 2003) whereas collagen X activates preferentially DDR2 (Leitinger & Kwan 2006). Collagen VIII activates DDR1 but it is not yet known whether it can induce DDR2 activation (Hou et al. 2001). DDR activation is strictly dependent on the collagen triple-helix, as heat-denatured collagens such as gelatin,
cannot promote DDR activation (Vogel et al. 1997; Shrivastava et al. 1997; Leitinger 2003). The amino acid sequence present in collagens I-III responsible for binding to both DDRs was identified in our lab in collaboration with Richard Farndale’s group, as the six amino acid motif, GVMGFO, where O is hydroxyproline (Konitsiotis et al. 2008; Xu et al. 2011a). To identify DDR binding motifs in collagen, they applied a useful tool, the so-called “Toolkit peptides”, which are synthetic triple-helical peptides consisting of overlapping sequences encoding for the amino acids present in the triple-helical regions of collagens II or III (Farndale et al. 2008). Further structural studies provided atomic level details regarding the collagen-DDR interaction. An amphiphilic trench present in the surface of the DS domain that consists of five protruding loops was found to be the location of the fibrillar collagen binding site, as shown by the crystal structure of a complex between the DDR2 DS domain and a GVMGFO-containing-triple-helical collagen-mimetic peptide (Ichikawa et al. 2007; Carafoli et al. 2009).

While we have atomic-level detail of the DDR-collagen interaction, not much is known about how the DDRs interact with native ligands in tissues. Most studies so far have used collagens in molecular form (as single triple helices, soluble collagen, see Chapter 1) as DDR ligands. However, when cells are incubated with triple-helical collagens in neutral pH at 37°C, collagens spontaneously aggregate and form higher-ordered structures (Kadler et al. 2008). Current knowledge on whether DDR autophosphorylation is induced solely by triple-helical collagens or higher structures (small fibrils) that form during the cell-collagen incubation at 37°C is limited. However, experiments using triple-helical collagen peptides that encompass the GVMGFO motif have shown efficient DDR activation by these peptides (Konitsiotis et al. 2008; Xu et al. 2011a). Since peptides are unlikely to form fibrillar structures, it follows that collagens do not need to be in fibrillar form to activate the DDRs. The question whether DDRs can undergo autophosphorylation induced by higher collagen supramolecular assemblies, such as fibrils or fibers, has not been addressed in detail. This is attributed to the fact that fibrils are insoluble, large and consequently not easy to work with (Hulmes 2002; Canty & Kadler 2005).
Collagen I is the most abundant collagen and a ligand for both DDR1 and DDR2. Answering whether collagen I fibrils or fibers can act as functional DDR ligands is important since fibrils and fibers represent the native forms by which collagen I is present in tissues (Myllyharju & Kivirikko 2004). Collagen I is implicated in many diseases and co-exists in many tissues in which the DDRs are also prominently expressed (Bonnans et al. 2014; Leitinger 2014; Rammal et al. 2016). Answering whether collagen I fibrils can serve as functional DDR ligands, and therefore contribute to the progression of DDR-related diseases, will help understand more in depth the DDR-collagen interaction and ultimately aid the attempt to utilize the DDRs as therapeutic targets for the diseases they participate in (Borza & Pozzi 2014; Leitinger 2014; Multhaupt et al. 2016). Therefore, in this part of the PhD, we promoted collagen I fibrillogenesis and analysed the DDR-collagen I fibril interaction in more detail. More specifically, we tried to answer whether collagen I fibrils can serve as functional DDR ligands that induce transmembrane signaling and whether the collagen I fibril-induced DDR autophosphorylation is dependent on the collagen I source. Moreover, we performed some preliminary signaling assays to determine whether DDR activation by collagen I fibrils promotes the same signaling pathways as soluble collagen I.

To analyse the DDR-collagen I fibril interaction first I optimized in vitro generated fibrillogenesis using fibrillogenesis protocols applied for rat-tail collagen I (Silver 1981; Harris et al. 2013). Collagen I fibrillogenesis occurs spontaneously when collagen solution is warmed up and the pH is neutral (Kadler et al. 1996; Kadler et al. 2008). In vivo, triple-helical collagen is secreted from cells (as procollagen) and then C- and N-propeptides are cleaved by members of the Bone Morphogenetic Proteinase 1/tolloid-like and the ADAMTs family, respectively, producing the mature collagen molecule (tropocollagen)(Banos et al. 2008; Bonnans et al. 2014; Kadler et al. 2007). Tropocollagen molecules then spontaneously aggregate to form higher structural orders, fibrils (Banos et al. 2008). The enzyme lysyl-oxidase indirectly participates in the formation of fibrils, forming covalent bonds between the soluble collagen I molecules (Canty & Kadler 2005; Myllyharju & Kivirikko 2004). When fibrillogenesis is generated in vitro though, soluble collagen I aggregates to form
fibrils only due to temperature increase, as lysyl oxidase is not present (Zeugolis et al. 2009; Kadler et al. 1996). These fibrils are therefore not covalently cross-linked.

Traditionally, *in vitro* fibrillogenesis is performed by incubating soluble collagen I with a solution whose constituents induce fibrillogenesis. More specifically, collagen I fibrillogenesis *in vitro* is either induced in a rapid manner by collagen incubation with the fibrillogenesis solution, which results in a change to a specific pH and ionic strength, at a certain temperature (Holmes & Kadler 2006; Harris et al. 2013) or slowly by dialysis of collagen I against neutral pH and physiological saline conditions at room temperature (Harris & Reiber 2007). Alternatively, *in vitro* fibrillogenesis can be promoted enzymatically by incubation of soluble collagen I with procollagen C-proteinase, an enzyme responsible for cleavage of C-propeptide and consequently for collagen I fibrillogenesis (Kadler et al. 1987; Kadler et al. 1996).
Results

3.1 Collagen I fibrillogenesis in vitro

The first aim of this PhD was to explore whether collagen I fibrils can activate the DDRs. Published literature has shown that the DDRs are activated by soluble collagen, presented in triple-helical form (Vogel et al. 1997; Shrivastava et al. 1997). However, as mentioned above, it is not known whether materials containing only fibrils can induce DDR activation. To this end, I optimized fibrillogenesis protocols that I obtained from published literature. I used two types of collagen I; rat-tail collagen I that was either commercially available from Sigma, from here on termed collagen I (Sigma), or purified and provided to us by Dr. Hansen, from here on termed collagen I (Hansen). Tendon rat-tail collagen I was chosen because it consists a very common collagen I source that produces high yields of collagen I (Rajan et al. 2006). The reason for using two different rat-tail collagen I solutions will be explained later on in Chapter 3.1.

First, in vitro fibrillogenesis was performed using commercially obtained rat-tail collagen I (Sigma). I decided to follow the protocol published by Harris et al. (2013) because it was simple, very straightforward and cheap. However, Harris et al. (2013) only used two collagen concentrations, so I optimized their protocol by testing more collagen concentrations for in vitro fibrillogenesis as mentioned in Silver (1981). Collagen I fibrillogenesis depends on the fibrillogenesis buffer (1xPBS with 100-437mM NaCl), incubation time (1h-24h), collagen I concentration (25-250μg/ml) and incubation temperature (23°C or 37°C) (Harris et al. 2013; Silver 1981). Optimal conditions were verified using the same protocol, but results are shown only for optimization of collagen I concentration. The steps followed were the following: in order to eliminate any aggregated material present in the starting material, collagen I was first diluted to its final concentration in Eppendorf tubes using the optimal fibrillogenesis buffer (1xPBS with 437mM NaCl) and centrifuged to precipitate
aggregated collagen. The supernatant, which should contain mostly soluble collagen I, was incubated for 24h (optimal incubation time) at 37°C (optimal temperature) in order for fibrillogenesis to take place. Afterwards, fibrils were centrifuged and the pellet was solubilized in sample buffer that contained urea. The supernatant, which should contain soluble collagen that was not incorporated into fibrils, was incubated overnight at -80°C with ethanol in order to precipitate any soluble collagen I left. Aggregation of collagen was verified by SDS-PAGE. Figure 3.1A shows representative results for different collagen I concentrations incubated for 24h at 37°C. The majority of protein was recovered in the pellets for all collagen concentrations, suggesting that fibrillogenesis was complete with any of the collagen I concentrations used. Collagen I α1 chain (130kDa), α2 chain (115kDa) and dimers of α1 chain (235 kDa) and α2 chain (215 kDa) were strongly stained in the pellet samples, and only very faintly visible in the supernatant samples from the 50μg/ml and 100μg/ml collagen incubation conditions. There were also bands of higher molecular weight present in all samples, which we assumed to be insoluble aggregated material that was also present in the starting material (Figure 3.1A, lane 1).

In order to visualize the produced collagen I fibrils, fibrillogenesis was induced on coverslips instead of Eppendorf tubes. After centrifugation to eliminate aggregated material in the starting solutions, the supernatant was coated on coverslips and incubated for 24h at 37°C in fibrillogenesis buffer. Immunostaining results showed that collagen I was present, but it had not formed fibrils, indicating that fibrillogenesis had not occurred (data not shown). We reasoned that aggregated material present in the starting material may be required to nucleate fibrillogenesis. In order to investigate whether aggregated material was important for collagen I fibrillogenesis, collagen I was incubated with fibrillogenesis buffer on coverslips for 24h at 37°C but without any prior centrifugation, fixed and stained for collagen I. As shown in Figure 3.1B, at higher collagen I concentrations (100μg/ml and 250μg/ml) collagen seemed to aggregate (staining was fuzzy without any obvious fibrillar staining present), whereas at lower collagen concentrations (25μg/ml) collagen I formed structures that resembled fibrils of very small size. Structures that appeared like collagen I fibrils were present when collagen I was used at 50μg/ml. However,
fibrillogenesis was not complete, as there were places on the coverslips where soluble collagen I staining, which is visualized as a diffused layer of collagen I staining (see Figure 3.1B, 25µg/ml collagen), or aggregates were present (see Figure 3.1B, 100µg/ml collagen).
Figure 3.1: In vitro fibrillogenesis of commercial collagen I (Sigma). Collagen I fibrillogenesis was performed in vitro using commercial rat-tail collagen I (Sigma) incubated at 37°C for 24h either in Eppendorf tubes (A) or on coverslips (B). (A) Collagen I was diluted to its final concentration using fibrillogenesis buffer, centrifuged and the pellet was solubilized in sample buffer containing urea. The supernatant was incubated at 37°C for 24h and the aggregates produced were centrifuged. Again, the pellet produced was solubilized in sample buffer, and the supernatant was precipitated with ethanol and solubilized in sample buffer. Samples were run on a 7.5% SDS-PAGE gel and stained with Instant Blue. An aliquote of the starting material was also loaded. Positions of a molecular weight standard are depicted on the left side of the gel in kDa. SM; starting material, AM; aggregated material = pellet before incubation at 37°C, P; pellet after incubation at 37°C, S; supernatant after incubation at 37°C, Insol.mat; insoluble aggregated material. (B) Collagen I was diluted to its final concentration using fibrillogenesis buffer, coated on coverslips and incubated for 24h at 37°C. Coverslips were then centrifuged and fixed. Collagen I was stained using an anti-collagen I antibody followed by Alexa 555 anti-rabbit IgG antibody. Images were taken using wide-field microscopy at 40x magnification. Scale bar=50μm. Data are representative of three independent experiments.
We reasoned that fibrillogenesis may not have been complete because of the lower purity of the starting material which contained a large proportion of high molecular weight bands (see Figure 3.1A lane 1). Therefore, we sought to use rat-tail collagen I where the amount of aggregates would be minimal. We received collagen I purified from rat tail from our collaborator, Dr Uwe Hansen. Again, fibrillogenesis was induced in Eppendorf tubes like in the case of collagen I (Sigma) (see Figure 3.2A). Insoluble aggregated material was again present but at a much lower proportion compared to collagen I (Sigma). In general, results were similar to those observed in collagen I (Sigma) fibrillogenesis: the majority of protein was recovered in the pellets when collagen was used at 50μg/ml or 100μg/ml concentration, suggesting that fibrillogenesis was complete. When tested at 250μg/ml though, collagen precipitated prior to fibrillogenesis. Collagen I α1 chain, α2 chain and dimers of α1 chain and α2 chain were strongly stained in the pellet samples, and only very faintly visible in the supernatant sample from the 50μg/ml and 100μg/ml collagen incubation conditions. Again, fibrillogenesis was also performed on coverslips and fibrils were visualized by immunofluorescence but without centrifugation prior to fibrillogenesis, like in the case of collagen I (Sigma) (see Figure 3.1B). As shown in Figure 3.2B, fibrils were present both at 50μg/ml and 100μg/ml. When fibrillogenesis was performed at 100μg/ml, collagen I fibrillogenesis seemed more complete, compared to fibrillogenesis at 50μg/ml collagen, as there were no areas on the coverslips that showed diffused staining, which would indicate presence of soluble collagen I, or any aggregated material. Furthermore, when used at 100μg/ml, collagen I was more uniformly coated on the coverslips. When coated at 250μg/ml fibrils seemed to aggregate, as the collagen I staining observed was more diffuse, with less obvious fibrillar structure pattern.
Figure 3.2: In vitro fibrillogenesis of collagen I (Hansen). Collagen I fibrillogenesis was performed in vitro using rat-tail collagen I (Hansen) incubated at 37°C for 24h either in Eppendorf tubes (A) or on coverslips (B). (A) Collagen I was diluted to its final concentration using fibrillogenesis buffer, centrifuged and the pellet was solubilized in sample buffer containing urea. The supernatant was incubated at 37°C for 24h and the aggregates produced were centrifuged. Again, the pellet produced was solubilized in sample buffer, and the supernatant was precipitated with ethanol and solubilized in sample buffer. Samples were run on a 7.5% SDS-PAGE gel and stained with Instant Blue. An aliquote of the starting material was also loaded. Positions of a molecular weight standard are depicted on the left side of the gel in kDa. SM; starting material, AM; aggregated material=pellet before incubation at 37°C, P; pellet after incubation at 37°C, S; supernatant after incubation at 37°C, Insol.mat; insoluble aggregated material. (B) Collagen I was diluted to its final concentration using fibrillogenesis buffer, coated on coverslips and incubated for 24h at 37°C. Coverslips were then centrifuged and fixed. Collagen I was stained using an anti-collagen I antibody followed by Alexa 488 anti-mouse IgG antibody. Images were taken using wide-field microscopy at 60x magnification. Scale bar=50μm. Data are representative of two independent experiments.
3.2 Collagen I fibrils from cell-derived matrix

Fibroblasts are responsible for secretion of ECM components such as collagen and fibronectin in vivo. Several fibroblastic or epithelial cell lines, primary or immortalized, are able to secrete ECM in cell-culture conditions (Beacham et al. 2007; Campbell et al. 2014). This CDM can be denuded from the cells that secrete it and is able to induce several cellular functions, normal or tumor-associated, when incubated with cell-lines (Cukierman et al. 2001; Campbell et al. 2014; Cukierman & Bassi 2010). In order to obtain results with a more physiologically relevant ligand, we generated CDM containing collagen I fibrils from NIH-3T3 cells, a mouse fibroblastic cell line. This cell-line was chosen because it is easy to handle and can secrete collagen I. NIH-3T3 cells can secrete collagen I when seeded at high density and cultured with 50μg/ml ascorbic acid for a period of time varying from 9-14 days (Beacham et al. 2007; Murad et al. 1981). Previous literature has also shown that CDM is very unstable and coverslip coating and cross-linking with gelatin and glutaraldehyde-ethanolamine respectively is necessary for CDM stabilization (Beacham et al. 2007). To verify this, 19mm coverslips were either coated with gelatin or coated with gelatin and cross-linked with glutaraldehyde-ethanolamine or left non-coated, in order to test which of the above conditions would help matrix stabilization on coverslips. NIH-3T3 cells were incubated for 9 days with complete medium containing 50μg/ml ascorbic acid in order to increase collagen hydroxylation and therefore collagen I fibril production (Murad et al. 1981). After 9 days of incubation, cells were lysed and removed, in order to produce a cell-free CDM. Coverslips were fixed and stained using an anti-collagen I antibody. As shown in Figure 3.3A, no collagen staining was obtained in non-coated coverslips or coverslips coated with gelatin. On the other hand, when coverslips were coated with gelatin and cross-linked with glutaraldehyde-ethanolamine, collagen I fibrils were present, suggesting that coverslip pre-treatment was necessary for the CDM to remain attached. Fibrils visualized had the expected structure, as shown from immunostaining images published by others (Cukierman et al. 2001; Cukierman & Bassi 2010).
Next, I performed a time course of CDM generation and tested for the presence of collagen I and fibronectin, which are the main components of fibroblast-generated CDM (Cukierman et al. 2001). Again, cells were incubated with complete medium containing 50μg/ml ascorbic acid for 5, 9 or 14 days and lysed. The CDM produced was stained for collagen I and fibronectin levels. As shown in Figure 3.3B, collagen I fibrils and fibronectin were already present from day 5 and had the expected structure; however, it appeared that their density increased with time. It is well-established that, collagen I fibrils and fibronectin co-localize in CDM (Kadler et al. 2008; Singh et al. 2010). To verify this in our system, CDM was co-stained using anti-collagen I and anti-fibronectin antibodies. As shown in Figure 3.3C, collagen I fibrils and fibronectin co-localized, as expected.
Figure 3.3: Cell-derived matrix characterization. (A) NIH-3T3 cells were seeded at 1x10^5 cells/ml on non-coated coverslips (NC), coverslips coated with gelatin (Gel) or coated with gelatin and cross-linked with glutaraldehyde-ethanolamine (Gel/Glut/Eth). Cells were incubated for 9 days with complete medium containing 50µg/ml ascorbic acid and then lysed to produce a cell-free cell-derived matrix. Coverslips were fixed and stained using an anti-collagen I antibody followed by Alexa 555 anti-rabbit IgG antibody. Data are representative of two independent experiments. (B) NIH-3T3 cells were seeded at 1x10^5 cells/ml on coverslips, incubated with complete medium containing 50µg/ml ascorbic acid and lysed after 5, 9 or 14 days of incubation. Next, coverslips were stained using an anti-collagen I antibody followed by Alexa 555 anti-rabbit IgG or an anti-fibronectin antibody followed by Alexa 568 anti-goat IgG antibody respectively. Data are representative of one experiment. (C) NIH-3T3 cells were seeded on coverslips, incubated with complete medium containing 50µg/ml ascorbic acid, lysed after 9 days of incubation and co-stained using an anti-collagen I and an anti-fibronectin antibody, followed by Alexa 555 anti-rabbit IgG and Alexa 568 anti-goat IgG antibodies. Data are representative of two independent experiments. Images were taken using wide-field microscopy at 40x magnification. Scale bar=50µm.
3.3 Purified equine fibrils

Another source of collagen fibrils I used in order to use a more physiologically relevant material, in comparison to fibrils generated in vitro, was equine (Horm) fibrils. Briefly, they are native collagen I fibrils from equine tendon and they have been used for studies related to platelet aggregation, which can only be stimulated by collagen I fibrils, not soluble collagen I (Nesbitt et al. 2003; Nieswandt & Watson 2003). Equine collagen I fibrils were coated onto 19mm coverslips at 10μg/ml and the next day, they were fixed and stained using an anti-collagen I antibody. As shown in Figure 3.4, equine fibrils were present and they had the expected structure (Harris & Reiber 2007; Chapman et al. 1990; Kadler et al. 1996).

In order to compare the size of the different collagen I fibrils generated, fibrillogenesis in vitro using collagen I (Sigma) or (Hansen) (see Section 3.1), fibrils obtained from CDM (see Section 3.2) and coating of equine fibrils (see Section 3.3) were performed in parallel. Coverslips were fixed and stained for collagen I levels using an anti-collagen I antibody. As shown in Figure 3.5, fibrils generated from collagen I (Sigma) seem to be of smaller size compared to fibrils generated from collagen I (Hansen). Fibrils obtained from CDM seem to have bigger size (longer length and increased diameter) compared to in vitro generated fibrils. Equine fibrils appear similar in size as those obtained from CDM.
**Figure 3.4: Equine fibril staining.** Coverslips were sequentially rinsed with 1M HCl:50% ethanol, 300mM NaCl and sterile water, then air-dried. Next, they were coated with equine fibrils at 10μg/ml overnight at 4°C. The next day, coverslips were fixed and stained using an anti-collagen I antibody followed by Alexa 555 anti-rabbit IgG antibody. Data are representative of two independent experiments. Images were taken using wide-field microscopy at 60x magnification. Scale bar=50μm.

**Figure 3.5: Immunostaining of collagen I fibrils from different sources.** Fibrils were generated *in vitro* on coverslips from collagen I (Sigma) or collagen I (Hansen), or were obtained from CDM. In addition, purified equine fibrils were also used to coat coverslips. Coverslips were fixed and stained using anti-collagen I antibody followed by Alexa 555 anti-rabbit IgG antibody. Data are representative of one experiment. Images were taken using wide-field microscopy at 60x magnification. Scale bar=50μm.
3.4 DDR1 is activated by collagen I fibrils from different sources: immunostaining results

After obtaining collagen I fibrils from different sources, we wanted to test whether they can induce DDR activation. Previous literature has shown that DDR activation by soluble collagen I is manifested by autophosphorylation (Vogel et al. 1997; Shrivastava et al. 1997). Traditionally, DDR activation has been tested by Western blotting, using cells grown as monolayers and soluble collagen added to these cells (Leitinger 2003; Vogel et al. 1997; Shrivastava et al. 1997). In order to explore DDR activation by fibrils we first needed to verify by immunofluorescence, using phospho-specific anti-DDR1 antibodies, whether we can induce DDR1 activation when cells are in suspension, as would be expected from previous literature (Vogel et al. 1997; Juin et al. 2014). DDR1 activation was assessed by immunofluorescence due to the fact that only collagen (Sigma) was available at the time, which did not induce complete fibrillogenesis in vitro. For that reason, Western blotting could not be used to assess DDR1 activation by fibrils, as we would not know whether the signal produced would be induced by fibrils or the soluble collagen I remaining on the coverslip. We focused on DDR1 activation because there are no reagents available to detect DDR2 by immunofluorescence, as there are no antibodies available that react with native DDR2.

In order to visualize DDR1 phosphorylation by immunofluorescence, Cos-7 cells were used, since they can spread well and phosphorylation signal can be easily visualized by immunostaining. Cos-7 cells were transfected with a full-length DDR1 expression vector, re-suspended with serum-free medium and seeded onto coverslips coated with collagen I or laminin for 90min at 37°C. Coverslips were fixed and co-stained for total DDR1 levels, by using an anti-DDR1 antibody which recognizes the extracellular region of DDR1 (Carafoli et al. 2012) and for DDR1 autophosphorylation, by using an antibody that recognizes phosphorylation of a specific tyrosine present in the IJM region of DDR1b, tyrosine 513 (PY513), which is known to work well in Western blotting assays (Xu et al. 2014). This was the only commercially available antibody at
the time, so cells were transfected with a DDR1b expression vector, as this tyrosine is present only in the DDR1b isoform, not in DDR1a. Later, more phospho-specific anti-DDR1 antibodies became available, such as DDR1 anti-792, which detects phosphorylation of tyrosine 792 which is present in the DDR1 activation loop (PY792) and DDR2 anti-740, which detects phosphorylation of tyrosine 740, present in the DDR2 activation loop (PY740). As shown in Figure 3.6, cells incubated with coverslips coated with soluble collagen I were phosphorylated, whereas cells incubated with coverslips coated with laminin did not show any phosphorylation signal, as expected.

In order to analyze whether collagen I fibrils can induce DDR1 activation, Cos-7 cells were seeded onto coverslips coated with immobilised collagen I fibrils from different sources: collagen I fibrils generated in vitro using collagen I (Sigma) or collagen I (Hansen), CDM and equine fibrils coated at 10μg/ml, and incubated for 90min at 37°C. Cells added onto coverslips with soluble collagen I or laminin were used as positive and negative controls respectively. After stimulation, coverslips were fixed and stained for PY513 and total DDR1 levels. As shown in Figure 3.7, cells incubated with laminin showed no phosphorylation whereas cells stimulated with soluble collagen I showed PY513 signal, as expected. PY513 signals were visualized in cells stimulated with fibrils from all fibrillar sources. Moreover, the PY513 signals obtained with fibrils were different in appearance from those obtained with soluble collagen: they appeared to align with fibrillar structures.

In a separate experiment, cells stimulated with collagen I (Sigma) fibrils, were also stained for phosphorylation of tyrosine 792, which is, as mentioned above, present in the DDR1 activation loop (PY792), and total DDR1 levels. As shown in Figure 3.8, cells incubated with laminin showed no PY792 signal whereas cells incubated with soluble collagen I showed phosphorylation signal, as expected. Cells incubated with collagen I (Sigma) fibrils showed PY792 signal, which had a pattern of linear structures, like in the case of PY513 signal (See Figure 3.7).

In order to obtain clearer images, I stained only DDR1 that was present at the cell surface and used confocal microscopy. DDR1b-expressing Cos-7 cells were incubated
with collagen I fibrils *in vitro* generated from collagen I (Sigma) or CDM and cells were fixed and stained for DDR1, without cell permeabilisation, and PY513 levels. As mentioned earlier, PY513 targets a specific tyrosine present in the IJM region of DDR1b, tyrosine 513 (PY513). As shown in Figure 3.9, cells incubated with soluble collagen I showed phosphorylation signals whereas cells incubated with laminin showed no signal, as expected. Cells incubated with *in vitro*-generated fibrils or CDM showed strong PY513 signal, as expected from the previous experiments. It was also noticeable that DDR1 re-distributed in the presence of collagen I fibrils. This DDR1 re-distribution was reminiscent of the structure of collagen I fibrils. Also, as expected, PY513 staining co-localized with DDR1 staining, which represented only surface DDR1 levels. On the other hand, when DDR1 was stimulated with soluble collagen I, DDR1 staining was diffused, as was the PY513 signal.
Figure 3.6: DDR activation by soluble collagen I of cells seeded onto collagen-coated coverslips. Cos-7 cells were transiently transfected with full-length DDR1 expression vector, cells were dissociated from 6-well plates, re-suspended in serum-free medium and seeded for 90 min at 37°C onto coverslips coated with laminin or soluble collagen I. Cells were then fixed and co-stained using an antibody that recognizes a phosphorylated DDR1b tyrosine, tyrosine 513 (PY513) followed by Alexa 555 anti-rabbit IgG antibody and an anti-DDR1 antibody followed by Alexa 488 anti-mouse IgG antibody. The experiment was repeated five times. The majority of cells showed a PY513 signal. Representative images are shown. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30μm.
Figure 3.7: Wide-field images show DDR1 activation by collagen I fibrils from different sources. Cos-7 cells were transiently transfected with full-length DDR1 expression vector and cells were dissociated from 6-well plates, re-suspended in serum-free medium and incubated for 90 min at 37°C on coverslips coated with laminin, soluble collagen I, collagen I (Sigma) fibrils, collagen I (Hansen) fibrils, equine fibrils or cell-derived matrix. Cells were then fixed, permeabilized and co-stained using an antibody that recognizes a phosphorylated DDR1b tyrosine, tyrosine 513 (PY513) followed by Alexa 555 anti-rabbit IgG antibody and an anti-DDR1 antibody followed by Alexa 488 anti-mouse IgG antibody. The experiment was performed three times. The majority of cells showed a PY513 signal. Representative images are shown. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30 μm.
Figure 3.8: Wide-field images show autophosphorylation of DDR1 activation loop induced by collagen I (Sigma) fibrils. Cos-7 cells were transiently transfected with full-length DDR1 expression vector and cells were dissociated from 6-well plates, re-suspended in serum-free medium and incubated for 90min at 37°C on coverslips coated with laminin, soluble collagen I or collagen I fibrils. Cells were then fixed, permeabilized and co-stained using an antibody that recognizes a phosphorylated tyrosine present in the DDR1 activation loop, tyrosine 792 (PY792) followed by Alexa 555 anti-rabbit IgG antibody and an anti-DDR1 antibody followed by Alexa 488 anti-mouse IgG antibody. The experiment was performed once. The majority of cells showed a PY792 signal. Representative images are shown. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30μm.
Figure 3.9: Confocal images show DDR1 activation by collagen I fibrils from different sources. Cos-7 cells were transiently transfected with full-length DDR1 expression vector and cells were dissociated from 6-well plates, re-suspended in serum-free medium and incubated for 90 min at 37°C onto coverslips coated with laminin, soluble collagen I, collagen I (Sigma) fibrils or cell-derived matrix. Cells were then stained using an anti-DDR1 antibody followed by Alexa 488 anti-mouse IgG antibody, with no prior cell permeabilisation. Next, cells were fixed, permeabilized and stained using an antibody that recognizes a DDR1 phosphorylated tyrosine, tyrosine 513 (PY513) followed by Alexa 555 anti-rabbit IgG antibody. The experiment was performed once. The majority of cells showed a PY513 signal. Images are merged z-stacks of the PY513 and DDR1 signals shown as maximum projections. Representative images are shown. Images were taken using confocal microscopy at 60x magnification. Scale bar=30μm.
3.5 DDR1 is activated by collagen I fibrils or CDM: Western blotting results

DDR1 activation by collagen I fibrils was also analysed by Western blotting. To this end we used HEK-293 cells, which are often used in signaling experiments with transfected receptors, as they produce only minimal levels of endogenous receptors and no levels of endogenous DDR proteins. These cells are also the cells of choice for detecting DDR phosphorylation by Western blotting and have been previously used by our lab for this purpose (e.g. Leitinger 2003; Noordeen et al. 2006; Xu et al. 2011a). HEK-293 cells were transfected with the DDR1a or the DDR1b expression construct and stimulated either with collagen I (Hansen) fibrils and CDM respectively for 90 min at 37°C. The reason for using different DDR1 constructs was that the DDR1a construct was shown to be working better when used for Western blotting assays compared to the DDR1b construct after a certain point. Western blotting of cell lysates was performed, probing for total phosphorylation levels using 4G10 antibody, an antibody commonly used for detection of phosphorylated tyrosines present in any protein species (Tinti et al. 2012), or phospho-specific DDR1 signals (PY513 or PY792) and DDR1 levels. The signal detected using 4G10, PY513 or PY792 was quantified relative to the respective DDR1 levels. Signals are shown as fold increase relative to that detected in the negative control: cells incubated with laminin, which was set to a value of 1.0. As shown in Figure 3.10A, cells incubated with laminin showed only background phosphorylation whereas cells incubated with soluble collagen I showed a strong phosphorylation signal detected with 4G10, PY513 or PY792, as expected. Cells incubated with in vitro generated collagen I fibrils showed a clear 4G10 signal and lower PY513 and PY792 signals. As shown in Figure 3.10B, cells incubated with laminin showed no phosphorylation whereas cells incubated with soluble collagen I showed strong phosphorylation signal, 4G10, PY513 or PY792, as expected. In order to demonstrate that phosphorylation and DDR1 signals are due to the transfected cells added on the CDM, and do not derive from residual cells within the CDM, a coverslip that contained only CDM and no cells (cell-derived matrix-no cells) was lysed and loaded next to the cell lysates. Lysate
from CDM showed no phosphorylation or DDR1 signal, as expected. Cells incubated with CDM showed 4G10, PY513 and PY792 signals, like in the case of cells incubated with collagen I (Hansen) fibrils. DDR1 levels were similar in all wells. Even though there was a clear phosphorylation signal induced both by in vitro generated fibrils and CDM, it was weaker than that induced by the respective soluble collagen. This could be due to the fact that fibrils, in both cases, do not coat the whole area of the surface of the coverslip, in comparison to soluble collagen I, which would result in fewer binding sites for DDR1 on the coverslips. Another plausible hypothesis for the lower phosphorylation signal induced by in vitro generated fibrils and CDM could be that there are fewer DDR1 binding sites on the surface of collagen I fibrils compared with soluble collagen I.
### A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Alpha-4G10</th>
<th>Alpha-PY513</th>
<th>Alpha-PY792</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-derived matrix-no cells</td>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
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<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Soluble collagen I</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- **Alpha-4G10**: Reveals bands at 130 and 100 kDa.
- **Alpha-PY513**: Shows bands at 130, 100, and 70 kDa.
- **Alpha-PY792**: Displays bands at 130, 100, and 70 kDa.

### B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Alpha-4G10</th>
<th>Alpha-PY513</th>
<th>Alpha-PY792</th>
</tr>
</thead>
<tbody>
<tr>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Soluble collagen I fibrils</td>
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<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- **Alpha-4G10**: Reveals bands at 130 and 100 kDa.
- **Alpha-PY513**: Shows bands at 130, 100, and 70 kDa.
- **Alpha-PY792**: Displays bands at 130, 100, and 70 kDa.
Figure 3.10: DDR1 is activated by in vitro generated collagen I (Hansen) fibrils or CDM. HEK-293 cells were transiently transfected with full-length DDR1a ((A) probed for 4G10 or PY792 signal) or DDR1b ((A) probed for PY513 signal, (B) probed for 4G10, PY513 or PY792 signal) expression vector. Cells were re-suspended, seeded onto wells coated with laminin, soluble collagen I or in vitro generated collagen I (Hansen) fibrils (A) or CDM (B) and incubated for 90 min at 37°C. Cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates were probed for general tyrosine phosphorylation levels (4G10), phosphorylation of DDR1 tyrosine 513 (PY513) or phosphorylation of DDR1 tyrosine 792 (PY792), as indicated (upper blots). Blots were stripped and re-probed for total DDR1 levels (lower blots). The experiment where 4G10 signal was detected was performed more than three times with similar results. The other two experiments where PY513 and PY792 signals detected were performed one time. In order to demonstrate that phosphorylation and DDR1 signal were detected from the DDR1-transfected cells added (all cells transfected with DDR1b expression vector), an extra well that contained only CDM, with no cells, was lysed and analysed by Western blotting (cell-derived matrix-no cells). Quantitation of phosphorylation signal relative to total DDR1 levels was performed for every blot using Image J. The increase of phosphorylation signal after incubation of cells with soluble collagen I or in vitro generated collagen I (Hansen) fibrils (A) or CDM (B) is shown relative to baseline levels induced by laminin, which is set to a value of 1.0. The experiment was performed once. The molecular weight of the marker is depicted at the left side of the blots in kDa.
3.6 DDR1 is activated by collagen I fibrils with the same kinetics as by soluble collagen I

In Sections 3.4 and 3.5 I showed that DDR1 is activated by collagen I fibrils. However, we did not investigate whether fibrils activate DDRs with the same slow kinetics as soluble collagen I does (Vogel et al. 1997; Shrivastava et al. 1997). To this end, I performed a time-course assay: cells were transfected with a full-length DDR1 expression vector, re-suspended in serum-free medium and seeded onto wells coated with soluble collagen I (Hansen) or collagen I fibrils for 15, 30, 45, 60 or 90 minutes at 37°C. In this experiment cells were transfected with DDR1a expression vector and showed DDR1 autophosphorylation upon incubation with in vitro generated fibrils (see Figure 3.10A, first blot). As mentioned earlier, DR1a construct was shown to be working better when used for Western blotting assays compared to the DDR1b construct after a certain point. Cells incubated with laminin were used as a negative control. As shown in Figure 3.11A, cells incubated with soluble collagen I or collagen I fibrils started to produce a phosphorylation signal at 15 minutes which increased with time. It was also observed that the signal produced from fibril stimulation was weaker than that obtained after incubation with soluble collagen I, as was already noted in Figure 3.10. Our data suggest that soluble collagen I and in vitro generated fibrils induce DDR1 activation with similar kinetics. DDR1 levels were similar in all wells. Figure 3.11B depicts quantitation results of the phosphorylation signal from three independent experiments. Based on this observation, the next question we wanted to answer was whether collagen I fibrils induce phosphorylation signal with lower strength compared to soluble collagen I.
Figure 3.11: DDR1 activation kinetics is similar when stimulated with collagen I (Hansen) fibrils or soluble collagen I (A) HEK-293 cells were transiently transfected with full-length DDR1a expression vector. Cells were re-suspended with serum-free medium, seeded onto wells coated with laminin, soluble collagen I or in vitro generated fibrils and incubated for 15, 30, 45, 60 or 90 min at 37°C. After stimulation, cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates were probed for general tyrosine phosphorylation (4G10, upper blot). The blot was stripped and re-probed for total DDR1 levels (lower blot). The molecular weight of the marker is depicted at the left side of the blots in kDa. The experiment was repeated three times with similar results. (B) Quantification of total phosphorylation levels relative to total DDR1 levels from three independent experiments using image J. The signals produced by soluble collagen I or collagen I fibrils are compared to unstimulated cells incubated with laminin (LN) for 90 min, which was set to a value of 1.0.
3.7 DDR1 is activated by collagen I fibrils with the same strength as by soluble collagen I

As shown in Figure 3.10A, soluble collagen I induces stronger phosphorylation signal compared to collagen I fibrils, when tested by Western blotting. Western blotting detects phosphorylation of a large number of cells added to each well. Soluble collagen I coats the whole surface of the coverslip/well. This is not the case for fibrils, which only cover a proportion of the coverslip/well. We hypothesized that the signal induced by soluble collagen I was stronger because a higher number of cells (or more receptors per cell) could bind to collagen, and therefore become phosphorylated. To test whether the different forms of collagen induced a difference in signal strength at the level of individual cells, transfected HEK-293 cells were re-suspended and incubated on coverslips coated with soluble collagen I or fibrils (Sigma), for 30 minutes or 90 minutes. Coverslips were then fixed and stained for PY513 and surface DDR1 levels. As shown in Figure 3.12A, PY513 signals were present when cells were stimulated with soluble collagen I or fibrils for 30 or 90 minutes. There was a stronger signal after 90min of incubation compared to 30min, as expected. Figure 3.12B depicts quantification results from three independent experiments. In total, 180 cells were quantitated (approximately 45 cells per condition). As shown, fibrils activate DDR1 with similar strength as soluble collagen I. Therefore, it seems that the stronger phosphorylation signals produced by soluble collagen I that are detected by Western blotting, are likely due to the fact that fibrils do not coat the whole area of the coverslip, in comparison to soluble collagen I.
A

30min incubation

PY513 | DDR1
---|---
Soluble Collagen I

90min incubation

PY513 | DDR1
---|---
Collagen I Fibrils

B

30min incubation

90min incubation

NS

Py relative to DDR1

Incubation buffer

Soluble collagen I | Collagen I Fibrils
Figure 3.12: DDR1 is activated by collagen I (Hansen) fibrils with the same strength as by soluble collagen I (A) Cos-7 cells were transiently transfected with full-length DDR1b expression vector, re-suspended in serum-free medium and incubated for 90min at 37°C onto coverslips coated with laminin, soluble collagen I or collagen I fibrils. Cells were then stained using an anti-DDR1 antibody followed by Alexa 488 anti-mouse IgG antibody, with no previous permeabilisation. Next, cells were fixed, permeabilized and stained using an antibody that recognizes a DDR1 phosphorylated tyrosine, tyrosine 513 (PY513) followed by Alexa 555 anti-rabbit IgG antibody. The experiment was performed once. The majority of cells showed a PY513 signal. Representative images are shown. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30μm. (B) Quantification of PY153 signal relative to total DDR1 levels from three independent experiments using image J. Quantitated in total 180 cells using Image J. Statistical analysis was performed using unpaired t-test.
3.8 DDR1 activation by collagen I fibrils is not inhibited by blocking of β1 function

A subfamily of integrins, which consist of β1 subunit bound to α1, α2, α10 or α11 subunit, function as receptors for collagen, (as well as laminin) and they are essential in cell adhesion (Leitinger 2011; Theocharis et al. 2016). Published literature has shown that they recognize specific amino acid sequences present on the surface of triple-helical collagen structure (Hynes 1992; Leitinger 2011; Barczyk et al. 2010; Humphries et al. 2006). The DDRs can promote or inhibit integrin function (Wang et al. 2006; Xu et al. 2012; An, Lin, et al. 2016). However, previous literature has shown that DDR1 activation by soluble collagen I is independent of β1 integrin function (Vogel et al. 2000). Experiments were conducted in T47D cells which express endogenous DDR1. More specifically, T47D cells were pre-incubated with antibodies that blocked either β1 or α2 integrin function, and stimulated with soluble collagen I on coated wells. DDR1 was immunoprecipitated and analysed by Western blotting. Results showed that DDR1 could still be autophosphorylated by soluble collagen I (Vogel et al. 2000). We wanted to analyse whether DDR1 activation by collagen I fibrils is similarly independent of β1 integrin function. To this end, Cos-7 cells were seeded on 6-well plates, transfected with DDR1 construct, serum-starved and re-suspended. The reason for using Cos-7 cells for this experiment is that they were easier manipulated compared to T47D cells, since they can spread well and phosphorylation signal can be easily visualized by immunostaining (see Chapter 3.4). Prior to stimulation, half of the cells were pre-incubated in complete medium for 30 minutes at 37°C with an antibody that blocks β1 integrin function, P5D2 antibody (Thodeti et al. 2009; Xu et al. 2012). Cells were then seeded onto coverslips coated with laminin, soluble collagen I or collagen I fibrils (Sigma), fixed and stained for PY513 and surface DDR1 levels.

As shown in Figure 3.13A, blocking of β1 integrin function had no effect on DDR1 phosphorylation induced by soluble collagen I, as cells with or without P5D2 pre-incubation showed similar PY513 signals, as expected from published work (Vogel et
However, as shown in Figure 3.13B, blocking of β1 integrin function affected phosphorylation induced by collagen I fibrils (Sigma). Cells with P5D2 pre-incubation showed either lower PY513 signal or no PY513 signal, in comparison to cells without P5D2 pre-incubation which were phosphorylated, as in previous experiments. Blocking of β1 integrin function also affected the size of cells stimulated with soluble collagen I or collagen I fibrils, possibly because the cells could not adhere properly. Cells with P5D2 pre-incubation presented different morphology when incubated with fibrils, which was similar to the one acquired when cells were incubated with laminin. Quantification of PY513 signal relative to surface DDR1 levels from three independent experiments is shown in Figure 3.13C. In total, 160 cells were quantitated (approximately 40 cells per condition). Results confirmed those depicted in Figures 3.13A and B respectively, and showed no significant difference in phosphorylation levels between cells with or without P5D2 incubation when cells were seeded onto soluble collagen I but a decrease in phosphorylation in the presence of P5D2 when cells were seeded onto fibrils.

As a complementary method that allows monitoring of many more cells, I used Western blotting to assess DDR1 activation with or without P5D2 incubation. HEK-293 cells were seeded on 6-well plates, transfected with DDR1a construct, serum-starved and re-suspended. As before, prior to stimulation, half of the cells were pre-incubated for 30 minutes with P5D2 antibody, and then cells were incubated on wells coated with laminin, soluble collagen I or collagen I fibrils (Hansen), lysed and lysates were analysed by Western blotting (see Section 3.5). As shown in Figure 3.13D, cells incubated with collagen I (Hansen) fibrils, with or without P5D2 pre-incubation, showed 4G10 signal of similar intensity. This result was different to the one shown by immunostaining. A likely explanation would be that blocking of β1 integrin affects cell adhesion, which is well documented in the literature (Xu et al. 2012; Hynes 2002). Since non-adherent cells are washed off in the protocol of the immunofluorescence assay but they are kept and lysed in the protocol for the Western blotting analysis, this could account for the difference in results if the cells that are activated by fibrils detach easier than those activated by soluble collagen.
B

Image of immunofluorescence experiments showing the expression of PY513 and DDR1 in cells with and without P5D2. The images are labeled as 'No Ab' and 'P5D2'. The 'Merge' column shows the combined images.

C

Bar graphs showing the comparison of Soluble collagen I and Collagen I fibrils between No Antibody and P5D2. The x-axis represents the antibody conditions, and the y-axis shows the PY relative to DDR1. The graphs indicate a non-significant difference (NS) for Soluble collagen I and a significant difference (*) for Collagen I fibrils.
**Figure 3.13: Blocking of β1-integrin function does not affect DDR1 activation (A, B)** Cos-7 cells were transiently transfected with full-length DDR1 expression vector, cells were dissociated from 6-well plates, re-suspended in serum-free medium and incubated for 90min at 37°C onto coverslips coated with laminin, soluble collagen I (A) or (Sigma) collagen I fibrils (B). Cells were then stained using an anti-DDR1 antibody followed by Alexa 488 anti-mouse IgG antibody, with no previous permeabilisation. Next, cells were fixed, permeabilized and stained using an antibody that recognizes a DDR1 phosphorylated tyrosine, tyrosine 513 (PY513) followed by Alexa 555 anti-rabbit IgG antibody. The experiment was performed three times with similar results. Representative images are shown. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30μm.  

**C** Quantification of PY153 signal relative to total DDR1 levels from three independent experiments using image J (total 160 cells). Statistical analysis was performed using unpaired t-test. *p<0.05  

**D** HEK-293 cells were transiently transfected with full-length DDR1a expression vector, dissociated from the plate, seeded onto wells previously coated stimulated with soluble collagen I or collagen I fibrils. Prior to stimulation half amount of cells were incubated with 10μg/ml P5D2 antibody for 30min at 37°C. After incubation, cells with and without P5D2 antibody were stimulated with soluble collagen I or (Hansen) collagen I fibrils for 90min at 37°C. Cells incubated with laminin for 90min at 37°C was used as a negative control. After stimulation, cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates depicted at the upper blot were probed for 4G10 signal. After imaging, the blot was stripped and re-probed for total DDR1 levels. The increase of phosphorylation signal after incubation of cells with soluble collagen I or in vitro generated collagen I (Hansen) fibrils is shown relative to baseline levels induced after incubation with laminin, which is set as 1. The molecular weight of the marker is depicted at the left side of the blots in kDa. The experiment was performed one time.
3.9 Endogenously expressed DDR1 is activated by collagen I fibrils

So far, all experiments were performed using transiently transfected cells over-expressing full-length DDR1 protein. In order to verify the previous results, we applied a cell-line with endogenous DDR1 levels, where every cell would have the same DDR1 expression levels. To this end, in this experiment, DDR1 activation was tested using a breast cancer cell line with endogenously expressed DDR1 levels, T47D cells. T47D cells were previously used as a model cell line in which DDR1 was shown to be activated by soluble collagen I (Vogel et al. 1997; L’hote et al. 2002). T47D cells were seeded on 6-well plates again, serum-starved and re-suspended as usual (see Section 3.5). Cells were seeded for 90 min at 37°C onto wells coated with laminin, soluble collagen I or in vitro generated fibrils using collagen I (Hansen). In both Figures 3.14A and 3.14B, cells incubated with soluble collagen I were phosphorylated, whereas cells incubated with laminin showed no phosphorylation, as expected. Collagen I fibrils induced phosphorylation signal (4G10 or PY513) of the same strength as soluble collagen I, which was not the case when transient transfected cells were tested (see Figure 3.10). This result was observed both in DDR1 immunoprecipitates (Figure 3.14A) and in cell lysates (Figure 3.14B). DDR1 levels were similar in all wells.
Figure 3.14: Endogenously expressed DDR1 is activated by collagen I (Hansen) fibrils. T47D cells were dissociated from 6-well plates, re-suspended in serum-free medium and incubated for 90min at 37°C on wells coated with laminin, soluble collagen I or in vitro generated collagen I fibrils using collagen I (Hansen). (A) Cells were lysed and DDR1 was immunoprecipitated with monoclonal antibody 7A9 which recognizes the DDR1 extracellular region and then analysed by SDS-PAGE and Western blotting. The upper blot depicted was probed for general phosphorylation levels (4G10). The blot was stripped and re-probed for total DDR1 levels (lower blots). The experiment was performed once. (B) Cells were lysed and lysates were analysed by SDS-PAGE and Western blotting. Lysates in the upper blots were probed for 4G10 and PY513 signal respectively. The blots were stripped and re-probed for total DDR1 levels (lower blots). The experiments were repeated two times with similar results. The molecular weight of the marker is depicted at the left side of the blots in kDa.
3.10 Collagen I fibrils induce low DDR2 autophosphorylation

As mentioned earlier, the reason why we initially studied only DDR1 activation by collagen I fibrils was that there were no available antibodies that detected DDR2 autophosphorylation at the time we started working on this project. At a later point, an antibody that detects phosphorylation of a specific tyrosine, tyrosine 740, located in the DDR2 activation loop (PY740) became available. We therefore repeated key experiments to explore DDR2 autophosphorylation by collagen I fibrils. First, DDR2 activation was tested by immunofluorescence using two different sources of collagen I fibrils: collagen I (Hansen) and equine fibrils. Hence, Cos-7 cells were seeded on 6-well plates, transfected with a DDR2-FLAG expression vector, serum-starved, re-suspended and seeded onto coverslips coated with laminin, soluble collagen I or collagen I fibrils (Hansen or equine). Cells were fixed and stained for PY740 and FLAG levels. The FLAG tag was present in the intracellular region of DDR2, at the C-terminus following the kinase domain. Due to the intracellular location of the epitope, we could only image total DDR2 levels (after cell permeabilisation), not surface levels. The reason why we used this construct (DDR2-FLAG expression vector) was that there are no suitable available antibodies for immunofluorescence applications to detect native DDR2 (all available anti-DDR2 proteins detect denatured protein only).

As shown in Figure 3.15A, DDR2-expressing cells incubated with soluble collagen I showed PY740 signal, whereas DDR2-expressing cells incubated with laminin showed no PY740 signal, as expected. Cells incubated with collagen I (Hansen) fibrils showed a range of signal strengths, with some DDR2-positive cells giving very low signals and some giving quite strong signals, while their total DDR2 levels seemed similar. However, the surface DDR2 levels could vary, with cells with higher DDR2 levels on the surface better able to bind collagen and hence result in higher phosphorylation levels. When cells were seeded onto equine fibrils however, I could not detect any PY740 signal in any DDR2-positive cells that interacted with the fibrils (Figure 3.15B). This could be due to the fact that equine fibrils cover only a very small part of the
coverslip, so only a few cells interacted with them. Due to time constraints this experiment was only performed once, and only 11 DDR2-positive cells were found to bind to fibrils. In order to conclude that equine fibrils do not induce DDR2 autophosphorylation we would need to analyze a much higher number of cells.

DDR2 activation by collagen I fibrils was also tested by Western blotting. HEK-293 cells expressing wt-DDR2 were stimulated with \textit{in vitro} generated fibrils using collagen I (Hansen), in parallel to experiments conducted on DDR1-expressing cells. Cell lysates were analysed by Western blotting. As shown in Figure 3.15C, DDR2-expressing cells incubated with soluble collagen I showed strong 4G10 and PY740 signal, as expected, whereas cells incubated with laminin showed only baseline phosphorylation levels. Cells stimulated with collagen I fibrils showed very low 4G10 and PY740 signal compared to the respective positive control. DDR2 levels were similar in all the wells. It seems that collagen I fibrils induce low DDR2 autophosphorylation, compared to soluble collagen I and compared to the signals obtained with DDR1-expressing cells stimulated with collagen I fibrils. It also seems that cells incubated with \textit{in vitro} generated collagen I fibrils induce low DDR2 autophosphorylation when analyzed by Western blotting but strong signal when analysed by immunofluorescence. This could be due to the fact that there are fewer DDR2 binding sites present on the fibrillar surface. This leads to fewer DDR2-expressing cells binding to fibrils and lower total DDR2 phosphorylation signal induced, when analyzed by Western blotting. But individual DDR2-expressing cells that manage to bind to \textit{in vitro} generated fibrils could get strongly phosphorylated.
**Figure 3.15: Collagen I fibrils induce low DDR2 phosphorylation signal.** Cos-7 cells were transiently transfected with full-length DDR2-Flag expression vector, re-suspended in serum-free medium and incubated at 37°C for 90min on coverslips coated with laminin, soluble collagen I, collagen I (Hansen) fibrils (A) or equine fibrils (B). Cells were fixed, permeabilised and stained using an anti-collagen I antibody followed by Alexa 555 anti-rabbit IgG2b antibody, an antibody that recognizes phosphorylation of tyrosine 740 (PY740) followed by Alexa 647 anti-rabbit IgG antibody and an anti-FLAG antibody followed by Alexa anti-mouse 488 IgG1 antibody. (A) The experiment was repeated three times with similar results. (B) The experiment was performed once. Results shown are representative. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30μm. (C) HEK-293 cells were transiently transfected with full-length DDR2 expression vector, re-suspended in serum-free medium and seeded for 90min at 37°C on wells coated with laminin, soluble collagen I or (Hansen) I fibrils. Cells were lysed and lysates were analysed by SDS-PAGE and Western blotting. Lysates were probed for 4G10, PY740 or DDR2 signal respectively. The experiment was repeated three times with similar results. The molecular weight of the marker is depicted at the left side of the blots in kDa.
3.11 Signaling molecules recruited by DDR1 or DDR2 activation by collagen I fibrils

DDR activation is manifested by autophosphorylation of tyrosines present in the DDR activation loop and in the IJM region. Those tyrosines serve as docking sites for several signaling molecules that are activated after DDR autophosphorylation. However, it is not yet known in detail how those signaling molecules serve as regulators for the cellular functions they participate in (Leitinger 2014).

ERK1 and ERK2 are key signaling molecules and represent two isoforms of serine/threonine kinases that regulate the MAP kinase pathway (Roskoski 2012). They are very common signaling molecules present in almost every cell-line, they phosphorylate a variety of substrates and thus they participate in several cellular functions such as cell proliferation and differentiation (Saba-El-Leil et al. 2016). DDR1 can promote or inhibit ERK phosphorylation: smooth muscle cells, megakaryocytes and transfected HEK293 cells show increased ERK phosphorylation after DDR1 activation by collagen (Hilton et al. 2008; Lu et al. 2011; Abbonante et al. 2013). On the other hand, DDR1 activation can suppress ERK phosphorylation in mesangial cells (Curat 2002). There is limited knowledge regarding adaptor molecules that use DDR2 phosphorylated tyrosines as docking sites, as well as on DDR2-induced signaling pathways. However, ERK phosphorylation after DDR2 activation has been shown in two independent studies using B16BL6 murine melanoma cancer cells and MDA-MBA-231 breast cancer cells respectively (Zhang et al. 2013; Poudel et al. 2015). However, whether EKR phosphorylation is induced by DDR2 activation has not been shown in HEK293 cells.

Akt is a serine/threonine kinase that participates in downstream signaling of the PI-3 kinase pathway (Engelman et al. 2006). Akt phosphorylation has been shown to regulate several cellular functions such as cell survival and proliferation and has been closely related to cellular transformation and cancer (Vivanco & Sawyers 2002; Engelman et al. 2006). Recent studies aim to use Akt inhibitors as a potential
therapeutic approach to minimize its activity and thus its tumorigenic effects (Liu et al. 2009). DDR1 was shown to induce Akt phosphorylation in mouse embryonic stem cells (Suh & Han 2011) but whether Akt phosphorylation is induced by DDR2 activation has not yet been established.

We performed signaling assays to test whether collagen I fibrils can activate the same signaling molecules as soluble collagen I does. To this end, we analysed whether DDR1 or DDR2 phosphorylation by fibrils would result in downstream phosphorylation of ERK and Akt signaling molecules. HEK-293 cells (expressing DDR1 or DDR2) were in parallel stimulated with in vitro generated collagen I (Hansen) fibrils, soluble collagen I or laminin (see Section 3.5). In the case of Akt phosphorylation, cells were also stimulated with FBS, which served as positive control. Cells were lysed and lysates were analysed by Western blotting.

As shown in Figure 3.16A, DDR1 or DDR2 cells showed a 4G10 signal when incubated with soluble collagen I. DDR1 cells also showed 4G10 signal when incubated with fibrils, whereas DDR2 cells did not, as expected from the previous experiments. DDR1 cells stimulated with soluble collagen I showed a clear phosphorylation signal of the lower ERK band (42kDa) and a weaker phosphorylation signal of the upper ERK band (44kDa). DDR1 cells stimulated with fibrils showed very weak ERK phosphorylation (pERK), which was only 1.3-1.4 higher than control levels. DDR2 cells incubated either with soluble collagen I or collagen I fibrils showed baseline ERK phosphorylation levels only. Total ERK levels were similar in DDR1 and DDR2-transfected cells. Signal quantitation indicated that the ratio of pERK signal produced by DDR1-expressing cells between soluble collagen I and collagen I fibrils was similar to the ratio of the 4G10 signal between soluble collagen I and collagen I fibrils, indicating that phosphorylated DDR1 receptors lead to similar signaling responses regardless of the mode of the ligand binding (to soluble collagen I or collagen I fibrils). In the case of DDR2 cells, the weak 4G10 signal produced by fibrils was matched by no significant increase in pERK signal.
Similarly to ERK signaling results (see Figure 3.16A), DDR1 or DDR2-transfected cells incubated with soluble collagen I showed Akt phosphorylation (pAkt), whereas pAkt was very weak in cells incubated with fibrils, which was expected in the case of DDR2 cells since 4G10 signal was also very weak, as shown in Figure 3.16B. Again, the ratio of pAkt signal strength between soluble collagen I and collagen I fibrils was similar to the 4G10 signal ratio. Total Akt levels were similar in DDR1 and DDR2-transfected cells. Cells were also stimulated with FBS, to demonstrate that the antibody that detects pAkt is working.
Figure 3.16: Signaling molecules recruited by DDR1 or DDR2 activation by collagen I (Hansen) fibrils. HEK-293 cells were transiently transfected with full-length DDR1a or DDR2 expression vectors, dissociated from the plate, seeded onto wells coated with laminin, soluble collagen I or collagen I fibrils and incubated for 90 min at 37°C. Cells were lysed and lysates were analysed by SDS-PAGE and Western blotting. (A) Blots were probed for general tyrosine phosphorylation levels (4G10) or ERK phosphorylation (pERK), as indicated. The blots were stripped and re-probed for total DDR1 or DDR2 or ERK levels (corresponding lower blots). The experiment was repeated three times with similar results. (B) Cells in the first two lanes were incubated with FBS, which serves as a positive control for Akt phosphorylation. Blots were probed for general tyrosine phosphorylation levels (4G10) or Akt phosphorylation (pAkt), as indicated. The blots were stripped and re-probed for total DDR1 or DDR2 or Akt levels (corresponding lower blots). Quantitation of phosphorylation signal relative to total protein levels was performed for every blot using Image J. The increase of phosphorylation levels (A, B) or ERK phosphorylation (A) or Akt phosphorylation (B) after incubation of cells with soluble collagen I or in vitro generated collagen I (Hansen) fibrils is shown relative to baseline levels induced after incubation with laminin, which is set to a value of 1.0. The experiment was repeated two times with similar results. The molecular weight of the marker is depicted at the left side of the blots in kDa.
This chapter’s aim was to analyse in greater depth the interaction between DDRs and collagen I fibrils, one of the most abundant constituents of the ECM (Kadler et al. 2007). To determine whether collagen I fibrils can serve as functional DDR ligands, we first generated fibrils by \textit{in vitro} fibrillogenesis using collagen I. In order to characterize more extensively the DDR-collagen I fibril interaction, collagen I fibrils from different sources were used: \textit{in vitro} generated using tendon rat-tail collagen I (Sigma) or collagen I (Hansen) (Figures 3.1B and 3.2B respectively), fibrils obtained after collagen secretion from NIH-3T3 mouse fibroblastic cells (Figure 3.3) or native fibrils from equine tendon (Figure 3.4).

As immunostaining data show (see Figures 3.1B and 3.2B), \textit{in vitro} generated fibrils from collagen I (Sigma or Hansen) had the expected overall architecture (Wall et al. 2005). Current knowledge suggests that \textit{in vitro} generated collagen I fibrils are unipolar, they have two smoothly tapered ends and the characteristic 67nm D-banded pattern (Kadler et al. 1996; Kadler et al. 2008; Hulmes 2008). The data we acquired by wide-field microscopy allowed only qualitative validation of fibrillar characteristics, since fibrillar polarity, diameter, orientation or the characteristic D-band pattern cannot be determined by immunofluorescence. Fibrils isolated from tendon tissue display a wide range of diameter and length reaching up to 500nm and several micrometers respectively, depending on the developmental stage they were isolated from (Banos et al. 2008; Gautieri et al. 2011). Traditionally, characteristics related to fibrillar structure or size have been visualized using scanning electron microscopy or transmission electron microscopy (Kadler et al. 1996; Orgel et al. 2001; Harris et al. 2013). Due to time restraints, we applied immunostaining to gain insight on the fibrillar characteristics. An interesting future experiment would be to analyse more in depth the characteristics of the fibrils we produced using electron microscopy.
In vitro fibrillogenesis using collagen I (Hansen) appeared to be complete (when used at 100 μg/ml), in agreement with the experimental results obtained from Harris et al. (2013), where the protocol was optimized from. On the contrary, in vitro fibrillogenesis induced by collagen I (Sigma) was not complete (in any of the concentrations tested), allowing some proportion of collagen I to remain in triple-helical or aggregated form, as shown by the immunostaining data (triple-helical collagen appeared as diffused staining whereas aggregated material appeared as staining of undefined morphology). A plausible hypothesis for incomplete fibrillogenesis could be lower purity of collagen I starting material. We speculate that such impurities could be the reason of artifacts (appearing as bright spots) present in immunostaining images stained for collagen (Sigma) fibrils (see Figure 3.1B, 50 μg/ml collagen I). Such artifacts were not visible in immunostaining images of collagen (Hansen) fibrils. Another observation based on our results was the presence of insoluble collagen (see Figure 3.1A and 3.2A respectively), as highly cross-linked, present in the gels stained using Coomassie stain. Those bands were much more visible in the gel stained for collagen (Sigma) α-chains in comparison to the gel stained for collagen (Hansen) α-chains (see Figures 3.1A and 3.2A starting material respectively).

As explained in Section 3.1, in vitro fibrillogenesis was first generated in Eppendorf tubes and then on coverslips. When generated in Eppendorf tubes, collagen I fibrillogenesis appeared to be complete, as there was no soluble collagen I present in the supernatant lanes in any of the collagen concentrations tested, as judged by Comassie staining of ethanol precipitated material (see Figure 3.1A). However, when we performed the same protocol on coverslips and stained for the presence of fibrils in the fibrillar fraction, we only imaged a diffusely stained layer, which is attributed to soluble collagen I (data not shown). We thus decided to induce in vitro fibrillogenesis on coverslips, without prior centrifugation of the collagen I aggregated material. The immunostaining data we acquired suggest that the aggregated collagen I material is vital for fibrillogenesis to occur on coverslips (see Figure 3.1B and 3.2B), since when the collagen I solution was centrifuged prior to fibrillogenesis, no fibrils were present on the coverslips (data not shown). A plausible hypothesis for
this could be that the aggregated material includes oligomers, which are required as seeding material for fibrillogenesis to take place. It is not known why Coomassie staining and immunostaining data showed this discrepancy. It could be attributed perhaps to the inability of ethanol to completely precipitate all soluble collagen present in the solutions used for fibrillogenesis in Eppendorf tubes.

A limitation of in vitro fibrillogenesis is that it cannot fully simulate the structure and biomechanical characteristics of native fibrils. Fibrils in vivo vary in terms of polarity, as they can be bipolar or unipolar but most importantly they associate with other ECM proteins such as proteoglycans and fibronectin that regulate their assembly (Kadler et al. 1996; Kadler et al. 2008; Singh et al. 2010). In order to obtain more physiologically relevant fibrillar material, I induced secretion of ECM using NIH-3T3 cells, a mouse fibroblastic cell line. Cukierman et al. (2001) demonstrated that ECM derived from fibroblastic cell-lines incorporates the biomechanical and physical properties of in vivo-generated matrix, including the characteristic fibrillar D-pattern (Cukierman et al. 2001). Therefore we chose CDM from NIH-3T3 cells to analyze DDR-fibril interactions. Our first aim was to verify that our CDM incorporated the basic features of the in vivo-generated matrix. Well-established evidence supports that fibronectin is a main component of such CDM and that it co-localizes with collagen I fibrils (Cukierman et al. 2001). Thus, we co-stained our CDM for collagen I and fibronectin levels and showed that collagen I fibrils and fibronectin co-localized in the CDM produced (see Figure 3.3C), mimicking their in vivo behavior. However, those images were acquired using wide-field microscopy, which transmits high out of focus light (Schermelleh et al. 2010). To effectively capture the collagen I fibril-fibronectin co-localization, high-resolution microscopy such as confocal microscopy or de-convolution of the wide-field images acquired should be performed. Other principal biomechanical factors of this cellular microenvironment that have been shown to affect cell behavior include stiffness and porosity of the matrix (Doyle et al. 2015; Friedl & Wolf 2010; Haycock 2011). Unfortunately we could not study those characteristics due to time constraints.
We also stained fibrils from all different sources in parallel in a separate experiment. As shown in Figure 3.5, \textit{in vitro} generated fibrils using collagen (Sigma) seem to have smaller size (diameter, length) compared to the rest of fibrils tested. \textit{In vitro} generated fibrils (Sigma) and (Hansen) appeared to be less organized compared to fibrils produced from CDM. This agrees with published literature, which suggests that collagen I assembly is regulated to a high extent by other ECM components such as fibronectin and integrins (Kadler et al. 2008; Banos et al. 2008; Sweeney et al. 2008). Equine tendon fibrils appeared to be bigger both in diameter and length compared to the rest of the fibrils generated. An interesting experiment would be to image our fibrils using second harmonic generation imaging (SHG). SHG has been used for imaging of fibrillar collagens and its main advantage is that it does not require staining of fibrils, while it offers 3D imaging (Strupler et al. 2007). However, its resolution is in the scale of micrometers (Strupler et al. 2007; Campagnola 2011). More recent studies have applied SHG in combination with atomic force microscopy or transmission electron microscopy to obtain higher resolution (Bancelin et al. 2014; Brown et al. 2013). Combination of SHG and transmission electron microscopy allowed imaging of fibrils with diameter as low as 30nm. However, this approach has only been applied for low-packed fibrils (Bancelin et al. 2014).

After producing collagen I fibrils, we aimed to answer whether they can activate DDRs. Our experiments were constrained mainly on studying the DDR1-collagen I fibril interactions due to the fact that when this PhD project was initiated there were very few DDR2 antibodies available. Our data, analysed by immunostaining or Western blotting assay, showed clear DDR1 activation by collagen I fibrils, which was obtained with any collagen source tested (Figures 3.7-3.10). The pattern of the phosphorylation signal was similar to the morphology of the respective fibrils used to induce DDR1 activation, indicating that DDR1 distribution re-organized along the collagen fibrils. Those data agree with the study published by Juin et al. (2014), who showed that DDR1 co-localizes with collagen I fibrils (Juin et al. 2014). While they do not show DDR1 activation by collagen I fibrils directly, they demonstrated that collagen I fibrils can re-organise DDR1. However, this study used polymerized collagen gels, which can comprise also soluble collagen I (Juin et al. 2014). Our
Western blotting data also showed that the 4G10, PY513 or PY792 signal induced by \textit{in vitro} generated fibrils (Hansen collagen) or CDM was weaker than the respective signal induced by soluble collagen I. This result could be attributed perhaps to fewer DDR1 binding sites present on the fibrillar surface, in addition to the fact that fibrils cover a much smaller area of the wells compared with soluble collagen. Another approach to further verify that the phosphorylated band is due to DDR autophosphorylation (when tested with the 4G10 antibody) would be to pre-incubated cells with a DDR1 inhibitor, DDR1-IN-1 (Kim et al. 2013). If the band was not present after pre-incubation with this inhibitor, we could claim even strongly that collagen I fibrils induce DDR1 phosphorylation.

A number of studies have shown that collagen I fibrils have a variety of ligands \textit{in vivo}. Collagen I fibrils integrate binding sites for more than 50 ligands present in extracellular matrices (reviewed in Kadler et al. 2008; Sweeney et al. 2008; Banos et al. 2008; Singh et al. 2010; Di Lullo et al. 2002). We tested DDR1 activation using CDM produced by NIH-3T3 fibroblasts, which has been shown to mimic the \textit{in vivo} ECM (Cukierman et al. 2001), and is thus expected to contain additional collagen binding partners. As mentioned earlier, our immunostaining data showed that collagen I fibrils co-localized with fibronectin (see Figure 3.3C). Published literature has shown that fibronectin binds to the region of collagen I that overlaps with one of the DDR2 binding sites, in the so-called “Cell interaction domain” (Sweeney et al. 2008; Konitsiotis et al. 2008). As mentioned earlier, the only known functional DDR2 binding site present on collagens I-III is the GVMGFO motif, which is highly conserved between DDR1 and DDR2 (Konitsiotis et al. 2008; Xu et al. 2011a). There have been identified more DDR2 binding sites present on collagens II and III, but they do not induce DDR2 autophosphorylation (Konitsiotis et al. 2008; Xu et al. 2011a). Furthermore, DDR1 binding is limited to one binding site that contains the GVMGFO motif. Based on the above, we speculated that perhaps fibronectin may hinder DDR1 fibril-induced activation, if it blocks DDR binding to collagen. Another consideration was that the main DDR-activating binding site in fibrillar collagens, GVMGFO, may not be accessible on the surface of collagen fibrils or fibers. Orgel et al. (2006) using X-Ray diffraction data obtained a 3D structure of a collagen I fiber from rat tail
collagen and based on this model Perumal et al. (2008) suggested that segments D4 and D5 are on the surface of the fiber and thus supporting that the GVMGFO motif is partially hidden (Orgel et al. 2006; Perumal et al. 2008). However, these data are controversial, and according to Herr and Farndale (2009), the collagen I molecule adopts a different orientation, leaving segments D1 and part of D2 and D3 on the fibrillar surface. In that case, the GVMGFO motif would be fully accessible (Herr & Farndale 2009). Taking the above into account, we analysed DDR1 phosphorylation both by immunostaining and Western blotting using the CDM we produced. Interestingly, both immunostaining and Western blotting data showed that DDR1 was activated by CDM and that DDR1 activation was not inhibited by the presence of fibronectin. These findings indicate that fibronectin, a collagen organizer, does not hinder DDR1 activation by collagen I fibrils even though collagen I fibrils and fibronectin associate so closely (Ledger et al. 1980; Kadler et al. 2008). Furthermore, our findings implicate that the GVMGFO motif is accessible on the surface of fibrils. However, we cannot exclude that DDR1 activation attributed to collagen I fibrils present in CDM could also be induced by other components of the cellular microenvironment of the CDM such as soluble collagen I that has not yet aggregated to fibrils, or collagen IV, which has been found to be present in CDM produced by WI-38, a human lung fibroblastic cell-line, in low concentrations (Soucy & Romer 2009).

A possible experiment to test whether other collagen ligands might inhibit DDR1 activation would be to perform competition binding experiments, using SPARC or vWF, since previous studies identified that these proteins bind to collagen I via the GVMGFO motif (Giudici et al. 2008; Lisman et al. 2006). However, while vWF has only one binding site on collagen I (Lisman et al. 2006) it should be taken into account that SPARC has several binding sites on the collagen I surface, as shown by two independent studies (Giudici et al. 2008; Wang et al. 2005).

To gain insight on whether fibrillar size can affect DDR activation, experiments where fibrillar size is adjusted could be applied. Collagen V has been shown to act as a collagen I fibril nucleator factor (Hansen & Bruckner 2003; Kadler et al. 2008). Also,
molecules such as thrombospondin 2 (Bornstein et al. 2000; Kyriakides et al. 1998) or SLRPs have been shown to decrease fibrillar size (Hedbom & Heinegård 1993; Danielson et al. 1997; Kvist et al. 2006). However, the dimensions of the fibrils produced would have to be measured using electron microscopy or atomic force microscopy (Stamov et al. 2013). A more straight-forward and simple approach to test whether the fibrillar diameter influences the ability of fibrils to induce DDR activation is to induce fibrillogenesis in vitro using a variation of pH values (Harris et al. 2013; Ghazanfari et al. 2016). Increase of pH from relatively neutral (6.9-7.4) to relatively basic (7.6-8.1) has been shown to induce a 10-fold increase of fibrillar diameter in chick cornea (Bard et al. 1993). Dehydration of collagen fibrils would also be an interesting experiment to test whether DDR activation would be affected, as dehydration would increase the stiffness of the fibrils produced (Bhadriraju et al. 2009). Dehydration of fibrils is a very simple procedure, during which fibrils are incubated in a laminar flow for 24-48h with no solution (McDaniel et al. 2007).

The DDRs have been associated with many diseases, including cancer (Rammal et al. 2016; Leitinger 2014). CDM can be produced not only from healthy but also from tumor-associated fibroblasts (Cukierman & Bassi 2010). Tumor-associated fibroblasts have been extensively studied in relation to the matrix they produce. It has been shown that they produce fibronectin and collagen I fibrils that are far more dense and tightly-packed in comparison to those produced by normal fibroblasts, including NIH-3T3 fibroblasts (Amatangelo et al. 2005). Such fibrils would lead to ECM with different biomechanical characteristics (Amatangelo et al. 2005). An interesting experiment would be to test whether these alterations on the fibrils produced would reflect on the DDR1 activation and perhaps activate different signaling molecules compared to normal collagen I fibrils.

Several studies have shown that DDRs and integrins can co-operate or antagonize each other, regarding signaling pathways and subsequent cellular process (Leitinger 2014; Valiathan et al. 2012). Two independent studies using MDCK cells, have shown that DDR1 expression can inhibit integrin-induced cell migration and adhesion (Wang et al. 2006; Yeh et al. 2009). On the other hand, studies using pancreatic cell-lines
have shown that the DDRs can work synergistically with integrins in order to induce EMT and cell scattering (Shintani et al. 2008). In HEK-293 cells, the DDRs were shown to modulate the activation state of α1β1 and α2β1 integrins (Xu et al. 2012). Nevertheless, Vogel et al. (2000) showed that DDR1 autophosphorylation can occur independently of β1 integrin function. To this end, they used T47D cells pre-incubated with antibodies that block α2 or β1 integrin function. In our study, we tested the DDR1-integrin interaction, using collagen I both in soluble and fibrillar form. We showed that DDR1 activation by collagen I fibrils occurs independently of β1 integrin function. Moreover, we demonstrated that DDR1 autophosphorylation is independent of the β1 integrin-fibril interaction (see Figure 3.13A-D).

Surprisingly, there is little information in the literature on the integrin-collagen I interaction when collagen I is in fibrillar form. Previous literature has identified the integrin binding motifs present on soluble collagen I using synthetic triple-helical collagen peptides: GFOGER where O is hydroxyproline was identified as a high-affinity binding motif for α1β1, α2β1 and α11β1 (Knight et al. 1998; Knight et al. 2000; Zhang et al. 2003). GLOGER was also identified as a α1β1 moderate-affinity binding motif (Xu et al. 2000). GROGER and GMOGER were also identified as α2β1 high- and moderate-affinity binding motifs respectively (Siljander et al. 2004; Raynal et al. 2006). However, as mentioned earlier, those studies were conducted using triple-helical peptides. How integrins interact with fibrils remains still unclear. A study by Jokinen et al (2004), studied the interactions of α1β1 and α2β1 integrins with collagen I fibrils and soluble collagen I, using both recombinant integrins and integrins expressed on the surface of Chinese hamster ovary cells. The α1I and the α2I recombinant integrins, which include the α1 and α2 ligand binding site respectively, consisted of the amino-acids 123-338 and 124-339 respectively tagged in the N-terminus with a GST construct. Using solid phase binding assay Jokinen et al. (2004) demonstrated that both α1I and α2I proteins bound weaker to fibrils in comparison to soluble collagen I. When tested using immunoelectron microscopy though, they saw that α2β1 bound to fibrils with the same avidity as to soluble collagen I. Finally, they showed that different level of collagen aggregation resulted in different cellular function: Chinese hamster ovary cells transfected with α2β1
integrins incubated with fibrils formed cellular projections whereas cells incubated with soluble collagen I formed lamellipodia. They also showed that α2β1-transfected cells spread with the same efficiency on collagen I fibrils and soluble collagen I whereas α2β1-transfected cells spread on soluble collagen I with higher efficiency (Jokinen et al. 2004). A recent study by Woltersdorf et al. (2017) using immunoelectron microscopy showed that human soluble β1 integrins encompassing the extracellular integrin region can only bind to soluble collagens II, IX or XI but not to authentic cartilage fibrils. Based on their results, the authors suggested that integrins interact with collagen fibrils via non-collagenous components present in the articular cartilage (Woltersdorf et al. 2017).

At the beginning of this PhD we attempted to study the interactions of cartilage fibrils with DDR2. As mentioned earlier, articular cartilage contains complexes of collagens II/IX/XI or II/XI (Eyre 2002; Cremer et al. 1998). DDR2 is present on the surface of chondrocytes, the only cellular constituent of articular cartilage (Lin Xu et al. 2005). A number of studies have shown that in early stages of OA DDR2 is up-regulated, causing degradation of the collagen II network present in the articular cartilage via MMP-13 over-expression. It has been supported that this up-regulation is due to DDR2 interaction with collagen II (Lin Xu et al. 2005; Xu et al. 2007; Xu et al. 2010). We therefore wanted to analyse the ability of cartilage fibrils to induce DDR2 activation. Our collaborator, Dr. Uwe Hansen, provided us with evidence, based on immunoelectron microscopy, showing that recombinant DDR2 protein, consisting of the extracellular DDR2 region fused to the Fc region of human IgG1 antibody (Xu et al. 2011a), can bind to fibrils reconstituted from collagen complexes II/IX/XI and II/XI. Based on those data, we tried to reconstitute cartilage fibrils using soluble collagens II, IX or XI from chick cornea provided to us by Dr. Hansen. Using Western blot assays, we showed that DDR2 could be activated by collagens II/IX/XI in fibrillar form. However, further experiments established that fibrillogenesis was not complete. Due to the inability to produce collagen complexes II/IX/XI purely in fibrillar form, we changed the focus of the project and worked on the interactions of DDR1 with collagen I fibrils.
Another observation obtained from our data was that DDR1 can serve as an adhesion receptor. As shown in Figure 3.13, even when β1 integrin function was hindered, cells managed to adhere onto soluble collagen I or collagen I fibrils, suggesting that DDR1 can also serve as an adhesion receptor. However, we noticed that cells could not spread very well onto collagen when they were pre-incubated with the P5D2 antibody. This could be due to blocking of the β1 integrin function, however, it could also be due to steric hindrance promoted by the P5D2 antibody. To exclude the second, we could incubate cells with β1 si-RNA and then test for DDR1 autophosphorylation. Our results agree with previously published literature, where it was demonstrated that DDR1 can contribute to integrin-mediated cell-adhesion (Xu et al. 2012). This is supported by two independent studies using primary mesangial cells derived from mice or human pituitary adenoma cells showed that DDR1 knock down resulted in lower cell adhesion in comparison to controls (Curat 2002; Yoshida & Teramoto 2007).

Collagen I fibrils appear to mediate weak DDR2 autophosphorylation as shown by Western blotting data using in vitro generated fibrils. Data by others have attributed G0/G1 cell cycle arrest to weak DDR2 activation by collagen I fibrils, using human melanoma and fibrosarcoma cell lines A2058 and H1080 (Wall et al. 2005). However, the authors do not describe in much detail the fibrillogenesis protocol they followed (Wall et al. 2005). We used immunostaining to analyse DDR2 activation by fibrils, which showed that a variety of immunofluorescence intensities were produced (see Figure 3.15A). However, all cells that remained on the coverslips were activated, showing that collagen I fibrils serve as a functional DDR2 ligand. It was surprising though, that cells incubated with equine fibrils showed no DDR2 autophosphorylation (see Figure 3.15B). This could be due to the fact that fibrils did not occupy a large area of the coverslip therefore only few cells would have come into contact with the fibrils. A future experiment could be to include an increase in molarity of immobilized equine fibrils. Another reason for the discrepancy of results obtained using in vitro fibrils and equine fibrils could be that equine fibrils offer additional DDR2 binding sites that promote an inactive DDR2 conformation, similar to what was found in the studies performed by Konitsiotis et al. (2008) and Xu et al.
Using triple-helical collagen-mimetic peptides derived from collagen II and collagen III, it was found that DDR2 has binding sites on these fibrillar collagens in addition to the GVMGFO motif. Triple-helical peptides encompassing these additional binding sites were not able to promote DDR2 activation, in contrast to GVMGFO-containing peptides (Xu et al. 2011a). However, those studies are limited in the sense that they tested only DDR binding to peptides derived from collagens II and III, not collagen I (Konitsiotis et al. 2008; Xu et al. 2011a).

Finally, we started to analyze whether DDR activation by collagen I fibrils can induce the same signaling pathways as soluble collagen I. Our data were consistent with data obtained by Hilton et al. 2008: ERK was phosphorylated after DDR1 stimulation with soluble collagen I in transfected HEK293 cells. Moreover, our data showed that DDR1 activation by collagen I fibrils can induce weak ERK phosphorylation, of approximately half signal strength compared to soluble collagen I. On the other hand, DDR2-transfected cells showed no ERK phosphorylation when incubated with soluble collagen I. Regarding phosphorylation of DDR2-expressing cells incubated with collagen I fibrils, we cannot conclude whether there is any association between ERK-DDR2 phosphorylation since DDR2 activation was very weak.

Another signaling pathway that appeared to be activated with DDR activation was the PI-3 kinase/Akt pathway. Published literature has shown that DDR1 activation in cancer cells and mouse embryonic cells can activate Akt (Suh & Han 2011; Ongusaha et al. 2003). Our results showed that phosphorylation of DDR1 tyrosines, induced by incubation with soluble collagen I or collagen I fibrils, leads to Akt phosphorylation in HEK-293 cells. DDR2 activation by soluble collagen I, in HEK-293 cells also identified Akt as a DDR downstream signaling molecule. Future experiments could include testing whether already known DDR1 downstream signaling molecules activated after DDR1 phosphorylation with soluble collagen I serve as signaling molecules for collagen I fibril-induced DDR1 activation using similar approach as for soluble collagen I. Such downstream signaling molecules could include SHP-1 or SHP-2, members of the Stat family or of the nuclear factor κB pathway, which have already
been known to get activated after DDR1 incubation with soluble collagen I (Abbonante et al. 2013; Wang et al. 2006; Leitinger 2014).

Not much is known about different functional consequences of DDRs interacting with fibrils in comparison to soluble collagen. Several studies have provided clues as to the cellular functions of the DDRs, but they were all undertaken with soluble collagen I as a DDR1 ligand. For example, in MDCK cells, migration was down-regulated as a consequence of DDR1 activation, whereas it was promoted in other cell lines such as leukocytes (Wang et al. 2006; Kamohara et al. 2001). In smooth muscle cells, DDR1 activation was shown to promote collagen remodeling in vitro (Hou et al. 2001; Hou et al. 2002). It would be interesting to see if the signal induced by collagen I fibrils can induce the same cellular functions. Furthermore, in the case of DDR1-induced cell migration, we would also need to test whether DR1 shedding takes place, since Shitomi et al. 2015 showed that shedding of DDR1 is necessary for DDR1-induced cell migration. Little is known about whether soluble collagen and fibrils induce different cellular behavior that is regulated by the DDRs. As mentioned earlier, a study by Wall et al. (2005) showed DDR2 promotes cell cycle arrest in human melanoma and fibrosarcoma cell lines A2058 and H1080 after incubation with collagen I fibrils but not with soluble collagen I (Wall et al. 2005). However, the exact protocol followed to induce in vitro fibrillogenesis was not accessible. In the publication it is referred to as fibrillar collagen gel, so one could assume that fibrillogenesis might not be complete and soluble collagen or aggregated material could be present in the fibrillar gel. Another study by Bhadriraju et al. (2009) showed that DDR2 activation by collagen I fibrils, but not by soluble collagen I, down-regulated Focal Adhesion Kinase in A10 smooth muscle cell line. Again, fibrils were produced as thin films, using alkanethiol surfaces (Bhadriraju et al. 2009). Such approaches do not assure that the gels produced include strictly collagen only in fibrillar conformation. To conclude, a major unresolved question that needs to be answered is whether DDR activation by collagen I fibrils induces different signaling pathways and subsequently different cellular outcomes compared to DDR stimulation by triple-helical collagens.
As mentioned earlier, many studies have applied collagen gels as a source of highly cross-linked collagen I material. Those approaches utilize compounds such as aldehydes, isocyanates, carbodiimide, epoxides or quinones to enhance the level of cross-linking between collagen molecules and thus synthesize robust fibrils (Yu et al. 2016; Zeugolis et al. 2009). These chemicals have been shown to affect the morphology of fibrillar surface and increase the fiber diameter (Zeugolis et al. 2009). Such fibrillar gels have been applied to test whether they can promote cellular processes such as cell migration, fibroblast contraction or tumor cell migration and whether such cellular processes are affected by the stiffness and the density of the gel produced (Cukierman & Bassi 2010). Fibrillar gels have been shown to acquire several of the in vivo ECM characteristics (Cukierman & Bassi 2010; Pedersen & Swartz 2005). However, we cannot exclude that such gels will not include collagens in soluble form and thus any cellular response cannot be solely attributed to the presence of collagen in its fibrillar form.
Chapter 4: DDR interactions with collagen VI tetramers and microfibrils

Introduction

Collagen VI is an abundant ECM protein present in many tissues such as skin, adipose tissue and skeletal muscles (Cescon et al. 2015). Findings from several studies demonstrate an architectural role for collagen VI, suggesting that it is vital for tissue integrity and linking of cells to other ECM components such as decorin, biglycan, hyaluronan and matrilin-1 (Wiberg et al. 2003; Specks et al. 1992; Wiberg et al. 2001). Collagen VI is a heterotrimer, consisting of three different α-chains forming a short triple-helical region flanked by large globular domains (Baldock et al. 2003; Hulmes 2008). 6 genes have been identified that code for the collagen VI α-chains (Fitzgerald et al. 2013). The number and type of globular domains that flank the triple-helical region vary, depending on the particular α-chains, but they all include one or more vWF A domains (Fitzgerald et al. 2013). Collagen VI is synthesized intracellularly in the form of triple-helical monomeric collagen. Monomeric collagen then assembles anti-parallel to produce dimers and dimers subsequently bind anti-parallel inducing the formation of tetramers (Baldock et al. 2003; Cescon et al. 2015). Tetramers are then secreted from cells and assemble laterally in order to produce the final collagen VI structure, microfibrils (Furthmayr et al. 1983; Kadler et al. 2007). Microfibrils, also called beaded microfilaments, is the final structure by which collagen VI is present in extracellular matrices. They consist of multiple units of triple-helical regions flanked by N- and C-globular domains or else called “bead regions”. This pattern appears with a D-period of 110nm (Baldock et al. 2003; Cescon et al. 2015).

Collagen VI constitutes a major component of articular cartilage, where collagen VI is located in the pericellular matrix (Buckwalter & Mankin 1998; Aigner 2003). The only
cellular component of articular cartilage are chondrocytes (Lin Xu et al. 2005). Detailed studies have shown that DDR2, which is present on the chondrocyte surface, plays a pathogenic role in OA progression (Aigner 2003; Li et al. 2007). Extensive studies by Xu et al. (2003, 2005, 2007, 2010) using mouse models or tissue samples from human OA patients showed that DDR2 up-regulation in chondrocytes induces activation of MMP-13, which causes degradation of the collagen II network and further DDR2 up-regulation. This cascade of events is thought to result in OA appearance (Xu et al. 2003; Lin Xu et al. 2005; Xu et al. 2007; Xu et al. 2010). The molecular details of the early stages of OA are still unknown, but several studies imply that degradation of proteoglycans located around chondrocytes in the pericellular matrix, enables the interaction of DDR2 with collagen II fibrils, secretion of MMP-13, degradation of the collagen II network and thereby the occurrence of OA (Xu et al. 2011b; Holt et al. 2012).

Another tissue where collagen VI is present in increased levels is the adipose tissue, where it is produced by adipose stem cells (Divoux & Clement 2011; McCulloch et al. 2015). It is suggested that collagen VI participates in its correct structural organisation, as reduction of collagen VI levels is associated with disorganized tissue (Alexopoulos et al. 2009). Collagen VI also forms an abundant component of skeletal muscles (Bonnemann 2011). It also participates in muscle regeneration, as null collagen VI mice were shown to have reduced ability for self-renewal of muscle stem cells (Urciuolo et al. 2013). Collagen VI mutations have severe impacts in bones, as they can lead to diseases such as Bethlem and myosclerosis myopathy (Jobsis et al. 1996; Merlìni et al. 2008). On a functional level, collagen VI participates in cell apoptosis via the JNK pathway (Cheng et al. 2011). However, it has been linked to promotion of breast cancer (Khan et al. 2009). In skeletal muscles, collagen VI has been demonstrated to participate in the correct mitochondria function and the protection of cells from apoptosis (Irwin et al. 2003; Grumati et al. 2010). Even though collagen VI participates in cell-survival pathways in skeletal muscles, and its deletion can result in cell apoptosis, it has not been clarified yet which transmembrane receptors are responsible for the transmission of collagen VI-induced signals inside the cells (Cescon et al. 2015).
While the interactions of the DDRs with fibrillar collagens are well established, much less is known about DDR interactions with non-fibrillar collagens. Dr. Uwe Hansen provided us with evidence based on immunoelectron microscopy showing that recombinant DDR2 protein consisting of the extracellular DDR2 region fused to the Fc region of human IgG1 (Xu et al. 2011a) can bind to collagen VI microfibrils. Based on this evidence, and intrigued by the literature that shows DDR2 is not activated by intact pericellular matrix (Vonk et al. 2011; Xu et al. 2010; Xu et al. 2011b), which contains collagen VI, we decided to test whether collagen VI can serve as a functional DDR ligand. Therefore, the aim of this chapter, was to analyse the interactions of both DDR1 and DDR2 with collagen VI tetramers and microfibrils. Particularly, we aimed to test whether collagen VI tetramers or microfibrils can bind to DDRs and induce DDR autophosphorylation. Furthermore we wanted to identify whether the triple-helical region of collagen VI tetramers or microfibrils was responsible for DDR activation. Finally, we tried to answer whether collagen VI binds to DDR2 using another binding site than the one used by fibrillar collagens.
Results

4.1 DDRs bind to collagen VI tetramers or microfibrils

In this project, we tested collagen VI tetramers and microfibrils isolated from bovine cornea, provided by Dr. Uwe Hansen, University of Muenster, for their interactions with DDR1 or DDR2 protein. As mentioned in Chapter 4 Introduction, preliminary immunoelectron experiments conducted by Dr. Hansen showed that collagen VI microfibrils can bind to DDR2-Fc protein. To isolate collagen VI from bovine cornea the tissue was homogenized with 10mM Tris-HCl, 400mM NaCl and 10mM CaCl₂, pH 7.4 and subsequently treated with protease inhibitors (Hansen et al. 2012). Next, the homogenized sample was digested overnight with bacterial collagenase type I, filtrated and finally fractionated using a Superose 6 column. A proportion of the purified collagen VI microfibrils was subjected to dialysis against 100mM sodium citrate pH 4.0 at 4°C in order for collagen VI microfibrils to dissociate into collagen VI tetramers. SDS-PAGE and Western blotting were applied to verify the presence of collagen VI tetramers and microfibrils and to verify that the material was free of contaminants (Hansen et al. 2012). The collagen VI isolation and purification procedure applied by Hansen et al. (2012) is very similar to that used by other groups (Baldock et al. 2003; Kielty et al. 1998). Apart from bovine cornea, collagen VI has been previously isolated from bovine ligaments or aorta, or mouse articular cartilage (Kiely et al. 1998; Baldock et al. 2003; Godwin et al. 2016). An additional source of purified collagen VI was from conditioned medium of HT1080 cells (Baldock et al. 2003).

To investigate whether the isolated collagen VI tetramers or microfibrils can act as DDR1 or DDR2 ligands, we applied solid phase binding assays using DDR1-Fc or DDR2-Fc construct respectively. Early studies that focused on DDR interactions with collagens I-III used DDR constructs fused C-terminally to the Fc fragment of human IgG1 sequence encompassing the DDR1 ectodomain (DDR1-Fc1) or the DDR2 DS
domain (DS2-Fc) (Leitinger 2003). The DDR2 construct containing the DDR2 ectodomain was not secreted from cells. However, DDR2 constructs encompassing the DDR2 ectodomain fused N-terminally to a His-tag and a Myc epitope (His-DDR2) were secreted from HEK293-EBNA cells (Leitinger 2003; Leitinger & Kwan 2006; Konitsiotis et al. 2008). In order to have comparable constructs, Xu et al. (2011a) produced new DDR constructs consisting of the DDR1 or DDR2 ectodomain fused to the same linker, the Fc fragment of human IgG2 sequence (DDR1-Fc and DDR2-Fc respectively, see Section 2.2.21) (Xu et al. 2011a). These constructs were used in this thesis. In the first step of the solid phase binding assay collagens were coated overnight at 4°C onto 96-well plates. The next day, wells were incubated with DDR1-Fc or DDR2-Fc, and binding was detected with anti-Fc antibodies conjugated to HRP. As shown in Figure 4.1, both DDRs bound to collagen I but did not bind to casein or BSA respectively, as expected. Both DDR1-Fc and DDR2-Fc bound to collagen VI tetramers or microfibrils. However, it is evident that DDR2-Fc bound to collagen I with higher affinity compared to DDR1-Fc. This result was consistent throughout this project. Moreover, DDR2-Fc bound with higher affinity to collagen VI tetramers or microfibrils than to collagen I, whereas DDR1-Fc bound to collagen I to a greater extent (and possibly with higher affinity) compared to collagen VI tetramers or microfibrils.
Figure 4.1: DDR1-Fc (A, B) or DDR2-Fc (C) binds to collagen VI tetramers or microfibrils. Collagens were coated at 10μg/ml onto 96-well plates and incubated overnight at 4°C. The next day wells were incubated with a DDR1-Fc (A, B) or DDR2-Fc (C) construct for 3h at room temperature and then an anti-Fc antibody conjugated to HRP was added to the wells. Absorbance was measured at 492nm. (A, B) Casein was used as negative control. Data are representative of two independent experiments. (C) Collagen IV was used as a negative control. Data show average means from three independent experiments. SEM error bars are depicted.
4.2 DDR1, but not DDR2 is activated by collagen VI tetramers or microfibrils

Data reported from several studies have shown that collagen binding to DDRs usually results in receptor autophosphorylation and that fibrillar collagens serve as functional ligands for both DDRs (Vogel et al. 1997; Shrivastava et al. 1997). However, the DDRs show collagen specificity for non-fibrillar collagens; collagen IV binds and activates only DDR1 whereas collagen X can serve only as a DDR2 ligand (Vogel et al. 1997; Leitinger 2003; Leitinger & Kwan 2006; Shrivastava et al. 1997). After demonstrating that both DDRs can bind to collagen VI, we investigated whether collagen VI can induce DDR autophosphorylation. To this end, HEK-293 cells were transfected with full-length DDR1 or DDR2 expression vector, serum-starved for 16h and then incubated with fresh medium that contained serial dilutions of collagen VI tetramers or microfibrils. Cells were incubated with the respective solution for 90min at 37°C before cell lysis. Lysates were analysed by SDS-PAGE and Western blotting. As shown in Figure 4.2A, DDR1-expressing cells incubated with collagen I showed strong 4G10, PY513 (phosphorylation of a specific tyrosine present in the intracellular juxtamembrane region of DDR1b, tyrosine 513) or PY792 signals, as expected. Cells stimulated with collagen VI tetramers showed strong phosphorylation signals (4G10, PY513 or PY792), even at low collagen VI concentrations (2-5μg/ml). Figure 4.2B depicts results from cell stimulation with collagen VI microfibrils. Again, cells stimulated with collagen VI microfibrils showed strong phosphorylation signals (4G10, PY513 or PY792), with clear signals from 2μg/ml of collagen VI microfibrils. Therefore we can conclude that collagen VI is a potent ligand for DDR1.

Figure 4.3A and B depict results from DDR2-transfected cells stimulated with collagen VI tetramers and microfibrils respectively. Cells incubated with collagen I showed strong 4G10 or PY740 signals, as expected. In sharp contrast to the situation with DDR1, however, DDR2-expressing cells incubated with collagen VI tetramers or microfibrils did not show any consistent phosphorylation signal even when
incubated with high collagen VI concentrations (100μg/ml and 50μg/ml respectively). These results were consistently obtained, since nine independent repeats showed no DDR2 activation by different batches of collagen VI. This was a very unexpected result, considering the previous literature on DDR collagen binding and receptor activation (Shrivastava et al. 1997; Vogel et al. 1997; Leitinger 2003; Leitinger & Kwan 2006). However, as mentioned in the Introduction to this chapter, in mice, DDR2 was shown to be inactive when surrounded by intact pericellular matrix, which is rich in collagen VI (Xu et al. 2011b). In this context, our results are in agreement with these findings.
Figure 4.2: DDR1 is activated by collagen VI tetramers or microfibrils. HEK-293 cells were transiently transfected with full-length DDR1a (4G10 and PY792 signals) or DDR1b (PY513 signal) expression vector, serum-starved for 16h and incubated with 2, 5, or 10μg/ml collagen I or 2, 5, 10, 20, 50μg/ml collagen VI tetramers (A) or microfibrils (B) for 90min at 37°C. Non-stimulated cells were used as a negative control. After stimulation, cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates depicted were probed for general tyrosine phosphorylation (4G10), phosphorylation of DDR1 tyrosine-513 (PY513) or phosphorylation of DDR1 activation loop tyrosine-792 (PY792), as indicated. After imaging, the blots were stripped and re-probed for total DDR1 levels (lower blots). The molecular weight of the marker is depicted at the left side of the blots in kDa. The experiments probed for 4G10 signal were repeated three times with similar results whereas the experiments probed for PY513 or PY792 signals were performed once. Quantification of 4G10, PY513 or PY792 signals, normalised to DDR1 levels, are shown relative to baseline levels of non-stimulated cells, which is set as 1.0. These values are shown underneath the respective blot. (C, D) Quantification of phosphorylation levels (4G10) after stimulation with collagen VI tetramers (C) or microfibrils (D) relative to total DDR1 levels from two and three independent experiments respectively using image J. Statistical analysis was performed, comparing unstimulated cells with cells incubated with collagen I (2, 5, 10μg/ml) or collagen VI microfibrils (2, 5, 10, 20, 50μg/ml) using unpaired t-test. All results showed statistical significance, **p<0.005, *p<0.05. SEM error bars are depicted. CI, collagen I; CVIT, collagen VI tetramers; CVIM, collagen VI microfibrils.
Figure 4.3: DDR2 is not activated by collagen VI tetramers (A) or microfibrils (B). HEK-293 cells were transiently transfected with full-length DDR2 expression vector, serum-starved for 16h and incubated with 2, 5, or 10μg/ml collagen I or 2, 5, 10, 20, 50, 100μg/ml collagen VI tetramers (A) or 2, 5, 10, 20, 50μg/ml microfibrils (B) for 90min at 37°C. Non-stimulated cells were used as a negative control. After stimulation, cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates were probed for general tyrosine phosphorylation (4G10), for phosphorylation of DDR2 activation loop tyrosine-740 (PY740) or for total DDR2 levels, as indicated. Quantification of 4G10 and PY740 signal, normalised to DDR2 levels, is shown relative to baseline levels of non-stimulated cells, which is set as 1.0. These values are shown underneath the respective blot. The molecular weight of the marker is depicted at the left side of the blots in kDa. The experiments were performed one time (PY740) or three times (4G10) with similar results. (C) Quantification of phosphorylation levels (4G10) after stimulation with collagen VI microfibrils relative to total DDR2 levels from three experiments using image J. Statistical analysis was performed, comparing unstimulated cells with cells incubated with collagen I (2, 5, 10μg/ml) or collagen VI microfibrils (2, 5, 10, 20, 50μg/ml) using unpaired t-test. Results were found not statistically significant. SEM error bars are depicted. CI, collagen I; CVIT, collagen VI tetramers; CVIM, collagen VI microfibrils.
4.3 Immunostaining confirms that DDR1, but not DDR2 is activated by collagen VI tetramers or microfibrils

To confirm the results obtained by Western blotting, we used immunostaining. Cos-7 cells were seeded onto coverslips, transfected in parallel with full-length DDR1b or DDR2-FLAG expression vectors, serum-starved for 16h and then incubated with medium containing collagen VI tetramers or microfibrils. Cells were fixed, permeabilized and stained using an antibody that reacts both with DDR1 and DDR2: it detects phosphorylation of tyrosine 796 present in the DDR1 activation loop or the equivalent tyrosine 740 present in the DDR2 activation loop respectively. As shown in Figure 4.4A, DDR1 cells incubated with collagen I showed strong PY796 signal whereas non-stimulated cells showed no PY796 signal, as expected. Cells incubated with collagen VI tetramers or microfibrils also showed strong PY796 signals. Figure 4.4B depicts immunostaining results of DDR2-transfected cells incubated with collagen VI tetramers or microfibrils. Results were similar as those obtained from Western blotting. While collagen I induced strong PY740 signals, cells incubated with collagen VI tetramers or microfibrils showed no PY740 signal.
Figure 4.4: DDR1, but not DDR2 is activated by collagen VI tetramers or microfibrils. Cos-7 cells were transiently transfected with wt-DDR1b (A) or DDR2-FLAG (B) serum-starved for 16h and incubated for 90min at 37°C with 10μg/ml soluble collagen I, collagen VI tetramers of collagen VI microfibrils. Non-stimulated cells (NS) were used as a negative control. Cells were then fixed, permeabilized and co-stained using an antibody that recognizes phosphorylation of activation loop tyrosines, tyrosine 796 in DDR1 or tyrosine 740 in DDR2 (PY796 and PY740) followed by Alexa 555 anti-rabbit IgG antibody and a mouse anti-DDR1 (A) or mouse anti-FLAG (B) antibody followed by Alexa 488 anti-mouse IgG antibody. The experiment was performed once. Representative images are shown. At least 18 cells were imaged for each condition. Images were taken using wide-field microscopy at 60x magnification. Scale bar=30μm.
4.4 Only native, not denatured collagen VI tetramers or microfibrils can bind to the DDRs and induce DDR1 autophosphorylation

Our data so far support that collagen VI tetramers and microfibrils can serve as DDR ligands. As mentioned earlier, detailed studies using synthetic triple-helical peptides encompassing overlapping sequences coding for the triple-helical region of collagen II or III identified the GVMGFO motif as a key binding site for both DDR1 and DDR2 (Konitsiotis et al. 2008; Xu et al. 2011a). Earlier findings showed that gelatin, which is heat-denatured collagen I, cannot act as a functional DDR ligand (Vogel et al. 1997; Shrivastava et al. 1997; Leitinger 2003). Only collagen that adopts the triple-helical conformation can bind and activate DDRs. The underlying reason was realized from a crystallographic study by Carafoli et al. (2009) that identified an amphiphilic trench on the surface of the DDR2 DS domain into which the GVMGFO motif is accommodated. They further revealed that the leading and middle chains of the triple-helix are responsible for the ability of collagens I-III to bind to DDR2. Because the DDR binding site needs residues from two different collagen α-chains, it follows that denatured collagens I-III cannot act as DDR ligands (Carafoli et al. 2009). We hypothesized that this would also be the case for collagen VI. To this end, I performed solid phase binding assays where I coated wells with native or denatured collagen VI tetramers or microfibrils. Collagens were denatured by incubation for 30min at 70°C before being coated onto 96 well-plates overnight at 4°C. The next day, wells were incubated with a DDR1-Fc or DDR2-Fc construct and binding was detected with anti-Fc antibodies conjugated to HRP. As shown in Figure 4.5A, DDR1-Fc did not bind to denatured collagen I, as expected. DDR1-Fc bound to collagen VI tetramers or microfibrils when present in native form but did not bind to them when they were denatured. Similarly, in Figure 4.5B, DDR2-Fc did not bind to denatured collagen I, whereas it bound to native collagen I, as expected. DDR2-Fc bound to collagen VI tetramers or microfibrils when present in native form but did not interact with them when they were denatured.
DDR1 activation by native vs denatured collagen VI tetramers or microfibrils was analysed by Western blotting of cell lysates. HEK-293 cells were transfected with DDR1a expression vector, serum-starved for 16h and then incubated with medium that contained native or denatured collagen VI tetramers or microfibrils for 90min at 37°C. Next, cells were lysed and lysates were analysed by SDS-PAGE and Western blotting. As shown in Figure 4.6, cells incubated with native collagens showed 4G10 signal, as expected, while cells incubated with denatured collagens only showed background 4G10 signals, demonstrating that DDR1 activation by collagen VI requires its native conformation.
Figure 4.5: DDR1-Fc (A) or DDR2-Fc (B) binds specifically to native collagen VI tetramers and microfibrils. Collagens were coated at 10μg/ml onto 96-well plates and incubated overnight at 4°C. In order to denature collagens, collagen I and collagen VI tetramers or microfibrils were incubated for 30min at 70°C prior to coating. The next day wells were incubated with a DDR1-Fc (A) or DDR2-Fc (B) construct for 3h at room temperature and then an anti-Fc antibody conjugated to HRP and substrate were added to the wells. Absorbance was measured at 492nm. The experiment was performed one time.
Figure 4.6: DDR1 is activated specifically by native collagen VI tetramers or microfibrils. HEK-293 cells were transfected with full-length DDR1α expression vector, serum-starved for 16h and incubated with 10μg/ml native (N) or denatured (D) collagens for 90min at 37°C. Non-stimulated cells were used as a negative control. Denatured collagens were incubated for 30min at 70°C prior to cell stimulation. Cells were lysed and lysates were analysed by SDS-PAGE and Western blotting. Lysates were probed for general tyrosine phosphorylation levels (4G10) or phosphorylation of DDR1 activation loop tyrosine 792 (PY792) (upper blots). After imaging, blots were stripped and re-probed for total DDR1 levels (lower blots). The molecular weight of the marker is depicted at the left side of the blots in kDa. The experiment was performed one time.
4.5 DDR1 binds and is activated by the triple-helical region of collagen VI tetramers and microfibrils

Collagen VI monomers (another term used for collagen in triple-helical form) consist of short triple-helical regions flanked by large N- and C-globular domains (Furthmayr et al. 1983; Baldock et al. 2003). As mentioned in the Introduction to this chapter, collagen VI microfibril assemblies consist of tetramers that associate laterally (Furthmayr et al. 1983; Hulmes 2008). Studies using automated electron tomography support that the D-period of this conformation is 110nm, the length of the triple-helical region is 60.3nm whereas the length of the N- and C-globular domains flanking the collagenous domain (bead region) is 52.6nm (see Figure 1.5B) (Baldock et al. 2003). It is evident that the triple-helical region of collagen VI is much smaller compared to the fibrillar triple-helical region, which is 300nm (see Section 1.1) (Banos et al. 2008). Collagen VI monomers reach approximately 500kDa molecular weight (triple-helical region is 336 amino acids approximately) whereas the size of the N- and C-globular domains vary depending on the number of vWF type A they contain, each of which has 21kDa molecular weight (200 amino acids) (Baldock et al. 2003; Cescon et al. 2015; Lamandé et al. 2006). The C-terminal of α3-chains is 88kDa (Lamandé et al. 2006). So far we showed that native, full-length collagen VI tetramers or microfibrils bind to both DDRs and induce DDR1, but not DDR2 autophosphorylation. Furthermore, heat denaturation of collagen VI abolished DDR binding and DDR1 activation, implying that the DDR binding site is contained in the triple-helical regions of collagen VI, and that binding to this region is sufficient to induce DDR1 autophosphorylation. To test this assumption, our collaborator, Dr. Uwe Hansen, provided us with collagen VI tetramers or microfibrils that consisted only of the triple-helical region, with no globular domains present (pepsinized collagen VI tetramers or microfibrils). First, we analysed DDR1 binding to pepsinized collagen VI tetramers or microfibrils by solid phase binding assays: 96-well plates were coated with collagen VI tetramers or microfibrils, full-length or pepsinized, then incubated with a DDR1-Fc construct and finally with an anti-Fc antibody conjugated to HRP. As shown in Figure 4.7A, DDR1-Fc bound to pepsinized collagen
VI tetramers, however with considerably lower affinity compared to full-length collagen VI tetramers. Similarly, as shown in Figure 4.7B, DDR1-Fc also bound to pepsinized collagen VI microfibrils. Interestingly, DDR1-Fc bound to pepsinized microfibrils with similar affinity as to full-length microfibrils. However, pepsinized microfibrils were tested very quickly after they were received whereas pepsinized tetramers were used after several months of storage at 4°C. We therefore reasoned that the lower binding signals obtained with pepsinized tetramers might be due to this material not being very stable and that it likely lost its DDR binding capacity after prolonged storage at 4°C.

We next analysed whether pepsinized collagen VI can induce DDR1 autophosphorylation. To this end, DDR1b-expressing HEK-293 cells were incubated with medium that contained collagen VI tetramers or microfibrils, full length or pepsinized. Western blotting of cell lysates showed that cells incubated with pepsinized collagen VI tetramers or microfibrils showed increased 4G10 signals, albeit not as strong as the signal induced by full-length tetramers or microfibrils (Figure 4.8). However, the data were not as robust as those obtained with full-length collagen VI. The experiment was repeated five times, with pepsinized tetramers inducing DDR1 phosphorylation only twice, whereas pepsinized microfibrils activated DDR1 only one time. It was noticed that the positive signals were obtained when the pepsinized collagen VI was used soon after purification, while it lost its ability to induce DDR1 phosphorylation when stored at 4°C for more than two months. Overall, pepsinized collagen VI from three different batches was tested. The first batch of pepsinized collagen VI was used at several time points, varying from one month to more than three months after obtained. The next two batches were used within one to two weeks after storage at 4°C. However, they were stored at 4°C for some time before being shipped to us. This further supported our hypothesis that the pepsinized material was not very stable and likely lost its binding capacity after prolonged storage at 4°C.
Figure 4.7: DDR1-Fc binds to the triple-helical region of collagen VI tetramers (A) or microfibrils (B). Casein, collagen I and collagen VI tetramers, full-length or pepsinized, (A) or collagen VI microfibrils, full-length or pepsinized, (B) were coated at 10μg/ml onto 96-well plates and incubated overnight at 4°C. The next day wells were incubated with a DDR1-Fc construct for 3h at room temperature and then an anti-Fc antibody conjugated to HRP was added to the wells. Absorbance was measured at 492nm. Casein was used as negative control. (A) The experiment was performed one time. Pepsin. Collagen VI Tetramers; pepsinized collagen VI tetramers (B) Data are representative of two independent experiments. Pepsin. Collagen VI Microfibrils; pepsinized collagen VI microfibrils
Figure 4.8: DDR1 is activated by the triple-helical region of collagen VI tetramers or microfibrils. HEK-293 cells were transiently transfected with full-length DDR1b expression vector, serum-starved for 16h and incubated with 10μg/ml collagen VI tetramers or microfibrils, 10μg/ml pepsinized collagen VI tetramers (Pep. Collagen VI tetramers) or 10, 20μg/ml pepsinized collagen VI microfibrils (Pep. Collagen VI microfibrils) for 90min at 37°C. Non-stimulated cells were used as a negative control. After stimulation, cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates depicted at the upper blot were probed for general tyrosine phosphorylation (4G10). After imaging, the blot was stripped and re-probed for total DDR1 levels (lower blot). Quantification of 4G10 signal, normalised to DDR1 levels, after incubation of cells with collagen I or collagen VI tetramers or microfibrils, full-length or pepsinized, is shown relative to baseline levels of non-stimulated cells, which is set as 1.0 is shown underneath the respective blot. The molecular weight of the marker is depicted at the left side of the blots in kDa. The experiment was repeated five times, pepsinized tetramers induced DDR1 phosphorylation two times whereas pepsinized microfibrils induced DDR1 phosphorylation one time.
4.6 Collagen I and collagen VI do not compete for binding to DDR2

As shown in Sections 4.1 and 4.2, DDR2 can bind to collagen VI tetramers or microfibrils but collagen VI tetramers or microfibrils cannot induce DDR2 autophosphorylation. These data raised the possibility that collagen VI and collagen I have different binding sites on DDR2. As explained earlier, the binding pocket for the DDR binding motif on fibrillar collagens, GVMGFO, has been defined for DDR2 using structural biology (Carafoli et al. 2009). Carafoli et al. (2009) solved the crystal structure of the DDR2 DS domain bound to a synthetic triple-helical peptide containing the GVMGFO motif. This revealed that five protruding loops in the DS domain form a trench that accommodates the GVMGFO motif (Carafoli et al. 2009). In order to analyse whether DDR2 binds to collagens I and VI in the same binding mode, I analysed whether collagen I and collagen VI would compete for binding to DDR2-Fc using solid phase binding assay. In order to verify our assay, competition between ligands that are known to use the same binding pocket was tested first; we verified whether collagen II would compete with collagen I for binding to DDR2-Fc, as we would expect them to, because they both contain the GVMGFO motif as their main binding site for binding to DDR2 (Konitsiotis et al. 2008). To this end, 96-well plates were coated with 10μg/ml collagen II overnight at 4°C. The next day, DDR2-Fc was pre-incubated with serial dilutions of collagen I for 30min at room temperature. DDR2-Fc was used at 5μg/ml since that was found to be its half-maximal binding concentration in our solid phase assay that analysed DDR2 binding to collagen I (see Figure 4.1B). A proportion of DDR2-Fc without collagen I pre-incubation was used as a positive control. DDR2-Fc was also pre-incubated with serial dilutions of denatured collagen I for 30min at room temperature, which served as a specificity control. DDR2-Fc, with or without collagen I pre-incubation, was added onto collagen II-coated wells and absorbance was measured at 492nm. As shown in Figure 4.9A, DDR2-Fc without collagen I pre-incubation bound to collagen II, as expected. When DDR2-Fc was pre-incubated with native collagen I, DDR2-Fc binding efficiency to collagen II decreased with increasing collagen I concentrations. Pre-incubation with
denatured collagen I, on the other hand, did not reduce binding of DDR2-Fc to collagen II. These data show competition between two ligands that bind to the same binding site in DDR2.

After establishing that the competition assay was working, I applied it to analyse whether collagen I competes with collagen VI for binding to DDR2-Fc. In this case, 96-well plates were coated with collagen VI overnight at 4°C. Again, the same steps were applied as for collagen II competition assay with collagen I: DDR2-Fc was pre-incubated with serial dilutions of native or denatured collagen I. DDR2-Fc was added to the wells and absorbance was measured at 492nm. As shown in Figure 4.9B, DDR2-Fc without collagen I pre-incubation bound to collagen VI, as expected. However, in this case, pre-incubation with native collagen I, did not reduce binding of DDR2-Fc to collagen VI, even when high collagen I concentrations were used (100μg/ml). These data indicate that collagen VI binds to DDR2-Fc by occupying a different binding site on DDR to the one used by the fibrillar collagen motif GVMGFO.
Figure 4.9: Collagen I competes with collagen II for binding to DDR2-Fc (A), but does not compete with collagen VI (B). 96-well plates were coated with collagen II (A) or collagen VI (B) at 10μg/ml and incubated overnight at 4°C. 5μg/ml DDR2-Fc was then pre-incubated with 10, 25, 50 or 100μg/ml soluble collagen I, native or denatured, for 30min at room temperature. Next, DDR2-Fc, with or without collagen I pre-incubation, was added onto 96-wells and incubated for 3h at room temperature. Finally, an anti-Fc antibody conjugated to HRP and substrate were added to the wells. Absorbance was measured at 492nm. Data show average means from three independent experiments. Statistical analysis was performed using unpaired t-test. Absorbance was compared between DDR2 pre-incubated with serial dilutions of collagen I and DDR2 without pre-incubation. *p<0.05, **p<0.005. SEM error bars are depicted.
4.7 Collagen VI binds to a DDR2 protein that carries a mutation in the collagen I binding site

Another approach to verify that collagen VI binds to DDR2-Fc using another binding site than those used by the GVMGFO motif, was to perform binding assay with a DDR2 mutant that has reduced binding affinity to collagen I, DDR2-W52A-Fc (Carafoli et al. 2009). DDR2-W52A-Fc has a point mutation in a key ligand binding residue in the binding pocket for fibrillar collagens, Trp52. Mutation of Trp52 to Ala abolishes the ability of the construct to bind to collagen I (Ichikawa et al. 2007; Carafoli et al. 2009). We tested whether collagen VI can bind to DDR2-W52A-Fc. 96-well plates were coated with collagen VI microfibrils or collagen I, wells were incubated with 5μg/ml of the respective DDR2 construct and absorbance was measured at 492nm. Data show that DDR2-Fc bound to collagen I and collagen VI with similar affinities (see Figure 4.10A), as expected from my earlier experiments, and that DDR2-W52A-Fc had reduced binding affinity to collagen I (see Figure 4.10B), as expected from the published data (Ichikawa et al. 2007; Carafoli et al. 2009). However, DDR2-W52A-Fc was still able to bind to collagen VI to a considerable extent (see Figure 4.10B). These data show that Trp52 is not essential for binding to collagen VI and thus further reinforce the notion that collagen VI binds DDR2 at a different site than the fibrillar collagen binding pocket.
Figure 4.10: Solid phase binding assay of DDR2-Fc (A) and DDR2-W52-Fc (B) to collagen I or collagen VI. Collagens were coated at 10μg/ml onto 96-well plates and incubated overnight at 4°C. The next day wells were incubated with a DDR2-Fc (A) or DDR2-W52A-Fc (B) construct for 3h at room temperature and then an anti-Fc antibody conjugated to HRP was added to the wells. Absorbance was measured at 492nm. BSA was used as negative control. Data are representative of three independent experiments.
4.8 Collagen VI inhibits DDR2 phosphorylation by collagen I

Based on results in Sections 4.6 and 4.7, DDR2 seems to have different binding sites for collagen I and collagen VI. Furthermore, based on the those results, we would expect that collagen VI binding to DDR2 may not block DDR2 autophosphorylation by collagen I. Published literature has shown that a collagen-like protein produced in bacteria containing the DDR binding site (GVMGFP, without hydroxylation of the proline) can bind to DDR1 and DDR2 but does not activate the DDRs (An et al. 2016). This was shown by experiments in which DDR1- or DDR2-expressing cells were incubated with the collagen-like protein at 23°C or 37°C and analysis of cell lysates by SDS-PAGE and Western blotting. This led to the conclusion that the collagen-like protein functions as a DDR inhibitor (An, Abbonante, et al. 2016). Consequently, the next question we wanted to answer was whether collagen VI would inhibit DDR2 activation by collagen I. To this end, HEK-293 cells transfected with full-length DDR2 expression vector were serum-starved for 16h and then incubated with serial dilutions of collagen VI microfibrils in serum-free medium for 30min at 37°C. Next, cells (with or without pre-incubation with collagen VI) were seeded onto wells coated with laminin or collagen I and incubated for 90min at 37°C. Cell lysates were analyzed by SDS-PAGE and Western blotting. As shown in Figure 4.11A, cells without collagen VI pre-incubation showed strong 4G10 signal when stimulated with collagen I, as expected. The signal strength of cells pre-incubated with serial dilutions of collagen VI was inversely proportional to the collagen VI concentration used and was abolished when cells were incubated with 100μg/ml collagen VI microfibrils. These data indicate that collagen VI could serve as a regulating factor for DDR2 activation induced by fibrillar collagens. However, they were somewhat unexpected based on the data presented in Section 4.6 where I showed that in the context of the isolated DDR2 ectodomain collagen VI did not compete with collagen I for binding to DDR2. It should be noted, however, that in this context DDR2-Fc was pre-incubated with collagen I, then added to plates coated with collagen VI, whereas in this section full-length DDR2 on cells was pre-incubated with collagen VI and then added to plates.
coated with collagen I. A plausible hypothesis could be that collagen VI binding to DDR2 induces an inhibitory conformation that does not allow collagen I binding (as implied by the results in this section) but that collagen I binding to DDR2 does not induce such a conformation and still allows collagen VI to bind. However, it is also possible that results obtained with the isolated ectodomain and the full-length receptor are not directly comparable.

Our results indicate that collagen VI could serve as a limiting factor for DDR2 activation in articular cartilage, particularly in the pericellular matrix, where it would shield chondrocytes from activation by collagen II fibrils, such as has been speculated in the work of Yefu Li (Xu et al. 2011b; Xu et al. 2010; Li & Xu 2015). Published literature has shown that degeneration of the proteoglycan network induces DDR2 activation and OA progression (Xu et al. 2011b). Perhaps, additionally, collagen VI binds to DDR2 and keeps it in an inactive state.
Figure 4.11: Collagen VI microfibrils inhibit DDR2 phosphorylation induced by collagen I in three independent experiments (A, B, C). HEK-293 cells were transfected with a full-length DDR2 expression vector, serum-starved for 16h, re-suspended in serum-free medium and a proportion of cells was pre-incubated with 10, 25, 50 or 100µg/ml collagen VI microfibrils for 30min at 37°C. Cells without collagen VI pre-incubation were used as a positive control. Cells with or without collagen VI pre-incubation were seeded onto wells coated with 10µg/ml laminin or collagen I and incubated for 90min at 37°C. Cells were lysed and lysates were analysed using SDS-PAGE and Western blotting. Lysates on the left blot were probed for general phosphorylation levels (4G10) and blots on the right blot were probed for total DDR2 levels. Quantification of 4G10 signals, normalised to DDR2 levels, after incubation of cells with collagen I, is shown relative to baseline levels of non-stimulated cells, which is set as 1.0 is shown underneath the respective blot. The molecular weight of the marker is depicted at the left side of the blots in kDa. All three independent experiments are shown.
Discussion

In this part of the PhD, we studied the interactions of collagen VI tetramers or microfibrils with DDR1 and DDR2. First, using solid phase binding assays, we demonstrated that both DDR1 and DDR2 bind to collagen VI in the form of tetramers or microfibrils (see Figure 4.1A-C). We showed that consistently collagen VI tetramers or microfibrils bound to DDR2-Fc with somewhat higher affinity compared to collagen I, whereas collagen I bound with higher affinity to DDR1-Fc than collagen VI tetramers or microfibrils. It was also apparent that collagen VI tetramers or microfibrils bound with higher affinity to DDR2-Fc in comparison to DDR1-Fc. We also showed that binding of DDR1-Fc and DDR2-Fc to collagen VI tetramers or microfibrils depends on the native conformation of collagen VI, as denatured collagen VI did not bind to DDR1-Fc nor to DDR2-Fc (see Figure 4.5A and B respectively). We further showed that both collagen VI tetramers and microfibrils induced DDR1 autophosphorylation even at low concentrations (2μg/ml, 18.3nM) (Figure 4.2A and B). This result was also verified by immunostaining, where Cos-7 cells were incubated with 10μg/ml collagen VI tetramers or microfibrils in solution, with the use of an antibody that targets a tyrosine present in the DDR1 activation loop, tyrosine 796 (see Figure 4.4A). However, incubation of HEK-293 cells with the DDR1-IN-1 compound, which inhibits DDR1 autophosphorylation (Kim et al. 2013), prior to cell stimulation with collagen VI tetramers or microfibrils could further enhance our conclusion; if the characteristic 120kDa band was not present after incubation with the inhibitor it would further suggest that collagen VI can induce DDR1 autophosphorylation, however, if the band was present then it could be a result of another 120kDa protein phosphorylated, such as Focal Adhesion Kinase (Panetti 2002).

Collagen VI is present in several tissues, such as cornea, tendon, skin, articular cartilage, kidneys and blood vessels (Cescon et al. 2015). DDR1 is also found in most of these tissues and has been shown to be functional. For instance, DDR1 receptor on smooth muscle cells and macrophages participates in atherosclerosis progression,
by regulating both inflammation and fibrosis (Franco et al. 2008; Franco et al. 2009). Published literature has shown that atherosclerosis progression is contained in mice with DDR1 knockdown. Franco et al. (2008, 2009) demonstrated that low-density lipoprotein receptor deficient mice with knock-down of DDR1 protein were associated with containment of the atherosclerotic lesion area and increased synthesis of elastin and collagens I and III by smooth muscle cells in vitro. Furthermore, DDR1 deletion resulted in decreased macrophage presence in the atherosclerotic plaque at both early and later stages of the disease (Franco et al. 2008; Franco et al. 2009). Another two studies by Hou et al. (2001, 2002) using smooth muscle cells isolated from DDR1-null mice showed that invasion, migration and secretion of MMP-2 and MMP-9 were decreased in comparison to wild-type cells stimulated with collagen I (Hou et al. 2001; Hou et al. 2002; Ferri et al. 2004). An early study by Katsuda et al. (1992) using immunoelectron microscopy showed that collagen VI was localized in the same region with collagens I and III, the thickened intima of a human atherosclerotic plaque, in every stage of atherosclerosis (Katsuda et al. 1992). Our data supporting that collagen VI can serve as a DDR1 functional ligand may offer further understanding on the progression of atherosclerosis and the pathogenic role of DDR1 in this disease. While we do not know which collagen ligands are most important in the pathogenesis of atherosclerosis, our data open up the possibility that collagen VI-induced DDR1 activation may contribute to the regulation of fibrosis or inflammation, by promoting macrophage proliferation or matrix degradation. However, further experiments would need to be conducted to analyse the role of collagen VI in atherosclerosis, since other collagens such as collagens I, III and IV are also present in the blood vessels (Xu & Shi 2014). It would also be important to study whether DDR1 on macrophages and smooth muscle cells can be activated by collagen VI, since macrophages and smooth muscle cells have been shown to participate in atherosclerosis progression (Franco et al. 2008; Franco et al. 2009). To address this point, we would again incubate smooth muscle cells or macrophages with several dilutions of collagen VI and test whether DDR1 is activated, like in the case of HEK-293 cells.
Unlike DDR1, DDR2 was shown not to be activated by incubation with collagen VI tetramers or microfibrils (see Figure 4.3A and B), even when incubated with high collagen VI concentrations (100μg/ml). This result was also verified using immunostaining analysis, as DDR2-transfected cells incubated with collagen VI tetramers or microfibrils showed no phosphorylation signal when incubated with an antibody that recognizes phosphorylation of a specific tyrosine present in DDR2 activation loop, tyrosine 740 (see Figure 4.4B). As mentioned earlier, collagen VI is present in articular cartilage, particularly in the pericellular matrix surrounding chondrocytes, where DDR2 is present as a cell-surface receptor (Lin Xu et al. 2005; Aigner 2003). Published literature has shown that when pericellular matrix is intact, DDR2, which has a critical role in OA progression, is not activated (Xu et al. 2011b; Xu et al. 2010). Xu et al. (2010) studied OA progression in mice heterozygous in DDR2 gene, promoted either by producing heterozygous collagen XI gene or by destabilization of the medial meniscus. OA progression, which was evaluated using the Mankin scoring system and histology, was studied in relation to the expression levels of DDR2 and MMP-13, which were evaluated using immunohistochemistry. They showed that lower DDR2 and MMP-13 expression was related to attenuated OA appearance in mice heterozygous for both collagen XI and DDR2, compared to their wild type littermates (Xu et al. 2010). This result was obtained with mice either heterozygous for collagen XI or with destabilized meniscus. Finally, they also showed that OA appearance synchronized with proteoglycan and collagen II network degradation (Xu et al. 2010). The same group, in a later study, using a “tetracycline-regulated gene expression system”, which can alter the expression of the DDR2 gene without altering the stages of mouse development or the structure of the pericellular matrix, created transgenic mice and showed similar results as in their previous study: during OA progression DDR2 expression increased, as shown by immunohistochemistry and real-time PCR (Xu et al. 2011b). Furthermore, MMP-13 levels increased, as shown by immunohistochemistry. Additionally, they co-localized DDR2 and MMP-13 proteins (Xu et al. 2011b). Another study by Vonk et al. (2011) concluded that intact pericellular matrix inhibits DDR2 activation and they also suggested that DDR2 activation is linked to MMP-13 over-expression. First, they isolated chondrons, which are chondrocytes surrounded by pericellular matrix, and...
chondrocytes (with no pericellular matrix surrounding) from milk goats’ articular cartilage. These cells were incubated with immobilized soluble collagen I or collagen II, and MMP-13 and DDR2 gene expression levels were measured using real time PCR (Vonk et al. 2011). There was no elevation of MMP-13 or DDR2 gene expression when collagens I or II were incubated with chondrons, whereas collagens I or II induced increased MMP-13 and DDR2 gene expression when incubated with chondrocytes. Furthermore, when the DDR2 gene was silenced, this resulted in decreased MMP-13 gene expression levels (Vonk et al. 2011). Our Western blotting and immunostaining analysis (see Figure 4.3 and 4.4B respectively) agree with published literature in the sense that collagen VI, which is the main collagen found in the pericellular matrix, does not induce DDR2 autophosphorylation. So far, nobody has directly studied whether the pericellular matrix can induce DDR2 phosphorylation. It would be important to verify our results with cells that express endogenous DDR2, such as chondrocytes.

Even though we showed that collagen VI binds to the DDRs, we were not able to determine the specific collagen VI binding site in DDR1 or DDR2. Our results suggest, however, that the collagen VI binding site is different than the binding site for fibrillar collagens. In order to locate the collagen VI binding site, future experiments could include solid phase binding assays using DDR1 or DDR2 constructs constructed in our lab, lacking certain DDR domains. More specifically, two constructs lacking the DS-like (His-DS-DDR1) or DS domain (His-ΔDS-DDR1), both tagged in the N-terminus with a His tag and a Myc epitope, could be applied in solid phase binding assays to identify whether collagen VI binds to the DDR1 DS-like or DS domain respectively (Leitinger 2003; Xu et al. 2011a). With relevance to DDR2, the DS2-Fc construct, which consists mainly of the DDR2 DS domain (C-terminally tagged with human IgG1 Fc sequence), could be used to analyse whether collagen VI binds to the DDR2 DS or DS-like domain. A construct consisting only of the DDR2 DS-like domain could not be obtained, as the protein was not secreted from cells (Leitinger 2003; Xu et al. 2011a).

As mentioned earlier, our results indicated that collagen VI tetramers or microfibrils can bind strongly to DDR2 without inducing DDR2 autophosphorylation, which was
an unexpected result, since all other collagen types that were shown to bind the DDRs also induced DDR autophosphorylation (Leitinger 2011; Leitinger 2014). A study conducted by Leitinger et al. (2006) showed that the globular domain of collagen X represents another example of a DDR ligand that does not induce DDR kinase activation. More specifically, the authors demonstrated that both collagen X triple-helical region and non-collagenous C-terminal domain can bind to DDR2. However, only the triple-helical region could act as a functional DDR2 ligand, not the C-terminal non-collagenous domain (Leitinger & Kwan 2006). Another series of experiments, conducted by An et al. (2016) showed that a bacterial collagen-like protein (Scl2) can bind to both DDRs, as demonstrated by solid phase binding assays. This bacterial protein was engineered to contain the fibrillar DDR binding site, GVMGFP, (with proline instead of hydroxyproline). However, this protein was not able to induce DDR activation, even when tested at very high bacterial collagen concentrations (800μg/ml), at 23°C or 37°C (An et al. 2016). The reasons for the inability of the bacterial protein to activate the DDRs are unclear. Even though the molecular mechanism of how ligand binding results in DDR activation remains unanswered, a plausible hypothesis for the previous data is that binding of bacterial collagen to DDRs cannot promote DDR clustering in the required fashion or perhaps clustering cannot result in DDR activation. However, it cannot be ruled out that the synthetic peptide concentrations applied to activate the DDRs might not have been high enough, since collagen peptide-induced DDR1 activation takes place using much higher concentrations (6-12μM) in comparison to native collagens (0.5-1μM) (Konitsiotis et al. 2008; Xu et al. 2011a).

Our next aim, after identifying that collagen VI can promote DDR1 autophosphorylation, was to determine whether the collagen VI collagenous domain was responsible for DDR1 activation. We first demonstrated that both pepsinized tetramers and microfibrils could bind to DDR1-Fc (see Figure 4.7A and B). Our preliminary data also suggested that pepsinized collagen VI tetramers or microfibrils can serve as functional DDR1 ligands, even at low concentrations (10μg/ml) (see Figure 4.8). However, when the material was re-used several weeks later in an attempt to demonstrate reproducible results, the data we produced were not
conclusive (see Section 4.5). We hypothesized that this was due to the material not being stable enough in pepsinized form. This is in contrast to intact collagen VI, which appears to be very stable when stored at 4°C. The results we obtained with intact collagen VI (both solid phase binding assay and Western Blotting results) were consistent, even when the material was used years after its isolation and storage at 4°C. On the contrary, pepsinized material appears to be much less stable since binding results obtained by solid phase assays showed progressively less strong signals, and subsequent receptor activation results were negative. Protein stability of pepsinized collagen VI appears to be dependent on the α3-chain stability, as α3-chain appears to be more sensitive to pepsin-digestion compared to the other two α-chains. As a result, it is possible that pepsin-digestion could have resulted in limited amount of α3-chain, which in turn can affect the stability of the pepsinized collagen VI monomer (Lamande et al. 1998). If we received more stable pepsinized material, it would also be interesting to test whether the characteristic 120kDa phosphorylated band would disappear, as we would expect, after incubation of cells with the DDR1-IN-1 reagent, which has been shown to inhibit specifically DDR1 autophosphorylation (Kim et al. 2013).

We also analysed more in depth the DDR2-collagen VI microfibrils interaction. Using a competition solid phase binding assay, we demonstrated that collagen VI microfibrils do not compete with collagen I for binding to DDR2-Fc (see Figure 4.9B). This suggests that collagen I and collagen VI have different binding sites on DDR2. While DDR2-W52A binds only minimally to collagen I, this construct was able to bind much more strongly to collagen VI, suggesting that Trp52, a key residue in the DDR2-collagen I interaction, is not required for the DDR2-collagen VI interaction (Carafoli et al. 2009; Ichikawa et al. 2007). However, in some assays the DDR2-W52A-Fc protein showed unexpected high binding to collagen I (data not shown). The reasons for this are unclear at present. However, the DDR2-W52A-Fc construct was purified several years ago while DDR2-Fc was purified at the beginning of this PhD project (see Section 2.20.21). Both constructs (DDR2-Fc and DDR2-W52A-Fc) were centrifuged prior to solid phase assay to precipitate any aggregated material that might have been present. To obtain more robust results, the experiment would need to be
repeated with fresh DDR2-W52A-Fc protein. In order to avoid any association of soluble collagen I to higher-order aggregates, we could also repeat the experiment using collagen mimetic triple-helical peptides.

Finally, since our data suggested that collagen VI binds to DDR2 occupying a different binding site to the fibrillar collagen binding site, the next step would be to map the DDR1 and DDR2 binding site on collagen VI. Toolkit peptides encompassing overlapping amino acids of the collagen VI triple-helical region could be applied to identify possible binding motifs, like in the case of collagens II and III (Konitsiotis et al. 2008; Xu et al. 2011a). The reason for constructing peptides encompassing only the triple-helical region of collagen VI is that so far, no globular regions have been found to promote DDR activation. However, due to the fact that collagen VI is a heterotrimeric collagen, synthesis of such triple-helical peptides would be very difficult to be accomplished. Mapping of DDR2 DS domain residues that interact with a triple-helical peptide encompassing the fibrillar collagen binding site was possible using crystallography (Carafoli et al. 2009; Carafoli et al. 2012). Provided we could synthesize triple-helical peptides encompassing the collagen VI DDR binding site or use collagen VI consisting only of the triple-helical region, the same approach could be followed in order to map the collagen VI binding sites on DDR1 and DDR2.

Since collagen VI can bind strongly to DDR2 without inducing DDR2 autophosphorylation, we hypothesized that it could serve as an inhibitor of DDR2 autophosphorylation. To test this hypothesis, we pre-incubated cells with collagen VI to test whether it can inhibit collagen I-induced DDR2 phosphorylation. The results we obtained were very interesting, since we showed that collagen VI microfibrils can inhibit DDR2 activation by collagen I (see Figure 4.11A-C). However, our competition solid phase binding data showed that both collagens I and VI can bind to DDR2 (see Figure 4.9B). Since in the competition assay collagen VI was coated on the wells, whereas in the activation assay collagen VI was in solution, a plausible hypothesis could be that collagen VI binding promotes a DDR2 conformation that does not allow collagen I binding. On the other hand, collagen I binding still allows collagen VI to bind to DDR2. To verify this hypothesis, we would need to repeat the competition
assay using collagen VI in solution and immobilized collagen I. We did not test this hypothesis using DDR2 activation assays though, because pre-incubation of DDR2 with collagen I would promote receptor autophosphorylation. However, published literature has shown that collagen VI microfibrils can adopt more complex conformation after interaction with several ECM components such as SLRPs and matrillin-1 (Wiberg et al. 2001; Wiberg et al. 2003; Fresquet et al. 2010). Such interactions could be facilitated by integrins, particularly α1β1 integrin which has been shown to interact directly with collagen VI (Godwin et al. 2016; Loeser et al. 2000; Tulla et al. 2001). Collagen VI microfibrils could remain attached to such molecules when isolated, like in the study conducted by Beecher et al. (2011). Using mass spectrometry, they analysed the bovine cornea collagen VI microfibril fraction isolated by gel filtration chromatography and found that along with collagen VI, a small proportion of decorin protein was also present (Beecher et al. 2011). In that case, when collagen VI would be incubated with cells where also integrins would be present, bovine cornea collagen VI microfibrils could form higher-order complexes, and thus interact with cells in a different form than when coated on wells (as in the case in the competition solid phase assays).

Our data suggest that collagen VI may protect chondrocytes from collagen II-induced DDR2 activation, suggesting that collagen VI degradation could serve as a prerequisite for DDR2 activation, which would attenuate OA progression. However, further experiments would need to be conducted to make the hypothesis that collagen VI can act as a DDR2 inhibitor more robust. As mentioned earlier, previous studies suggest that intact pericellular matrix surrounding chondrocytes inhibits DDR2 activation and thus OA progression (Xu et al. 2011b). The pericellular matrix consists mainly of laminin, fibronectin, aggrecan, decorin, biglycan, cartilage oligomeric matrix protein and collagen VI (Li & Xu 2015). Manning et al. (2016) have shown that knockdown of DDR2 in the articular cartilage of adult mice can play a protective role against OA progression. Furthermore, they suggested that DDR2 can serve as a therapeutic target against OA progression (Manning et al. 2016). Several studies have identified several molecules with broad or restricted specificity that can serve as DDR inhibitors. For instance, imatinib and ponatinib are compounds with
broad target spectrum. Among their targets are tyrosine kinases such as DDRs (Canning et al. 2014; Day et al. 2008). Recently, molecules that can selectively inhibit DDR function have been discovered, such as DDR1-IN-1 and DDR1-IN-2 (Kim et al. 2013), which are type II inhibitors, 7rj and 7rh (Gao et al. 2013), which are pyrazoloura based molecules and compounds 2a, 4a and 4b (Richters et al. 2014). Compounds 2a, 4a and 4b have been also shown to inhibit DDR2 activation (Richters et al. 2014). Murray et al. (2015) using fragment-based drug discovery have further identified selective DDR1 and DDR2 inhibitors (Murray et al. 2015). However, whether or not these inhibitors could be developed into a drug therapy that could be administered for OA is not clear. Current possible methods of delaying OA progression include targeting molecules such as MMP-13 (Hellio Le Graverand-Gastineau 2009), Hif-2α (hypoxia inducible factor 2α)(Saito & Kawaguchi 2010) or Zip8, a Zn²⁺ reporter (Kim et al. 2014). However, more studies need to be conducted regarding the effects of silencing such molecules, both in cellular and tissue level.

An interesting future experiment would be to analyse the collagen VI-collagen I-DDR2 interaction using chondrocytes, as they express endogenous DDR2 protein levels, they are the only cell type present in articular cartilage and they are related to OA appearance (Goldring 2012). However, this can be tricky as chondrocytes tend to lose their phenotype and de-differentiate if cultured in monolayers even for a few passages (Schulze-Tanzil et al. 2004; Ma et al. 2013). This obstacle could be overcome if 3D pellet cultures were used. Protocols to obtain such 3D cultures are easy and straight-forward. Moreover, pellet cultures are formed very quickly so results are not compromised by collagens produced by the chondrocytes (Zhang et al. 2004). Another reason to use chondrocytes would be that there is a plethora of integrin receptors present on the chondrocyte surface, including α1β1, α2β1 and α10β1 integrins, which have been shown to interact directly with collagen VI (Tulla et al. 2001; Loeser et al. 2000; Woltersdorf et al. 2017). Integrins could facilitate collagen VI assembly into their final higher-order structure and thus promote a more physiologically-relevant final collagen VI conformation. Several studies by Wiberg et al. (2001, 2002, 2003) showed that collagen VI microfibrils bind with high affinity with SLRPs, particularly with biglycan or decorin (Wiberg et al. 2001; Wiberg et al.
They further showed that biglycan can regulate collagen VI assembly into higher-order conformations (Wiberg et al. 2002). Finally, they demonstrated that complexes of matrillin-1 with biglycan or decorin can link collagen VI microfibril molecules to aggrecan or collagen II (Wiberg et al. 2001; Wiberg et al. 2002; Wiberg et al. 2003). Other studies demonstrated that collagen VI networks can bind to other matrix components such as matrillin-1, perlecan or aggrecan (Wiberg et al. 2003; Vincent et al. 2007; Fresquet et al. 2010). Since those molecules affect collagen VI final structure, it would be very interesting if we could pre-incubate mixtures of collagen VI microfibrils with components such as SLRPs or matrilins-1 and cells, and test whether collagen VI could still inhibit collagen I-induced DDR2 autophosphorylation. This would allow us to imitate a more physiologically relevant collagen VI structure and test whether the interaction of collagen VI with the non-collagenous ECM components would block the DDR2 binding site on collagen VI and thus its ability to hinder collagen I-induced DDR2 activation.

In our experiments, we used collagen VI from bovine cornea, a common collagen VI source. Using collagen VI that has been isolated from articular cartilage would be a more physiologically relevant material for our collagen I-collagen VI-DDR2 interactions, since it is not clear whether collagen VI from cartilage and cornea have the same α-chain composition. Previous literature has shown that in mouse cornea, apart from the collagen VI α1-α3 chains, also α4-α6-chains are expressed (Gara et al. 2011). These α4-α6 chains could replace the α3-chain (Godwin et al. 2016). Therefore in cornea, collagen VI could consist of any of the α1, α2 and any of the α3-α6-chains. However, this is not the case for mouse articular cartilage, since α4-α6 chains have not been yet detected (Gara et al. 2011). This differential expression of α4-α6 chains suggests that perhaps articular cartilage presents different chain composition in comparison to cornea, and thus collagen VI from articular cartilage would be a more appropriate material to test the collagen VI-DDR2 interactions. However, the presence of collagen VI α4-α6 in bovine cornea is controversial, since another study by Beecher et al. (2011) using mass spectrometry analysis did not identify α4-α6 chains in bovine cornea (Beecher et al. 2011). Fitzgerald et al. (2008)
though identified α6-chain in human articular cartilage. Furthermore, they showed that collagen VI microfibrils consisting of α1-α3 chains were located around chondrocytes in articular cartilage, whereas collagen VI containing the α6-chain was localized further away, in the territorial matrix (Fitzgerald et al. 2008). This differential localization of α-chains even within human articular cartilage further suggests the necessity to test DDR2 activation by collagen VI isolated by articular cartilage, due to the α6-chain presence, which may result in different collagen VI final structure (Fitzgerald et al. 2008).

So far, our data suggest that DDR2 inactivity in intact articular cartilage could be attributed to its binding to the collagen VI microfibrils network acting as a DDR2 inhibitor, rather than the pericellular matrix presence restricting DDR2 from coming in contact with collagen II. However, such a hypothesis could only be verified by conducting further experiments many of which were mentioned in this discussion.
Chapter 5: General Discussion

This PhD thesis provides insight into the interactions of DDRs with collagen I fibrils generated in vitro and collagen VI. Furthermore, in this thesis I tested whether ECM secreted from a mouse fibroblastic cell-line could induce DDR autophosphorylation. Previous studies suggested that DDRs interact with or are activated by collagen I in fibrillar form (Wall et al. 2005; Juin et al. 2014; Coelho et al. 2017; Bhadriraju et al. 2009). However, in these studies, collagen I fibrils were generated in the form of collagen gels where the level of integration of soluble collagen I into fibrils had not been tested. My experiments suggest that collagen I fibrils can induce strong DDR1 autophosphorylation signals. Furthermore, collagen I fibrils induce DDR1 activation with the same kinetics as soluble collagen I, and the cellular location of DDR1 phosphorylation signals produced is reminiscent to the structure of the collagen I fibrils; therefore the location of phosphorylation is most likely where the fibrils bind to the cells. I also show that DDR2 is phosphorylated by fibrils to a much lower extent compared to DDR1.

The DDRs were also tested for their interactions with collagen VI tetramers and microfibrils. My analysis revealed that collagen VI tetramers and microfibrils bind to both DDRs; they promote however only DDR1 autophosphorylation. Collagen VI induces strong DDR1 phosphorylation even at very low concentrations, indicating that collagen VI is a potent ligand for DDR1. My data further suggested that collagen VI microfibrils bind to DDR2 occupying another binding site than the fibrillar collagen binding site. Finally, my preliminary data show that collagen VI can inhibit collagen I-induced DDR2 activation.

As mentioned earlier, my data showed that collagen I fibrils serve as functional ligands for both DDR1 and DDR2. However, they induce much stronger DDR1 phosphorylation signal than DDR2. Previous literature had shown that soluble collagen I serves as functional ligand for both DDRs (Vogel et al. 1997; Shrivastava et
As mentioned in Chapter 1, previous publications using triple-helical peptides encompassing the fibrillar binding motif for DDRs, the GVMGFO motif, have shown that collagen in the form of single triple-helices is sufficient for DDR activation (Konitsiotis et al. 2008; Xu et al. 2011a). Due to the fact that triple-helical peptides cannot assemble into higher-order aggregates, we concluded that soluble collagen is sufficient for DDR activation. However, it was not clear whether fibrils could also serve as functional DDR ligands.

Apart from DDRs, collagen I can also bind to other receptors, such as GPVI. In this case though, GPVI binds to both soluble and fibrillar collagen I but it is activated only by fibrillar collagen I. Early experiments conducted by several groups such as Morton et al. (1995) and Kehrel et al. (1998) showed that triple-helical peptides consisting of (GPO)_{10} triplets were able to bind to GPVI. LAIR-1 has been also shown to bind to both soluble collagen I and collagen I fibrils. Published literature using K562 cancer cells transfected with LAIR-1 protein identified soluble collagens XVIII and I-III as LAIR-1 ligands (Lebbink et al. 2006). Additionally, collagen I fibrils were also identified as LAIR-1 ligands, when incubated with K562 cells over-expressing LAIR-1 or PBMC cells expressing endogenous LAIR-1 levels (Lebbink et al. 2006). In this study, after completion of *in vitro* fibrillogenesis, the proportion of collagen I that was not incorporated into fibrils was isolated by centrifugation and removed (Lebbink et al. 2006). Finally, integrins constitute a major collagen receptor class. To date, there is a large amount of papers studying the interaction of integrins with soluble collagen. It is surprising though that we know so little about how integrins interact with fibrillar collagen. To date, there are two publications studying the interactions of integrins with fibrillar collagen I. In the first one, Jokinen et al. (2004) using solid phase binding assays and immunoelectron microscopy tested the binding of fibrillar and soluble collagen I with the α1I and the α2I recombinant integrins and integrins expressed on the surface of Chinese hamster ovarian cancer cells. Even though fibrillar collagen bound weaker to the recombinant integrins compared to soluble collagen I, immunoelectron data demonstrated that both collagen I fibrils and soluble collagen I bound strongly to α2β1 integrins (Jokinen et al. 2004). In the second study, Woltersdorf et al. (2017) showed that fibrillar collagen I, IX and XI
isolated from the conditioned medium of cultured chick embryo chondrocytes, did not manage to bind directly to human soluble β1 integrins. On the contrary, when present in soluble form, they bound strongly to β1 integrins. Furthermore, they suggested that the integrin-fibrillar collagen interaction was facilitated via articular cartilage non-collagenous proteins (Woltersdorf et al. 2017). Finally, OSCAR is another collagen-binding receptor. Literature so far has shown that OSCAR can bind to triple-helical peptides (Zhou et al. 2016). However, nothing is known about its interactions with fibrils.

A cell type with which collagen I was shown to functionally interact as both soluble collagen and fibrils is smooth muscle cells. Two early studies demonstrated that collagen I-induced smooth muscle cell functions depended on the final collagen I form (Koyama et al. 1996; Henriet et al. 2000). Koyama et al. (1996) and Henriet et al. (2000) using human arterial smooth muscle cells and human melanoma cancer cells respectively, showed that collagen gels generated from bovine collagen can inhibit cell proliferation. More specifically, they demonstrated that incubation of cells with collagen gel generated from bovine collagen I did not allow the cells to overcome the G1 phase of the cell cycle (Henriet et al. 2000; Koyama et al. 1996). In contrast, soluble collagen I (Koyama et al. 1996) in addition to denatured collagen I, (Henriet et al. 2000) promoted proliferation of smooth muscle cells. Both studies demonstrated that inhibition of cell cycle progression was due to up-regulation of cdk2 inhibitor p27kip1 in an α2β1 integrin-dependent way. Fibrils generated from rat-tail collagen I were also shown to inhibit proliferation of airway smooth muscle cells, whereas incubation of smooth muscle cells with soluble collagen I promoted their proliferation (Nguyen et al. 2005). In another study, primary human uterine leiomyoma smooth muscle cells incubated with fibrillar collagen I showed lower proliferation rate and phosphorylation of focal adhesion kinase in comparison to soluble collagen I incubation (Koohestani et al. 2013). Furthermore, when cells were incubated with soluble collagen I, the MAPK signaling pathway was activated. Finally, there were distinct differences in the morphology of cells, depending on whether they were incubated with fibrillar (star-like shape) or soluble (spindle-like shape) collagen I (Koohestani et al. 2013).
An interesting future experiment would be to test whether collagen I fibrils can promote different DDR-related signaling pathways to soluble collagen I. A plausible experiment would be to test whether collagen I fibrils, like soluble collagen I, can induce MMP secretion. In an early study Vogel et al. (1997) demonstrated that soluble collagen I-induced DDR2 activation in human fibrosarcoma HT1080 cells resulted in increased secretion of MMP-1 (Vogel et al. 1997). Two studies from Hou et al. (2002) showed that soluble collagen I-induced DDR1 autophosphorylation in mouse aorta smooth muscle cells resulted in increased MMP-2 and MMP-9 activity (Hou et al. 2002). DDR2-mediated MMP-2 secretion and activity was also demonstrated in skin fibroblasts after incubation with soluble collagen I (Olaso et al. 2002). Finally, DDR2-mediated MMP-1 and MMP-13 expression after incubation with soluble collagen I was demonstrated in synovial fibroblasts (Su et al. 2009). MMPs are key regulators of a number of diseases, including atherosclerosis (Jacob 2003; Khokha et al. 2013; Schuliga 2015). Smooth muscle cells and macrophages have been implicated in atherosclerosis progression, particularly with inflammatory processes, during which both smooth muscle cells and macrophages have been shown to produce MMPs. More specifically, vascular endothelial cells can produce collagenases (MMP-1), vascular endothelial cells, smooth muscle cells and fibroblasts can produce gelatinases (MMP-2 and MMP-9) whereas immune cells can additionally secrete collagenases MMP-8 and MMP-13 (Maxova et al. 2010; Jacob 2003). MMP-12 and MMP-13 can be secreted from airway smooth muscle cells (Schuliga 2015). Several publications have demonstrated an important role for MMPs in cancer progression. For example, MMP-9 is implicated in lung cancer (El-Badrawy et al. 2014) whereas MMP-1 and MMP-9 in breast cancer (Benson et al. 2013). DDRs have also been shown to play pathogenic roles in those types of cancer (Rammal et al. 2016; Valiathan et al. 2012; Borza & Pozzi 2014; Leitinger 2014). It would be interesting if we could incubate smooth muscle cells, macrophages or cancer cell lines which express DDR1 or DDR2 with collagen I fibrils, and test whether MMP secretion is DDR-dependent. Furthermore, MMP activity could be detected, in the case of MMP-9 using gelatin zymography, whereas for MMP-1 the most suitable approach would be collagen or casein zymography (Hu & Beeton 2010). As mentioned in previous chapters, DDR2 up-regulation by collagen II results in MMP-
over-expression and destruction of the collagen II network (Xu et al. 2003; Lin Xu et al. 2005; Xu et al. 2007; Xu et al. 2010). Our data suggested that collagen VI presence inhibits DDR2 activation induced by collagen I. It would be interesting if we could pre-incubate chondrocytes with collagen VI, add them on collagen I-coated wells and test MMP-13 mRNA levels using quantitative PCR and MMP-13 activity using collagen zymography (Hu & Beeton 2010). Furthermore, incubation of collagen VI with chondrocytes isolated from patients with OA (Lagana et al. 2014; Tew et al. 2008; López-Ruiz et al. 2017) at an earlier or a later stage of the disease could also provide more insight in the role of collagen VI in OA progression, particularly whether collagen VI could incorporate a protective role against early or later stages of OA.

My data further indicated that pre-incubation of DDR2 with collagen VI microfibrils inhibits collagen I-induced DDR2 autophosphorylation. Collagen I has been shown to participate in promotion of inhibitory signals. For example, in vitro assays using colon and breast cancer cells have identified that collagens serve as LAIR-1 functional receptors, promoting inhibitory signals in immune cells (Lebbink et al. 2006). Another study by Olde Nordkamp et al. (2014), using ELISA assays showed that LAIR-1-Fc protein, which consists of the LAIR-1 ectodomain fused to the Fc region of the IgG antibody, was able to bind to immobilized soluble collagen I (Olde Nordkamp et al. 2014). Their ability to induce inhibitory signals, which so far has only been demonstrated in in vitro assays, is attributed to the presence of immunoreceptor tyrosine-based inhibitory motifs in their cytoplasmic region (Kang et al. 2016).

Collagen I has also been shown to inhibit the apoptosis of cancer cells. It was shown that, in the presence of soluble collagen I, synthesis of free radicals by HeLa cells was inhibited, allowing the intracellular glutathione levels, which serve as substrate for cyto-protective enzymes, to remain stable and thus block cell apoptosis (He et al. 2002). However, when cells were incubated with BSA or gelatin, cells were still able to produce free radicals. Furthermore, low collagen I levels further inhibited lipid peroxidation, as a consequence of low levels of free radicals (He et al. 2002). Finally, previous studies have shown that both fibrillar and soluble collagen I can inhibit cell cycle progression (Cho et al. 2005; Wall et al. 2005; Koohestani et al. 2013; Koyama
et al. 1996; Schocklmann et al. 2000). As mentioned earlier (see Chapter 3, Discussion), Wall et al. (2005) showed that arrest of cell cycle progression, particularly in the G₀/G₁ phase, of human melanoma and fibrosarcoma cell lines A2058 and H1080 respectively, is attributed to cell incubation with collagen I fibrils. Another study by Koohestani et al. (2013) using primary human uterine leiomyoma smooth muscle cells also showed cell cycle arrest at the G₀/G₁ phase after incubation with polymerized collagen I (Koohestani et al. 2013). Koyama et al. (1996), as mentioned earlier in this Chapter, identified that incubation of human arterial smooth muscle cells with polymerized collagen gels results in inhibition of cell cycle progression, particularly in the G₁/S phase (Koyama et al. 1996). Schöcklmann et al. (2000) using mesangial cells showed that polymerized collagen I abolished their proliferation. More specifically, incubation of cells with fibrillar collagen I resulted in decreased expression of cyclins D1 and E and lower activation or MAPK signaling pathway resulting in cell cycle arrest at the phase G₁/S (Schocklmann et al. 2000). Finally, another study by Cho et al (2005), using soluble bovine collagen I showed that it suppresses proliferation of Raw264.7 macrophages by promoting cell cycle arrest at the phase G₁/S. Using signaling experiments they showed that the PI-3 kinase pathway, the JNK1 pathway and the MAP kinase pathway were involved in collagen I-induced inhibition of cell cycle progression by suppressing the expression of cyclins D1, A and B1 respectively (Cho et al. 2005). Fibrillar collagen I has further been shown to act as an inhibitor for other receptors, such as VEGFR-2, a member of the RTK family (Mitola et al. 2006). Human endothelial cells from umbilical cord veins were isolated and incubated with soluble collagen I. It was evident that VEGFR-2 autophosphorylation was blocked in the presence of collagen I, whereas VEGFR-2 was able to phosphorylate when incubated with negative control (vitronectin). The authors furthermore showed that collagen I blocks VEGFR-2 autophosphorylation via the SHP-2 signaling pathway, which is recruited to tyrosine 1173 and promotes endocytosis of the receptor (Mitola et al. 2006). However, they did not identify the receptor that interact with fibrillar collagen I and result in dephosphorylation of VEGFR-2. Another recent study by Chen et al. (2010) also showed that polymerized collagen gel inhibits VEGFR-2 phosphorylation. The authors using human aortic umbilical vein and saphenous vein endothelial cells, showed that VEGFR-2 co-
immunoprecipitated with β1 integrins, suggesting that those two receptors form a stable complex, resulting in the regulation of VEGFR-2 activation (Chen et al. 2010).

In conclusion, this thesis has provided a further insight in the interactions of DDRs with their solely ligand, collagens. DDRs, which are unique receptors since they are activated by a component of the ECM, and they can remain activated for hours upon stimulation, participate in several diseases in a collagen-dependent way but the details of their interactions with collagens are still unkown. Even though their functions in developed tissues are still not clarified, we are aware that they are essential during development (Leitinger 2014; Fu et al. 2013). The results of this PhD further provide deeper understanding of the DDR-collagen interactions, showing that DDRs can get phosphorylated not only by soluble collagen I, but also by fibrils. Furthermore, my data show that DDRs can get preferentially activated, like in the case of collagen VI, where only DDR1 is activated, not DDR2. Understanding how DDRs interact with collagens and identifying which collagen types can serve as functional ligands, will promote the design of compounds that can serve as therapeutic targets in the diseases the DDRs participate in.


Borochowitz, Z. et al., 1993. Spondylo-meta-epiphysseal dysplasia (SMED), short limb-hand


Carafoli, F. & Hohenester, E., 2013. Collagen recognition and transmembrane signalling by


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Doyle, A.D. et al., 2015. Local 3D matrix microenvironment regulates cell migration through spatiotemporal dynamics of contractility-dependent adhesions. *Nature Communications*, 6, p.8720. Available at: http://dx.doi.org/10.1038/ncomms9720.


Franco, C. et al., 2008. Discoidin domain receptor 1 (ddr1) deletion decreases atherosclerosis by accelerating matrix accumulation and reducing inflammation in low-density lipoprotein receptor-deficient mice. Circulation research, 102(10), pp.1202–1211.


Harris, J.R., Soliakov, A. & Lewis, R.J., 2013. In vitro fibrillogenesis of collagen type I in varying


Kyriakides, T.R. et al., 1998. Mice that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. The Journal of cell biology, 140(2), pp.419–430.


Luo, Y. et al., 2017. The minor collagens in articular cartilage. Protein & cell.


Orgel, J.P.R.O., Persikov, A. V & Antipova, O., 2014. Variation in the helical structure of


Poudel, B. et al., 2013. Induction of IL-12 production by the activation of discoidin domain receptor 2 via NF-kappaB and JNK pathway. Biochemical and biophysical research communications, 434(3), pp.584–588.


Raynal, N. et al., 2006. Use of synthetic peptides to locate novel integrin alpha2beta1-binding motifs in human collagen III. The Journal of biological chemistry, 281(7),


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## Appendix

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