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TENOgenic MODULating INsider factor: systematic assessment on the functions of tenomodulin gene

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ABSTRACT

Tenomodulin (*TNMD*, *Tnmd*) is a gene highly expressed in tendon known to be important for tendon maturation with key implications for the residing tendon stem/progenitor cells as well as for the regulation of endothelial cell migration in chordae tendineae cordis in the heart and in experimental tumour models. This review aims at providing an encompassing overview of this gene and its protein. In addition, its known expression pattern as well as putative signalling pathways will be described. A chronological overview of the discovered functions of this gene in tendon and other tissues and cells is provided as well as its use as a tendon and ligament lineage marker is assessed in detail and discussed. Last, information about the possible connections between *TNMD* genomic mutations and mRNA expression to various diseases is delivered. Taken together this review offers a solid synopsis on the up-to-date information available about *TNMD* and aids at directing and focusing the future research to fully uncover the roles and implications of this interesting gene.

Abbreviations: 3D, three dimensional; Ad-EGFP, adenovirus vector encoding enhanced green fluorescent protein; APOE, apolipoprotein E; Aqp1, aquaporin 1; Arg, arginine; bHLH, basic helix-loop-helix; BMDSC, bone marrow-derived stem cell; BMP, bone morphogenetic protein; BMSCs, bone marrow stromal cells; C, cysteine; C2C12, mouse myoblast cell line;C57BL/6, C57 black 6; CA region, Cornu Ammonis region; cDNA, complementary deoxyribonucleic acid; Chm1, chondromodulin-1; Col, collagen; COMP, cartilage oligomeric matrix protein; COS-7, African green monkey fibroblast-like kidney cell line; CS, mutant with deleted cleavage site; CTC, chordae tendineae cordis; CTD, C-terminal domain deletion mutant; C-terminal, carboxyl-terminal; *cTnmd*, chicken tenomodulin; Cys, cysteine; DNA, deoxyribonucleic acid; E, embryonic day; EC domain, mutant with entire extracellular portion of Tnmd deleted; Egr, early growth response protein; Eya, eyes absent transcription factor; FCR, flexor carpi radialis; FGF, fibroblast growth factor; FL, full length tenomodulin; GSK-3, glycogen synthase-3; H5V, mouse embryonic heart endothelial cells; HH, Hamburger-Hamilton stage; hPDL, human periodontal ligament; *hTNMD*, human tenomodulin; Htra3, HtrA serine peptidase 3; HUVEC, human umbilical vein endothelial cell; I, isoleucine; ICC, immunocytochemistry; IFM, interfascicular matrix; IHC, immunohistochemistry; ISH, in situ hybridization; I-TASSER, iterative, threading assembly refinement; K, lysine; kb, kilo base; kDa, kilodalton; KO, knockout; MA, microarray; Mkx, Mohawk; MMP, matrix metalloproteinase; mRNA, messenger ribonucleic acid; MSC, mesenchymal stem cell; *mTnmd*, mouse tenomodulin; N, asparagine; NB, Northern blot; NIH3T3, mouse embryonic fibroblasts; OIR, oxygen-induced retinopathy; p53, tumour protein 53; PBS, phosphate-buffered saline; PCDH19, Protocadherin 19; PCR, polymerase chain reaction; PDL, periodontal ligament; PNGaseF, peptide-N-glycosidase F; Q, glutamine; qPCR, quantitative PCR; RCAS-cScx, replication-competent avian sarcoma-leukosis virus-copy Scleraxis; RNA, ribonucleic acid; rs, reference small nucleotide polymorphism; RT-PCR, reverse transcriptase-polymerase chain reaction; Sca-1, stem cells antigen-1; Scx, Scleraxis; SMA, smooth muscle actin; SNP, small nucleotide polymorphism; Sox, sex determining region Y; SUMO, small ubiquitin-like modifier; TGF, transforming growth factor; Thbs, thrombospondin; *TNMD*, *Tnmd*, tenomodulin; TSPC, tendon stem/progenitor cell; UTR, untranslated region; V, valine; VEGF, vascular endothelial growth factor; WB, western blot; WT, wildtype.

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Contents

1. Introduction

Tendons are dense connective tissues arranged in a hierarchical manner. Mature tendons are normally characterized by low cellular density and this is one of the most obvious features when looking at tendon histological preparations. About 90–95% of the cellular content of tendon comprises tendon-specific cell types described in the literature as tenoblasts and tenocytes, the latter being the terminally differentiated form (Kannus, 1997; Chuen et al., 2004). During development newborn tendons have a very high cell-to-matrix ratio with tenoblasts of various shapes and sizes aligned in long parallel chains. Following tendon maturation, the ratio between cells and matrix gradually decreases, with tenoblasts transforming from an ovoid to a spindle and elongated shape, specific for their differentiated counterparts, tenocytes (Ippolito et al., 1980).

Although the general knowledge about the differentiated cells residing in tendon tissue has been developing in the latest years, still little is known about their precursors. Stem/progenitor cells of mesenchymal origin, such as the tendon lineage, are of great interest in order to understand tendon development and healing processes. In 2007, Bi et al. (2007)demonstrated that human and mouse tendons harbour a unique cell population which has universal stem cell characteristics such as clonogenicity, self-renewal capacity and multipotency. Compared to bone marrow stromal stem cells, these tendon-derived cells expressed high levels of Scleraxis (*SCX*), cartilage oligomeric matrix protein (*COMP*), tenascin-C and tenomodulin (*TNMD*), all tendon-related factors, thus identifying their origin. Additionally, these isolated cells showed the ability to regenerate tendon-like tissue after extended expansion in vitro and transplantation in vivo. However, the fact that the cells of this population showed heterogeneity in their stem properties and the possibility of containing tendon progenitor cells as well, made the authors name them- tendon stem/progenitor cells (TSPCs).

The repair of musculoskeletal tissues often recapitulates the cellular and molecular events of development. Thus, understanding the process of tendon development can significantly help to develop novel repair strategies. So far, the knowledge on the ontogeny of the tendon lags far behind other mesodermal tissues due to both the lack of specific markers exclusive to the tendon lineage. However, the identification of the basic helix-loop-helix (bHLH) transcription factor *Scx* as a specific and early marker of tendon progenitors during embryonic development (Cserjesi et al., 1995; Schweitzer et al., 2001) and the study of mice harbouring genetic mutations leading to tendon phenotypes, reviewed in Tozer and Duprez (2005) and Liu et al. (2011), have provided some insights into the onset of the tendon lineage and the molecules involved in this process. Using knockout mouse models, further transcription factors were identified to be essential for tendon differentiation and development such as Mohawk (*Mkx*), *Egr1* and *Egr2* (Ito et al., 2010; Lejard et al., 2011; Guerquin et al., 2013).

Tenomodulin was discovered in 2001 by Brandau et al. (2001) and Shukunami et al. (2001) as a gene sharing high homology with chondromodulin-1 (CHM1, alternative names chondromodulin-1 and alternative abbreviations, CHM-I, LECT1, BRICD3 and MYETS1) (Hiraki et al., 1991). Both research groups described high expression in tendons, explaining the rationale behind its name. It was hinted to be useful as a tendon-specific marker, which later on became an established marker for the mature tendon and ligamentous lineage.

Despite great progress in deciphering tendon development, the exact molecular pathways orchestrating tendon progenitor specification and differentiation still remain largely unknown. Therefore, further studies are required to identify novel tendon-specific markers, to understand their roles and to elucidate the molecular cascades occurring during tendon development and maintenance.

In view of the above, the focus in this review is on *TNMD*, one of the best so far tendon-specific marker genes. We carried out a systematic review analysis on all available articles in the PubMed databank. The examination was performed by searching tenomodulin under its full name, but also by its alternative names and abbreviations (tendin and myodulin, *TNMD* and *TeM*). A summary of the selection of articles for this review is shown in Fig. 1.

2. Tenomodulin

2.1. Gene discovery and nomenclature

TNMD was found independently by Brandau et al. (2001) and Shukunami et al. (2001). Brandau's team named it tendin with the justification that it is highly expressed in tendons and ligaments. Shukunami's team, on the other hand named it tenomodulin, since it shares high homology to chondromodulin-I, but is expressed in tendons, hence postulating a similar modulatory function in these tissues. Tenomodulin also circulated around the literature very briefly under the name of myodulin (Pisani et al., 2004; Pisani et al., 2005). The authors gave the alternative name myodulin based on detecting *Tnmd* mRNA in Northern blot analysis of rat gastrocnemius muscles. As previously mentioned, its abbreviations in the literature are found under *TNMD* and *TeM*.

2.2. Gene and protein structure

TNMD, *Tnmd* belongs to the new family of type II transmembrane glycoproteins with a highly conserved cleavable C-terminal cysteine-rich domain (Brandau et al., 2001; Shukunami et al., 2001). The *TNMD*, *Tnmd* gene consists of seven exons localized on the X chromosome (Fig. 2) and accounts for an approximately 1.4 kb transcript and a predicted protein consisting of 317 amino acids (Brandau et al., 2001; Shukunami et al., 2001). Analysis of primary amino acid sequences

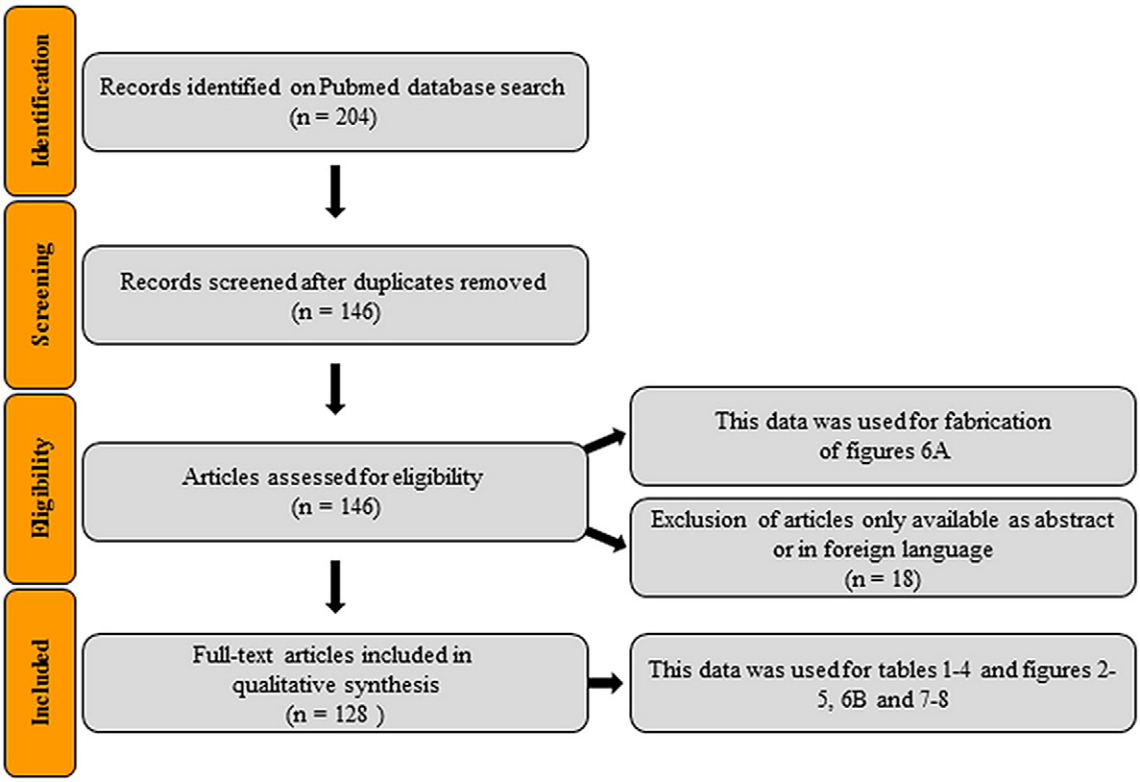

Fig. 1. Flow chart of the search strategy and study selection used in this systematic review.

reveals several structural features in the putative TNMD, Tnmd protein (Fig. 3).

The gene is composed of seven exons among which the second encodes for the transmembrane domain and the last for the C-terminal cysteine-rich domain that is conserved across species (Fig. 3A). Unlike the Chm1 precursor, the Tnmd protein does not include a furin cleavage site, although a putative protease recognition sequence (Arg-Xxx-Xxx-Arg) was identified at the position 233–236 (Barr, 1991; Shukunami et al., 2005). The extracellular part, prior the cleavage site, contains a BRICHOS extracellular domain (Fig. 3B). This domain consists of a homologous sequence of approximately 100 amino acids containing a pair of conserved cysteine residues (Sanchez-Pulido et al., 2002). BRICHOS has been found in several unrelated to Tnmd genes involved in dementia, respiratory distress and cancer. It has been suggested that it participates in post-translational processing, however the function remains unclear (Sanchez-Pulido et al., 2002). In contrast to Chm1, which has two N-glycosylation sties and one O-glycosylation site (Neame et al., 1990), Tnmd is absent of these three sites, while it contains two N-glycosylation sites at position 94 and 180 (Brandau et al., 2001). Protein analyses in eye and periodontal ligament revealed full length Tnmd protein as a double band of 40 and 45 kDa (Shukunami et al., 2005; Komiyama et al., 2013). It has been experimentally proven that the 45 kDa band corresponds to the glycosylated Tnmd, while the 40 kDa band refers to the non-glycosylated Tnmd (Komiyama et al., 2013).

Tnmd contains a highly conserved C-terminal cysteine-rich domain (Shukunami et al., 2001), making up the part of the protein sharing the highest resemblance to Chm1 (77% similarity/66% identity) (Brandau et al., 2001). This domain contains C-terminal hydrophobic tail with eight Cys residues forming four disulphide-bridges, which are well conserved across vertebrate species (Shukunami et al., 2005; Shukunami et al., 2006; Kondo et al., 2011) as shown in Fig. 3. A smaller cyclic structure forming by single Cys280–Cys292 disulphide bridge in Tnmd has been shown to exert an anti-angiogenic function, while the other three disulphide-bridges are speculated to hold this cyclic structure and the C-terminal hydrophobic tail separated from each other to avoid the formation of intramolecular aggregates (Miura et al., 2012). In certain tendon tissues, such as Achilles tendon and chordae tendineae cordis (CTCs), 16 kDa cleaved C-terminal part of Tnmd was detected in the collagenous extracellular matrix, which suggested tissue-specific Tnmd secretion (Docheva et al., 2005; Kimura et al., 2008).

Since TNMD and its homologue CHM1 are a novel class of proteins it is very relevant to clarify the existence of isoforms as well as their exact protein structure and function. A study dealing with human tendon cells and tissues, and using the I-TASSER molecular modelling programme, has suggested that the *TNMD* gene has 3 possible isoforms I, II and III with molecular weights of 37.1, 20.3 and 25.4 kDa, respectively (Qi et al., 2012). However, further validation by carrying out Northern blot analyses as well as specific PCR designed to clearly discriminate

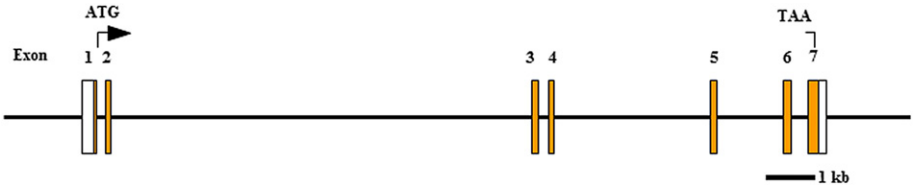

Fig. 2. Schematic representation of the human *TNMD* gene. White boxes represent 5′′ and 3′′ UTR sites, while orange boxes represent exons. Abbreviations: ATG, start codon; kb, kilo base; TAA, stop codon.

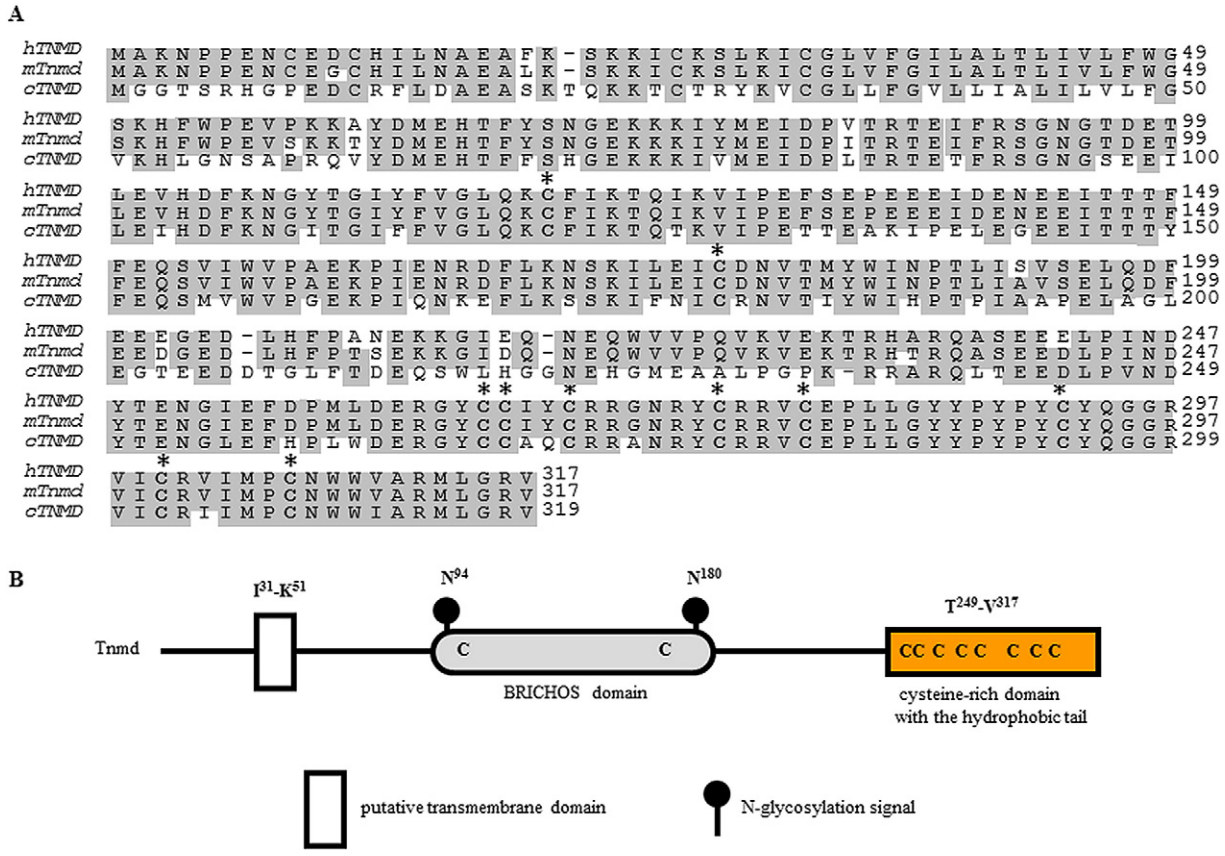

Fig. 3. A comparison of the deduced amino acid sequences of tenomodulin proteins and structural features of the human TNMD. (A) Predicted amino acid sequences of human (Shukunami et al., 2001, and Yamana et al. 2001), mouse (Brandau et al., 2001; Shukunami et al., 2001, and Yamana et al. 2001), and chick (Shukunami et al., 2006) tenomodulin. The conserved amino acid residues are shaded and gaps were introduced for optimal alignment. Conserved cysteine residues are indicated in bold with an asterisk. The chicken sequence shares 62% homology with the mouse and human orthologs. The conserved amino.GenBank accession numbers for the aligned sequences are as follows: human *TNMD*, AF234259.1; mouse *Tnmd*, AF219993.1; chick *TNMD*, AY156693. (B) Human TNMD protein includes a type II transmembrane domain at the N terminus, a BRICHOS domain (Sanchez-Pulido et al., 2002) and a C-terminal cysteine-rich domain. The TNMD protein contains two N-glycosylation sites within the BRICHOS domain. Abbreviations: C, cysteine; *cTnmd*; chicken tenomodulin; *hTNMD*, human tenomodulin; I, isoleucine; K, lysine; *mTnmd*, mouse tenomodulin; N, asparagine; T, threonine; V, valine; *TNMD*, *Tnmd*, tenomodulin.

and demonstrate individual isoforms is necessary. With regards to mouse, Brandau et al. (2001) (Northern blot analyses on multiple mouse tissues with *Tnmd* cDNA probe for exons 4–7) and Shukunami et al. (2001) (Northern blot with 8 different mouse tissues and cDNA probe encoding two thirds of the entire coding region) did not detect *Tnmd* isoforms. Similar findings were observed in Northern blot investigations on samples from wild type and knockout mice with full cDNA probe and western blotting analyses of mouse tissues and anti-Tnmd antibodies (Docheva et al., 2005; Shukunami et al., 2006; Takimoto et al., 2012; Komiyama et al., 2013). This data suggests that *TNMD* isoforms might be species-specific. Interestingly, the programme-based study predicted for each isoform a very different protein function: isoform I to be a cytosine methyltransferase; isoform II a SUMO-1-like SENP-1 protease; and isoform III to be an α-syntrophin, pleckstrin homology domain scaffolding protein. A possible explanation behind the suggestion that each isoform functions entirely different could be that the novel and unstudied protein domain structures of TNMD and CHM1 causes the available prediction platforms to search for minimal homology scores, hence identifying totally different proteins. Despite of the many open questions left, the study of Qi et al. (2012) strongly urges for further analyses on the TNMD isoforms and exact protein domain structure and function.

2.3. Expression pattern

TNMD is a protein predominantly expressed in tendons and ligaments. The first Northern blot analysis on new-born mouse tissue showed the highest expression of *Tnmd* in skeletal muscle (Shukunami et al., 2001), diaphragm and eye, even though an overall weak signal was visible in almost all other screened tissues (Brandau et al., 2001). Following in situ hybridization and western blotting studies revealed that the signal from muscle tissue is mainly due to expression of *Tnmd* in tendons and ligaments as well as in the muscle sheet epimysium (Brandau et al., 2001; Docheva et al., 2005; Kimura et al., 2008; Komiyama et al., 2013; Sato et al., 2014). In a temporal expression analysis in mice the presence of the *Tnmd* transcript was weekly detected already at embryonic day E9.5 in developing limb buds (Brandau et al., 2001); however a recent paper has firmly reported very strong *Tnmd* mRNA expression at day E14.5 corresponding to the differentiation stage of tendon progenitors (Havis et al., 2014). Furthermore, also tendon-like structures in the heart, namely chordae tendineae cordis, demonstrated *Tnmd* messenger and protein existence in these areas (Kimura et al., 2008). Interestingly, several papers have found *Tnmd* expression in the maxilla-facial region. Komiyama et al. (2013) reported high Tnmd expression, by immunohistochemical analysis, in periodontal ligaments at 3 and 4 postnatal weeks, marking the molar eruptive and post-eruptive phases when occlusal forces are transferred to the teeth and the surrounding tissues. Watahiki et al. (2008) discovered a specific expression of *Tnmd* in mandibular condylar cartilage at 1 week in rats. The masseter muscle is compartmentalized by a laminar structure, which was shown to express by in situ hybridization *Tnmd* in mouse embryos at E12.5 to E17.5 (Sato et al., 2014). Furthermore, Oshima et al. (2003) revealed *Tnmd* expression at E15.5 in skin and eye using Northern blotting. The exact localization of *Tnmd* mRNA in the eye was shown by in situ

hybridization in the sclerocornea, tendon of the extraocular muscle, ganglion cell layer, lens fibre cells, inner nuclear layer cells and pigment epithelium of the retina (Oshima et al., 2003). *TNMD* expression was also found by PCR in human subcutaneous adipose tissue and adipocytes (Saiki et al., 2009). Last, in situ hybridization showed *Tnmd* expression in various parts of the adult mouse brain such as the dentate gyrus, CA regions of the hippocampus, neurons in the cerebral nuclei, cerebellum, Purkinje cells and neuronal cells in the cerebellar nucleus (Brandau et al., 2001). However, a general notion should be taken that *Tnmd* expression outside dense connective tissues, such as tendons and ligaments, is very low. The main expression sites of *TNMD*, *Tnmd* are summarized in Table. 1.

2.4. Putative signalling pathway

TNMD is an established marker for the mature tendon and ligament lineage (Shukunami et al., 2006) due to its high expression in these tissues, segregating uncommitted from committed cells of this lineage. In order to decipher upstream and downstream effectors of *Tnmd* many knockout mouse models with tendon phenotypes have helped in understanding which factors or pathways affect *Tnmd*. Similarly, the loss of *Tnmd* allows suggestions on which molecules might be downstream effectors. It is important to emphasise that the most of the following studies show correlations between *Tnmd* expression or function to other genes and not a direct link in a common signalling cascade. In

Table 1
Expression pattern of *TNMD*, *Tnmd*.

Species	Experimental source	Methods	Expression domains	References
Human	Musculoskeletal tissues	Real-time PCR	Tendon, fat, muscle in	Jelinsky et al. (2010)
	CTC from patients	IHC	Elastin-rich mid layer of CTC	Kimura et al. (2008)
	Adipose tissue	DNA-MA, Real-time PCR	Lean and obese adipose subcutaneous tissue, more specifically mammary glands, omental fat, tongue superior part and main corpus, but also urethra, coronary artery, oral mucosa, ovary, skeletal muscle, bone marrow, colon, substantia nigra and pituitary gland	Saiki et al. (2009)
	Cultured cells	ICC	Bone marrow-derived MSCs expressing Scx	Alberton et al. (2012)
		PCR	TSPCs	Bi et al. (2007)
Porcine	Pig heart and eyes	RT-PCR, WB	Chordae and eye	Kimura et al. (2008)
Rabbit	1-Week-old Japanese white rabbits	ISH	Achilles tendon	Yukata et al. (2010)
	12-week-old Japanese white rabbits	PCR	CTC interstitial cells	Kimura et al. (2008)
	Rotator cuff healing model in 19–21-week-old rats	Real-time PCR ISH	Healing tissues at 2-, 4-, 6-, 8-, 12-weeks, but significantly upregulated in animals treated with FGF-2 at weeks 4, 6, 8 and 12 compared to normally healing tendons Midsubstance of tendon at 2- and 8-weeks	Tokunaga et al. (2015)
Rat	Achilles tendon healing model with 9–10-week-old rats	RT-PCR	Upregulation in the normally loaded Achilles tendon compared to the animal where normal loading of the muscle-tendon unit was prevented by intramuscular Botox injection	Eliasson et al. (2009)
Mouse	Embryos	NB	Whole embryos at E15.5 and E17.5	Oshima et al. (2003)
			High expression in skeletal muscle, diaphragm and eye, weak signals in brain, lung, liver, heart, kidney, skin, thymus and perichondrium- and periosteum-free rib cartilage	Brandau et al. (2001)
		IHC	Secondary lens fibres and retina at E16.5	Oshima et al. (2003)
		ISH	Tendon of extrinsic ocular muscle, sclera, equatorial lens, epithelial cells at E16.5	Oshima et al. (2003)
			Tendons at E14.5, E15.5, and E16.5	
			CD31- negative regions of tendons at E16.5. Strong signals in limb tendons and cruciate ligaments, tendons of the masculus flexor digitorum, ligament intercostal, diaphragm, cortex, medulla of the thymus at E17.5	Shukunami et al. (2008)
		PCR	Heart from E14.5 to E18.5	Kimura et al. (2008)
	New-born	IHC	Patellar ligament, anterior and posterior cruciate ligament, Achilles tendon of *Sox9*$^{Cre/+}$;*R26R*	Sugimoto et al. (2013)
		RT-PCR	Heart	Kimura et al. (2008)
	2- and 3-week-old mice	IHC	Peridontal ligaments	Komiyama et al. (2013)
	4-Week-old mice	NB/WB	Whole eye, skin	Oshima et al. (2003)
		PCR	Whole eye, cornea, retina, sclera with ocular muscles	
		ISH	Cornea, sclera, retina	
			Tendon, epimysium of skeletal muscle	Shukunami et al. (2001)
		WB	Skeletal muscle	Oshima et al. (2004)
		IHC	Achilles tendon	Docheva et al. (2005)
		NB	High in tendon, low in eye and muscle	
	Cultured cells	PCR/IHC	TSPC	Bi et al. (2007); Alberton et al. (2015)
Chick	Embryos (from HH stage 23–41)	ISH	Tendon primordia and tendons	Shukunami et al. (2006)
			Tendon primordia and tendons, ligaments and perichondrium	Shukunami et al. (2008)
			Hind limb tendons at HH stage 30	Takimoto et al. (2012)
		NB	Whole embryos from HH stage 25 to stage 35, tendon, skeletal muscle, heart, skin and eye at HH stage 45	
	Cultured cells	NB	Tenocytes isolated from leg tendons at HH stage 41	Takimoto et al. (2012)

Abbreviations: CTC, chordae tendineae cordis; DNA, deoxyribonucleic acid; E, embryonic day; HC, immunohistochemistry; HH, Hamburger Hamilton; ICC, immunocytochemistry; ISH, in situ hybridization; MA, microarray; MSC, mesenchymal stem cell; NB, Northern blot; PCR, polymerase chain reaction; RT, reverse transcriptase; Scx, Scleraxis; TSPC, tendon stem/progenitor cell; WB, western blot.

Fig. 4, we have summarized the so far reported, mostly indirect, links between *Tnmd* and putative upstream and downstream factors.

Brent et al. (2005) reported that in *Sox5* and *Sox6* double knockout mice, where skeletal elements failed to undergo normal chondrogenic differentiation, the domain of *Scx* is broadened and accompanied by the expression of *Tnmd*. These observations proposed that *Tnmd* might be a direct target of *Scx* transcriptional activities. Towards the same suggestion led also an in vitro experiment where over-expression of *Scx* triggered the up-regulation of *Tnmd* in cultured chicken tenocytes (Shukunami et al., 2006). Additional evidence came from the study of Murchison et al. (2007) which reported the phenotype of the *Scx* knockout mice. These mice are characterized by severe tendon defects, ranging from a dramatic failure in progenitor differentiation to the formation of small and poorly organized force transmitting tendons. Interestingly, in these mice the screening of the expression of several genes known to be expressed in tendons revealed the complete loss of expression of *Tnmd* and *type XIV collagen* at E16.5 (Murchison et al., 2007). Another study in 2012 validated that by overexpression of *Scx* in mesenchymal stem cells *Tnmd* expression is significantly upregulated in a cell type which normally does not express *Scx* nor *Tnmd* (Alberton et al., 2012).

Another factor participating in *Tnmd* signalling is the growth differentiation factor *myostatin*. The deletion of *myostatin* in mice resulted in small, brittle and hypocellular tendons (Mendias et al., 2008), a phenotype similar, in term of cell density, to the one found in the *Tnmd*-deficient mice (Docheva et al., 2005). Moreover, these mice showed a decrease in *Scx* and *Tnmd* expression (Mendias et al., 2008) and *myostatin* stimulation of fibroblasts led to *Scx* and *Tnmd* mRNA upregulation, suggesting *myostatin* as an upstream factor in the *Tnmd* pathway by first inducing *Scx* expression.

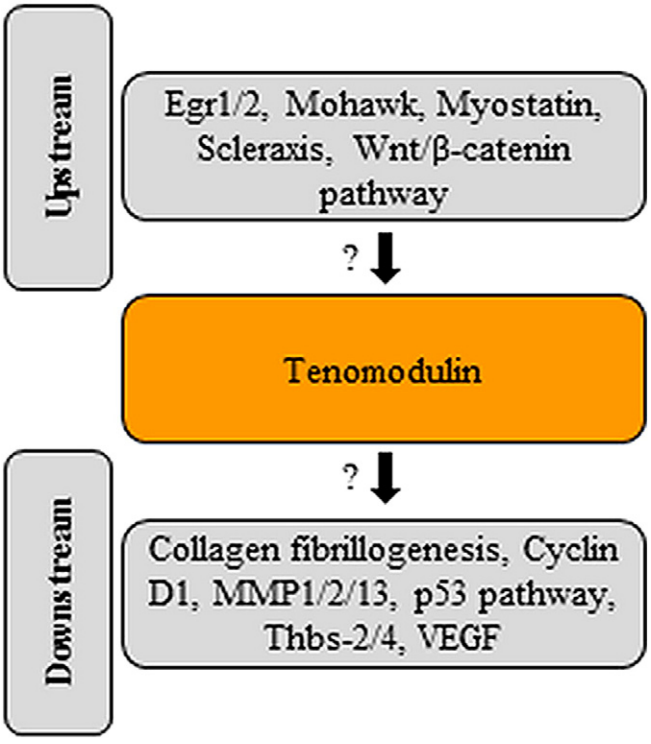

Fig. 4. Summary of putative upstream and downstream factors in Tnmd-related signalling. The figure is based on studies showing mostly correlations, indicated by the question marks, between *TNMD* expression or function to other genes and not a direct link in a common signalling cascade. Based on Alberton et al. (2012); Alberton et al. (2015); Brent et al. (2005); Docheva et al. (2005); Frolova et al. (2014); Kimura et al. (2008); Kyriakides et al. (1998); Lejard et al. (2011); Liu et al. (2010); Mendias et al. (2008); Miyabara et al. (2014); Murchison et al. (2007); and Shukunami et al. (2006). Abbreviations: Egr, early growth response protein; MMP, matrix metalloproteinase; Thbs, thrombospondin; VEGF, vascular endothelial growth factor.

The expression of an *Egr*-like transcription factor (Frommer et al., 1996) in tendon precursor cells of *Drosophila* motivated the generation of *Egr1* and *Egr2* mutant mice and chicks in 2011 for investigation of their involvement in vertebrate tendon development (Lejard et al., 2011). This study concluded that *Egr1* and *Egr2* expression in tendon cells was associated with the upregulation of *Col1a1* from E11.5 onwards by trans-activating its proximal promoter as well as other collagens in tendon cell differentiation in embryonic limbs. Furthermore, the authors established that *Egr* genes were activated by muscle-derived *FGF4* and are able to induce *de novo* expression of *Scx*. Moreover, both *Egr1* and *Egr2* knockouts were characterized by reduced *Col1a1* transcripts together with decreased number of collagen fibrils (Lejard et al., 2011). It would be interesting to investigate if loss of *Egr1*/2 also affects *Tnmd* expression.

The absence of the *Mohwak* (*Mkx*) homebox gene in mouse led to an abnormal tendon phenotype including an effect on *Tnmd* expression (Liu et al., 2010). These mice exhibited tendons with inferior size compared to normal mice. On a molecular level, *Mkx* mutants revealed significantly lower *Tnmd* levels as well as *collagen I* and *fibromodulin* (Liu et al., 2010). *Tnmd* mRNA was downregulated in *Scx* and *Mkx* mutant mice around E16.5 (Murchison et al., 2007; Liu et al., 2010). Interestingly, *Scx* mutant mice show tendon defects as early as E13.5, while the tendon phenotype of *Mkx* mutants is first visible at E16.5 despite that *Scx* expression is sustained in these animals (Murchison et al., 2007; Liu et al., 2010). This clearly suggests that in tendon development *Scx* acts prior to *Mkx*, but with regards to regulation of *Tnmd* both *Scx* and *Mkx* can perform independently from each other as upstream factors.

The use of gene silencing experiments was valuable for understanding why bone marrow-derived stem cells (BMDSCs) from horse, which in culture have very low amounts of *TNMD* compared to tendons, showed similar *TNMD* expression when cultured in collagen gels containing a glycogen synthase kinase-3 (GSK-3) inhibitor (Miyabara et al., 2014). Application of inhibitor or small interference RNA corresponding to GSK-3α/β into BMDSCs resulted in nuclear translocation of β-catenin and this drove the expression of *TNMD*. Since the levels of *Scx* and *Mkx* were unaffected in the above conditions, it points out that the Wnt/β-catenin signalling works independent from these transcription factors (Miyabara et al., 2014).

Next, a *Tnmd* knockout mouse model study revealed that loss of *Tnmd* results in reduced proliferation and earlier onset of senescence in TSPCs (Alberton et al., 2015). The study compared tenogenic markers in both genotypes of which no differences were observed, except for the absence of *Tnmd* transcript in mutant mice. However, analysis of TSPCs self-renewal showed that *Tnmd* knockout TSPCs had significantly lower clonogenic ability as well as proliferated less after passage 3 and reached an earlier plateau compared to wildtype TSPCs. This was further accompanied with significant downregulation of the proliferative marker *Cyclin D1* in *Tnmd* knockout TSPCs. Another striking feature of the *Tnmd* knockout TSPCs is that at an earlier passage they show signs of senescence, revealed by β-galatosidase staining, and the number of senescent cells was always larger in the knockouts. In addition a profound upregulation of *p53* was detected in the knockout cells. Taken together, these findings suggest that loss of *Tnmd* results in reduced proliferation and premature ageing of the TSPCs (Alberton et al., 2015) and secondary to this there is a distorted gene expression balance (*Cyclin D1* and *p53*). This finding is in line with the analyses of the *Tnmd* knockout mouse model, which revealed reduced cellular density and proliferation in vivo (Docheva et al., 2005). In addition, *Tnmd* mutants showed an abnormal collagen fibril phenotype with pathologically thicker fibrils, resembling signs of premature tendon matrix ageing. In sum, it can be suggested that collagen type I, proliferation and senescent-related factors are belonging to the putative downstream effectors. Still, at this stage of the research it is not possible to clarify how exactly *Tnmd* is regulating the above factors. Since Tnmd shares high homology with *Chm1*,

it was obvious to actually consider *Chm1* as a possible compensatory factor. However, the study of *Tnmd* expression in *Chm1* null mice and vice versa of *Chm1* expression in *Tnmd*-deficient mice, showed no up-regulation of neither of the genes, suggesting that the loss of these genes is likely to be compensated by other factors (Brandau et al., 2002; Docheva et al., 2005).

Interestingly, both *Thrombospondin-2* (*Thbs-2*) and *Thbs-4* knockout mice both display tendon phenotypes consisting of abnormally large collagen fibrils (Kyriakides et al., 1998; Frolova et al., 2014), a similar outcome to the collagen fibril phenotype of *Tnmd* deficient mice. These observations are open invitations for exploring if *Thbs* members and Tnmd are participating or connected in the same molecular cascade.

Last, the study analysing ruptures of human CTCs revealed that in the affected area TNMD expression is downregulated, but VEGF-A and several MMP (MMP1, 2 and 13) expressions are upregulated. Furthermore, tube formation and mobilization of human coronary artery endothelial cells were dramatically inhibited when treated with conditioned media from CTC or NIH3T3 cells that were transfected with the *Tnmd* C-terminal domain. On the contrary, when cultured with conditioned media from CTC cells treated with *Tnmd* siRNA their capability forming ability was recovered. Hence, these experimental findings confirm an antiangiogenic role of Tnmd in CTCs (Kimura et al., 2008).

We strongly stress that in future research it would be essential to decipher what are the exact binding partners of *Tnmd*. This may help us to explain, for example, opposing effects such as stimulating the proliferation of tendon-derived cells, but inhibiting vascular cells. At present, we speculate that *Tnmd* might be a co-factor regulating the function of a growth factor or growth factor receptor and hence, depending on the availability, act as a stimulator or inhibitor.

2.5. Tenogenic differentiation cascade

The up-to-date paradigm underlining the putative tendon cell commitment and differentiation process is still very controversial and not fully understood; however the involvement of Tnmd in several steps of this process became very apparent in recent studies. Fig. 5 represents the molecular orchestra of tendon cell differentiation and importantly, it marks the participation of *Tnmd* in specific commitment steps. In early tendon development, embryonic mesenchymal progenitors at E10.5 commit into the tendon lineage by first elevating the *Scx* transcription factor in developing sclerotomes of the somites, mesenchymal cells in the body wall and limb buds in mouse embryo (Brent et al., 2003). Furthermore, follow up studies suggested that the initial *Scx* expression is mediated by transcription factors *Pea3* and *Erm*, which are activated by FGF signalling (Brent and Tabin, 2004; Eloy-Trinquet et al., 2009). Among the FGF family the critical members in mice are FGFs 4 and 6, and in chick FGFs 4 and 8. Pryce et al. (2009) reported that in addition to FGFs, TGFβ (*Tgfβ*) signalling is also critical in tendon progenitor differentiation via direct induction of *Scx* expression. In this paper, the tendon phenotypes of *Tgfb2* −/−;*Tgfb3* −/− double mutant, or the corresponding receptors, revealed that TGFβ signalling acts later than FGF signalling since the tendon phenotype was first manifested at E12.5, while the tendon condensation and *Scx*-positive tendon cells were normal at E11.5.

Next, a breakthrough paper based on transcriptome analysis comparing expression profiles of a variety of genes in *Scx*GFP-positive tendon cells derived from different embryonic tendon development stages, resulted in more clear picture of the molecular events during tendon cell differentiation (Havis et al., 2014). By comparing cells from E11.5, corresponding to tendon progenitors, to cells from E14.5,

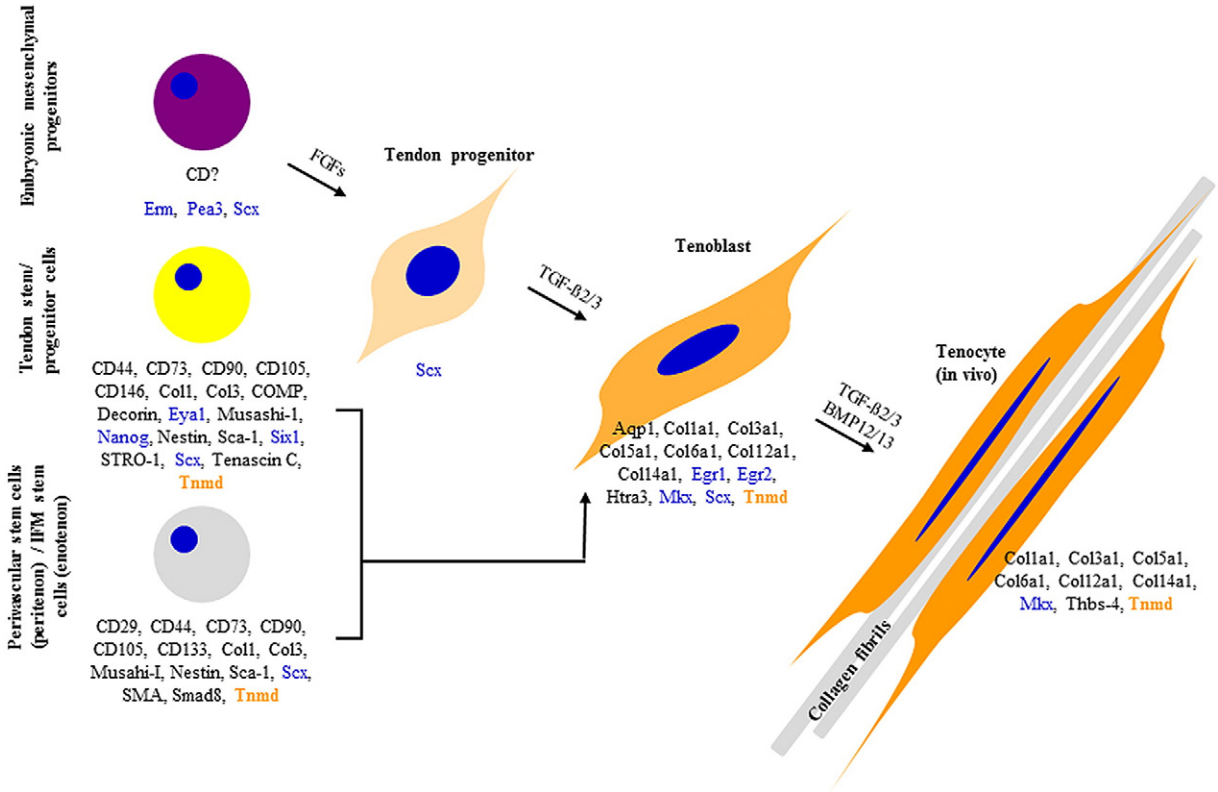

Fig. 5. Current model of the tenogenic cascade and *TNMD* involvement. *TNMD* is marked in orange, transcription factors in blue and other genes in black. Based on Alberton et al. (2015); Berasi et al. 2011; Eloy-Trinquet et al. (2009); Havis et al. (2014); Huang et al. 2015; Lee et al. (2015); Liu et al. (2014); Mienaltowski et al. (2013); Pryce et al. (2009); Shen et al. (2013); and Tempfer et al. (2009). Abbreviations: Aqp1, aquaporin 1; BMP, bone morphogenetic protein; Col, collagen; COMP, cartilage oligomeric matrix protein; Egr, early growth response protein; Eya, eyes absent transcription factor; FGF, fibroblast growth factor; Htra3, HtrA serine peptidase 3; IFM, interfascicular matrix; Mkx, Mohawk; Sca-1, stem cells antigen-1; Scx, Scleraxis; SMA, smooth muscle actin; TGF, transforming growth factor; Thbs, thrombospondin; Tnmd, tenomodulin.

corresponding to tendon differentiated cells (tenoblast), the authors found a significant upregulation of *Tnmd*, *Col1a1*, *Col3a1*, *Col5a1*, *Col6a1*, *Col12a1* and *Col14a1* and two novel and unrelated so far to tendon genes, namely *aquaporin 1* (*Aqp1*) and *HtrA serine peptidase 3* (*Htra3*). With regards to the further maturation of tenoblasts towards tenocytes the following studies suggested the essential contribution of the *Tnmd*, *Mkx*, *Erg 1/2*, and *Thbs-4* (Docheva et al., 2005; Liu et al., 2010; Lejard et al., 2011; Alberton et al., 2012; Barsby et al., 2014; Onizuka et al., 2014). The Scx expression in tenocytes remains at present debatable. Scx signals are visible in the mature cells in ScxGFP reporter mice (Sugimoto et al., 2013). However, from this transgenic model is not clear how strong the levels of endogenous Scx in these cells are.

With regards to tendon differentiation during tendon postnatal homeostasis/maintenance or at times of tendon healing, the commitment cascade is still very elusive. Cumulatively the few articles providing some information on this topic are suggesting that the above process might be mediated by the adult TSPCs or perivascular stem cells or tendon intra-fascicular matrix stem cells, or even a mixture of all these cell types depending on the circumstances (Bi et al., 2007; Tempfer et al., 2009; Rui et al., 2010; Mienaltowski et al., 2013; Liu et al., 2014; Alberton et al., 2015; Docheva et al., 2015; Lee et al., 2015). Mienaltowski et al. (2013) actually proposed that in tendons there is a regional distribution of different stem/progenitor cells. Specifically, they analysed two subpopulations; one originating from the peritenon and the other from the tendon proper of mouse Achilles tendons. Comparison between these subpopulations revealed that the cells from the tendon proper are more proliferative and exhibit higher levels of tendon-related markers, such as *Tnmd* and *Scx*, while peritenon-derived cells showed increased vascular and pericyte markers. As illustrated in Fig. 5 these adult cell types share some marker gene expression; however their identity, density, exact location and distribution are still not fully understood. Importantly, *Tnmd* expression has been strongly connected with the adult TSPC cell type (Bi et al., 2007; Alberton et al., 2015).

Taken together, even though our knowledge is being gradually enriched about what genes are expressed in tendon precursor cells, still very little is known whether they originate from embryonic-, TSPC- or the perivascular cell lineages as well as how exactly the various molecular factor are interwoven in the progression of the tendon differentiation programme. For these reasons it is highly important to identify further markers exclusive to tendons and their residing stem, progenitor and mature cells. Specifically for the *Tnmd* signalling pathway a detailed analysis on the expression of other genes in loss-of-function experimental models may clarify the compensatory mechanisms. Chromatin immunoprecipitation can rule out which transcription factors directly interact with the *Tnmd* promoter, while pool-down assays with *Tnmd* antibody might help identifying possible directly binding molecules. Last, since tendons are mechano-sensitive tissues, follow up studies can aim at testing if and how *Tnmd* is regulated in vitro and in vivo by mechanical stimuli.

2.6. Tenomodulin functional analyses

Ever since the discovery in 2001, *TNMD* gained gradual attention in the tendon research field with an immense rise of publications just in the recent years as depicted in Fig. 6A. Altogether 146 articles and abstracts on Pubmed have been published covering *TNMD* until the end of 2015. After exclusion of articles only available in abstract form and foreign language articles, we can group the remaining 128 full-text publications into four categories; namely into studies looking into functions of *TNMD*, articles using *TNMD* as a tendon marker, research observing correlations between *TNMD* mutations and a variety of diseases, and lastly reviews Fig. 6B.

From this analysis it becomes obvious that most of the studies utilize *TNMD* in research as a tendon marker. In contrast, very little research, 16 full-text studies, were conducted on determining the functional role of *TNMD* in tendons and other tissues. A summary and detail comments on the so far known *TNMD* functions are shown in Table 2, which is organized in chronological appearance of the articles from the discovery of *TNMD* in 2001 to the end of 2015.

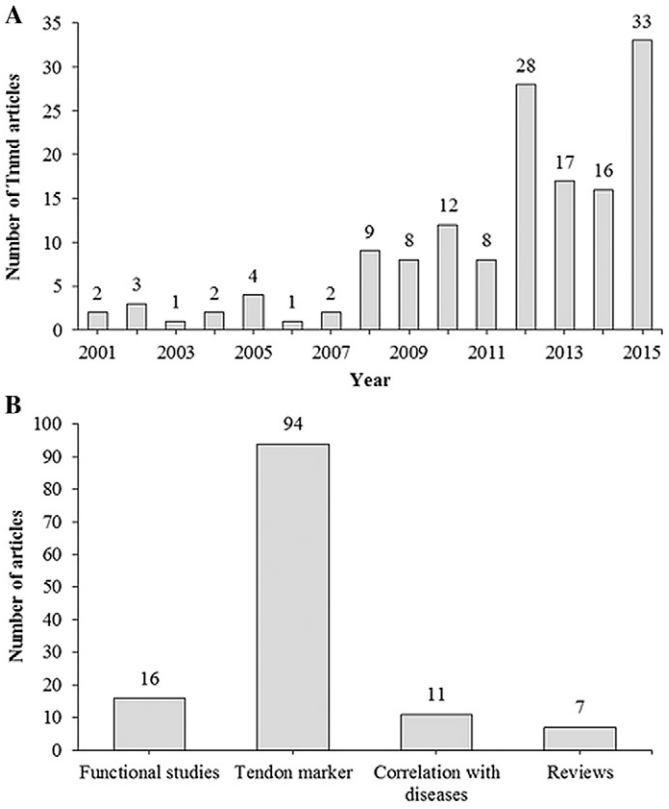

Fig. 6. Research articles published annually including tenomodulin. (A) All articles published on Pubmed covering tenomodulin and its alternative names tendin and myodulin as well as its abbreviations *TNMD* and *TeM*. (B) Distribution of the published research on tenomodulin into four main categories. This figure only includes research published in English and full-text research articles.

From this table we see major breakthroughs in the functions of *TNMD* not just in tendons, but also in other tissues and cells. In tendons it proves to have beneficial functions for the maintenance of healthy and functional tissue, because its loss results in distorted collagen fibrillogenesis, reduced cell density and overall premature tendon ageing. *Tnmd* exerts a positive effect on TSPCs by sustaining their stem cell like features such as promoting self-renewal and delaying senescence, and the proliferative effect can be carried out exclusively by the Tnmd C-terminal cysteine-rich domain (Alberton et al., 2015). Another discovered function of *Tnmd* is the contribution to proper adhesion of periodontal fibroblasts, which have high gene expression (Komiyama et al., 2013).

Intriguingly, the proliferative influence of *Tnmd* was shown to be dependent on the cell type. For example, human retinal endothelial cells transduced with Tnmd exhibited a reduction in proliferation. In addition, Tnmd lowered the angiogenic migration of human umbilical vein endothelial cells (HUVECs) as reported by Oshima et al. (2003) and Oshima et al. (2004). Transduction of *Tnmd* in human melanoma cells resulted in suppression of tumour growth in ectopic in vivo model, due to decreased vessel density most likely because of reduced endothelial cell migration (Oshima et al., 2004). With respect to organismal vessel formation in vivo it is clear that lack of *Tnmd* does not affect angiogenesis in tendon and retina development since the knockout model revealed no major differences to wildtype mice (Docheva et al., 2005). Still, the latter finding is open for discussion because a study with recombinant Tnmd has shown an obliterating effect on retinal

Table 2
Summary of the discovered functions of *TNMD*, *Tnmd*.

Reference	Experimental model	Study type	Findings and functions of Tnmd
Brandau et al. (2001)	Northern blot analysis of new born mouse RNA; ISH on E17.5 mouse embryos and adult mouse brain; cloning of human and mouse *Tnmd* cDNA; genomic analysis of human Tnmd organization	Genomic study and in vivo	- Strong expression of 1.5 kb *Tnmd* transcript in diaphragm, skeletal muscle and eye; low expression in brain, liver, lung, kidney, heart, thymus and perichondrium- and periosteum-free ribcage; *Tnmd* transcript found in tendons and ligaments but also in the brain, spinal cord, liver, lungs, bowel, thymus and eye of E17.5 mouse embryos; high expression of *Tnmd* mRNA in the dentate gyrus, CA regions of the hippocampus, neurones in the cerebral nuclei, Purkinje cells and neuronal cells in the cerebellar nucleus, neurons in the anterior and posterior horn of the spinal cord and neurons from all cell layers in the cerebral cortex of adult mice. - Mouse and human *Tnmd* share 89% homology, similarity (54%) and identity (31%) to *Chm1*, especially with the C-terminal (77% and 66%); 7 exons found.
Shukunami et al. (2001)	Cloning of mouse *Tnmd*; Northern blot analysis of mouse tissues; ISH of mouse skeletal muscle	Genomic study and in vivo	- Cloning of *Tnmd* full-length reveals novel protein of 317 amino acid residues; predicted amino acid sequence revealed 33% overall identity with mouse Chm1 precursor; structural features include singly transmembrane domain at the N-terminal region and the putative angiogenic domain with eight cysteine residues; Tnmd lacked a hormone-processing signal compared to Chm1, suggesting it may function as a type II transmembrane protein on cell surface. - *Tnmd* transcript of 1.4 kb detected in skeletal muscle; *Tnmd* expression not associated with muscle fibres, but rather with epimysium and tendon.
Oshima et al. (2003)	Northern blot analysis on mouse embryos; ISH on eyes of mouse embryos; human retinal endothelial cells (HRECs) transduced with *Tnmd*; human umbilical vein endothelial cells (HUVECs) co-cultured with HRECs transduced with *Tnmd*	In vivo and in vitro	-*Tnmd* mRNA expression detectable from day E15.5 and present in eye and skin; localization on *Tnmd* transcript on the extraocular muscle, sclerocornea, lens fibre cells, ganglion cell layer, inner nuclear layer cells and pigment epithelium of the retina. - Effective autocrine suppression of cell proliferation and capillary-like morphogenesis of retina vascular endothelial cells; conditioned media from soluble *Tnmd*-overexpressing cells also showed marked inhibitory effect on angiogenesis.
Oshima et al. (2004)	Adenoviral expression system to force expression of *Tnmd* C-Terminal in HUVECs; C57BL/6 mouse model subcutaneously injected with melanoma tumour cells; histological analysis of melanoma tumour cells injected into mice; transduction of *Tnmd* into BL-6 melanoma cells	In vivo and in vitro	- Transduction of *Tnmd* into HUVECS impaired tube formation of HUVECs cultured in Matrigel; conditioned media from COS7 cells transfected with *Tnmd* impaired tube formation of HUVECs; transformation of HUVECS with *Tnmd* downregulated VEGF synthesis to 4–50% of normal levels; migration of HUVECs in response to VEGF was significantly affected after *Tnmd* transduction. - Formed tumours were 46% smaller compared to Ad-EGFP-transduced melanoma cells; visible inhibition of angiogenesis in implanted tumours and decreased microvessel density; no effect on growth rate.
Pisani et al. (2004)	Northern blot analysis on gastrocnemius muscle of rats; RT-PCR analysis of tendons separated from soleus muscle and tendon-free soleus muscle of rats and Tnmd in primary human muscle cells derived from single satellite cells; quantitative Northern blot analysis of C2C12 mouse cell line; overexpression of Tnmd fused to FLAG peptide and immunofluorescence to FLAG; western blot analysis of myodulin-FLAG fusion protein; co-culture experiment of C2C12 mouse myoblast with H5V cells; proliferation of WT muscle cells and Tnmd-FLAG C2C12 cells compared; Tnmd expression in hind limb suspension model in rats	In vivo and in vitro	-Tnmd mRNA found in muscle fibres and tendons; Tnmd transcript found at the myoblast proliferating stage and myotube differentiated stage; Tnmd transcript found in C2C12 cells without any significant difference in expression levels between proliferating and differentiated stages; evidence of a muscle cell surface protein. - Tnmd-FLAG fusion protein = 44 kDa, FLAG = 1 kDa so mass of Tnmd = 43 kDa. Calculated mass = 37.047. - Tnmd has an active role in invasive action of endothelial cells, without evidence of extracellular Tnmd secretion; no differences observed in proliferative capacity between WT muscle cells and Tnmd-FLAG C2C12 cells, even with the addition of FGF-2; soleus muscle mass decrease resulted in capillary loss resulting in three-fold Tnmd downregulation and 2-fold upregulation the in opposite condition.
Docheva et al. (2005)	Generation of the Tnmd KO mouse model; Northern blot analysis of Tnmd in new-born thymus, eye, tendon, muscle and heart tissues; oxygen-induced retinopathy model (OIR); electron microscopy of Achilles tendons; immunohistochemical analysis of collagens, decorin, lumican, aggrecan and matrilin-2	In vivo	- Successful generation of *Tnmd* KO mouse line validated by lack of *Tnmd* transcript; high Tnmd expression in tendons and at lower levels in eye and muscle. - Probing of tendon extracts with an antibody raised against the C-terminal domain of Tnmd, detected a protein signal of 16 kDa corresponding to the cleavable C-terminal cysteine-rich extracellular domain; suggestion that C-terminal cysteine rich domain of Tnmd is rapidly cleaved in vivo - No changes in retinal vascularization seen in both genotypes after OIR. - Loss of Tnmd expression resulted in decreased tenocyte proliferation and reduced tenocyte density in vivo. - No changes in extracellular matrix protein production like collagen type I, II, III and VI and decorin, lumican, aggrecan and matrilin-2; collagen fibrils showed significantly increased average diameters

(continued on next page)

Table 2 (*continued*)

Reference	Experimental model	Study type	Findings and functions of Tnmd
Pisani et al. (2005)	Yeast transformation with mouse Tnmd vector including C-terminal region and western blotting; purification of Tnmd in yeast by column purification; co-culturing C2C12 and H5V cells together with modified yeast	In vitro	- Mouse Tnmd could be expressed at the plasma membrane of *Saccharomyces cerevisiae* in an N-glycosylated state. - Murine Tnmd can be purified from yeast; detection of three different bands of which one shows Tnmd in a glycosylated state (65 kDa). - Mouse Tnmd expressed in yeast fully functional by increasing invasive potential of C2C12 cells in H5V co-culture system.
Shukunami et al. (2006)	ISH on developing chick embryos; retroviral expression of Scx in chick tenocytes and chondrocytes; misexpression of RCAS-cScx by electroporation into hind limb	In vivo and in vitro	- At stage 23, Scx expression in the syndetome has extended to the tail region and *Tnmd* detectable in anterior eight somites; at stage 25, *Tnmd* and *Scx* detectable in regions adjacent to the myotome; at stage 32 and later, *Scx* and *Tnmd* displayed similar expression profiles in developing tendons. - Upregulation of *Tnmd* in tenocytes but not chondrocytes; no induction of generation of additional tendons but resulted in upregulation of *Tnmd* in tendons at stage 33 and later.
Watahiki et al., 2008	Quantitative PCR of mandibular condylar cartilage and tibial growth plate cartilage of 1- and 5 week old rats; IHC analysis on mandibular-, condylar- and tibial cartilage of 1- and 5- week old rats	In vivo	- Mandibular condylar cartilage showed higher mRNA levels of *Tnmd* than tibial growth plate cartilage; mandibular condylar cartilage 1-week-old rats presented higher *Tnmd* mRNA levels than 5-weeks-old rats; *Tnmd* was found on condylar cartilage except on fibrous layer; Tnmd only detected in the hypertrophic layer and part of articular cartilage.
Shukunami et al. (2008)	In situ hybridization on developing mouse at E14.5; immunohistochemical analysis on developing mouse forelimb; ISH on avascular mesenchyme and vertebrae of developing chick	In vivo	- mRNA localized in developing tendons which were co-localized to *Scx* expression; Tnmd staining spotted in cartilage and localization restricted to the PECAM-1 negative region of the developing tendons; first identified in posterior regions of limb mesenchyme at HH stage 23 and later expressed in attachment sites connecting cartilage and muscle; at HH stage 30, distinct expression pattern adjacent to MyoD-positive domain marking the myotome and partially overlapping with *Scx* expression; *Tnmd*-positive domains were lacking blood vessels; *Tnmd* expression exclusive to connective tissues including intervertebral region, perichondrium of vertebrae and ligaments; *Tnmd* only identified in peripheral region where thick bundles of collagen fibres were present.
Wang et al. (2012)	OIR in mice; injection of commercially produced recombinant Tnmd into eye for analysis of blood vessel patterns	In vivo	- In OIR model, fewer central non-perfused areas were observed in Tnmd-injected eyes than in PBS-injected eyes; significantly less nuclei in new blood vessels breaking in each retinal cross-section of Tnmd-treated- compared to PBS-treated mice; suggestion of potential role of Tnmd in prevention and treatment of ocular neovascularization.
Qi et al. (2012)	I-TASSER protein three-dimensional conformation modelling prediction; human and porcine tenocytes; knock- down of human FCR cells; Tnmd western blotting of pig, horse and human tendon cells; ICC on Tnmd isoforms overexpressed in COS-7 cells	Computer analysis and in vitro	- Three *TNMD* transcripts with molecular weights of 37.1, 20.3 and 25.4 kDa for isoforms I, II and II respectively proposed and verified by western blot from human cells; isoforms I and II localized perinuclear and isoform III on cytoplasm; predicted function of *TNMD* I = cytosine methyltransferase, II = SUMO–1-like SENP-1 protease, III = α-syntrophin, pleckstrin homology domain scaffolding protein. - Knockdown of all isoforms resulted in reduced cell proliferation and downregulation of myostatin and Scleraxis.
Komiyama et al. (2013)	Tnmd IHC on WT mouse molars; FL-, CTD-, CS-, BRICHOS-, and EC Tnmd overexpression in NIH3T3 and hPDL cells; cleavage of N-glycans and inhibition of glycosylation in vitro analysis by adding tunicamycin and digesting cell lysates with PNGaseF respectively; analysis of adhesion capacity of Tnmd KO mouse fibroblasts	In vivo and in vitro	- Tnmd expression is related to time of tooth eruption at 2–3 weeks of age; Tnmd localized in Golgi apparatus, microtubules and plasma membrane of PDL cells. - 45-kDa Tnmd protein is likely to be glycosylated form;40-kDa protein the non-glycosylated Tnmd protein. - *Tnmd* overexpression resulted in enhanced adhesion of PDL and NIH3T3 cells to collagen I and increased surface area; Tnmd KO fibroblasts showed decreased cell adhesion; BRICHOS domain or CS region seem responsible for Tnmd-mediated improvement of cell adhesion in Tnmd overexpressing NIH3T3 cells.
Miyabara et al. (2014)	Wnt in vitro model; equine BMSCs cultured in collagen gel; nuclear translocation of ß-catenin in glycogen synthase kinase-3 inhibitor-treated BMSCs	In vitro	- mRNA expression of Tnmd in equine BMSCs increased when Wnt pathway was activated, comparable to levels in tendon. - Concluded regulation of tenomodulin expression via Wnt/ß-catenin signalling.
Sato et al. (2014)	Tnmd messenger analysis using qPCR of masseter muscle (MM) from C57/BL6 mice; ISH of Tnmd transcript MM	In vivo	- Level of *Tnmd* RNA increased from E12.5-E17.5 and decreased after birth; CD31 and VEGF mRNA levels in the MM remained constant from E12.5-E18.5 and low after birth; *Tnmd* mRNA found in the middle region of the MM at E12.5 as well as in the muscle-bone junction from E14.5 onwards.

Table 2 (*continued*)

Reference	Experimental model	Study type	Findings and functions of Tnmd
Alberton et al. (2015)	Self-renewal comparison between WT and Tnmd KO TSPC; transfection of full-length and C-terminal domain of Tnmd in Tnmd KO mouse TSPCs; multipotential comparison on TSPCs	In vitro	- *Tnmd* KO TSPCs show reduced proliferative capacity. - C-terminal alone restores proliferation in *Tnmd* KO TSPCs. - Loss of *Tnmd* results in premature and increased senescence of TSPCs compared to WT. - Lack of *Tnmd* does not affect multipotential in TSPC.

Abbreviations: Ad-EGFP, adenovirus vector encoding enhanced green fluorescent protein; BMSCs, bone marrow stromal cells; C2C12, mouse myoblast cell line;C57BL/6, C57 black 6;CA region, Cornu Ammonis region; cDNA, complementary deoxyribonucleic acid; Chm1; chondromodulin-1; COS-7, African green monkey fibroblast-like kidney cell line; CS, mutant with deleted cleavage site; CTD, C-terminal domain deletion mutant; E, embryonic day; EC domain, mutant with entire extracellular portion of Tnmd deleted; FCR, flexor carpi radialis; FGF-2, fibroblast growth factor-2; FL, full length Tnmd; H5V, mouse embryonic heart endothelial cells; HH, Hamburger-Hamilton stage; hPDL, human periodontal ligament; ICC, immunocytochemistry; IHC, immunohistochemistry; ISH, in situ hybridization; I-TASSER, iterative, threading assembly refinement; kb, kilo base; kDa, kilodalton; KO, knockout; mRNA, messenger ribonucleic acid; NIH3T3, mouse embryonic fibroblasts; OIR, oxygen-induced retinopathy; PBS, phosphate-buffered saline; PDL, periodontal ligament; PNGaseF, peptide-N-glycosidase F; qPCR, quantitative PCR; RCAS-cScx, replication-competent avian sarcoma-leukosis virus-copy Scleraxis; RNA, ribonucleic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; Scx, Scleraxis; SUMO, small ubiquitin-like modifier; Tnmd, tenomodulin; VEGF, vascular endothelial growth factor; WT, wildtype.

vessels when Tnmd was injected in the vitreous body in vivo (Wang et al., 2012). In this study, however, information and validation of the produced Tnmd were not included and at present the protein is not available for purchase from the producer company. Last, the study on CTCs demonstrated that in this tissue type Tnmd prevents vascularization (Kimura et al., 2008).

Now that some *Tnmd* functions have been exposed it is also equally important to decipher in follow up studies how exactly *Tnmd* exerts these. As already discussed previously, the way to deliver the necessary explanations is via narrowing down the exact signalling pathway of *Tnmd*. This will not only allow a complete understanding of the role of *Tnmd* in the domains where is expressed, but will also enable concrete manipulation of its effect in possible clinical applications.

2.7. Tenomodulin as lineage marker

As observed in Fig. 6 most of the full-text research papers containing *TNMD* have used it as a gene marker for the tendon and ligament lineage. Table 3 summarizes these studies as all findings were clustered into 6 categories. Table 3 makes it evident that most research looks at TNMD on a messenger level with the use of PCR to demonstrate that the used cell types are or have differentiated into tendon-like cells. Protein data on the other hand is shown very rarely. Based on our experience, it is the protein data that is most difficult to gain, but it is an essential proof of the true involvement of TNMD in the investigated model. We and our Japanese collaborators have produced self-made antibodies and examined their specificity using *Tnmd* knockdown or knockout samples (Docheva et al., 2005; Shukunami et al., 2006; Komiyama et al., 2013). It is therefore crucial for researchers to carefully validate the antibody specificity.

Interestingly, several wound healing models, namely in skin, rotator cuff and patella tendons, revealed differential *Tnmd* expression. In skin, *Tnmd* expression significantly downregulated between day 1 and 3 after skin incision, while in the tendon defect studies *Tnmd* expression significantly upregulated in the period between 1–2 weeks in the patella and between 4–12 weeks in the rotator cuff that was FGF-*2* treated (Kameyama et al., 2015; Omachi et al., 2015; Tokunaga et al., 2015). This data strongly motivates further investigations on the exact roles of *Tnmd* in tissue repair.

2.8. Tenomodulin correlations to various diseases

In recent years, research mostly conducted on a genomic level by single nucleotide polymorphism (SNP), has presented very interesting correlations between *TNMD* and a variety of diseases. Specifically, *TNMD* was selected as a candidate gene for obesity, type 2 diabetes, metabolic syndrome, Alzheimer's disease and age-related macular degeneration, etc. The stated association studies are summarized in Table 4, and the concrete *TNMD* SNPs are shown in Fig. 7.

Associations with obesity, type 2 diabetes and metabolic syndrome were investigated in two study populations; the Finnish diabetes prevention study and the metabolic syndrome in men (Tolppanen et al., 2007; Tolppanen et al., 2008a; Tolppanen et al., 2008b). In these studies several gender-specific SNPs were identified; however it is still not exactly known how these SNPs affect *TNMD* transcription, splicing or protein amino acid sequence. Intriguingly most of the identified SNPs are found in introns and not in exons or near exon–intron splice sites. Only SNP rs2073162 is found on exon 3, suggesting that the key SNPs might have a long-distance effect on transcriptional control. A follow up study has revealed that *TNMD* expression is increased in obesity and down-regulated during weight-reducing diet (Saiki et al., 2009). A similar finding was also reported by Kolehmainen et al. (2008), the authors found a strong downregulation of the *TNMD* gene along with weight reduction. Moreover, the expression of *TNMD* was associated with several characteristics of the metabolic syndrome, but the mechanisms by which *TNMD* may be involved in insulin sensitivity remain elusive. Other three studies have looked into correlations between *TNMD* SNPs, or expression, to metabolic syndrome and reported certain links to serum lipoproteins levels and inflammatory factors (Tolppanen et al., 2008a; Tolppanen et al., 2008b; Gonzalez-Muniesa et al., 2013). One circulated idea is that an increase in *TNMD* expression during obesity might exert a protective mechanism aimed at limiting growth of new blood vessels in periods of adipose tissue expansion. There are still many open questions to these studies: for example, which cell type expresses *TNMD* mRNA and how abundant is the protein; if and how *TNMD* regulates the vascular formation and can it even directly affect adipose cells; how Tnmd might be involved in cholesterol metabolism or can affect systemic immune mediators.

Tolppanen et al. (2011) suggested *TNMD* as an interesting candidate gene for Alzheimer's disease since they detected associations between *TNMD* SNPs and known risk factors for this disease. The sequence variation of *TNMD* was not connected with the prevalence of Alzheimer's disease when the results were adjusted to the *APOE* genotype, but the marker rs5966709 was linked with disease risk among women with *APOE ε4*-allele. These results suggest that the effect of *APOE* may be modified by *TNMD*. Brandau et al. (2001) has shown by in situ hybridization a *Tnmd* messenger in multiple areas of the adult mouse brain, however it is not clear if the protein is expressed, hence it would be very interesting to follow up on the above studies and explain possible functions of TNMD in the brain and brain diseases.

Age-related macular degeneration can be divided into atrophic and exudative forms, the latter being more common and accounting for approximately 80% of age-related macular degeneration cases. Since dysregulated neovascularization is involved in the pathogenesis of age-related macular degeneration, Tolppanen et al. (2009) also investigated the associations of *TNMD* SNPs with this condition and found rs1155974, rs2073163 and rs7890586 were correlating with higher risk in women. This study further supported the theory of a disrupted

Table 3
The use of *TNMD*, *Tnmd* as a lineage marker.

Study type	Experimental models	*TNMD* expression found by							Number of articles	References
		PCR	ISH	MA	NB	WB	ICC	IHC		
Cell phenotypisation (proliferation or differentiation) with growth factors/gene overexpression	In vitro using mouse, rat, dog and human cells	21	2		2	9	7		26	Chen et al. (2015); Li et al. (2015); Liu et al. (2015); Wada et al. (2015); Zhang et al., 2015; Mohanty et al. (2014); Shimada et al. (2014); Zhang et al., 2014; Fong et al. (2013); Gulati et al. (2013); Qiu et al. (2013); Rothan et al. (2013); Rui et al. (2013); Shen et al. (2013); Xia et al. (2013); Alberton et al. (2012); Lovati et al. (2012); Sassoon et al. (2012); Takimoto et al. (2012); Backman et al. (2011); Lee et al. (2011); Schneider et al. (2011); Haddad-Weber et al. (2010); Park et al. (2010); Violini et al. (2009); Yeh et al. (2005)
Characterization of tendon cells in culture	In vitro using mouse, rat, rabbit and human cells	13			1	1	2	1	17	Kato et al. (2015); Zhang et al., 2015; Mienaltowski et al. (2013); Al-Sadi et al., 2011; Busch et al. (2012); Inoue et al. (2012); Lee et al. (2012) Leone et al. (2012); Mazzocca et al. (2012); Tan et al. (2012a); Tan et al. (2012b); Wagenhauser et al. (2012); Hasegawa et al. (2010); Pauly et al. (2010); Rui et al. (2010); Itaya et al. (2009); Yeh et al. (2008)
Mechanical stimulation on cells and tendons	In vitro and in vivo with mouse and rat cells and tendons	3							3	Xu et al. (2015); Zhang and Wang (2013); Eliasson et al. (2009)
Tissue engineering, 3D combination of stem cells with biomaterials and/or growth factors	In vitro, ex vivo and in vivo using mouse, rat, rabbit, dog, pig, sheep, horse, cow and human cells	24				3	2	3	26	Brown et al. (2015); Herchenhan et al. (2015); Musson et al. (2015); Otabe et al. (2015); Pawelec et al. (2015); Qin et al. (2015); Theiss et al. (2015); Wagenhauser et al. (2015); Wang et al. (2015); Barsby et al. (2014); Klatte-Schulz et al. (2014); Mitani et al. (2014); Mizutani et al. (2014); Moshaverinia et al. (2014); Subramony et al. (2014); Tang et al. (2014); Younesi et al. (2014); Erisken et al. (2013); Chen et al. (2012); Kishore et al. (2012); Omae et al. (2012); Peach et al. (2012); Tong et al. (2012); Zhu et al., 2010; Bashur et al. (2009); Omae et al. (2009)
Developmental analyses	In vivo using zebrafish, mouse, chicken and sheep	5	2					1	7	Murata et al. (2015); Russo et al. (2015); Chen and Galloway (2014); Liu et al. (2012), Liu et al. (2010); Lorda-Diez et al. (2009); Brandau et al. (2002)
Tendon healing and expression analyses in tissues	In vivo using mouse, rat rabbit, horse and human tissues	14	1	1					15	Kameyama et al. (2015); Omachi et al. (2015); Rubio-Azpeitia et al. (2015);Tokunaga et al. (2015); Kaux et al. (2014); Carvalho Ade et al. (2013); Johansson et al. (2012); Kaux et al. (2012); Mendias et al. (2012); Watts et al. (2012); Scott et al. (2011); Watts et al. (2011); Jelinsky et al. (2010); Minogue et al. (2010); Mendias et al. (2008)

Abbreviations: 3D, three dimensional; ICC, immunocytochemistry; IHC, immunohistochemistry; ISH, in situ hybridization; MA, microarray; NB, Northern blot; PCR, polymerase chain reaction; WB, western blot.

balance between stimulators and inhibitors of neovascularization in the pathogenesis of exudative age-related macular degeneration. However, functional studies are needed to reveal the exact mechanisms and involvement of *TNMD* in these associations.

Due to the presence of *TNMD* in tendinous structures *TNMD* was in the spotlight also in heart-related studies. In fact, the anatomical organization of the CTCs, which connect the papillary muscle to the atrioventricular valves, is very similar to tendons but of much smaller size. Their rupture is a well-known cause of mitral regurgitation and cardiac valvular syndromes. At the base of these failures is the abrogated avascularity of the cordis (Kimura et al., 2008; Hakuno et al., 2011). The study by Kimura et al. (2008) strongly confirmed that the local absence of TNMD leads to enhanced angiogenesis, VEGF-A and MMPs activation following the rupture of the heart chordae tendineae. In the above study *TNMD* knockdown experiments have suggested that *TNMD* can directly inhibit endothelial cell migration, but the receptor or molecular agent through which Tnmd acts has not been found.

The associations with juvenile dermatomyositis were studied by Chen et al. (2008) revealing that *TNMD* mRNA was upregulated during the chronic inflammatory phase. The authors suggested that TNMD might regulate contractility of vascular smooth muscle cells and speculated that interventions that diminish the anti-angiogenic remodelling may be beneficial for children with longer duration of untreated juvenile dermatomyositis. However, there is little information describing how exactly *TNMD* is involved in vasculature loss and tissue remodelling.

Last, a case report study of two female patients with intellectual disability and seizures related to female-restricted epilepsy with mental retardation found a genomic deletion at PCDH19, spanning the *TNMD* gene (Vincent et al., 2012). Despite being a case study, it would be very interesting, after validation of TNMD protein expression in the brain, to examine if *Tnmd* knockout animals exhibit a brain phenotype and altered behaviour.

In summary, now that it is apparent that certain SNPs linked to several health conditions are located in the *TNMD* locus and that changes in the *TNMD* levels are associated with at least three different disease it becomes almost obligatory in future research to prove whether or not these associations have a critical role in the establishment of these diseases and especially how the SNPs affect *TNMD* gene expression or protein production and function. One way to continue is to challenge the Tnmd knockout strain by crossing it with mouse strains harbouring a concrete disease phenotype. We believe that the uncovering of the

Table 4
Summary of studies correlating *TNMD* with various diseases.

Diseases	Study model	TNMD analysis	Main conclusions	References
Obesity	Longitudinal study, 166 men and 341 women with overweight and impaired glucose tolerance	*TNMD* SNPs	rs5966709 and rs4828037 were associated with adiposity in women; rs11798018 was related with adiposity in men.	Tolppanen et al. (2007)
	Longitudinal study, 68 obese cases, subcutaneous adipose tissue, omental adipose tissue	*TNMD* mRNA	*TNMD* was highly expressed in adipose tissue and increased in obesity and was down-regulated during diet-induced weight loss in men and women.	Saiki et al. (2009)
	Longitudinal study, 28 weight reduction cases and 18 controls, fat tissue	*TNMD* mRNA	Strong downregulation of *TNMD* expression after long-term weight reduction in men and women.	Kolehmainen et al. (2008)
Type 2 diabetes	Longitudinal study, 166 men and 341 women with overweight and impaired glucose tolerance	*TNMD* SNPs	rs2073163 and rs1155974 were linked with 2-h plasma glucose in men; rs2073163, rs1155974, and rs2073162 were associated with type 2 diabetes in men; only rs2073162 associated with 2-h plasma glucose in women.	Tolppanen et al. (2007)
Metabolic syndrome	Cross-sectional study, random sample of 5298 men	*TNMD* SNPs	rs2073162 was related with serum lipoproteins in a BMI-dependent manner in men; no women were involved in the research.	Tolppanen et al. (2008a)
	Longitudinal study, 9 lean and 9 obese male high fat cases, subcutaneous abdominal adipose tissue	*TNMD* mRNA	Expression of *TNMD* was linked with some relevant metabolic syndrome features in men; no women were involved in the research.	Gonzalez-Muniesa et al. (2013)
	Cross-sectional study, 166 men and 341 women with overweight and impaired glucose tolerance	*TNMD* SNPs	rs2073163, rs1155974, and rs2073162 were connected with acute phase reactants in men; rs5966709 and rs4828037 were associated with inflammatory factors in men and women; rs2073163, rs4828038, and rs1155974 were related with inflammatory factors in women.	Tolppanen et al. (2008b)
Alzheimer's disease	Cross-sectional study, 526 Alzheimer's disease cases and 672 controls	*TNMD* SNPs	rs5966709 was connected with risk of Alzheimer's disease among women with AOPE ε4-allele; none of the SNPs associated with Alzheimer's disease in men.	Tolppanen et al. (2011)
Age-related macular degeneration	Cross-sectional study, 307 age-related macular degeneration cases and 168 controls	*TNMD* SNPs	rs1155974 and rs2073163 were associated with a risk of age-related macular degeneration in women; none of the SNPs associated with prevalence of age-related macular degeneration among men.	Tolppanen et al. (2009)
Rupture of the chordae tendineae cordis	Cross-sectional study, 12 normal and 16 ruptured chordae tendineae cordis (CTC), specimens obtained from autopsy or surgery, immunohisto-chemical analysis	TNMD protein	TNMD was locally absent in the rupture area, where abnormal vessel formation, strong expression of vascular endothelial growth factor-A and matrix metalloproteinases, and infiltration of inflammatory cells were observed, but not in the normal or non-ruptured CTC.	Kimura et al. (2008)
Juvenile dermatomyositis	Cross-sectional study, 31 girls with Juvenile dermatomyositis and 4 healthy controls, muscle biopsies	*TNMD* mRNA	*TNMD* was up-regulated during the chronic inflammatory state of the disease, and down-regulated when disease duration was short in girls, no boys were involved in the research.	Chen et al. (2008)
Intellectual disability and seizures	Case report, two female patients, deletions spanning PCDH19	*TNMD* gene	Genomic deletions spanning the *TNMD* gene in female-restricted epilepsy with mental retardation.	Vincent et al. (2012)

Abbreviations: APOE, apolipoprotein E; CTC, chordae tendineae cordis; mRNA, messenger ribonucleic acid; PCDH19, Protocadherin 19; rs, reference SNP; SNP, single nucleotide polymorphism; TNMD, tenomodulin.

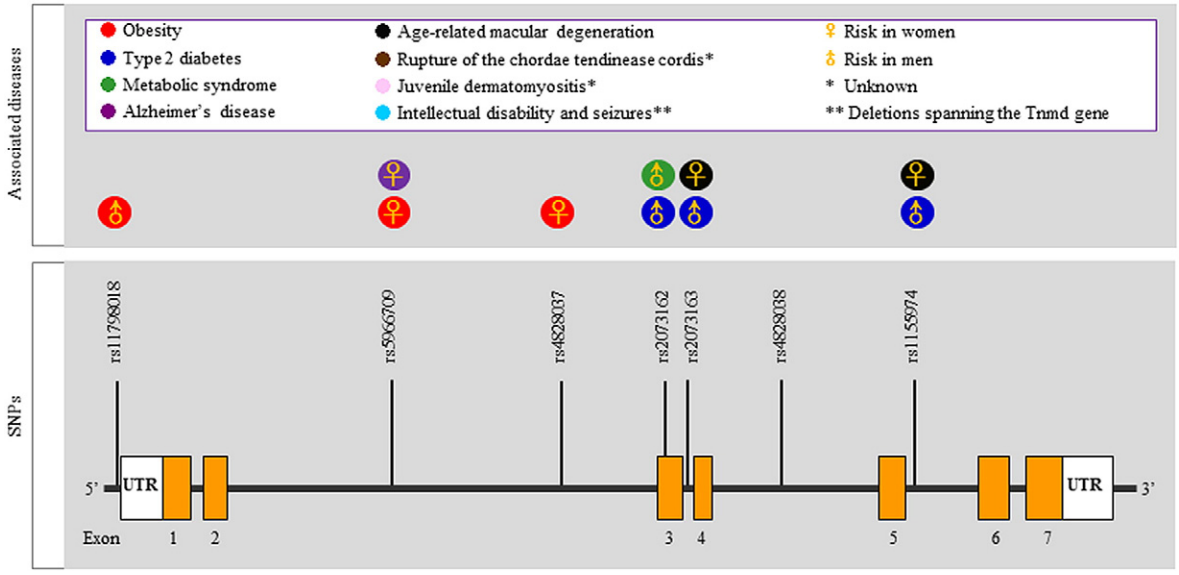

Fig. 7. Single nucleotide polymorphism (SNPs) and other putative mutations in the *TNMD* gene locus correlating with various diseases. Orange boxes represent exons, while the connecting black lines are introns. Abbreviations: rs, reference SNP; SNPs, small nucleotide polymorphisms; UTR, untranslated region.

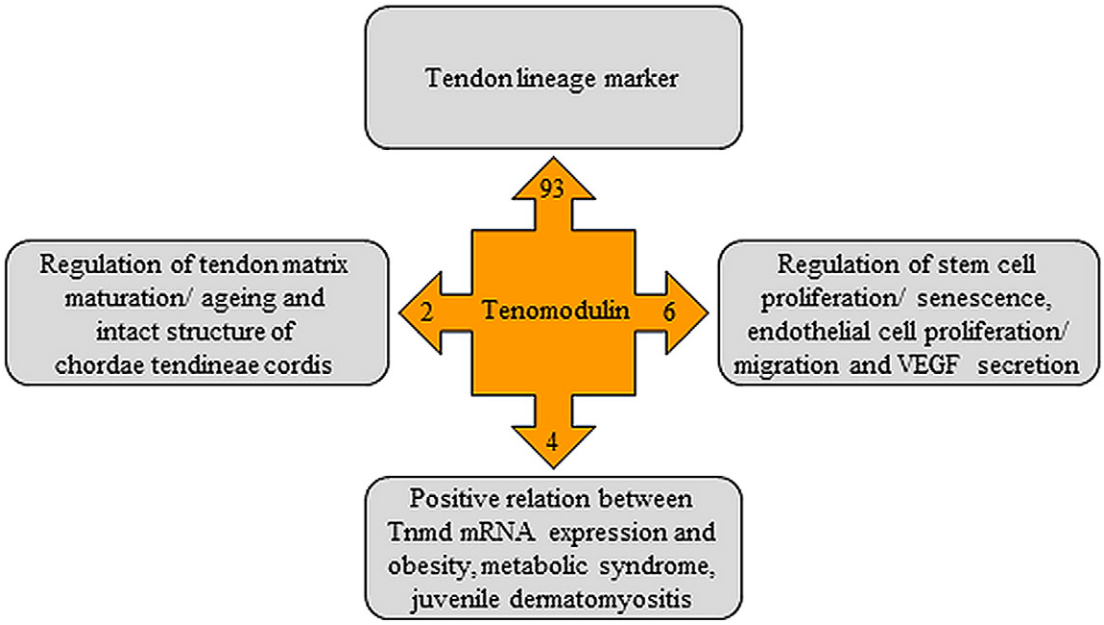

Fig. 8. Schematic summary of *TNMD* known functions. Abbreviations: mRNA, messenger ribonucleic acid; Tnmd, tenomodulin; VEGF, vascular endothelial growth factor.

exact *TNMD* functions will be essential for improving our understanding of the mentioned clinical conditions, which subsequently will permit the development of appropriate counterstrategies.

3. Conclusion and future perspectives

Since its discovery in 2001, *TNMD* has gained significantly more attention as demonstrated in the increasing number of articles in the last 15 years. The alternative names have been condensed to the single name tenomodulin with its corresponding abbreviation as *TNMD*. It is a gene bearing high and only homology to *Chm*1, but still exhibits important differences, such as the absence of a furin cleavage signal, different glycosylation sites and expression pattern. Although the predominant expression of *TNMD* is found in tendons and ligaments, it is also shown to be expressed in other tissues such as parts of the eyes, cordae tendineae cordis, muscle sheaths, brain, fat and skin. The presence of isoforms needs to be carefully verified in different species. Another very important future goal should be the better understanding of the TNMD protein domain structure. The *TNMD* signalling pathway is still very elusive as up to date only few upstream factors, such as *Scx* and *Mkx*, have been identified. With regards to downstream effectors, there is a complete lack of knowledge on the direct protein binding partners of TNMD. The full clarification of TNMD signalling must become central in follow up research, because by only elucidating *TNMD* modes of action we would be able to specifically manipulate its effect in tendons and other tissues. We have summarized the current discovered functions of *TNMD* in Fig. 8. Firstly, *TNMD* has been strongly justified as the best tendon and ligament marker in more than 90 different studies. Next, a combination of in vivo and in vitro investigations has revealed its positive role on tendon/ligament cell and tissue functions as well as is negative effect on vessels in specific regions of the heart and in tumour models. Last, we are just starting to comprehend the potential involvement of *TNMD* in various diseases such as obesity and metabolic syndrome, where *TNMD* expression is positively correlated to an advanced disease state. However, since most of the disease-associated studies are based on identification of SNPs in *TNMD* gene locus it is very important to find out how exactly these mutations translate into these diseases. In sum, we believe that this review not only summarizes the current state of affairs of our knowledge of *TNMD* gene, protein, expression and functions, but also will excite international researchers to further study this mysterious tenogenic modulating insider factor in terms of complete deciphering of its signalling pathway, contribution to certain pathologies as well as possible development of therapeutic strategies.

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