Inter-Molecular Interactions Of Thrombospondins Drive Their Accumulation In Extracellular Matrix

Dae Joong Kim¹⁺, Elena D. Christofidou²⁺, Douglas R. Keene³, Marwah Hassan Milde¹, Josephine C. Adams¹,²,⁴#

¹Dept. of Cell Biology, Lerner Research Institute and ⁴Dept. of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Cleveland Clinic Cleveland, OH 44195, USA; ²School of Biochemistry, University of Bristol, Bristol BS8 1TD, UK; ³Micro-Imaging Center, Shriners Hospital for Children, Portland, OR 97239, USA

*Equal contribution

#Author for correspondence:
Prof. Josephine Adams,
School of Biochemistry,
University of Bristol,
Bristol BS8 1TD, UK.
Email: jo.adams@bristol.ac.uk

+current address: Department of Cell Biology & Physiology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA.
²current address: Henry Wellcome Building, Dept of Cell Physiology and Pharmacology, College of Medicine, University of Leicester, Leicester LE1 9HN, UK.

Running title: Thrombospondins in extracellular matrix
Keywords: Extracellular matrix, Cell-matrix interactions, Thrombospondins, L-type lectin domain

ABBREVIATIONS: COMP, cartilage oligomeric matrix protein; ECM, extracellular matrix; EDHB, ethyl-3,4-dihydroxybenzoate; EGF, epidermal growth factor; FN, fibronectin; mRFP, monomer red fluorescent protein; PSACH, pseudoachondroplasia; TSP, thrombospondin; TSR, thrombospondin type 1 domains

ABSTRACT
Thrombospondins participate in many aspects of tissue organisation in adult tissue homeostasis, and their dysregulation contributes to pathological processes such as fibrosis and tumour progression. The incorporation of thrombospondins into extracellular matrix (ECM) as discrete puncta has been documented in various tissue and cell biological contexts, yet the underlying mechanisms remain poorly understood. We find that collagen fibrils are disorganised in multiple tissues of Thbs1⁻/⁻ mice. In investigating how thrombospondins become retained within ECM and thereby impact on ECM organisation, we identify that accumulation of thrombospondin-1 or thrombospondin-5 puncta within cell-
derived ECM is controlled by a novel, conserved, surface-exposed site on the thrombospondin L-type lectin domain. This site acts to recruit thrombospondin molecules into ECM by inter-molecular interactions in \textit{trans}. This mechanism is fibronectin-independent, can take place extra-cellularly, and is demonstrated to be direct \textit{in vitro}. The \textit{trans} intermolecular interactions can also be heterotypic, e.g. between thrombospondin-1 and thrombospondin-5. These data identify a novel concept of concentration-dependent, inter-molecular “matrix-trapping” as a conserved mechanism that controls the accumulation and thereby the functionality of thrombospondins in ECM.

\section*{INTRODUCTION}

Thrombospondins (TSPs) are multidomain, extracellular, calcium-binding glycoproteins that are conserved from sponges to human (Bentley and Adams., 2010; Ozbek et al., 2010). The single TSP of \textit{Drosophila} has vital roles in integrin-dependent ECM organisation at developing muscle/tendon attachment sites (Chanana et al., 2007; Subramanian et al., 2007). Mammals encode five TSPs in two structural subgroups, A and B. TSP1 and TSP2 in subgroup A are trimeric and are transient, low-abundance components of adult connective tissue that are elevated within the extracellular matrix (ECM) in healing wounds, tumour stroma or atherosclerotic lesions. Important roles identified from knockout mice include the inhibition of angiogenesis, modulation of wound healing, onco-protection, tissue-specific phenotypes and increased susceptibility to experimental disease models (Adams, 2001; Bornstein et al., 2004; Zhang and Lawler, 2007, Adams and Lawler, 2011). In human populations, susceptibility to premature heart disease correlates with certain polymorphisms in \textit{THBS1} or \textit{THBS2} (Burke et al., 2010; Topol et al., 2001). In contrast, subgroup B TSPs, comprising TSP3, TSP4 and TSP5 (also known as cartilage oligomeric matrix protein (COMP)), are pentameric proteins that are lifelong ECM constituents in cartilage, bone, tendon and blood vessel walls (Hankenson et al., 2010). TSP4 contributes to susceptibility to cardiovascular disease (Frolova et al., 2010; Stenina et al., 2003; Topol et al., 2001) and mutations in \textit{THBS5/COMP} are causal for pseudoachondroplasia (PSACH) and certain forms of multiple epiphyseal dysplasia (Posey et al., 2008).

Some of these multiple roles are mediated by domains or motifs that are specific to individual family members. For example, inhibition of angiogenesis, binding of matrix
metalloproteases, or binding of latent transforming growth factor β (TGFβ), by TSP1 and TSP2, derive from their multi-functional TSP type 1 domains (TSRs) that are specific to subgroup A TSPs. Latent TGFβ activation is mediated by a TSR motif, RFK, that is unique to TSP1 (Bein and Simons, 2000; Bentley and Adams; Crawford et al., 1998; Jimenez et al., 2000; Schultz-Cherry et al., 1995; Yang et al., 2000). In contrast, the extent to which the complex roles of TSPs in tissue organisation and physiology depend on shared mechanisms remains poorly understood. Identification of such mechanism(s) at molecular level would be an important step toward novel strategies to modulate physiological or pathological activities of TSPs across multiple tissue contexts.

TSPs exhibit complete conservation of domain architecture in their C-terminal region, that forms a single tertiary structure built from contiguous epidermal growth factor (EGF) domains, thrombospondin type 3 repeats and a C-terminal, L-type lectin domain (L-lectin domain) (Carlson et al., 2005; Kvansakul et al., 2004; Tan et al., 2009, Bentley and Adams, 2010) (see also domain diagrams in Figures 2 and 4). Thus, the C-terminal region and especially the L-lectin domain are of great interest for understanding potential fundamental shared roles of TSPs. The L-lectin domain of TSP-1 has been implicated in signaling by the transmembrane immunoglobulin superfamily member, CD47 (Gao et al., 1996; Isenberg et al., 2006). However, CD47 is specific to amniotes and therefore cannot be the mediator of evolutionarily conserved activities of the L-lectin domain (Bentley and Adams., 2010). In contrast, activities attributed to the L-lectin domain of TSP5 include binding to various collagens and matrilin-3 (Posey and Hecht, 2008).

This laboratory identified previously that the C-terminal region of TSP1 in trimeric form is necessary and sufficient to mediate incorporation as discrete puncta into ECM (Adams et al., 2008). This finding opened a major question on the mechanism of ECM incorporation of TSP1 and the potential conservation of this activity within the TSP family. Here, we identify a novel process in ECM organisation, in which a conserved site on the L-lectin domain controls ECM accumulation of TSP puncta through the trans-association of TSP molecules. We propose the novel concept that “matrix trapping” of secreted TSPs according to their local concentration is a central mechanism by which TSPs can flexibly associate with, and impact on, the interstitial ECM of multiple tissues.
RESULTS

The absence of TSP1 impacts connective ECM organisation in vivo

To address whether the absence of TSP1 has biological consequences for ECM organisation, we examined by electron microscopy the connective ECM of tail tendons and dermis in healthy, sex- and age-matched wild-type or Thbs1-/- mice. Tail tendons of wild-type mice contained, as expected, highly organised bundles of parallel collagen fibrils. In contrast, the fibrils of Thbs1-/- mice were of variable widths and irregularly packed, with blending, splaying and even splitting of fibrils (Fig. 1A). In cross-section, the collagen fibrils of Thbs1-/- mice were irregular in profile and included fibrils that appeared larger than those of control mice with apparent merging of adjacent fibrils (Fig. 1A over-view; higher magnification in Fig. 1B). Similar enlargement and disorder of collagen fibrils was apparent in the dermal ECM of Thbs1-/- mice (Fig. 1A; higher magnification in Fig. 1B). The images presented compare 6.5 week old male mice; equivalent phenotypic differences were present in samples from 9 week old female mice (unpublished observation).

Quantitative measurements substantiated these observations: the mean cross-sectional area of collagen fibrils was increased in Thbs1-/- mice in both tissues, corresponding to mean fibril diameters of 124 nm for wild-type dermis, 138 nm for Thbs1-/- dermis, 192 nm for wild-type tail tendon and 207 nm for Thbs1-/- tail tendon. Thbs1-/- tail tendons contained more large fibrils (>70000nm²) and fewer intermediate fibrils (40000-70000nm²) (Fig. 1C). In both tissues, the range of fibril areas was increased in Thbs1-/- mice, as was the representation of large fibrils (upper quartiles in Fig. 1D, 1E). The frequency of small fibrils (lowest quartile) was unchanged (Fig. 1D, 1E). Thus, absence of TSP1 impacts on the organisation of collagen fibril ECM in different tissues.

Molecular determinants within the L-lectin domain control ECM deposition of a TSP1 C-terminal mini-trimer

To begin to identify the mechanism by which TSP1 contributes to collagenous ECM organisation, we turned to a model assay that measures ECM (defined in the assay as the insoluble material remaining after extraction of cell cultures with 20mM ammonium hydroxide) accumulation of a mRFP-tagged, TSP1 C-terminal mini-trimer protein
(mRFPovTSP1C) (Fig. 2A). This protein contains the domains necessary and sufficient for ECM incorporation (Adams et al., 2008). Endogenous TSP1 accumulates in ECM in the form of arrays of small puncta, and cells transfected to express mRFPovTSP1C also secrete and deposit this protein in the form of puncta in their ECM. A control, secreted mRFPo protein does not enter the ECM (Adams et al., 2008). ECM accumulation of mRFPovTSP1C depends in part on the double calcium ion-binding site in the L-lectin domain, motif D1001DD (Adams et al., 2008) (Fig. 2B). In other L-type lectin domains, for example those of mammalian endoplasmic reticulum cargo transport proteins, ligand-binding is mediated jointly by a calcium ion-binding site and an adjacent surface-exposed loop (Satoh et al., 2007). The structurally homologous portion of the TSP1 L-lectin domain is the β7/β8 loop (Fig. 2B), which contains eight surface-exposed residues with externally facing side groups. The possible functional significance of each of these for ECM incorporation activity was examined by alanine or charge-reversal point mutations. Mutation of T1032 or Y1040 had no effect on ECM incorporation and mutation of N1033 or Q1038 had minor effects (Fig. 2C). Mutation of either Y1029 or W1030 decreased ECM deposition by about 50% (Fig. 2C). The strongest effects were obtained upon mutation of either D1031 (either to alanine or with charge reversal to arginine) or R1036; these mutations decreased ECM deposition by around 70% (Fig. 2C). The transfections under matched conditions yielded equivalent percentages of mRFP-positive cells upon expression of either wild-type mRFPovTSP1C or the point mutant proteins (Fig. S1A). Quantification of immunoblots of the conditioned media from multiple independent experiments demonstrated that the point mutant proteins were present in conditioned media at equivalent levels to the wild-type protein (Fig. S1B,C). Thus, the reduced incorporation of some mutants into ECM is due to specific impairment of post-secretion processes.

To achieve more complete inhibition of ECM deposition we next tested combined point mutations. These included pairwise combinations of point mutations within the β7/β8 loop, or combinations of these with the D1001DD to A1001AA mutation (termed AAA) that partially blocks ECM incorporation (Adams et al., 2008 and Fig. 2D). Combinations such as Y1029A/D1031A or D1031R/R1036E did not further inhibit ECM incorporation (Fig. 2D and unpublished observations), and the AAA/D1031R or AAA/R1036E combinations had similar effects to AAA alone. However, accumulation of mRFPovTSP1C/AAA/D1031R/R1036E in ECM was diminished to about 10% of wild-type (Fig. 2D). The residual deposits were amorphous and covered much smaller areas
than those formed by mRFPOvTSP1C. Thus, both puncta assembly and ECM accumulation were impaired. The transfections under matched conditions yielded equivalent percentages of mRFP-positive cells upon expression of either wild-type mRFPOvTSP1C or the combination point mutant proteins (Fig. S1D). The combination mutant proteins were present in conditioned media at equivalent levels to wild-type mRFPOvTSP1C (Fig. S1E,F). The reduced ECM accumulation of the AAA/D1031R/R1036E mutant was confirmed in a second cell type, CHO (Fig. S1G,H). Residues D1031 and R1036 are surface-exposed on opposite sides of the β7/β8 loop near its outer tip (Fig. 2E). We examined by homology modeling if either mutation is predicted to affect the tertiary structure of the L-lectin domain. Molecular dynamics modeling showed that the alterations to the sidegroups at the D1031 and R1036 positions, or the D1001DD to AAA mutation, did not affect the folded protein conformation (Fig. 2F and data not shown). The AAA mutation does not affect secondary structure (Adams et al., 2008).

The novel L-lectin domain site mediates ECM incorporation of intact TSP-1

Having identified this novel site in the L-type lectin domain, our next question was to test the functional significance of the AAA/D1031R/R1036E mutations (abbreviated AAARE) in the context of full-length TSP1 (Fig. 3A). ECM deposition was visualised with two antibodies, reactive with epitopes in the laminin G-like N-terminal domain (N) or the type 3 repeats (T3), respectively. Very similar results were obtained with each antibody. ECM accumulation of TSP1/AAARE was decreased by 60-70% relative to wild-type TSP1, as established by immunofluorescence (Fig. 3B-3D) or immunoblotting of the ECM (Fig. 3E, 3F, shown for the T3 antibody only). ECM deposition of fibronectin remained unaltered under the different conditions (Fig. 3E, 3G). Because the N-terminal half of TSP1 contains multiple heparin-binding sites and conformation flexibility of the TSP-1 molecule has been documented (Calzada et al., 2008; Tan et al., 2006) we considered whether heparin-binding sites in the N and TSR domains might work together in intact TSP1 to increase the avidity of ECM incorporation. We compared directly the ECM deposition activity of TSP1, TSP1/AAARE, No (aa 1-297 of TSP-1), NovTSR (aa 1-530), and NoC mini-trimer (aa1-297 plus 531-1152) (Fig. 3A), all detected with an antibody to the N domain. Only the NoC protein had similar ECM incorporation activity to TSP1 itself (Fig. 3B, 3C). ECM deposition of No or NovTSR was only seen in small patches (Fig. 3B), and represented no more than 20% of the level of either TSP1 or NoC (Fig. 3C), even though all the proteins were expressed
equivalently (Fig. 3D). These results define that the vWF_C and TSR domains do not mediate ECM incorporation of TSP1 whereas the N domain contributes at low level.

A homologous L-lectin domain site controls ECM accumulation of TSP5/COMP

The DDD motif is invariant in vertebrate TSPs and the R1036 position is also very strongly conserved (Fig. 4). To identify whether the newly identified L-type lectin domain site is important for ECM accumulation of other TSPs, we examined the pentameric family member, TSP5, that is a component of cartilage ECM and has a simpler domain organization without the N-terminal, vWF_C, or TSR domains (Posey et al., 2008) (Fig. 5A). Endogenous TSP5 was incorporated into ECM of chondrosarcoma cells as arrays of puncta, some of which align with fibrillar elements (Fig. 5B). Ectopic TSP5 deposited by COS7 cells also appeared as arrays of puncta (Fig. 5B). Mutation of Q623 or R628 individually, (the residues in TSP5 equivalent to D1031 and R1036, Fig. 4), had little or no effect on ECM accumulation (Fig. 5C). An R628E mutant, alone or in combination with Q623E, reduced ECM accumulation by around 20% (Fig. 5C). The TSP5/D593DD/AAA mutant was 70% reduced for ECM accumulation and inclusion of the Q623E and R628E mutations with D593DD/AAA resulted in a statistically significant further decrease in ECM deposition to 10% of wild-type TSP5, with residual deposits appearing amorphous with greatly diminished puncta (Fig. 5B, 5C). The decreased deposition of TSP5 mutants in ECM was confirmed by immunoblotting of ECM (Fig. 5D). The transfections under matched conditions yielded equivalent percentages of V5-positive cells for the wild-type or mutant TSP5 proteins (Fig. S2A). The mutant TSP5 proteins oligomerised correctly (Fig. S2B, oligomerisation shown for TSP5 only) and were present at equivalent levels to wild-type TSP5 in conditioned media (Fig. S2C). We conclude that the DDD site has general functional significance for ECM deposition and puncta assembly of TSPs with more varied roles of the “D” and “R” positions. Interestingly, many TSP5 mutations that are causal for PSACH result in buildup of mutant TSP5 and other ECM molecules within the endoplasmic reticulum of chondrocytes, suggesting that although secretion is impaired, ECM interaction site(s) are not (Merritt et al., 2007). COS cells can secrete these mutant TSP5 proteins (Chen et al., 2004), therefore we analysed the ECM accumulation of a prevalent PSACH mutation, TSP5/ΔD469 (Posey et al., 2004). This protein was secreted effectively (Fig. S2C) and accumulated as puncta in ECM to the level of wild-type TSP5, clarifying that the ΔD469 PSACH mutation does not intrinsically impair ECM accumulation activity (Fig. 5C).
The mechanism of ECM accumulation of TSP molecules depends on L-lectin domain-dependent inter-molecular recruitment

On the basis of the general importance of the novel L-lectin domain site for ECM accumulation of TSP1 and TSP5, our next question was to identify how this site mediates ECM-incorporation. We first considered that the TSP L-lectin domain might bind a sugar group. However, tests of mRFPovTSP1C against the Functional Glycomics consortium microarray of 406 glycans did not reveal any significant binding either in absence or presence of calcium ions (data not shown). We next considered that ectopic expression of the TSP1 or TSP5 proteins might affect cell proliferation or viability, thereby altering parameters of ECM deposition indirectly. However, expression of wild-type or mutant TSP1 or TSP5 proteins did not result in altered cell growth over the time-period of the experiments (Fig. S3).

We therefore initiated pilot comparative proteomics of the ECM deposited by cells expressing mRFPo, mRFPovTSP1C or mRFPovTSP1C/AAARE to identify any changes in the composition of the ECM. The analysis focused on bands that appeared enriched upon SDS-PAGE analysis of the ECM from cells expressing mRFPovTSP1C. Surprisingly, the protein most enriched in the ECM of cells expressing mRFPovTSP1C was endogenous TSP1, as identified by peptides from the N-terminal domain that is not included in mRFPovTSP1C (Fig. 2A). From one protein band enriched in the ECM from cells expressing mRFPovTSP1C, 17 peptides from TSP1 were obtained, of which 11 were derived from the N-terminal domain, aa 1-240. The corresponding region of the lane in which ECM from cells expressing mRFPovTSP1C/AAARE was run yielded 19 peptides from TSP1, of which 1 was derived from the N-terminal domain. Minor enrichment of perlecan and decorin was also noted in the analysis (unpublished observations). This pilot finding raised the novel hypothesis that the incorporation of TSP1 into ECM depends on a trans-acting property of the L-lectin domain; for example, mediation of inter-molecular interactions. To test this idea, we first compared the dose-response characteristics for ECM incorporation of mRFPovTSP1C or mRFPovTSP1C/AAARE. ECM incorporation of mRFPovTSP1C was dose dependent, whereas mRFPovTSP1C/AAARE incorporation was minimal at all amounts of transfected plasmid tested (Fig. 6A). Conditions for intermediate incorporation of mRFPovTSP1C (48% of maximum) were chosen to test how its expression affected accumulation of endogenous TSP1 into ECM. Under each experimental condition, the
ectopically expressed proteins were detected in conditioned media (Fig. 6B, 6C), and the low level of endogenous TSP1 in conditioned media was unchanged (Fig. 6B, 6D). Endogenous TSP1 in ECM was identified specifically by immunoblot with antibody to the N-terminal domain, and, as reported previously, was barely detectable in the ECM of untransfected COS7 cells (Fig. 6E, lane 1 and quantified in Fig. 6F). In agreement with the pilot proteomics, endogenous TSP1 accumulated in the ECM of cells expressing mRFPovTSP1C but not in the ECM of cells expressing control mRFPo or mRFPovTSP1C/AAARE (Fig. 6E, lanes 2-4, 6F). Similar results were obtained by immunoblotting with antibody to the type 3 repeats of TSP1 (Fig. 6E). As expected, mRFPovTSP1C accumulated in ECM and mRFPovTSP1C/AAARE did not (Fig. 6E, 6G). The elevated ECM content of endogenous TSP1 in the presence of mRFPovTSP1C had specificity, because ECM deposition of endogenous fibronectin was unchanged irrespective of the transfected protein (Fig. 6E, 6H). Immunofluorescence of isolated ECM confirmed that endogenous TSP1 was specifically enriched only in the ECM of mRFPovTSP1C-expressing cells. Examples of colocalised or closely opposed TSP1 puncta and mRFPovTSP1C puncta were observed (Fig. 6I, arrowed). The lack of complete co-localisation is probably because it will be a random event whether a TSP1 molecule interacts extracellularly with another TSP1 molecule or with an mRFPovTSP1C molecule, or vice versa. These data are consistent with a mechanism of L-lectin domain-dependent recruitment of endogenous TSP1 as puncta into the insoluble ECM.

Because of the high conservation of the L-lectin domain site across the TSP family (Fig. 4), we examined whether TSP5 also had trans-recruitment activity. Since TSP5 is a natural “mini-TSP” with no N domain, an additional expression construct for full-length TSP5 with Flag and myc tags was developed (Fig. S4A, S4B). Wild-type and mutant TSP5 with V5 tags, or FlagTSP5Myc, were each expressed ectopically in separate cell populations to avoid co-translational co-oligomerisation. Cells were then trypsinised, washed extensively and plated 24h later, either as single populations of cells expressing each protein, or pairwise 1:1 mixtures of cells transfected with the different TSP5 expression plasmids. Wild-type and mutant V5-tagged proteins were present at very similar levels in conditioned media from each experimental condition, yet only TSP5.V5 was significantly enriched in ECM after co-culture (Fig. 7A, 7B). FlagTSP5Myc was also secreted effectively, yet was enriched in ECM only when TSP5.V5 was also present in the medium. In the presence of TSP5/Mut.V5, deposition
of FlagTSP5Myc was not significantly altered from the single expression control (Fig. 7A, 7C). Thus, the L-lectin domain motif of TSP5 also functions in inter-molecular recruitment of TSP5 molecules into ECM.

Because TSP1 is present at low levels in cartilage and tendon along with TSP5 (DiCesare et al., 1994) and Thbs1-/− mice have altered collagen fibril organisation in tendons (Fig. 1), we tested whether TSP1 and TSP5 could recruit each other into ECM in a L-lectin domain-dependent manner. Under transfection conditions for low-level ECM deposition of either TSP1 or TSP5.V5, conditioned media and ECMs were analysed with the appropriate specific antibodies. ECM incorporation of TSP5.V5 was strongly and specifically increased in the presence of expressed TSP1 (Fig. 7D, right upper panel; quantified in Fig. 7E). Incorporation of TSP5/AAAEE.V5 was minor in the presence of TSP1 and was barely detectable in the presence of TSP1/AAARE (Fig. 7D, right upper panel; Fig. 7E). TSP1 was incorporated at similar levels into ECM in the presence of either TSP5.V5 or TSP5/AAAEE.V5 (Fig. 7D; quantified in Fig. 7F). In contrast, TSP1/AAARE incorporated weakly when co-expressed with either wild-type or mutant TSP5.V5 (Fig. 7D, right lower panel; Fig. 7F). Thus, ECM recruitment can also take place between trimeric and pentameric TSPs and the putative inter-molecular recruitment activity of the L-lectin domain motif is important in this process.

**TSP molecules undergo direct interactions in trans**

To elucidate the mechanism underlying this intermolecular activity of the L-lectin domain of TSPs, we first considered the possible role of fibronectin as a bridging molecule between TSP molecules in the ECM. Fibronectin enhances matrix assembly of several ECM components in cell culture (Sabatier et al., 2009) and is present in ECM in our experiments (e.g., Fig. 4). However, addition of a 10–fold excess of fibronectin to the cells (based on quantification of endogenous fibronectin in ECM by immunoblots calibrated against known amounts of purified fibronectin), did not alter the amount of TSP1 incorporated into ECM. Addition of excess soluble collagen I was also ineffective (unpublished observations).

To test for possible direct, L-lectin domain motif-dependent interactions between TSP1 molecules, an ELISA-type protein-binding assay was set up with TSP1. Collagen I, which binds relatively weakly to TSP1 (Cockburn and Barnes, 1991), RFP control protein, or wild-type or mutant mRFPovTSP1C proteins were absorbed to wells,
blocked, overlaid with purified full-length TSP1, and the binding of TSP1 detected specifically with antibody to TSP1 N-domain. TSP1 binding to collagen I or mRFPovTSP1C was significantly elevated over basal binding to RFP, whereas binding to mRFPovTSP1C/AAARE was not. Interaction with mRFPovTSP1C was inhibited in the presence of EDTA, demonstrating the importance of calcium-replete TSP1 for the interaction (Fig. 8A).

TSPs secreted by cells also undergo trans-interactions in solution

Because the results of the above binding assay implicated that TSPs may also undergo trans-interactions in solution, their interactions were investigated further in the conditioned media of transfected cells. Upon immunoprecipitation of endogenous TSP1 (Fig. 8B), mRFPovTSP1C, but not mRFPovTSP1C/AAARE, was co-immunoprecipitated (Fig. 8C). Collagen I was not detected in the immunoprecipitates (unpublished observation). Similarly, after mixing conditioned media from matched numbers of transfected cells expressing different tagged forms of wild-type or mutant TSP5 and immunoprecipitating for FlagTSP5Myc (Fig. 8D), wild-type TSP5.V5 was effectively co-immunoprecipitated whereas TSP5AAAEE.V5 was not, even though both proteins were present in the same amounts in the conditioned media (Fig. 8D, 8E). Thus, L-lectin domain-dependent, TSP-TSP inter-molecular interactions in trans can occur directly and in solution, i.e., prior to incorporation into the insoluble ECM.

This point was investigated further in relation to extra-cellular interactions of endogenously expressed TSPs. As documented, COS7 cells secrete a low level of TSP1 which is insufficient for ECM incorporation (Fig. 3) into their ECM (Fig. 3), or express TSP5 (see below), were treated for 48h with filtered conditioned media from mouse skeletal myoblasts C2C12 cells that endogenously express TSP1 (Adams et al., 2008) or from rat chondrosarcoma (RCS) cells that endogenously express TSP5 (Fig. 5). The isolated ECMS were then stained with antibodies for detection of TSP1 or TSP5. In comparison to the control condition in which neither TSP1 or TSP5 were detected in ECM, punctate arrays of TSP1 were present in the ECM after treatment with either C2C12 or RCS medium. ECM deposition of TSP5 was detected only after treatment with RCS medium (Fig. 8F). These results demonstrate that the concept that inter-molecular interactions of TSPs facilitate their incorporation into ECM is relevant to endogenously-produced TSPs and further establish that this step can take place extra-cellularly.
DISCUSSION

We demonstrate a novel molecular process of prospective general importance for ECM incorporation of TSPs. TSPs do not form structural fibrils in the ECM and their functional significance for ECM organisation has remained poorly understood and difficult to study. We demonstrate that the presence of TSP1 in collagenous ECM is relevant to collagen fibril organization in vivo. In the absence of TSP1, collagen fibrils in multiple mouse tissues are disorganised and enlarged, indicative of improper fibril organisation. These phenotypes are clearly detectable in unchallenged mice, even though levels of TSP1 in post-natal dermis or tendon are low under normal physiological conditions (Kannus et al., 1998; Pablos et al., 1998). Similar alterations in collagen fibril packing and connective ECM organisation have been described in tissues from Thbs2-/- or Thbs5-/- mice (Kyriakides et al., 1998; Posey et al., 2008, Alford et al., 2013, 2014). Thbs2-/- mice have also been shown to suffer from skin laxity and weakened tail tendons (Kyriakides et al., 1998). Thbs4-/- mice also show enlarged collagen fibrils in tendons and have reduced limb muscle grip strength (Frolova et al., 2014). Loss of function of Drosophila TSP results in altered distribution of tiggrin, an ECM component required for proper ECM function at muscle-tendon attachment sites, and embryonic lethality (Subramanian et al., 2007). Thus, TSPs have significant conserved roles as modulators of ECM organisation.

In analyzing the mechanisms by which TSPs impact on ECM, a first requirement is to understand the molecular process by which TSPs accumulate in ECM. This process involves the deposition of TSPs as discrete, nano-scale puncta. We identify a novel site on the conserved TSP L-lectin domain in which specific residues of the β7/β8 loop work together with the calcium ion-binding DDD motif. We demonstrated the role of this novel site with regard to ECM incorporation of trimeric TSP1 and pentameric TSP5 and identified that the underlying molecular mechanism depends on L-lectin domain-mediated, inter-molecular interactions in trans between TSP molecules. Both homotypic and heterotypic TSP interactions can occur. Evidence is that: 1), ECM accumulation of endogenous TSP1 puncta is increased in the presence of mRFPovTSP1C mini-trimer, but not with mRFPovTSP1C/AAARE; 2), ECM accumulation of TSP5 puncta depends on the equivalent motif in the TSP5 L-lectin domain, and this also has inter-molecular recruitment activity; 3), ECM accumulation of
TSP5 is increased specifically in the presence of TSP1 yet this is not apparent for TSP5 mutated at the L-lectin domain site; 4), in vitro, direct binding in trans between TSP1 molecules depends on the L-lectin domain site; 5), physical association of TSP1 and mRFPOvTSP1C, or between differently-tagged forms of TSP5 in conditioned media is promoted by the L-lectin domain site. 6), ECM incorporation of endogenous TSPs can also take place from conditioned media. With regard to the studied heterotypic interaction between TSP1 and TSP5, an interesting anomaly is the recruitment of TSP1 to ECM by both TSP5 and TSP5/AAAEE. Possibly, the additional L-lectin domain avidity provided by a pentameric cluster compensates for lack of appropriate charge or cation-dependent interactions at the ECM incorporation site of a trimer. Alternatively, the C-terminal located heparin-binding patch of TSP5 (Tan et al., 2009) might be particularly accessible to co-operate with glycosaminoglycan-binding by the N-domain of TSP1.

These unexpected findings lead to the novel concept that TSP molecules undergo inter-molecular interactions via their L-lectin domain sites and that this activity is important for their ECM incorporation and assembly into puncta. Our model proposes that interactions between L-lectin domains of separate TSP molecules can be initiated in solution and lead to formation of multi-protein assemblies that incorporate in ECM in the form of TSP puncta (Fig. 8D).

We also demonstrate that TSP1 impacts on collagen fibril organisation in multiple tissues. Current knowledge of mechanisms of TSP/collagen interactions is sparse; most data are from studies of TSP5. In tendon and cartilage ECM, TSP5 associates with collagen fibril bundles with preference for the gap region; in vitro it promotes collagen fibrillogenesis (Halasz et al., 2007; Sodersten et al., 2005). In the dermis, TSP5 clusters are associated with collagens XII and XIV in the dermal-epidermal junction zone (Agarwal et al., 2012). The novel site we have identified is distinct from a collagen binding peptide identified in the TSP5 L-lectin domain (Holden et al., 2001), or known sites of disease-causing mutations in any TSP. We speculate that collagen interactions might further stabilise the ECM accumulation of TSP puncta by binding to a face of the L-lectin domain distinct from the site that we have identified here, or to another TSP domain. This remains a question for further investigation.
The identification of this novel mechanism has broad implications in consideration of the biological roles of TSPs. TSP1 and TSP2 are present at very low levels in adult mammalian tissues, yet are elevated extracellularly due to release by multiple cell types during wound repair or in contexts such as atherosclerotic lesions, breast tumour stroma or fibrosis (Agah et al., 2002; Frolova et al.; Yee et al., 2009). The process of “Matrix-trapping” of TSPs into ECM according to L-lectin domain mediated, trans-interactions dependent on their local concentration would imply that increased levels of TSPs will be associated with a change in physiological presentation to localized, insoluble ECM deposits. Intrinsically, the activities and binding interactions of other TSP domains will become clustered and contribute to micro-environmental heterogeneity within the ECM. This model can explain how TSPs can participate in many tissues that vary widely in ECM composition. The possibility for heterotypic, as well as homotypic, L-lectin domain interactions demonstrates that “matrix trapping” can be a general principle in the TSP family. Conceptually, co-expression of several TSPs can enable inter-molecular interactions between TSPs and for the lowest expressed TSPs to be recruited into ECM by more abundant TSPs. To date, efforts to translate aspects of TSP biology to the clinic have focused on properties fulfilled by TSPs in solution (Isenberg et al., 2009; Zhang and Lawler, 2007). We predict that the previously unknown basic mechanism identified here has important roles in TSP and ECM biology. Knowledge of this process brings new potential for approaches to control the localization and functionality of TSPs within the diverse ECMs of tissues.

MATERIALS AND METHODS

Cell lines and materials
Cos-7 cells, Chinese hamster ovary (CHO) cells, rat chondrosarcoma cells (RCS) and C2C12 mouse skeletal myoblasts were cultured in DMEM containing 10% fetal calf serum (FCS). All cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. pCEP-pu/mRFpovTSP1C, pCDNA3/TSP1 and pCDNA3/TSP5.V5His plasmids were as described (Adams et al., 2008). TSP5 cDNA containing the ΔD469 mutation was the gift of Jack Lawler. Site-directed mutagenesis was carried out on TSP1 or TSP5 cDNA templates by PCR-based mutagenesis using oligonucleotides as listed (Table S1) and the QuikChange II XL site-directed mutagenesis kit (Stratagene). All oligonucleotides were synthesised by Sigma-Genosys; oligonucleotides for mutagenesis reactions were HPLC-purified. DNA sequences were confirmed by automated DNA sequencing by CCF Genomics Core or MWG Europe. Primary antibodies used included: rabbit polyclonal antisera to DsRed (Clontech), RFP (Abcam), fibronectin (Sigma), or...
TSP5/COMP (Kimiya Biomedical Company); Rabbit monoclonal to TSP5 (EPR2689, Abcam); mouse monoclonal antibodies to human TSP1 (MAI, Lawler et al., 1985) (gift of Jack Lawler, Harvard), A6.1 (Abcam); to FLAG tag (M2, Sigma), V5 tag (Clontech), or 6His tag (Genscript or AbCam) for immunoblotting; goat polyclonal anti-V5-FITC (AbCam) for immunostaining and goat anti-TSP1 N-domain (Santa Cruz). Alkaline-phosphatase-conjugated or FITC-conjugated secondary antibodies were from Applied Biosystems and ICN, respectively.

Bioinformatics and protein structure analysis
Predicted effects of point mutations of the TSP1 L-lectin domain on its structure were examined by homology modeling using the SWISS-MODEL server (swissmodel.expasy.org) and the Swiss-PDB Viewer program (Guex and Peitsch, 1997). Template structures were generated by substituting the point mutations into the protein sequence. Models were submitted to the Dali server (http://ekhidna.biocenter.helsinki.fi/dali_server) to compare their structural relationship to PDB 1ux6. PyMOL (http://www.pymol.org) was used to illustrate the models.

ECM Accumulation
Transfection of cells and epifluorescence microscopy scoring of mRFP-tagged proteins in ECM were carried out as described (Adams et al., 2008). Full-length TSP1 proteins were detected by immunostaining with antibodies reactive with epitopes in the N-terminal domain (N-20, MAII), or type 3 repeats (A6.1) (Annis et al., 2006). TSP5 tagged with V5 epitope was detected with anti-V5-FITC. Two coverslips were scored for each condition per experiment. The percentage of transfected cells was measured after fixing in 2% paraformaldehyde and staining nuclei with 4,6-diamidino-2-phenylindole (DAPI). Transfected cells and total number of nuclei per field were counted from 20 random fields per coverslip, either by direct scoring of cells expressing mRFP-tagged proteins, or by indirect immunofluorescence with the appropriate primary antibody. ECM deposition was scored from 30 fields per coverslip by morphometry and the mean area of deposition of each mutant protein was normalized to the appropriate wild-type control. Co-localisation of mRFPOvTSP1C and endogenous TSP1 was examined by isolating ECM from untransfected cells or cells transfected with mRFP-tagged proteins, staining with antibody to the N-terminal domain of TSP1, and examining by epifluorescence microscopy with appropriate filters. Deposition of endogenous TSPs from conditioned media was analysed by plating 2.5 x 10^5 COS7
cells on glass coverslips in p60 dishes. After 3h, cells were either continued in standard media or exposed to 72h conditioned media from C2C12 or RCs cells, from which any cellular material had been removed by centrifugation at 1200rpm for 5 mins and filtration through low-protein binding, 0.22micron pore filter units (Millipore). 48h later ECM was isolated from all samples and stained by indirect immunofluorescence for either TSP1 or TSP5, or with FITC-conjugated secondary antibody only. For SDS-PAGE analysis of ECM, 5.3x10^5 COS-7 cells per 90mm dish were transfected with 0.5 to 6μg of plasmid and cultured for 60 hours. ECM was isolated by treatment with 20mM NH₄OH for 5mins, copious washing with water, and scraping into hot SDS-PAGE sample buffer containing 100mM DTT for analysis by immunoblot. Digital scans of immunoblots were analysed with NIH ImageJ for normalization of band intensities. Molecular mass markers are indicated in kDa on all immunoblot panels. Between 3 and 8 independent experiments were carried out.

**Thrombospondin secretion and immunoprecipitation**

Three days after transfection, TSP proteins were collected from conditioned media on TALON metal affinity resin (Clontech) or heparin-Sepharose (Amersham Biosciences, Sweden) as previously described (Adams et al., 2008). Beads were washed three times in TBS containing 2mM CaCl₂ and protease inhibitor cocktail (Pierce) and collected by centrifugation. TSP1 was also immunoprecipitated from conditioned medium with TSP1 N-20 antibody bound to Protein G-agarose (Zymed). TSP5 was immunoprecipitated with FLAG or V5 antibodies. Bead-bound proteins were solubilised in boiling SDS-PAGE sample buffer with or without 100mM DTT, and analysed by Western blotting with appropriate antibodies and ECL detection for their expression level and oligomerisation status. Digital scans of the blots were analysed in NIH ImageJ for normalization of band intensities.

**Protein purification**

For purification of recombinant TSP proteins, COS-7 cells were adapted over 1 month to serum-free VP-SFM medium (Gibco) supplemented with 4 mM glutamine. 4.4 × 10⁶ COS-7 cells were seeded per p150 mm dish (Corning) and transfected 1 day later with 24μg plasmid per dish using 60μl of Polyfect transfection reagent (Qiagen) according to manufacturer’s instructions. 20ml of fresh VP-SFM medium was added to each dish after transfection; medium was harvested and refed with fresh VP-SFM medium each day up to a total of 50ml/dish. Protease inhibitor VIII cocktail (Calbiochem) was added
to harvested conditioned media, the media centrifuged, and the supernatants passed over 300μl TALON metal affinity resin columns at 4°C. Each column was washed three times with 10ml TBS/CaCl$_2$ and eluted three times with 300μl of TBS/CaCl$_2$ containing 1M imidazole, over 1.5h. Purified proteins were dialyzed using Disp-Biodialyzer (The Nest Group Inc) in dialysis buffer (0.8X TBS with 2mM CaCl$_2$) and concentrated as needed by centrifugation in 50,000Da cutoff Amicon Microcon Ultra YM filter units (Millipore). Protein integrity and oligomerisation was checked on SDS-PAGE gels. Trimeric mRFPo (control) and mRFPovTSP1C (test) at 200 μg/ml in TBS containing either 2mM CaCl$_2$ or 1mM EGTA were assayed for glycan binding on the Consortium for Functional Glycomics’ glycan microarray (v3.2, 406 glycans) (Smith et al. 2010) by CFG Protein-Glycan Interaction Core H. Binding was analysed directly by RFP fluorescence (excitation 594 nm).

**TSP1 binding assay**

Rat tail collagen I (1μg/well; Sigma), or mRFPovTSP1C, mRFPovTSP1C/AAARE or RFP control proteins (all at 30nM) were coated in triplicate onto polystyrene 96-wells overnight at 4°C in TBS containing 2mM CaCl$_2$. After blocking with 1% BSA in TBS containing 2mM CaCl$_2$ and 0.1% Tween 20, 30nM platelet TSP1 (Cell Sciences) was added for 1h with rocking. Wells were washed three times with TBS containing 2mM CaCl$_2$ and 0.01% Tween 20, incubated with goat N-20 antibody to TSP1 N-terminal domain for 1h, washed again, incubated with rabbit anti-goat IgG-HRP (Dako) for 1h, washed again and color developed with TMB substrate (Pierce). A450nm was measured in a Molecular Devices plate-reader. The mean value from BSA blocked wells was subtracted as background from the other results. Data were normalised on TSP1 binding to mRFPovTSP1C-coated wells. Three independent experiments were carried out.

**Electron Microscopy and analysis of collagen fibrils**

C57Bl/6 Thbs1-/- mice were originated as described (Lawler et al., 1998) and were the gift of Jack Lawler, Harvard. Tissue samples were obtained from wild-type or Thbs1-/- mice that had been euthanised in the laboratory of Takao Sakai at CCF. Mice were housed and sacrificed according to CCF IRB-approved procedures. For transmission electron microscopy, tail tendons or small (2mm) squares of skin tissue were dissected from four 6.5 or 9 week old C57Bl/6 male or female wild-type or Thbs1-/- littermate mice, fixed for 3h at 4°C in 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1M
sodium cacodylate containing 0.05% CaCl$_2$ and 0.05% tannic acid (all from Electron Microscopy Science), and washed three times over 45min at 4°C in 0.1M sodium cacodylate or DMEM containing 0.05% CaCl$_2$. Tissues were rinsed in DMEM, post-fixed in 1% OsO$_4$ in DMEM for 2h, then dehydrated and embedded in Spurr’s epoxy. 70nm ultrathin sections were mounted onto formvar-coated single hole grids and observed using a FEI Tecnai G2 TEM operated at 120kV. For samples from dermis, images were taken from matched planes of section in the upper, middle and deep dermis from wild-type and Thbs1-/- samples. For all samples, areas of individual fibrils were measured from cross-sectional views in high power (52900x) electron micrographs using NIH Image J software. Eight fields were scored for each tissue sample from each mouse. All fibrils within each image were scored (430-700 fibrils/field in dermis and 200-350/field in tendon). Data were analysed by frequency distribution and descriptive statistics.

Proliferation assay
Cell proliferation and viability were measured using CyQUANT Direct reagents (Invitrogen) according to manufacturer's instructions.

Statistical analyses
Data are based on 3 independent experiments, unless otherwise stated. Statistical analyses were by unpaired Student’s t-test or one-way ANOVA, with $p = 0.05$ taken as the threshold for significance.

ACKNOWLEDGEMENTS
We thank the Protein-Glycan Interaction Core (Emory University School of Medicine, Atlanta) of the Consortium for Functional Glycomics funded by NIGMS GM62116, for analysis of samples on the glycan microarray; Kate Heesom for proteomic analysis; Takao Sakai and Keiko Sakai at CCF for provision of mouse tissue, and CCF Genomics core and MWG for DNA sequencing. Supported by NIH GM068073 and HL077107, BHF studentship 09/038/2787 to EDC and MRC K018043.

AUTHORS CONTRIBUTIONS
JCA designed the study with DJK and EDC and analysed data. DJK, EDC, MHM performed experiments, analysed data and prepared figures. DRK conducted electron microscopy of collagen fibrils and contributed to data analysis. The manuscript was
written by JCA with DJK and EDC. All authors approved the submitted version and declare that they have no commercial, financial or other conflicts of interest.

REFERENCES


Figure 1. Tissues of Thbs1-/- mice contain disorganised collagen fibrils.

A, B, Transmission electron microscope images of collagen fibrils in the tail tendons or dermis of wildtype or Thbs1-/- C57BL/6 male mice at 6.5 weeks of age. Arrowheads indicate splaying and splitting of collagen fibrils (in longitudinal section) and irregular, enlarged profiles of fibrils (in cross-section) in Thbs1-/- mice. Bars = 500 nm (A), 200 nm (B).

C, Frequency histogram of the cross-sectional areas of tail tendon fibrils. In each wildtype or Thbs-/- sample, 8-10 images were scored containing 250-650 fibrils.
per image. D, E. Box and whisker plots demonstrate that the range of fibril areas and representation of fibrils with large areas (upper quartile) is increased in tail tendon (D) or dermis (E) of *Thbs1*−/− mice.
Figure 2. Identification of joint roles of the β7/8 loop and double calcium ion binding site in the L-lectin domain of TSP1 in ECM incorporation of mRFPOvTSP1C.

A, schematics of the domain organisation of the TSP1 polypeptide, mRFPOvTSP1C and the control mRFPo. SP = signal peptide, N= N-terminal domain; o= oligomerisation domain, vWF_C = von Willebrand Factor type C domain, TSR = TSP type 1 domain, EGF = EGF-like domain. B, Structure of the L-type lectin domain of TSP1 from PDB.

C, bar graph showing the ECM deposition, normalised on mRFPOvTSP1C (mean ± sem) for different mutant TSP1 variants. D, similar bar graph for another set of mutant TSP1 variants.

E, molecular model showing the positions of D1031 and R1036 in the wild-type (WT) TSP1 protein. F, similar model for the mutant AAA/D1031R/R1036E TSP1 protein.
1UX6. The ECM incorporation activity site includes the DDD motif and β7/β8 loop and is at the outer tip of the domain, remote from the RGD motif that is in the last type 3 repeat. 

C. Identification of single point mutations that decrease ECM accumulation activity of mRFPovTSP1C, scored as described in Materials and Methods. 

D. Identification of a multiple point mutation that maximally inhibits ECM accumulation of mRFPovTSP1C. In each graph, data are shown as mean +/- sem. * = p ≤ 0.001 and ** = p ≤ 0.0001 relative to wild-type control. 

E,F. Enlarged view of the region of the TSP1 L-lectin domain around the novel ECM incorporation activity site, showing the polypeptide backbone and sidegroups of the identified key residues in wildtype (E), and homology modeling of the AAARE mutations (F).
Figure 3. Major role of the L-type lectin domain site in ECM accumulation of intact TSP1.

A. The domain organisation of TSP1 showing the domains included in different constructs. N= N-terminal domain, o = oligomerisation domain, v = von Willebrand factor_C domain; C= TSP C-terminal region. B-D, ECM accumulation activity of TSP1
compared to TSP1/AAARE mutant and N- and C-terminal mini-trimers. COS-7 cells were transfected with the indicated constructs and ECM accumulation quantified 4 days later by staining all samples with antibody to TSP1 N domain. B, examples of the ECM deposition patterns of the TSP1-derived proteins. Bars = 20 μm. C, quantification of ECM deposition relative to wild-type TSP1 from multiple immunofluorescence experiments. D, Equivalent expression of the proteins. Cells were fixed in 2% PFA, permeabilised and stained with antibody to TSP1 N domain. In C, * = p<0.01 relative to wild-type control. E, Immunoblots demonstrating reduced ECM accumulation of TSP1/AAARE mutant. UT = untransfected control cells. F, G, quantified data on TSP1, TSP1/AAARE and fibronectin accumulation in ECM from multiple immunoblots. In C,D, F,G, data are shown as mean ±/+ sem. In F, * = p<0.001 relative to wild-type in ECM.
Figure 4. Multiple sequence alignment of the DDD motif and β7/β8 loop region of the TSP L-lectin domain from vertebrate thrombospondins. A TCOFFEE multiple sequence alignment of the region corresponding to human TSP1 aa1001-1037, from the thrombospondins of representative vertebrates is presented in Boxshade 3.2. Black
shading indicates identical residues, grey shading indicates conservative substitutions
and white background indicates unrelated amino acids. The DDD motif is in green and
the residues corresponding to D1031 and R1036 of human TSP1 are in red. Sequences
used for the alignment correspond to those in Bentley and Adams (2010), with
correction of the Xt5 sequence on the basis of expressed sequence tags.
Key: Dr = Danio rerio; Gg = Gallus gallus; Hs = Homo sapiens; Mm = Mus musculus; Tn
= Tetraodon nigriviridis; Tr = Takifugu rubripes; Xt = Xenopus tropicalis.
Figure 5. The L-type lectin domain site has conserved activity in ECM accumulation of...
thrombospondin5.

**A**, Domain organisation of TSP5. Key as in Fig. 1 legend. **B-D**, ECM accumulation activity of wild-type TSP5 compared with point mutations of its L-type lectin domain. **B**, immunofluorescence detection of endogenous TSP5 puncta in ECM produced by RCS cells (left panel), or V5-tagged forms of TSP5 expressed in COS-7 cells (right-hand 3 panels). **C**, quantified comparison of ECM deposition of TSP5, the L-type lectin domain mutants, and a PSACH mutant, TSP5ΔD469. All proteins were detected with antibody to V5 epitope-tag. **D**, a representative V5 immunoblot and quantified analysis of ECM accumulation of TSP5/AAA and AAAEE mutants from V5 immunoblots. In C and D, data are shown as mean +/- sem, from 4 experiments. *** = p<0.001 by one way ANOVA vs. TSP5.V5.
Figure 6. A trans-acting inter-molecular mechanism of the L-type lectin domain site controls ECM accumulation of TSP1.
A, Demonstration of experimental conditions for specific concentration-dependent ECM accumulation of mRFPovTSP1C. ECM was analysed 3 days after transfection by immunoblot for the 6His tag. CM = conditioned media. B-D, Mini-trimers and endogenous TSP1 are present in conditioned media at equivalent levels under each experimental condition; E-G, endogenous TSP1 (detected with N-terminal domain-specific TSP1 antibody, also antibody A6.1 to T3 repeats) is enriched only in the ECM of mRFPovTSP1C-expressing cells. ECM incorporation of fibronectin was unaltered (E and H). In C, D, F and G, data are shown as mean -/+ sem. In F and G, * = p<0.0001 vs. wild-type value by one way ANOVA. I, Specific colocalisation of endogenous TSP1 (detected with N-terminal domain specific antibody; FITC panel) with expressed mRFPovTSP1C (RFP panel) within ECM. Arrowhead indicates example of colocalisation and arrows an apposition of puncta.
Figure 7. Inter-molecular ECM accumulation activity is conserved in TSP5 and also acts heterotypically.

**A-C**, L-lectin domain-dependent co-recruitment of TSP5 molecules into ECM. Transfections were as indicated. TSP5.V5 and TSP5/AAAEE.V5 are present at equivalent levels in conditioned media (CM), but only TSP5.V5 is enriched in ECM upon co-culture with cells expressing FLAGTSP5myc. Similarly, FLAGTSP5myc is enriched in ECM only in the presence of TSP5.V5. **A**, immunoblot; **B, C**, quantification.
and normalised data, shown as mean +/- sem. **D-F**, L-lectin domain dependent co-recruitment of TSP1 and TSP5 into ECM. **D**, Transfection conditions were as indicated and full-length TSP5 proteins were detected by V5 tag (upper panels). Full-length TSP1 was detected with antibody to its N-terminal domain (lower panels). **E, F**, quantified and normalized data, shown as mean +/- sem. In B and C * p < 0.05; ** = P ≤ 0.001; *** = p ≤ 0.0001 by one-way ANOVA against the TSP5.V5 plus FlagTSP5myc ECM condition. In E, *= p ≤ 0.001 by one-way ANOVA against the TSP1 plus TSP5.V5 ECM condition.
**A**

![Bar chart showing the normalized binding of TSP1 to different conditions.](chartA)

**B**

- **IP: TSP1 (N)**
  - Blot: TSP1 (N)
  - Relative Protein Level (mean ± SEM)
  - WT, Mut
  - Transfection

**C**

- **Blot: 6His**
  - CM, Co-IP
  - Relative Protein Level (mean ± SEM)
  - WT, Mut

**D**

- **Blot: Flag**
  - CM, ab: Flag, IP: Flag
  - Relative Protein Level (mean ± SEM)
  - +WT/V5, +Mut/V5, +WT/V5, +Mut/V5
  - Transfection

**E**

- **Blot: V5**
  - CM, ab: V5, Co-IP
  - Relative Protein Level (mean ± SEM)
  - +WT/V5, +Mut/V5, +WT/V5, +Mut/V5

**F**

- 2dary ab only, + control CM, +C2 CM, + RCS CM
- anti-TSP1, anti-TSP5

**G**

- Extracellular Secretion
- TSP1C
- TSP1
- TSP5
- Intermolecular interactions in trans via L-lectin domain motifs
- ECM accumulation of TSP molecular assemblies as puncta
**Figure 8.** The L-lectin domain site functions in direct interactions between TSP molecules.

**A,** Inter-molecular interactions of TSP1 molecules *in vitro.* Each column represents the mean, bars indicate sem.

**B, C,** Physical association of TSP1 molecules *in trans* detected by co-immunoprecipitation and immunoblotting from conditioned media (n=2 experiments, **= p< 0.01).**

**D, E,** Physical association of TSP5 molecules *in trans* detected by co-immunoprecipitation and immunoblotting from conditioned media pooled from cells expressing different tagged forms of TSP5 (*= p< 0.05).**

**F,** Demonstration that incorporation of ECM puncta of endogenous TSP1 or TSP5 can be driven extracellularly. ECMs were prepared from COS7 cells after 48h in standard media, or with exposure to conditioned media from C2C12 or RCS cells, and stained with antibodies to TSP1 or TSP5. Images are representative from 3 independent experiments. **G,** Model for the mechanism of initiation and ECM accumulation of TSP puncta by TSP1-TSP1, TSP5-TSP5, or heterotypic TSP1-TSP5 inter-molecular interactions. Key: L= L-type lectin domain, N= N-terminal domain. See Discussion for details.