RESEARCH PAPER

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Spatial organization of the tenascin-C microenvironment in experimental and human cancer

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The extracellular matrix (ECM) molecule tenascin-C (TNC) promotes tumor progression. This has recently been demonstrated in the stochastic murine RIP1-Tag2 insulinoma model, engineered to either express TNC abundantly or to be devoid of TNC. However, our knowledge about organization of the TNC microenvironment is scant. Here we determined the spatial distribution of TNC together with other ECM molecules in murine RIP1-Tag2 insulinoma and human cancer tissue (insulinoma and colorectal carcinoma). We found that TNC is organized in matrix tracks together with other ECM molecules of the AngioMatrix signature, a previously described gene expression profile that characterizes the angiogenic switch. Moreover, stromal cells including endothelial cells, fibroblasts and leukocytes were enriched in the TNC tracks. Thus, TNC tracks may provide niches for stromal cells and regulate their behavior. Given similarities of TNC rich niches for stromal cells in human insulinoma and colon cancer, we propose that the RIP1-Tag2 model may be useful for providing insights into the contribution of the tumor stroma specific ECM as promoter of cancer progression.

Introduction

The tumor microenvironment is characterized by stromal and tumor cells that express a tumor specific extracellular matrix (ECM), growth factors, cytokines and matrix remodeling enzymes that altogether provide signaling information to cells thus regulating tumor cell survival and proliferation as well as invasion and metastasis. Yet, the coordination of the spatial cross-talk between cells and their ECM in the tumor microenvironment is poorly understood. Collagens (Col), laminins (LM), oncofetal fibronectin (FN), tenascin-C (TNC) and other ECM molecules are highly expressed in cancer tissue and are now known to contribute to tumor progression.[1-3] In particular, high collagen abundance and crosslinking was documented to generate mechanical tissue stiffening that triggers integrin signaling and lung metastasis in murine breast cancer models.[4]

TNC is expressed during embryonic organ development and in stem cell niches of the adult organism, yet is only poorly detectable in normal resting tissues.[5] TNC becomes reexpressed in tissue remodeling conditions found in wound healing, inflammation and cancer. High TNC expression correlates with worsened survival prognosis in glioma patients and reduced time of metastasis free survival of breast cancer patients suggesting that TNC promotes tumor progression.[2,6] This hypothesis was recently proven in the well-established pancreatic neuroendocrine RIP1-Tag2 mouse model in which SV40Tag drives multistage tumorigenesis, characterized by consecutive appearance of hyperplasia, angiogenesis, carcinoma and metastasis.[7] Hence, in mice engineered to either lack the TNC protein or to overexpress transgenic TNC in pancreatic islets, it was demonstrated that TNC promoted multiple events linked to tumor progression such as survival,

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Table 1. Comparison of TNC tracks in RIP1-Tag2 tumors with melanoma channels and thymic conduits

	ECM tracks	ECM channels	Conduits
Model/tissue	*RIP1-Tag2 insulinoma*	*Malignant melanoma*	*Thymus*
ECM molecules	TNC, FN, LM, LMα5, LMγ2, Tsp1 pro-ColIII, ColIV, ColV	TNC, FN, ColI	TNC, LMα3, LMβ3, LMγ2 ColI, pro-ColIII, ColIIV, ColIVI, ColXII
	No LMα1	No LMγ2, ColIV	Fibrillin-1/2, Nidogen, Perlecan No LMα1
Juxtaposed cells	Endothelial cells, fibroblasts, leukocytes, erythrocytes	Melanoma cells, erythrocytes No endothelial cells	Thymocytes
Suspected function	Niche for tumor and stromal cells	Dissemination route	Transport of chemokines, antigens Lymphocyte education
	Modulation of tissue stiffness		Thymocyte migration cue Mechanical stability
	Dissemination route Role in angiogenesis Role in immune evasion		

TNC rich ECM tracks in RIP1-Tag2 tumors are compared to ECM rich channels in malignant melanoma [15]and thymic conduits.[22] ECM components and cells that are in juxtaposition and thus presumably interact with this ECM are listed. The suspected functions are noted.

proliferation and invasion of tumor cells as well as angiogenesis and lung micrometastasis.[8]

TNC affects tumor angiogenesis by promoting the angiogenic switch leading to more, albeit leaky blood vessels.[8] TNC is one of the most highly induced ECM molecules of the so called AngioMatrix signature, a matrisomal gene expression profile comprising 110 genes encoding ECM and matrix remodeling enzymes,[9] that is turned on during the angiogenic switch.[10] Yet, it is unknown whether TNC forms spatial networks together with other ECM components of the angiogenic switch profile.

In the RIP1-Tag2 model it was further shown that TNC promotes Wnt signaling by alleviation of DKK1 inhibition. Cell adhesion to TNC blocked actin stress fiber dependent expression of DKK1.[8] In breast cancer models it was seen that in addition to Wnt signaling, Notch signaling was enhanced by TNC.[11] Altogether these results suggested that TNC generates a protumorigenic signaling permissive microenvironment. However, it is unknown whether and how TNC orchestrates such a microenvironment. Thus information regarding the spatial organization of TNC is important to further understand the multiple effects of this ECM molecule in cancer.

Until today only very limited information is available about how TNC is organized in tumor tissue. Originally TNC was described to surround groups of tumor cells in breast cancer.[12] Thereafter, TNC was repeatedly shown to be expressed around tumor blood vessels,[3,13] together with oncofetal FN[3] or LMγ2.[14] A TNC containing ECM network has also been observed in malignant melanoma.[15] In melanomas, differential gene expression analysis combined with tissue staining revealed an upregulation of TNC together with other ECM molecules including FN and ColI in invasive and metastatic tumors. TNC was localized in channel-like structures that did not represent blood or lymphatic vessels and were suspected to play a role in melanoma dissemination.[15] Whether similar TNC containing channels also exist in other cancers is unknown.

Other ECM networks rich in e.g. ColIV, ColVI and LMγ2 and ECM remodeling metalloproteases had been observed in vasculogenic mimicry (VM) where tumor cells generate a vessel like network, devoid of CD31 positive endothelial cells.[16] VM is linked to shortened 5 year survival in several cancers and is suspected to play a role in tumor evasion upon anti angiogenic therapies.[17] Yet, molecular screens did not identify TNC as VM candidate. [16,18,19]

In secondary lymphoid organs, in particular in the thymus LMs (LMα3, LMβ3, LMγ2), collagens (ColI, pro-ColIII, ColIV) and Fibrillin-1/2 are found to form reticular fibers.[20-22] In the thymus these reticular fibers, coined conduits, have been shown to coexpress TNC that is suggested to wrap around the core region of collagens and LMs.[22]

To improve our understanding about the tumor promoting role of TNC, more information about its spatial organization is needed. Here we address this question in RIP1-Tag2 tumors, human insulinoma and in colorectal carcinoma (CRC) by comparative microscopy. We found TNC organized in matrix arrays forming tracks together with other AngioMatrix ECM molecules. Moreover, stromal cells including endothelial cells, fibroblasts and immune cells were enriched in the TNC tracks which presumably provide signaling niches promoting tumor angiogenesis and metastasis.

Results

Tenascin-C containing ECM tracks in RIP1-Tag2 insulinoma

Little is known to date about TNC organization and how it forms networks with other ECM molecules in RIP1-Tag2 tumor tissue. Therefore we analyzed TNC expression in tumors of 12 week old RIP1-Tag2 mice together with total LMs, LMα5, LMγ2, pro-ColIII, ColIV, ColV, FN and thrombospondin-1 (Tsp1) by immunofluorescence staining, conventional and

confocal microscopy. TNC was found to be expressed in spatially defined arrays that we coined tracks. Representative pictures are shown in **Fig. 1, Fig. S1 and 2** and **Movies 1–5**. Upon staining of other ECM molecules, we observed that TNC tracks are heterogeneous, as described in more detail below.

Costaining of TNC and LM, with a pan-LM antibody that recognizes LMβ1 and/ or LMγ1 containing LMs, revealed that LMs are not only abundantly expressed on their own at the tumor periphery and within the tumor tissue, they are also expressed adjacent to TNC. Thus, TNC and LMs form matrix networks of variable size (μm range) with TNC in the core region and LMs on the outside (**Fig. 1A-E, Fig. S1A**). Upon staining for LMα5 and LMγ2, 2 LM chains highly abundant in several cancer tissues,[23-25] a similar pattern was seen for LMγ2 (**Fig. S1C**). In contrast to LMα5 and LMγ2, no expression of LMα1, another LM isoform highly expressed in CRC (Mammadova-Bach et al., submitted), was observed in RIP1-Tag2 tumors (data not shown). Confocal analysis confirmed the localization of TNC and LMs in networks that are reminiscent of channels (**Fig. 1C and D, Movie 1**). Upon DAPI staining, we observed nucleated cells localized within the TNC track (**Fig. 1C and E**).

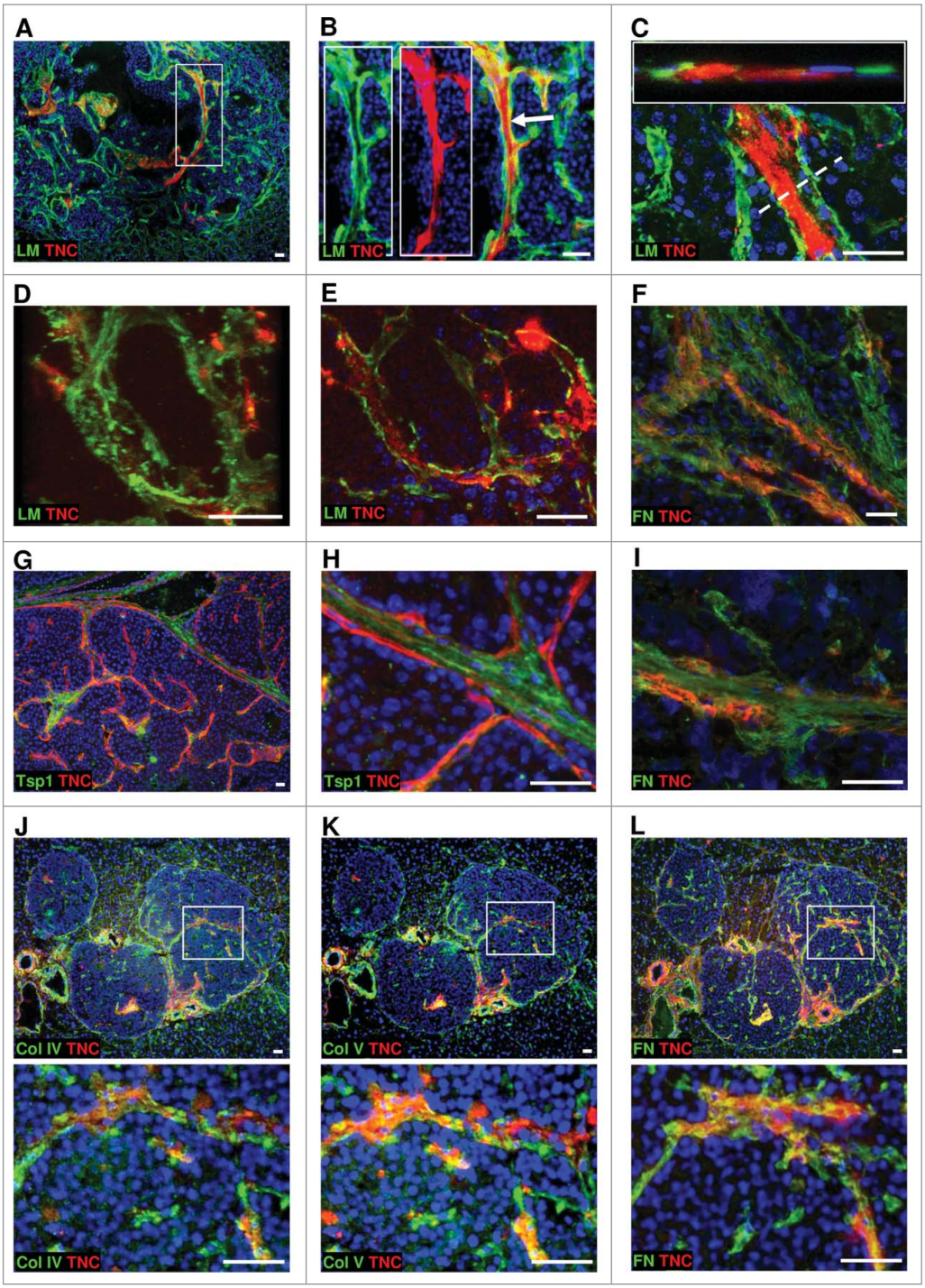

Figure 1. Costaining of TNC with other ECM molecules in RIP1-Tag2 tumors (**A–E**) Immunodetection of LMs (green) and TNC (red) with specific antibodies. LMs and TNC are forming tracks that run across the tumor tissue; some of the LM tracks are filled with TNC (**A**), (**B**) higher magnification of (**A**). DAPI stained nuclei were observed inside the tracks (**C**). TNC tracks are represented as 3D channels upon reconstruction of confocal images (**D**, **E and Movie 1**). (**F, I, and L**) Fibrillar FN and TNC are found to form juxtaposed track networks. (**G and H**) Tsp1 is expressed inside TNC tracks. ColIV (**J**) and ColV (**K**) are forming networks together with TNC as seen in the lower panels that represent magnifications. Scale bars: 20 μm, nuclei are visualized with DAPI.

In addition to TNC, FN, Tsp1, several laminins and collagens are upregulated in the AngioMatrix signature during the angiogenic switch in RIP1-Tag2 tumorigenesis[10] and thus may play a role together with TNC in promoting tumor progression.[8] Whether these ECM molecules form distinct or common tracks with TNC is unknown. Therefore, we determined their expression in conjunction with TNC. We confirmed expression of FN and TNC as juxtaposed fibrillar networks (**Fig. 1F and I**).[3] Tsp1 also formed tracks that were juxtaposed and occasionally aligned with

TNC (**Fig. 1G and H**). In contrast to LM, the relative orientation of Tsp1 was different and characterized by the arrangement of TNC around Tsp1 and the presence of additional TNC tracks within the Tsp1 core (**Fig. 1H**).

Upon staining for pro-ColIII, ColIV, ColV and FN together with TNC we noticed that TNC is present in close vicinity to all 3 analyzed collagen types (**Fig. 1J and K, Fig. S1D**). Moreover, in adjacent sections we observed that TNC tracks contained not only ColIV and ColV, but also FN (**Fig. 1L**) suggesting that TNC is present in complex networks composed of multiple ECM molecules.

Altogether, these results showed that in tumors of RIP1-Tag2 mice TNC is expressed in multi-molecular tracks comprising several LMs, among them LMα5 and LMγ2, pro-ColIII, ColIV, ColV, FN and Tsp1. As seen by confocal microscopy for LM/TNC tracks we showed that they can form channels.

Analysis of tenascin-C tracks by 3D reconstruction microscopy

To further analyze the TNC tracks and a possible connection to the tumor vasculature we perfused RIP1-Tag2 mice with FITC-coupled dextran particles and analyzed thick tumor sections (50 mm) by costaining for TNC and LMs and performing 3D reconstruction microscopy (**Fig. S2, Movie 2–5**). Again we observed that TNC and LMs formed adjacent tracks within the tumor tissue. The LM matrix appeared as short interrupted aggregates that did not form a continuous basement membrane. Thus the LM network seen here is presumably not part of a basement membrane surrounding a functional blood vessel. The absence of a tube like FITC-dextran signal further supports this hypothesis (**Fig. S2**). The 3D view revealed organization of both ECM molecules into channel-like tracks (**Fig. S2, Movie 2–5**). Nevertheless, a weak FITC-dextran signal was detectable in the TNC/LM tracks suggestive of a lumen that had been reached upon perfusion (**Fig. S2B–D**).

Tenascin-C tracks and abundance of stromal cells reveal parallels in experimental murine and human insulinoma

We had previously shown that TNC is expressed in human insulinoma where the highest TNC levels had been observed in the rare cases of metastatic disease.[8] Here we analyzed TNC expression in more detail and observed that in human insulinoma TNC is also expressed in tracks as seen in RIP1-Tag2 tumors (**Fig. 2A, Fig. S3**), and that TNC tracks can be connected to blood vessels suggestive of a continuum with the circulation (**Fig. 2A**).

Next, we wanted to know whether TNC tracks are potentially providing a microenvironment for stromal cells. Therefore, we searched for the presence of fibroblasts, endothelial cells and immune cells by immunostaining with specific antibodies. We found highly abundant vimentin and αSMA expressing fibroblasts, Mts1/FSP1/S100A4 expressing cells, leukocytes (CD45) and activated macrophages (F4/80) in TNC tracks of RIP1-Tag2 tumors (**Fig. 2B, E–G**). In addition to fibroblasts, endothelial cells (CD31) were also found to be embedded into the TNC tracks in the RIP1-Tag2 tumors as well as in the human insulinoma (**Fig. 2C**). It is remarkable that only few of the analyzed stromal cells are present outside the TNC containing tracks suggestive of TNC tracks providing a hospitable microenvironment (**Figs. 2B–G, Fig. S4**).

Tenascin-C tracks in human colorectal carcinoma

To address TNC organization in human CRC we stained paraffin embedded or PFA fixed tissue with an anti TNC antibody. We observed that TNC is not homogenously expressed throughout the tumor tissue but is locally organized in multiple, parallel aligned tracks (**Fig. 3A**). This TNC network is reminiscent to that seen in human insulinomas (**Fig. 2A**). Upon costaining with a pan-LM antibody matrix tracks containing TNC and LMs seem again to be organized in adjacent layers forming a channel (**Fig. 3B, Movie 6**) as seen in RIP1-Tag2 insulinoma (**Fig. 1B–E, Movie 1**). Analysis of sagittal sections revealed abundance of both ECM molecules in confined stromal microdomains underneath the tumorigenic epithelium. A more highly magnified view showed that TNC is expressed together with LMs in apparently mixed as well as aligned networks. This stromal region is delimitated by a LM matrix arranged beneath the colonic epithelium representing the basement membrane (BM) (**Fig. 3C and D**). Immunofluorescence staining further revealed that TNC can be secreted at the tip of a nascent track. Upon costaining, CD31 expressing endothelial cells were seen to be embedded into the TNC track (**Fig. 3E and F**). Similar to the human insulinoma, αSMA and vimentin positive fibroblasts were abundant in the TNC tracks in human CRC tissue (**Fig. 3G–J**). We also addressed TNC expression and abundance of αSMA and vimentin positive fibroblasts in intestinal carcinomas spontaneously arising in mice genetically engineered to express activated oncogenic KRAS in intestinal epithelia, combined with a targeted mutation of the APC tumor suppressor (pvillin-$Kras^{V12G}$/$Apc^{1638N/wt}$).[26] Again, stromal cells were not seen outside the TNC tracks but within, suggesting that these TNC tracks represent a hospitable microenvironment for stromal cells encompassing endothelial cells and fibroblasts in murine experimental and human intestinal cancer (**Fig. 3K and L**).

Tumor cells contribute to expression of tenascin-C in tracks as a microenvironment for stromal cells

Stromal cells are a major source of TNC in the lung metastatic breast murine 4T1 breast cancer grafting model.[27] Moreover, during lung metastasis in the MDA-MB231 xenograft model tumor cell-derived TNC was crucial for survival until stromal cells in the lung tissue expressed TNC.[11] Here we wanted to know how tumor cell-derived TNC is organized and whether it may provide a microenvironment for stromal cells. Therefore, we had generated immune compromised RAG2 knockout (KO) mice lacking the TNC protein (RAG2KO/TNCKO) by breeding and used these mice (and their littermate controls) for tumor cell engraftment. We chose to use the well-established cecum orthotopic grafting model employing SW480 human CRC cells. Grafted SW480 cells formed well differentiated tumors in the cecum (**Fig. S5A and B**). We observed TNC tracks in the arising tumors (**Fig. S5C, D, and F**). Whereas the TNC signal intensity

and abundance of TNC tracks was high in tumors of the control host the signal was weak in tumors of the TNCKO host (**Fig. S5D and F**). This result suggested that in this grafting model TNC is predominantly expressed by stromal cells in track like networks as seen in RIP1-Tag2 tumors. Also tumor cell derived TNC is organized in tracks together with LMs (**Fig. 4**, **Fig. S5E and F**). By immunostaining we addressed whether tumor derived TNC also generated niches for stromal cells. Upon immunostaining for vimentin and CD31, respectively, we observed that fibroblasts and endothelial cells were poorly present in TNC free areas, yet they were highly abundant in close vicinity or within the TNC rich matrix (**Fig. 4**).

Our results showed that whereas TNC is predominantly expressed by stromal cells, tumor cells can also express TNC which is also organized in a track like pattern in the CRC grafting model. Similar to stromal cell-derived TNC, tumor cell-derived TNC tracks also provided a microenvironment for fibroblasts and endothelial cells.

In summary, by using the RIP1-Tag2 mouse model we show that TNC is expressed together with up to 9 other ECM molecules in confined microenvironments in a track like pattern. Stromal cells including fibroblasts, endothelial and immune cells are enriched in and embedded into the TNC tracks. Although stromal cells are the major source of TNC, tumor cells also express TNC. In human insulinoma and CRC TNC is organized into tracks together with LMs, thus providing a microenvironment for stromal cells and suggesting the relevance of our results for insulinoma progression and potentially other human cancers.

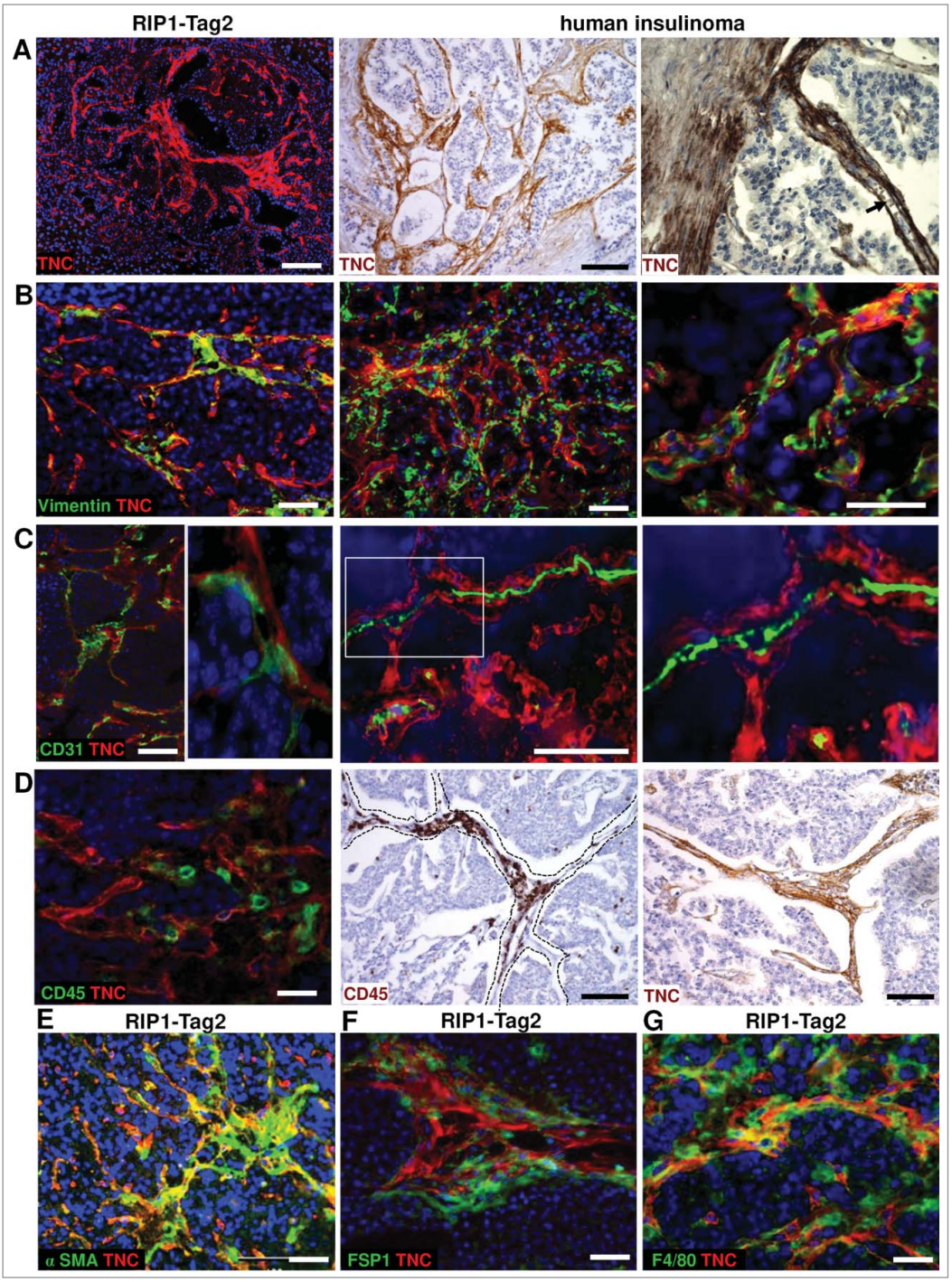

Figure 2. Characterization of TNC tracks in RIP1-Tag2 tumors and comparison with human insulinoma (**A**) TNC is forming tracks in RIP1-Tag2 tumors and in human insulinoma. (**B**) TNC tracks are juxtaposed to vimentin expressing fibroblasts, and CD31 positive endothelial cells (**C**). (**C**) Insert of higher magnification. Leucocytes are visualized with an antibody against the common leucocyte antigen CD45. This immunostaining result shows that immune cells are expressed in close contact to the TNC tracks (**D**). αSMA, FSP1 and F4/80 positive cells are also found in close vicinity to the TNC tracks (**E-G**). Scale bars: 20μm, nuclei are visualized with DAPI.

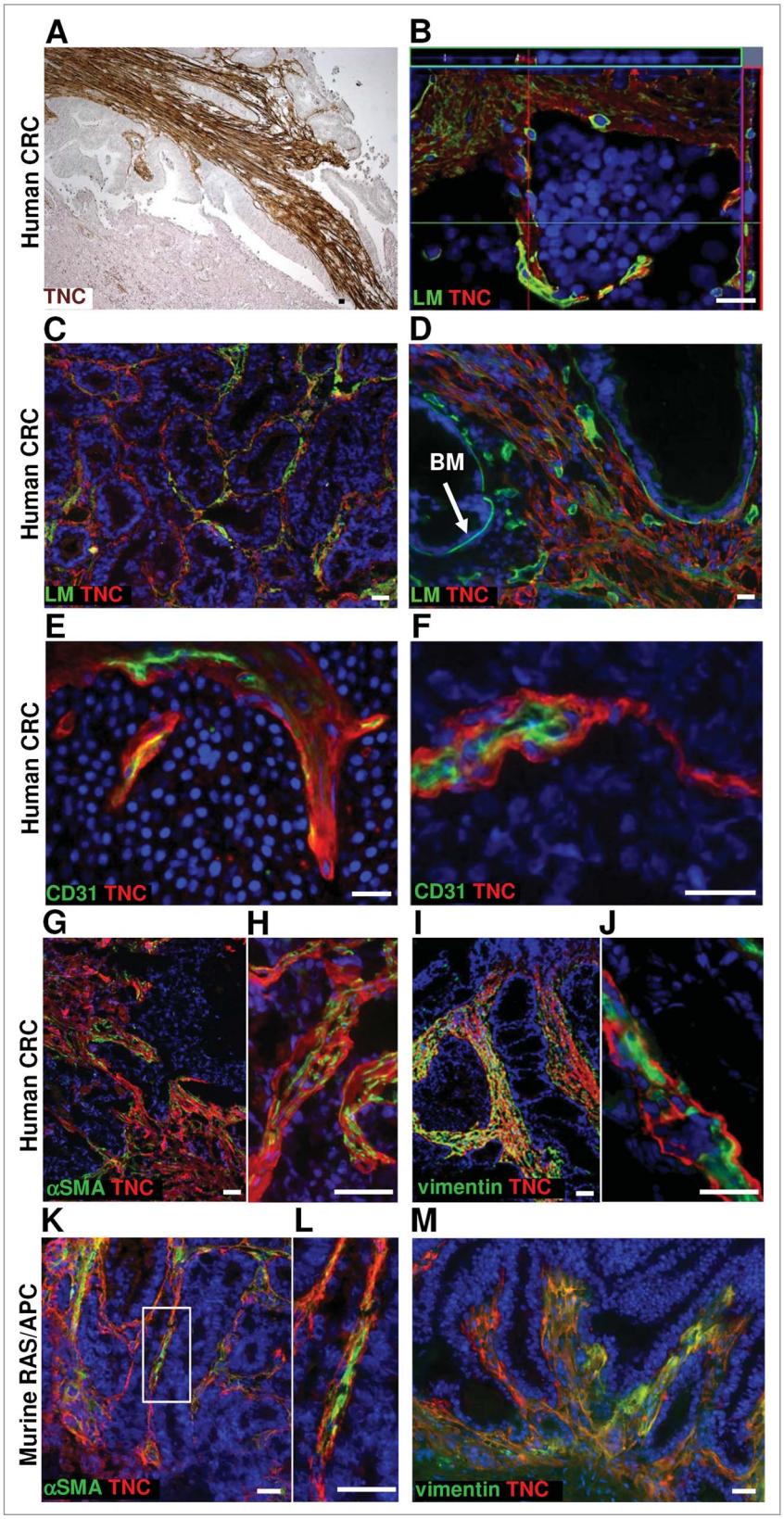

Figure 3. Localization of ECM in human and murine intestinal cancer (**A**) Similar to insulinoma, TNC is forming tracks in human colorectal cancer tissue where TNC is again found inside a LM channel (**B**) and in juxtaposition to LM matrix (**C and D**). (**D**) Arrow points at the basement membrane (BM). In colorectal cancer, TNC is expressed in close contact to CD31 (**E**), αSMA (**G**) and vimentin (**I**) positive cells. These cells seem to be localized inside the TNC tracks (**F, H, and J**). (**H, J, and L**) higher magnification view. In mouse RAS/APC tumors, a similar organization is observed as illustrated for αSMA (**K**) and vimentin (**L**) positive cells embedded into TNC rich matrix. Scale bars: 20 μm, nuclei are visualized with DAPI.

Discussion

In the RIP1-Tag2 tumorigenesis model we previously demonstrated that TNC promotes multiple events in tumor progression and in particular angiogenesis and metastasis.[8] TNC is one of the most highly expressed molecules in the AngioMatrix signature that has negative prognostic value for survival of glioma and colon cancer patients which suggests that this signature is relevant for tumor progression.[10] However it was not known whether any of these ECM molecules potentially form common networks, in particular together with TNC. Therefore, here we determined expression and organization of TNC in RIP1-Tag2 tumors together with other ECM molecules and compared some of these features to those of human insulinoma and CRC. We observed that in RIP1-Tag2 insulinoma TNC is expressed locally in arrays (that we had coined tracks) together with other ECM molecules, LMs (e.g., LMα5, LMγ2), FN, collagens (pro-ColIII, ColIV, ColV) and Tsp1. However not all ECM networks contained TNC. We also observed TNC/LM tracks in human insulinoma and CRC, suggesting clinical relevance of our results for human cancer progression.

We hypothesize that the TNC containing ECM tracks potentially could induce local tissue stiffening and thus may contribute to enhanced metastasis formation. TNC may reduce cellular tension generated in the vicinity of the tracks since TNC is a poorly adhesive substratum for most cells. Accordingly, upon binding to TNC, actin polymerization and stress fiber stabilization is impaired ([13,14,] reviewed in [2]).

This "slippery" TNC ground may also promote cancer cell dissemination. Indeed tumor

invasiveness was increased *in vivo* upon forced expression of additional transgenic TNC in RIP1-Tag2 mice.[8] Importantly, TNC channels were observed only in metastatic melanoma, not in non-metastatic tumors, which supports the possibility that TNC containing dissemination tracks actively promote metastasis.[15,28] However, whether TNC is an important component was unknown. Our results suggest that TNC tracks, representing the most abundant TNC network in RIP1-Tag2 tumors, are crucial, as lung micrometastasis was increased upon overexpression of transgenic TNC. In good accordance, the least lung micrometastases were seen in the TNC deficient background.[8] In further support, also in human insulinoma, which feature abundant TNC tracks (this study), the highest TNC levels had been observed in the metastatic cases.[8]

The results presented here raise the possibility that TNC tracks which we had shown to be enriched by endothelial cells, fibroblasts and immune cells, provide a hospitable microenvironment. Whether the TNC tracks impact on the described pro-angiogenic activities of these stromal cells is a likely possibility that needs to be addressed in the future.

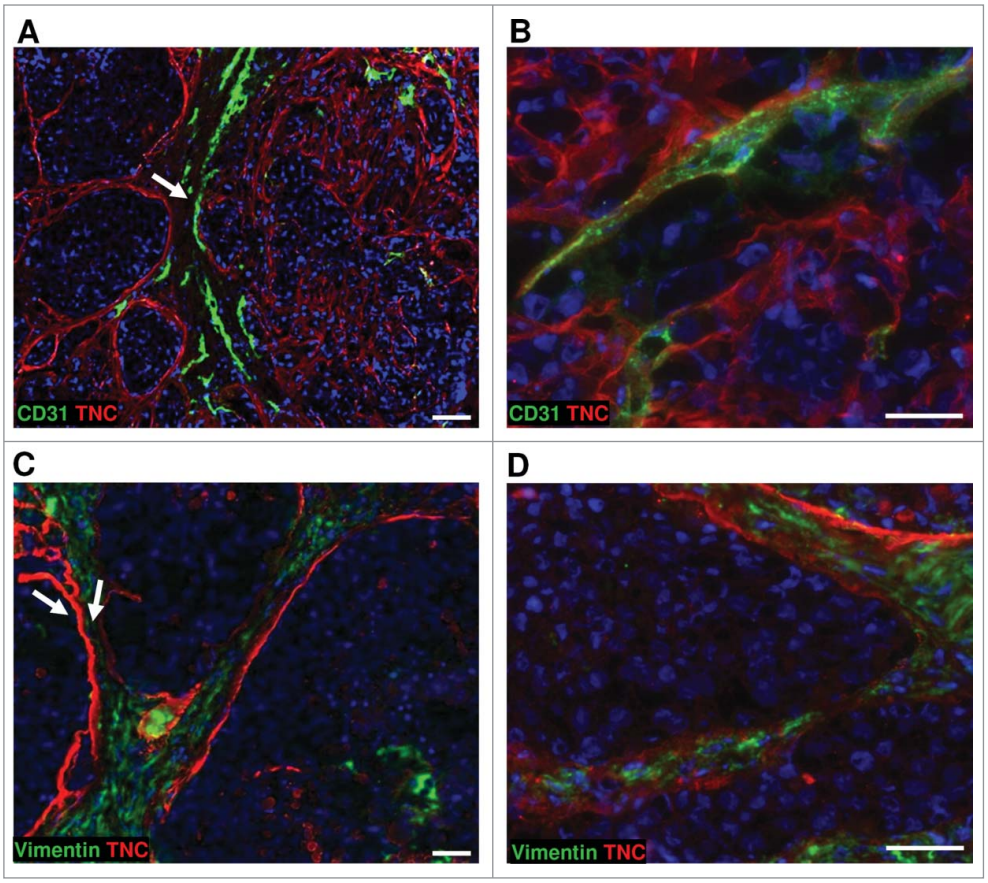

Figure 4. Expression of CD31, vimentin and TNC in orthotopically grafted SW480 tumors SW480 cells had been grafted into the cecum of double KO RAG2KO/TNCKO mice devoid of the TNC protein. Upon tissue sectioning and staining, tumor cell-derived TNC expression is detected showing organization into tracks (**A–D**). In close contact to the TNC tracks, CD31 positive (**A and B**) as well as vimentin positive cells (**C and D**) are observed. Scale bars: 20 μm, nuclei are visualized with DAPI. Arrows point at an array of endothelial cells in close contact with TNC (**A**) and at a TNC track expressed by SW480 cells (**B**).

In breast cancer and in human glioma TNC was shown to be coexpressed with TNW another tenascin family member in a track-like pattern.[29,30] In malignant melanoma TNC formed channels together with FN and ColI that did not represent blood or lymphatic vessels.[15] Other laminin and collagen containing tracks, coined conduits, have been described in secondary lymphoid organs. Except for the thymus, where TNC formed conduits together with several laminins, collagens and other ECM molecules (suggesting that these structures represent multimolecular ECM networks), it is unknown whether conduits in lymph nodes or the spleen contain TNC. We had previously hypothesized that during tumorigenesis a genetic program for conduits may be turned on out of context leading to the formation of TNC tracks.[3,31] This possibility is supported by similarities in the molecular composition of the TNC tracks in RIP1-Tag2 tumors with those of the melanoma channels and the thymic conduits. A common determinant is TNC that is present in all 3 entities (**Table 1**). Fibril-forming collagens (e.g., Col III, Col IV) as well as LMγ2 are present in the RIP1-Tag2 tracks as well as in the conduits. In contrast, neither ColIV nor LMγ2 had been seen in the TNC channels of malignant melanoma.[15] Thus TNC tracks found in RIP1-Tag2 tumors share molecular features with reticular fibers and ECM channels of malignant melanoma.

Previously it had been shown that tumor cells can evade tumor surveillance due to molecular mimicry of lymph nodes through expression of CCL21 and CCR7.[32] Thus, TNC tracks found in cancer tissue may share common features with conduits in the thymus, providing a physical niche promoting lymph node mimicry. TNC tracks might also generate spatially confined niches to promote cancer promoting signaling such as Wnt and Notch as seen in breast cancer and RIP1-Tag2 tumors.[2,8,11]

Insulinomas are mostly benign tumors but can also become malignant with metastasis to regional lymph nodes, lung and liver.[33] Thus, it is clinically important to predict progression to malignant cancer to improve the therapeutic management, and the onset of anti-cancer treatment. Here we documented parallels of the murine RIP1-Tag2 model to human insulinoma and human CRC, exemplified in the formation of LM rich TNC

tracks as a microenvironment for stromal cells. Thus, we suggest that, extending beyond published work,[8] more information gained on the microenvironment in the RIP1-Tag2 model could be useful for understanding human insulinoma progression. TNC channels and intensity of TNC staining was a better marker for propensity of primary melanomas to metastasize than the Breslow score of tumor thickness.[15] Thus it is an intriguing possibility that a combined expression of TNC together with other ECM molecules found in the TNC tracks could have predictive and prognostic value. Since TNC tracks are not targeted by cytotoxic drugs they may create alternative routes for tumor cell dissemination and potentially play a role in enhanced tumor metastasis formation upon anti-angiogenic drug treatment. For a potential future therapeutical intervention it will be important to determine which cells express TNC and other track ECM molecules.

Altogether, here we described that TNC tracks are of heterogeneous appearance in respect to molecular composition, network alignment, spatial orientation and 3D organization. We observed that a few up to multiple TNC tracks are often aligned in a parallel pattern. These TNC confined microdomains were characterized by association with other ECM molecules and represented niches for stromal cells. We found indications for a potential connection of TNC tracks to blood vessels raising the possibility that TNC tracks may play a role in tumor vessel formation.

Material and Methods

Human specimens

Human insulinoma material was obtained from the Klinikum rechts der Isar der TUM (Munich, Germany) or the Hôpital de Hautepierre (Strasbourg, France) with prior patient informed written consent. Patients underwent surgical resection at the Department of Surgery, Klinikum rechts der Isar, Munich, Germany (between 1991 and 2011) and at the Hôpital de Hautepierre, Strasbourg (between 1994 and 2007). Tissue specimens were embedded in Tissue-Tek (Sakura, Labonord), frozen on dry ice and stored at −80°C, or fixed in formalin and embedded in paraffin. The median age of patients from Group 1 was 52 years (35 to 82 years, 5 male and 2 female patients) and that from Group 2 was 46 years (13 to 69 years, 2 male and 5 female patients).

Human colorectal carcinoma (CRC) tissue was obtained from the Department of Visceral Surgery (Hôpitaux Universitaires de Strasbourg), under the institutional review of the French ethic committee and approved by the CCPPRB "Est-IV" ethic committee. Tissue from 10 human CRC were obtained from surgical specimens. Tumor tissue was directly embedded in Tissue-Tek (Sakura, Labonord) and stored at −80°C, or fixed in formalin and embedded in paraffin.

Murine cancer models

Experiments comprising animals were performed in accordance with the ethical rules for the care and use of animals for research (INSERM agreement E67-482-21). RIP1-Tag2 mice were maintained by in-house breeding.[7] Drinking water was supplemented with 5 % glucose (w/v) at the age of 10 weeks. Tumor tissue of RIP1-Tag2 mice was analyzed at the age of 12 weeks.

We had generated immune compromised Rag2KO/TNCKO mice lacking the TNC protein by breeding Rag2KO mice (C57Bl6 strain, gift from T. Rolink, University Basel)[34] with TNCKO mice (gift from R. Fässler, MPI Munich)[35] upon crossing for 10 generations into the C57Bl6 strain. Genotyping was performed by PCR using the following primers, Rag2 KO (Fwd: 5′-TTG GGA GGA CAC TCA CTT GCC AGT-3′, Rev 5′-GCA ACA TGT TAT CCA GTA GCC GGT-3′, Rag2 KO 5′-GCT TCC TCT TGC AAA ACC ACA CTG-3′), TNCKO (Fwd wt: 5′-CTG CCA GGC ATC TTT CTA GC-3′, Fwd TNCKO: 5′-CTG CTC TTT ACT GAA GGC TC-3′, Rev: 5′-TTC TGC AGG TTG GAG GCA AC-3′).

Rag2KO and Rag2KO/TNCKO mice were used for orthotopic grafting of SW480 human colon adenocarcinoma cells expressing TNC into the cecum wall (4 million cells in 100 μl PBS). The human CRC cell line SW480, obtained from the American Type Culture Collection (Rockville, MD, USA), was maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 100U/ml penicillin and 0.1 mg/ml streptomycin, 40mg/ml gentamycin sulfate (Invitrogen, France). Grafted SW480 cells had formed tumors in the cecum in the TNCKO host (5/5) with similar frequency as in the wildtype host (5/5). Tumors were dissected after 8 weeks and further analyzed as described below.

Tumor tissue from mice was either fixed in 4% paraformaldehyde (PFA) for 2 hours followed by immersion in 20% sucrose overnight and embedded in Tissue-Tek (Sakura, Labonord). Alternatively, freshly isolated tissue was embedded in Tissue-Tek, immediately frozen on dry ice and stored at −80°C.

Mice heterozygous for a chain termination mutation in the mouse Apc gene (Apc^{1638N}), were mated with transgenic pvillin-$KRAS^{V12G}$ mice that expressed human oncogenic KRAS in intestinal epithelia, on a C57Bl/6N background, as previously described in detail.[26] Mice were bred at a barrier facility at the ZPF (Zentrum für Präklinische Forschung) at TUM, Munich, Germany, in accordance with national and institutional guidelines. The macroscopic study of the intestinal tract and processing of intestinal lesions for microscopic analysis by cryosectioning was carried out as described.

Dextran perfusion and 3D-reconstruction microscopy

Twelve week old RIP1-Tag2 mice were anesthetized, the chest was opened, and the vasculature was perfused by injection of 150 μl FITC-conjugated dextran (containing 7.5 mg dextran, 2000 kDa, Sigma, Schnelldorf, Germany) into the left heart chamber. Circulation was allowed for 5 minutes before pancreas preparation, followed by fixation in 4% PFA for 2 hours, incubation in 20% sucrose overnight, embedding into Tissue-Tek and freezing on dry ice. Cryosections (50 μm) were stained as described below and Z-series acquisitions were performed with a Zeiss Axio Imager.Z2 microscope equipped with the ApoTome module and AxioVs40 software (Carl Zeiss Microimaging) with a 3D analysis tool.

Histopathological and immunohistochemical analysis of mouse and human tumor tissue

Tissue sections were prepared for standard hematoxylin/eosin (HE) staining. Paraffin sections (6μm) or cryosections (7 μm) were processed by immunohistochemistry (IHC) or immunofluorescence (IF) staining, respectively. For IHC labeling tissue sections were either treated with a Ventana Benchmark automate (Ventana Medical Systems) or revealed after incubation with the primary antibody (**Table S1**) with biotinylated secondary antibodies (Vector Laboratories), amplified with the ABC Elite Vectastain kit and developed with the DAB kit from Vector Laboratories, followed by staining with hematoxylin. For IF detection, frozen sections of human or murine tumor tissue were incubated overnight at 4°C directly with the primary antibody (see **Table S1**). Polyclonal anti TNC antibody TNC1.2 was generated by immunization of a rabbit with purified recombinant human TNC expressed in HEK293 cells.[36] Bound antibodies were visualized upon 1 hour incubation at room temperature with anti-mouse, -rabbit, -chicken or -rat secondary antibodies conjugated with fluorophores DyLight-488 (Molecular Probe), Cy3 or Cy5 (Jackson ImmunoResearch, UK). Cell nuclei were stained with DAPI. Images of stained sections were acquired with an Axioskop2 plus light microscope (Zeiss), fluorescence Axio Imager.Z2 (Zeiss) or a Zeiss LSM 780 confocal microscope. Control sections were processed as above with omission of the primary antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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