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The Alternative Matrisome: alternative splicing of ECM proteins in development, homeostasis and tumor progression

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Running title: Alternatively spliced ECM proteins

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Abbreviations:

RNA binding protein (RBP)

ribonucleoprotein complex (RNP)

splicing regulatory element (SRE)

heterogeneous nuclear ribonucleoprotein (hnRNP)

TIMP metalloproteinase inhibitor 1 (TIMP1)

FN type III domain (FNIII)

fibronectin (FN)

tenascin-C (TNC)

matrix metalloproteinase (MMP)

thrombospondin-2 (TSP2)

osteopontin (OPN)

cancer-associated fibroblast (CAF)

lysyl oxidase-like (LOXL)

Abstract

The extracellular matrix (ECM) is a fundamental component of the tissue of multicellular organisms that is comprised of an intricate network of multidomain proteins and associated factors, collectively known as the matrisome. The ECM creates a biophysical environment that regulates essential cellular processes such as adhesion, proliferation and migration and impacts cell fate decisions. The composition of the ECM varies across organs, developmental stages and diseases. Interestingly, most ECM genes generate transcripts that undergo extensive alternative splicing events, producing multiple protein variants from one gene thus enhancing ECM complexity and impacting matrix architecture. Extensive studies over the past several decades have linked ECM remodeling and expression of alternatively spliced ECM isoforms to cancer, and reprogramming of the alternative splicing patterns in cells has recently been proposed as a new hallmark of tumor progression. Indeed, tumor-associated alternative splicing occurs in both malignant and non-malignant cells of the tumor environment and growing evidence suggests that expression of specific ECM splicing variants could be a key step for stromal activation. In this review, we present a general overview of alternative splicing mechanisms, featuring examples of ECM components. The importance of ECM variant expression during essential physiological processes, such as tissue organization and embryonic development is discussed as well as the dysregulation of alternative splicing in cancer. The overall aim of this review is to address the complexity of the ECM by highlighting the importance of the yet-to-be-fully-characterized “alternative” matrisome in physiological and pathological states such as cancer.

Keywords: Alternative splicing, extracellular matrix, matrisome, alternative isoforms, cancer, development, homeostasis

Introduction

The extracellular matrix (ECM) is a complex and dynamic network of macromolecules that provides a mechanically-tuned bioactive scaffold upon which cellular constituents of tissues adhere, migrate, divide and differentiate. The A dynamically evolving matrix has proven to be fundamental for the regulation of physiological processes, such as tissue morphogenesis [1] and wound healing [2], and it plays a determinant role in disease progression, as observed in fibrosis, osteoarthritis and cancer [3–5]. Over the past decade, molecular and proteomic profiling of ECM and ECM-associated proteins has shed light on the matrisomal landscape of numerous cell and tissue types [6,7].

Most ECM proteins are encoded by mRNA transcripts that undergo considerable alternative splicing. As the distribution of splicing factors varies across organs, developmental stages and diseases, the production of alternate mRNA transcripts is tissue and context specific. In cancer, reprogramming of the cellular alternative splicing pattern is now considered to be a new hallmark of tumor progression [8,9]. Among cancer-associated cellular reprogramming events, it is now recognized that expression of specific ECM splicing variants represents a key step in stromal activation and disease progression [10]. In this review we will discuss various studies that support a role for ECM splicing variants in embryonic development and tissue homeostasis, then highlight the implication of their misexpression in cancer progression. Key mechanisms and regulators of splicing variant biogenesis will also be addressed. A final section will describe a step-by-step procedure for the basic analysis of ECM splicing variants in cancer using online databases.

Overview of alternative splicing and its modulation from the ECM perspective

Alternative splicing is a co-transcriptional event affecting over 95% of human genes [11]. It consists of intron removal and joining of exons or their skipping depending on multiple interactions of splicing factors with the splicing machinery (spliceosome) (see **Box 1**). Alternative splicing allows for the production from a single gene of various mature mRNAs that, once translated into different protein variants, increase proteome diversity and impact cellular functions ([12] for review).

In contrast to constitutive splicing, alternative splicing can be modulated and the final outcome of the splicing reaction (combination of exons in the mRNA) results from opposing effects of splicing factors on the splicing machinery (**Fig. 1A**) and the recognition of weaker splicing sites, compared to constitutive splicing sites [13]. Moreover, some splicing factors can exhibit either “splicing enhancing” or “splicing inhibitory” activities, depending on their post-translational modifications and/or interactions, thus turning alternative splicing into a dynamic process that is tightly linked to cellular state and signaling events. In cells, the selection of exons to be included and/or introns to be retained

during alternative splicing reactions can occur through 5 main types of splicing events (see **Fig. 1B**). In the case of ECM genes, alternative splicing reprogramming can profoundly affect transcripts and lead to the translation of functionally distinct proteins with different conformations and biochemical features, as schematized in (**Fig. 1C**)

In addition to splicing factors, other RNA Binding Proteins (RBPs) can modulate splicing reactions by interacting with splicing factors [14] and **Box 1**. On a larger scale, the interaction of multiple RBPs with splicing factors generates complex Splicing Regulatory Networks (SRNs) that create tissue- and cell process-specific alternative splicing patterns ([15] for extensive review).

Alternative splicing Box

Spliceosome: Large RiboNucleoProtein complex (RNP), composed of small nuclear RNAs (snRNA), that binds to associated protein factors creating 5 small nuclear ribonucleoprotein subunits U1, U2, U4, U5, and U6 that dynamically interact forming a larger complex: the spliceosome.

Splicing sites: *cis*-acting sequences located on the pre-mRNA, flanking and defining a potential intron. The consensus sequence of these motifs is variable but contains preponderantly dinucleotides 'GT' in the 5' SS (donor site) and 'AG' in the 3' SS (acceptor site). These 2 sites will be recognized by the U1 and U2 spliceosome subunits, respectively, for initiation of the splicing reaction.

Splicing regulatory elements (SRE): *cis*-acting sequences in the transcript which regulate the constitutive or alternative splicing reaction by recruiting specific *trans*-acting splicing factors that either enhance or silence the splicing reaction via regulation of spliceosome assembly. Depending on their location and interacting factors, SRE are called either Exonic/Intronic splicing enhancers (ESE/ISE) or Exonic/Intronic splicing silencers (ESS/ISS). Recruitment of splicing factors and/or additional RNA binding proteins (RBPs) on these sites is an essential part of alternative splicing regulation.

Splicing factors: RBPs that recognize splicing regulatory elements of the pre-mRNA and further interact with the spliceosome machinery exerting enhancing or inhibitory activities. Well described families of splicing factors include: heterogeneous nuclear ribonucleoproteins (hnRNPs) largely involved in the inhibition of the splicing reaction and members of the serine/arginine (SR)-rich protein family which have been mostly described as splicing enhancers.

Interacting RBPs: additional RBPs that indirectly participate in the splicing reactions by interacting with splicing factors. For example, the RBP SAM68 was shown to interact with the splicing factor hnRNPA1/2 in response to DNA damage and to cooperate with it in the production of the pro-apoptotic (short) isoform of the apoptosis regulator Bcl-x, termed Bcl-xS [16]

At the cellular level, alternative splicing is tightly regulated by signals that arise from the extracellular environment. For example, in trabecular meshwork cells, mechanical stretching significantly increases the expression of transcripts containing the fibronectin type III (FNIII) domain D of tenascin-C (TNC), the long NC3 isoform of collagen type XII, the V1 isoform of versican and several other variants of ECM proteins [17]. This mechanically-regulated tuning of the matrix is important in the context of tissue stiffening that occurs in disease states such as fibrosis and cancer (reviewed in [18]). Also, fluid pressure and flow in the arterial endothelium has been shown to trigger splicing of the fibronectin (FN)

gene (*FN1*) and promote the inclusion of the pro-angiogenic FNIII Extra Domains EDB and EDA (also known as EIIIA or FNIII EDA and EIIB or FNIII EDB, respectively) [19].

In addition to physical signals, biochemical changes in the extracellular environment are also important for regulation of alternative splicing. For instance, extracellular acidification favors accumulation of the 6kb mRNA of the TNC gene (*TNC*) in fibroblasts whereas the 8kb transcript is preferentially expressed above pH7 [20]. Also, in mesangial cells elevated glucose levels have been correlated with increased expression of the long isoform of this glycoprotein [21]. These changes linked to metabolism occur in the microenvironment in many normal and pathological conditions.

Receptor activation by growth factors, cytokines and ECM components is also a crucial modulator of alternative splicing. For example, TGF β signaling in human podocytes mediates alternative splicing of the Extra Domain A of FN [22]. In endothelial cells IL-1 can induce splicing of the CS1 domain of FN, thus mediating VLA-4 (α v β 4 integrin) adhesion [23]. Lastly, ECM splicing variants themselves can trigger signaling events that modulate alternative mRNA splicing. Recent evidence showed that the secreted ECM1a isoform of the extracellular matrix protein-1 (ECM1), through interactions with integrin α X β 2, increases expression of the splicing factor hnRNPLL, leading to enhanced ECM1 transcript splicing and ECM1a protein expression [24].

Alternative splicing pattern variation in response to extracellular stimuli can be explained mechanistically by post translational modification of splicing factors and their RBP partners, as they are often downstream targets of receptor signaling cascades causing changes in their splicing activities. Of note, EGFR or FGF-2 signaling are for instance known to induce SR splicing factor phosphorylation and nuclear translocation [25,26]. Also, hnRNP A1 splicing factor can be ubiquitinated downstream of EGF stimulation [27]. Lastly, ERK-dependent phosphorylation of the associated splicing RBP, SAM68 (Src associated in mitosis, of 68 kDa), leads to increased inclusion of the exon v5 of the glycoprotein CD44 and expression of its secreted oncogenic isoform over its plasma membrane bound isoform [28].

Cells adapt to their surrounding microenvironment by modulation of alternative splicing in response to extracellular stimuli. Such adaptation includes ECM variant production and remodeling. In the next section, we will discuss findings that highlight the importance of ECM variant production for embryonic development and organ homeostasis, focusing on “core matrisome” proteins, as well as some key interacting and remodeling partners. Due to size limitations, this review will not cover the extensive literature on alternatively spliced Matrisome-associated secreted factors (hormones, cytokines, and growth factors) and their receptors.

Matrisome splicing variants in cell physiology: implications for embryonic development and tissue homeostasis

Studies aimed at characterizing sets of ECM proteins date back to more than 80 years. It was not until the late 80's that George Martin proposed the term basement membrane "matrisome" to describe supramolecular complexes composed of the basement membrane constituents type IV collagen, laminin, heparan sulphate proteoglycan and nidogen [29]. Two decades later, in an effort led by Richard Hynes, Alexandra Naba and colleagues, the concept of "matrisome" was refined to include more than 1000 genes encoding structural ECM components, remodeling partners and associated factors (see <http://matrisomeproject.mit.edu/> for the latest Matrisome database).

Matrisome-coding genes are most often long and display multi-exon structures predicted to generate a wide range of alternatively spliced transcripts that encode either very few or up to dozens of isoforms (RefSeq (NCBI), The Human Protein Atlas and [30]). Although it is known that not all predicted isoforms result in translated proteins, systematic analysis of actively translated splicing variants is still greatly lacking. Thus, the prevalence of splicing variants of ECM components and their potential importance in physiological (examples in **Table 1**) and pathological contexts (examples in **Table 2**) is largely underestimated. Interest in alternatively spliced variants is often sparked by the discovery of a critical role for a particular isoform during development caused by mutations affecting alternative splicing of the gene, or by large-scale analysis of splicing pattern changes in disease.

ECM splicing variants during development

The embryonic ECM is structurally, mechanically, biochemically, and functionally different than the ECM of adult tissues. Developmentally regulated transcripts can be found in both the core matrisome and matrisome-associated gene sets. Despite the largely ignored functional implications of these splicing variants during embryonic development, certain isoforms exert critical roles that, if perturbed, have dramatic consequences on tissue physiology and organ formation (see **Table 1**).

Among alternatively spliced core matrisome transcripts, those encoding collagens are of particular interest. Collagens are the most abundant proteins in mammals and represent essential constituents of connective tissues. These trimeric molecules associate with different ECM proteins, creating a wide range of 3D structuring and signaling environments that drive and sustain tissue organization and homeostasis. Their expression is both tissue-specific and developmentally-regulated and, to date, 42 different collagen genes coding for 28 collagens have been identified [31]. In addition to this molecular diversity, the complexity of collagen architecture and functions arise from extensive alternative splicing of each collagen gene. A significant number of developmental abnormalities are linked to

mutations in collagen genes that lead to their aberrant splicing. One well known example of collagen splicing dysregulation during development involves the *COL11A1* gene in which a splicing mutation induces a deletion of the last 54 bp of the mRNA causing Marshall syndrome [32]. Interestingly, a recent study on a cohort of 867 patients affected by Osteogenesis Imperfecta (OI), mainly caused by mutations in genes encoding type I collagen, revealed that among 29 variants from 34 OI probands, 17 were generated by aberrant splicing [33].

Aberrant splicing leading to the expression of pathological isoforms is not the only example of collagen alternative splicing during development. Indeed, splicing of the *COL2A1* gene provides an interesting model of ‘normal’ alternative splicing regulation during cartilage development [34]. To date 4 isoforms of type II procollagen have been identified: IIA, encoded by the longest and most abundant transcript that includes the 2nd exon; IIB, encoded by a shorter transcript that lacks the 2nd exon; and IIC and IID, encoded by transcripts generated respectively by alternative usage of 5’ and 3’ splice sites within the 2nd exon (**Fig. 2**). Initially, undifferentiated chondrocytes express the IIA isoform, but they switch to expression of the IIC isoform during early steps of differentiation. Finally, expression of the IIB isoform, lacking exon 2, is produced exclusively by fully differentiated cells [35–37]. Although the precise biological consequences of this sequential isoform production during development have not been established, procollagen II splicing has been shown to be co-transcriptionally regulated by the RPB TIA-1, a modulator of cytokine expression following injury [38]. This finding has led authors to propose a role for procollagen II splicing during tissue repair [39]. A subsequent study, performed on transgenic mice constitutively expressing the IIA procollagen isoform, revealed that the IIA isoform can form hetero fibrils with type XI collagen, thus modifying the ECM structure of cartilage and resulting in the formation of a cartilage with thinner fibrils [40]. Despite no apparent tissue abnormalities, this “minor” defect suggests that procollagen II splicing is a regulator of collagen XI assembly with a possible implication in cartilage homeostasis [41].

Alternative splicing of ECM glycoproteins, a core matrisome sub-class comprised of nearly 200 genes, also plays a fundamental role during embryogenesis. Indeed, the adhesive glycoprotein FN with its spliced variants are known to be key regulators of vascular development [42]. Although alternative splicing of the *FN1* gene at three different sites can yield more than 20 alternative transcripts [43], 2 classes of FN proteins have been defined. First, the insoluble “cellular” FNs which harbor the alternatively spliced Extra Domains EDB and/or EDA, each corresponding to a FN Type III repeat (FNIII), and the soluble “plasma” FN which is devoid of these domains (**Fig. 2**). Whereas plasma FN is produced continuously by the liver, cellular FN is expressed during embryonic development and at sites of tissue remodeling (e.g. wound healing, fibrosis) [44,42] where its expression is critical for both processes. In mice, deletion of both Extra Domains of FN induces multiple early cardiovascular defects leading to

embryonic lethality [42], whereas the single removal of EDB or EDA results in viable and fertile mice [45,46], suggesting that compensatory mechanisms exist.

The adhesion-modulating glycoprotein TNC is another example of a developmentally-regulated multi-modular protein for which over 500 possible alternatively spliced isoforms exist (see [47]). Similar to FN, TNC is expressed mainly during embryonic development and transiently upregulated during inflammation and tissue repair. TNC is composed of 4 domains, including one central region which features a string of FNIII repeats (**Fig. 2**). In addition to 8 constitutively expressed FNIII repeats, 9 additional FNIII repeats (A1-4, B, AD2, AD1, C, D) can be incorporated in the protein as a result of extensive alternative splicing events (reviewed in [48]). Depending on the inclusion of these spliced FNIII repeats, the size of TNC ranges from 230/260 to 190/200 kDa commonly named “long” and “short” isoforms, respectively. Short isoforms are mostly restricted to areas of cell condensation and differentiation during organogenesis, whereas long variants, containing one or more alternatively spliced FNIII repeats, have been associated with regions of active tissue remodeling, cell migration and division [49]. Interestingly, the combinatorial expression of short and long TNC variants (at least 20) in mouse embryo neurospheres, driven by the Pax6 transcription factor, was proposed to have a significant role in neural stem/progenitor cell physiology [50]. Very similarly, the heavily-glycated multiplexin collagen XVIII (*COL18A1*) shows differential patterns of both transcription-specific and alternative splicing-dependent isoform abundance, e.g. in the kidney, though the exact mechanisms sustaining proper balancing of the isoforms remain largely unknown [51].

During morphogenesis, expression of other glycoprotein variants also contributes to the formation of “alternative” ECM networks with different properties supporting distinct tissue organizations. For example, the glycoprotein fibulin-1 features at least 4 structurally distinct alternatively spliced variants (from A to D) with differential ECM binding affinities (**Fig. 2**). The fibulin-1C variant displays a 100-fold higher affinity for nidogens than the fibulin-1D variant [52]. During *Caenorhabditis elegans* development these 2 variants are incorporated in distinct basement membrane assemblies sustaining different functions [53,54]. Fibulin 1C regulates cell shape and adhesion in the developing pharynx, intestine, gonad, and muscle, while the fibulin-1D variant plays a unique role in assembly of body wall-muscle connections in the pharynx.

The proteoglycan sub-class of glycoproteins also exhibits multiple splicing variants that are of particular interest during development. Versican for example is a large proteoglycan found in ECM of highly hydrated tissues [55] and is widely expressed during development (reviewed in [56]). The *VCAN* gene encodes 4 classical isoforms generated by alternative splicing of the exons coding its glycosaminoglycan binding regions (GAG α and GAG β). V0 is the full-length transcript, V1 has only the

GAG β domain, V2 has only GAG α , and V3 lacks both domains (**Fig. 2**). In addition to the main isoforms, a V4 variant encoding only a portion of the GAG β domain was described in a human breast cancer lesion [57] and a C-terminal-truncated versican variant was detected in dorsal root ganglia extracts from a rat spared nerve injury model of neuropathic pain [58].

In a mouse model, imbalanced expression of versican due to constitutive deletion of exon 7 of the gene was also shown to prevent the expression of V2 and V0 isoforms, resulting in severe heart formation defects [59]. In the brain, versican V0/V1 and V2 isoforms are shown to negatively regulate axon growth while V0/V1 isoforms cause this phenotype on peripheral axons in the developing chick hindlimb by inducing defasciculation and axon stalling [60]. The V2 isoform impairs axon growth in nodes of Ranvier neurons by peri-axonal ECM remodeling. [61]. This is an interesting example of how ECM splicing isoforms can induce similar cell phenotypes through apparently distinct mechanisms. It is also noteworthy that this function of versican variants could be of essential importance for the sculpting of neuron networks during the pruning phase of brain development [62].

Matrisome-associated proteins, of which 751 have been recognized to date, are also functionally important during development. Active morphogenesis requires extensive remodeling and mechanical tuning of the provisional embryonic ECM by both non-proteolytic and proteolytic mechanisms to ensure proper integration of tissue forces and transmission of biochemical information.

Amongst ECM-degrading proteases, members of the ADAMTS family have been extensively studied for their role in versican proteolysis and provisional matrix remodeling in different developmental contexts (see [63] for review). Splicing variants of several ADAMTS proteases have been described, yet in most instances the functional consequences of the alternative splicing events have not yet been investigated in detail. Alternative splicing of *ADAMTS17*, one of the two ADAMTS proteases mutated in a rare connective tissue and ocular disorder (Weill-Marchesani syndrome) was shown to give rise to two novel isoforms (ADAMTS17A and ADAMTS17B) with altered proteolytic activity and cellular localization [64]. Another metalloproteinase, the disintegrin and metalloproteinase ADAM12, displays variant-specific maturation pathways. ADAM12 is a metalloproteinase that can be synthesized either in the form of a single pass transmembrane protein (ADAM12-L), or a shorter secreted enzyme (ADAM12-S) (**Fig. 2**). Both proteinases are implicated in cell fusion, growth and tissue repair [65]. Whereas expression of ADAM12 is restricted to developing and healing tissues, elevated plasma levels of ADAM12-S is an early marker of preeclampsia [66] and trisomy 18 [67].

Splicing variants of matrisome transcripts maintain ECM balance and cell homeostasis

Remodeling and fine tuning of the ECM is not restricted to embryonic development. Indeed, ECM structure and composition constantly evolves throughout life to assure cell and tissue homeostasis which, in turn, require a balanced response to environmental signals. Alternative splicing of ECM components offers a unique means of attaining such equilibrium, considering that splicing variants can display isoform-dependent counterbalancing functions.

Versican for instance is important for maintaining the balance between cell proliferation and cell death in fibroblast populations. Indeed, expression of the V1 variant of versican was shown to enhance cell proliferation and reduce apoptosis, while expression of the V2 variant reduced cell proliferation and resistance to apoptotic stimuli [68]. In arterial smooth muscle cells, overexpression of the short V3 variant enhanced cell adhesion and reduced cell migration and proliferation, in contrast to V1 overexpression. [69].

TNC is also known for its variant-specific functions that impact pro- and anti-adhesive interactions of cells with their ECM [70]. For example, alternative splicing of the central FNIII domains of TNC can confer unique proteolytic susceptibilities to the protein which lead to the generation of degradation products with indirect or direct effects on cell adhesion. For example, insertion of the FNIII A3 repeat introduces a matrix metalloproteinase (MMP) cleavage site that is absent in the constitutive FNIII region lacking this repeat [71]. Functionally, the expression of MMP-specific TNC degradation products has been associated to increased aggrecanase activity and ECM degradation in arthritic cartilage [72].

During inflammation, alternative splicing was found to participate in differential regulation of the adhesive activity of TNC in stromal and immune cells. Whereas TNC as a substratum is known to inhibit fibroblast adhesion, it was found to support myeloid cell adhesion [49]. Moreover, in this study by Giblin and Midwood, expression of a short isoform of TNC harboring the FNIII AD2-AD1 domains was found to be transiently induced upon activation of resting myeloid cells with inflammatory stimuli. The authors proposed that the FNIII AD2-AD1 domains harbor a novel immunoregulatory site that tunes the pro-inflammatory activity of TNC, tempering inflammation in healthy tissue and enabling immune cell infiltration in damaged tissue or in certain pathological states.

Besides inflammation, the process of aging on itself impacts matrix homeostasis as it is characterized by exacerbated ECM degradation versus production, as well as changes in composition and altered mechanical properties [73]. ECM remodeling enzymes such as transglutaminases display increased expression and activity during aging [74,75]. Of interest, the tissue transglutaminase 2 (TGM2) gene has multiple TG2 mRNA isoforms generated by intron retention or exon splicing at various sites [76] and **Fig. 2**), which produces truncated proteins with different C-terminal sequences and functional characteristics. For instance, loss of the C-terminal domain in some TG2 variants impedes their Ca^{2+} -

dependent regulation and increases their GTPase activity [77]. Interestingly, C-terminal isoforms of TG2 are mostly detected in autoimmune diseases such as multiple sclerosis [78] or age-related degeneration such as Alzheimer's disease, where they are detected in brain samples of post-mortem Alzheimer's disease patients and co-localize with tau in neurofibrillary tangles in hippocampal neurons. Dysregulated TGM2 variants and their increased activity is suspected to be a cause of tau aggregation and subsequent long-term chronic stimulation and neurodegeneration [79]. It is noteworthy that splicing isoforms of transglutaminase, as well as ECM remodeling enzymes, are most often linked to inflammatory and neoplastic conditions (see next section).

Dysregulation of alternative splicing in cancer from the matrix perspective

During the past decade, studies linking alternative splicing and cancer have revealed the importance of alternative splicing dysregulation and its impact on cancer hallmarks as discussed in [80,81]. Alternative splicing reprogramming is now proposed to be a key metastatic tumor-promoting event. [82] Moreover, the transcriptome diversity generated by cancer-specific alternative splicing events can be profiled for use as diagnostic/prognostic tools, mined for discovery of novel biomarkers and selective drug targets for improved therapeutic intervention [9]. Interestingly, a recent study exploring the TCGA database reported that 119 genes encoding early splicing factors (such as U2) carry putative cancer-driving mutations in over 33 tumor types [87]. Such mutations, identified as hotspot or loss of function mutations, occur mainly in the coding sequence of these factors and often on residues implicated in the recognition of splicing sites of their target RNA. Consequently, mutated splicing factors display impaired recognition of splicing sites on their target RNAs, resulting in abnormal inclusion/exclusion of exons in RNAs encoding oncogenes and tumor suppressors.

Hypoxia, for example, is known to induce alternative splicing reprogramming in multiple cell types and diseases including cancer [88]. Deregulated alternative splicing under hypoxic conditions can result from effects on the splicing machinery, miRNA/lncRNA splicing regulators and hypoxia-sensitive signal transduction affecting both hypoxia-inducible factor (HIF)-dependent and-independent pathways [83]. With regard to matrisomal proteins, alternative splicing of the *Vegfa* gene (reviewed in [84]) has important implications for neovascularization of hypoxic tissue by generating matrix-tethered and freely diffusible isoforms of VEGF-A that tune the bioavailability of the protein. Inflammation and ECM remodeling in the tumor stroma also greatly affect cell signaling pathways that deregulate splicing factor expression and activity. For example, increased expression/activation of TGF β and downstream phosphorylation of SMAD3 in tumors promotes the interaction of SMAD3 with the RBP, PCBP1, which ultimately regulates the alternative splicing of CD44. CD44 is a surface glycoprotein receptor for hyaluronic acid that interacts with a number of matrisome components (e.g. osteopontin, collagens,

matrix metalloproteinases). By favoring the expression of the mesenchymal isoform, designated CD44s, over the epithelial isoform of the protein, CD44E, tumor cells are pushed towards a mesenchymal phenotype [85]. In mouse mammary epithelial cells, exposure of cells to MMP3, a matrix metalloproteinase overexpressed in many cancers (see below), releases hnRNP A1 splicing factor from the exon 3b of Rac1 transcripts leading to the expression of the pro-EMT isoform of Rac, Rac1b [86]. Tumor-associated changes in tissue mechanics, notably increases in density and stiffness of the ECM, can also trigger splicing changes. In gastric cancer cells, interaction with stiff matrices induces stiffness-dependent alternative splicing of the pro-EMT isoform of CD44 [87]. In addition to triggering splicing changes, tumor ECM proteins are themselves targets of dysregulated alternative splicing events (**Table 2**), which further push “splicing switches” towards stromal activation and tumor invasiveness.

Implication of ECM splicing variants in tumor progression

Historically, the implication of ECM splicing variants in cancer has been indirectly addressed in instances where known splicing isoforms of well characterized ECM components were found to exhibit tumor-associated expression patterns. For example, the so-called oncofetal isoforms of FN and TNC were shown in the early 90’s to be abnormally re-expressed in tumors [88,89]. Two decades later, the emergence of cancer “omics”, together with the in silico definition of the matrisome, led to the progressive identification of numerous ECM splicing variants across multiple tumors, and to the correlation of their expression with tumor stage and malignancy (see below and **Table2**). Nonetheless, these large-scale studies rarely delved into the mechanisms underpinning the specific roles of these splicing variants in tumor progression. Rather, analyses were often limited to their classification as prognostic biomarkers. Recent advances in single-cell profiling and quantitative proteomics have transformed the ability to examine the previously underappreciated expression of alternative matrisomal proteins in the tumor setting, beyond the “classical” matrisome. Understanding their function and underlying molecular mechanisms remains a major challenge for future ECM research.

ECM splicing variants impact tumor matrix organization

ECM remodeling during tumorigenesis and metastatic expansion is characterized by increased deposition of matrix components (e.g. collagens, FN, TNC) and their rearrangement by proteolytic and crosslinking enzymes that, collectively, lead to the formation of a stiff fibrotic stroma [5]. Splicing variants of multiple collagen types have been identified and several reports have linked their expression to neoplastic conditions. Profiling of the alternative splicing landscape in pancreatic ductal adenocarcinoma (PDAC) revealed that the collagen genes were amongst the most sensitive to splicing reprogramming events [90]. Another study on alternative splicing in PDAC reported tumor-specific expression of splicing variants of the COL6A3 chain of collagen VI harboring additional N-terminal von

Willebrand factor type A (vWF-A) modules [91]. Collagen VI is a microfibril-associated collagen upregulated in multiple malignancies. Although specific mechanisms by which alternative splicing of collagen VI could affect ECM arrangement have not been elucidated, the COL6A3 chain is known to play a crucial role in collagen VI assembly (see [92]). Collagen XI, a fibril-forming collagen that regulates collagen fibrillogenesis, is also upregulated in neoplasms. In human breast cancer, analysis of collagen XI mRNA splicing variants (A, B, C, E) revealed differential expression of the COL11A1 variants A and E, with modified N-terminal domains that potentially modify N-terminal proteolysis of the protein and ECM arrangement. Moreover, expression of the variant E transcript was associated with lymph node involvement and metastasis [93]. Given the importance of collagens for ECM organization, it is likely that the regulated expression of these (and other unidentified-to-date) splicing variants has major consequences for tumor matrix remodeling.

Upregulation of alternatively spliced cellular FN isoforms is also a characteristic of ECM in tumors and in the premestatic tumor niche ([94] and references therein). As cellular FN isoforms are among the first matrix proteins deposited, their assembly determines the subsequent deposition of other matrisome proteins [95]. Inclusion of EDA/EDB FNIII repeats in the FN molecule has been reported to enhance fibrillar assembly [94,96] through conformational changes in the molecule that alter its self-association properties and interactions with other ECM molecules and cellular receptors [97–99]. Recently, a computational image analysis pipeline based on confocal images of FN was developed to quantitatively assess the geometrical organization of alternatively spliced FN networks assembled by fibroblasts presented with the EDB and/or EDA variants [94]. This graph-based description of fibers using Gabor filters allowed for comparison of network features, such as fiber orientation, connectivity, thickness, heterogeneity, and pore density. FN assemblies containing Extra Domains, especially the EDA FNIII repeat, were found to be more branched and displayed smaller pores than plasma-like FN, and the presence of EDB resulted in a mixed set of attributes. Further molecular insights into extra domain-specific interactions and functions will be necessary to explain these results and their consequences. However, such approaches highlight the potential of computational image processing methods to explore the diversity of ECM structural features and to establish predictive links between ECM patterns and disease outcomes or response to treatment.

Aside from alternative splicing of core ECM components, abnormal degradation or crosslinking of the matrix by dysregulated splicing of matrisome-associated proteins also critically contributes to ECM reorganization in tumors. For example, several ECM-remodeling variants of ADAM metalloproteinases have been identified (reviewed in [100]). Alternative splicing of ADAMs 9,12, and 15 proteinases yields two major isoforms, a long transmembrane form and a short secreted form lacking the EGF-like, transmembrane and cytoplasmic domains (**Fig. 2**). Expression of the transmembrane proteinases are

involved in ectodomain shedding of membrane proteins, whereas short secreted isoforms have been proposed to cleave extracellular substrates and ECM components that may not be targeted by the membrane-spanning isoforms [101]. Notably, the short ADAM9-S isoform expressed by liver myofibroblasts, localized at the invasive front of tumors, has been implicated in cancer progression through proteolytic cleavage of laminins [102].

Finally, a recent survey of mutations within the tumor matrisome revealed several alterations potentially affecting splicing sites within core-matrisome proteins (principally collagens) and other factors, suggesting a larger impact of alternative matrisome splicing in cancer than currently known [103].

ECM splicing variants modulate stromal cell physiology

Alternative splicing of ECM components not only impacts the architecture of tumor matrix but also affects stromal cell physiology, thus contributing to the establishment and development of the tumor niche. Such effects include (i) induction of tumor angiogenesis, (ii) modulation of the immune environment and (iii) generation of cancer-associated fibroblasts (CAFs) and their ECM.

i) induction of tumor angiogenesis

An angiogenesis-promoting role for spliced matrisome isoforms in tumor angiogenesis can be illustrated by the upregulation of a thrombospondin-2 (TSP2) variant in endothelial cells of tumor-associated blood vessels. In a study by the group of Detmar [104], vascular endothelial cells of bladder cancer were found to express elevated levels, compared to normal bladder tissue, of a short form of thrombospondin-2 (sTSP2) lacking the anti-angiogenic thrombospondin type 1 repeat domain. Functional analyses confirmed that sTSP2 did not inhibit angiogenesis. Rather, sTSP2 antagonized the angiogenesis-inhibiting effects of full-length TSP2 thus supporting a model in which switches in alternative splicing patterns can produce matrix molecules with distinct or antagonistic functions.

FN variants containing EDA and/or EDB FN III repeats are well established markers of cancer-associated vessels [105,106]. This observation has been exploited to develop anti-tumor targeting therapies and imaging tools, as discussed in [107]. Surprisingly, the individual knock-out of FN containing either EDA or EDB in mouse models has no effect on tumor angiogenesis [45], suggesting the existence of functional redundancy of these isoforms. However, the double knock-out of EDB/EDA mice results in severe cardiovascular defects leading to embryonic lethality, despite the presence of plasma FN lacking the extra FNIII domains [42]. In aggregate, these intriguing observations point to the complex functional interplay of FN splicing variants and illustrate the difficulty of dissecting variant-specific functions in physiological and disease processes.

Finally, versican expression has been positively correlated with early stages of inflammation and vessel density in the tumor stroma ([108] and references therein). In glioblastoma, highly vascularized brain tumors, the role of versican V2 in tumor angiogenesis was investigated by ectopic expression in U87 glioblastoma cells. Following injection of versican V2-overexpressing U87 cells in nude mice, a striking enhancement of angiogenesis was observed. This phenotype was attributed to an increased expression of FN [109], known to enhance angiogenesis in orthotopic grafts of glioblastomas [110].

(ii) modulation of the immune environment

Alternative splicing has recently been pointed out as a crucial process for the fine tuning of immune responses (reviewed in [111]). Accumulating evidence has extended its importance to the modulation of the inflammatory microenvironment of tumors [112]. However, investigations of immune-related alternative splicing in the tumor environment are more focused on signaling molecules, cytokines and cytokine receptors than on splicing of core matrisome components [113]. Osteopontin (OPN), the multi-isoform glycoprotein also known as early T-lymphocyte activation 1 (ETA-1) provides an interesting example of the immunomodulatory potential of alternatively spliced matrix components. During inflammation, OPN induces cytokine secretion and functional responses in different leucocyte populations affecting cell adhesion, differentiation, migration and proliferative potential, as discussed [114]. In the tumor setting, differential expression of OPN variants has been observed in various tumors [115]. iOPN, a non-secreted intracellular isoform of OPN lacking the signal sequence, is of particular interest as it has been implicated in signal transduction pathways as a scaffolding protein downstream of innate immune receptors (e.g. Toll-like receptors) [116]. In a mouse model of melanoma in which the gene encoding OPN was invalidated (OPN KO), specific reconstitution of mice with iOPN knock in natural killer (NK) cells increased the NK cell population and reduced metastatic dissemination [117].

(iii) Cancer associated fibroblasts (CAFs)

Intratumoral fibroblasts constitute a prominent cellular component of stromal tissue. Excellent literature reviews have compiled numerous studies describing the origin, phenotypes, and functions of these important cells during their journey through the progression of precancerous lesions to invasive disease [118–120]. As major builders of the tumor ECM, CAFs are central actors of matrisome diversification through alternative splicing events. Indeed, de novo expression of oncofetal variants of matrisomal proteins (e.g. FN, TNC, versican), discussed above in the context of alternative splicing in development, is a characteristic feature of stromal reprogramming in the tumor microenvironment. In the case of oncofetal FN, assessment of variant expression and localization in the ECM of cells and tumor tissue is possible with isoform-specific antibodies, as mentioned above. Proteomic analysis of

the matrisome of CAFs from head and neck tumors has been used to validate and further characterize the relative expression of EDB- and EDA-containing isoforms [121].

In triple negative breast cancer, CAFs were shown to produce a short form of the glycoprotein periostin, likely corresponding to 2 of the periostin isoforms, PN1 and PN3, that contain POSTN exon 17 (the C-terminal part). This short fragment is able to bind tightly to Wnt3a and it was detected in and around cancer cells, rather than in the stroma, suggesting that this short fragment originating from CAFs could be taken up by cancer cells in which it triggers pro-proliferative signaling [122]. Another example of how alternatively spliced matrix variants in CAFs can exert opposing influences involves the glycoprotein fibulin-1. A comparative study between normal breast-associated fibroblasts and tumor-associated fibroblasts revealed that isoforms C and D of fibulin-1 are specifically downregulated in CAFs [123]. In a mouse xenograft model these splicing variants were shown to reduce the growth of fibrosarcomas [124–126]. Hence, decreased production of the anti-proliferative fibulin-1 variants by CAFs was proposed to favor tumor growth.

ECM splicing variants drive tumor invasion

In addition to modifying tumor tissue architecture and regulating stromal cell physiology, splicing variants of matrisome proteins can actively influence tumor progression by enhancing the motile and invasive potential of tumor cells.

The fine modulation of the migratory phenotype of cells by ‘variants’, and subsequent changes in interacting components and signaling cascades is surprisingly frequent in multiple tumors. For example, in esophageal squamous cell carcinoma, a truncated variant of lysyl oxidase-like (LOXL), LOXL2 (LOXL2 Δ e13), with impaired deamination activity was shown to promote tumor cell motility and invasion by activating an alternative MAPK8-dependent signaling cascade that differs from the FAK, AKT, and ERK signaling pathway activated by full length LOXL2 [127]. Overexpression of the secreted splicing isoform of ADAM9 (ADAM9-S) in breast cancer was also shown to promote breast cancer cell migration by excreting proteolytic activities on ECM substrates, in contrast to the full length protein ADAM9-L that is locally restricted at the cell membrane [128].

In HPV-negative head and neck squamous cell carcinoma, for example, a large screen for splicing events identified a novel LOXL2 variant with insertion of an exon between its canonical exon 1 and 2 as a major splicing event associated to the disease. [129]. Later, functional investigations showed that this LOXL2 variant enhanced cell proliferation, migration, and activation of the FAK/AKT pathway in HPV-negative HNSCC cells [130]. Gene set enrichment analysis associated the expression of the variant with increased expression of laminin subunit gamma-1 (*LAMC1*) and integrins (*ITGB1*, *ITGB3*), which have been shown to increase the invasiveness of tumor cells [131,132].

Since the identification and characterization of TNC in the 1980s, a large number of studies have analyzed the expression of its alternatively spliced isoforms in cancer tissue and cultured cells. Earlier works examined the 'short' (Mr 180-190 kDa) and 'long' (ranging from 210 to 330kDa) forms of TNC, of which the latter were predicted to contain one or more alternatively spliced FNIII repeats (**Fig. 2**). Subsequently, detection of specific domains became possible. The reader is referred to Giblin and Midwood [48] for a detailed review of alternatively-spliced TNC isoforms and the results from studies of their expression and roles in cancer. TNC expression is often observed at the tumor invasion front and associated with migration and metastasis. Whereas expression is largely localized to the stromal compartment, TNC can also be upregulated in malignant cells. For example, in head and neck cancer the detection of TNC transcripts in carcinoma cells by single-cell RNA sequencing was associated to a partial EMT program induced in epithelial tumor cells at the tumor-stroma interface, presumably by tumor-stroma interactions, and associated to nodal metastasis [133]. A detailed and systematic analysis of TNC splicing in tumors is still lacking, but alternative splicing profiling and the use of isoform-specific antibodies together with other advanced molecular technologies should shed light on the nature of the TNC variants expressed and their respective functions.

In a study focused on the large and heavily glycosylated protein mucin-1, stable transfection of highly tumorigenic mouse mammary tumor cells (DA3 cells) with a truncated genomic fragment of human MUC1 led to the induction of an EMT phenotype [134]. This genomic fragment devoid of the cytoplasmic C-terminal part of the *MUC1* gene produced 4 splicing variants, similar to human mucin-1 splicing isoforms. Hence, cells expressing the truncated mucin-1 isoforms displayed over-activation of ERK and PI3K pathways, a significant decrease in their epithelial markers including E-cadherin and increased expression of mesenchymal markers such as vimentin. Interestingly, these cells displayed higher proliferative rates, enhanced invasive capacities and marked increase in expression of FN and its cellular receptor $\alpha 5\beta 1$ [134]. In pancreatic cancer, the splicing variant of mucin-4, MUC4/X, lacking the highly glycosylated tandem repeat domain, was found to be significantly overexpressed in patient samples, and overexpression of the isoform in mucin-4 null pancreatic cancer cells over activated the integrin- $\beta 1$ /FAK/ERK signaling pathway leading to increased invasion and metastasis in both in vitro and in vivo models [135].

Lastly, it is important to note that the splicing variants cited above (schematized in **Fig. 3**) only represent the 'tip of the iceberg' of the alternative tumor ECM. As large-scale analysis and detection methods progress, a plethora of new ECM variants are identified with each study. However functional investigations on molecular mechanisms and differential pathways triggered by splicing variants expression are still preliminary. An interesting starting point however is the identification of potential

“splicing isoforms” at play in a specific context (tumor) using the extensive resources available from massive databases facilitating study design and technical boundaries (see next section).

A no-code workflow for evaluating alternative splicing of matrisome genes in cancer

Analyzing alternative splicing across thousands of transcripts in multi-cancer studies is a computational-intensive task, requiring a solid command of local and server-side programming languages as well as in-depth knowledge of the sequencing process from sample preparation to its actual sequencing, QC-ing, mapping and identification. Typically, these tasks are best handled by data analysts and bioinformaticians but, with a reasonable compromise on the amount of genes that can be analyzed and the depth of such analysis, any researcher can approach this topic using free web-based resources.

Here we will provide a simple “workflow” to assess alternative splicing of matrisome genes across cancer genomes, using only open access resources and a web browser. Readers should keep in mind, however, that this workflow is by no means equivalent to the possibilities offered by ad-hoc bioinformatics analysis, and interested researchers should definitely consult a data analyst before drawing hasty conclusions. Also, all the tools presented in this workflow use The Cancer Genome Atlas (TCGA) [136] data, ensuring some degree of interconnectivity across data. It is clear that, if one aims at analyzing different data sources, one will need to write quite a sensible amount of code lines (or, again, contact an analyst!).

For illustrative purposes, we will analyze the same set of data through (probably) the two most-widely used browser interfaces for alternative splicing analysis, namely the **SpliceSeq** tool developed at MD Anderson Cancer Center (link [here](#))¹ [137] and the **TCGA Splicing Variants DB (TSVdb)** from Zhejiang University (link [here](#))² [138]. Additionally, we will make use of other integrative resources such as the **Xena** Browser (link [here](#))³ [139] and **CBioPortal** (link [here](#))⁴ [140] for additional evaluation of possible connections between alternative splicing and mutations, gene expression and clinical parameters.

EVALUATING ALTERNATIVE SPLICING IN A GENE OF INTEREST USING SPLICESEQ

- 1) Select a matrisome gene. For example, the typical Type-I collagen gene *COL1A1*.

¹ <https://bioinformatics.mdanderson.org/TCGASpliceSeq/>

² <http://www.tsvdb.com/plot.html>

³ <https://xenabrowser.net/>

⁴ <https://www.cbioportal.org/>

- 2) Navigate to the **SpliceSeq** page and select **Single Gene** as the analysis mode from the top-left menu.
- 3) Input *COL1A1* in the **Search Gene** box and press **Search** without changing the default parameters. The page will become unresponsive while the analysis runs and, at completion, will be updated with the results.
- 4) The output can be generally divided into 4 sections, though minor differences can occur owing to the different sources and types of data available for the query gene. From left to right and top to bottom, the following panels should appear:
 - a table of splicing events, per junction/localization and type of alternative splicing event, across cancer types,
 - a boxplot of the “percent spliced-in” (PSI) across cancer types (with or without comparison with normal samples, see the checkbox on top of the graph),
 - aggregated information on gene/protein/exons from various sources (such as NCBI and Uniprot)
 - an interactive splice graph, allowing the visualization of the frequency of exon-exon junctions (with or without novel splice events, see the checkbox on the top-left of the graph) by raw counts or average observation totals normalized by exon length and million-aligned-reads (OPKM).
- 5) Evaluate the results. Following the example of *COL1A1*:
 - possible splicing events are grouped into **Alternate Acceptor (AA)**, **Alternate Donor (AD)**, **Exon Skip (ES)**, **Retained Introns (RI)**, **Mutually Exclusive Exons (ME)**, **Alternate Promoter (AP)** and **Alternate Terminator (AT)**. If the user feels unfamiliar with any of these terms, there is an exhaustive frequently asked questions (FAQ) section on the website. With default parameters, several alternative splicing events are found across cancers for *COL1A1*, all of the ES type.
 - The results are, by default, ordered by increasing exon number but they can be sorted by, for example, frequency in a given cancer type.
 - Also, results can be filtered by **Tumor Type**, analysis parameters (**Filter**) and tumor/normal (**Tumor**). More on this later.
 - Clicking a row (that is, selecting one specific alternative splicing event type) will highlight that event, zooming it in the PSI boxplot and marking the spliced exons in red in the graph below.
 - Data cannot be downloaded as they are from this view, unfortunately. A workaround is to highlight and copy/paste them into a spreadsheet for further analyses though this is

not a standard practice. Note, however, that the local Java web start application of SpliceSeq³ allows basic grouped analysis.

- 6) Tweak the parameters to evaluate more stringent or subtle events. With default values, SpliceSeq runs with **PSI 50%** and **minor splice expression 10%**. These values can be changed via the **Filter** tab. The PSI value acts as a threshold on the baseline exon presence. In fact, PSI is the ratio of reads indicating the presence of an exon versus the total reads covering the same genomic area. Hence, a PSI 50% means that eventual alternative splicing events will only be drawn for genomic areas with a 50% coverage (the exon is present in 50% of all the reads for that area). Decreasing the PSI value allows the retrieval of infrequent events that occur only on “rare” exons, such as those influenced by the tissue of origin [141] and present only in a few reads out of total. Conversely, the minor splice expression filter gates out all splicing events whose frequency is below the specified threshold. Decreasing the value increases the total splicing events found, with a concurrent decrease in the “reliability” of the results as single events begin to surface. Without an exhaustive exploratory Pan-Cancer and/or cancer-specific analysis of the frequencies of each event, selecting the best filter combination is a painful trial and error event ridden with uncertainty and should not be attempted unless clear indications can be mined, e.g., from the literature.
- 7) After some exploratory analysis on the single gene tab, one can download the PSI values for the gene of interest via the **PSI Data Download** tab. Note that leaving the “Gene HUGO symbols” and “TCGA Samples” will retrieve all genes and all samples in a given cancer type, respectively. The user can otherwise select a cancer type, one or more genes, one or more specific samples, and any type of splicing event. Also, the user can select thresholds for the event. Importantly, the thresholds in this tab do not match with those in the single gene mode, hence users need to take care of setting the values that make most sense!

EVALUATING ALTERNATIVE SPLICING IN A GENE OF INTEREST USING TSVDB

- 8) Navigate to **TSVdb** page and press **START** (or choose **INSTRUCTION** for a quick introduction to the methods behind the analysis).
- 9) Users will be prompted to **choose a tumor type** among those with data available.
- 10) Now **input a gene**, again *COL1A1* as from the previous section.
- 11) Users will be finally prompted with a choice to **visualize exon- or junction-level** data, depending on the users’ focus on single exon’s or exon-exon junctions’ abundance.

- 12) Finally, depending on the clinical data available for the tumor type of interest, users will **choose clinical data**. These will be useful later to perform grouped analyses. After this selection, the page will start to update compiling the results and will become browsable again at completion.
- 13) The output page is intended for easy at-a-glance visualization of the results, though it takes a bit of experience before the intended simplicity can be appreciated. Briefly, the structure of the gene is shown on the left margin of the page (rotated 90° clockwise) and the normalized frequency of reads per exon or junction is graphically reported as a ribbon plot across the samples. Note that various isoforms or consolidated transcripts are reported on the left side in darker colors, together with a fictional reference transcript (union of all) in grey.
- 14) Each ribbon is connected to its exon or junction by a straight line. Clicking the ribbon highlights the connector and the exon/junction of origin, making it simpler to trace back counts to the genomic element.
- 15) Clicking the exon/junction at *each* of the transcripts given will open a further analysis pop-up page. In it, the expression of counts for the given exon/junction divided by the clinical covariate chosen before (see step 12) are shown as boxplots or other graphs. Also, along the upper margin of this pop-up, users can select to visualize patient survival depending on above- or below-the-average expression of the exon/junction. Despite the various visualizations per-transcript, no statistical analysis is launched automatically (nor can be launched by user input) and the users can only download the graphs (though in various formats).
- 16) Data can be downloaded for the whole gene by closing the pop-up and clicking **Download Data** in the upper left corner.

GENE EXPRESSION AT THE EXON LEVEL AND GENOMICS OF ALTERNATIVE SPLICING

- 17) Navigate to the **Xena Functional Genomics Explorer**. In the leftmost column (**Select a Study to Explore**), choose the TCGA study you are interested in. The browser will propose several studies as you type in “TCGA”. Make sure to select those named “TCGA” (published studies from their original hubs, same data as those for alternative splicing) and not GDC TCGA studies. Move to the bottom of the page and press **DONE**.
- 18) In the central column (**First Variable**, defaults to genomic variables) users input matrisome gene(s) of interest (for example, *COL1A1*) and, just below, press **show advanced**. There,

select **exon expression RNAseq** from the IlluminaHiSeq platform. Move to the bottom of the page and press **DONE**.

- 19) A second, rightmost column will open (**Second Variable**, defaults to genomic variables). This is accessory, but various other genomic or phenotypic variables could be added. Note that at least a second variable (even if the user will not use it) is needed for the download button to activate.
- 20) In the **First Variable** column (notice the heatmap-like visualization that opens when fetching the required data), hover to the top-right column and a three-dots button will appear. Several possibilities for visualization and editing the query open from there. Also, users can **download** the data and match with exon- or junction-level alternative splicing data by patient (sample) ID, using for example a spreadsheet software.
- 21) Navigate to **CBioPortal**. In the main tab, choose a tumor of interest. Again, take care of selecting a study matching those present in SpliceSeq or TSVdb. Once the tumor is selected, press the **Query By Gene** button.
- 22) The second tab from the top should read **Select Genomic Profiles**. Here, selecting **Mutations** and **Putative copy-number alterations from GISTIC** will enable retrieval of genomics event that might, in *cis*, be responsible for alternative splicing. In the next tab (**Select Patient/Case Set**), make sure that **Samples with mutation and CNA data** is selected.
- 23) In the **Enter Genes** tab, finally, input gene(s) of choice (e.g., *COL1A1*) and press the **Submit Query** button.
- 24) Several tabs will appear along the header of the frame as the analysis completes. For example, the tab **Mutations** lists those matching the query and, if one is lucky, a potential splicing-affecting mutation could be found, whose coordinates match the exon-exon junction of interest!

Past, present, and future perspectives

Alternative splicing was discovered in 1977 by Susan Berget, Claire Moore, and Phillip Sharp working on the sequence arrangement in the 5' terminus of adenovirus 2 mRNA [142]. Soon thereafter, other mRNAs were examined for alternative splicing and interestingly, transcripts of the *FN1* gene were among of the first mRNAs investigated [143], as FN was known as a multi-form protein since the late 50's [144]. Interest in studying alternative splicing rose significantly in the early 90's when repeated

observations showed that EST libraries for one gene were found to be more diversified than expected [145,146].

However, a set of challenges quickly stood in the way of alternative splicing studies, particularly in the case of ECM-related genes for which the multi-modular structure and extended length represented technical obstacles that remain challenging to overcome today. The paucity of isoform specific antibodies, or difficulty in some cases to express and purify full length multi-subunit proteins, have limited studies to shortened RNA transcripts (mini genes) or reporter fusions of truncated proteins that, unfortunately, do not fully reflect the conformational features and functional properties of endogenous isoforms. Molecular detection of isoform transcripts by qPCR also lacks contextual information. Nonetheless, technological advances such as Riboseq [147], long read sequencing [148] CRISPR/Cas9 genome editing [149] are rapidly paving the way for improved expression, and functional interrogations of splicing isoforms. Single-cell sequencing and spatially compatible approaches such as spatial transcriptomics [150], and BaseScope [151] have also improved the characterization of ECM isoforms at the level of whole tissues. All of these approaches, together with powerful bioinformatics tools for alternative splicing profiling, are now expanding our knowledge about ECM splicing variants in physiological and pathophysiological contexts and opening new horizons in translational research. Expression patterns of ECM splicing variants are already utilized in early diagnosis of some cancers and mounting evidence suggests that ECM splicing variants could represent specific pharmacological targets, as well as ideal candidates for splice-correction therapies [152].

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Figure Legends

Figure 1. Alternative splicing mechanisms and consequences

(A) Schematic representation of the splicing reaction for an exon (center). Competing factors (SR and hnRNP family proteins) regulate spliceosome complex activity. Functions of splicing factors can be further modulated by interactions with additional RNA binding proteins. Outcome of the splicing reaction results from the addition of all enhancing and inhibiting activities. In the case of the central exon, positive outcome of the splicing reaction leads to the excision of neighboring introns and retention of the exon in the mature RNA (upper dashed lines), whereas negative outcome of the splicing reaction results in the excision of both neighboring introns as well as the central exon leading to exon removal from the mature RNA (lower dashed lines). **(B)** Representation of 5 main types of alternative splicing outcomes. **Exon skipping** : is the most prevalent type of AS [153] for which a specific exon is not included in the mRNA. For example, the synthesis of the plasmatic form of fibronectin is generated by skipping both EDA and EDB exons in the mRNA sequence [154]. **Mutually exclusive exon usage** : Alternative splicing results in the inclusion of one or another specific exon as for FGFR2 receptor isoforms, for which either exon IIIb or IIIc, but never both, is included in the mRNA [155]. **Alternative splice site selection** occurs when multiple *cis*-regulatory elements are present at the 5' or the 3' end of an exon, resulting in longer or shorter exons from the same transcript. For example, the isoforms IIC and IID of procollagen II are generated by alternative 5' and 3' splice sites within the 2nd exon, respectively [41] **Intron retention**: when part of an intron is still retained in the mature mRNA transcript. The gene encoding TIMP metalloproteinase inhibitor 1 (TIMP1) displays retention of its 3rd intron during colon cancer progression [156]. **(C)** Main consequences of alternative splicing on a theoretical pre-mRNA and its splicing isoforms that will be translated into different proteins harboring or lacking specific features : Total or partial inclusion/exclusion of exons can affect (i) the presence of ECM domains and/or repeats (e.g. FNIII), (ii) regulatory domains and catalytic sites (e.g. C-terminal Ca^{2+} regulatory domain of TGM2), (iii) RNA stability, protein truncation, protein fragment production (e.g. short isoforms of TNC).

Figure 2. Examples of alternative ECM isoform structures and functions

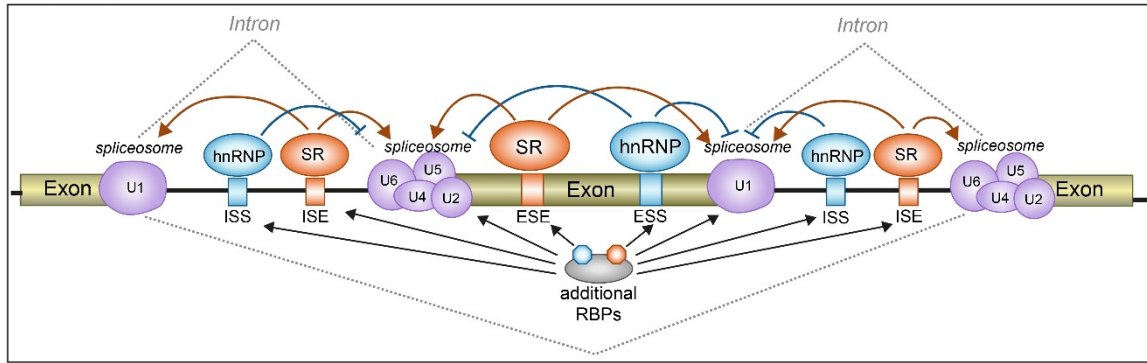
Non-exhaustive examples of physiological effects / phenotypes resulting from alternative splicing of ECM related genes (discussed in Matrisome splicing variants in cell physiology). For simplification only a part of the RNA with spliced exons is represented. Dashed lines: additional exons before or after the exon, full lines: 5' or 3' ends of the RNA (no additional exons), E: exon. On schemes, local ECM variant deposition patterns are speculative for some of the chosen examples (COL1A, TNC, VCAN, ADAM12). Concerning COL1A, antibodies directed against the IIA variant detected expression of this variant in

human embryonic cartilage [34]. Regarding FN variants, both EDB and EDA isoforms are detected in angiogenic blood vessels [106,107]. In the case of fibulins during *C. elegans* development, integration of the Fibulin-1D variant in ECM networks at mechanosensory neuron and uterine attachments is perlecan dependent while the Fibulin-1C variant incorporates ECM networks at mechanosensory neuron attachments via laminins [58]. Regarding ADAM12 isoforms, although both long intracellular and short extracellular isoforms have been described, specific functions of long and short isoforms are still unclear in healthy tissues [65]. Lastly, in Alzheimer disease short isoform(s) of TGM2 (lacking the Ca^{2+} domain) are known to be deposited along tau aggregates within neurofibrillary tangles [79].

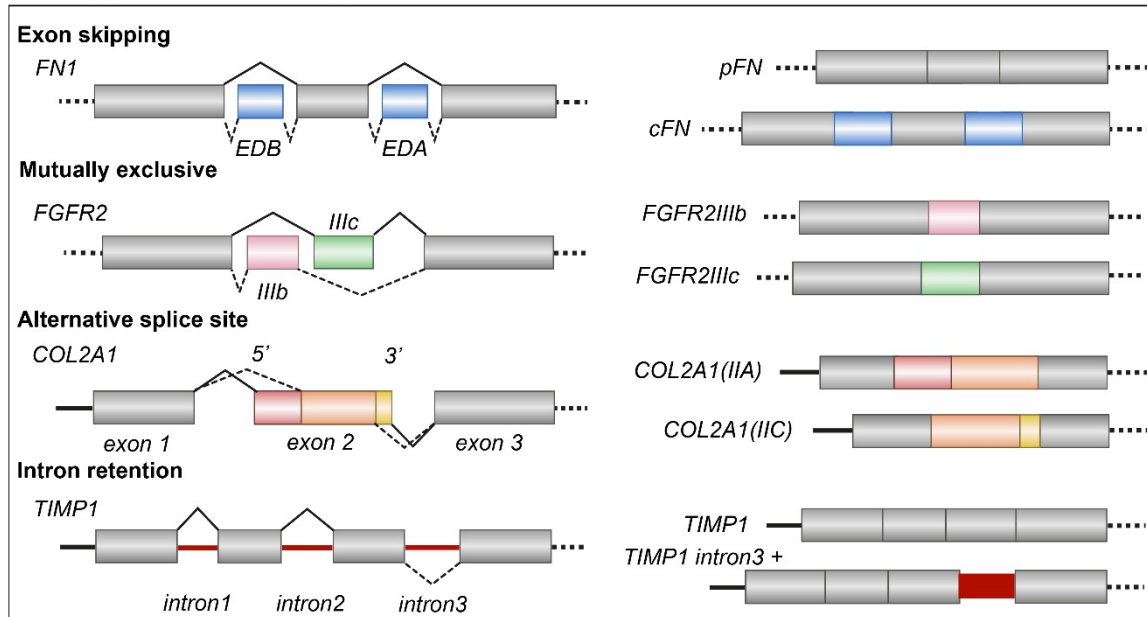
Figure 3. ECM alternative isoforms in cancer

Schematic representation of a tumor with its surrounding stroma. Changes in the ECM include increased expression/deposition, rigidification, switch in isoform composition and re-expression of oncofetal ECM isoforms. Some misregulated ECM splicing isoforms in tumors are represented (discussed in Dysregulation of alternative splicing in cancer).

A. Splicing reaction



B. Main types of alternative splicing



C. Main consequences of alternative splicing

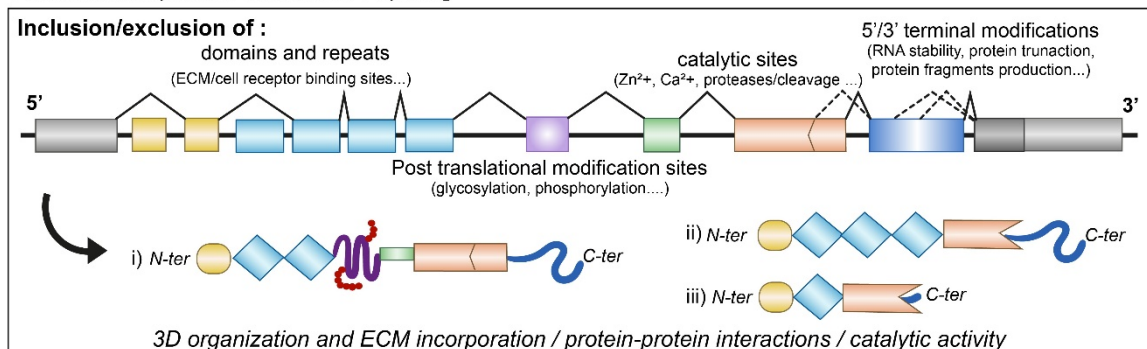


Figure 1. Alternative splicing mechanisms and consequences

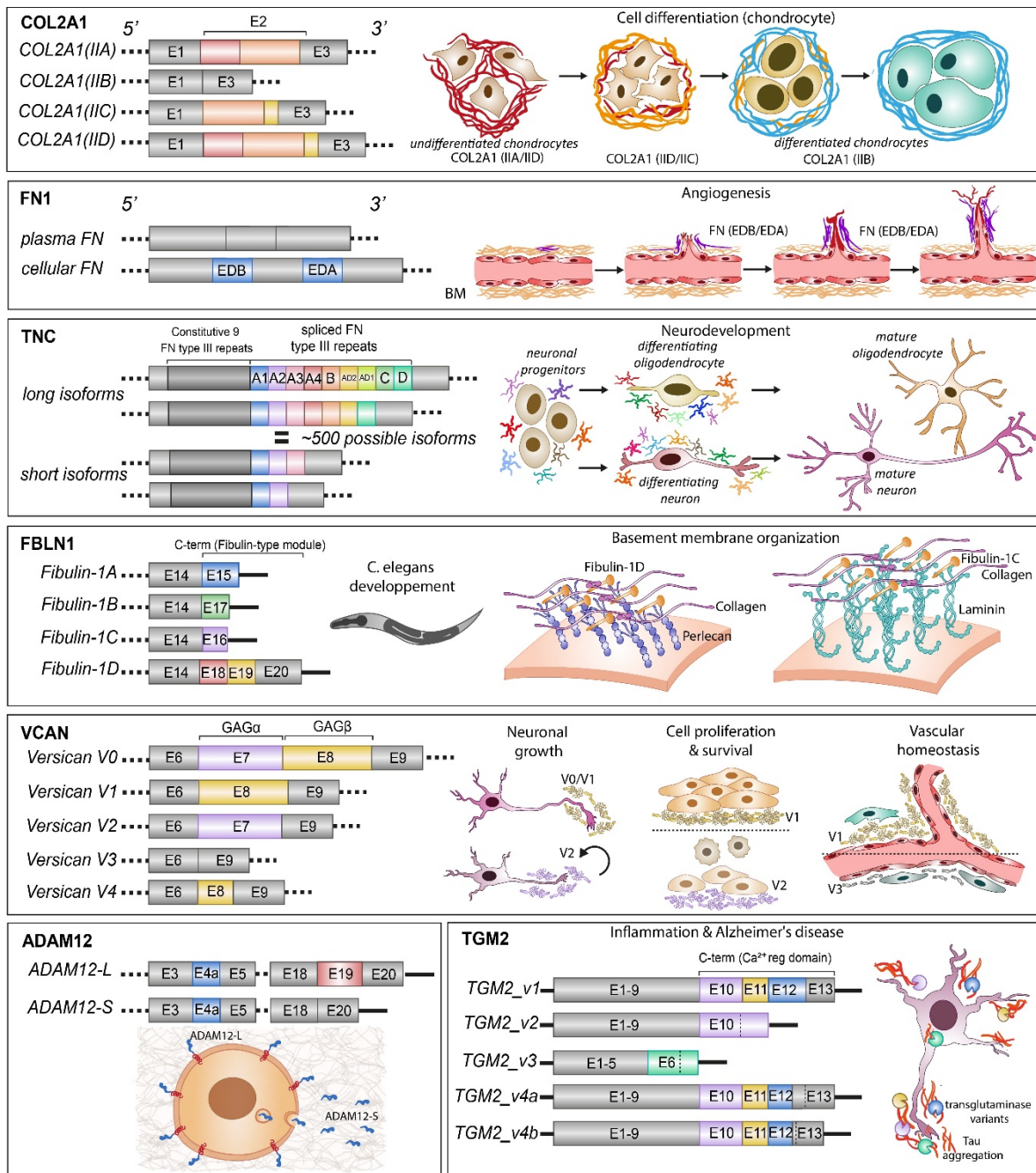


Figure 2. Examples of alternative ECM isoform structures and functions

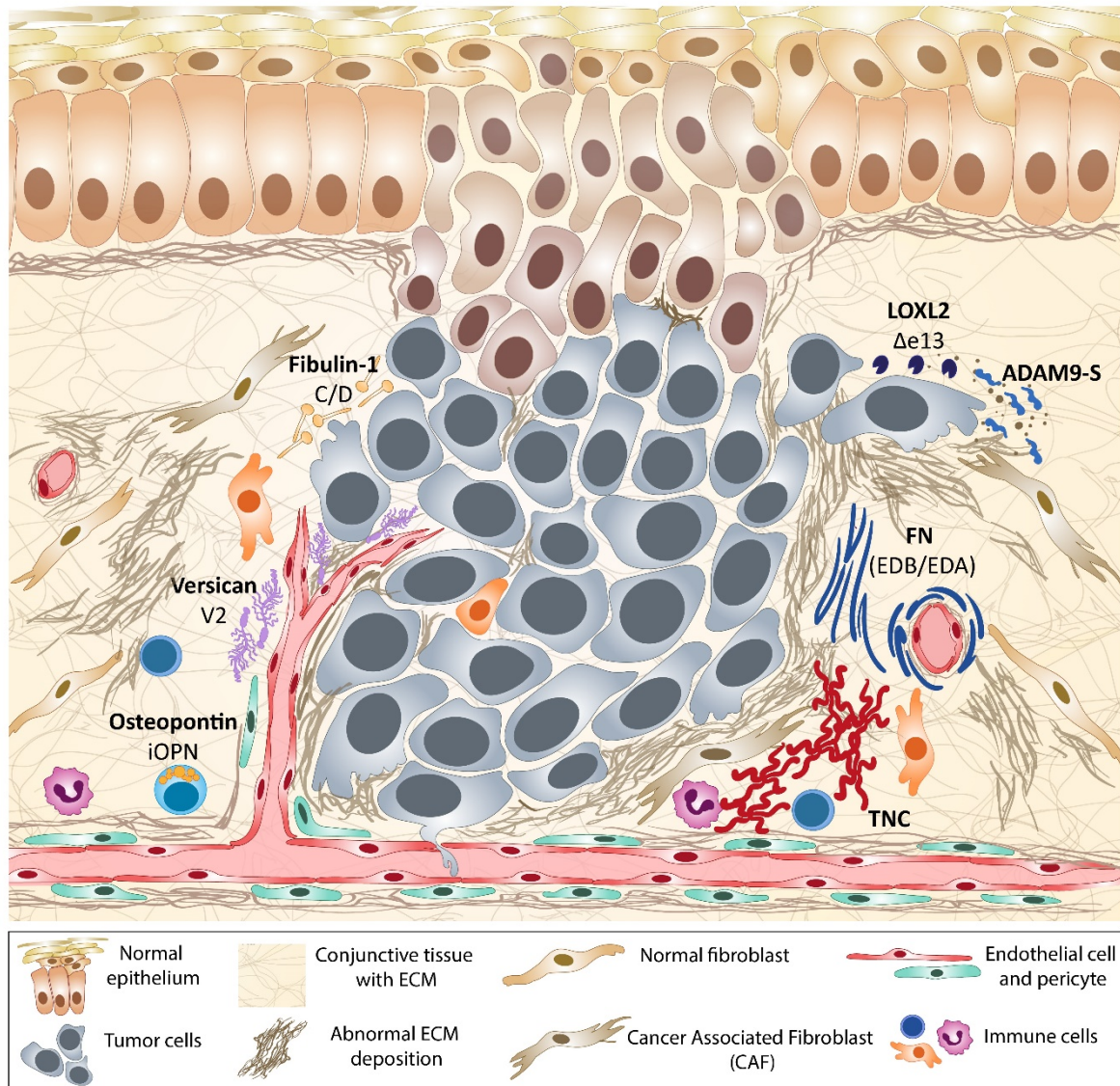


Figure 3. ECM alternative isoforms in cancer

Table 1 Examples of ECM splicing isoforms in development / tissue homeostasis

| ECM class | Protein | Gene symbol | Variants | Physiological / functional relevance | Reference |
|----------------|--|-------------|---|---|-----------------|
| Core matrisome | Aggrecan | ACAN | G3 splicing variant | G3 variant shows differential binding to ECM molecules | [157] |
| | Amelogenin (amelogenesis imperfecta 1, X-linked) | AMELX | M180; LRAP | Expressed during tooth enamel formation | [158–160] |
| | Collagen type XI α 1 chain | COL11A1 | Multiple variants | Tissue-specific and developmentally regulated expression in cartilage and bone formation | [161–163] |
| | Collagen type II α 1 chain | COL2A1 | IIA/IIB/ IIC/IID | Developmentally regulated during cartilage development (see text) | [36,40,164] |
| | Collagen type VI α 3 chain | COLVIA3 | Differential inclusion of exons A9, A8 and A6 | Developmentally regulated, might regulate cell adhesion (<i>in vitro</i>) | [165] |
| | Fibulin 1 | FBLN1 | Isoforms A to D | Developmentally regulated (H. Sapiens and C. elegans) incorporates in different ECM network (C. elegans) | [53,54,166] |
| | Fibronectin 1 | FN1 | FN-EDB; FN-EDA | Cell adhesion/critical for embryonic angiogenesis | [42,167] |
| | | | FN-CS1 (variable region) | Cell adhesion, inflammation, immune cell recruitment | [168,169] |
| | NEL-like 2 (chicken) | NELL2 | NELL-TSP, cNELL2 | Neuron specific glycoprotein, in vitro NELL-TSP blocks the secretion of the full-length NELL. In glial cells, cNELL2 is retained in the cytoplasm and activates PKC activation (regulation of neurite outgrowth). | [170,171] [170] |
| | Tenascin C | TNC | Multiple isoforms | Developmentally regulated, isoform specific functions (see review) | [48,172] |
| ECM regulators | Versican | VCAN | VCAN-V0, V1; V2; V3; V4 | Inhibition of axonal growth; negative regulation of cell adhesion | [60,173] |
| | Disintegrin and metalloproteinase domain-containing protein 12 | ADAM12 | ADAM12-La, Lb and S | La and Lb isoforms show different cellular processing, isoform S might play an important role in cell-matrix interactions between the placental syncytium and the maternal interface | [174,175] |
| | Heparanase | HPSE | XHpaL and XHpaS | Splicing of exon4 generates a short, developmentally regulated secreted isoform. Differential roles of the variants on Xenopus embryonic development. | [176] |

Table 2 Examples of ECM splicing isoforms in cancer

| ECM class | Protein | Gene symbol | variant | cancer/cell type | effect/observed phenotype | references |
|----------------|---|-------------|----------------------------|--|--|--------------------------------|
| Core matrisome | Collagens type XI α 1 chain and type VI α 3 chain | COL11A1 | COL11A1-A, B, C and E | Breast cancer tissue sample | Statistical association of COL11A1 A and E variants expression with lymph nodes and metastasis | [93] |
| | | COL6A3 | COL6A Exon3,4 and 6 | Pancreatic ductal adenocarcinoma | Expression of COL6A3 with inclusion of exons 3, 4 and 6 specifically in tumor lesions | [91] |
| | Fibronectin | FN1 | FN-EDA/EDB | Multiple cancer and cancer cell lines | Overexpression in tumors, prognosis marker, tumor angiogenesis marker, cancer therapy target (see review) | [167],(review) |
| | Fibulin-1 | FBLN1 | FBLN-1C, 1D | Human fibrosarcoma derived cell line | Downregulated in malignant cells. Variant D overexpression delayed cell growth and migration | [125] |
| | | | | ER positive ovarian cancer cell line and breast cancer cell line | Estrogen dependent overexpression of Fibulin1C, estrogen dependent destabilization of Fibulin-1D variant. High 1C:1D ratio associated with malignancy | [177,178] |
| | Mucin | MUC1 | multiple variants | Breast, cervical, prostate cancer, ovarian tumors | Multiple roles in carcinogenesis, associated with malignancy, variant-specific therapy target | [134,179–188] [189](review) |
| | Osteopontin | OPN | OPNa,OPNb, OPNc,OPN4, OPN5 | More than 12 cancer types | Various level of expression and correlation with prognosis. Isoform specific activation of signaling/migratory pathways. | [115,190–193] |
| | Periostin | POSTN | Variants I and II | Bladder carcinogenesis | Variant II and full length periostin reduce cell invasion and metastasis, while variant I is lacking suppressive activity. Decreased expression of periostin and its variants in tumor tissues | [194] |
| | Slit homolog 2 protein | SLIT2 | Slit2- Δ E15 | Lung cancer | Both canonical and Δ E15 isoforms are highly down regulated in tumors, Δ E15 over expression inhibits tumor growth in a mouse model | [195] |
| | | | | | Both canonical and Δ E15 isoforms reduces endothelial cell migration with a greater effect caused by Δ E15 isoform. Vessel normalization in lung cancer model. | [196] |

| | | | | | | |
|----------------|--|--------|--------------------------------|---|---|----------------|
| | Tenascin C | TNC | TNC-L and TNC-S | Thyroid and oral squamous cell carcinoma | Various expression and extracellular staining, prevalence of the large splicing variant in most cancer cell lines. | [197] |
| | | | High molecular weight isoforms | Bladder cancer | Marker of prognosis and invasion | [198,199] |
| | | | TNC-L | Pancreatic cancer | High co-expression with annexin A2 and marker of poor prognosis | [200] |
| | Thrombospondin | THBS-2 | TSP2 short isoform | Invasive bladder cancer | Differential anti-angiogenic properties between isoforms | [104] |
| | Versican | VCAN | VCAN-V1 | Gliomas Chondrosarcoma | V1 has pro-proliferative and pro-migratory effects compared to V3 Enhanced ECM deposition and cell motility compared to V3 | [201] [202] |
| | | | VCAN-V2 | U87 Glioblastoma cell line | Over expression of V2 in U87 cell enhances Fibronectin expression and angiogenesis upon injection in immunocompromised mice | [109] |
| | | | VCAN-V3 | Human and canine melanoma cell lines / human melanoma | Overexpression of V3 reduces cell growth in-vitro. In-vivo reduction of tumor development. | [203,204] |
| | | | VCAN-V4 | Breast cancer tissue | Over expressed in primary breast cancer tissues | [57] |
| ECM regulators | Lysyl oxidase-like (cross-linking enzymes) | LOXL1 | LOXL1-D4 | Breast cancer and MCF12F cells | Pro-oncogenic activity on MCF12-F cell line. differential expression pattern from the main isoform | [205] |
| | | LOXL2 | LOXL2 5'UTR variant | HPV-negative HNSCC cells and primary tumor | Enhanced proliferation, migration, and invasion, activation of the FAK/AKT pathway | [130] |
| | | | LOXL2 Δ13 | Esophageal squamous cell carcinoma cells | Enhanced cell migration and invasion compared to full length LOXL2 | [127] |
| | | | LOXL2Δ72 | Esophageal squamous cell carcinoma (human ESCC cell line) | Enhanced cell migration and invasion | [206] |
| | | LOXL4 | splv1 / splv2 | Ovarian and breast carcinomas | Regulation of tumor progression in a mouse model, up-regulates the expression of onco-miRNAs. | [207] |
| | | TGM2 | TGM2 v1 to v4 | Multiple cancer cell lines | High correlation of variant expression in cancer cell lines | [208] |
| | | | | | | |

| | | | | | | |
|--|-------------------|--------|----------------------------------|--|--|----------------|
| | Metalloproteinase | ADAM9 | ADAM9-S | Activated hepatic stellate cells | Production of soluble form of ADAM9-S promoting invasion of carcinoma cell lines | [102] |
| | | | ADAM9-L /ADAM9-S | Cancer cell lines and breast cancer tissues | Differential effect on tumor progression | [128] |
| | | ADAM12 | ADAM12-L /ADAM12-S | Multiple cancers | Cell proliferation, receptor activation, stromal ECM remodeling | [209] (review) |
| | | ADAM15 | ADAM15A/B | Human mammary carcinoma and MDA-MB-435 cells | Correlation with poor clinical outcomes, ADAM15A enhances cell migration and adhesion/ ADAM15B reduces cell adhesion | [210] |
| | | HPSE | Heparanase Splicing variant (T5) | Renal cell carcinoma biopsies | Enhancing cell proliferation, anchorage independent growth and tumor xenograft development, overexpressed in renal cell carcinoma biopsies | [211,212] |

