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Mechanisms of Tenascin-C dependent tumor migration and metastasis

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Abstract (in English)

A high TNC expression correlates with lung metastagenicity and was shown to promote experimental lung metastasis, but the underlying mechanisms are poorly understood. In this thesis, I used several *in vitro* and *in vivo* models to study the roles of TNC in tumor migration and metastasis. I used the stochastic MMTV-NeuNT and a novel associated syngeneic orthotopic breast cancer model engineered to present abundant and no/low TNC. I observed that TNC promotes tumor onset and increases lung metastasis. I further revealed an important role of stromal TNC in metastasis and that TNC is a prominent component of parenchymal metastasis and lymphovascular invasions (LVI). I document that LVI are nests of proliferating circulating tumor cells, that are enveloped by a layer of endothelial cells and fibroblasts, contain platelets and express distinct layers of TNC, fibronectin and laminin. Moreover, TNC increases platelet abundance and endothelial cell layering of LVI. TNC also elevates tumor cell survival in LVI, partial epithelial-to-mesenchymal transition (EMT) and breaching into the lung parenchyma. In cultured cells through TGF-β signaling, TNC induces EMT, cell migration and survival. The detailed characterisation of the composition and role of LVI provides novel opportunities for anti-cancer treatment suggesting platelets, endothelial and FSP1+ cells as well as TGF-β as novel targets to prevent extravasation from the LVI. I also described a novel tumor cell autonomous signaling pathway in osteosarcoma and glioblastoma cells where TNC inhibits actin stress fibers and YAP through integrin α9β1. This is required for TNC to promote amoeboid-like tumor cell migration. In support for a potential biological role of this mechanism, I observed that expression of YAP target genes is elevated in a glioblastoma model upon knockdown of TNC. The provided link could have clinical relevance as a YAP signature comprising CTGF, Cyr61 and Cdc42EP3 at lower levels, resembling the TNC effect *in vitro*, correlates with worst survival of cancer patients. This indicates that even low levels of TNC can be dangerous and could account for cancer progression. Integrin α9β1may be a good target for blocking TNC promoted migration in osteosarcoma and glioblastoma and potentially also other cancers including breast cancer. In summary, the results of my thesis have provided insight into the roles of TNC in metastasis suggesting that TNC contributes to extravasation by impacting on survival, endothelialization, EMT and migration. Moreover, I have identified TGF-β signaling and integrin α9β1 as important pathway and molecule, respectively to be employed by TNC. Whether both

molecule/pathway play a similar role in the investigated models of breast cancer, osteosarcoma and glioblastoma remains to be seen.

Abstract (in French)

Introduction :

Les métastases sont la principale cause de décès chez les patients atteints d'un cancer. Lors du développement métastatique, les cellules tumorales disséminées (CTD) doivent franchir certaines étapes clés avant de coloniser des organes distants de la tumeur primaire. Ces étapes incluent l'invasion du tissu environnant cette tumeur, l'intravasation dans la circulation, la survie face aux forces de cisaillement intravasculaire, l'infiltration de tissus distants, l'échappement au système immunitaire, l'adaptation à la nouvelle niche de soutien, la survie en tant que ''graines latentes'' initiatrices de tumeurs et éventuellement l'expansion rapide en remplaçant le tissu hôte. Bien que l'ensemble de ces multiples événements soit très peu efficace, les CTD ayant réussi à coloniser de nouveaux organes ne peuvent pas être traités du fait du manque d'efficacité des thérapies actuelles sur le long terme (Massagué et al., 2016). Nous devons donc acquérir de meilleures connaissances sur les mécanismes moléculaires dans le but de développer de nouveaux traitements anticancéreux persistants.

L'invasion locale du tissu environnant est une étape précoce du processus métastatique, et un déterminant clé du potentiel métastatique des cellules tumorales. Les CTD peuvent développer des morphologies et des caractéristiques migratoires distinctes, dans le but de s'adapter à des conditions environnementales spécifiques, ainsi que de franchir les barrières tissulaires (Friedl et al., 2011). Le microenvironnement tumoral (MET) comprend un réseau dynamique de protéines de la matrice extracellulaire (MEC) incluant un ensemble cellulaire tel que des fibroblastes, des cellules dérivées de la moelle osseuse, des cellules endothéliales ainsi que des cellules immunitaires infiltrantes (Balkwill et al., 2012), en plus de facteurs solubles (Gattazzo et al., 2014). Ces cellules stromales apportent des signaux mécaniques et chimiques aux cellules tumorales et remodèlent la MEC. Les stimuli mécaniques comme la rigidité matricielle, la topographie de la MEC, l'ouverture de pores, le maintien d'un certain stress, les contractions matricielles, couplés aux stimuli chimiques de sources autocrines et paracrines influencent les propriétés migratoires des cellules tumorales (Polacheck et al., 2013). Tous ces signaux agissent simultanément sur les cellules cancéreuses, impliquant que la migration tumorale soit une réponse intégrée, induite par ces multiples stimuli.

Des études sur tissus humains ainsi que de l'imagerie intra vitale utilisant des modèles murins (Clark et al., 2015) ont montré une grande diversité dans la migration des CTD ; ce qui inclue la migration de type amiboïde ou de type transition épithélio-mésenchymateuse (TEM) des cellules isolées, ainsi que des flots multicellulaires et des migrations collectives. Plusieurs facteurs intrinsèques régulant le mode de migration et la morphologie ont été identifiés via des études *in vitro*. Lorsque les cellules migrent individuellement, une morphologie allongée, une contractilité plus faible et une adhésion améliorée favorisent une migration de type mésenchymateuse. A l'inverse, une morphologie arrondie, accompagnée d'une augmentation de la contractilité sous contrôle de la signalisation Rho-dépendante va favoriser une migration de type amiboïde (Sanz-Moreno et al., 2008). Dans le cas de migration collective, une hausse de l'expression des cadhérines dans les interactions cellules-cellules ou l'augmentation des liens cellules-MEC via les intégrines sont impliquées (Friedl et al., 2011). Bien que des progrès significatifs aient été réalisés dans la compréhension des mécanismes moléculaires et des voies de signalisation régissant les différents modes de migration, les connections entre migration de cellule isolée, flot cellulaire et migration collective restent peu connues.

La ténascine-C (TNC) est une glycoprotéine matricellulaire sécrétée (190-300kDa) de la MEC dont l'expression est élevée durant le développement embryonnaire, à l'inverse d'une quasi absence dans la plupart des tissus adultes. La TNC est cependant exprimée de nouveau lors de traumas tissulaires et joue un rôle important dans la réparation tissulaire, ainsi que dans des conditions pathologiques où son expression importante, durant des inflammations chroniques, des fibroses ou encore des cancers, accroit la sévérité de la pathologie (Midwood et al., 2016). L'expression de la TNC est corrélée avec un faible taux de survie et stimule la prévalence métastatique pulmonaire du cancer du sein (Oskarsson et al., 2011). Il a été montré que la TNC, considérée comme une partie du réseau de MEC spécifique de la tumeur, accroit la malignité tumorale en augmentant la survie cellulaire, la prolifération et la migration. Dans ces processus, la TNC dirige la signalisation d'adhésion cellulaire ainsi que les propriétés biomécaniques du tissu (Saupe et al., 2013, Midwood et al., 2016). La TNC est capable d'induire la migration en stimulant des processus de type TEM dans les cellules cancéreuses mammaires (Katoh et al., 2013). De plus, il a été récemment montré que le domaine C-terminal de type fibrinogène (FBG) de la ténascine-X (TNX), qui est une structure conservée et présente chez la TNC, interagit physiquement avec le complexe latent TGF-β et induit la TEM sur des cellules en culture (Alcarz et al.,

2014). Même si nous savons que la TNC joue un rôle important dans le développement métastatique du cancer du sein (Oskarsson et al., 2011), nous ne comprenons que trop peu les mécanismes moléculaires impliquant la TEM et/ou la signalisation via des facteurs de croissance.

Lors de la migration d'une cellule isolée, la TEM est un processus rigide impliquant de profondes altérations dans la transcription génique (Stephan et al., 2012). Par opposition, la transition mésenchymo-amiboïde (TMA) fait intervenir de rapides changements dans le mode de migration qui apparait comme un retour aux propriétés basales de la TEM de manière réversible et transitoirement régulée (Paňková et al., 2010). Les états de polymérisation de l'actine déterminent comment les cellules migrent dans un mode mésenchymateux (dépendant des fibres de stress actiniques) ou amiboïde (indépendant de la polymérisation actinique ou de l'inhibition des fibres de stress) (Bergert et al., 2012). Sachant que le substrat de TNC modifie la formation de fibres de stress (Huang et al., 2001, Saupe et al., 2013), nous nous sommes demandés comment une TEM spécifique riche en TNC peut forcer les cellules tumorales à migrer de façon amiboïde.

Notre hypothèse est que la TNC pourrait jouer différents rôles dans la migration des cellules cancéreuses et par conséquent dans le développement métastatique. Considérant l'actine comme un réservoir de facteurs de croissance, la TNC pourrait induire la TEM ainsi que la survie et l'extravasation des cellules tumorales. Cependant, des cellules cancéreuses individualisées localement pourraient répondre à la TNC en initiant des changements rapides menant à un phénotype migratoire de type amiboïde. L'objectif de cette thèse a été d'étudier comment la TNC stimule le développement métastatique dans le cancer du sein au niveau cellulaire et moléculaire en utilisant des modèles tumoraux et cellulaires.

Objectifs et résultats :

Objectif 1 : Identification des mécanismes cellulaires et moléculaires en lien avec le rôle pro-métastatique pulmonaire de la TNC dans le modèle NeuNT de cancer mammaire.

Pour comprendre les rôles de la TNC dans les métastases pulmonaires, nous avons analysé les conséquences de la non-expression de la TNC dans un modèle murin de cancer du sein dépendant d'ErbB2 (MMTV-NeuNT). Nous avons montré que la perte de la TNC retarde significativement l'apparition de tumeurs mammaires primaires sans pour autant impacter la croissance tumorale. Cependant, la perte de la TNC réduit fortement le développement de métastases pulmonaires du fait de l'induction de phénomènes apoptotiques dans ces métastases. Nous avons aussi constaté un rôle de la TNC stromale dans l'induction des métastases, notamment au niveau de la survie cellulaire, dans le modèle syngénique de greffe orthotopique NT193. De plus, nous avons déterminé l'effet de la TNC sur l'invasion lymphovasculaire (ILV) qui est caractéristique de ce modèle et représente une forme particulière de CTD. Puisque l'ILV est une cause majeure de décès des patients cancéreux, notre caractérisation cellulaire et moléculaire de cette ILV présenterait un intérêt pour le cancer du sein. Nous avons constaté que l'ILV représente une masse compacte de cellules tumorales épithéliales positives pour CK8/18. Cette masse de cellules tumorales est entourée d'une couche de TNC suivie d'une couche de fibronectine, de laminine puis enfin une couche externe de cellules endothéliales. De plus, des thrombocytes ont été retrouvées attachées à ces ILV. Enfin, nous avons observé que l'abondance en thrombocytes et en cellules endothéliales était diminuée en absence de TNC. Un autre mécanisme important induisant potentiellement le développement métastatique pulmonaire est la TEM. L'analyse des ILV a révélé un marquage intense de la vimentine (néanmoins pas d'αSMA) en présence de TNC. A l'inverse, très peu de vimentine a été observée dans les ILV de souris n'exprimant pas la TNC. *In vitro*, la TNC induit la TEM sur les cellules NT193 en impliquant certainement les voies moléculaires activées par TGF-β, car la TEM était bloquée en présence d'inhibiteurs du récepteur I de TGF-β. Par ailleurs, nous avons trouvé que la signalisation via TGF-β induite par la TNC implique les voies canoniques Smad2/3 phosphorylés ainsi que non-canoniques Akt et Erk1/2 phosphorylés. Dans son ensemble, cette étude nous apprend que les ILV seraient des cibles potentielles des traitements anti-cancéreux. En particulier, le ciblage des cellules endothéliales et des thrombocytes représenteraient une opportunité thérapeutique intéressante pour réduire l'effet stimulant de la TNC sur le développement métastatique pulmonaire.

Objectif 2 : Expliquer les conséquences et les mécanismes sous-jacents de l'arrondissement cellulaire induit par la TNC.

Etant une part importante de la MEC spécifique à la tumeur, la TNC joue divers rôles dans la stimulation de la malignité tumorale. La TNC module les propriétés tissulaires, l'adhésion cellulaire, la signalisation pro-migratoire ainsi que l'expression génique (Chiquet-Ehrismann et al., 2014). Plusieurs molécules contrôlant la migration et l'adhésion cellulaire sont régulées par la TNC (Ruiz et al., 2004) ; cependant les mécanismes entrant en jeu sont peu connus. En utilisant les bases de données publiques disponibles sur l'expression des ARNm ainsi que la méthode de ''Gene Set Enrichment Analysis'' (GSEA), nous avons observé une corrélation inverse entre l'expression de gènes cibles de MKL1 ainsi que de YAP et de l'expression de gènes sous-régulés par la TNC, laissant suggérer un rôle de répresseur de la TNC sur l'activité de MKL1 et/ou YAP, deux senseurs des fibres de stress actiniques. Nous avons identifiés trois classes de gènes dérégulés sur un substrat anti-adhésif constitué de fibronectine (FN) et TNC ; gènes étant en liens avec uniquement MKL1, uniquement YAP ou bien MKL1 et YAP ensembles. Nous avons observé que l'expression de ces gènes est diminuée par la TNC via la répression de MKL1 et la rétention cytoplasmique de YAP. L'expression de la TNC elle-même est réduite suite à celle de MKL1 et YAP, suggérant une boucle de rétro-contrôle potentiellement intéressante au niveau thérapeutique. De plus, nous avons constaté que la TNC stimule la migration de type amiboïde dans un espace tridimensionnel. Nous avons aussi identifié l'intégrine α9 (ITGA9) comme étant un gène en amont de la répression médiée par MKL1 et YAP, dépendant de la TNC. L'intégrine α9β1, ainsi que l'abolition de l'activité de YAP, sont nécessaires à la migration de type amiboïde induite par la TNC. Par conséquent, la diminution de la tension cellulaire par la TNC pourrait être importante pour contrebalancer les tensions présentes au sein du tissu tumoral et pour faciliter la migration de type amiboïde. Ce que nous avons observé présente un réel intérêt clinique car notre nouvelle combinaison tri-génique CTGF, Cyr61 et Cdc42EP3, dont la sous-expression est liée à YAP, corrèle avec une dégradation globale de l'état et une diminution de la survie sans métastase des patients atteints de glioblastome ou d'ostéosarcome, respectivement dans un contexte de faible expression de la TNC. De ce fait, une faible quantité de TNC est déjà capable de stimuler la malignité cancéreuse en induisant la migration de type amiboïde. Sachant que le ciblage de YAP apparait actuellement comme une stratégie anti-cancéreuse, nos données suggèrent que dans les cancers présentant un lien entre la faible expression de la signature tri-génique dépendante de YAP et un pronostic négatif,

le ciblage de YAP serait dans ce cas contre-indiqué. Au contraire, le ciblage de l'intégrine α9β1 pourrait diminuer la migration de type amiboïde et le développement métastatique.

Conclusion :

En tant que part importante de la MEC spécifique de la tumeur, la TNC joue différents rôles dans la migration lors des processus métastatiques. Agissant potentiellement comme un réservoir de facteurs de croissance, la TNC peut induire la TEM via des signaux dépendant de TGF-β. *In vivo*, la TNC stromale stimule la survie et l'extravasation des cellules cancéreuses mammaires de la circulation aux organes cibles des métastases, où la TNC semble induire la TEM. De plus, la TNC au niveau local peut modifier la migration tumorale via l'induction de la migration de type amiboïde, qui dépend de l'intégrine α9β1 et de l'inactivation de YAP. Ces informations traitant de l'effet de la TNC sur la migration tumorale devrait être exploitables par la médecine personnalisée afin de mieux contrer la formation de métastases cancéreuses.

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Contribution to manuscripts

This thesis has provided results for 2 manuscripts. One of them is in revision in Cancer research, the other one is ready to submit. In addition, I'm involved in one manuscript published in Cell reports. One more manuscript from my PhD work (results not shown here) is in preparation now.

1. Tenascin-C promotes tumor cell migration through integrin α9β1 inhibiting YAP

Zhen Sun*, Anja Schwenzer*, Tristan Rupp*, Devadarssen Murdamoothoo, Olivier Lefebvre, Annick Klein, Thomas Hussenet, and Gertraud Orend#

* equal contribution # corresponding author.

In revision in Cancer research

2. Stromal tenascin-C increases breast cancer lung metastasis by impacting on lymphovascular invasions

Zhen Sun*, Inés Velazquez-Quesada*, Devadarssen Murdamothoo, Constance Ahowesso, Alev Yilmaz, Catherine Bourdon, Marie-Pierre Chenard, Gerlinde Averous, Michael van der Heyden, Christiane Arnold, Annick Klein, Anna Brown, Olivier Lefebvre, Thomas Hussenet, Szuszana Herzgovic, Gerhard Christofori, Pierre Mangin, Renate Kain and Gertraud Orend#

* equal contribution # corresponding author.

Ready to submit

3. Tenascin-C orchestrates glioblastoma angiogenesis by modulation of pro and anti angiogenic signaling

Rupp T, Langlois B, Radwanska A, Koczorowska MM, **Z Sun**, Hussenet T, Lefebvre O, Murdamoothoo D, Arnold A, Klein A, Biniossek ML, Hyenne V, Naudin E, Velazquez-Quesada I, Schilling O, Van Obberghen-Schilling E, Orend G, Cell Rep. 2016. 17(10):2607-2619

4. Employing a novel orthotopic syngeneic ErbB2-driven breast cancer model reveals Tenascin-C regulating tumor immunity

Zhen Sun, Inés Velazquez-Quesada, Devardarssen Murdamoothoo, Constance Ahowesso, Szuszana Herzgovic, Renate Kain, Patricia Simon-Assmann, Michaël van der Heyden, Christiane Arnold, Annick Klein, Anna Brown, Thomas Hussenet, Marie-Pierre Chenard, Olivier Lefebvre and Gertraud Orend*

In preparation

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Abbreviations

APC: adenomatous polyposis coli gene product

CAF: cancer associated fibroblasts

CAFs: cancer-associated fibroblasts

CSCs: cancer stem cells

CSF-1: colony-stimulating factor 1

CTCs: circulating tumor cells

CTLs: cytotoxic T cells

DCs: dendritic cells

DTCs: disseminated tumor cells

ECM: extracellular matrix

ECs: endothelial cells

EDNRA: endothelin receptor type A

EDNRB: endothelin type B receptor

EGF: epidermal growth factor

EMT: epithelial- to-mesenchymal transition

FAP: fibroblast activation protein

FGF: fibroblast growth factor

FSP-1: fibroblast-specific protein-1

GBM: glioblastoma

GSK3: glycogen synthase kinase 3

HGF: hepatocyte growth factor

HIFα: hypoxia-inducible factor 1-alpha

HUVECs: human umbilical vein endothelial cells

ICAM1: intercellular adhesion molecule 1

IFN: interferon

IL-6: interleukin-6

KD: knockdown

KO: knock out

LAP: latency associated peptide

LATS1: Large tumour suppressor homologue

LECs: lymphatic endothelial cells

LLC: large latent complex

LLC: Lewis lung carcinoma cells

LOX: lysyl oxidase

LPAR: lysophosphatidic acid receptor

LTBP: latent TGF-β binding protein

LVI: Lymphovascular invasion

MAPK: mitogen-activated protein kinase

MCP1: monocyte chemotactic protein

MDSCs: myeloid-derived suppressor cells

MKL1: megakaryoblastic leukemia 1

MLC: myosin light chain

MLCK: MLC kinase

MMPs: metalloproteinases

MSCs: mesenchymal stem cells

NK: natural killer cells

NKT: natural killer T cells

NMuMG: normal murine mammary gland epithelial cells

NSCLC: non-small cell lung cancer

PDGF: platelet-derived growth factor

PDGFR: platelet-derived-growth factor receptor

PDX: patient-derived xenograft

PECAM1: platelet endothelial cell adhesion molecule

PGs: proteoglycans

PLOD2: procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2

PVN: perivascular niche

PYK2: proline-rich tyrosine kinase 2

RCCs: renal cell carcinoma cells

SDF1A: stromal cell-derived factor-1α

SRF: serum response factor

TAMs: Tumor-associated macrophages

TCRs: T cell receptors

TEAD: TEA domain family members

TEM: transendothelial migration

TGF- β: transforming growth factor beta

Th: helper T cells

TME: tumor microenvironment

TNC: tenascin-C

TNFα: tumor necrosis factor alpha

TNX: tenascin-X

TRAIL: tumour necrosis factor (TNF)-related apoptosis-inducing ligand

Treg: T regulatory cells

VAP1: vascular adhesion protein 1

VCAM1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor

YAP: yes activating protein

αSMA: alpha-smooth muscle actin

1. Introduction

1.1 Tumor metastasis

Metastasis is the leading cause of death in cancer patients. During metastasis, disseminated tumor cells (DTCs) must overcome several key steps to colonize distant organs, which include invading surrounding tissue from the primary tumor, intravasation into the circulation, surviving from shear stress in the circulation, infiltrating distant tissue, evading immune defenses, adapting to supportive niches, surviving as latent tumor-initiating seeds and eventually extensively expanding thus replacing the host tissue (**Fig. 1**). Although these multiple events are highly inefficient altogether, disseminated tumor cells that have colonized to distant organs cannot be treated since current treatments fail to have long lasting responses (Massagué et al., 2016).

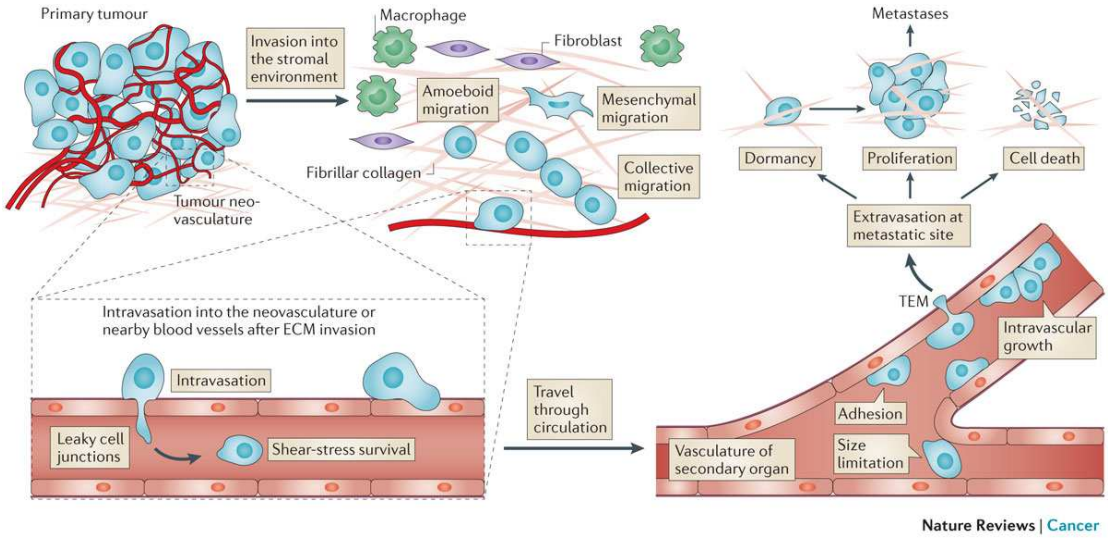

Figure 1. Metastatic dissemination (Reymond et al., 2013). During the initial phase of metastasis, a limited number of tumor cell invade the surrounding tissue from the primary tumor by using several types of migration. Some invading cells can migrate towards their neighbouring blood vessel and enter into the circulation either as a single cell or as cell clusters. This may occur through an intact endothelium or through leaky site of tumor vessels and is called “intravasation”. Surviving circulating tumor cells (CTCs) can then reach to different locations in the body which depends on the circulatory patterns and distinct capillary walls in different organs. After the trapping and adhesion to the

endothelial cells (ECs), CTCs leave the circulation which is called “extravasation” and colonize to the parenchymal tissue of the target organ. Most of the cancer cells that extravasate will undergo cell death. Some of the surviving cancer cells can then enter a state of dormancy or proliferate within this new microenvironment and prepare for a second wave of metastasis.

1.1.1 The inefficiency of metastatic colonization

Although millions of tumor cells can disseminate from the primary tumor, some patients who have recovered from cancer treatment never get a relapse or do so after a long disease free latency period (Massagué et al., 2016). The number of circulating tumor cells has been shown far to exceed the number of metastatic lesions (Nagrath et al., 2007). DTCs can be present in bone marrow of breast cancer patients for a long time and only half of these patients eventually develop metastasis (Braun et al., 2005) indicating that only a minority of DTCs can survive and form macrometastases altogether indicating that tumor cell dissemination, seeding and metastatic outgrowth is very inefficient. Consistent with clinical observations, some experimental mouse models also demonstrate this inefficient process. For example, the vast majority of melanoma cells that are injected in the portal vein fail to form micrometastases in the liver, and only 0.02% form macrometastases (Cameron et al., 2000). A large number of intravenously or arterially injected breast tumor cells die after lodging in the target organs like lungs, brain, liver or bone marrow (Minn et al., 2005). In addition, tumor stem cells which will trigger metastasis initiation often undergo apoptosis after reaching the lung (Malanchi et al., 2012). Both mouse model and clinic observations suggest that the DTCs’ survival and tumor initiating activities are important elements of metastasis which need to be comprehensively studied.

1.1.2 Tumor cell infiltration

The invasion and migration of cancer cells in tissues and the circulation are the early steps in the metastasis process. Cytoskeletal regulation within cancer cells (Hall et al., 2009), adhesive interactions between cells and secretion of ECM-degrading metalloproteinases (MMPs) (Kessenbrock et al., 2010) prompt the invasion and migration of the cancer cells through the surrounding stromal tissue. Studies on human tissues as well as intravital imaging using mouse models (Friedl et al., 2011; Clark et al., 2015) have revealed a great

diversity in DTCs migration which includes single cell migration, multicellular streaming and collective migration. (**Fig. 2**)

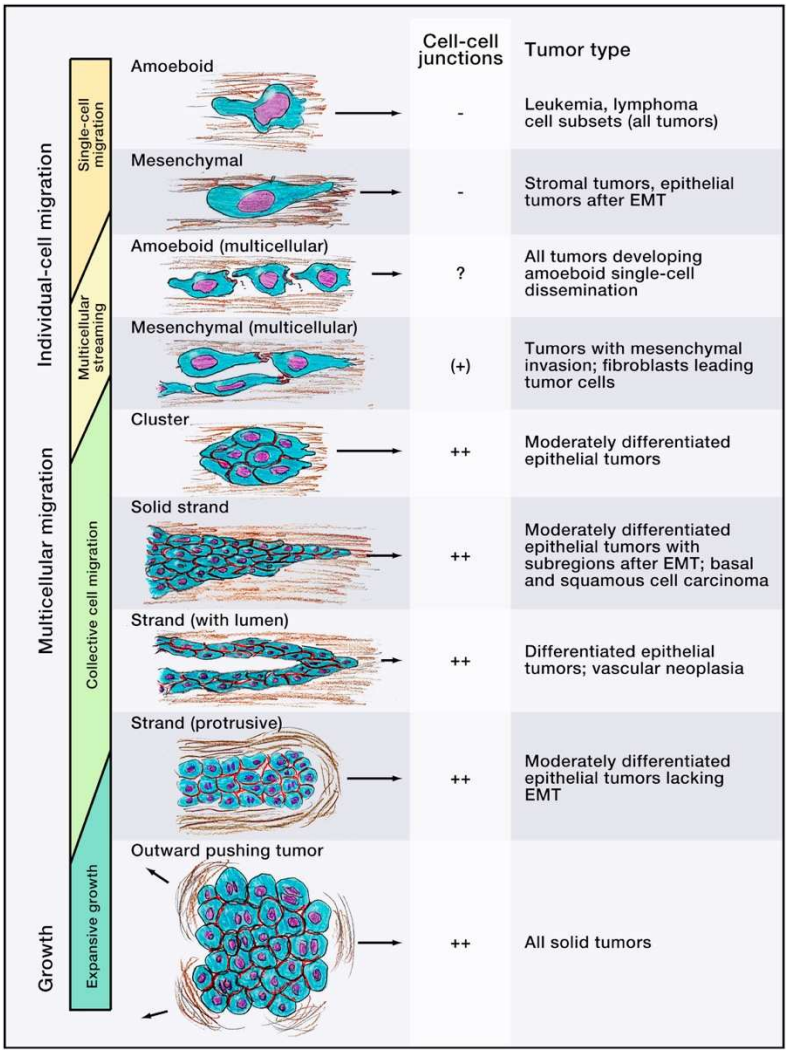

Figure 2. Modes of tumor cell migration (Friedl et al., 2011). The switch between these migration modes occurs upon alterations of cell-cell interactions, cell-ECM adhesion, or cytoskeletal contractility.

In single cell migration, cells lose their interaction with their neighbors and migrate individually. There are two main well characterized migration modes when cell migrate as single cells either in an amoeboid-like or mesenchymal-like manner. Amoeboid-like migration is characterized by a rounded cell shape, increased cell contractility, under control of the Rho/ROCK-pathway and ECM degradation independence. Cells squeeze

through gaps in the ECM fibers with a high velocity ranging from 2 – 25 μm/min where they adapt their shape to the pre-existing space (Paňková et al., 2010; Clark et al., 2015). The exact nature of the cell–ECM attachment mode in amoeboid migration is poorly understood and needs further investigation. The elongated cell shape, obvious leading edge with one or more leading pseudopods, traction forces generation by contractile stress fibers and a ECM degradation favor mesenchymal-like migration and drives cell migrate in 3D matrices with a lower velocity of approximately 0.1 - 0.5 μm/min (Paňková et al., 2010; Clark et al., 2015).

Multicellular streaming is characterized by loosely- or non- adherent cells that migrate one after another by using the same path which is often seen as a chain or swarm-like tissue invasion with a speed of 1 - 2 μm/min. Occurring in hematologic and solid tumors, the streaming cells have a longer and straighter path compared to single migratory cells (Friedl et al., 2011; Kedrin et al., 2008).

In collective migration, cell-cell adhesions are retained (Clark et al., 2015). Cells can move either as a solid strand with a leader cell at the tip that generates traction forces and pericellular proteolysis for invasion of the tissue structure (Gaggioli et al., 2007, Khalil et al., 2010). Alternatively, multicellular buds consisting of many cells that protrude collectively and thereby mechanically push towards the tissue stroma (Ewald et al., 2008). Collectively migrating cells can display mesenchymal or epithelial phenotypes, and the phenotypes may differ between ‘leader’ and ‘follower’ cells (Khalil et al., 2010; Cheung et al., 2013). Collective migration is typically the slowest mode of cancer cell migration (0.01 - 0.05 μm/min) (Clark et al., 2015).

Several factors regulating the mode of migration and cancer cell morphology have been identified by both in vitro and in vivo studies. For example, cancer cells can undergo epithelial- to-mesenchymal transition (EMT) in which cells lose their intercellular adhesion and gain a mesenchymal phenotype which can enhance their invasiveness. TGF-β, Wnt, Notch, HIF1α and TNF-α signaling are Important in this process (Kishor et al., 2016). The ECM properties can also influence the cancer call migration mode. For example, high density of collagen network or inhibition of ECM degradation can promote amoeboid migration. The protease-independent mechanism of amoeboid migration are only plausible when the collagen network is devoid of covalent cross-links (Sabeh et al., 2009). Interestingly, collagen properties can also have an impact on collective migration. A recent

study demonstrated that high collagen density can induce a switch from single-cell to collective migration which display mesenchymal and proteolytic-dependent phenotypes (Haeger et al., 2014).

Despite significant progress in unveiling the underlying molecular mechanisms and signaling pathways for the different migration modes, the switch between single-cell, streaming and collective migration remains largely unknown.

1.1.3 Intravasation

Before entering into the bloodstream, cancer cells can shed from primary tumors as single cells or in clusters. It has been demonstrated that polyclonal clusters of circulating tumor cells (CTCs) have an increased metastatic potential and correlate with worse overall survival in breast cancer patients compared with single CTCs (Acteo et al., 2014).

To intravasate, tumor cells need to pass through the tissues for reaching the blood vessel. The tumor vasculature is believed to have weak cell–cell endothelial junctions through which cancer cells can enter into the bloodstream (Weis et al., 2011). Factors that locally reduce endothelial barrier function, such as TGF-β or VEGF can increase the number of cancer cells entering into blood vessels and contribute to metastasis (Anderberg et al., 2013). Some tumor invasion related proteins such as the invasive isoform of the actin-regulatory protein mammalian enabled homologue (MENA; also known as ENAH), actin nucleation promoter neural Wiskott–Aldrich syndrome protein (NWASP) or membrane-type-4 matrix metalloproteinase (MT4MMP; also known as MMP17) are also involved in intravasation (Roussos et al., 2011; Gligorijevic et al., 2012; Chabottaux et al., 2009) suggesting that modulating cell shape has an impact on invasion. In the bloodstream, shear forces, the innate immune system and oxidative stress have a big impact on the tumor cell behavior. To protect them from the unfriendly environment, tumor cells can undergo reversible metabolic changes that increase their ability to tolerate oxidative stress (Le Gal et al., 2015). Tumor cells can also interact with platelets in the blood to undergo EMT in order to withstand shear forces (Labelle et al., 2011) amongst other haphazardous triggers.

1.1.4 Extravasation

The pattern of the blood circulation in different organs can influence the CTCs' lodging location. In most organs, the venous circulation enters into the right ventricle of the heart and then into the lungs. Besides, the portal venous circulation from the gut drains into the liver. These two main circulation patterns in our body contribute to the high incidence of metastasis in these organs (Denève et al., 2013). In addition, through the arteriovenous shunts, tumor cells can also reach to their target organs through the arterial circulation (Massagué et al., 2016). It is now largely accepted that the anatomic architecture of the blood flow is not sufficient to fully describe the patterns of metastatic tumor spread. The seed and soil hypothesis was also another important way to describe the tumor spread. Differential tumor cell adhesion to organ-derived microvessel endothelial cells and organ parenchymal cells, differential invasion of basement membranes and organ tissues, and differential responses to organ-derived growth-stimulatory and-inhibitory factors all appear to be important factors in explaining the organ preference of metastasis (Nicolson et al., 1988).

To extravasate, tumor cells need to attach to ECs first and then migrate towards the parenchyma of the target organ through the endothelial junction, a process called transendothelial migration (TEM). Cancer cells have been shown to slow down and then arrest in capillaries of a similar diameter to that of the cells indicating that they first become physically restricted and then form stable attachments (Kienast et al., 2010). A wide range of ligands and receptors contribute to the attachment process, including selectins, integrins, cadherins, CD44 and immunoglobulin (Ig) superfamily receptors (Reymond et al., 2013). CTCs that lodge in the microvasculature can extravasate by retracting the endothelial cells that line vascular walls or outgrow from the emcompassed endothelial lining within the lumen to form an embolus that eventually ruptures the vessel (Lapis et al., 1988).

As to single tumor cell TEM, two routes have been described: paracellular TEM, in which tumor cells can squeeze between the adjacent ECs by disrupting their cell junctions, and transcellular TEM defined as the migration of cancer cells directly through the EC body (Carman et al., 2008). In paracellular TEM, αvβ3 integrin on cancer cells is specifically implicated in TEM and has been suggested to interact with platelet endothelial cell adhesion molecule 1(PECAM1), which localizes to EC junctions (Bauer et al., 2007). Cancer cells can also secrete various factors to open EC junctions through signaling pathways such as VEGF (Hoeben et al., 2004), TGF-β1(Drabsch et al., 2011), 12(S)

hydroxyeicosa- tetraenoic acid (12(S)-HETE) (Uchide et al., 2007), CXCL12(Lee et al., 2004), Angiopoietin-like protein 4 (Padua et al., 2008) and others. The signaling in ECs during the junction opening induced by TEM is also well described. Cancer cells promote activation of RAC1, ROCK and/or p38 MAPK in ECs. These pathways increase myosin light chain (MLC) phosphorylation, stress fibre formation and actomyosin-mediated tension on EC junctions (Reymond et al., 2013). Cancer cells can also increase ERK-induced activation of the tyrosine kinase SRC, and/or they can activate the PI3K subunit p110α that functions upstream of the proline-rich tyrosine kinase 2 (PYK2); these pathways induce phosphorylation and disassembly of the vascular endothelial cadherin (VE-cadherin) - β-catenin complex and therefore induce EC junction opening (Reymond et al., 2013). Although not many, there is few evidence of transcellular TEM in vitro. In breast cancer, MLC kinase (MLCK) is involved in transcellular TEM, since blocking MLC phosphorylation prevents transcellular intravasation but not paracellular intravasation (Khuon et al., 2010). Further studies are needed to prove whether this route is used by other cancer cells and whether it exists *in vivo.*

Besides the interaction between tumor cells and EC, the interplay between cancer cells and their microenvironment in the bloodstream involving stromal cells and platelets also plays a crucial role in extravasation. (See more details in 1.2.). After extravasation, DTCs enter into the parenchyma of target organs to initiate colonization. Colonizing cancer cells must develop resistance to immunity and other host-tissue defenses in order to survive. Settlement in supportive niches enables them to survive and retain their stem-like tumor-initiating capacity. This metastatic niche will be introduced in details below (1.2).

1.1.5 Latent and overt metastasis

After the settlement in the target organ, cancer cells can enter a latent state as single cells or as micrometastases. During the latent state, disseminated cells must achieve long-term survival, which can last from months to decades. This long tumor latent state is also nominated as tumor dormancy (Goss et al., 2010). Clinically, detection of DTCs in bone marrow of a patient with no overt manifestation of metastasis after successful treatment indicated that a fraction of DTCs survive therapy and remain within the tissu despite no evidence of disease (Uhr et al., 2011). However, persisting DTCs may be of bad prognosis for the patinet as DTCs could be the origin of future metastases after a long latency period (Ghajar et al., 2016). Biologically, tumor dormancy encompasses two distinct states:

population-level dormancy and cellular dormancy (Goss et al., 2010). In cellular dormancy, isolated DTCs enter a state of proliferative quiescence in which cells exit the cell cycle and stay in a G0 state. In population-level dormancy, micrometastases stop to grow due to blood supply limitations and/or immune surveillance. Like the “angiogenic switch”, the “dormancy switch” could be the first obstacle that must be overcome for quiescent DTCs to grow out to from metastasis. Dormant DTCs are found in close association with vascular basement membranes, which is usually described as a dormancy induction perivascular niche (PVN) (Ghajar et al., 2013). However, the PVN that is rich in type I collagen (Barkan et al., 2010) and fibronectin (Aguirre-Ghiso et al., 2001) also showed an inhibitory effect in DTCs’ dormancy. Therefore, understanding tissue-specific perivascular heterogeneity between different tissues will provide a good opportunity to determine whether there are general properties of the PVN that can be exploited to maintain tumor dormancy and to eradicate dormant tumor cells, or whether a more precise approach has to be taken to target specific organ PVNs (Ghajar et al., 2013).

When the cancer cells leave their dormant state, proliferation within the organ parenchyma completes the metastatic process (Reymond et al., 2013). To continue growing, the micrometastasis overtake the local tissue microenvironment by developing a vascular network and evading the destruction by host defenses. The cells can then invade blood vessels, enter the circulation and produce additional metastases as so called secondary metastasis (Talmadge et al., 2010).

1.2 The tumor microenvironment

The tumor microenvironment (TME) comprises a dynamic network of extracellular matrix (ECM) proteins and a host of associated cells including fibroblasts, bone marrow-derived cells, endothelial cells, and infiltrating immune cells (Balkwill et al., 2012) as well as soluble factors (**Figure 3**, Gattazzo et al., 2014). The interplay between tumor cells and their TME has a great impact on tumor initiation, progression and patient prognosis (Joyce et al., 2009). To understand how the tumor cell drives the construction of its own niche as well as how this niche influences the metastasis is important to understand better for developing therapeutic strategies to eradicate metastasis and prevent tumor resurrection.

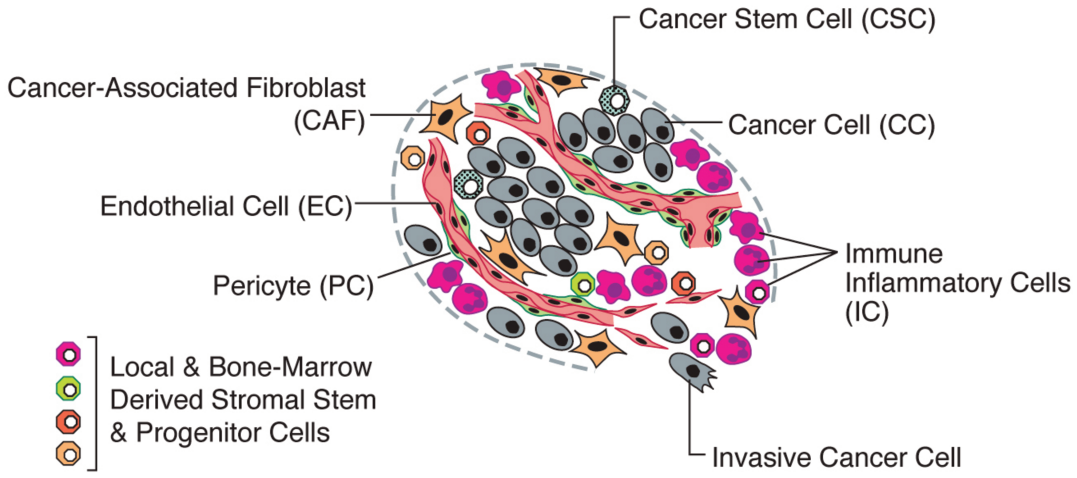

Figure 3 Cellular composition of the tumor microenvironment according to (Hanahan and Weinberg, 2011). A solid tumor is constituted of distinct cell types that contribute to tumor growth and progression. Notably, inflammatory immune cells present in tumors can have both tumor-promoting as well as tumor-killing properties.

1.2.1 Clinical evidence of TME and tumor progression

Chronic inflammation generally correlates with a high incidence of cancer and may be considered as evidence for an important role of the tumor microenvironment in cancer progression. In a large study of 19,486 patients who were diagnosed with inflammatory bowel disease, 2 841 of which exhibited long-standing extensive colitis, the increased risk of colorectal cancer in these two groups was 2.2- and 7.0-fold to the patients without inflammatory bowel disease, respectively (Beaugerie et al., 2013). In a retrospective cohort study of 417 cancer-free patients diagnosed with cirrhosis, 27% developed liver cancer in a median 148 months' surveillance (Sangiovanni et al., 2004). Furthermore, one meta-analysis reported that in 16.1% of cancers, tumor initiation can be directly attributed to different infection agents, including viruses, bacteria and parasites, in which a chronic inflammatory response is usually ongoing (de Martel, C et al., 2012). These unsolved inflammatory responses may accumulate and activate different stromal cells and modulate the local microenvironment from hemostasis to a pro-tumorigenic niche (Barcellos-Hoff et al., 2013).

On the contrary, it is also important to note that a dysfunctional immune system can also correlate with high incidence of cancer. In an analysis of 25,914 female immunosuppressed organ transplant recipients, the incidence of cancer was significantly higher than predicted, including lung, gastrointestinal, reproductive and skin cancers (Stewart et al., 1995). In contrast, breast cancer incidence decreased in this cohort, showing the paradoxical trait of immune responses. Furthermore, a cohort of 122,993 individuals with AIDS revealed increased incidence of not only AIDS-related cancers (for example, Kaposi's sarcoma) but also non–AIDS-related cancers including rectum, rectosigmoid, anus, trachea, bronchus, lung, brain and central nervous system (Gallagher et al., 2001). A similar retrospective analysis (Vajdic et al., 2009) has indicated that adequate immune functions may be anti-tumorigenic, opposite with evidence that supported pro-tumorigenic functions for inflammation (Grivennikov et al., 2010). Because of this complexity and paradox, therapeutic opportunities for tumor progression lie in pliancy of the tumor stroma, which is context and function dependent.

1.2.2 TME in primary tumor growth

Immune cells

Tumor-associated macrophages (TAMs) and T cells are the most frequently found immune cells within the tumor microenvironment which does not exclude that other immune cells of the innate and adaptive immune system also play a role.

TAMs can support tumor growth, invasion and therapeutic resistance in multiple cancer types by secreting proteases like cysteine cathepsins (Shree et al., 2011). TAMs can also facilitate the invasion and intravasation of tumor cells through a paracrine signaling loop that involves tumor-derived colony-stimulating factor 1 (CSF-1) and macrophage-derived epidermal growth factor (EGF) (Condeelis et al., 2006).

T cells are other important immune cells involved in tumorigenesis. There are two major groups of matured T cells based on the T cell receptors (TCRs) they express: γδ and αβ. According to their effector functions αβT cells are further classified as CD8+ cytotoxic T cells (CTLs) and CD4+ helper T (Th) cells, which include Th1, Th2, Th17, T regulatory (Treg) cells, as well as natural killer T (NKT) cells (Grivennikov et al., 2010). Importantly, T cells can exert both tumor-suppressive and tumor-promoting effects depending on their

effector functions and context (DeNardo et al., 2009l; Wang et al., 2016). Increased T cell numbers, specifically activated CTLs and Th1 cells, correlate with better survival in over 20 different cancer types (Fridman et al., 2012). Correspondingly, mice lacking T cell cytotoxic effector pathways have also been shown to be more susceptible to spontaneous chemical carcinogenesis (Swann et al., 2007). However, there is also evidence that many of the T cell subsets found in solid tumors are involved in tumor progression and metastasis, including IFNγ-producing Th1 cells, Th2 cells and Th17 cells (Grivennikov et al., 2010).

The capacity of the immune system to control and shape cancer is the result of three processes which can occur individually or in sequence: (i) elimination - linked to cancer immunosurveillance, in which immunity functions as an extrinsic tumor suppressor in a naive host; (ii) equilibrium – where expansion of transformed cells is held in check by immunity; and (iii) escape – where tumor cell variants with dampened immunogenicity or the capacity to attenuate immune responses grow into clinically apparent cancers (Koebe et al., 2007). Evidence for tumor immunosurveillance come from the presence of tumor infiltrating T and B lymphocytes that recognize tumor antigens and the observation that for tumor patients with increased infiltration of activated T cells have a better prognosis (Dunn et al., 2004). Such infiltration is even more noticeable in tumors that have a mutated phenotype or have instable microsatellites in which tumor antigens exhibit greater differences from normal tissue or their counterparts (Buckowitz et al., 2005).

In most tumors, the presence of tumor-infiltrating lymphocytes is insufficient for eradicating tumor cells. Cancer cells constantly edit and modulate the host antitumor immune response and the host immune response shapes tumor immunogenicity and clonal selection. During this process the balance between antitumor and tumor-promoting immunity can be in favor of tumor regression (Grivennikov et al., 2010). Before a tumor undergoes immune escape, an equilibrium phase happens between tumor growth and immune destruction. The equilibrium process was first observed in clinical cases of organ transplantation with undetected cancer within the transplanted organ. In the recipient outgrowth of the cancer was observed where the immunesuppressed condition to favor acceptance of the transplanted organ may have promited tumor outgrowth (MacKie et al., 2003). It has also been shown that tumor cells in equilibrium are unedited but become edited when they spontaneously escape immune control and tilt the balance in favor of tumor growth (Koebe et al., 2007). The aspect of immune editing is described below.

In the escape process, cancer cells can edit expression of tumor antigens toward lower immunogenicity and can also remodel the tumor microenvironment to become immunosuppressive. The suppressive activity of myeloid-derived suppressor cells (MDSCs) is one of the most prevalent mechanisms in immune evasion in patients. MDSCs can infiltrate into the developing tumors where they function as a promoter of tumor vascularization (Talmadge et al., 2013) and as inhibitor of immunosurveillance, including blocking antigen presentation by dendritic cells (DCs) (Gabrilovich et al., 2012), T cell activation (Mazzoni et al., 2002), M1 macrophage polarization (Sinha et al., 2005) and NK cell cytotoxicity (Liu et al., 2007). Regulatory T (Treg) cells are another TME cell type that has various immune suppresive capacities. Treg can suppress tumor-associated antigen presentation and can also interfere with cytotoxic T cell function by inhibiting cytolytic granule release (von Boehmer et al., 2013). Tumor-associated Treg accumulate by multiple mechanisms such as peripheral recruitment, proliferation of cells in the TME or differentiation of progenitors in response to tumor-secreted factors which determines their heterogeneity (Blatner et al., 2012).

Other immune cells also affect tumorigenesis. B lymphocytes and mast cells are important contributors to immune-mediated tumor growth (Ammirante et al., 2010). Neutrophils can play both tumor-promoting and suppressing functions, depending on their differentiation status and the presence of TGF-β (Fridlender et al., 2009). Conventional macrophages and dendritic cells are important for antigen presentation and T cell activation during immunosurveillance as well as for cytokine production during immunosuppression in established tumors (Grivennikov et al., 2010). The distinct functions of different immune cell populations in the tumor microenvironment during tumorigenesis renders immune based therapy challenging and unpredictable.

Endothelial cells and pericytes

Angiogenesis is now accepted as a hallmark of cancer (Hanahan et al., 2011). Oxygen and nutrients from the bloodstream can be transported to support the tumor progression through this process. But blood vessels also play a role in metastasis and in regulating dormancy of metastasizes cells. Tumor vascularization requires the co-operation of multiple TME cell types, including vascular endothelial cells (which form tight adhesions to ensure vessel integrity), pericytes (which provide vessel coverage and indicate vessel

maturity) and BM-derived precursor cells, whose differentiation is often regulated by hypoxia (Hanahan et al., 2011). In addition to the cell types comprising the normal vessels, accessory cells, including TAMs, cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs) also contribute to tumor angiogenesis by releasing a plethora of pro-angiogenic signals into the TME (Hanahan et al., 2011). Lymphangiogenesis is another type of vascularization in tumors, and lymphatic vessels represent an alternate route for cancer cell dissemination (Alitalo et al., 2012). High levels of VEGF-C and VEGF-D produced by activated macrophages correlate with increased peritumoral lymphangiogenesis in human cervical cancer (Schoppmann et al., 2002). Moreover, myeloid cell populations can have a critical impact on lymphatic endothelial cells (LECs) by not only modulating their signaling properties but also their transdifferentiation into functional LEC-like cells themselves (Zumsteg et al., 2009).

Cancer associated fibroblasts (CAF)

Fibroblasts are the most common cells in connective tissue with multiple functions. They can synthesize extracellular matrix (ECM) and basement membrane components, regulate differentiation events in associated epithelial cells, modulate immune responses and mediate homeostasis (Kalluri et al., 2006). In the TME, cancer-associated fibroblasts (CAFs) are highly present and are distinct from normal fibroblasts. For instance, when co-injected with CAFs, normal prostate epithelial cells give rise to intraepithelial neoplasia in mice but not when co-injected with normal fibroblasts (Olumi et al., 1999). In breast cancer, CAFs induce a mesenchymal-like phenotype and promote metastasis of both premalignant and malignant mammary epithelial cells, whereas normal fibroblasts confer an epithelial-like phenotype and suppress metastasis (Dumont et al., 2013). These effects indicate that CAFs need to be considered as an entirely different cell type than normal fibroblasts, one with potent promoting functions on tumorigenesis.

It is unclear where CAFs arise from during tumor progression (Marsh et al., 2013). Some studies have suggested that they are developed from an endothelial-to-mesenchymal transition. Lineage-tracing experiments in mouse melanomas and pancreatic neuroendocrine tumors showed that CAFs in these tumors were of endothelial cell origin (Zeisberg et al., 2007). EMT and bone marrow recruitment are also reported as possible mechanisms for the generation of CAFs. In prostate cancer and breast cancer, tumor cells

of epithelial origin can undergo an EMT process to generate a mesenchymal-like cell population that expresses CAF markers (Petersen et al., 2003; Orr, B et al., 2012). The heterogeneity within CAF populations requires reliable markers. Native fibroblasts stain for vimentin and, once activated, stain positive for alpha-smooth muscle actin (αSMA). Other CAF markers include fibroblast activation protein (FAP), fibroblast-specific protein-1 (FSP-1), stromal cell-derived factor-1α (SDF1A), and paladin (von Ahrens et al., 2017).

Growth factors and cytokines that are present in the surrounding environment can trigger CAF accumulation in the TME. TGF-β, monocyte chemotactic protein (MCP1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and secreted proteases have all been involved in CAF activation (Kalluri et al., 2006). Moreover, induction of the YAP transcription factor was required for the ability of CAFs to remodel the ECM by stiffening matrix to support tumorigenesis and tumor invasion. Matrix stiffening further enhances YAP activation, thus establishing a feed-forward self-reinforcing loop that helps to maintain the CAF phenotype (Calvo et al., 2013). After activation, CAFs can act as a reservoir of chemokines, growth factors and pro-inflammatory factors that support tumorigenesis and tumor metastasis.

Other cell types

There are several other stromal cells that are emerging with a potential role in cancer progression. For instance, adipocytes and their progenitors, contribute to tumor growth in ovarian cancer and its metastasis to omentum, an organ primarily composed of adipocytes (Nieman et al., 2011). Moreover, stromal progenitor cells from endogenous adipose tissue can differentiate into pericytes that provide a supportive tumor microenvironment for tumor growth (Zhang et al., 2012), demonstrating the plasticity of stromal cells in the TME. The autonomic nerves may also influence cancer progression, given the association between perineural invasion and neurogenesis in several tumor types, including colorectal and prostate cancers (Liebig et al., 2009; Magnon et al., 2013).

Given the heterogenous composition and emergence of non-canonical stromal cell types in cancer, more comprehensive and tailored combination therapies targeting stromal cells may have a better benefit in managing primary tumor growth and progression into metastasis.

1.2.3 The tumor microenvironment in the circulation

Once tumor cells evade the host immune defenses and intravasate into the circulation, the interplay between cancer cells and the microenvironment in the blood is underway. Most of these interactions enhance survival of tumor cells and in consequence increase extravasation by protecting tumor cells form immune surveillance. This can involve resistance to shear stress within the circulation and attachment of cancer cells to the blood vessel wall as a first step toward extravasation.

Platelets

Platelets have an important role in the microenvironment of the bloodstream, where they aggregate around cancer cells almost immediately after they enter the bloodstream (Labelle et al., 2011). Cancer cells increase coagulation through expression of thrombin and release of microparticles, and inhibition of coagulation greatly limits cancer metastasis in many experimental models (Gil-Bernabe et al., 2013). In addition, platelet inhibition and depletion has been shown to reduce tumor metastasis in vivo (Camerer et al., 2004). By enhancing fibrin deposition, platelets can protect cancer cells from shear stress and impede immune cell surveillance such as NK cell - mediated cytotoxicity (Palumbo et al., 2005). Platelets can also release a series of active molecules like TGF-β and ATP which promote tumor cell extravasation and metastatic seeding (Schumacher et al., 2013; Labelle et al., 2011). In the meantime, by increasing adhesion of cancer cells to ECs, by expressing P-selectins, platelets can further increase tumor cell extravasation and metastasis in the target organ (Coupland et al., 2012).

Leukocytes

Leukocytes can also increase the number of cancer cells binding to the endothelium and facilitating their transmigration. Chemokines and cytokines produced by cancer cells can attract and activate leukocytes and, platelets aggregated around cancer cells can attract leukocytes thus enforcing the impact of platelets and leukocytes on cancer cells (Dole et al., 2005). L-selectin expressed on leukocytes contributes to cancer cell survival in the lung, but whether it specifically affects platelet aggregation or initial tumor cell embolization

and extravasation is unknown (Laubli et al., 2006). In vitro, neutrophils can potentiate melanoma cell adhesion to the endothelium and promote transendothelial migration (TEM) through the interaction of αLβ2 and αMβ2 integrins that are expressed on neutrophils, with intercellular adhesion molecule 1 (ICAM1) that is expressed on both ECs and melanoma cells (Li et al., 2012; Wu et al., 2001). In vivo, the interaction between cancer cell expressed ICAM1 and β2 integrin on neutrophils increases melanoma cell anchoring to the endothelium (Huh et al., 2010). Leukocytes can also secrete inflammatory cytokines that contribute to cancer cell extravasation, for instance, neutrophils can facilitate melanoma cell arrest on the EC and undergo TEM involving endogenously produced interleukin-8 (IL-8) (Dong et al., 2005).

Monocytes

Besides neutrophils, monocytes and macrophages also contribute to cancer cell extravasation. Cancer cell derived cytokines CCL2 and VEGF can induce the recruitment of monocytes and opening ECs junction, which promote breast tumor cell extravasation and metastasis outgrowth (Qian et al., 2009). Moreover, adhesion of cancer cells can induce EC activation, which leads to the recruitment of myeloid cells that then promote the metastatic process (Qian et al., 2010). As an example, platelets that are attached to cancer cells on ECs in the mouse lung induce expression of endothelial cell surface molecules — such as vascular cell adhesion molecule 1 (VCAM1) and vascular adhesion protein 1 (VAP1) — that promote attachment of monocytes or macrophages to ECs and promote cancer cell survival during the first step of the extravasation process (Ferjancic et al., 2013).

Together, these findings suggest that disruption of adhesion signaling between stromal and tumor cells may be a new approach to targeting circulating tumor cells. For that more detailed information about these interactions is needed.

Most of the previous studies are based on the behavior of single tumor cells across the vessel wall. However, it has been reviewed that the aggregated tumor cells in the circulation indeed have a better resistance to the immune system and can increase the number of cells that arrest due to blood vessel size restriction (Reymond et al., 2013). Moreover, these polyclonal clusters of circulating tumor cells have an increased metastatic potential in comparison to single CTCs which correlates with worse overall survival of

breast cancer patients (Aceto et al., 2014). However, due to limited in vivo models, the composition and function of the microenvironment in these tumor cell clusters remains largely unknown. Hence, one aim of my thesis was to characterize the specific microenvironment in CTC clusters.

1.2.4 Metastatic niches

In the primary tumor, the microenvironment can suppress immune surveillance through immune editing by tumor cells. However, once the disseminated tumor cells reach the distant organ, a new and unfriendly niche is encountered which causes death of DTCs (Chambers et al., 2002). In this new microenvironment, immune surveillance again can act as gatekeeper where cytotoxic T cells and natural killer (NK) cells are involved (Eyles et al., 2010). For example, in mice with deficient CD8+T and NK cell responses, metastasis was accelerated (Bidwell et al., 2012). Targeting the negative regulator such as tyrosine kinase receptors Tyro3, Axl and Mer on NK cells suppressed metastasis (Paolino et al., 2014). Neutralization of pro-apoptotic NK-cell-derived tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) increased hepatic metastasis in mice (Takeda et al., 2001). Furthermore, by using immune checkpoint inhibitors, which represent an emerging immune therapeutic approach durable long-lasting responses in metastatic menaloma and other tumor types have been observed (Postow et al., 2015; Sharma et al., 2015). Thus, immunity in the stroma is a major defence against metastasis initiation and it is worth and urgent to better understand tumor immunity for developing long lasting therapies against cancer metastasis.

For many cancers, cancer stem cells (CSCs) represent a distinct population that can be prospectively separated from the remainder of tumor cells. It was shown that CSC have clonal long-term repopulation and self-renewal capacity (Kreso et al., 2014). When CSCs spread to distant sites, the native stem-cell niches of the host tissue may facilitate their survival and help to keep their tumor initiating potential (Oskarsson et al., 2014). For example, human prostate cancer cells target the hematopoietic stem cell niche to establish footholds in the mouse bone marrow during metastasis (Shiozawa et al., 2011). Breast tumours that are rich in a CXCL12-secreting mesenchymal stroma are primed for metastasis in the CXCL12-rich microenvironment of the bone marrow (Zhang et al., 2013).

Besides the native stem cell niche, perivascular niches can support DTCs that disperse over the vessel basement membrane after extravasation. In a real-time imaging *in vivo* brain metastasis model, the extravasated cancer cells including breast-cancer, lung-cancer and melanoma cells persistently contacted the microvessels and induced perivascular growth (Kienast et al., 2010). It has also been shown that extravasated tumor cells can spread to capillaries and proliferate to form a sheath that eventually remodels the co-opted capillary network through expression of the cell-adhesion molecule L1CAM in the metastatic cells (Valiente et al., 2014).

DTCs can set up an ad hoc niche by producing components of stem-cell niches (Massagué et al., 2016). The extracellular matrix (ECM) protein tenascin-C (TNC) is produced by metastatic breast cancer cells and can act as a metastatic niche component to amplify Notch and Wnt signaling thus supporting metastasis outgrowth (Oskarsson et al., 2011). Breast-cancer stem cells can also induce stromal periostin expression which maintains cancer cell stemness properties by recruiting Wnt factors thus promoting metastasis (Malanchi et al., 2012). The collagen-crosslinking enzymes lysyl oxidase (LOX) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) secreted by cancer cells can modify the collagen organization in the target organs, stiffen the extracellular matrix and thus promoting metastatic growth (Massagué et al., 2016). Versican, a large chondroitin sulphate proteoglycan, which is identified in the conditioned medium from Lewis lung carcinoma (LLC) cells can activate macrophages to secrete interleukin-6 (IL-6) and tumour necrosis factor (TNF-α) to establish a pro-inflammatory microenvironment that drives metastatic growth (Kim et al., 2009). Furthermore, in the glioma perivascular niche, through activation of CD44 signaling, osteopontin promotes stem cell-like properties and radiation resistance in cancer cells (Pietras et al., 2014). A novel-screening platform capable of measuring tumor cell's responses to ECM has further uncovered ECM's promoting effects on metastasis (Reticker-Flynn et al., 2012). Taken together, these studies point at the importance of DTCs in ECM remodeling as a mechanism to enhance metastasis. Thus targeting the prometatastatic ECM niche could be a therapeutic approach. Yet, little is known about the characteristics of this niche which requires intensified future research.

1.3 Extracellular matrix (ECM)

The ECM is a complex three-dimensional meshwork of highly cross-linked fibrous proteins. There are two types of ECM networks based on their location and composition: the interstitial matrix, which provides structural scaffolding for tissues and regulates tissue tensile strength; and the basement membrane, which separates the epithelium from the adjacent stroma. In mammals, the core matrisome (**Hynes et al., 2012**) is composed of around 300 proteins such as collagen, proteoglycans (PGs) and glycoproteins. Collagens are the main structural proteins of the ECM and can provide tensile strength to the ECM. PGs, such as aggrecan, versican, perlecan and decorin, are interspersed among the collagen fibrils. They can not only provide structural strength but also can confer space-filling and lubrication of the ECM. Glycoproteins, such as laminins, elastin, fibronectins, thrombospondins, tenascins and nidogen, have a varity of functions (Hynes et al., 2012). Rather than their role in ECM assembly, they can not only function as ligands of cell surface receptors but also act as a reservoir of growth factors that can be released. In addition, cleavage of glycoproteins can form different fragments which may have distinct functions that could be different to that of the full-length protein. The complete matrisome further comprises ECM-affiliated proteins. These ECM binding proteins are growth factors, cytokines, mucins, secreted C-type lectins, galectins, semaphorins, plexins and ECM-modifying enzymes that are involved in crosslinking (Bonnans et al., 2014). The composition, abundance and structure are different in pathological and healthy conditions. In tumor progression, ECM has multiple functions such as mediating tissue stiffness, promoting angiogenesis, regulating cancer migration and releasing growth factors (Bonnans et al., 2014). The role of ECM in the tumor microenvironment is schematically depicted in **Figure 4** (**Lu et al., 2012**).

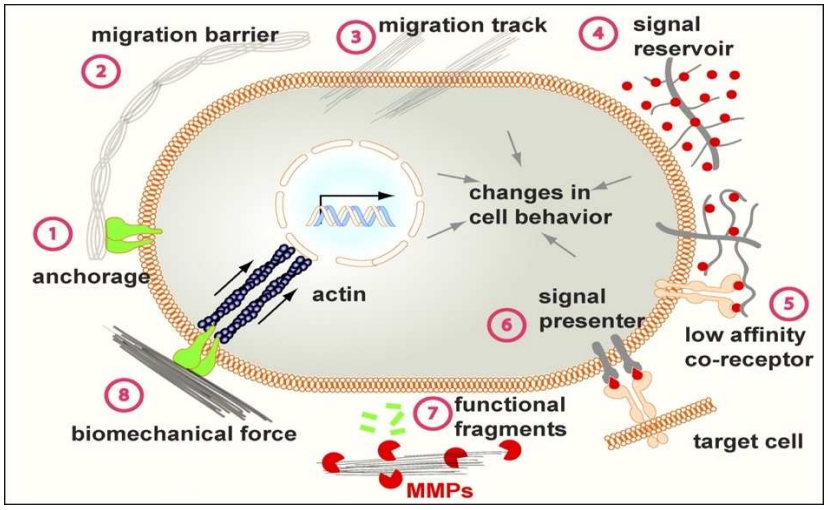

Figure 4. Role of ECM in the tumor microenvironment Cells can anchor to the ECM (e.g. the basement membrane) through cell surface receptors (1). For migrating cells the ECM can function as a barrier (2) or facilitate migration by serving as migration track (3). ECM components have been demonstrated to bind growth factors therefore acting as a reservoir for growth factors (4) or enabling the establishment of a growth factor gradient. In addition, the ECM can function as co-receptor for growth factor binding (5) or in presenting growth factors (6). Intrinsic domains of the ECM can bind itself to cell surface receptors (7). Cells sense the biomechanical properties, including stiffness, of the surrounding ECM and react by changing actin stress fibre contractility that results in altered target gene expression (8) (Lu et al., 2012).

1.3.1 Tenascin-C (TNC)

TNC is a secreted glycoprotein of the ECM, which consists of several domains with distinct functions. The assembly domain followed by the heptad repeats at the N-terminus mediates the trimerization of the molecule. A hexamaric structure is finally formed by 2 trimers during secretion. Followed by the assembly domain, from N- to C- terminus, there are epidermal growth factor (EGF)-like repeats, Fibronectin type III repeats (FNIII) and a fibrinogen-like globe (FG). In human TNC, FNIII 1–8 are conserved, but the 9 additional FNIII repeats between FNIII 5 and FNIII 6 are alternatively spliced in or out, thus potentially providing up to 511 different isoforms (Midwood et al., 2016; Van Obberghen-Schilling et al., 2011; Orend et al., 2006) (**Figure 5**).

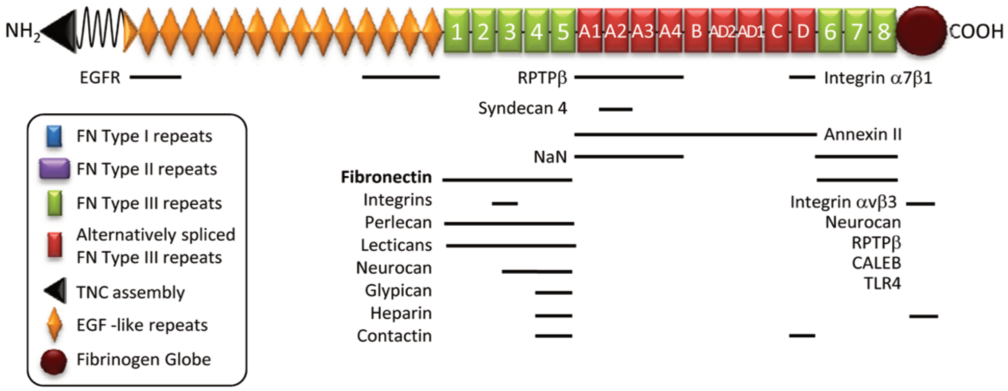

Figure 5. Domain structure of tenascin-C and potential binding partners Tenascin-C is a modular molecule composed of an oligomerization domain, EGF-L and FN type III (constant and alternate) repeats, and a Fbrinogen like domain. Binding sites for interacting molecules are indicated **(**Van Obberghen-Schilling et al., 2011; Orend et al., 2006**).**

1.3.2 TNC expression

TNC is highly expressed during development, however, TNC is generally repressed in adult tissues except in a few connective tissues that provide tensile strength (Flück et al., 2000) and certain stem cell niches including the bulge of hair follicles, crypts of the intestine and bone marrow (Chiquet-Ehrismann et al., 2014). Under some pathological conditions such as wound healing, inflammation and cancer, TNC is re-expressed. In the wound healing process TNC is transiently expressed during ECM remodeling at wound closure. This is different to rheumatoid arthritis and cancer, where TNC expression is deregulated leading to sustained and high expression. Hence high TNC levels correlate with poor survival in multiple cancer types such as breast cancer, lung cancer, head and neck cancer, melanoma, colorectal cancer and gliomas (Lowy et al., 2015).

TNC expression can be triggered by hypoxia, mechanical strain, and cytokines such as TGF-β, EGF, bFGF, PDGF-BB, CTGF and TNF-α (Orend et al., 2006). In addition, signaling through Ras/MAPK, Notch, Wnt/β-catenin, ROS/NFκB, ERK1/2, Rho/ROCK, PI3K/Akt and TLR4/NFκB induce TNC expression. Several validated transcription factors can bind to the TNC promoter and induce TNC expression such as Brn2, c-Jun, NFkB, Prx1 Smad3/4, Sp1, Ets1,2 CBP/p300, Sox4, RBPJk, MKL1/MAL/MRTF1, MEF2c with scleraxis and EWS-ETS (Van Obberghen-Schilling et al., 2011**;** Asparuhova et al., 2011). Furthermore, the microRNA-335 (miR-335) has been shown to be an important negative regulator of TNC expression. This suppression is often lost in cancer cells due to low levels of miR-335 (Tavazoie et al.,2008).

1.3.3 TNC isoforms

TNC isoforms are derived from alternative splicing from the Fibronectin type III repeats. Different TNC isoforms have distinct functions and are highly context dependent. However, the regulation of the TNC isoforms remains poorly understood (reviewed in Midwood et al., 2016).

Sam68 and SRSF6 are reported as regulator of alternative TNC splicing, their overexpression gives rise to a larger TNC splice form (Moritz et al., 2008; Jensen et al.,

2014). The cellular microenvironment plays important role in TNC alternative splicing. Mitogenic stimulation is reported to induce modification of TNC mRNAs (Borsi et al., 1994). Only small variation pH of the culture medium (ranging from pH 7.2-6.9) can remarkably modify the alternative splicing of TNC (Borsi et al., 1995). Cytokines like interferon gamma (IFN-α) slightly increase the proportion of large TNC isoforms, whereas, tumor necrosis factor alpha (TNF-α) or TGF-β favoured expression of smaller tenascin-C transcripts (Latijnhouwers et al., 2000; Zhao et al., 1995). Despite this scatchy knowedge, what effects the different TNC splice forms have in tumor initiation, progression, and metastasis formation is largely unknown and requires future investigations.

1.3.4 Cellular sources of TNC in tumors

Both stromal and tumor cells can express TNC in different tumor stages. In the stroma, activated fibroblasts are a main source of TNC. This was shown by injecting t4T1 mammary tumor cells in TNC wildtype and TNC knock out (KO) mice. It was found that TNC produced by S100A4+ stromal cells are important for metastatic colonization (O'Connell et al., 2011). It was also reported long time ago that the conditioned medium from MCF-7 cancer cells can induce TNC expression resting fibroblasts, yet the molecular nature remained obscure (Chiquet-Ehrismann et al., 1989). Angiogenic blood vessels are another stromal source of TNC. Whereas resting blood vessels do not express TNC, TNC is highly induced during angiogenesis in tumors (Seaman et al., 2007; Langlois et al., 2014). In addition, human umbilical vein endothelial cells (HUVECs) can express TNC upon incubation with conditioned medium derived from renal cell carcinoma (RCC) cells (Galler et al., 2012). In gliomas TNC staining overlaps with staining for desmin-positive cells, indicating that pericytes could be one source of TNC in these tumors (Martina et al., 2010). Besides stromal TNC, tumor cell derived TNC also plays an important in tumor progression. By using immunohistochemical analysis and in situ hybridization, TNC was found to be expressed by a variety of cancer cells as e.g. in breast cancer (Yoshida et al., 1997), colon cancer (Hanamura et al., 1997), oral squamous cell carcinoma (Hindermann et al., 1999) and melanoma (Herlyn et al., 1991). In a PDX mouse model of human breast cancer, TNC deficiency in the tumor cells significantly impaired metastasis initiation and outgrowth in the lung. In particular, when tumor nests in the lung reached a critical size smooth muscle actin (α-SMA) expressing myofibroblasts become the main source of TNC

(Oskarsson et al., 2011). This observation revealed distinct properties of stromal cell and cancer cell derived TNC. However, what is the relative contribution of these two sources of TNC in tumor initiation, progression, invasion and survival in the circulation was unknown.

1.3.5 Multiple and divers effects of TNC on cell behavior

Adhesion

Regulation of the cell adhesion is the earliest and best descripted characteristics of TNC (Chiquet-Ehrismann et al.,1986). TNC can block fibronectin mediated cell adhesion through competition with sydecan-4, a transmembrane heparan sulfate proteoglycan that operated together with integrin α5β1 (Huang et al., 2001). FAK and RhoA signalling are hence blocked in the presence of TNC, inhibiting polymerisation of actin and stress fibre formation (Ruiz et al., 2004). Different TNC splice forms have different abilities to bind to fibronectin and display different roles in mediating the anti-adhesive effect of TNC (Chiquet-Ehrismann et al., 1991). For example, smaller TNC isoforms bind more to annexin-II and are more than the larger TNC isoforms (Giblin et al., 2015). The anti-adhesive effect of TNC can even be enhanced by additional stimuli such as activation of endothelin receptor type A (EDNRA) signaling. On the contrary, activation of signaling by lysophosphatidic acid receptor (LPAR), platelet-derived-growth factor receptor (PDGFR), endothelin type B receptor (EDNRB) or cleavage of TNC by metalloprotease meprinβ abolished the anti-adhesive effect of TNC altogether suggesting that the adhesivenss of a TNC substratum is largely context dependent (Midwood et al., 2016).

Proliferation and Survival

Human breast cancer section analysis revealed that a high TNC expression correlates with proliferation (Tsunoda et al., 2003). In vitro evaluation of the roles of TNC demonstrated that TNC increases tumor cell tumor cell prpliferation (Huang et al., 2001; Yoshida et al., 1999). However, in anchorage-dependent fibroblasts, TNC causes cell cycle arrest and cdk2 inactivation by interfering with fibronectin/syndecan-4 interactions (Orend et al., 2003). More specifically, a TNC-derived peptide, TNIIIA2, highly enhances

platelet-derived growth factor (PDGF)-dependent cell proliferation through potentiated and sustained activation of integrin α5β1 (Tanaka et al., 2014). Although with a low affinity to EGFR, the EGF-like repeats of TNC can elicit mitogenesis, EGFR autophosphorylation and cell proliferation (Swindle et al., 2001).

In vitro, by enhancing fibroblast growth factor receptor (FGFR) and by blocking bone morphogenic protein (BMP4) signaling, TNC can promote survival of stem cells (Garcion et al., 2004). TNC can also induce resistance to apoptosis in tumor cells through activation of ERK/NFκB signaling (Shi et al., 2015). However, it was also shown that EGF-like domains of TNC can be proapoptotic for cultured smooth muscle cells (Wallner et al., 2004). In the PDX breast cancer model, TNC is reported not affecting proliferation but promoting survival of metastatic breast tumor cells (Oskarsson et al., 2011; O'Connell et al., 2011).

Migration, Invasion and EMT

By using a wound healing and a chemotactic transwell migration assay, TNC was reported to promote cell migration in various cell types including cancer cells (Orend et al., 2006; Yoshida et al., 1999; Tsunoda et al., 2003; Saupe et al., 2013; Toshimichi et al., 2015), fibroblasts (Tamaoki et al., 2005), smooth muscle cells (Ishigaki et al., 2011) and endothelial cells (Castellon et al., 2002, Rupp et al., 2016).

It has also been demonstrated that TNC can induce tumor cell invasion in various in vitro models. In a glioblastoma (GBM) xenograft model, knockdown (KD) of endogenous TNC in tumor cells abolished cell migration on a two-dimensional substrate and tumor cell invasion in surrounding brain tissue, although tumor growth and proliferation was not affected (Hirata et al., 2009). TNC can also promote tumor invasion through the interplay between tumor cells and myofibroblasts. TNC and scatter factor/hepatocyte growth factor (SF/HGF) produced by myofibroblasts in vitro is reported to provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac (De Wever et al., 2004). By overexpression of TNC isoforms, containing TNIII D, TNIII BD or TNIII BAD1D domains, an increased tumor cell invasion was observed in breast cancer cells (Adams et al., 2002; Guttery et al., 2010; Hancox et al., 2009). TNC is also reported to increase matrix metalloproteinase (MMPs) expression which is thought to play an important role in cell

migration and invasion. Ilunga and colleagues observed that upon co-stimulation of human breast cancer cells with TGF-β and TNC, MMP-9 expression and cancer cell invasion were enhanced (Ilunga et al., 2004). TNC can also increase the invasiveness of glioma cells through the downstream production of MMP-12 (Sarkar et al., 2010).

EMT is reported to play an important role in cellular motility and it was suggested to be a mechanism of embryonic development and cancer metastasis (Lamouille et al., 2014). TNC has been shown to be highly expressed in hair follicles, teeth or mammary glands during embryogenesis (Chiquet-Ehrismann et al., 1986). In addition, a close correlation between TNC expression and gastrulation and formation of the neural crest, endocardial cushion, and secondary palate indicate a potential role of TNC in EMT (Jones et al., 2000). In human breast cancer tissue, TNC was found to be co-expressed with vimentin a classical EMT mesenchymal marker (Dandachi et al., 2001). In colorectal cancer tissue, TNC was also found to be positive correlated with an EMT signature (Takahashi et al., 2013). Cell detachment and disruption of cell-cell contacts were observed upon additon of condition medium containing TNC or upon growth on a TNC substratum (Chiquet-Ehrismann et al., 1989; Martin et al., 2003). It has been shown that TNC can induce an EMT-like morphological change in MCF-7 cells involving binding to αvβ6 and αvβ1 integrins (Katoh et al., 2013). It has also been shown that this TNC induced EMT-like change is associated with focal adhesion kinase (FAK) phosphorylation by SRC. Moreover, combined treatment with TGF-β1 reinforced these phenotypic changes (Nagaharu et al., 2011). In addition, the fibrinogen-like (FBG) domain of the matrix glycoprotein tenascin-X (TNX) which has some homologies to the same domain in TNC can interact physically with the small latent TGF-β complex and induce EMT in normal murine mammary gland (NMuMG) epithelial cells involving integrin α11β1 (Alcaraz et al., 2014). However, the mechanism of how TNC impacts on EMT is poorly investigated. One aim of this thesis was to study the signaling pathways involved in TNC-induced EMT and metastasis.

Angiogenesis

Angiogenesis is a fundamental step in the transition of tumors from a benign to a malignant state. It was reported that perivascular TNC around blood vessels significantly correlated with a shorter disease-free time in 86 glioblastoma patients (Herold-Mende et al., 2002). It was also reported that GBM with strong perivascular TNC staining had more

microvascular density than tumors with low TNC expression (Behrem et al., 2005). In a cohort of 63 non-small cell lung cancer (NSCLC) patients, serum TNC was found positive correlated with intra-tumor microvessel density (Ishiwata et al., 2005). These clinical observations suggest a pro-angiogenic role of TNC in cancer progression. Experimentally, when Tanaka and his colleagues injected A375 human melanoma cells into mice with and without TNC, they found a more abundant capillary net in tumors grown in WT mice than in TNKO mice. When melanoma cells were co-cultured with mesenchyme from WT and TNCKO mice, a higher VEGFA level was observed by ELISA in TNC expressing conditions (Tanaka et al., 2004). In vitro, TNC has also been demonstrated to have multiple roles in endothelial cell proliferation, adhesion, migration, invasion and tube formation (Delaney et al., 2006; Castellon et al., 2002; Ishiwata et al., 2005; Martina et al., 2010).

The molecular mechanisms underlying the impact of TNC on angiogenesis are under intense investigated. Integrins α2β1 and αvβ3 expressed on endothelial cells can interact with TNC and induce a sprouting phenotype (Sriramarao et al., 1993; Canfield et al., 1995). Recently, TNC has been reported to promote the angiogenic switch and the formation of more but leaky blood vessels which involves Wnt signaling presumably through repression of Dickkopf1 (Saupe et al., 2013; Langlois et al., 2014). Induction of Ephrin-B2 by TNC in GBM cells triggered survival and tubulogenesis in endothelial cells via a paracrine mechanism (Rupp et al., 2016). It was also shown that a direct contact of endothelial cells with TNC induces apoptosis. This Janus actvity of TNC on endothelial cells could explain that TNC enhances the formation of new but leaky tumor vessels (Rupp et al., 2016). In summary, these studies showed an important role of TNC in tumor angiogenesis. Whether the described mechanisms potentially also apply to other contexts where TNC plays a role such as in inflammatory conditions and metastasis remains to be seen.

Immunosurveillance

By crossing TNCKO with MMTV-PyMT mice Talts and colleagues generaed compound mice to investigate the roles of TNC on breast cancer lung metastasis, yet the number and size of metastasis were similar to that in mice with WT levels of TNC. Yet, the organization of the tumors was different. Whereas large tumor cell nests were surrounded by thick layers of ECM including TNC, these tumor nests were smaller in the TNCKO

condition. This observation was accompanied by more F4/80-positive monocytes/ macrophages in stroma of TNCKO tumors. Interestingly, no F4/80-positive cells were found inside the tumor cell nests (Talts et al., 1999). These findings indicate a potential role of TNC as a barrier to prevent infiltration of tumor nests by inflammatory cells. T cell activity may also be affected by TNC expression. In a prostate cancer model TNC was shown to potentially protect cancer stem-like cells from immune surveillance by inhibiting T-cell receptor-dependent T-cell activation, proliferation, and cytokine production (Jachetti et al., 2015). In lung cancer TNC has been observed to inhibit tumor-infiltrating lymphocyte proliferation and cytokine secretion, thereby potentially promoting tumor immune evasion and tumor recurrence (Parekh et al., 2005). In addition, it has been shown that the TNC FNIII1-5 domain can block T-cell adhesion to fibronectin through inhibition of integrins α5β1 and α4β1 (Hauzenberger et al., 1999). FNIIIA1A2 domain has also been shown to inhibit T-cell activation in vitro which provides insights into potential immunosuppressive activities of TNC (Puente Navazo et al., 2001).

Potential impact on biomechanical tissue properties

TNC networks can have similarities with reticular fibers in lymphoid organs (Spenlé et al., 2015). These networks may represent areas of high tissue stiffeness and thus may alter biomechanical properties of cancer tissue (Imanaka-Yoshida et al., 2014). Recently, by orthotopically injecting GBM cells expressing high or low levels of TNC into immune compromised mice, Miroshnikova and colleagues found that tumors with low TNC levels were softer and had lower machanosignaling as revealed by reduced pFAK and pMLC2 suggesting that TNC may be involved in enhancing ECM stiffness and through that potentially increased glioma progression (Miroshnikova et al., 2016).

1.4 TNC regulating cellular signaling

1.4.1 Biomechanical signaling

Given that actin stress fibers which can be modulated by TNC are required for mechanotransduction, how do these mechanical signals affect gene expression and impact biological outcome? The actin polymerization state and associated mechanical

signaling can be interpreted by the cell through two co-transcription factors, megakaryoblastic leukemia 1 (MKL1) (Asparuhova et al., 2011) and yes activating protein (YAP) (Zhao et al., 2008).

MKL1

MKL1 (also termed MRTF-A or MAL) is regulated by extracellular mitogenic signals or mechanical stimulations. Under poorly adhesive conditions, cells fail to polymerize actin and do not form actin stress fibers where MKL1 binds to globular G-actin monomers and remains sequestered in the cytoplasm. In consequence MKL1 cannot reach nuclear serum response factor (SRF) or SAP binding sequences to induce gene transcription (Olson et al., 2010).

YAP

The YAP and TAZ (transcriptional co-activator with PDZ-binding motif; also known as WWTR1) are transcriptional coactivators which have been shown to sense the mechanical signals and can be regulated by actin cytoskeleton dynamics (Dupont et al., 2011; Zhao et al., 2012). YAP/TAZ can shuttle between the cytoplasm and the nucleus, where they can recognize and interact with several transcription factors, and in particular TEA domain family members (TEAD) as well as RUNX2, T-box 5 (TBX5) and p73 (Pan et al., 2010; Zhao et al., 2010; Halder et al., 2011).

YAP/TAZ have instrumental roles in cancer cell behavior such as proliferation, survival and maintaining stemness properties. For example, activation of YAP/TAZ promotes tumor cell proliferation (Camargo et al., 2007; Chan et al., 2008). This YAP/TAZ induced cell cycle transcription program comprises activation of factors involved in replication licensing, DNA synthesis and repair, control of cyclins for S-phase entry, and completion of mitosis (Zanconato et al., 2015). YAP/TAZ also displays a promoting role on tumor cell survival. Zhao and his colleagues observed that increased YAP/TAZ activity can overcome anoikis induced cell death (Zhao et al., 2012). YAP/TAZ can also drive the expression of BIRC5 and BCL2L1 and inhibit the mitochondrial-induced apoptosis pathway (Rosenbluh et al., 2012). In adition, YAZ/TAZ plays a very important role in

activation, expansion and maintenance of cancer stem cells (CSC) (Basu-Roy et al., 2015; Cordenonsi et al., 2011). The link of YAP/TAZ and CSCs determine its role in tumor initiation, cell plasticity, drug resistance and metastasis (Zanconato et al., 2016). Not only impacting on tumor cells, YAP is also involved in matrix remodeling in the tumor microenvironment. For example, YAP can elevate tissue stiffness by CAFs through increasing expression of MYL9 and MYH10. Conversely, stiff matrices and a contractile actin cytoskeleton can work together as a positive feedback loop to activate YAP (Calvo et al., 2013). YAP/TAZ is also intensively investigated in human cancers. An elevated expression of YAP correlates with high grade tumors and poor prognosis in a variety of human cancers such as lung cancer, breast cancer, colorectal cancer, liver cancer, gliomas and others (Zanconato et al., 2016).

Because of the multiple roles in tumor biology, the upstream regulators of YAZ/TAZ are now under intense investigation. The Hippo pathway is the best known pathway regulating YAZ/TAZ. Large tumour suppressor homologue 1 (LATS1) and LATS2 (which are the mammalian homologues of D. melanogaster Warts) which are the two main components of Hippo pathway can phosphorylate YAP/TAZ, thereby promoting their cytoplasmic retention and degradation (Halder et al., 2011).

YAP/TAZ are also directly regulated by mechanical cues such as ECM stiffness, cell shape, and cell adhesion. YAP/TAZ are localized in the nucleus and transcriptionally active in cells under an adequate adhesive strength, a stiff extracellular matrix (ECM) or stretching between stiff micropillars. On the contrary, YAP/TAZ can be relocalized to the cytoplasm and functionally inhibited in cells cultured on a small adhesive area, a soft ECM or on top of bendable micropillars (Dupont et al., 2011). Since mechanotransduction is tightly linked to the integrity of the actinomyosin cytoskeleton, YAP/TAZ are also reported to be regulated by cytoskeletal dynamics. YAP/TAZ activity is diminished when F-actin is disrupted or RhoA pathway is inhibited (Dupont et al., 2011). On the other hand, triggering F-actin polymerization and stress fibers formation by over-expression of activated diaphanous protein (DIAPH1) increased YAP/TAZ activity (Dupont et al., 2011). In addition, inhibiting expression of ROCK, MLCK and myosin expression results in YAP/TAZ inactivation (Dupont et al., 2011; Sansores-Garcia et al., 2011).

The G-actin to F-actin ratio is an important regulator of myocardin-related transcription factors MKL1, the nuclear exclusion and activity inhibition of which is determined by its

binding status to G-actin (Olson et al., 2010). However, YAP/TAZ are not regulated by G-actin to F-actin ratio, as expression of non-polymerizable G-actin has no effect on YAP/TAZ activity both in vitro or in vivo. Moreover, increasing the amount of F-acin by F-actin stabilization did not have an effect on YAP/TAZ activity suggesting that stress fibers instead of F-actin polymerization are needed for YAP/TAZ activity (Dupont et al., 2011).

It has been shown that TNC can modulate cellular tension by impairing actin stress fiber formation and through that regulates gene expression which may impact on cell behavior and tumor malignancy (Ruiz et al., 2004). One aim of my thesis was to investigate if TNC can regulate YAP or MKL1 activities through impairing actin stress fibers.

1.4.2 Growth factor binding related signaling

Being more than a mechanical scaffold, ECM can also act as a reservoir of different growth factors to produce different cellular responses. For example, fibronectin can bind a varity of growth factors that play essential roles in fibrosis and tumor progression such as: latent TGF-β (Horiguchi et al., 2012), Wnt (Bentzinger et al., 2014) vascular endothelial growth factor (VEGF) (Wijelath et al., 2006), hepatocyte growth factor (HGF) (Rahman et al., 2005), fibroblast growth factor (FGF)-2 (C. Bossard, 2004, Cancer Research), platelet-derived growth factor (PDGF) (Smith et al., 2009) among others. These collaborative interactions can enhance the availability and efficiency of growth factor signaling which can be exploited for target therapies in many diseases. Here I will introduce two growth factors that play instrumental roles in tumor progression as well as their interaction with ECM molecules.

TGF-β signaling

Transforming growth factor-beta (TGF-β) is a family of cytokines that includes bone morphogenetic proteins (BMPs), activin and inhibin, nodal and growth differentiation factors (Massagué et al., 1998). Under normal conditions, TGF-β plays an important role in embryogenesis and tissue homeostasis. During pathological conditions such as fibrosis, inflammation and cancer, TGF-β expression is disrupted and may induce aberrant cell proliferation, survival, differentiation and ECM molecules synthesis (Blobe et al., 2000).

Pro-TGF- β forms a homodimer after synthesis and binds to latent TGF-β binding protein (LTBP). After intercellular proteolytic cleavage from its propeptide dimer (latency associated peptide (LAP), a large latent complex (LLC) is formed which consist of LAP, LTBP and TGF-β molecules (Rifkin et al., 2005). After secretion, due to the affinity of LTBP to ECM, TGF- β (as part of LLCs) can incorporates into the ECM. Active TGF- β can be released by proteolytic degradation or conformational modification of the LLCs or by mechanical stretching and binding to their receptors (Hinz et al., 2015). TGF- β induced signaling acts through a canonical Smad dependent pathway and non-canonical pathways including mitogen-activated protein kinase (MAPK) pathways, PI3K / Akt pathways and RhoA activation. Activated phospho-Smad2/3 can bind with to transcription cofactors and induce TGF-β target gene expression (Neuzillet et al., 2013).

Many ECM molecules such as fibrillins, fibulins, fibronectin and some proteoglycans have been reported to be involved in binding to LTBP and activate TGF- β signaling (Horiguchi et al., 2012). Recently, Alcaraz and colleagues found that the fibrinogen-like (FBG) domain of the matrix glycoprotein tenascin-X (TNX) can activate latent TGF-β into an active molecule by changing the conformation of the small latent TGF-β complex and subsequently induce EMT in mammary epithelial cells (Alcaraz et al., 2014). It has also been reported that TNC deficiency can attenuate TGF-β-mediated fibrosis following murine lung injury indicating a potential role of TNC in latent TGF-β activation (Carey et al., 2010). In addition, the fifth fibronectin type III-like (TNCIII) domains of TNC was shown to bind various growth factors including TGF-β1(De Laporte et al., 2013). Intriguingly, it has been demonstrated that TGF-β can induce TNC expression in different types of cells including fibroblasts, pericytes as well as tumor cells (Islam et al., 2014; Rupp et al., 2016; Calon et al., 2012) suggesting a positive feedback loop of regulation.

All these observations indicate that TNC may act as a resevoir of TGF-β and regulate multiple physiological and pathological processes through TGF-β signaling, however, the molecular mechanism such as the specific latent TGF-β complex binding site of TNC, involved integrin as well as the impact on cellular behavior after the TNC-induced TGF-β signaling is largely unknown.

Wnt signaling

The Wnt family of signaling molecules constitutes a large number of cysteine-rich secreted glycoproteins that regulate a variety of cellular processes such as development, homeostasis, regeneration, stem cell pluripotency, cell polarity and migration (Clevers et al., 2012; Niehrs et al., 2012). There are two Wnt signaling pathways: canonical and non-canonical signaling. In the canonical Wnt pathway also refered to as “Wnt/β-catenin pathway”, Wnt ligands bind to the frizzled receptors and LRP5/6 co-receptors leading to formation of a “destruction complex”, composed of Axin2, Glycogen synthase kinase 3 (GSK3) and adenomatous polyposis coli gene product (APC) which leads to β-catenin stabilisation. In the absence of a Wnt ligand, this complex can degrade the β-catenin protein by phosphorylation and ubiquitination. When the Wnt signalling is activated β-catenin phosphorylation will be inhibited and β-catenin will translocate into the nucleus and induce target gene expression through binding to the TCF/Lef family of transcription factors (Astudillo et al., 2014). This Wnt/β-catenin signaling can be regulated by several extracellular proteins such as dickkopf family of Wnt/β-catenin inhibitors and R-spondin family of Wnt/β-catenin agonists (Cruciat et al., 2012). In the non-canonical pathway also refered to as “β-catenin independent signaling”, the Wnt ligands can interact with frizzled receptors together with several co-receptors, including Strabismus/Vangl2, Ryk, Ror2, Ptk7 and activate several intracellular proteins, such as small GTPases Rho and Rac, JNK, CaMK among others (Cruciat et al., 2012).

The interplay between Wnt signaling and ECM especially the presenting role of ECM as a reservoir remains largely unknown. It has been reported that fibronectin, together with Wnt 7a can induce Wnt signaling through a non-canonical pathway and regulate the symmetric division of satellite stem cells (Bentzinger et al., 2014). TNC has been reported to decrease Dkk1 expression (a canonical pathway inhibitor) and induce Wnt/β-catenin signaling (Saupe et al., 2013; Hendaoui et al., 2014). Many other ECM molecules like collagen (Hendaoui et al., 2012), lamnin (Ritié et al., 2012) or periostin (Zhang et al., 2016) have been observed in regulating Wnt signaling, however, most of them are indirect interactions with Wnt components. The interactions between ECM and Wnt component needs to be further investigated.

2. Aims

My thesis was based on preliminary results obtained by two previous PhD theses in the laboratory. Ines Velazquez-Quesada observed that TNC participates in tumor initiation and in lung metastasis colonization in an ErbB2- driven transgenic breast cancer mouse model. She also established a cell line that serves as surrogate model for investigating the molecular and cellular mechanism of TNC promoted lung metastasis. I wanted to address the roles of TNC in each step of tumor progression. I also wanted to know how TNC promotes breast cancer lung metastasis at cellular and molecular level. Acting as a growth factor reservoir, ECM molecules may induce EMT and promote tumor cell dissemination. However, locally singularized tumor cells may also respond to ECM molecules by undergoing rapid changes towards a rounded amoeboid-like migratory phenotype. Based on preliminary results obtained by Anja Schwenzer who found that TNC-induced cell rounding impacts on MKL1 and YAP target gene expression, I wanted to understand the molecular mechanisms of TNC-induced cell rounding and its potential impact on cell migration.

Aim 1. Identify the cellular and molecular mechanisms of the promoting role of TNC in NeuNT breast tumor lung metastasis

Aim 2. Elucidate the consequences and underlying molecular mechanisms of TNC-induced cell rounding

3. Manuscript 1

Stromal tenascin-C increases breast cancer lung metastasis by impacting on lymphovascular invasions

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Running title: Tenascin-C promotes survival in lymphovascular invasions and metastasis

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Abstract

In breast cancer high expression of the extracellular matrix (ECM) molecule tenascin-C (TNC) correlates with shortened metastasis-free survival but the underlying mechanisms are poorly understood. To gain more insight we used the stochastic MMTV-NeuNT and a novel associated syngeneic orthotopic breast cancer model with abundant and no/low TNC. We observed that TNC increases tumor onset and lung metastasis. We revealed an important role of stromal TNC as prominent component of lymphovascular invasions (LVI). As LVI are precursors of parenchymal metastasis, cause thromboembolism and correlate with bad survival prognosis, they may be a good anti-cancer target. Yet, little was known about their cellular and ECM composition which we determined here. We document that LVI are nests of proliferating circulating tumor cells containing platelets, enveloped by fibroblasts and endothelial cells as well as TNC, fibronectin and laminin. In LVI, TNC increases platelet abundance, endothelial cell layering, tumor cell survival, epithelial mesenchymal transition (EMT) and breaching into the lung parenchyma. In cultured cells, through TGF-β signaling, TNC induces EMT and survival which could explain TNC-enhanced ErbB2-driven breast cancer lung metastasis. Thus, TNC in LVI may offer novel opportunities for cancer diagnostic and targeting.

Intoduction

Breast cancer is the most common cancer among women worldwide (Ferlay et al., 2012). Most breast cancer patients die from metastasis despite earlier diagnosis and improved treatment. For increasing breast cancer patient survival a better knowledge of metastasis is required. The tumor microenvironment (TME) comprising tumor and stromal cells, soluble factors and extracellular matrix (ECM) promotes metastasis (Hanahan et al., 2011). One extracellular matrix (ECM) molecule that promotes lung metastasis is tenascin-C (TNC) (Midwood et al., 2016). TNC plays multiple roles in cancer as recently demonstrated in a stochastic neuroendocrine tumor model with abundant and no TNC. In this model TNC enhanced survival, proliferation, invasion, angiogenesis and lung metastasis (Saupe et al., 2013). In breast cancer, high expression of TNC correlates with shortened patient survival (Yoshida et al., 1995). Although some downstream pathways (Notch and Wnt) (Oskarsson et al., 2011) and molecular candidates (VEGFA) (O`Connell et al., 2011) were identified to play a role in TNC-enhanced breast cancer lung metastasis our knowledge of how TNC promotes metastasis is poor. Moreover, controversial results from breast cancer models with abundant and no TNC blurr the view on the roles of TNC in breast cancer. Whereas TNC promoted lung metastasis in the 4T1 and PDX grafting models (O`Connell et al., 2011; Oskarsson et al., 2011), no promoting effect was seen in the stochastic PyMT model (Talts et al., 1999).

To gain a better understanding of the roles of TNC in breast cancer metastasis we have chosen the well described MMTV-NeuNT model which recapitulates features of Her2-driven breast cancer (Muller et al., 1988), a tumor type that represents about 20% of breast cancer (Zhang et al., 2007). Another advantage of this model is its stochastic nature and an extended tumor latency of several months thus allowing to generate a relevant tumor

microenvironment (TME). In addition to parenchymal metastasis, lymphovascular invasions (LVI) have been described in the MMTV-Neu model (Bouchard et al., 1989). The presence of LVI correlates with worsened cancer patient survival (Soerjomataram et al., 2008). In addition to causing thromboembolism and organ failure LVI were shown to progress into parenchymal metastasis where TGF-β signaling is instrumental (Siegel et al., 2003). Thus, a better knowledge of LVI may offer novel anti-cancer targeting opportunities. Yet, little was known about the cellular and molecular composition of LVI.

Here, we generated MMTV-NeuNT mice to lack the TNC protein (TNCKO) and compared tumor onset and lung metastasis with that in WT tumor mice. We identified an important role of TNC in increasing tumor onset and lung metastasis. We identified stromal derived TNC as an integral component of LVI promoting progression into parenchymal metastasis. We describe LVI as nests of circulating tumor cells (CTC) within arterioles of the metastatic lung that are enveloped by TNC and other ECM molecules. In LVI, TNC enhances survival, endothelialization, partial EMT and breaching into the lung parenchyma. We identified TGF-β signaling as driving TNC-induced EMT and migration *in vitro*. This information offers opportunities for imaging and targeting of LVI and in particular to block TNC actions in metastasis of Her2-positve breast cancer.

Results

Tenascin-C accelerates tumor onset

We generated MMTV-NeuNT tumor mice lacking TNC (TNCKO) by breeding and compared tumorigenesis with mice expressing wildtype (WT) levels of TNC. By immunoblotting and immunofluorescence staining (IF) of the primary tumors we found TNC expressed in matrix tracks (**Fig. S1A**) as shown in other cancers (Spenlé et al., 2015). No TNC protein was found in TNCKO tumors (**Fig. S1A, B**). We compared tumor latency and observed that in WT mice, tumors were first palpable at 135 days. At 195 days all mice had tumors. Tumor latency was largely delayed in TNCKO mice where tumors were first palpable at 175-322 days (**Fig. 1A**). All mice developed multiple tumors with no difference in numbers between genotypes (data not shown). Mice were sacrificed 3 months after first tumor palpation or when tumor size reached the ethical limit. Again, no difference in tumor size between genotypes was noted (**Fig. 1B**).

Tenascin-C enhances lung metastasis

We assessed lung metastasis by a stereological analysis of the left and biggest lung lobe and noticed no difference in the number of metastasis between genotypes (**Fig. 1D-F**). Yet, we found a higher metastatic index in WT mice as the metastatic surface was bigger in lungs of WT than in TNCKO mice (**Fig. 1C, E**). As LVI have been described in MMTV-Neu as precursor of parenchymal metastasis (Siegel et al., 2003), we searched for LVI by immunohistochemistry (IHC) and IF and observed them in arterioles of the lung (**Fig. 1F**). Surface measurement revealed that LVI were bigger in WT than in TNCKO mice (**Fig. 1F,**

G). A reduced LVI size could be due to reduced proliferation and/or increased apoptosis which we addressed by staining for Ki67 and cleaved caspase-3, respectively followed by quantification. It is remarkable that cells in the LVI proliferate, yet we did not see a difference in the number of Ki67+ cells between genotypes (**Fig. 1J, K**). In contrast, cleaved caspase-3+ cells were less abundant in WT than in TNCKO LVI, suggesting that TNC promotes survival (**Fig. 1H, I**). Next we addressed a potential role of TNC on apoptosis and proliferation in the parenchymal metastasis by staining lung parenchymal tissue. Similar to LVI, we observed no difference in proliferation but noticed less apoptosis in WT than in TNCKO tissue (**Fig. S1C-F**). In summary, these observations revealed (i) a role of TNC in accelerating tumor onset. Moreover, TNC promotes survival in (ii) LVI and (iii) parenchymal metastasis which could explain the (iv) higher metastatic burden of WT tumor mice.

Stromal tenascin-C enhances lung metastasis

We wanted to know whether TNC expressed by tumor or stromal cells is promoting lung metastasis. Therefore, we used the NT193 orthotopic grafting model, that we noticed to display LVI. In this model NT193 cells were injected into the mammary gland of an immune competent host leading to spontaneous lung metastasis (Sun, Velazquez-Quesada et al., in preparation). We downregulated TNC by shRNA technology in NT193 cells and grafted sh control (shC) and shTNC cells into a WT and TNCKO host, respectively. We confirmed TNC knockdown (KD) in the cells and tumors by western blot and IF (**Fig. S2A-C**). We observed that tumor bearing mice developed lung metastasis. As for the MMTV-NeuNT model, no difference in metastasis incidence between conditions was seen (**Fig. 2A**). Yet, upon quantification of the lung metastatic surface we noticed that the index of shC cells-

derived metastasis was higher in WT than in TNCKO mice. Moreover, irrespective of the cell gentotype, there was a tendency towards more metastasis in the WT than in the TNCKO host (**Fig. 2B**). Next, we determined the surface of LVI and found that LVI derived from shC tumor cells grown in a WT host were significantly bigger than those in a TNCKO host (**Fig. 2C**). Assessing survival and proliferation by tissue staining revealed that cells in LVI proliferated, yet independent of TNC which is similar to the MMTV-NeuNT model (**Fig. 2D, E**). In contrast, we saw the lowest apoptosis index when shC cells were grafted into a WT host in comparison to a TNCKO host (**Fig. 2F, G**). Altogether, these results demonstrate that stromal TNC is important for enhancing survival of cells in metastasis.

LVI are nests of CTC surrounded by a layer of stromal cells expressing tenascin-C

As our results suggest a role of TNC on LVI, we characterized LVI by hematoxylin/eosin (HE) and IF. We found LVI in arterioles of the lung (**Fig. 3A, S3A-C**). As seen by HE, in some LVI the core region was necrotic. LVI eventually occluded the vessel lumen, which did not cause apparent necrosis of the lung tissue (**Fig. S3B, C**). We wanted to know whether LVI express TNC which we addressed by IF. We saw abundant TNC expression at the rim yet not within ErbB2+ CTC in the center of the LVI (**Fig. 3B**). We also noticed the absence of TNC in CTC extravasating from the LVI into the lung parenchyma indicating that TNC may not be a component of the “soil” of parenchymal metastasis as previously had been suggested in another breast cancer model (Oskarsson et al., 2011) (**Fig. 3B**).

To assess the origin of TNC, we stained LVI from the NT193 grafting model with antibodies against TNC and αSMA (expressed in perivascular cells delineating the arteriole wall) (**Fig. 3C**). Upon grafting of shC cells the corresponding LVI expressed TNC when cells were grafted into a WT host, yet not when grafted in a TNCKO host (**Fig. 3C, S3D**). Also in LVI

derived from shTNC cells (generating tumors with largely reduced TNC levels (**Fig. S2B, C**)) we found some TNC expressed in LVI when cells were grafted in a WT yet not in a TNCKO host (**Fig. S3D**). These results reveal that stromal cells express TNC in LVI where tumor cell derived TNC appears to promote expression of TNC by stromal cells as the TNC signal was stronger in a WT host when shC cells were grafted in comparison to shTNC cells (**Fig. S3D**). To further address the stromal source of TNC we co-stained LVI with an antibody against TNC and Fsp1, a marker of fibroblasts and myeloid cells, that previously were described as source of TNC in another breast cancer model (O`Connell et al., 2011). We observed partial overlap of TNC and Fsp1 signals suggesting that Fsp1+ cells are a likely source of TNC (**Fig. 3D**). As in the genetic model, in the grafting model LVI often occluded the vessels (without apparent effects on the lung tissue, data not shown) (**Fig. 3E, Fig. S3E**). Co-staining revealed at most a weak signal overlap of TNC with αSMA, yet strong overlap with Fsp1 (**Fig. 3D, E**). Together, these results suggest that Fsp1 expressing cells are likely candidates to express TNC in LVI of both models. This result also demonstrates similarities between the genetic and grafting model.

In LVI tenascin-C increases abundance of endothelial cells and platelets

As very little was known about the cellular and ECM composition of LVI, we characterized LVI by HE and IF on sequential lung tissue sections. In particular, we used antibodies specific for tumor cells (CK8/18, ErbB2), endothelial cells (CD31), platelets (CD41), leukocytes (CD45), activated macrophages (F4/80), fibronectin (FN) and laminin (LM). We observed that ErbB2 and CK8/18+ CTC form a tightly packed “tumor nest” that is enveloped by a layer of TNC followed by layers of FN and LM (**Fig. 4A** panel b, e). Endothelial cells are present at the border of the LVI as a monolayer, as detected by IF and HE with characteristically flat endothelial cell nuclei (**Fig. 4A, S4A**). Neither FN, LM or TNC nor endothelial cells or fibroblasts were found within the tumor nests of LVI from

MMTV-NeuNT mice (**Fig. 4A**). Leukocytes were not directly associated with the LVI but were present at the basal side of the arteriole wall facing the lung parenchyma (**Fig. 4A** panel c). In addition, we found that activated macrophages were abundant at the lung vessel wall when LVI were present but not in other vessels (**Fig. 4A** panel f). By analyzing more than 20 LVI from a total of 6 WT mice, we noticed that all LVI were similarly organized. Moreover, LVI of the NT193 model showed a similar organization of TNC, fibroblasts and endothelial cells, again revealing similarities between models (**Fig. 3C, E, Fig. S4A, B**).

As LVI were also present in the lung vasculature of MMTV-NeuNT mice with a TNCKO we asked whether TNC had an impact on the organization of LVI. Staining for LM did not reveal differences between genotypes suggesting that TNC does not impact on the generation of LVI nor on the deposition of LM at the rim of the CTC nest (**Fig. 3C, 4B, D**).

Staining for CD31 revealed two layers of endothelial cells, one derived from the arteriole and one directly surrounding the LVI, which is seen in both models (**Fig. 4A, S4B**). We noticed that some LVI of TNCKO mice lacked the LVI endothelial layer. We quantified the abundance of LVI with an endothelial cell layer and noticed more LVI with an endothelial layer in WT than in TNCKO mice, suggesting that TNC positively impacts on endothelial cells (**Fig. 4B, C**). Moreover, we observed a continuum between the LVI endothelial layer and the endothelium of the lung vasculature reminiscent of fusion of both endothelial layers (**Fig. 4A,** panel b, e). Whereas the arteriole wall is covered by pericytes as they stained for αSMA we noticed also FSP1+ cells in walls of arterioles that host LVI (**Fig. 3C, D**). In addition, while activated macrophages were rarely seen within the lung parenchyma nor in arterioles or capillaries we noticed strong staining in those arterioles with LVI (**Fig. 4A** panel f). These findings suggest that the arteriole endothelium may be activated in the presence of LVI and potentially plays a role in endothelial layer fusion leading to LVI ingestion as documented in **Fig. S4**.

LVI are known to be associated with vessel occlusion through thromboembolism where platelets are instrumental (Meikle et al., 2016). Whether TNC has an impact on platelets we addressed by staining for CD41 and RAM1 (recognizing Gp1b (Mangin et al., 2009)), respectively. We found platelets as integral component of LVI surrounded by LM and an endothelal cell layer, suggesting a role of platelets in the generation of LVI (**Fig. 4D, S4E-G**). We also noticed an overlap of CD41 and TNC expression where the TNC signal was fibrillar and that of platelets was dot like (**Fig. S4G**). By quantification of CD41 we found less platelets in LVI of TNCKO mice than in WT mice suggesting a potential role of TNC in platelet attachment which is in agreement with observations made in another model (Schaff et al., 2011) (**Fig. 4E**).

Altogether, our detailed IF analysis allowed us to deduce the organization of LVI (**Fig. 4F**). We demonstrated that LVI are “organelle-like” structures where a CK8/18 positive core of CTC together with platelets is enveloped by distinct layers of stromal cells. Whereas FSP1+ cells face CTC, an endothelial monolayer is present at the LVI border. Due to overlay of signals with TNC, it is likely that Fsp1 expressing cells (and platelets) are a source of TNC. In contrast, lack of signal overlay suggests that CTC do not express TNC. Moreover, the discrete ECM layering of TNC and LM/FN suggests that endothelial cells and FSP1+ cells express LM and FN whereas CTC do not. Analysis of LVI from mice or tumor cells expressing TNC (or not) suggests that TNC (i) is not required for LVI formation, (ii) nor enveloping of CTC by ECM (LM and FN), yet that (iii) TNC promotes the abundance of endothelial cells and (iv) platelets thus, altogether potentially impacting on the properties of CTC within LVI.

Tenascin-C promotes extravasation of CTC from LVI into lung parenchyma

LVI were described as precursors of parenchymal metastasis in another MMTV-Neu model (Siegel et al., 2003). In support of this notion, we observed that the relative abundance of parenchymal metastasis increased over time in the MMTV-NeuNT model. Whereas no parenchymal metastases were seen early after tumor palpation, we clearly noticed parenchymal metastases at later time points (**Fig. S5A**). When we compared the ratio of LVI to parenchymal metastasis between genotypes of MMTV-NeuNT mice at the end of the experiment we found more parenchymal metastasis in lungs of WT mice (**Fig. 5A**). We made a similar observation in the NT193 grafting model and saw more parenchymal metastasis when shC cells were grafted in a WT compared to a TNCKO host (**Fig. 5B**). Interestingly, at the site of extravasation, TNC is absent whereas in the parenchymal metastasis TNC is expressed (**Fig. 3B, Fig. S5B-E**). These results suggest that TNC plays a role in the progression of LVI into parenchymal metastasis but probably not in preparing the soil.

Partial EMT in tenascin-C-expressing LVI and mixed mesenchymal and epithelial features in parenchymal metastasis

As LVI are described as precursor of parenchymal metastasis the question arises how CTC enter the lung parenchyma. Our IF analysis revealed a continuum of CD31+ layers surrounding the LVI and the lung vessel wall which is reminiscent of a potential fusion of both endothelial layers (**Fig. 4A** panel b, e) which is in agreement with a previously proposed mechanism described in another breast cancer model (Sugino et al., 2002) presumably leading to LVI ingestion (**Fig. S4C**). Another possibility of extravasation is EMT which we addressed by IF tissue analysis for CK8/18 and ErbB2 (combined with TNC) or E-cadherin (together with vimentin), where we observed cells leaving LVI and

invading the parenchymal lung tissue (**Fig. 3A, B, 6A**). While all CTC inside LVI expressed CK8/18, E-cadherin and ErbB2, some cells also co-expressed vimentin. Other cells at the invading front only expressed vimentin (**Fig. 6A**). This phenotype is reminiscent of partial EMT and collective tumor cell invasion of tissue (Cheung et al., 2016) (**Fig. 3B, 6A, S6A**). By quantifying the vimentin signal inside the LVI we noticed more vimentin expressing cells within WT than TNCKO LVI (**Fig. 6B, C, Fig. S6A, B**). FN and TNC are known EMT markers (Sundquist et al., 2017), yet we did not see expression of either of these molecules within the tumor nests and thus not in vimentin expressing cells (**Fig. 3B, 4A**). We also investigated expression of mesenchymal (vimentin, αSMA) and epithelial cell markers (ErbB2, CK8/18) in parenchymal metastatic tissue and observed that cells had a mixed phenotype expressing both epithelial and mesenchymal markers (**Fig. S5C-E**). Our observations suggest that TNC promotes breaching of cells from LVI into the lung parenchyma and EMT. Thus, it is possible that extravasation involves EMT by TNC.

Tenascin-C induces EMT in cultured cells involving TGF-β signaling, promoting cell migration and survival

In the MMTV-Neu model, extravasation from LVI into the lung parenchyma was shown to be promoted by TGF-β signaling which correlated with enhanced lung metastasis (Siegel et al., 2003). So far, we have demonstrated that TNC increases LVI breaching and parenchymal lung metastasis. Given a role of TGF-β (Xu et al., 2009) and TNC (Nagaharu et al., 2011) in EMT, and of TNC promoting partial EMT in LVI (our result), we asked whether TNC may promote tumor cell extravasation through EMT potentially involving TGF-β signaling. To address these possibilities, we treated NT193 cells with TNC in monolayer or spheroid cultures and observed loss of E-cadherin and gain of vimentin

expression (**Fig. 7A, Fig.S7C**). This observation suggests that TNC induces EMT in NT193 cells. We confirmed a change in expression of several EMT markers by qRTPCR and western blot. In particular, we saw increased mRNA levels for snail, slug, ZEB1, vimentin, PAI-1, MMP9 and TNC. On the contrary mRNA levels for E-cadherin were reduced (**Fig. 7B**). As determined by western blot, protein levels of vimentin and E-cadherin were increased and reduced, respectively upon treatment with TNC (**Fig. 7C**).

Next, we compared cell morphology and gene expression of cells treated with TNC or TGF-β Similar to TNC, TGF-β induces EMT in NT193 cells as seen by gain of a mesenchymal phenotype, loss of E-cadherin and gain of vimentin expression as well as increasing expression of several mesenchymal markers at mRNA level (**Fig. 7A, Fig. S7A, B**). By using the TGF-β signaling inhibitor GW788388 (GW) (Akhurst et al., 2012) we determined whether TNC-induced EMT involves TGF-β signaling. GW reverted the mesenchymal to an epithelial phenotype as demonstrated by high expression of E-cadherin and low expression of vimentin in cells treated with TNC as was seen for TGF-β (**Fig. 7A, B, Fig. S7A, C**). These observations suggest that TNC-induced EMT in NT193 cells involves TGF-β signaling. This possibility is further supported by nuclear expression of p-Smad2 (a marker of canonical TGF-β signaling) upon TNC treatment (**Fig. 7C, S7B**).

We determined downstream signaling upon TNC treatment by western blot and observed that both canonical and non-canonical, TGF-β signaling is induced by TNC as levels of p-Smad2, p-Akt and p-Erk1/2 were increased in comparison to control conditions which were similar to treatment with TGF-β. Again, TNC signaling was TGF-β dependent as it was blocked with GW (**Fig. 7C, S7B**).

As we saw platelets inside LVI and platelets are known to induce EMT (Labelle et al., 2011) we considered the possibility of a potential role of platelets in EMT of NT193 cells.

Therefore, we investigated EMT in NT193 cells upon addition of platelets. We saw that upon adhesion to NT193 cells, platelets induced EMT as E-cadherin levels dropped and vimentin expression increased (**Fig. 7D**). Similar to TNC, also platelet-induced EMT was blocked with GW suggesting that platelet-induced EMT is TGF-β signaling dependent in NT193 cells (**Fig. 7D**).

To address whether TNC-induced EMT has an impact on cell migration, we used a wound closure assay and saw increased migration of cells with TNC (over control conditions), which was similar to TGF-β treatment (**Fig. 7E, F**). Again, this effect was blocked with GW indicating that TNC-induced EMT enables NT193 cell motility throughTGF-β signaling.

As EMT provides tumor cells with survival resistance against chemical drugs (Singh et al., 2010), we determined whether TNC impacts on apoptosis induced by staurosporine. Indeed, pre-treatment of NT193 cells with TNC for 24 hours rendered cells more resistant against staurosporine-induced apoptosis as measured by a cleaved caspase-3/7 activity assay. The TNC effect was similar to that of TGF-β and was reverted by GW suggesting that TNC-induced EMT protects against apoptosis by staurosporine where TGF-β signaling is important (**Fig. 7G, Fig. S7D**).

Finally we addressed whether TNC potentially also induces Wnt signaling, a pathway that was shown to be promoted by TNC in other models (Saupe et al., 2013; Hendaoui et al., 2014). Indeed, expression of the Wnt marker Axin2 was increased by TNC as shown by qRTPCR, yet this did not occur with TGF-β. Moreover, GW did not have an effect on Axin2 expression (**Fig. 7H**). Given that GW blocked TNC-induced EMT, this result shows that TNC-induced EMT occurs despite increased Axin2 levels and suggests that Wnt signaling most likely does not play a role in TNC-induced EMT of NT193 cells.

In summary, our results have shown that (i) TNC-induced EMT involves canonical and non-canonical TGF-β signaling (ii) where platelets (known to contain TGF-β) may be a source of TGF-β as platelets also induced EMT in NT193 cells in a TGF-β signaling dependent manner. TNC-induced EMT promotes (iii) cell migration and (iv) apoptosis resistance against staurosporine (v) which occurred in a TGF-β signaling dependent manner. These observations may provide an explanation how TNC promotes survival and extravasation of CTC in LVI.

Mechanism of TNC-induced TGF-β signaling

So far, we have shown that TNC induces EMT in a TGF-β signaling dependent manner which raises the question how TNC activates this pathway. Previously, it was shown that TGF-β binds to the third fibronectin type III domain (FNIII3) in TNC (De Laporte et al., 2013). We considered the possibility that TNC activates TGF-β signaling through potentially bound TGF-β. Therefore, we looked for TGF-β in the TNC solution by western blot. Whereas 10 ng TGF-β (representing a dosage of 5 ng/ml that induces EMT in NT193 cells) was easily detectable, no TGF-β was found in two distinct TNC preparations (at an amount inducing EMT) (**Fig. 7I**). We addressed the kinetics of TNC- and TGF-β-induced signaling by western blot and observed that TNC immediately and transiently induced phosphorylation of Smad2, Akt and Erk1/2 that peaked between 1 – 2 hours which was similar to TGF-β. As we had seen increased phosphorylation of these molecules 24 hours after treatment with TNC and TGF-β, respectively, we conclude that both molecules induce also a sustained signaling.

In conclusion, TNC activates TGF-β signaling immediately and with a similar kinetics as TGF-β itself suggesting a potential direct mechanism. As TGF-β was not detectable in the used TNC preparation it remains to be determined how TNC activates TGF-β signaling.

Discussion

It is well established that the tumor stroma promotes metastasis (Hanahan et al., 2011). The stromal component and ECM molecule TNC plays an important yet poorly understood role in breast cancer as its high expression correlates with shortened metastasis-free and overall survival in breast cancer patients (Yoshida et al., 1995; Oskarsson et al., 2011). Here, we used an ErbB2-driven genetic and an associated novel syngeneic orthotopic breast cancer model with abundant and no/low TNC to address the roles of TNC in breast cancer onset and progression.

We identified several effects of TNC in increasing tumor onset and lung metastasis. Stromal cell derived TNC plays a particular role in LVI and their progression into parenchymal metastasis by impacting on endothelial cells, platelets and CTC. TNC enhances CTC survival, LVI endothelialization, EMT of CTC and breaching into the lung parenchyma. Moreover, TGF-β signaling is driving TNC-induced EMT, migration and drug resistance *in vitro* which could be relevant *in vivo* as TGF-β signaling is instrumental for lung metastasis formation in the MMTV-Neu model (Siegel et al., 2003).

The presence of LVI correlates with worsened patient survival as LVI cause lung thromboembolism and are precursors of parenchymal metastasis (Soerjomataram et al., 2008). Although the existance of LVI is long known (Winterbauer et al., 1968; Kane et al., 1975) their composition and role in lung metastasis is still poorly understood partially due to the lack of relevant murine models that recapitulate human cancer. Here, we provide a

novel and relevant ErbB2-driven syngeneic orthotopic grafting model with spontaneous formation of LVI that can be used to address the role of candidate cells and molecules by genetic engineering of the host and the tumor cells. We engineered TNC expression and observed many similarities between the genetic MMTV-NeuNT and the orthotopic syngeneic grafting model. In particular, we found spontaneous lung metastasis, formation of LVI and a similar impact of TNC on survival and extravasation in both models. Thus the NT193 model may be a relevant model to investigate the roles of LVI in breast cancer lung metastasis.

By tissue staining we found that LVI are organized as tightly packed nests of CTC within blood vessels of the metastatic lung where cells proliferate thus presumably giving rise to huge LVI that eventually occlude the vessel. Yet, this does not have an apparent necrotising effect on the surrounding lung tissue. As the LVI containing vessels were positive for αSMA that is expressed in arterioles (as well as in larger contractile lymphatics (Wang et al., 2010)) and, in addition contain platelets which are absent from lymphatics, we conclude that LVI are mostly present in arterioles in this model. We further observed that LVI are enveloped by distinct layers of stromal cells, which are not found within the CTC nests. In contrast to αSMA+ cells, nor leukocytes, LVI are surrounded by an endothelial cell layer at the border and, a layer of FSP1+ myeloid cells adjacent to the CTC nest. FSP1+ cells are a likely source of TNC whereas a FN/LM layer that is distinct but adjacent to TNC is presumably expressed by the endothelial cells.

To address whether stromal and/or tumor cells express TNC we had grafted NT193 cells into a WT or TNCKO host and observed that TNC is expressed by the host which is

essential for promoting metastasis. This information is new and could be acquired thanks to our novel grafting model. By costaining of TNC with markers for stromal cells, we observed colocalisation of TNC with Fsp1+ cells yet not with αSMA+ cells, nor with endothelial cells, tumor cells or leukocytes. Fsp1+ cells have previously been shown to be a major source of TNC in a 4T1 breast cancer grafting model (O`Connell et al., 2011) and are also a likely provider of TNC in the MMTV-NeuNT and NT193 model. Tumor derived TNC might play a role in CTC which is in agreement with results from Oskarsson et al., (2011) and may even be instrumental in the birth of the LVI (see below, **Fig. S8**). Yet at the stage of an established LVI, tumor cells do not express TNC. When breaching into the lung parenchyma TNC does not even appear to be expressed (our result), whereas in parenchymal metastasis TNC was documented to be expressed at the rim of the lung nodule (Oaskarsson et al., 2011). Here we showed that TNC forms matrix tracks in the parenchymal tissue which is similar to the organization in the primary tumor (data not shown). We suggest that TNC may have a similar role in these matrix tracks as niches for immune cells and promoting angiogenesis (Spenlé et al., 2015) which needs to be further addressed in the future. Interestingly, tumor cells have a mixed epithelial and mesenchymal phenotype in the lung metastasis where vimentin expressing cells are in vicinity to TNC. Moreover, TNC promotes survival of tumor cells in the parenchymal tissue, altogether suggesting a potential role of stromal cell derived TNC in the outgrowth and dissemination of tumor cells in the lung parenchyma.

Platelets are known to promote lung metastasis by several mechanisms initiated by attachment of tumor cells to the vasculature of the metastatic organ (Coupland et al., 2012). We observed platelets as integral component of LVI where they are surrounded by a LM and endothelial cell layer suggesting that platelets may play a role in the birth of LVI.

Platelets have been described as source of TNC and indeed we see colocalization of CD41 with TNC supporting the possibility that platelets are a provider of TNC in the MMTV-NeuNT model. Platelets were also shown to bind to TNC (Schaff et al., 2011) and may play a role in attraction of platelets since we observed less platelets in LVI of MMTV-NeuNT/TNCKO mice. In this first step, it is presumably tumor cell derived TNC that attracts platelets (**Fig. S8**).

Several concepts of how LVI arise and how cells extravasate from there have been proposed (Sugino et al., 2002; Lapis et al., 1988; Mahooti et al., 2010). An endothelial cell layer is often found around LVI and may play an active role in extravasation. Different mechanism of origin of this layer have been proposed such as non-invasive budding (Sugino et al., 2002), transdifferentiation of tumor cells into cells with endothelial characteristics (Mahooti et al., 2010) and endothelialization of tumor cells upon attachment to the lung vasculature (Lapis et al., 1988). We have seen that TNC plays a role in the formation of a surrounding layer of cells that have endothelial properties as they had flat nuclei and were positive for CD31. In the absence of TNC we noticed more LVI that lack such an endothelial cell layer suggesting that TNC has a positive impact on these cells. Endothelial cells are not in direct contact with TNC thus presumably avoiding an inhibitory effect of TNC as seen in other models (Saupe et al., 2013; Rupp et al., 2016). Given that TNC can induce pro-angiogenic factors in tumor cells and carcinoma associated fibroblasts (Rupp et al., 2016), it will be interesting to determine through which pro-angiogenic factors TNC promotes enothelial cell layering of LVI. A potential candidate is pleotrophin, a molecule that was induced by TNC (Rupp et al., 2016) and was seen to be highly expressed in metastatic LVI-inducing MCH66 (but not in non-metastatic MCH) cells (Sugino et al., 2002). Our data do not support a role of tumor cells and their

transdifferentiation into endothelial cells as had been proposed (Mahooti et al., 2010) since stromal cell derived TNC promotes metastasis. Moreover, placement of platelets within LVI and the absence of αSMA+ cells from LVI argues against budding (Sugino et al., 2002) in this model because upon budding platelets are expected to be at the LVI boarder. Our observations support the model of Lapis et al., (Lapis et al., 1988) where endothelialization within arterioles is a driving mechanism in LVI-genesis and extravasation. A potential scenario of LVI formation in MMTV-NeuNT metastasis is presented in **Fig. 7J** and **Fig. S8**. In summary, we have shown that TNC enhances metastasis through promoting survival and EMT in CTC, and increasing abundance of platelets and endothelial cells (**Table S1**).

By a GOF approach TGF-β signaling was identified to drive lung metastasis in the MMTV-Neu model (Siegel et al., 2003). TGF-β signaling is also promoting EMT and through that may increase lung metastasis in the MMTV-Neu model (Siegel et al., 2003). In addition, TNC was shown to cooperate with TGF-β in inducing partial EMT in MCF7 cells (Nagaharu et al., 2011). Here, we addressed whether TNC impacts on EMT and TGF-β signaling. By IF we observed that some CTC in LVI expressed vimentin in addition to E-cadherin (ErbB2 or CK8/18) suggesting a partial EMT. Moreover, vimentin+ cells were also present at the invading front in agreement with the concept of collective invasion (Cheung et al., 2016). Vimentin+ cells were more frequent in TNC expressing LVI suggesting that TNC promotes EMT *in vivo*. TNC-enhanced EMT may account for more extravasation and parenchymal metastasis that we had noticed. These observations are in line with those of others who observed coexpression of TNC with vimentin in breast cancer tissue (Dandachi et al., 2001) and a correlation ot TNC with an EMT signature in colorectal carcinoma (Takahashi et al., 2013). In cultured NT193 cells we showed that TNC induces EMT as vimentin expression increased and E-cadherin expression decreased which was accompanied by induction of

several EMT markers (e.g. MMP9, PAI-1, snail and slug). We observed that TNC rapidly induced canonical (p-Smad) and non-canonical (p-AKT and p-Erk1/2) signaling in cultured NT193 cells with an immediate early response peaking at 1 - 2 hours and a late response at 24 hours. That TNC signaling and EMT were TGF-β dependent was demonstrated by using a TGF-βRI inhibitor that blocked TNC-induced signaling and EMT. Although TNC also induced Wnt signaling, as Axin2 was (slightly but significantly) induced, TNC-induced EMT was reverted with a TGF-βRI inhibitor (despite elevated Axin2) suggesting that TNC induces EMT through TGF-β and not Wnt signaling in this model. We addressed how TNC activates TGF-β signaling and considered that through binding TGF-β (De Laporte et al., 2013), TNC may activate this pathway. But we did not find TGF-β in the TNC solution that was added to the cells. As TNC induced TGF-β signaling rapidly it is unlikey that TNC activates this pathway through increasing expression of molecules in the pathway such as TGF-β receptors or ligands. It is rather likely that TNC activates TGF-β signaling by a direct mechanism that needs to be addressed in future studies.

An important question is whether LVI in human cancers are similarly organized as in this mouse tumor model. It has been reported that LVI are present in several primary tumors where they can be endothelialized (Sugino et al., 2004) which is phenocopied in murine mouse models (Sugino et al., 2002; Oshima et al., 2004). The presence of an endothelial layer in these LVI varies largely between tumor types and seems highest in kidney, liver and thyroid cancers. Our preliminary analysis of human breast, ovarian and pancreatic cancer tissue revealed the presence of a few LVI in each of these cancers where we rarely observed an endothelial layer nor platelets. We also did not observe TNC staining in these LVI (data not shown). As endothelialization is an important step in LVI formation in the MMTV-NeuNT model, where platelets and FSP1+ cells are presumably secreting TNC, it

might not be surprising that in human LVI without an endothelial layer and no platelets, TNC is absent. In addition, in human cancer presumably not all LVI are involved in the extravasation process. LVI extravasation occurs in the lung (and maybe other metastatic organs) which has not been addressed so far in human specimens. A prediction from our study is that highly aggressive LVI in arterioles are endothelialized and express TNC.

Detection of CTC could improve prediction of tumor relapse (Soerjomataram et al., 2008). Yet the current technology selects for smaller CTC cell clusters and thus may miss LVI due to size restriction and their attachment to the arteriole wall (Aceto et al., 2014). Our knowledge about LVI may be exploitable for non-invasive live imaging of LVI (e.g. by imaging of TNC). Targeting TNC expression (e.g. by eliminating FSP1+ cells) might be useful as we have seen that cell survival is compromised in the absence of TNC and, that TNC promoted drug resistance and cell migration through EMT. Elimination of platelets during the birth of a LVI would be another possibility, yet this is not feasible due to complications with bleeding. However, targeting endothelial layering or TGF-β signaling in LVI could be a feasible opportunity to block extravasation of CTC.

Altogether our study has revealed the composition of LVI and identified TNC as an important component of LVI that drives survival, endothelial layering, EMT and extravasation which could have diagnostic and targeting opportunities. Our results suggest that through its impact on endothelial cells and CTC, TNC may promote two mechanisms that enhance extravasation, (i) fusion of endothelial layers leading to ingestion of the LVI and (ii) EMT inducing invasion, survival and presumably drug resistance (**Fig. 7J, Fig. S8**). Finally, our novel orthotopic breast cancer model could be

relevant for improving our understanding of LVI and CTC in breast cancer metastasis as well as for testing drugs targeting metastasis.

Figures

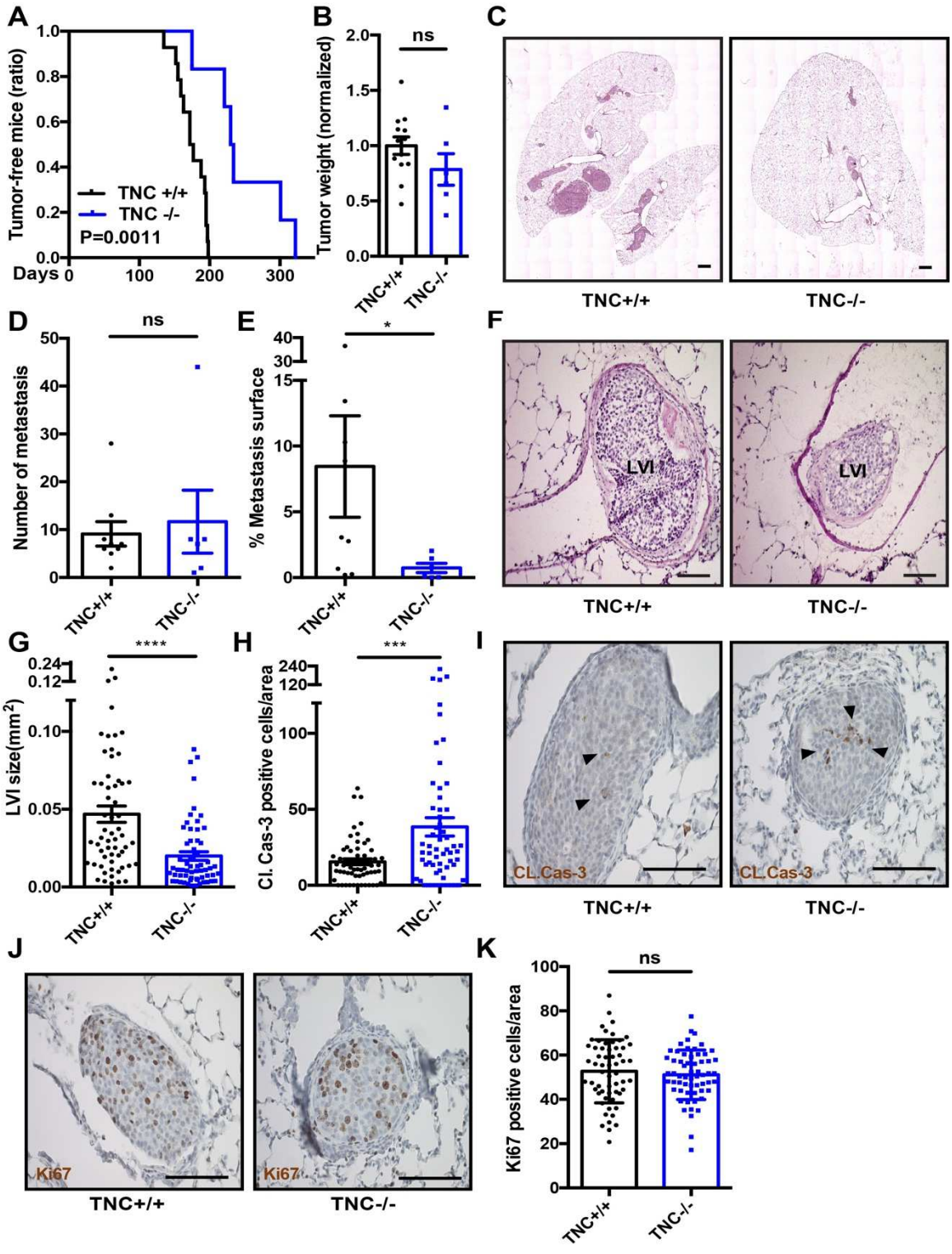

Figure 1. Reduced lung metastasis in the absence of TNC in MMTV-NeuNT mice

(A) Ratio of tumor-free mice is shown for NeuNT tumor mice with two (TNC +/+, N = 14 mice) and no (TNC -/-, N = 6) TNC alleles. The absence of TNC significantly delays tumor latency (TNC +/+ versus TNC -/-, p = 0.0011; Log-rank tests). **(B)** Tumor weight of NeuNT/TNC-/- tumor mice (N = 6) was determined and normalized to the mean of the control group (NeuNT/TNC +/+, N = 13). **(C)** Representative HE images of lung metastasis from MMTV-NeuNT/TNC +/+ and MMTV-NeuNT/TNC -/- mice that had been sacrificed 3 months after tumor detection. Scale bar: 1000 µm. **(D, E)** Number of lung metastases **(D)** and of the cumulated metastatic burden (metastatic area normalized to total lung area) **(E)** in lungs of MMTV-NeuNT/TNC +/+ (N = 9) and MMTV-NeuNT/TNC -/- (N = 6) mice. **(F, G)** HE stained lung tissue was used for LVI size determination (TNC +/+: N = 6 mice, n = 59 LVI; TNC -/-: N = 6 mice, n = 60 LVI). Scale bar: 100 µm. **(H-K)** IHC analysis for cleaved caspase-3 **(I)** and Ki67 **(J)** in LVI (TNC +/+, N = 6 mice, n = 59 LVI; TNC -/-, N = 6 mice, n = 60 LVI). Dots represent number of apoptotic **(H)** and proliferative cells **(K)** in LVI per area (0.1 mm^2), respectively. Arrowhead denotes cleaved caspase-3 positive apoptotic cell **(I)**. Scale bar: 100 µm. Mean ± SEM.

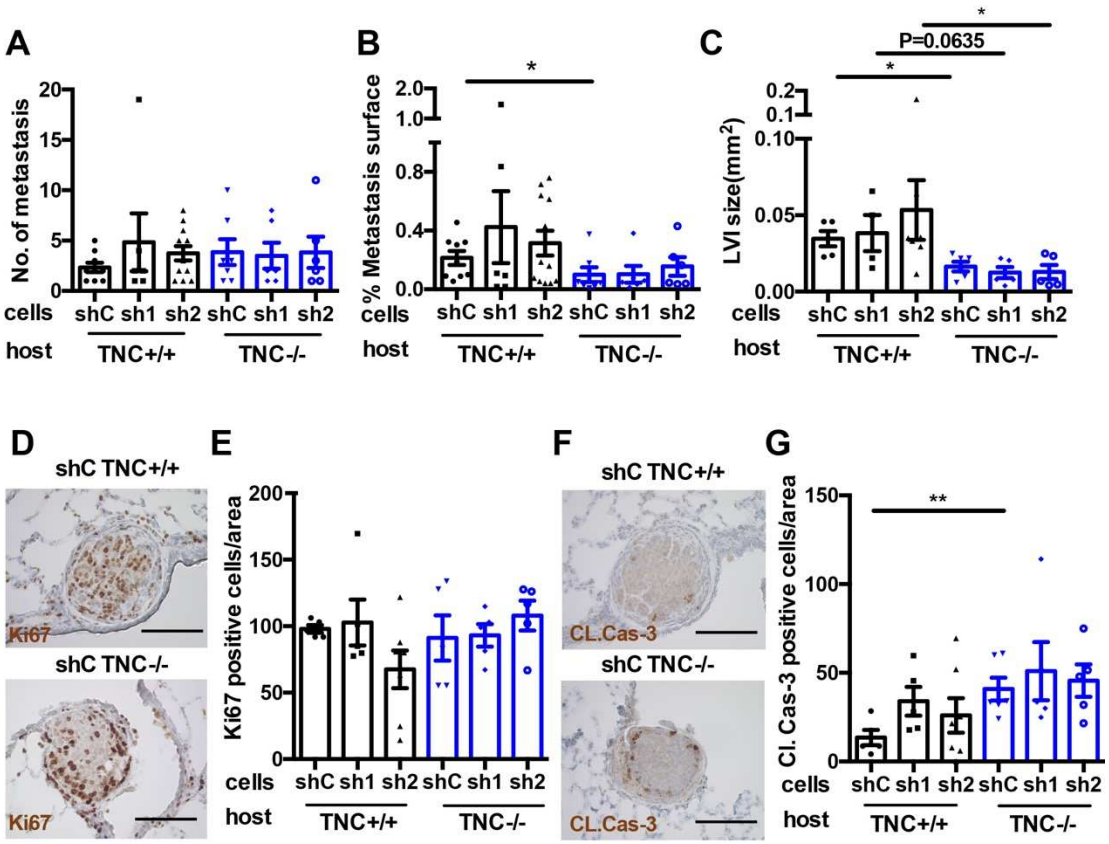

Figure 2. Stromal cell derived tenascin-C promotes LVI and lung metastasis in NT193 grafted tumor mice

(A-C) Quantification of the number of lung metastases **(A)**, cumulated metastatic burden (metastatic area normalized to total lung area) **(B)** and LVI size **(C)** in lungs of TNC+/+ and TNC-/- FVB hosts 3.5 months after engraftment of NT193 sh control (shC), sh1TNC and sh2TNC cells (shC in TNC +/+ mice (N = 5); sh1TNC in TNC +/+ mice (N = 4); sh2TNC in TNC +/+ mice (N = 7); shC in TNC -/- mice (N = 6); sh1TNC in TNC -/- mice (N = 5); sh2TNC in TNC -/- mice (N = 5). **(D-G)** IHC analysis for Ki-67 **(D)** and cleaved caspase-3 **(F)** in LVI of lungs from NT193 engrafted mice. Dots represent proliferative **(E)** and apoptotic cells in LVI **(G)** per 0.1 mm^2, respectively. Scale bar: 100 µm. Mean ± SEM.

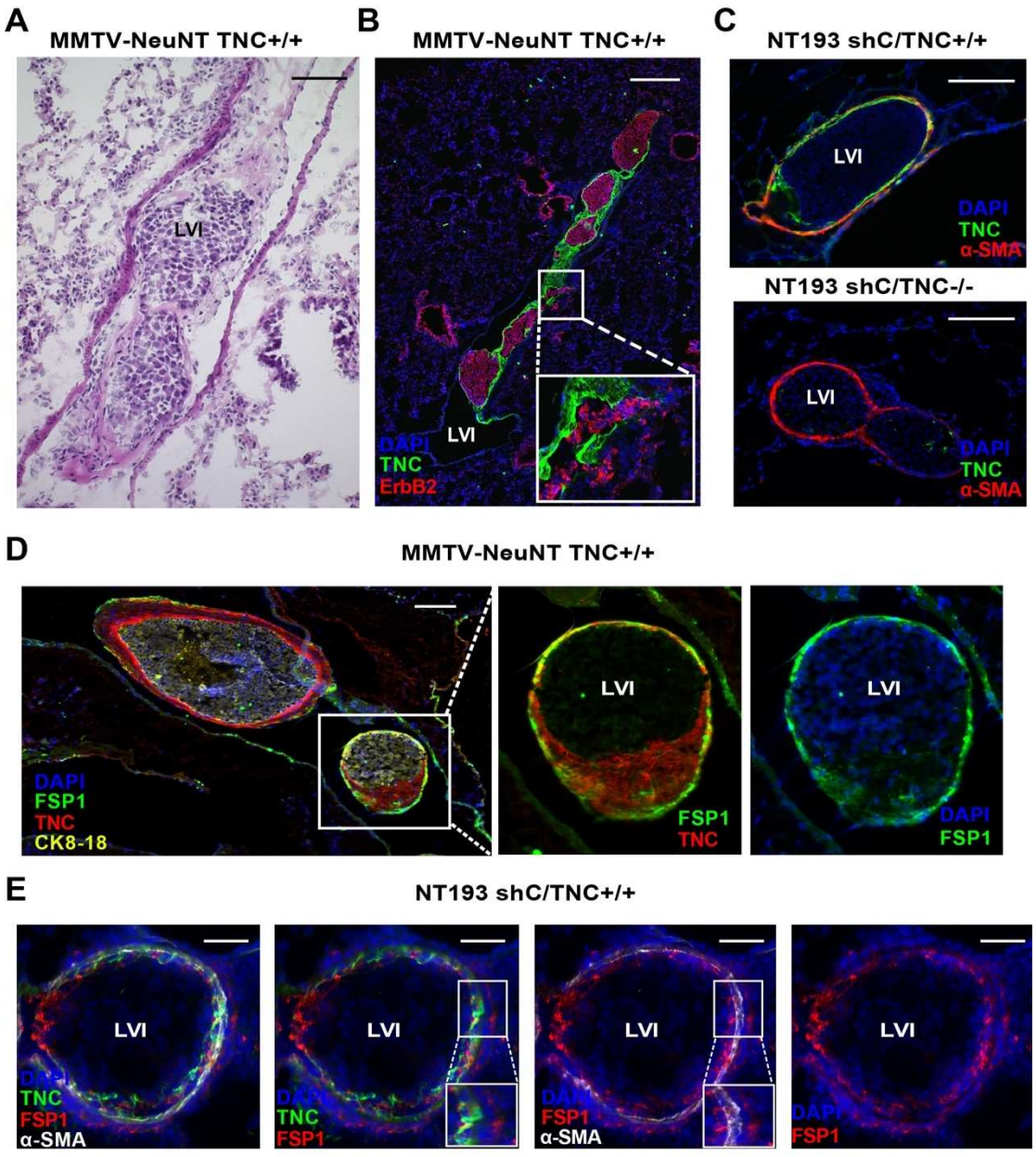

Figure 3. LVI are CTC clusters surrounded by stromal cell-derived tenascin-C

(A, B) Representative images of LVI in MMTV-NeuNT/TNC+/+ lungs upon HE and IF staining for the indicated molecules. **(B)** Note that TNC (green) is expressed around tumor cells in LVI (red, ErbB2). Cell nuclei stained with DAPI. Scale bar: 100 µm **(A)**, 500 µm **(B)**. **(C)** Representative IF images for TNC (green) in lung LVI of tumor cell grafts of NT193 shC cells in TNC+/+ and TNC-/- host. α-SMA staining (red) marks blood vessels. Note, that TNC is expressed in LVI of shC cells engrafted in TNC+/+ host, yet not in a TNC -/- host. Scale bar: 100 µm. **(D, E)** Representative IF images for FSP1+ myeloid cells and

TNC in LVI of MMTV-NeuNT/TNC+/+ **(D)** and NT193 lung tissue **(E)**. Scale bar: 100 µm **(D)**, 50 µm **(E)**. White square represents area of higher magnification.

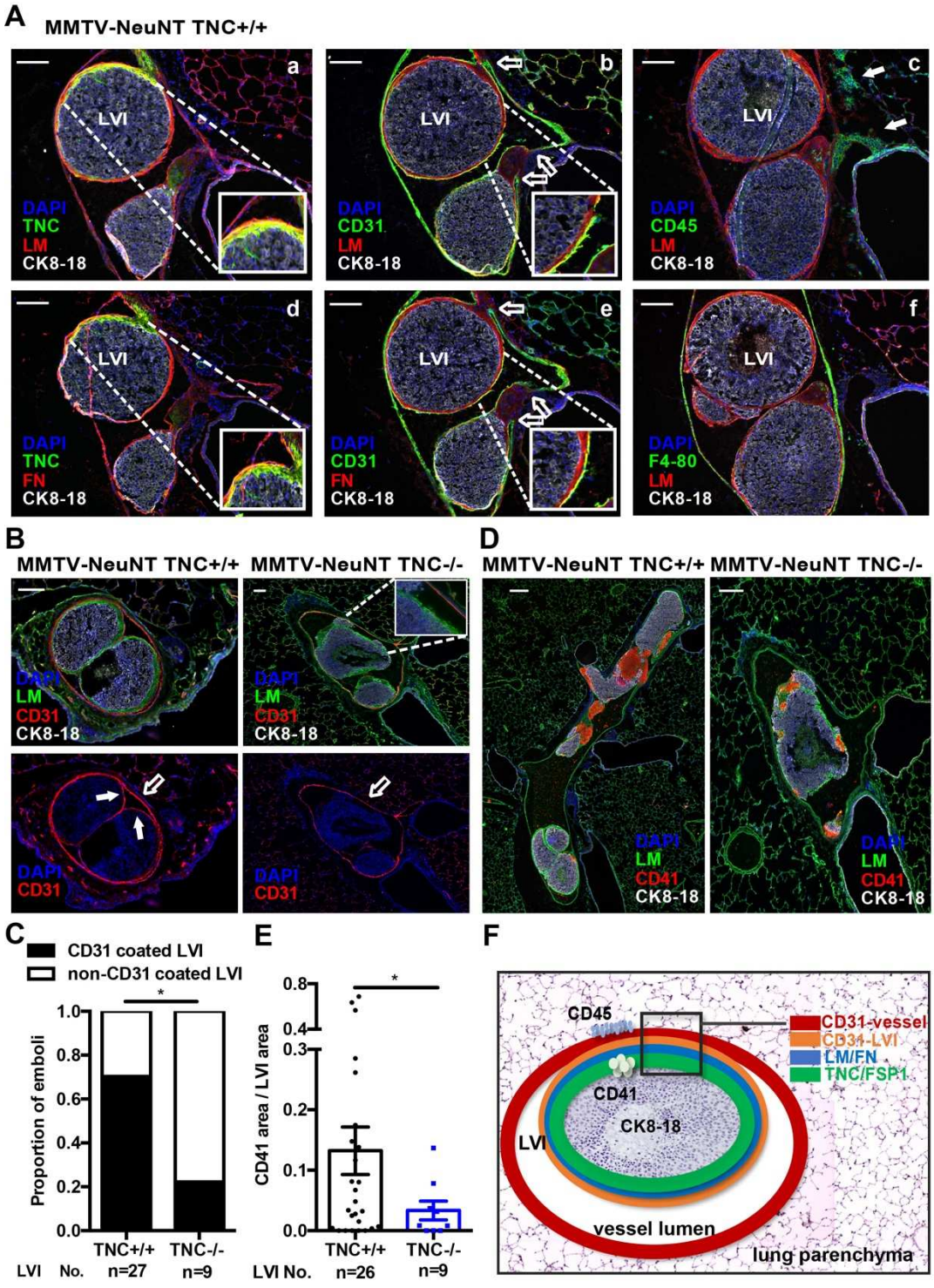

Figure 4. Architecture of LVI: CTC cluster is enveloped by ECM where tenascin-C increases abundance of endothelial cells and platelets

(A) Representative pictures of immunostainings for the indicated ECM molecules and cellular markers in MMTV-NeuNT/TNC+/+ mouse LVI. Note that tumor LVI are composed

of tightly packed cancer cells (CK8/18+) that are surrounded by FSP1+ myeloid cells and CD31+ endothelial cells which presumably express laminin (LM)/fibronectin (FN) and TNC, respectively. F4/80+ macrophages are seen at the lung arteriole that contains LVI yet not in the adjacent LVI-free capillary nor a bronchiole. White squares in each panel delineate the field shown at higher magnification. The empty arrows point at narrowing of endothelial layers reminiscent of fusion of the endothelial layers from the LVI and the lung vasculature. CD45+ leukocytes are not directly associated with the LVI but are present at the basal side of the vessel wall facing the lung parenchyma. Arrows point at CD45+ cells. Scale bar: 100 µm. **(B)** Representative IF images for CD31+ endothelial cells in lungs of MMTV-NeuNT/TNC+/+ and MMTV-NeuNT/TNC-/- mice. Arrows point at the endothelial cell layer that surrounds the tumor LVI. Note the absence of an endothelial layer in the TNC-/- LVI. The empty arrow points at a blood vessel wall. White square represents area of higher magnification. Scale bar: 100 µm. **(C)** Proportion of LVI with and without a CD31 layer for each genotype (TNC +/+ N = 6 mice, n = 27 LVI; TNC -/-, N = 4 mice, n = 8 LVI). **(D)** Representative IF images of LVI for CD41+ platelets together with LM in lungs of MMTV-NeuNT/TNC+/+ and MMTV-NeuNT/TNC-/- mice. Scale bar: 200 µm. **(E)** Platelet abundance (CD41 positive area normalized to the LVI area), TNC +/+ N = 6 mice, n = 26 LVI; TNC -/-, N = 4 mice, n = 9 LVI. **(F)** Schematic depicting the composition of tumor LVI. Note, that in LVI CTC are surrounded by an ECM capsule which contains consecutive layers of TNC and FN/LM. LVI contains an endothelial cell layer and platelets indicating their involvement in LVI formation. Leukocytes are present at the basal side of the vessel wall facing the lung parenchyma. Mean ± SEM.

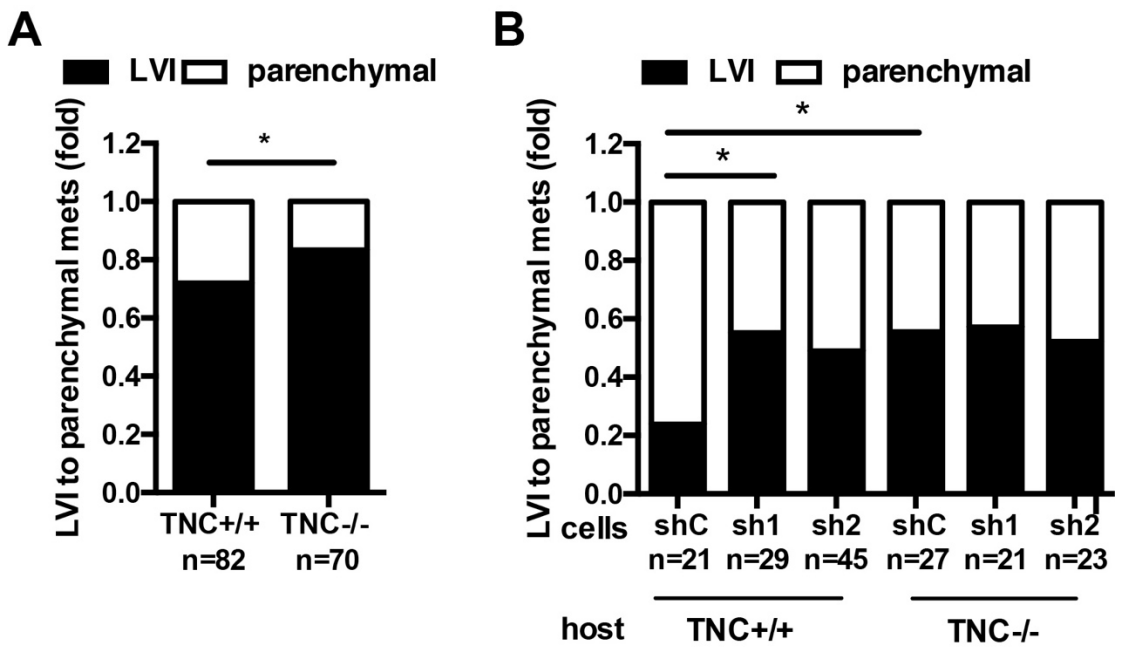

Figure 5. Tenascin-C promotes extravasation of CTC from LVI into lung parenchyma

(A, B) Proportion of LVI to parenchymal metastases in MMTV-NeuNT mice **(A)** (TNC +/+, N = 6 mice; TNC -/-, N = 6 mice) and in NT193 grafted mice **(B)** (shC in TNC +/+ mice (N = 5); sh1TNC in TNC +/+ mice (N = 4); sh2TNC in TNC +/+ mice (N = 7); shC in TNC -/- mice (N = 6); sh1TNC in TNC -/- mice (N = 5); sh2TNC in TNC -/- mice (N = 5). Mean ± SEM. Note, that (stromal cell derived) TNC increases parenchymal metastasis.

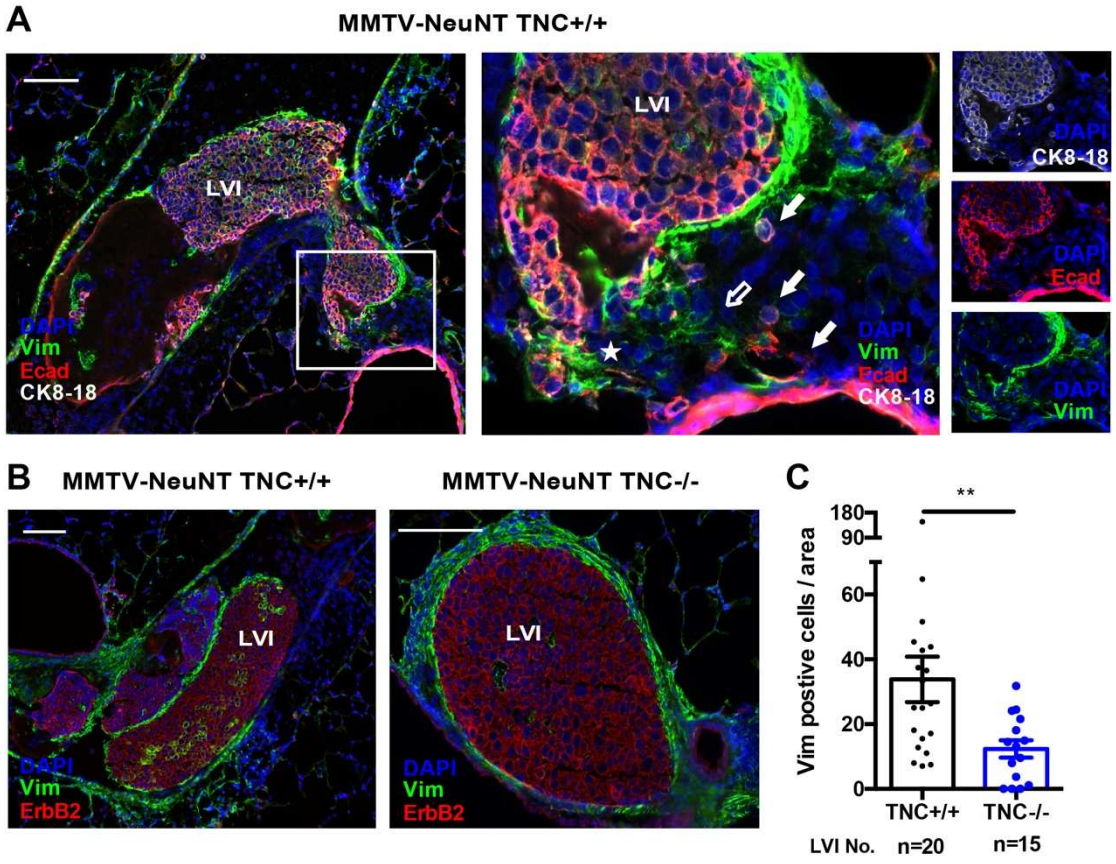

Figure 6. Partial EMT in LVI

(A) Representative IF images for vimentin (green), E-cadherin (red) and CK8/18 (white) in LVI of MMTV-NeuNT/TNC+/+ mice. White squares delineate areas of higher magnification. Note that tumor cells (CK8/18+) are leaving LVI and invade into the parenchymal lung tissue. Arrows point at single invading tumor cells with epithelilal characteristics (CK8/18+ and E-cadherin+) whereas the empty arrow points at invading mesenchymal vimentin+ and E-cadherin- cells. Star points at an event at the invading front reminiscent of collective tumor cell invasion. Scale bar: 100 µm. **(B)** Representative IF images of vimentin+ (green), ErbB2+ (red) cells in LVI of MMTV-NeuNT/TNC+/+ and MMTV-NeuNT/TNC-/- mice. Scale bar: 100 µm. **(C)** Quantification of tumor cells expressing both vimentin (green) and ErbB2 (red) normalized per LVI area (0.1 mm^2). MMTV-NeuNT (TNC +/+, N = 6 mice, n = 20 LVI and TNC-/-, N = 4 mice, n = 15 LVI).

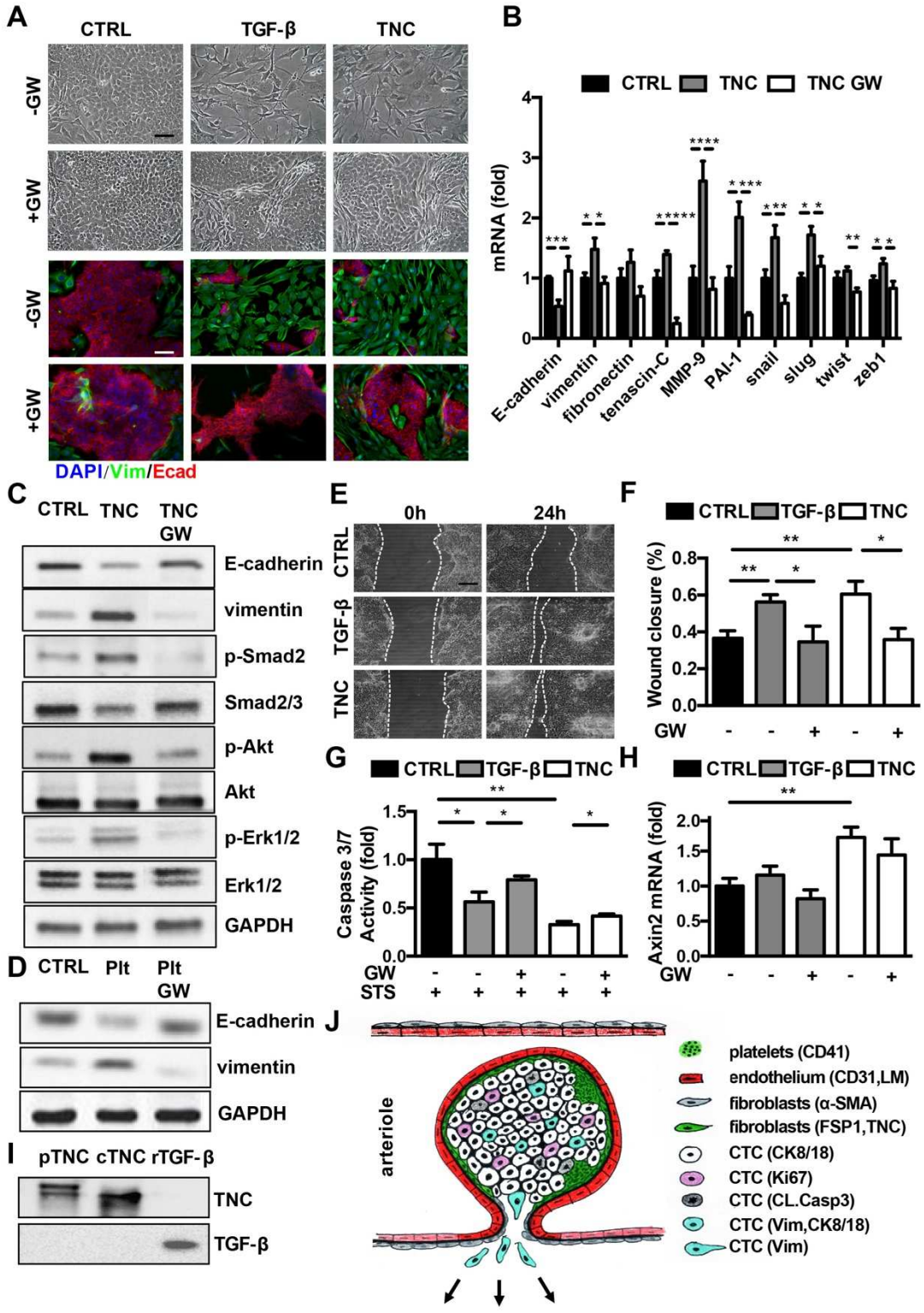

Figure 7. Tenascin-C induces EMT in cultured NT193 cells involving TGF-βRI, promoting cell migration and survival

(A) Phase contrast micrographs and IF images of E-cadherin (red) and vimentin (green) stained NT193 cells treated with 5 µM TGF-βR1 inhibitor (GW788388) prior to addition of TNC (10 µg/ml) and TGF-β (10 ng/ml) for 24h, respectively. Nuclei stained by DAPI. Scale bar: 20 µm. **(B)** Relative expression (fold change) of the indicated genes in NT193 cells upon treatment with 5 µM TGF-βR1 inhibitor (GW788388) and TNC (10 µg/ml) for 24h (n = 5, five independent experiments) with normalization to GAPDH. **(C)** Detection of E-cadherin, vimentin, phosphorylated Smad2, Akt, and Erk1/2, respectively and expression of total Smad2/3, Akt, Erk1/2 by immunoblotting with GAPDH as loading control (one representative result of three independent experiments is shown). **(D)** Detection of E-cadherin and vimentin expression by immunoblotting of lysates from NT193 cells treated with platelets for 24 h (n = 3, three independent experiments). **(E, F)** Wound closure of NT193 cells upon treatment as indicated, n = 14, five independent experiments with at least two replicates. Scale bar: 20 µm. **(G)** Assessment of staurosporine (STS) - induced apoptosis by measuring caspase-3/7 activity in NT193 cells treated as indicated, n = 9, three independent experiments in triplicates. **(H)** Fold change of Axin2 mRNA levels in NT193 cells upon indicated treatment, n = 5, five independent experiments. Normalization to GAPDH. **(I)** Detection of TGF-β by immunoblotting with two distinct TNC preparations; pTNC purified recombinant his-tagged TNC (Huang et al., 2001), 10 µg; cTNC commercial TNC, 10 µg) and rTGF-β (recombinant TGF-β 10 ng) as positive control. Mean ± SEM. **(J)** Summary cartoon of TNC impact on LVI formation facilitating extravasation. TNC promotes abundance of platelets suggesting a potential role in attachment to the arteriole wall. Platelets may play an early role in LVI-genesis since they are found inside the LVI wrapped by a LM and endothelial cells. TNC increases survival. TNC promotes endothelialization of LVI thus presumably enabling extravasation by endothelial layer fusion resulting in LVI ingestion (**Fig. S8**). Through EMT TNC may promote single cell and collective cell invasion and thus extravasation**.**

Supplementary figures

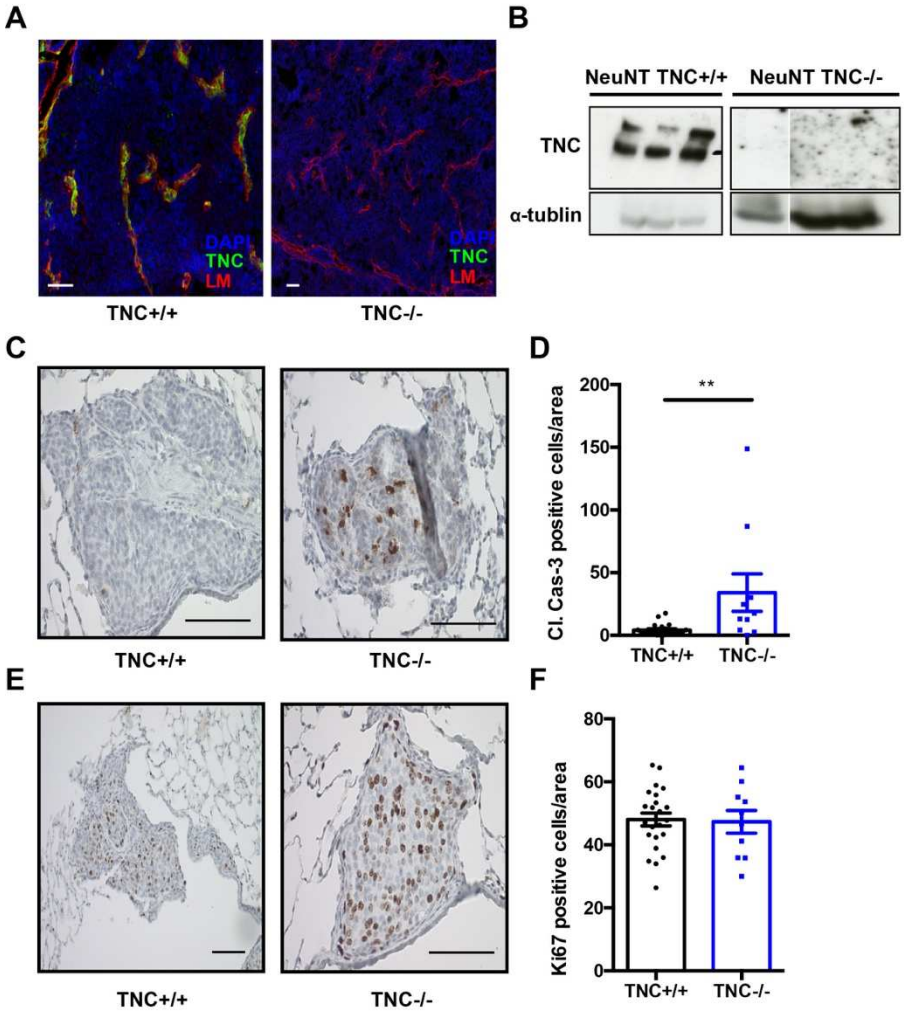

Figure S1. Tenascin-C promotes survival of cancer cells in parenchymal metastasis, yet not proliferation

(A) Representative IF images for TNC (green) and laminin (red) of primary tumors of MMTV-NeuNT/TNC+/+ and TNC-/- mice, respectively**. (B)** Representative immunoblot of TNC in MMTV-NeuNT tumors with α-tubulin as control. Note no detection of TNC in TNC-/- tumors. (**C-F)** IHC analysis for cleaved caspase-3 **(C)** and Ki67 **(E)** in parenchymal metastases (TNC +/+: N = 6 mice, n = 23 metastases; TNC -/-: N = 6 mice, n = 10 metastases). A dot represents the accumulated number of apoptotic **(D)** and proliferative cells **(F)** per area (0.1 mm^2) in parenchymal metastasis. Scale bar: 100 µm. Mean ± SEM.

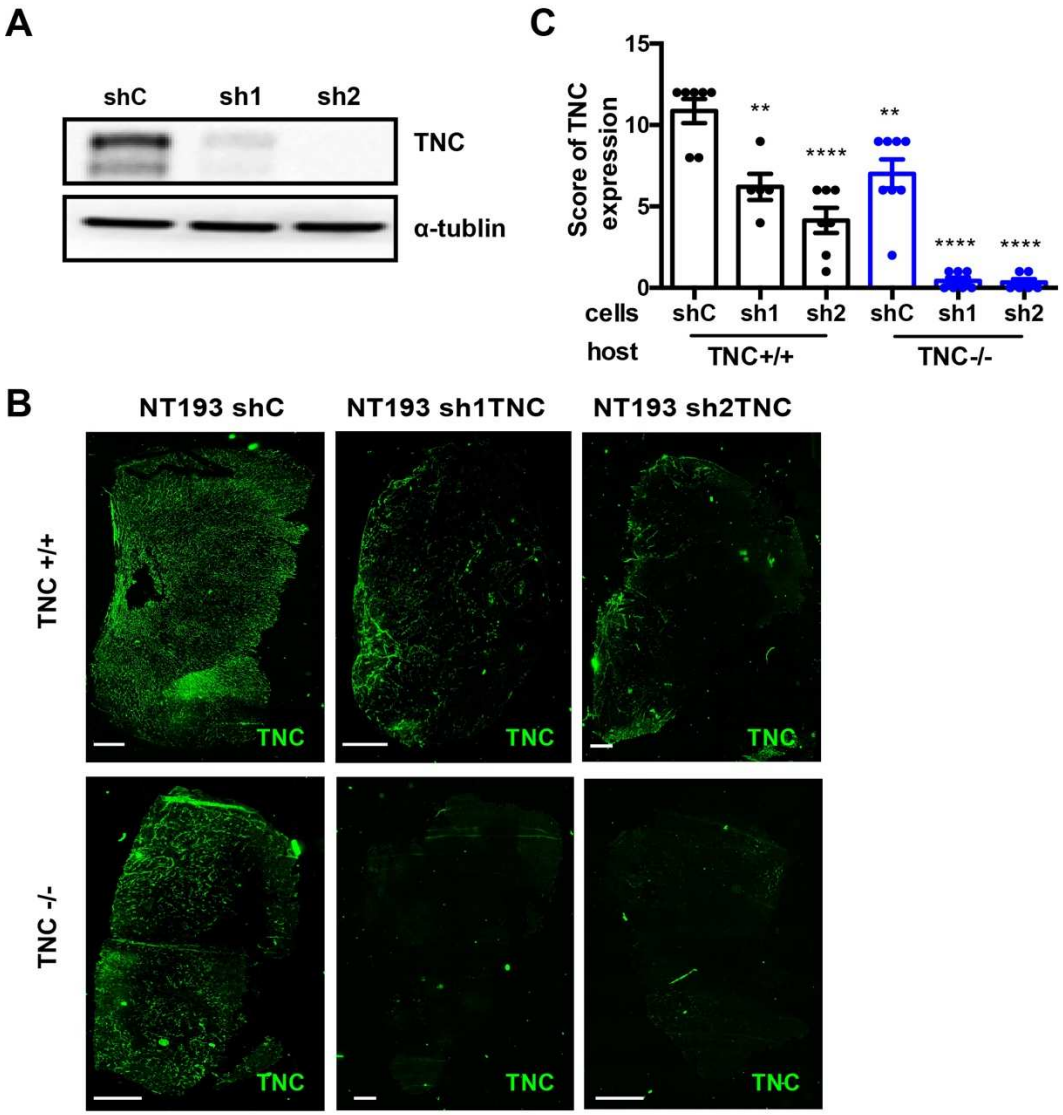

Figure S2. Knockdown of tenascin-C in NT193 cell in vitro and in tumor grafts

(A) Immunoblotting for TNC in cultured NT193 control (shC) and TNC KD cells (sh1 and sh2). Loading control, α-tubulin. **(B)** Representative mosaic IF images of TNC (green) in primary tumors, 3.5 months after engraftment of NT193 shC and TNC KD cells in the mammary fat pad of TNC+/+ and TNC-/- FVB mice, respectively. Scale bar: 1000 µm. **(C)** Quantification of the TNC score in the primary tumor of each grafting condition (see material and methods section for details) as means to determine TNC expression compared to TNC+/+ mice injected with shC cells. N = 6 mice for each grafting condition. Mean ± SEM.

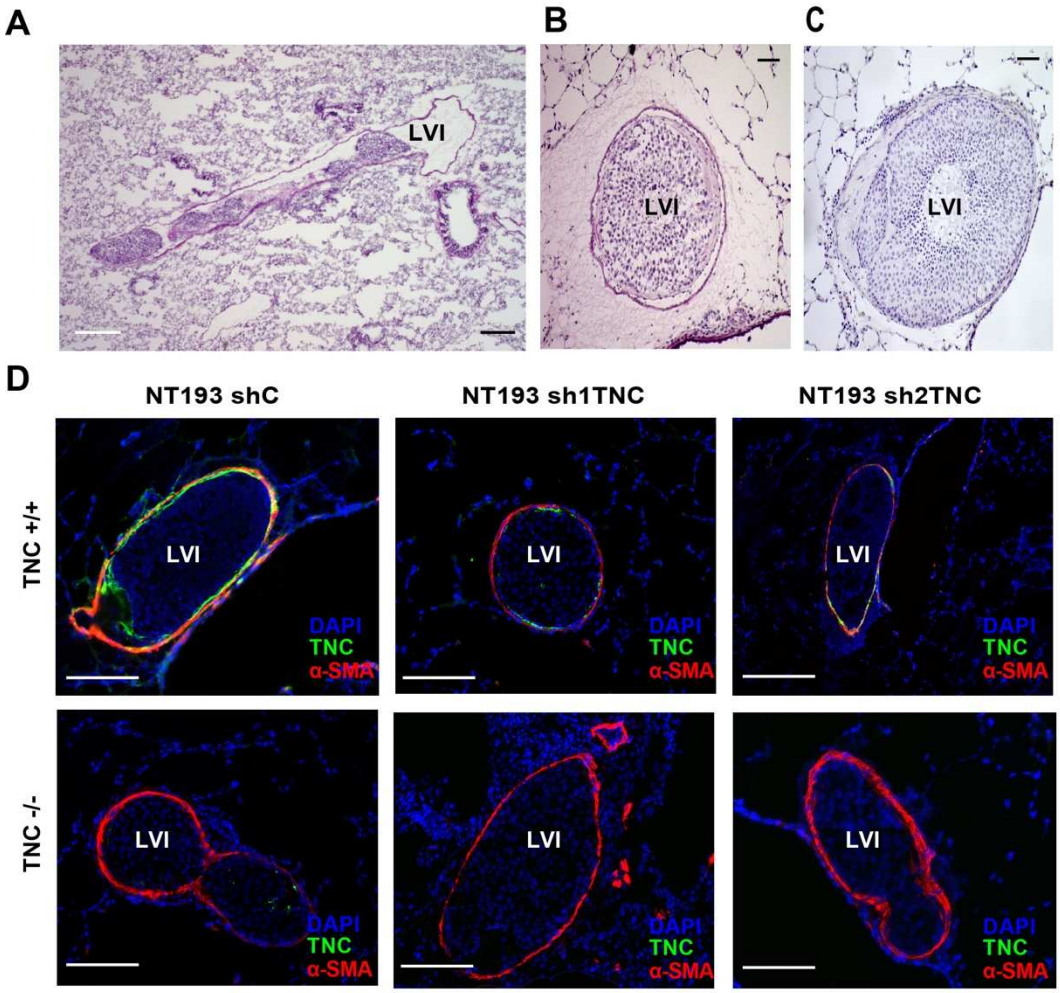

Figure S3. CTC in LVI are surrounded by a layer of stromal cell-derived tenascin-C

(A - C) Representative HE images of LVI in lung blood vessels from MMTV-NeuNT/TNC+/+ mice. **(B, C)** Note that LVI eventually can occlude the vessel lumen and that the central LVI area can be necrotic as indicated by the absence of nucleated cells **(C)**. Scale bar: 200 µm **(A),** 50 µm **(B, C)**. **(D)** Representative IF images of TNC (green) in LVI of lungs from NT193 cell grafted mice. αSMA staining (red) marks blood vessels. Note that TNC is expressed at the rim of LVI if the host (TNC+/+ FVB) expresses TNC yet not in a host (TNC -/-) that lacks TNC. In a TNC -/- host some TNC is expressed by the tumor cells. Scale bar: 100 µm.

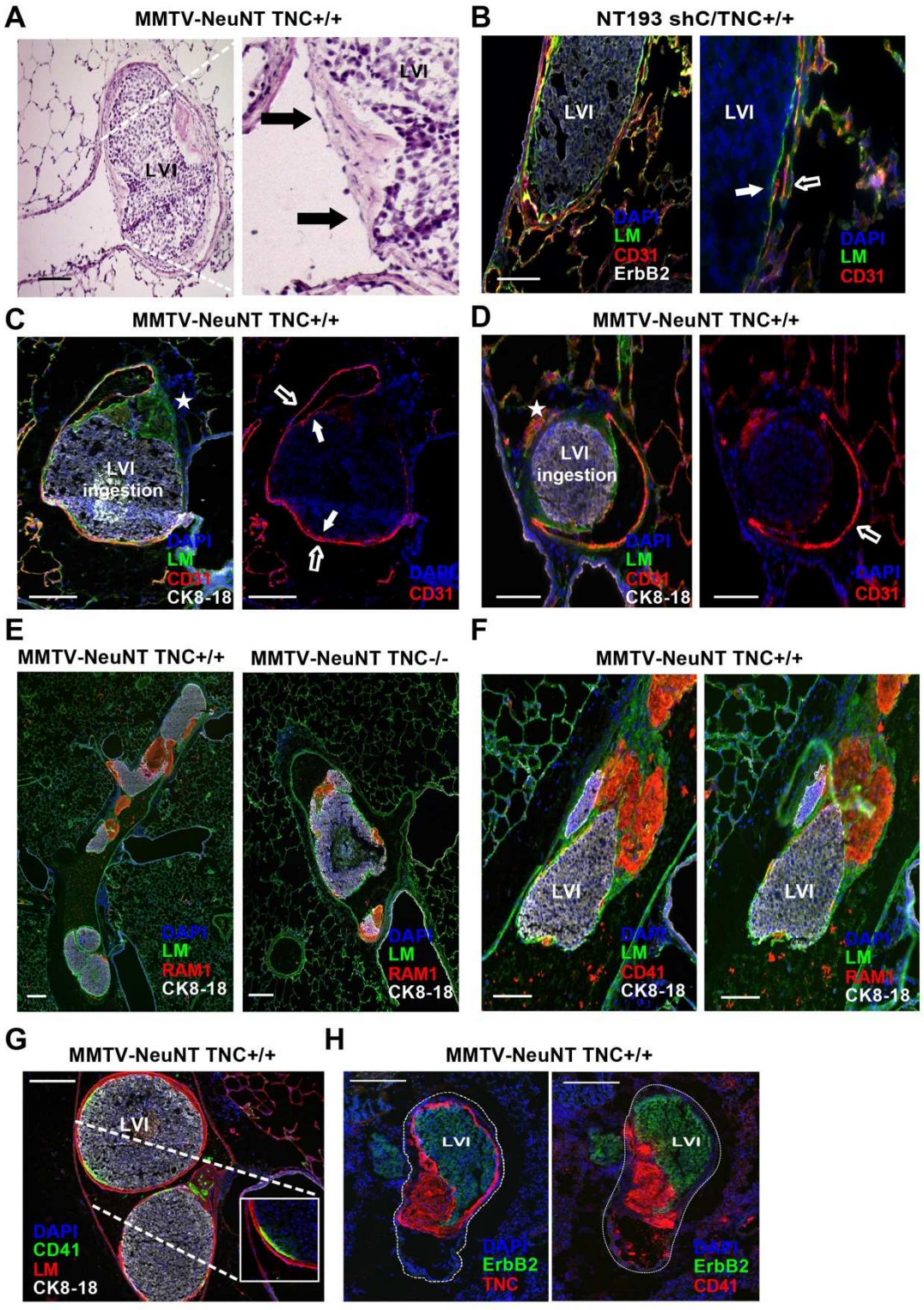

Figure S4. Presence of endothelial cells and platelets in LVI of lungs from MMTV-NeuNT and NT193 grafted mice

(A) Representative HE images of MMTV-NeuNT/TNC+/+ LVI. Note a layer of cells with flat nuclei at the LVI border (arrow). A higher magnification is shown in the right panel. Scale bar: 100 µm. **(B)** Representative IF images of CD31+ endothelial cells in lung LVI of NT193 grafted mice. A higher magnification is shown in the right panel. The filled arrow points at the layer of endothelial cells that surround the LVI, the empty arrow points at a blood vessel. Scale bar: 100 µm. (**C, D**) Representative IF images of CD31+ endothelial cells in the injesting LVI (**C**) and injested LVI (**D**) of MMTV-NeuNT/TNC+/+ mice. The filled arrow points at the layer of endothelial cells that surround the LVI, the empty arrow points at a blood vessel. Star points at the endothelial cell bare area of the invading LVI. Scale bar: 100 µm. **(E-H)** Representative IF images of RAM1 (Gp1b) or CD41 positive platelets together with laminin in LVI of MMTV-NeuNT/TNC+/+ and TNC -/- mice. Note that platelets and CTC are enveloped by a common laminin layer **(F, G)**. Scale bar: 200 µm **(E)**, 100 µm **(F, G)**. **(H)** Representative IF images of LVI for CD41+ platelets or TNC together with ErbB2+ tumor cells in lungs of MMTV-NeuNT/TNC+/+ mice. Scale bar: 100 µm.

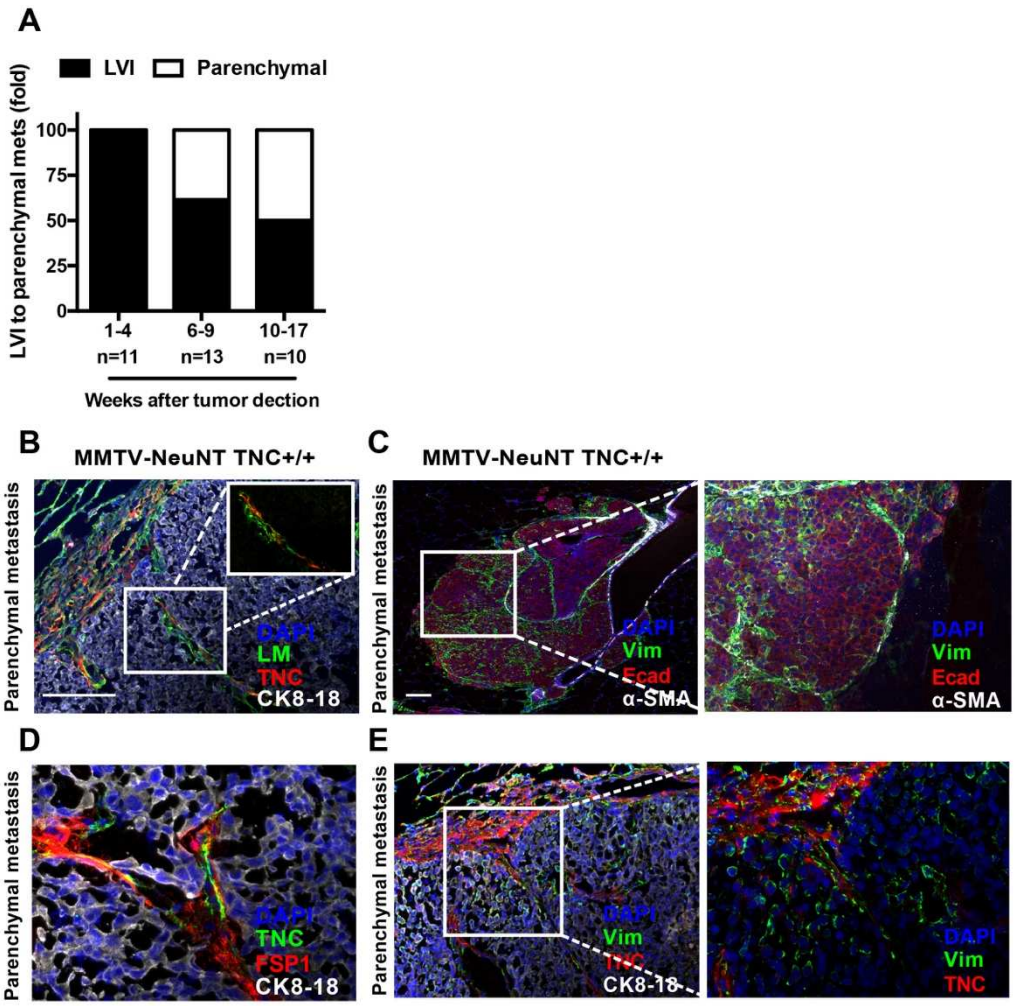

Figure S5. Kinetics of parenchymal lung metastasis and mixed mesenchymal and epithelial features of parenchymal metastasis in MMTV-NeuNT

(A) Proportion of LVI and parenchymal lung metastases in MMTV-NeuNT mice sacrificed at distinct time points after first tumor detection. 1 - 4 weeks, N = 3 mice; 6 - 9 weeks, N = 3 mice; 10 - 17 weeks, N = 3 mice. **(B-E)** Representative IF images of lung parenchymal metastases of TNC +/+ mice. White squares delineate fields of higher magnification. **(B)** Note that TNC (red) and laminin (green) form tracks inside and at the periphery of parenchymal metastases. Scale bar: 100 µm. **(C)** Note that FSP1 and TNC staining partially overlaps. Scale bar: 50 µm. **(D)** Note that cells have a mixed phenotype as indicated by expression of E-cadherin and vimentin. Scale bar: 100 µm. **(E)** Note close apposition of TNC to vimentin+ cells. Scale bar: 100 µm. Mean ± SEM.

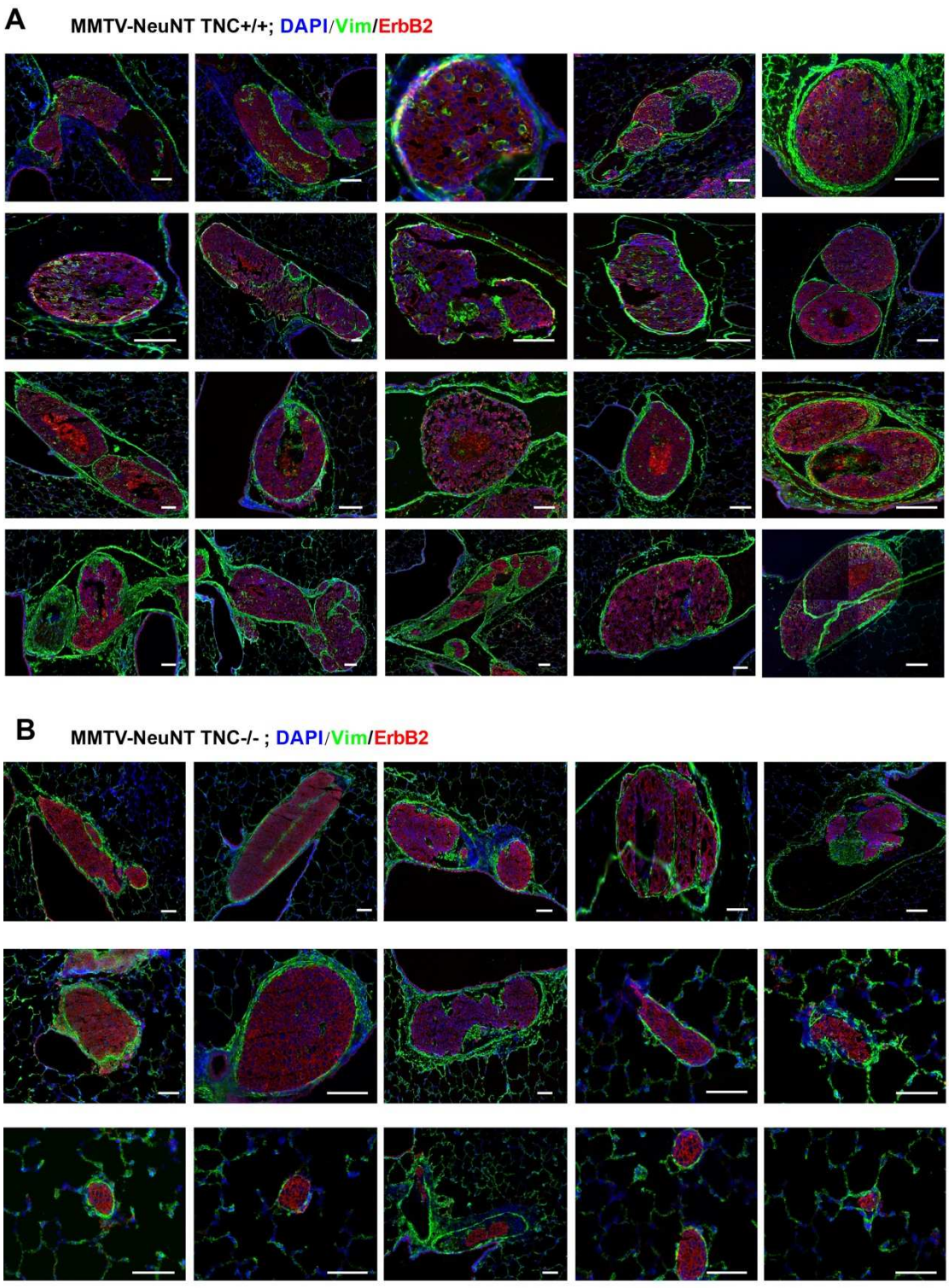

Figure S6. Partial EMT in LVI of MMTV-NeuNT lung tissue

(A, B) Representative IF images of vimentin+ (green) and ErbB2+ (red) cells in LVI of lung tissue from MMTV-NeuNT TNC +/+ (N = 6 mice, n = 20 LVI) **(A)** and TNC -/- mice (N = 4 mice, n = 15 LVI) **(B)**. Scale bar: 100 µm.

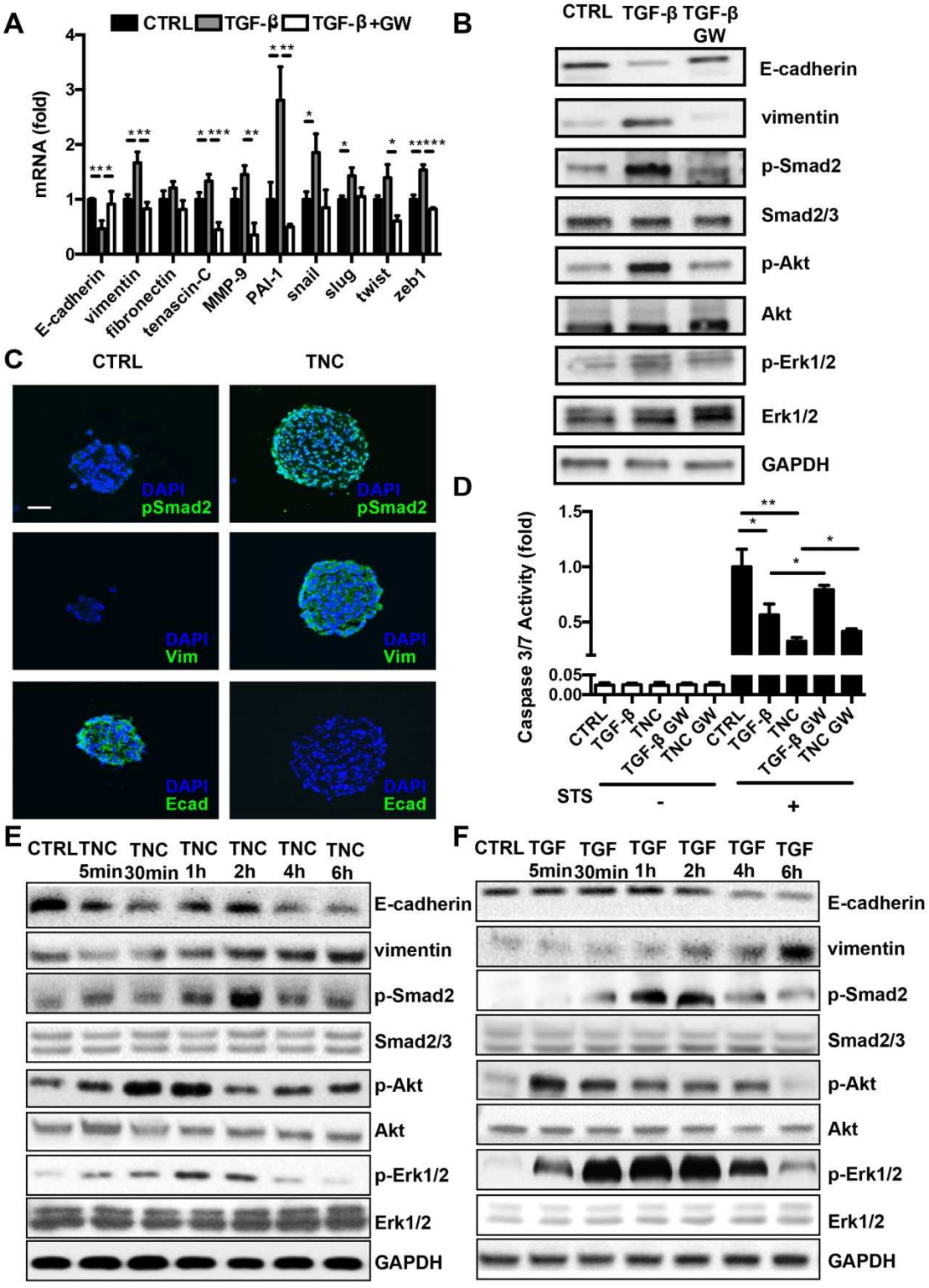

Figure S7. Role of TGF-β and TNC on EMT and cell behaviour of cultured NT193 cells

(A) mRNA expression levels of the indicated genes (fold change) in NT193 cells upon treatment with 5 µM TGF-βR1 inhibitor (GW788388) and TGF-β (10 ng/ml) for 24 h (n = 5, five independent experiments). Normalization to GAPDH. **(B)** Detection of E-cadherin, vimentin, phosphorylated Smad2, Akt, and Erk1/2, respectively and total Smad2/3, Akt, Erk1/2 by immunoblotting of lysates from NT193 cells treated as indicated. GAPDH was used as loading control (n = 3, three independent experiments). **(C)** IF images of E-cadherin, vimentin and phospho-Smad2/3 (green) of NT193 spheroids upon treatment with TNC (10 µg/ml). Cell nuclei stained with DAPI. Scale bar: 20 µm. **(D)** Assessment of staurosporine (STS)-induced apoptosis by measuring caspase-3/7 activity in NT193 cells treated as indicated, n = 9, three independent experiments in triplicates. **(E, F)** Detection of E-cadherin, vimentin, phosphorylated Smad2, Akt, and Erk1/2, respectively and total Smad2/3, Akt, Erk1/2 by immunoblotting of lysates from NT193 cells treated as indicated. GAPDH was used as loading control (n = 3, three independent experiments). Mean ± SEM.

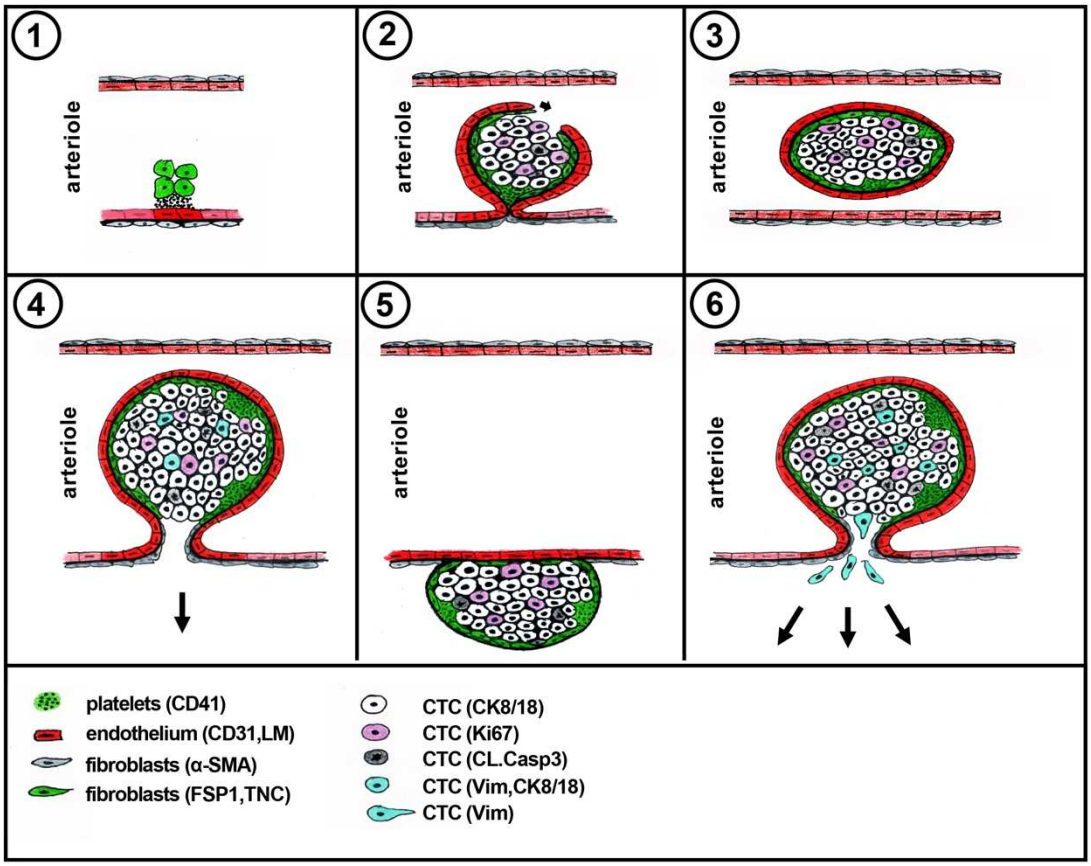

Figure S8. Summary cartoon of TNC enhancing parenchymal lung mestastasis by two mechanisms with LVI as critical precursors

1.- CTC are trapped by platelets at the lung arteriole wall. At this step CTC may express TNC which is consistent with results from another model where TNC expression in CTC was shown to be essential for breast cancer lung metastasis (Oskarsson et al., 2013). We have shown that TNC promotes abundance of platelets suggesting a potential role in platelet attachment which may involve a mechanism described in thrombus formation (Schaff et al., 2011). **2.-** Attachment may induce remodeling of the lung vessel endothelium triggering endothelialization, thus leading to a monolayer of endothelial cells around CTC, generating a LVI. In addition to endothelial cells also FSP1+ myeloid cells found underneath the LVI basement membrane (thick black lining) are involved in endothelialization. FSP1+ cells are already present in the arteriole wall where they do not express TNC which is in contrast to the LVI, where FSP1+ cells are positive for TNC (green) (**Fig. 3D, E**). Two possibilities are consistent with our observations. (i) The LVI may be released into the circulation (**step 3**, as we see "floating" LVI, **Fig. 3D, S3B**) and

then attach to another arteriole or (ii) LVI remain attached to the arteriole wall and continue to expand. **4.-** Walls of arterioles that are occupied by LVI are positive for activated macrophages (F4/80) indicating activation of the endothelium which may contribute to fusion of endothelial cells from the LVI and the arteriole. **5.-** An LVI may be ingested and grow into parenchymal metastasis (**Fig. S4C**). During expansion of an already attached LVI some CTC proliferate indicating stem like properties, other CTC die via apoptosis. Cell survival is promoted by TNC. **6.-** In addition to E-cadherin, some CTC express vimentin indicating partial EMT of CTC which is enhanced by TNC. TNC-driven EMT may involve TGF-β as TNC triggers EMT in cultured NT193 cells in a TGF-β-dependent manner (**Fig. 7**). Some cells at the invading front are exclusively vimentin+, altogether reminiscent of single cell and collective cell invasion. Altogether these events result in parenchymal metastasis which is increased by TNC and may involve TGF-β (Siegel et al., 2003). TNC enhances metastasis through several mechanisms promoting survival and EMT in CTC, increasing abundance of platelets and endothelial cells in LVI. Thus, targeting FSP1+ cells (to reduce TNC expression) as well as inhibiting TGF-β signaling downstream of TNC and endothelial cell remodeling (which is a current therapeutic vision, Vasudev et al., 2014) may be novel targeting opportunities to block LVI extravasation.

Material and methods

Mice

MMTV-NeuNT female mice (FVB/NCrl background) with a mutated constitutively active form of rat ErbB2 (NeuNT), expressed under control of the mouse mammary tumor virus (MMTV) regulatory region (Muller et al.,1988), were kindly provided by Gerhard Christofori (University of Basel, Switzerland). Mice expressing NeuNT develop multifocal breast adenocarcinoma and lung metastasis; TNC +/- mice in the 129/Sv genetic background were generously donated by Reinhard Fässler (Talts et al., 1999). Ten consecutive crosses with FVB/NCrl mice (Charles River) were done to homogenize the background. TNC +/- males were crossed with TNC +/- females to obtain TNC+/+ (WT) and TNC-/- (KO) littermates; MMTV-NeuNT mice (FVB/NCrl background) were crossed with TNC+/- mice to generate double-transgenic mice to obtain MMTV-NeuNT mice with a TNC+/+ and TNC-/- genotype. All mice were housed and handled according to the guidelines of INSERM and the ethical committee of Alsace, France (CREMEAS) (Directive 2010/63/EU on the protection of animals used for scientific purposes).

Cell culture

NT193 cells derived from a MMTV-NeuNT primary tumor (Arpel et al., 2014) were cultured in DMEM medium with 4.5 g/L glucose (GIBCO) supplemented with 10 % of inactivated fetal bovine serum, penicillin (10 000 U/ml) and streptomycin (10 mg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2.

Animal experiments

Tumor size was measured every 3 or 7 days with a caliper, and tumor volume was calculated using the formula $V = (a^2 * b)/2$, where b is the longest axis and a is the perpendicular axis to b. For the syngeneic mouse model**,** 10×10^6 NT193 cells were diluted in 50 µL PBS and injected orthotopically into the left fourth mammary glands of the indicated mice. Mice were euthanized at indicated time points and breast tumors and lungs were processed for histological analysis and western blot. Tissue was snap frozen in liquid nitrogen or embedded in O.C.T. (Sakura Finetek) as well as in paraffin for future analysis by qRT-PCR and immunostaining.

Tissue analysis

The sterelogical analysis of the lung metastasis (index and number) was done as published (Nielsen et al., 2001). Briefly, the left lung lobe was cut transversally into 2.0 mm thick parallel pieces, giving rise to a total of five to six pieces before paraffin embedding in parallel orientation, and cutting into 7 ¥ m thick sections. In cases where no metastasis was found, 8 to 10 additional sections separated by 200 µm were analyzed.

HE staining

Tissue from breast tumors and lungs were prepared and fixed overnight in 4% PFA, dehydrated in 100% ethanol for 24 h, embedded in paraffin, cut in 7 mm thick sections, dewaxed and rehydrated with 100% Toluene (2 washes of 15 min) then incubated in 100%–70% alcohol solutions (10 min each) followed by a final staining with hematoxylin (Surgipath) for 5 min and washing with tap water or followed by IHC. Sections were further processed with differentiation solution (1% HCl in absolute ethanol, for 7 seconds), followed by washing under tap water for 10 min. Sections were then incubated in eosin (Harris) for 10 seconds, rinsed and dehydrated in 70% - 100% alcohol baths with rapid dips in each bath before final wash in toluene for 15 min. Slides were mounted in Eukitt solution (Sigma).

Giemsa staining

Tissue was cut in 7 µm thick sections, dewaxed and stained with Giemsa (320310-0125, RAL) for 2 hours at 37°C. Sections were further processed in a 0.5% aqueous acetic acid solution, dehydrated and mounted with Eukitt solution.

Immunohistochemistry

Paraffin embedded tissue was rehydrated and the antigens were unmasked by boiling in 10 mM pH 6 citrate solution for 20 min. Cooled slides were washed and incubated in a peroxide solution (0.6% H_2O_2, 0.1% triton X-100 in PBS) to eliminate endogenous peroxidase activity. Non-specific binding sites were blocked with a blocking solution (5%

normal goat serum in PBS) for 1 hour at RT and then avidin/biotin receptors were blocked by using the avidin/biotin blocking kit as recommended by the manufacturer (Vector). Slides were incubated with the first antibody overnight at 4°C in a humidified container. Next day, slides were washed and incubated for 45 min at room temperature with a secondary antibody (coupled to biotin). The detection of peroxidase was done using the Elite ABC system (VECTASTAIN) with DAB (Vector) as substrate. Finally, tissue was stained with hematoxylin, dehydrated and mounted. Proliferation and apoptosis were quantified as events per area upon staining for Ki-67 and cleaved caspase-3, respectively.

Immunofluorescence

Tissue was air-dried and unspecific signal was blocked with blocking solution (5% normal goat or donkey serum in PBS) for 1 hour at RT. Tissue sections were incubated with the primary antibody overnight at 4°C in a humidified container. The following day the primary antibody was removed and tissue was incubated with a fluorescent secondary antibody for 1 hour at RT. Slides were washed and incubated with DAPI (Sigma) to visualize the nuclei (10 min at RT). Excess of dye was removed and tissue was mounted with FluorSaveTM Reagent (Calbiochem). Fluorescent signal was analyzed with a Zeiss Axio Imager Z2 microscope. The staining procedure (fixation, blocking, antibody dilution) and image acquisition setting (microscope, magnification, light intensity, exposure time) were kept constant per experiment and genetic conditions. Quantification of IF microscopic images was done by the ImageJ (National Institutes of Health) software using a constant threshold. The expression of TNC was scored according to the extent and intensity of the whole tumor mosaic picture. A typical fibrillar TNC staining with the MTn12 antibody in the stroma around the tumor cells was considered as positive signal (no signal with the secondary antibody alone). The extent of TNC staining was scored by the percentage of the positively stained area. The stained area in each region of interest was scored as 0 for staining less than 5 %, as 1 for 5–25 %, 2 for 25–50 %, 3 for 50–75 %, and 4 for more than 75 % of the stained area. The intensity of staining was scored as 0, 1, 2 and 3 representing no staining, mild (weak but detectable above control), moderate (distinct) and intense (strong) staining, respectively. The percentage of positively stained area and intensity of staining were multiplied to produce a weighted score according to Shi et al., (2015).

RT-qPCR analysis

Total RNA was prepared using TriReagent (Life Technologies) according to the manufacturer's instructions. RNA was reverse transribed (MultiScribe reverse transcriptase, Applied Biosystems) and qRT-PCR was done on cDNA (diluted 1:5 in water) on a 7500 Real Time PCR machine (Applied Biosystems) using SYBR green reaction mixture or Taqman reaction mixture (Applied Biosystems). Data were normalized by using a Taqman mouse GAPDH Endogenous Control (4333764T, Life Technology) and fold induction was calculated using the comparative Ct method (-ddCt). Primers used for qRT-PCR are listed in the **Table S1.**

Immunoblotting

Cell lysates were prepared in lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl,1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor (Roche) and Phosphatase Inhibitor Cocktail (Santa Cruz). Protein concentration was determined with a Bradford Assay (BioRad). After addition of Laemmli buffer (Biorad), 20-30 µg protein lysate was separated by SDS-PAGE in precasted 4-20 % gradient gels (Biorad), transferred onto nitrocellulose membranes (Biorad) using TransBlot Turbo™ Transfer System (Biorad), blocked with 5 % Blocking-Grade blocker (Biorad) in 0.1% Tween 20-PBS and incubated with the primary (overnight at 4°C) and secondary antibodies (1 hour at RT) in 1.5 % Blocking-Grade Blocker in 0.1 %Tween 20-PBS. Protein bands were detected with the Amersham ECL Western Blotting detection reagent (GE Healthcare) or SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher).

Immunofluorescence staining of cells

Cells were fixed in 4 % PFA for 10 minutes, permeabilized in PBS-Triton 0.1 % for 10 minutes, incubated with the primary antibody overnight at 4 °C, secondary antibody for 1 hour at RT, DAPI, mounted with FluorSaveTM Reagent (Calbiochem) and analyzed with an Zeiss Axio Imager Z2 microscope.

Antibodies

The primary antibodies used in this article are shown in **Table S2**. Secondary antibodies were ECL horseradish peroxidase-linked (1/1000): anti-rat (NA935) and anti-rabbit (NA934V) (GE Healthcare). Secondary goat or donkey antibodies were fluorescently labeled (1/1000): anti-mouse, anti-rabbit, anti-rat, anti-guinea pig and anti-goat IgG (Jackson Laboratory).

Treatment with TGF-β and GW788388

Cells were starved in the absence of serum overnight before treatment with human recombinant TGF-β1 (100-21, PEPROTECH), recombinant TNC (purified as described in Huang et al., (2001)) or the TGF-β type I receptor inhibitor GW 788388 (Selleckchem). Recombinant TNC (for detection of potentially bound TGF-β by immunoblot) was from Millipore (CC0651). Cells were pretreated with the inhibitor for 45 minutes prior to incubation with TNC, TGF-β and platelets, respectively. All reagents were diluted following the manufacturer's instructions.

Transduction of cells

Silencing of TNC in mouse cells was done by short hairpin (sh) mediated gene expression knock down (KD). Lentiviral particles with shRNA vectors (Sigma-Aldrich) specific for TNC were used. sh1, TRCN0000312137, sequence 5'-CCGGCCCGGAACTGAATATGGGATTCTCGAGAATCCCATATTCAGTTCCGGGTTTTTG-3'; sh2, TRCN0000312138, 5'-CCGGGCATCAACACAACCAGTCTAACTCGAGTTAGACTGGTTGTGTTGATGCTTTTTG-3'. Lentiviral particles encoding a non-targeting shRNA vector were used as a control (SHC202V, Sigma-Aldrich). Transduced cells were selected with normal medium supplemented with 10 µg/ml puromycin (ThermoFisher) and the selection pressure was maintained in all in vitro experiments.

Spheroid assay

NT193 cells were seeded at 5000 cells per 100 µL together with TNC (10 µg/ml) or PBS-Tween-20 (0.01%) in 96 well plates with round bottom pre-coated with 10 µg/ml of poly-HEMA (Sigma) for 24 hours to allow spheroid formation and then were embedded in OCT for further immunostaining analysis.

Wound healing assay

2×10^5 cells were grown to confluency in 24-well plates for 24 hours. Confluent cell monolayers were treated two hours with mitomycin-C (Sigma) at 2 µg/ml to inhibit proliferation before application of a scratch wound with a pipet tip. Cell debris was removed by PBS before addition of serm free medium supplemented with the indicated molecules. Images of the wounding area were acquired immediately after scratching and then in the same field after 24 hours. The relative wound closure was quantified by measuring the surface of the cell-free area at the time of injury and at the end point of the experiment.

Caspase3/7 activity assay

Caspase 3/7 activity assay (Promega) was performed according to the manufacturer's instructions. Briefly, 2000 cells/well were plated overnight in a 96-well plates. Cells were treated as described for the indicated time period and then cell apoptosis was induced by staurosporine (1 µg/ml, Sigma) for 24 hours. To measure caspase 3/7 activity, 75 µL of caspase Glo 3/7 reagent was added to each well for 1 hour with constant shaking at RT. Luminescence was measured using a TriStar² LB942 multidetection microplate reader.

Preparation of washed platelets

Blood was drawn from the abdominal aorta of adult FVB/NCrl mice anesthetized intraperitoneally with a mixture of xylazine (20 mg/kg, Rompun, Bayer) and ketamine (100 mg/kg, Imalgene 1000, Merial). Platelets were washed using ACD-anticoagulated whole blood as previously described by Cazenave et al. (2004).

Statistical analysis

The GraphPad Prism software (version 6) was used for graphical representations of data and statistical analyses to assess significance of observed differences. All parametric (unpaired Student t test with Welch's correction in case of unequal variance) and non-parametric tests (Mann-Whitney) were performed in a two-tailed fashion. To compare the proportion of LVI and parenchymal metastases, Fisher's exact test or Chi-square test was used. Mean ± SEM. p values < 0.05 were considered as statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Acknowledgement

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Table S1 Summary of potential roles of TNC in LVI of breast cancer lung metastasis

Observation	**Potential implication of TNC**
Continuous layers of LM/FN and TNC around CTC nest **S**imilar layering in WT and TNCKO	**L**ikely role of TNC in LVI **N**o impact of TNC on LM/FN layering
Fsp1+ cells at rim of LVI **F**sp1+ cells colocalize with TNC	**F**sp1+ cells likely source of TNC
Continuous CD31 monolayer at LVI border **C**onnection of CD31 layers of LVI and lung vasculature indicative of layer fusion **L**ess LVI CD31 layering in TNCKO **E**ndothelial cells not in direct contact with TNC	**T**NC promotes endothelial layering – by paracrine mechanism?
CD41 inside LVI - role in birth of LVI? **L**ess platelets in TNCKO **O**verlap of CD41 and TNC	**T**NC increases abundance of platelets **P**latelets likely source of TNC
Reduced LVI size in TNCKO **K**i67 staining inside LVI indicating CTC proliferation **K**i67+ cell numbers similar in TNCKO	**T**NC increases size of LVI **N**o impact of TNC on CTC proliferation
Cleaved caspase-3 across LVI - hypoxia unlikely trigger **M**ore apoptotic cells in TNCKO **N**o physical contact of apoptotic CTC with TNC	**T**NC promotes survival of CTC – by paracrine mechanism?
CTC breach into lung parenchyma **R**educed parenchymal metastasis in TNCKO	**T**NC promotes parenchymal metastasis **T**NC promotes extravasation – by paracrine mechanism?
Combined vimentin and E-cadherin expression in CTC **P**artial EMT at breaching front – collective invasion? **L**ess vimentin+ CTC in TNCKO	***I**n vivo* EMT promoted by TNC
EMT in NT193 cultures by TNC in TGF-β dependent manner **E**MT promoting migration **E**MT promoting drug-induced apoptosis	**E**MT through TGF-β signaling promoted by TNC **E**MT by TNC promoting survival **E**MT by TNC promoting extravasation?

Organization of LVI in the genetic MMTV-NeuNT and associated NT193 syngeneic orthotopic grafting model with abundant and low/no TNC is summarized. The presence and spatial organization of stromal cells, platelets and ECM, in the absence and presence of TNC (first column) and the deduced potential role of TNC in LVI (second column) are depicted. CTC, circulating tumor cells, EMT, epithelial-to-mesenchymal transition, Fsp1, marker of carcinoma associated fibroblasts/myeloid cells, Ki67, proliferation marker, KO, knockout, LVI, lymphovascular invasions, TNC, tenascin-C, TGF-β, transforming growth factor

Table S2. Primer sequences used for RT-pPCR		
GENE	**Forward primer**	**Reverse primer**
E-cadherin	CAGCCTTCTTTTCGGAAGACT	GGTAGACAGCTCCCTATGACTG
vimentin	CCAACCTTTTCTTCCCTGAAC	TTGAGTGGGTGTCAACCAGA
slug	CTCACCTCGGGAGCATACAG	GACTTACACGCCCCAAGGATG
twist	AGTGTTTGGCAGGGGACA	CCCATCCCCTGGGTATCT
zeb1	GCCAGCAGTCATGATGAAAA	TATCACAATACGGGCAGGTG
fibonectin	GATGCCGATCAGAAGTTTGG	GGTTGTGCAGATCTCCTCGT
tenascin-C	CAGGGATAGACTGCTCTGAGG	CATTGTCCCATGCCAGATTT
MMP9	ACGACATAGACGGCATCCA	GCTGTGGTTCAGTTGTGGTG
PAI-1	GGCACCTTTGAATACTCAGGA	TTTCCCAGAGACCAGAACCA
Axin2	CTGCTGGTCAGGCAGGAG	TGCCAGTTTCTTTGGCTCTT
snail	Tapman probe, Hs00195591_m1, ThermoFisher	

Table S3. List of antibodies					
Antigen	**Host**	**Antibody name**	**Source**	**Dilution**	**Application**
ErbB2	rabbit	MA5-13675	ThermoFisher	1/50	IF
Cytokeratin CK8/18	guinea pig	GP11	PROGEN	1/500	IF
E-cadherin	rat	13-1900	Life Technology	1/200 IF 1/1000 WB	IF, WB
vimentin	rabbit	2707-1	EPITOMICS	1/500 IF 1/1000 WB	IF, WB
TNC	rat	Mtn12	Aufderheide et al.	2µg/ml IF 0.4µg/ml WB	IF, WB
Pan-laminin	rabbit	Ln6 7S	Simo et al.	1/2000	IF
fibronectin	rabbit	F3648	Sigma-Aldrich	1/200	IF
CD31	rat	550274	BD pharmigen	1/200	IF
CD45	rat	550566	BD pharmigen	1/500	IF
FSP1	rabbit	Mts1 (S100a4)	Ambartsumian et al.	1/200	IF
αSMA	mouse	A2547	Sigma-Aldrich	1/400	IF
Caspase-3 cleaved	rabbit	9661	Cell Signaling	1/600	IHC
Ki-67	rabbit	RM-9106	ThermoFisher	1/600	IHC
CD41	rat	11024	Abcam	2µg/ml	IF
GPIbβ	rat	RAM.1	PH Mangin	3µg/ml	IF
Erk1/2	rabbit	9102	Cell Signaling	1/1000	WB
P-Erk1/2	mouse	9106	Cell Signaling	1/1000	WB
Akt	rabbit	4691	Cell Signaling	1/1000	WB
P-Akt	rabbit	4060	Cell Signaling	1/1000	WB
p-Smad2	rabbit	3108	Cell Signaling	1/1000	WB
Smad2/3	rebbit	3102	Cell Signaling	1/1000	WB
TGF-β	rabbit	3711	Cell Signaling	1/1000	WB

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4. Manuscript 2

Tenascin-C promotes tumor cell migration through integrin α9β1 inhibiting YAP

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Running title: **Through integrin α9β1 and inhibition of YAP tenascin-C promotes tumor cell migration**

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Abstract

Tenascin-C (TNC) plays multiple roles in cancer malignancy. Several signaling and adhesion controlling molecules are downregulated in tumor cells by TNC, yet the underlying mechanisms and consequences are poorly understood. Here we show that by modulating tumor cell adhesion TNC regulates gene expression. We describe a novel tumor cell autonomous signaling pathway where TNC inhibits actin stress fibers and YAP through integrin α9β1 which is required for TNC to promote tumor cell migration. In support, expression of YAP target genes is elevated in a glioblastoma model upon knockdown of TNC. The provided link could have clinical relevance as a signature comprising CTGF, Cyr61 and Cdc42EP3 at lower levels, resembling the TNC effect in vitro, correlates with worst survival of osteosarcoma and glioblastoma cancer patients. Our results suggest that TNC matters as soon as it is expressed where stimulation of tumor cell migration may be important for TNC promoting tumor malignancy. To our knowledge this is the first report that provides a full view on a signaling pathway initiated by tenascin-C, employing integrin α9β1 its impact on the actin cytoskeleton, YAP and gene expression thus promoting 3D migration. This information could be of diagnostic and therapeutic value.

Introduction

The extracellular matrix (ECM) molecule tenascin-C (TNC) that is highly expressed in the tumor microenvironment (TME) represents an active component of cancer tissue. Its high expression correlates with worsened patient survival prognosis in several cancer types (**1**). TNC promotes multiple events in cancer progression as recently demonstrated in a multi-stage neuroendocrine tumorigenesis model with abundant and no TNC. It was shown that TNC promotes tumor cell survival, proliferation, invasion and lung metastasis. Moreover, TNC promotes stromal events such as the angiogenic switch and the formation of more but leaky blood vessels which involves Wnt signaling activation and inhibition of Dickkopf1 (**2,3**), as well as Ephrin-B2 signaling in a glioblastoma (GBM) model (**4**). TNC networks that can have similarities with reticular fibers in lymphoid organs (**5**) may alter the biomechanical properties of cancer tissue (**6**) and in particular increase tissue stiffening (**7**). TNC also impairs actin stress fiber formation (**8**) and regulates gene expression which may impact on cell behaviour and tumor malignancy (**9**).

The actin polymerization state is interpreted by the cell through two co-transcription factors, megakaryoblastic leukemia 1 (MKL1) (**10**) and yes activating protein (YAP) (**11,12**). Under poorly adhesive conditions, cells fail to polymerize actin and do not form actin stress fibers. MKL1 binds to globular G-actin monomers and remains sequestered in the cytoplasm. In consequence MKL1 cannot reach nuclear serum response factor (SRF) or SAP binding sequences to induce gene transcription (**13,14**) and MKL1 dependent genes remain silent.

YAP and TAZ (transcriptional coactivator with PDZ-binding motif) proteins are integral parts of the Hippo signaling pathway that is important for organ growth control during development and often deregulated in cancer (**15**). Recently, YAP and TAZ were

demonstrated to transduce mechanical and cytoskeletal cues where actin stress fibers promote their nuclear translocation (**16**). Nuclear YAP/TAZ can activate gene expression e.g. through binding to the TEAD family of transcription factors (**16**), thus controlling gene expression upon cell adhesion.

Here, we analyzed the underlying mechanisms and consequences of poor cell adhesion by TNC. We demonstrate that TNC downregulates gene expression through inhibition of actin stress fibers which in turn abolishes MKL1 and YAP activities in tumor cells. TNC itself is downregulated by a negative feedback loop due to inactive MKL1 and YAP. We further show that integrin α9β1 and inactive YAP are instrumental for TNC to promote tumor cell migration in an autocrine and paracrine manner. Finally, poor expression of CTGF, Cyr61 and Cdc42EP3 identifies a group of osteosarcoma and glioblastoma patients with worst survival when TNC levels are below the median expression. To our knowledge this is the first report that provides a full view on a signaling pathway initiated by tenascin-C, employing integrin α9β1, its impact on the actin cytoskeleton, YAP and gene expression thus promoting 3D migration. This information could be of diagnostic and therapeutic value.

Results

TNC inhibits actin stress fiber formation on a mixed FN/TNC substratum

FN and TNC are often coexpressed and act as accomplices in cell adhesion where TNC can counteract the adhesive properties of FN (**8,17,18**). To set the stage for the subsequent mechanistic analysis, we determined how low cell adhesion to FN implemented by TNC affects actin dynamics and downstream gene expression in two tumor cell lines derived from GBM (T98G) and osteosarcoma (KRIB). We found that cells were round and adhered less on the FN/TNC substratum (**Fig. S1A-C**). Western blot upon fractionation into monomeric G-actin and polymerized F-actin revealed less F-actin in cells grown on FN/TNC compared to FN (**Fig. S1D-F**). Finally, by staining with TRITC-phalloidin we showed the absence of actin stress fibers on the FN/TNC substratum (**Fig. S1 G, H**).

A tenascin-C repression signature negatively correlates with MKL1 and YAP responsive gene sets

Since TNC inhibits actin stress fibers and actin stress fibers regulate MKL1 and YAP/TAZ functions (**10-12**), we asked whether TNC impairs MKL1 and/or YAP activities. Therefore, we searched for a potential correlated expression of genes that are regulated by TNC (**9**) and genes that are regulated by MKL1/SRF (**19**) or YAP/TAZ (**20**), respectively. We used publicly available mRNA expression data and Gene Set Enrichment Analysis (GSEA) and found that both gene sets are significantly negatively correlated with a gene signature that is downregulated by TNC in T98G cells (**Fig. 1A, B**) (**9**). By quantitative real time polymerase chain reaction (qPCR) we evaluated TNC substratum specific gene

expression and found that in contrast to c-Fos, a SRF/TCF target that is increased on FN/TNC, a selection of known MKL1 regulated genes (TPM1, TPM2, Zyxin, FosL1, Cdc42EP3, TNC) (**21-23**) and YAP regulated genes (CTGF, Cyr61, DKK1, Gli2) (**24**) was indeed lowered on the FN/TNC substratum (**Fig. 1C, D**). Whereas TAZ mRNA level was slightly enhanced in T98G, YAP mRNA and protein levels were not affected by the FN/TNC substratum (**Fig. 1C-E**). In contrast, MKL1 mRNA and protein levels were reduced on FN/TNC suggesting that TNC blocks expression of MKL1 but not of YAP (**Fig. 1C, D, F**).

TNC blocks MKL1 target gene expression through repression of MKL1

As mRNA levels of MKL1 and SRF were lower in both cell lines on the FN/TNC substratum in comparison to FN we wanted to know whether SRF dependent transcription is potentially impaired by TNC. Therefore, we measured SRF driven luciferase activity in cells grown on FN or FN/TNC. Surprisingly, we noticed similar activities on FN/TNC as on FN suggesting that TNC does not inhibit the SRF dependent function of MKL1 (**Fig. 2A, S2A**). To further investigate a potential impact of TNC on MKL1 functions, we used loss of function (LOF) and gain of function (GOF) approaches. First, we used two shRNAs to reduce MKL1 expression (**Fig. 2B-D, S2B-D**). Whereas expression of c-Fos, not a MKL1 target, was not altered, all other tested genes including TNC, a recently identified MKL1/SAP responsive gene (**14,23**), were downregulated (**Fig. 2C, S2C**). Second, upon overexpression of CA-MKL1 (**Fig. 2D),** we noticed high induction of SRF-luciferase activity indicating that cells are responsive to active MKL1 (**Fig. 2E**). CA-MKL1 significantly induced CTGF, Cdc42EP3, TNC, DKK1 and TPM1, yet not Cyr61 (**Fig. 2F, Fig. S2E**). To address whether TNC represses genes through impairment of MKL1 we compared gene

expression levels on FN/TNC to those in cells grown on FN upon transient expression of CA-MKL1. Whereas levels of Cdc42EP3, TNC, DKK1 (only T98G) and TPM1 significantly increased compared to transfected WT-MKL1, neither expression of CTGF nor Cyr61 was increased (**Fig. 2G, S2F**). These results suggest that TNC represses some genes such as TPM1, TNC, Cdc42EP3 and DKK1 through abolishing MKL1 function. In contrast, other genes such as CTGF and Cyr61 are repressed by another mechanism.

TNC represses genes in tumor cells through abolishing YAP by cytoplasmic retention

Since some YAP dependent genes (e.g. CTGF) were downregulated on the FN/TNC substratum (**Fig. 1C, D**), and a YAP/TAZ gene signature was significantly correlated with TNC downregulated genes (**Fig. 1A**), we considered that TNC may inhibit the YAP co-transcriptional function in tumor cells. We addressed this possibility by measuring TEAD dependent luciferase activity and found that this was indeed reduced in both cells grown on FN/TNC compared to FN (**Fig. 3A**). Since YAP protein levels were equal on FN/TNC to that on FN (**Fig. 1E**) TNC may impair nuclear translocation of YAP which we addressed by staining cells for YAP. Whereas YAP was nuclear in the large majority of both cells plated on FN, YAP remained mostly cytoplasmic in cells on FN/TNC even 24h after plating which resembles cells in the absence of fetal bovine serum, a condition that blocks YAP function (**Fig. 3B-D, S3A-C**) (**25**). Thus, on FN/TNC, nuclear translocation of YAP is abolished which could explain inactivation of YAP by TNC.

To determine regulation of genes by TNC through YAP in more detail we used LOF and GOF approaches by transiently expressing inhibitory (DN-YAP) or activating (CA-YAP)

YAP molecules (**Fig. 3E, F, S3D, E**). We addressed YAP transactivation function with a TEAD-luciferase assay and target gene expression, respectively and observed that CA-YAP increased TEAD-luciferase activity (**Fig. 3G, S3F**). CA-YAP also significantly increased CTGF, Cyr61, Cdc42EP3 and TNC gene expression. In contrast, neither DKK1 nor TPM1 were induced by CA-YAP (**Fig. 3H, S3G**). To investigate whether CA-YAP restored expression of TNC downregulated genes we measured target gene expression on FN/TNC upon expression of transiently transfected CA-YAP. Expression of Cyr61 and Cdc42EP3 was increased and for CTGF and TNC, expression was even restored to levels as on FN. Again, expression of DKK1 was poorly affected (**Fig. 3I, S3H**). These results suggest that TNC reduces expression of CTGF, Cyr61, and Cdc42EP3 by inhibiting YAP. Moreover, we showed that YAP regulates TNC expression.

Actin polymerization and stress fibers differentially affect tenascin-C regulated genes

We had seen that TNC impacts on the actin cytoskeleton and abolishes MKL1 and YAP target gene expression. Now we asked whether and how TNC-regulated genes respond to actin dynamics. We treated cells with Latrunculin B (LB) causing disassembly of actin filaments into monomeric G-actin (**26**), Jasplakinolide (Jasp) to stabilize F-actin (**27**) and LPA to induce actin stress fibers (**28**) (**Fig. 4A, B**) before measuring gene expression. We observed that LB blocked expression of all tested genes in both cells (**Fig. 4C, S4A**). Moreover, whereas Jasp blocked CTGF, Cyr61, Cdc42EP3, TNC and DKK1 expression, TPM1 was induced. These results suggest that F-actin is sufficient to drive TPM1 expression but not expression of the other five tested genes which may require actin stress fibers (**Fig. 4D, S4B**). Indeed, LPA induced expression of all these genes in both cells

(**Fig. 4E, S4C**). We conclude that actin stress fibers are important regulators of the TNC-repressed genes. To prove that the LPA effect is indeed due to its role in actin stress fiber formation (as LPA can also have other downstream effectors) (**29**), we treated cells with LPA together with LB generating G-actin and measured gene expression (**Fig. 4A**). We observed that LB abolished LPA induced expression of all tested genes and conclude that LPA bypasses TNC gene repression through its impact on actin stress fiber formation (**Fig. 4E, S4C**). Importantly, TNC expression itself requires actin stress fibers (**Fig. 4C-E, S4A-C**).

TNC downregulates YAP target gene expression through blocking actin stress fibers

Next, we wanted to know whether TNC impairs YAP target gene expression through blocking actin stress fibers. We used LPA to induce actin stress fibers and cell spreading on FN/TNC (**Fig. 5A**) and then investigated how this effect could be reverted. First, we measured YAP dependent luciferase activity in cells grown on FN/TNC. Indeed, LPA restored TEAD driven luciferase activity on FN/TNC to levels as on FN (**Fig. 5B**). LPA also increased expression of all six tested TNC target genes (CTGF, Cyr61, Cdc42EP3, DKK1, TNC, and TPM1) on FN/TNC (**Fig. 5D, E**) which is in contrast to very low expression of these genes on FN/TNC without LPA (**Fig. 1C, D, Fig. S5A-L**). Then, by siRNA mediated knockdown (**Fig. 5C**), we asked whether LPA restores gene expression on FN/TNC through YAP. Indeed, siYAP abolished expression of all LPA-restored genes on FN/TNC except TPM1 that was not reduced (**Fig. 5D, E, S5A-L**). This result suggests that TNC represses YAP target genes through inhibition of actin stress fibers. Altogether, we

identified three groups of genes that TNC represses through impairment of MKL1 (TPM1), YAP (CTGF, Cyr61) or MKL1 and YAP (Cdc42EP3, TNC and DKK1).

TNC promotes 3D migration through integrin α9β1 by blocking actin stress fibers and inactivating YAP

So far we showed that TNC impairs actin stress fiber formation and YAP dependent gene expression. We wanted to know whether this has an effect on cell migration. We monitored mobility by time lapse microscopy in KRIB cells and observed that the total migration distance was lower on FN/TNC than on FN and that the persistance was different (**Fig. 6A, B**, **Supplemental videos 1 and 2**). By using a Boyden chamber assay we observed that more cells moved to the other side of the filter when cells were placed on the FN/TNC substratum in comparison to FN, demonstrating that TNC promotes 3D transwell migration but not 2D migration (**Fig. 6C**). Subsequently, we investigated whether cells with depolymerized (LB) or polymerized actin (yet not forming actin stress fibers) (Jasp)) impact transwell migration on FN. We saw that LB and Jasp completely abolished migration of KRIB cells (**Fig. S6A**). As LPA restored cell spreading through induction of actin stress fibers we asked whether LPA had an impact on transwell migration. Indeed, LPA reduced migration of cells on FN/TNC to levels as on FN (**Fig. 6D, E**) suggesting that actin stress fibers counteract TNC induced transwell migration. Cells with a rounded cell shape can migrate in an amoeboid manner where active Rho-kinase (ROCK) is crucial (**30**). As cells are rounded by TNC, we wanted to know whether ROCK is involved in the TNC specific 3D migration which we addressed by chemically inhibiting ROCK. We observed that ROCK is required as Y27632 blocked migration from FN/TNC in the Boyden chamber experiment (**Fig. 6D**).

We had shown that LPA restores cell spreading on FN/TNC through its impact on actin stress fibers (**Fig. 4**) and now addressed a potential interdependence with YAP. Therefore, we used LPA to induce actin stress fibers on FN/TNC and then determined whether inhibition of MKL1 or YAP bypasses the LPA effect on migration (**Fig. 6E, F**). We found that knockdown of MKL1 did not alter migration (**Fig. 6F**). In contrast, knockdown of YAP in LPA treated cells increased transwell migration on FN/TNC to that of shCTRL cells (**Fig. 6E**). This was associated with siYAP bypassing the stress fiber inducing effect of LPA on FN/TNC (**Fig. 5A**, **Fig. S6B**). A link to actin stress fiber inhibition by TNC through blocking YAP is further supported in YAP GOF and LOF experiments. CA-YAP restored actin stress fibers on FN/TNC and abolished TNC induced transwell migration which was not the case with DN-YAP or WT-YAP (**Fig. 6G, Fig. S6C**). These results suggest that TNC promotes transwell migration through inhibition of YAP but not MKL1 and indicates a link of YAP to stress fiber formation.

We addressed whether syndecan-4 (**8**) or integrin α9β1 a receptor for TNC (**31**) are potential upstream regulators of TNC-induced migration. First, we lowered gene expression by siRNA and confirmed reduced expression of syndecan-4 and the α9 integrin chain (**Fig. 6H, S6D**). Reduced levels of syndecan-4 (mimicking cell rounding by TNC) (**32**) did not abolish LPA specific migration on FN/TNC suggesting that inactivation of syndecan-4 by TNC is not relevant for TNC transwell migration. In contrast, the integrin α9β1 knockdown induced actin stress fibers and abolished TNC specific transwell migration (**Fig. 6I, S6B**).

As TNC transwell migration occurs in the absence of actin stress fibers and, the knockdown of integrin α9β1 and of YAP impaired actin stress fibers and TNC migration, we wanted to know whether TNC downregulates YAP target genes through integrin α9β1 By qPCR we indeed observed that the α9β1 integrin knockdown increased

expression of all tested TNC target genes on FN/TNC reaching levels close to FN (**Fig. 6J**).

Next we addressed whether TNC expressed by the cells has an effect on transwell migration. We used control KRIB (shCTRL) and TNC knockdown (shTNC) cells (**Fig. S6E**) and found that less cells with a TNC knockdown moved through the uncoated filter than shCTRL cells (**Fig. 6K**). We conclude that TNC expressed by the tumor cell as well as offered as substratum induces ✔⏮🚲❒signaling and promotes 3D migration.

Finally, we tested whether TNC levels influence expression of YAP target genes in vivo. Since T98G cells are not tumorigenic we analyzed gene expression in tumors derived from xenografting human U87MG GBM cells with wildtype and knockdown levels of TNC. In this model TNC promoted tumor growth (**4**). We observed that whereas DKK1 levels were unaffected, CTGF, Cyr61, Cdc42EP3 and TPM1 were largely increased in TNC knockdown tumors in comparison to shCTRL tumors thus providing evidence for in vivo relevance of this novel TNC induced mechanism of gene regulation (**Fig. 6L**).

Altogether, our results showed that TNC promotes 3D transwell migration which requires ROCK and occurs in the absence of actin stress fibers. In contrast, a rounded cell shape per se or inhibition of actin stress fibers as implemented on FN upon treatment with LB or Jasp, respectively is not sufficient to promote transwell migration. TNC promotes 3D migration by signaling through integrin α9β1 that blocks actin stress fibers and inhibits YAP. In addition to surface adsorbed TNC, also endogenously expressed TNC promotes transwell migration suggesting an autocrine, in addition to a paracrine, integrin α9β1 TNC signaling loop. This mechanism may be relevant in tumors as we observed an increased expression of YAP target genes upon TNC knockdown in a GBM xenograft model.

Predictive value of TNC regulated genes CTGF, Cyr61, and Cdc42EP3 for cancer patient survival

By having established a link of TNC to enhanced migration through abolishing YAP activity we asked whether this information could be of relevance for cancer patients. Therefore, we analyzed survival and expression of a YAP signature (**33**) that was downregulated by TNC (**9**) in a publicly available mRNA expression data set of osteosarcoma patients (n = 53) (GSE 21257) (**34**) but noticed no link to survival (data not shown). Yet, when we used the three genes CTGF, Cyr61, and Cdc42EP3 together, that are strongly repressed by TNC in our cellular models, we noticed a shorter metastasis-free survival of patients with tumors exhibiting TNC expression below the median level (**Fig. 7A**). This was not the case in tumors with TNC levels above the median (**Fig. 7B**). Moreover, neither low nor high expression of each gene alone or in different combinations had any predictive value (data not shown). We also analyzed expression levels of the three-gene signature in a cohort of 46 GBM patient derived tumor xenografts (PDX) where the gene signature of the experimental tumors correlated with invasiveness and worsened overall patient survival of big GBM cohorts (GSE42669) (**35**). We observed that PDX tumors that had lower expression of CTGF, Cyr61, and Cdc42EP3 in a context of abundant but TNC expression below the median, represent a group of GBM patients with worsened progression-free survival (**Fig. S7A, B**). Low expression of either gene alone or in varied combinations had no relevance for patient survival (data not shown). Altogether we identified a short list of TNC downregulated genes with correlation to patient survival in osteosarcoma and GBM. More in-depth analysis is required to understand the mechanism of how a TNC-CTGF-Cyr61-Cdc42EP3 signaling context promotes tumor cell migration and malignancy.

Discussion

By using LOF and GOF approaches (**Table S1**), here we have shown a novel function of TNC in cancer by inhibiting gene expression through abolishing MKL1 and YAP transactivation function due to TNC blocking actin stress fibers. By blocking YAP (yet not MKL1 nor syndecan-4) TNC promotes tumor cell migration downstream of integrin α9β1 This mechanism could be relevant in cancer as we have documented that YAP target genes are increased in a GBM tumor model upon knockdown of TNC. We identified a three-gene signature comprising CTGF, Cyr61, and Cdc42EP3 that when expressed in tumors with levels of TNC below the median correlates with worse survival of osteosarcoma and GBM patients. These observations suggest that TNC matters in tumors as soon as it is expressed where promotion of tumor cell migration may be an important mechanism of malignancy (**Fig. 7C, S7C**).

As it was incompletely understood how TNC regulates cell adhesion and migration, we have revisited the effect of TNC on cell adhesion in context of FN. TNC competes syndecan-4 binding to FN, thus blocking integrin α5β1mediated cell adhesion and actin stress fiber formation (**8**) which results in a pro-tumorigenic gene expression profile (**9**). We had previously shown that through this mechanism the tumor suppressor like molecule TPM1 is downregulated. Restoring syndecan-4 function either through a peptide mimicking the syndecan-4 binding site in FN or by overexpressing syndecan-4 (yet not syndecans 1 or 2), TPM1 expression was turned on and induced cell spreading on the FN/TNC substratum. This was blocked upon knockdown of TPM1 (**9,32**). Now, we provide a link of TNC to TPM1 repression showing that TNC impairs MKL1. MKL1 itself is

repressed by TNC at mRNA and protein level. This mechanism is also relevant for TNC to downregulate other genes (Cdc42EP3, DKK1 and TNC). As SRF luciferase reporter activity was not reduced we consider that TNC may impair other MKL1 activities such as the SAP-associated MKL1 function (**14**), a possibility that is beyond the scope of this study.

In addition to MKL1 we identified YAP to be impaired by TNC through its inhibition of actin stress fibers. We identified CTGF, Cyr61, Ccd42EP3 as target genes of TNC that are regulated in a YAP dependent manner. We found TNC itself is a novel YAP regulated gene. Whether regulation of TNC and the other target genes is direct and occurs through the TEAD binding sites in the respective promoters needs to be seen in the future. Altogether, we identified three groups of genes that TNC represses through its impact on MKL1 (TPM1), YAP (CTGF, Cyr61) or MKL1 and YAP (Cdc42EP3, TNC and DKK1).

Our results suggest that TNC specific transwell migration has properties of amoeboid migration (**30**) as cells are rounded, have no actin stress fibers and do not form focal adhesions nor have active FAK and paxillin (**8,9,32,36,37**). Amoeboid migration depends on ROCK (**30**) which we have shown here (by chemical inhibition) to be required for TNC specific 3D migration. We have identified integrin α9β1 as novel upstream regulator of TNC-induced 3D migration. Although integrin α9β1 is known as receptor for TNC (**31**), little was known how binding to TNC affects cell behaviour. Here, we have demonstrated for the first time that integrin α9β1 is promoting 3D migration by TNC and link this effect to blocking actin stress fibers and inhibiting YAP.

We have shown that a direct interaction of tumor cells with TNC could be a key event in cancer malignancy by promoting motility through integrin α9β1 signaling (**Fig. 7C, S7C**).

Future studies need to address whether this signaling leads to TNC repression in tumor cells. Even though, TNC is copiously expressed by stromal cells including fibroblasts and immune cells (**38,39**) thus generating a TNC rich TME that may promote tumor malignancy by additional non-tumor cell autonomous events such as angiogenesis (**2,4**).

In tumor tissue TNC is often coexpressed together with FN and other ECM molecules forming matrix tracks that serve as niches for tumor and stromal cells (**5**). These ECM rich niches may have different stiffness properties than adjacent areas devoid of matrix as they offer many integrin binding opportunities for cells. In support, in GBM it was recently shown that high TNC is correlated with tissue stiffness (**7**). Here, we have demonstrated that TNC regulates its own expression by a negative feedback loop. TNC may also reduce cellular tension due to its anti-adhesive properties. Altogether, this may contribute to balancing stiffening and cellular tension in cancer cells thus potentially contributing to their survival.

We had investigated whether expression of TNC-downregulated genes correlate with cancer patient survival. We found that low expression of CTGF, Cyr61, and Cdc42EP3 (that are strongly repressed by TNC in vitro) correlates with worst survival of patients with osteosarcoma and GBM when TNC is below the median expression. It has to be noted that TNC levels below the median are still considerably high as normal tissue does essentially not express TNC. The presented correlation was unexpected since high TNC levels are known to correlate with bad patient survival (Midwood et al., 2011)(40). Generally lower TNC levels (as seen in lower grade gliomas (LGG) in comparison to GBM) correlate with better survival in patients with glioma and other cancers (**9,41**). Yet, some LGG patients are still at high risk to die of their cancer. We had shown that TNC together with other genes of the AngioMatrix (**3**) or together with Ephrin-B2 (**4**) allows to identify

LGG and GBM patients with worsened survival. Now our new result provides another opportunity to predict glioma patient survival when PDX expression data are available. Although tumors grown in a patient and in a mouse differ, it is remarkable that the expression data from the PDX tumors have survival predictive value for the GBM patients. It is tempting to speculate that in the PDX model of GBM the effect of TNC on migration is more pronounced than in *in situ* GBMs where stromal effects of TNC may mask the tumor cell autonomous role of TNC. Presumably therefore the levels of TNC together with the three-gene signature below the median did not correlate with patient survival in GBM expression data sets (not shown) while they have predictive value in the GBM PDX model.

As TNC expression is regulated by MKL1 and YAP, ablation of these activities may reduce TNC expression and subsequently TNC tumor promoting effects. Yet, our results suggest that lowering YAP activity also can promote tumor cell motility when TNC is present. In contrast, targeting integrin α9β1 signaling may not only reduce TNC expression but may also inhibit tumor cell migration and thus may represent a good anti-cancer targeting opportunity to counteract TNC effects.

Experimental Procedures

More details can be found in the supplemental information section.

Animal experiments

In the GBM xenograft model 5 x 10^6 U87MG control (shCTRL) and knockdown cells (shTNC) were diluted in 100 µl PBS and were injected subcutaneously in the left upper back of nude mice (Charles River); after 55 days, mice were sacrificed (**4**). Tumor tissue was directly frozen in liquid nitrogen and further analyzed by qPCR. Experiments with animals were performed according to the guidelines of INSERM and the ethical committee of Alsace, France (CREMEAS).

Cell culture

T98G (ATCC ® CRL-1690™) and KRIB cells (Berlin et al., 1993) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) 4.5g/l glucose with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin and 40u/ml gentamicin at 37°C and 5% CO_2. Cells were starved with 1% FBS overnight before drug treatment with 30µM LPA (H2O, Santa Cruz,), 5 µM LB (DMSO, Calbiochem), 2 µM Jasp (DMSO, Santa Cruz) and 10 µM Y27632 (DMSO, Selleck Chemicals) or seeding cells on FN or FN/TNC substrata for 24h (except other indicated time points) in DMEM containing 1%FBS.

Coating with purified ECM molecules

Purification of FN and TNC and coating of cell culture dishes was done using protocols as described (**2,8**). Briefly, FN and TNC were coated in 0.01% Tween 20-PBS at 1 μg/cm^2 before saturation with 10 mg/ml heat inactivated BSA/PBS.

RNA isolation and qPCR

Total RNA was isolated from transiently or stably transfected cells with or without prior growth on FN or FN/TNC for 24h by using TriReagent (Life Technologies) according to the manufacturer's instructions, reverse transcribed and used for qPCR with primers listed in supplementary **Table S1**. See details in supplemental information.

Immunoblotting

Cells were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), separated by polyacrylamide gel electrophoresis, blotted onto nictrocellulose membrane using the Trans-Blot Turbo RTA Mini Nitrocellulose Transfer Kit (BioRad) and incubated with primary and horseradish peroxidase coupled secondary antibodies before signal detection with the Amersham ECL detection reagent. See details in supplemental information.

Immunofluorescence staining

To quantify YAP nuclear localization, cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes and permeabilized in PBS-Triton 0.1% for 10 minutes, incubated with the anti-

YAP antibody and a secondary fluorophore coupled antibody and analyzed with a Zeiss Axio Imager Z2 microscope. At least 150 cells in duplicates per condition were quantified.

Lentiviral transduction of cells

The silencing of MKL1 and TNC was done by short hairpin (sh) mediated gene expression knockdown. Sequence information and clone IDs are shown in **Table S2**. MISSION lentiviral transduction particles (Sigma-Aldrich) or MISSION non-target shRNA control transduction particles (SHC002V, Sigma-Aldrich) with a MOI=1 were used, transduced cells were selected with 2.5 µg/ml puromycin for MKL1 knockdown or 10 ug/ml puromycin for TNC knockdown. Stable knockdown was determined at protein level by immunoblotting.

Transfection and RNAi

Plasmids encoding YAP and MKL1 molecules (see supplemental information) were transiently transfected (JetPEI, Polyplus) according to the manufacturer`s instructions. The siRNA Reagent System (sc-45064, Santa Cruz) for reducing expression of YAP, MKL1, integrin α9β1 and syndecan-4 was used according to the manufacturer's instruction. See details in supplemental information.

Luciferase reporter assay

Cells were transiently transfected (JetPEI, Polyplus, Strasbourg, France) with SRF and TEAD luciferase constructs (see supplemental information) together with the pRL-TK (TK-Renilla) plasmid for normalization. Upon lysis in passive lysis buffer (Promega) lysates

were analyzed using the Dual-Luciferase reporter assay system (Promega) and a BioTek Luminometer EL800. Firefly luciferase activity was normalized to internal Renilla luciferase control activity.

Migration assays

For 2D migration, 2 x 10^5 cells were seeded in a 50 mm lumox® dish (SARSTEDT). Real time phase contrast images of cells were taken with a Zeiss microscope (Axiovert observation) every 15 minutes for 24h. Migration of individual cells in the first 12h (10 cells in each field, 2 fields per condition) were analyzed with the ImageJ software. For the Boyden chamber transwell migration assays, 2 x 10^4 cells were plated onto the upper chamber of a transwell filter with 8 µm pores (Greiner Bio-one) that had been coated on the upper side with FN or FN/TNC. 10% FBS in the lower chamber was used as chemoattractant. After 24h cells were fixed with 4% PFA in PBS, stained with DAPI (Sigma D9542), fotographed and quantified using the ZEN Blue software (Zeiss). See details in supplemental information.

Patient survival analysis

The patient dataset, GSE21257 for Osteosarcoma and GSE42669 for GBM available in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/gds) were included in this study. Microsoft Excel was used to extract the expression values of a small number of genes (probesets) of interest and the clinical data from the data matrices were downloaded from GEO. The survival analyses were performed using SPSS23.0. Expression of genes was divided into high and low levels using the median level as the

cut-off point. The survival time of patients, stratified by TNC and CTGF-Cyr61-Cdc42EP3 expression was analyzed by the Kaplan-Meier survival procedure.

Statistical analysis

All experiments were performed at least three times independently with at least two to three replicates per experiment. For all data, Gaussian distribution was tested by the d'Agostino-Pearson normality test. Statistical differences were analyzed by unpaired t-test (with Welch's correction in case of unequal variance) or ANOVA one-way with Tukey post-test for Gaussian dataset distribution. Statistical analysis and graphical representation were done using GraphPad Prism. GSEA (**42**) was used to analyze enrichment of the YAP/TAZ/TEAD target genes (**20**) and MKL1 target genes (**19**) in the TNC specific gene expression signature (**9**). p-values < 0.05 were considered as statistically significant. Mean ± SEM. p values < 0.05 were considered as statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Author contribution

ZS, AS, TR and GO designed the experiments. ZS, AS, OL, TR, DM and AK performed experiments and ZS, AS, TR, OL and GO analyzed the data. TH provided scientific support. ZS, AS, TR and GO wrote the manuscript.

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Table S1

Summary of the impact of TNC on cell migration, actin stress fiber formation and CTGF expression

Substratum	Treatment	Transwell migration	Actin stress fibers	CTGF expression
FN	CTRL	Low	High	High
FN	LB	Low	Low	Low
FN	Jasp	Low	Low	Low
FN/TNC	CTRL	High	Low	Low
FN/TNC	LPA	Low	High	High
FN/TNC	ROCK inhibitor	Low	Low	x
FN/TNC	si- α9 integrin	Low	High	High
FN/TNC	LPA and si- α9 integrin	High	Low	Low
FN/TNC	LPA and si-YAP	High	x	Low
FN/TNC	CA-YAP	Low	High	High
FN/TNC	DN-YAP	High	Low	Low
FN/TNC	LPA and sh-MKL1	Low	x	x
FN/TNC	LPA and si-syndecan-4	Low	x	x
FN/TNC	sh-TNC	Low	x	High[1]

Tumor cell migration, actin stress fiber formation and CTGF expression (as read-out for YAP co-transcriptional activity) was assessed as low or high upon plating cells on FN or FN/TNC, respectively. Actin stress fibers as on FN or on plastic with 10% FCS is considered as “High”; no actin stress fibers, as without FCS is considered as “Low”; CTGF “High” values are in the range of cells on FN or in full medium, CTGF “Low” values are in the range of values on FN with LB. si, si-RNA mediated knockdown, sh, sh-RNA mediated knockdown. x, not determined. [1] in U87MG tumors (4).

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Figures

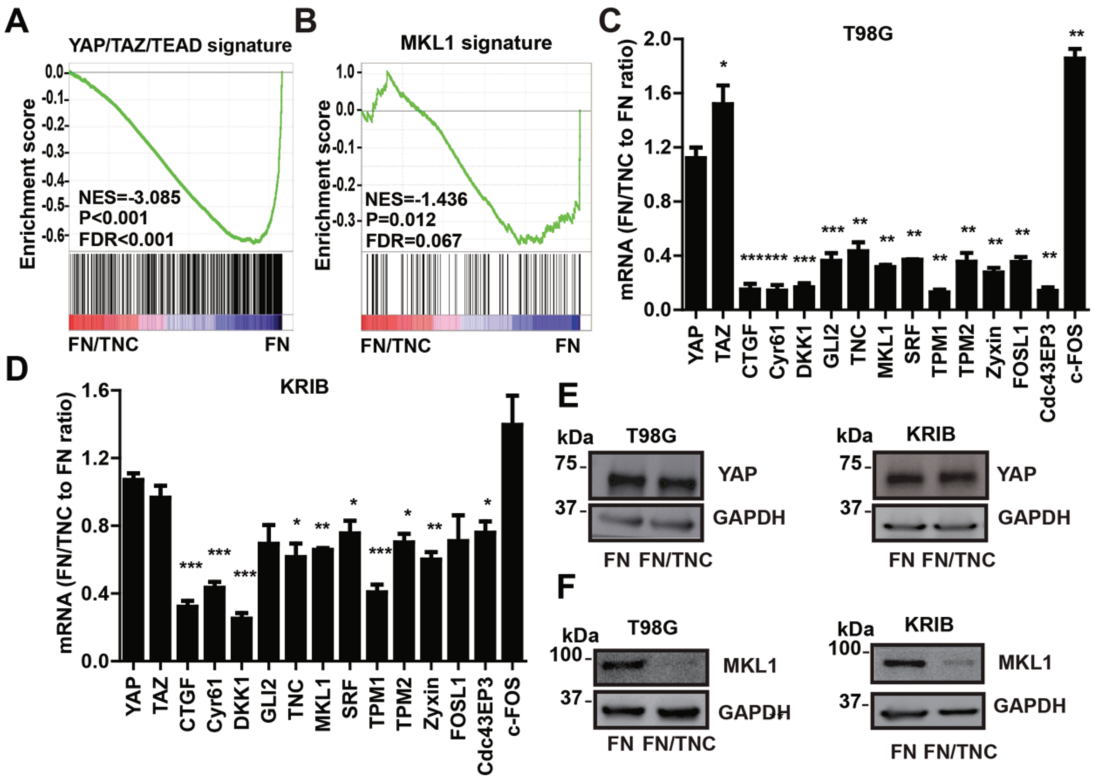

Figure 1. Impact of TNC on MKL1 and YAP target gene expression

Gene Set Enrichment Analysis reveals a significant anti-correlation between TNC and a YAP/TAZ **(A)** and a MKL1/SRF **(B)** gene expression signature, respectively. The Normalized Enrichment Score (NES) and the False Discovery Rate (FDR) q-value assessing the significance of enrichment are indicated. **(C, D)** Gene expression analysis by qPCR of selected genes in T98G **(C)** and KRIB cells **(D)** upon growth on FN or FN/TNC (n = 9) as relative ratio of values on FN/TNC versus FN. **(E)** Immunoblotting for YAP and MKL1 in T98G and KRIB cells on FN or FN/TNC. In all figures n = 9 and n = 6 represents 3 independent experiments with 3 replicates and 2 replicates, respectively. Mean ± SEM.

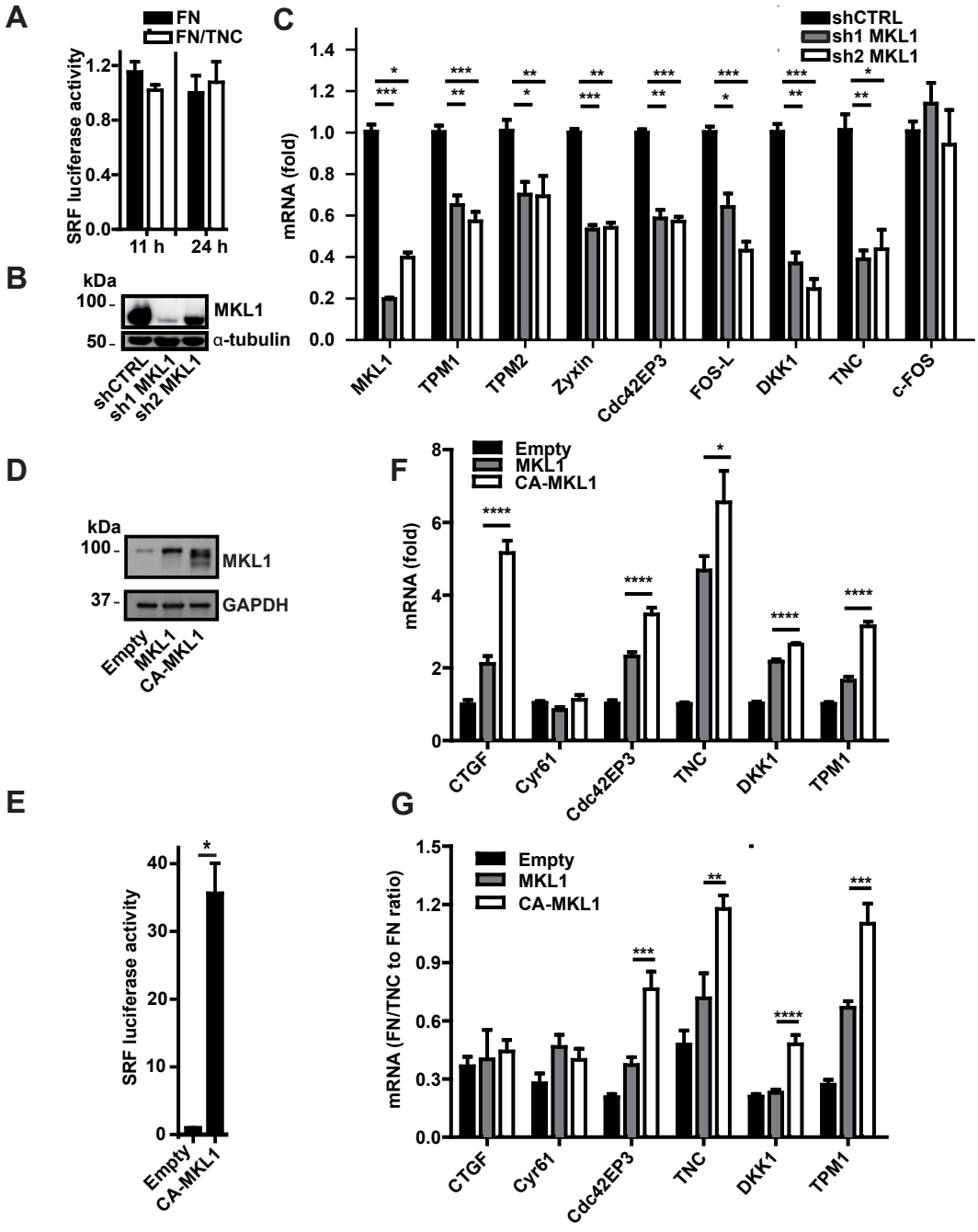

Figure 2. A FN/TNC substratum impairs MKL1 regulated gene expression

Analysis of MKL1 activity in T98G cells. **(A)** SRF reporter assay upon growth on FN or FN/TNC. **(B)** Immunoblotting for MKL1 and α-tubulin in control (shCTRL) or MKL1 knockdown (shMKL1) cells. **(C)** Gene expression analysis by qPCR of stably transfected shCTRL and shMKL1 cells. **(D)** Immunoblotting for MKL1 and GAPDH upon transfection

of empty vector (CTRL) and transfected MKL1 molecules. **(E)** SRF reporter assay upon transfection of CA-MKL1 or empty vector (CTRL). **(F, G)** Gene expression analysis by qPCR in cells transfected with empty vector (CTRL), WT-MKL1 or CA-MKL1 upon growth on plastic (**F**) or on FN or FN/TNC expressed as ratio of values FN/TNC versus FN (**G**). n = 9 except for **E**, n = 6. Mean ± SEM.

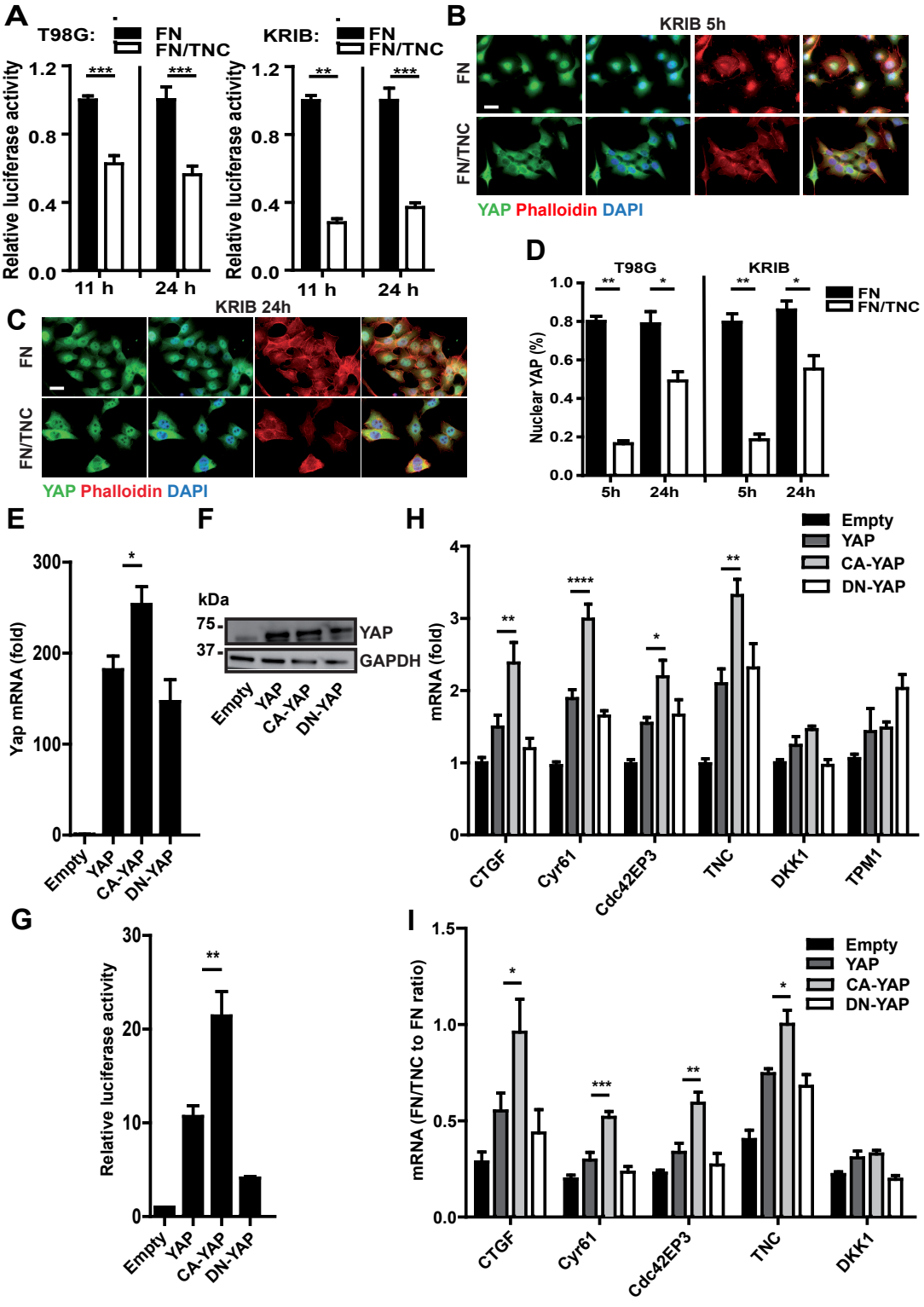

Figure 3. A FN/TNC substratum impairs YAP target gene expression by cytoplasmic retention of YAP

(A) TEAD luciferase assay of T98G and KRIB cells grown on FN or FN/TNC. **(B, C)** Representative images of YAP (green), polymerized actin (phalloidin, red) and nuclei (DAPI, blue) in KRIB cells upon growth on FN or FN/TNC. Scale bar, 5 µm. **(D)** Staining of T98G and KRIB cells on the indicated substrata and quantification of cells with nuclear YAP represented as percentage of all cells. **(E)** YAP expression by qPCR upon transfection of empty vector (CTRL) or YAP expression constructs in T98G cells. **(F)** Immunoblotting for YAP and GAPDH in T98G cells transiently transfected with YAP expression plasmids. **(G)** TEAD luciferase assay upon transfection of YAP expression plasmids in T98G cells. **(H)** Gene expression analysis by qPCR upon transient expression of YAP expression plasmids in T98G cells grown on plastic. **(I)** Ratio of gene expression on FN/TNC versus FN as determined by qPCR upon transient transfection of YAP expression plasmids. n = 9, except for **D** (n = 6) and **G** (n = 7, 3 experiments with at least duplicates). Mean ± SEM.

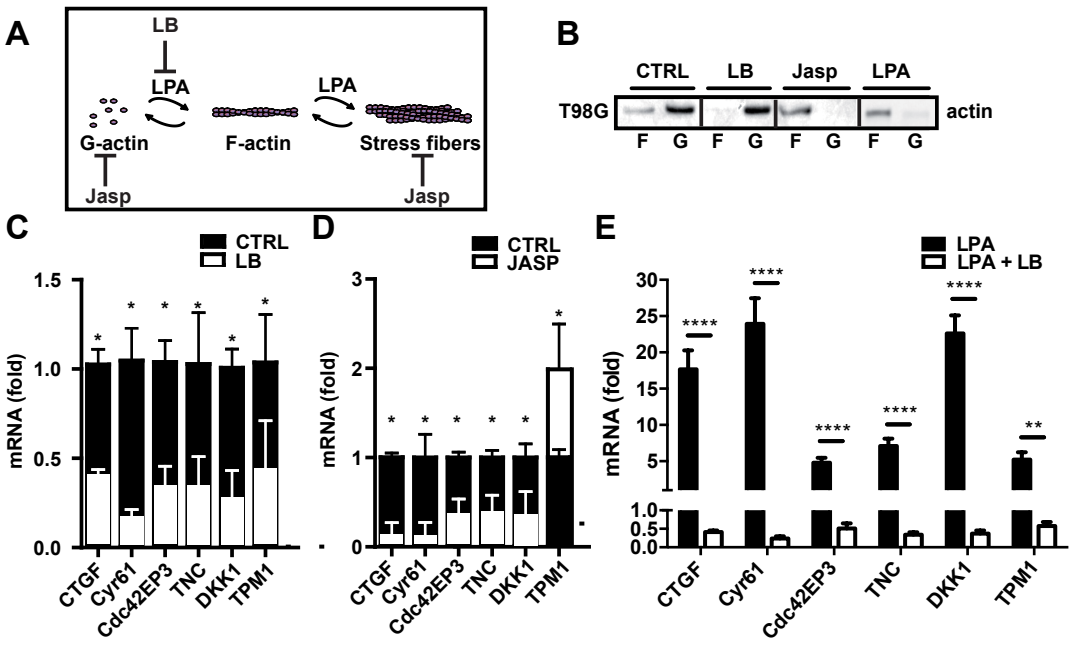

Figure 4. Actin polymerization dependent expression of TNC downregulated genes

(A) Schematic representation of the impact of LB, Jasp and LPA on actin polymerization. (**B**) Analysis of G-actin and F-actin by western blot upon treatment of T98G cells with LB, Jasp or LPA. (**C-E**) Gene expression analysis by qPCR of TNC target genes in T98G cells upon treatment with LB (**C**), Jasp (**D**) or LPA plus LB (**E**) after 5h (n ≧ 6, 3 experiments with at least duplicates). Mean ± SEM.

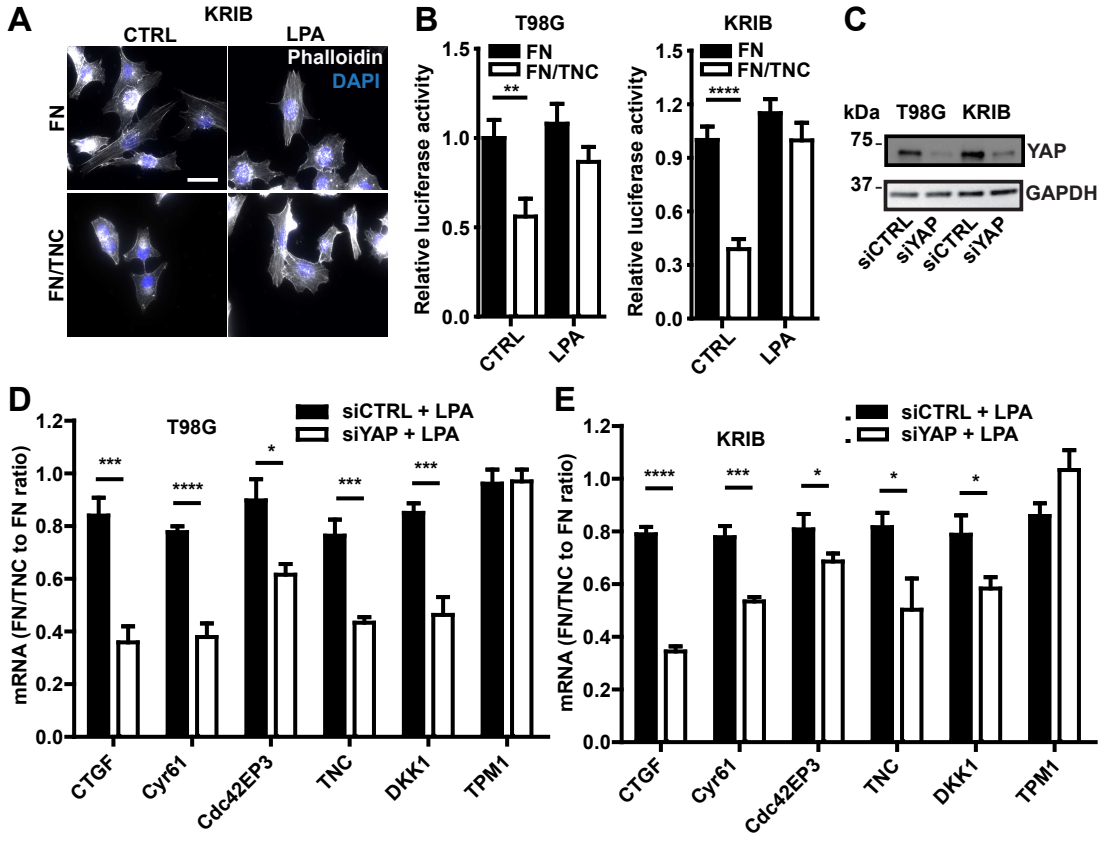

Figure 5. TNC downregulates YAP target gene expression through blocking actin stress fibers

(A) Representative image of polymerized actin (phalloidin, white) and nuclei (DAPI, blue) of KRIB cells on FN or FN/TNC with or without LPA treatment after 5h. Scale bar, 5 µm). **(B)** TEAD luciferase assay upon growth on FN or FN/TNC with or without LPA for 24h (n = 12, 4 experiments with triplicates). **(C)** Immunoblot for YAP upon YAP siRNA mediated knockdown. (**D, E)** Gene expression analysis in T98G (**D**) and KRIB cells (**E**) by qPCR upon treatment with LPA and siYAP and growth on FN or FN/TNC. Relative expression is depicted as ratio of values on FN/TNC versus FN, n = 9. Mean ± SEM.

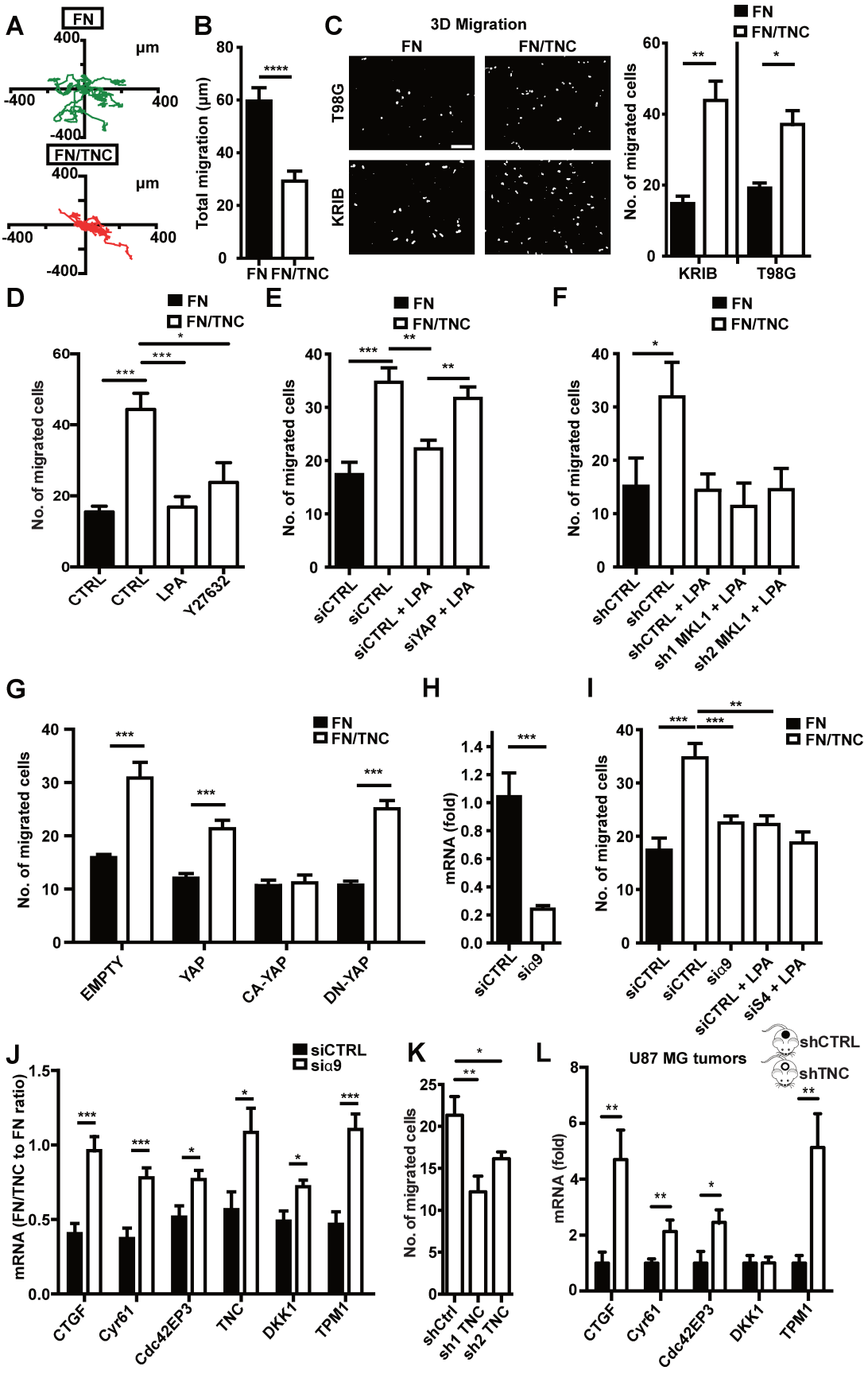
A
FN
400
μm
-400
400
-400
FN/TNC
400
μm
-400
400
-400
B
Total migration (μm)
80
60
40
20
0
****
FN FN/TNC
C
3D Migration
FN
FN/TNC
T98G
KRIB
FN
FN/TNC
No. of migrated cells
60
40
20
0
**
*
KRIB
T98G
D
FN
FN/TNC
No. of migrated cells
60
40
20
0
*
***
***
CTRL
CTRL
LPA
Y27632
E
FN
FN/TNC
No. of migrated cells
40
30
20
10
0
***
**
**
siCTRL
siCTRL
siCTRL + LPA
siYAP + LPA
F
FN
FN/TNC
No. of migrated cells
40
30
20
10
0
*
shCTRL
shCTRL
shCTRL + LPA
sh1 MKL1 + LPA
sh2 MKL1 + LPA
G
FN
FN/TNC
No. of migrated cells
40
30
20
10
0
***
***
***
EMPTY
YAP
CA-YAP
DN-YAP
H
mRNA (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
siCTRL
siα9
I
FN
FN/TNC
No. of migrated cells
40
30
20
10
0
***
***
**
siCTRL
siCTRL
siα9
siCTRL + LPA
siS4 + LPA
J
siCTRL
siα9
mRNA (FN/TNC to FN ratio)
1.5
1.0
0.5
0.0
***
***
*
*
*
***
CTGF
Cyr61
Cdc42EP3
TNC
DKK1
TPM1
K
No. of migrated cells
25
20
15
10
5
0
*
**
shCtrl
sh1 TNC
sh2 TNC
L
U87 MG tumors
shCTRL
shTNC
mRNA (fold)
8
6
4
2
0
**
**
*
**
CTGF
Cyr61
Cdc42EP3
DKK1
TPM1

Figure 6. TNC promotes migration through integrin α9β1 and requires inactive YAP

Assessment of 2D migration (**A, B**) showing the movement of individual KRIB cells during 12h live imaging (**A**) and 3D transwell migration 24h after seeding T98G and KRIB cells on FN or FN/TNC (**C**), and upon treatment with LPA or Y27632 (**D**), knockdown of YAP (**E**), MKL1 (**F**), integrin α9 (**I**), syndecan-4 (**I**) and TNC (**K**), respectively and, upon overexpression of YAP molecules (**G**) in KRIB cells. Assessment of successful knockdown of integrin α9 in KRIB cells (**H)** and mRNA levels of the indicated molecules upon knockdown of integrin α9 **(J)** expressed as ratio of values for FN/TNC versus FN. Scale bar, 20 μm, n = 6, except for **G** (n = 7, 3 experiments with at least duplicates) and **E**, **I**, **J** (n = 9). (**L**) mRNA levels of the indicated genes in U87MG tumors with control and knockdown levels of TNC (n = 9 mice in the control group, n = 6 mice in the shTNC group). Mean ± SEM.

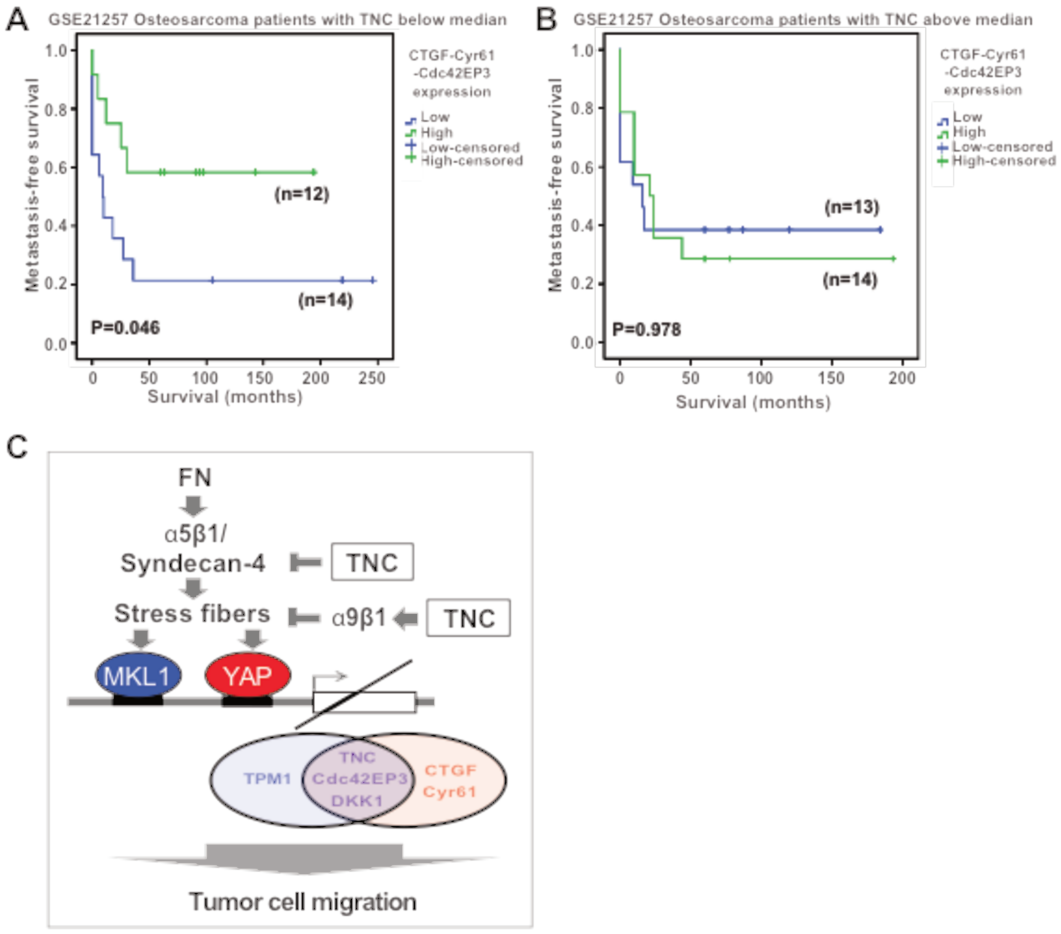

Figure 7 Kaplan Meier survival analysis in osteosarcoma patients and summary of TNC effects on actin polymerisation, gene expression and tumor cell migration

Kaplan-Meier survival analysis of patients with osteosarcoma upon stratification into tumors with low (**A**) and high (**B**) expression of TNC and combined high or low expression of CTGF, Cyr61 and Cdc42EP3 in each subgroup. The number of patients in each group is indicated within brackets, and p values indicate the significance of survival differences between the groups of individuals by log-rank test. (**C**) TNC impairs actin polymerisation and actin stress fiber formation by two mechanisms that involve competition with syndecan-4 binding to FN thus inhibiting integrin α5β1 signaling (which depends on syndecan-4 as coreceptor) (Huang et al., 2001, Cancer Res) and, through activation of integrin α9β1 by TNC (shown here). Upon actin stress fiber formation MKL1 and YAP are translocated to the nucleus where they regulate expression of downstream target genes. Genes are repressed by TNC through impaired MKL1 (TPM1), impaired YAP (CTGF, Cyr61) and impaired MKL1 and YAP (TNC, Cdc42EP3 and DKK1). Whereas TNC inhibits

mesenchymal-like migration through blocking syndecan-4, TNC increases 3D (amoeboid-like) migration through YAP. Activation of integrin α9β1 by a TNC substratum or, by TNC expressed by the tumor cell itself induces 3D migration in a paracrine and autocrine manner, respectively. This mechanism might be relevant for diagnosis and therapy, as patients with osteosarcoma that have low levels of CTGF, Cyr61 and Cdc42EP3 in a context of TNC levels below the median (yet abundant) have a worsened metastasis-free survival in comparison to high levels of TNC above the median. Thus targeting TNC is important as soon as it is expressed to block its impact on 3D migration of tumor cells.

Supplemental figures

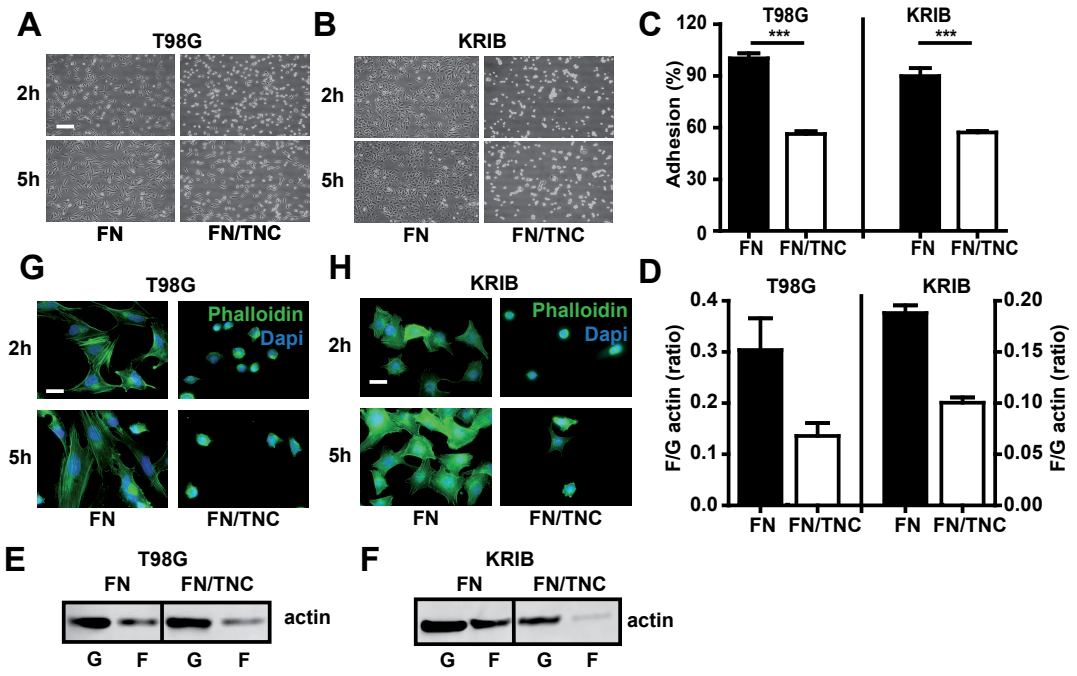

Figure S1. Impact of TNC on cell adhesion and actin polymerization

(A, B) Representative phase contrast pictures of T98G (**A**) and KRIB cells (**B**) upon seeding for 2h and 5h on FN or FN/TNC. Scale bar, 40 µm. **(C)** Quantification of adherent cells measured at OD 595 nm upon plating for 1h on FN or FN/TNC at 1 µg/cm^2 and staining with crystal violet, n = 12, 4 experiments with triplicates. Analysis of G-actin and F-actin in T98G (**E, D, G**) and KRIB cells (**F, D, H**) by immunoblotting upon growth on the indicated substrata for 2h in full medium and quantification of the signals as F/G actin ratio, n = 3 **(D).** Representative images of polymerized actin (phalloidin, green) and nuclei (DAPI, blue) of T98G (**G**) and KRIB cells (**H**) upon growth on FN or FN/TNC for 2h and 5h in full medium. Scale bar, 5 µm. Mean ± SEM.

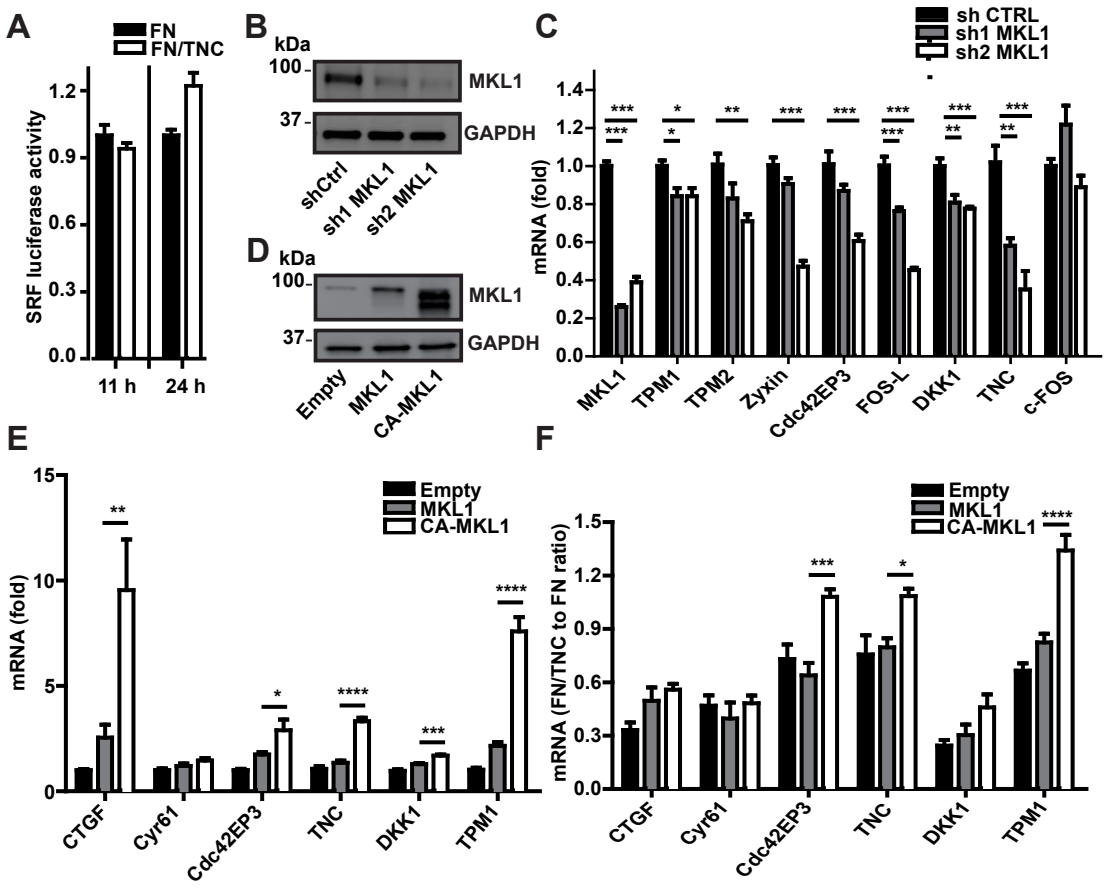

Figure S2 A FN/TNC substratum represses MKL1 and impairs MKL1 regulated gene expression in KRIB cells

Analysis of MKL1 activities in KRIB cells. **(A)** SRF reporter assay upon growth on FN or FN/TNC, n = 9. **(B)** Immunoblotting for MKL1 and α-tubulin in control (shCTRL) or MKL1 knockdown (shMKL1) cells. **(C)** Gene expression by qPCR in stably transfected shCTRL and shMKL1 cells, n= 6. **(D)** Immunoblotting for MKL1 upon expression of CA-MKL1. (**E, F)** Gene expression by qPCR in cells transfected with CA-MKL1, WT-MKL1 or empty vector (CTRL). Cells were grown on plastic in full medium (**E**) or upon plating on FN or FN/TNC, n = 9 (**F**). Mean ± SEM.

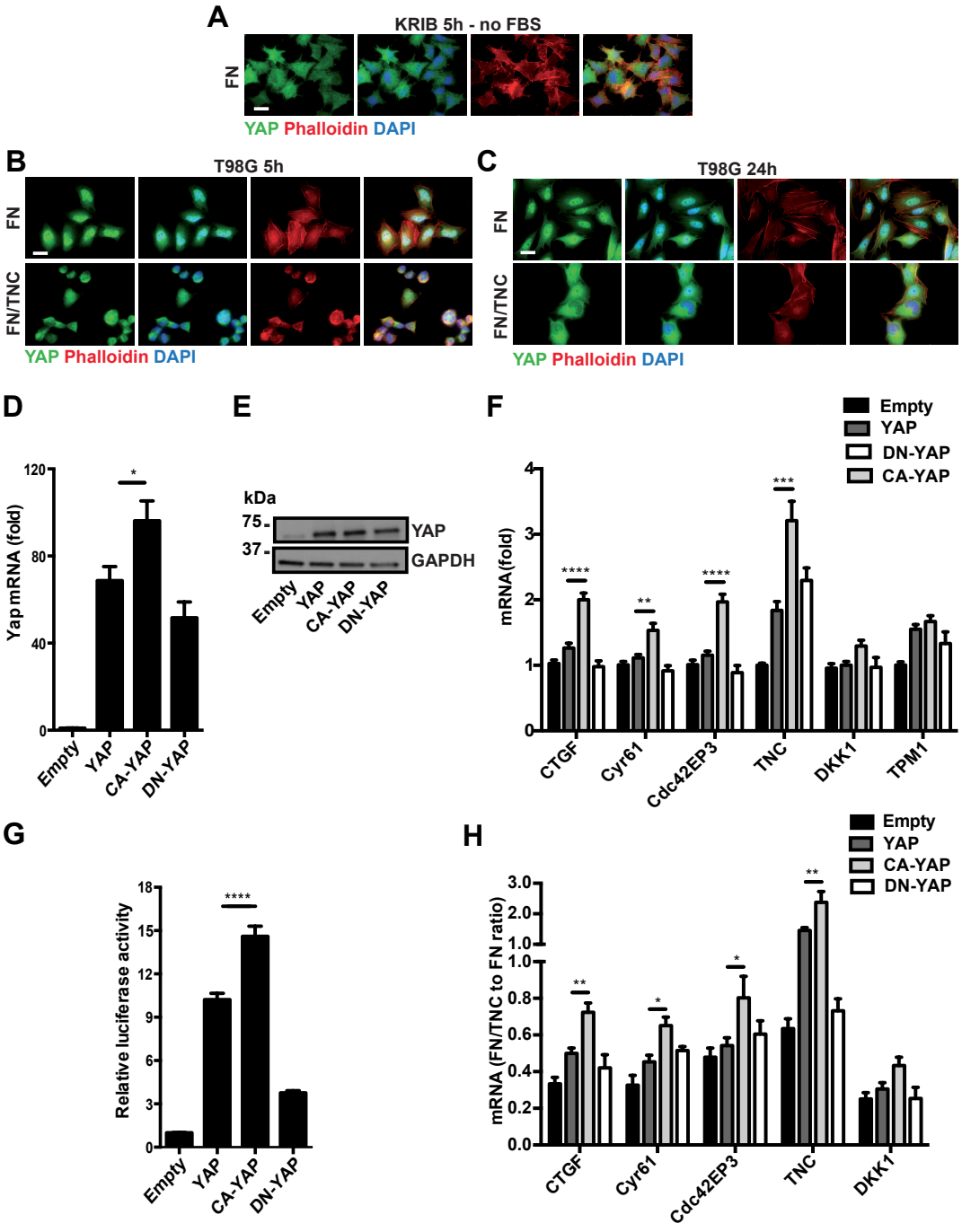

Figure S3. A FN/TNC substratum impairs YAP target gene expression by cytoplasmic retention of YAP in KRIB cells

(A - C) Representative images of KRIB (**A)** and T98G cells (**B, C**) upon immunostaining for YAP (green), polymerized actin (phalloidin, red) and nuclei (DAPI, blue) in absence of FBS for 5h (**A**) and in 1% FBS upon growth on FN and FN/TNC for 5h (**B**) and 24h (**C**).

Scale bar, 5 µm, n = 6. **(D, E)** YAP mRNA levels (qPCR) (**D**) and protein levels (western blot) (**E**) upon transfection of empty vector (CTRL) or YAP expression constructs, n = 9. **(G)** TEAD luciferase activity upon transfection of YAP molecules, n = 9. **(F, H)** Gene expression by qPCR upon transient expression of YAP molecules in cells grown on plastic (**F**) or matrix coating (**H**); ratio of gene expression on FN/TNC versus FN, n = 9 (**H**). Mean ± SEM.

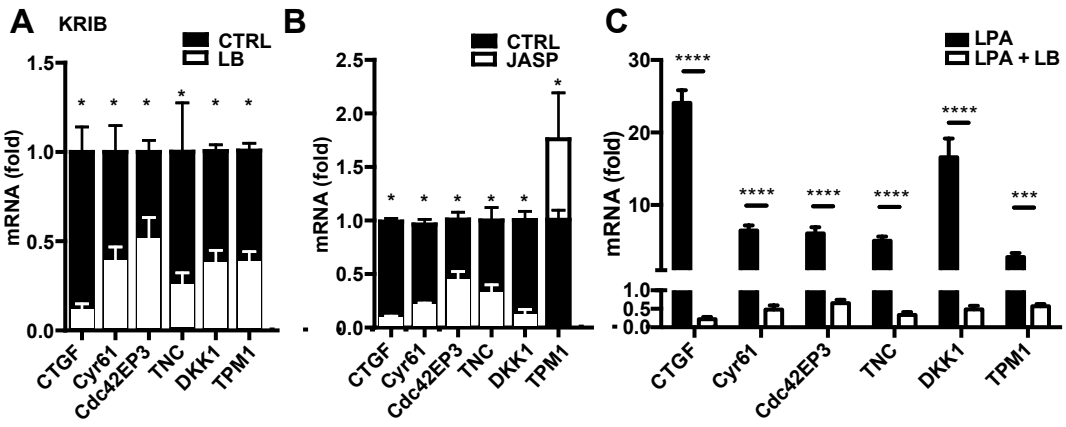

Figure S4. Actin polymerization dependent expression of TNC downregulated genes in KRIB cells

Gene expression analysis by qPCR in KRIB cells upon treatment with LB (**A**), Jasp (**B**) or LPA plus LB (**C**) (n ≧ 6, 3 experiments with at least duplicates). Mean ± SEM.

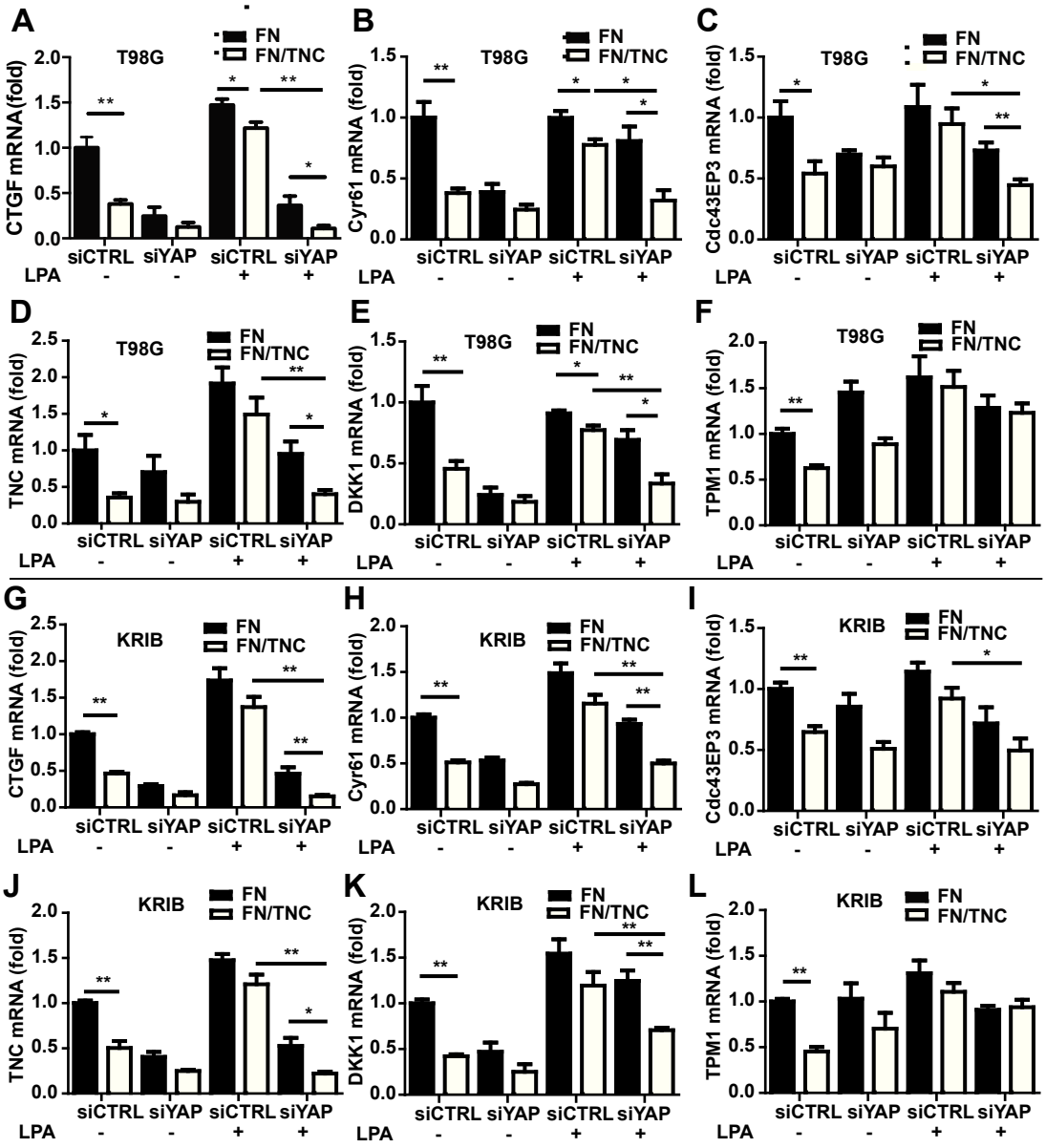

Figure S5 Target gene expression in KRIB and T98G cells plated on FN/TNC upon impacting on downstream signaling

Gene expression analysis by qPCR of T98G cells **(A – F)** and KRIB cells **(G – L)** upon growth on FN or FN/TNC together with LPA and siYAP for 24h, n = 9. Mean ± SEM.

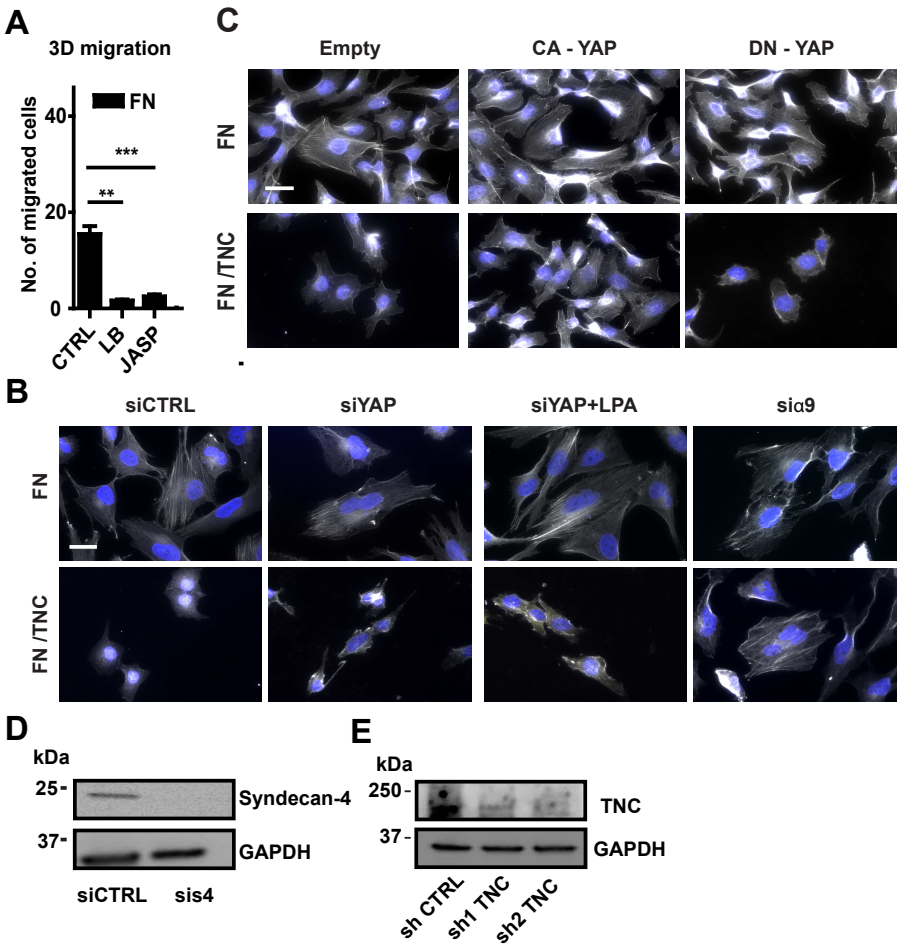

Figure S6 Transwell migration, gene expression and actin stress fiber formation in KRIB cells

(A) Transwell migration of KRIB cells upon plating on FN and treatment with LB or Jasp, n = 6. (**B, C**) Phalloidin staining of cells upon KD of YAP and integrin α9 (**B**) and expression of CA-YAP and DN-YAP (**C**). Scale bar, 5 µm. (**D, E**) Gene expression (immunoblot) of syndecan-4 (**D**) and TNC (**E**) upon KD of the respective molecule. Mean ± SEM.

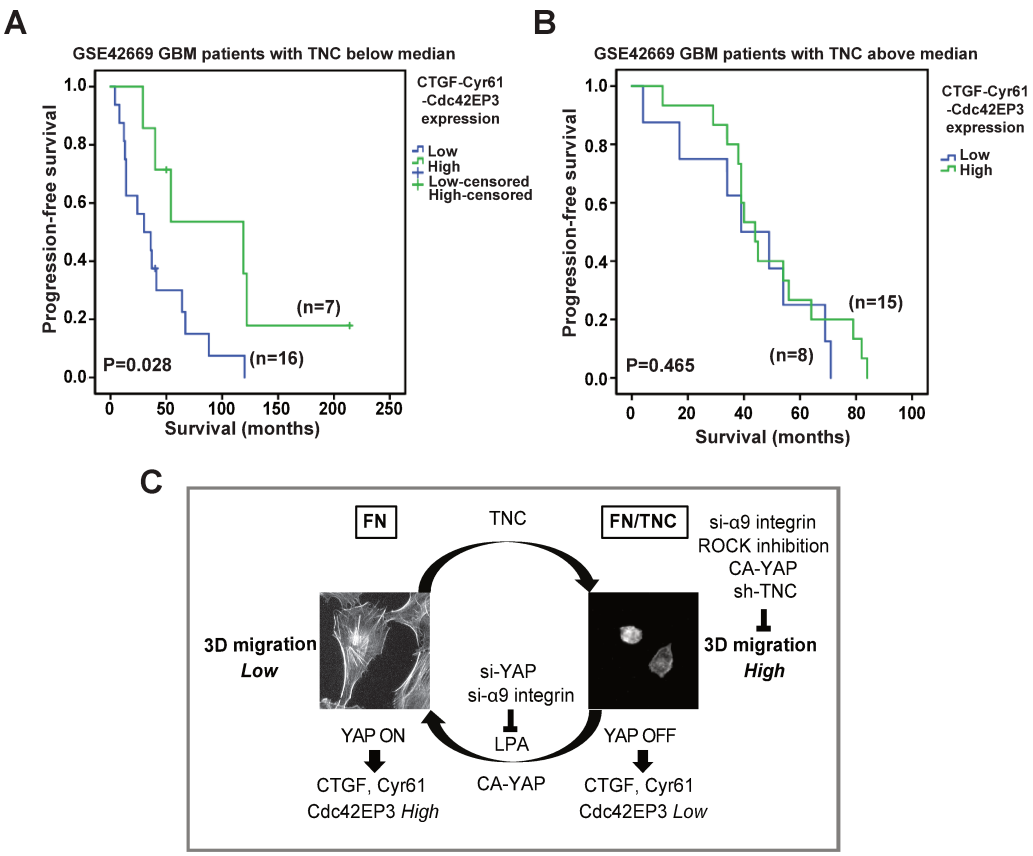

Figure S7 GBM Kaplan Meier survival analysis and summary of cell responses to inhibitors and LOF and GOF approaches

Kaplan-Meier survival analysis of patients with GBM (**A, B**) upon stratification into tumors with high and low expression of TNC and combined high or low expression of CTGF, Cyr61 and Cdc42EP3 in each subgroup. The GBM PDX (Joo et al., 2013) and GSE 42669 data were used here. In the TNC high and TNC low subgroups patients were stratified according to the median average expression of CTGF-Cyr61-Cdc42EP3 as high if the value was above the cutoff and as low if the value was below the cutoff. The number of patients in each group is indicated within brackets, and p values indicate the significance of survival differences between the groups of individuals by log-rank test. (**C**) Cells on FN have active YAP and high expression of CTGF, Cyr61 and Cdc42EP3 which correlates with lower 3D migration as on the FN/TNC substratum. On FN/TNC no actin stress fibers are formed and all treatment that restores actin stress fibers such as LPA and expression of CA-YAP abolish the TNC effect on transwell migration. Yet, inhibition of actin stress fibers on FN by Jasp is not sufficient to trigger high 3D migration. TNC specific cell

migration is dependent on integrin α9β1 endogenous and exogenous TNC and active ROCK as KD of α9, KD of TNC and inhibition of ROCK reduce this migration, respectively.

Supplemental information

for Sun et al., “Through integrin α9β1 blocking actin stress fibers and impairing YAP target gene expression tenascin-C promotes migration of tumor cells”

Supplemental experimental procedures

Supplemental Tables 1 and 2

Supplemental figure legends to Figures S1 – S7

Legends to supplemental videos 1 and 2

References to supplemental information

Supplemental experimental procedures

RNA isolation and qPCR

Total RNA was isolated from transiently or stably transfected cells with or without prior growth on FN or FN/TNC for 24h by using TriReagent (Life Technologies) according to the manufacturer's instructions. RNA was reverse transribed (MultiScribe reverse transcriptase, Applied Biosystems) and qPCR was done on cDNA diluted 1:5 in water on a 7500 Real Time PCR machine (Applied Biosystems) using SYBR green reaction mixture (Applied Biosystems). Data were normalized by using a Taqman human GAPDH Endogenous Control (4326317E, Life Technology) and fold induction was calculated using the comparative Ct method (-ddCt). Primers used for qPCR are listed in supplementary material **Table S1**.

Immunoblotting

Cells were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). Rabbit polyclonal antibodies against MKL1 (Abcam, ab49311 1:600), murine monoclonal antibodies against Actin (Abcam, ab14128, 1:1000), YAP (Santa Cruz, sc-101199, 1:1000), α-tubulin (EMD Millipore, CP06, 1:2000) and syndecan-4 (Santa Cruz, sc-12766, 1:200) and goat monoclonal antibody against GAPDH (Santa Cruz, sc-20357, 1:1000) were used. Secondary antibodies were ECL horseradish peroxidase linked whole anti-rabbit (NA934V), anti-mouse (NXA931) (GE Healthcare, Buckinghamshire, UK) and donkey anti-goat IgG (sc-2020, Santa Cruz, Biotechnology). Amersham ECL (RPN2106) or Amersham ECL-Plus Western blotting detection systems (RPN2132) (GE Healthcare, Buckinghamshire, UK) were used.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes and permeabilized in PBS-Triton 0.1% for 10 minutes. After blocking for 1h at room temperature in 5% NGS in PBS, cells were stained with anti-YAP antibody (Santa Cruz, sc-101199, 1:200) overnight at 4℃, secondary antibody for 1h and fluorescein conjugated phalloidin (Sigma P1591, 1/200) for 45 minutes at room temperature (RT). After staining of nuclei with DAPI (Sigma D9542), cells were embedded into FluorSaveTM Reagent (345789, Calbiochem) and analyzed with a Zeiss Axio Imager Z2 microscope. To quantify YAP nuclear localization, we calculated the percentage of cells with nuclear staining among all cells counting at least 150 cells in duplicate per condition.

Transfection and RNAi

For transient transfection (JetPEI, Polyplus) with plasmids encoding YAP-WT, constitutively active YAP (CA-YAP, S127A mutant) (**1**) and dominant negative non-TEAD interacting YAP molecules (DN-YAP, S127A-S94A mutant) (**1**) were used. MKL1-WT (pEF fl MAL HA) and CA-MKL1 (pEF HA-ΔN MAL) were both provided by Guido Posern (Halle-Wittenberg University, Halle, Germany). 3 x 10^5 cells were seeded in 6 well dishes the day before transfection with 3000 ng plasmid per well. Empty pCB6 was used for control transfection. Successful transfection was monitored by co-transfection of 200 ng pQCXIP-GFP. We used transient transfection, that reached about 30% of cells since cells died upon stable expression of active YAP molecules. The silencing of human YAP (sc-38637, Santa Cruz), human Integrin α9 (sc-75340, Santa Cruz) or human syndecan-4 (sc-36588, Santa Cruz) was done by small interfering (si) RNA and non-targeting siRNA (sc-37007,

Santa Cruz) as control. The siRNA Reagent System (sc-45064, Santa Cruz) was used according to the manufacturer's instruction.

Luciferase reporter assay

Cells were transiently transfected (JetPEI, Polyplus, Strasbourg, France) with the pGL3-5 x MCAT(SV)-49 plasmid (provided by I. Farrance, University of Maryland School of Medicine, Baltimore, U.S.A) encoding 5 x MCAT (TEAD binding sites) or 3DA.Luc plasmid (provided by Guido Posern, Halle-Wittenberg University, Halle, Germany) encoding c-fos derived SRF binding sites. The pRL-TK (TK-Renilla) plasmid was transfected at the same time for normalization. Cells were lyzed with passive lysis buffer (Promega) and lysates were analyzed using the Dual-Luciferase reporter assay system (Promega) and a BioTek Luminometer EL800. Firefly luciferase activity was normalized to internal Renilla luciferase control activity.

Fractionation into G- and F-actin

G- and F-actin was separated as described (**2**). Briefly, one million cells (1% FBS) were seeded into 6 well plates that had been coated with FN or FN/TNC. After 2h cells were lysed (20mM HEPES pH 7.7, 50mM NaCl, 1mM EDTA and 0.5% (v/v) Triton X-100). Upon centrifugation (100 000g, one hour at 4°C), the supernatants were collected (G-actin fraction), the pellets (F-actin fraction) were resuspended in the same buffer and sonicated, and both fractions were separated by SDS-PAGE (20µg protein) and stained for actin after immunoblotting.

Adhesion assay

Ninety six well plates (BD Bioscience) were coated with 1 µg/cm^2 of purified FN or FN and TNC (FN/TNC) followed by BSA in 4 replicates. Cells were plated for 1h at 37°C and then washed to remove non adherent cells. Cells were fixed with methanol (30 minutes at room temperature), washed and stained with 0.1% Cristal Violet (in H_2O). Pictures were taken before cells were lysed in 50 µl DMSO to measure the optical density (OD) at 595 nm.

Migration assays

For 2D migration, 2 x 10^5 cells were seeded in a 50 mm lumox® dish (SARSTEDT). Real time phase contrast images of cells were taken with a Zeiss microscope (Axiovert observation) every 15 minutes for 24h. Migration of individual cells were analyzed with the ImageJ software for the first 12h (10 cells in each field, 2 fields per condition). For the Boyden chamber transwell migration assays, 2 x 10^4 cells, suspended in medium containing serum-free DMEM (with different drugs), were plated onto the upper chamber of a transwell filter with 8 µm pores (Greiner Bio-one) that had been coated on the upper side with FN or FN/TNC. 10% FBS in the lower chamber was used as chemoattractant. After 24h cells were fixed with 4% PFA in PBS. Non-migrated cells on the upper side of the filter were removed with a cotton swab, and cells on the underside of the filter were stained with DAPI (Sigma D9542). Images were taken with 10x objective and cells were counted using the ZEN Blue software (Zeiss). For each experiment, the number of cells in eight random fields was counted, and three independent experiments with at least duplicates were analyzed.

Patient survival analysis

The patient dataset, GSE21257 (**3**) for Osteosarcoma and GSE42669 (**4**) for GBM available in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/gds) were included in this study. For each GEO data series, links are provided at the bottom of the page to the Series Matrix File(s), which contain the expression values for each gene (probeset) and survival information. Microsoft Excel was used to extract the expression values of a small number of genes (probesets) of interest and the clinical data from the data matrices were downloaded from GEO. The survival analyses were performed using SPSS23.0. Expression of genes was divided into high and low levels using the median level as the cut-off point. The CTGF-Cyr61-Cdc42EP3-low group consists of patients whose tumors expressed all three genes at low levels (below median), while the CTGF-Cyr61-Cdc42EP3-high group consists of patients whose tumors expressed the three genes at high levels (above median). The survival time of patients, stratified by TNC and CTGF-Cyr61-Cdc42EP3 expression was analyzed by the Kaplan-Meier survival procedure.

Supplemental Table S1, Primers used in qPCR

Gene	Forward primer	Reverse primer
DKK 1	GACCATTGACAACTACCAGCCG	TACTCATCAGTGCCGCACTCCT
YAP	CGCTCTTCAACGCCGTCA	AGTACTGGCCTGTCGGGAGT
TAZ	GGCTGGGAGATGACCTTCAC	AGGCACTGGTGTGGAACTGAC
CTGF	AGGAGTGGGTGTGTGACGA	CCAGGCAGTTGGCTCTAATC
Cyr61	AGCCTCGCATCCTATACAACC	TTCTTTCACAAGGCGGCACTC
GLI2	AGATGTTGTAAGAGAAGGTTTATG	CGTTAGCCGAATGTCAGC
TNC	CCTTGCTGTAGAGGTCGTCA	CCAACCTCAGACACGGCTA
TPM1	GCACCGAAGATGAACTGGACAA	CATCGGTGGCCTTTTTCTCTG

TPM2	CCAACAACTTGAAATCCCTGG	CTTTGGTGGAATACTTGTCCGC
Zyxin	CATGAAGTGTTACAAGTGTGAGGAC	AGTGTGGCACTTCCGACAG
FOSL1	AGTTACCCCAGGCCTCTGAC	CTTCCTCCGGGCTGATCT
Cdc42EP3	GCATCTCAGTCCAGCCAAG	GCCAGTCGGGGTACTGTTC
SRF	AGACGGGCATCATGAAGAAG	TGATCATGGGCTGCAGTTT
c-FOS	GTCCTTACCTCTTCCGGAGATGT	ACTAACTACCAGCTCTCTGAAGTGTCA
MKL1	TAGTGAGCGGAAGAATGTGC	ATCCCTTGGCTCACCAGTT
ITGA9	AAATGAGGAAAAAGATGGAGGTC	TCCAGGGTTGAGAGAGTTGG

Supplemental Table S2, TRC numbers and shRNA sequences

Gene	Name	Clone ID	Sequence (5'-3')
MKL1	Sh1	TRCN0000083563	CCGGGCGGAGAAATTTCAGCAGATTCTCGAGAATCTGCTGAAATTTCTCCGCTTTTTG
	Sh2	TRCN0000083565	CCGGGACTATCTCAAACGGAAGATTCTCGAGAATCTTCCGTTTGAGATAGTCTTTTTG
TNC	Sh1	TRCN0000230785	CCGGGGAGTACTTTATCCGTGTATTCTCGAGAATACACGGATAAAGTACTCCTTTTTG
	Sh2	TRCN0000230788	CCGGCCAGTGACAACATCGCAATAGCTCGAGCTATTGCGATGTTGTCACTGGTTTTTG

Legends to supplemental videos 1 - 2

Video 1. Assessment of KRIB cell mobility upon plating on FN in 1% FCS for 24h by time lapse microscopy.

Video 2. Assessment of KRIB cell mobility upon plating on FN/TNC in 1% FCS for 24h by time lapse microscopy.

References to supplemental information

1. Lamar JM, Stern P, Liu H, Schindler JW, Jiang ZG, Hynes RO. The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain. Proceedings of the National Academy of Sciences 2012; 109: E2441–E2450.

2. Posern G, Sotiropoulos A, Treisman R. Mutant actins demonstrate a role for unpolymerized actin in control of transcription by serum response factor. Molecular biology of the cell 2002; 13: 4167–4178.

3. Buddingh EP, Kuijjer ML, Duim RAJ, Bürger H, Agelopoulos K, Myklebost O, et al. Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents. Clin Cancer Res 2011; 17: 2110–2119.

4. Joo KM, Kim J, Jin J, Kim M, Seol HJ, Muradov J, et al. Patient-Specific Orthotopic Glioblastoma Xenograft Models Recapitulate the Histopathology and Biology of Human Glioblastomas In Situ. Cell Reports 2013; 3: 260–273.

5. Discussion and perspectives

In my thesis work, I provided results from a comprehensive *in vivo* and *in vitro* analysis addressing the roles of TNC in tumor cell migration and breast cancer lung metastasis. TNC is a secreted 190-300 kDa matricellular ECM glycoprotein that is highly expressed during development whereas in organs of the adult TNC expression is largely restricted to a few sites. TNC is highly expressed in cancer tissue where it may have multiple functions that are incompletely understood. High TNC expression correlates with poor survival in multiple cancer types such as breast cancer, lung cancer, head and neck cancer, melanoma, colorectal cancer and gliomas (Lowy et al., 2015). Whereas there is some evidence that supports a role of TNC in breast cancer lung metastasis outgrowth (Oskarsson et al., 2011), we poorly understand the roles of TNC in CTC and LVI which I have addressed here in detail in the first part of my thesis. I observed multiple effects of TNC that will be discussed in detail below. One importat finding is that TNC promotes EMT which involves TGF-β signaling that provides tumor cells motile and invasive properties.

In the second part of my thesis work I addressed a potential impact of TNC on single cell migration through its impact on the actin cytoskeleton. EMT leads to separation of cells that can migrate in a mesenchymal mode which is due a particular gene transcription program **(**Jahn et al., 2012). Under particular conditions, cells can adopt an amoeboid mode of migration which is obtained through mesenchymal-to-amoeboid transition (MAT). MAT involves rapid changes in the migratory mode that arises as a reply to the local properties of the TME and are reversible and transiently regulated (Paňková et al., 2010). The actin polymerisation state determines whether cells migrate in a mesenchymal (actin stress fiber dependent) or amoeboid (actin polymerisation independent or stress fiber inhibited) mode (Bergert et al., 2012). Since TNC as substratum impairs actin stress fiber formation (Huang et al., 2001, Orend et al., 2003, Saupe et al., 2013), we asked whether a TNC-rich substratum can force tumor cells to migrate in an amoeboid-like mode.

First manuscript

5.1 Stromal tenascin-C increases breast cancer lung metastasis by impacting on lymphovascular invasions

5.1.1. Tenascin-C accelerates tumor onset in the murine MMTV-NeuNT breast cancer model

The amplification of oncogene ErbB2, a member of EGF receptor family, is associated with 15 to 20% of human breast cancers. Currently, the expression of this gene is widely used as a prognostic marker and determines the choice of treatment (Ariga et al., 2005). Consistent with this observation, by constitutive activation of the rat homolog of ErbB2 (Neu) driven by the mouse mammary tumor virus promoter (MMTV), Muller and colleagues developed the MMTV-NeuNT transgenic mouse model harboring an activated form of Neu (NeuNT) where mice develop stochastically multifocal mammary adenocarcinomas that metastasize to the lung in about 15 weeks after pregnancy (Muller et al., 1988). Like the other ErbB2-induced breast cancer mouse models (Oshima et al., 2004; Guy et al., 1992; Siegel et al., 2003), the MMTV-NeuNT mouse model exhibits both intravascular and parenchymal metastasis which provides a good tool to comprehensively study breast cancer metastasis, in particular the role of LVI in context of TNC.

We generated MMTV-NeuNT tumor mice lacking TNC (TNC KO) by breeding and compared tumorigenesis with mice expressing wildtype (WT) levels of TNC where TNC is highly induced in tumorigenesis. We found that tumor latency was largely delayed in TNC KO mice in comparison to WT mice presuming that TNC may impact on events that trigger dysplasia. Yet, the number of dysplastic lesions was not higher in TNC WT mice (data not shown). We also found no statistically significant difference in total tumor numbers nor tumor mass between genotypes suggesting that TNC may not play a role in early lesions and progression into tumors, and/or that TNC is not required for promoting the growth of established tumors.

5.1.2. LVI can form large CTC clusters that may be underestimated in cancer diagnosis

CTC clusters have been observed in patients with cancers of different origin (Winterbauer et al., 1968; Kane et al., 1975; Cho et al., 2012; Molnar et al., 2001; Stott et al., 2010; Yu et al., 2013). It has been demonstrated that polyclonal clusters of CTCs in contrast to single CTCs have an increased metastatic potential and correlate with worsened overall survival in breast cancer patients (Aceto et al., 2013). Multiple microfluidic CTC isolation

technologies are currently applied in capturing CTC clusters from blood of both cancer patients and tumor mice (Cho et al., 2012; Stott et al., 2010; Yu et al., 2013). However, only small CTC clusters less than dozens of tumor cells can be captured by these technologies. Bigger CTC clusters appeara to be more rapidly cleared from the circulation than single CTCs, where the structural deformability of the aggregated cells within these clusters and the presence of vascular shunts within the circulation could explain the size limiting ability of current CTC capturing technologies (Aceto et al., 2013). Another explanation is also that LVI get stuck at the lung vessel wall and therefore cannot be captured. Thus CTC clusters larger than 100 cells which may have an even worse metastatic potential may be underestimated.

By analyzing HE stained lung tissue from MMTV-NeuNT mice, we found LVI in both small and big lung vessels, most likely arterioles. These cell clusters eventually occluded the vessel lumen and presumably can not be detected by the microfluidic CTC isolation technologies. In addition, we frequently found big LVI in lung vessels. In contrast, we eventually found some smaller CTC clusters in the primary tumor (data not shown) consistent with published data suggesting that small CTC clusters may be shed from the primary tumor navigating through the circulation and expanding at the distant target organ (Aceto et al., 2013).

5.1.3. CTC inside LVI expand where TNC can protect tumor cells form apoptosis

In order to dissect the roles of TNC in metastasis and in particularly in LVI, we assessed lung metastasis by a stereological analysis of the left and biggest lung lobe upon Giemsa staining and noticed no difference in the number of metastasis between genotypes, suggesting that TNC is not required for birth of a LVI. However, a higher metastatic index as indicated by a larger metastatic surface in lungs of WT mice in comparison to TNC KO and a bigger surface of LVI in WT than in TNC KO mice, suggest a promoting role of TNC in LVI expansion and lung metastasis outgrowth. The number of CTCs in the bloodstream far exceeds the number of metastatic lesions in patients, indicating that the vast majority of CTCs die in the bloodstream. Anoikis, a process occuring in most CTCs while in the bloodstream where epithelial cell lose adhesion-dependent survival signals, shear forces, attack by the innate immune system and oxidative stress may all contribute to killing of CTCs in the citculation. However, EMT, stromal-derived factors, or persistent

interepithelial cell junctions may provide survival signals that attenuate this apoptotic outcome and counterbalance the aforementioned proapoptotic events (Duda et al., 2010; Mani et al., 2008; Robson et al., 2006; Yu et al., 2012). In order to determine whether the promoting role of TNC in LVI expansion occurs through triggering survival or proliferation signaling in LVI, we quantified cleaved caspase-3 and Ki67 stained cells in LVI of both genotypes and found that cells in LVI proliferate, yet we did not see a difference in the number of Ki67 positive cells between genotypes. That CTCs proliferated indicates that some CTCs may have stemlike properties. In contrast to proliferating cells, cleaved caspase-3 positive cells were less abundant in WT than in TNC KO LVI. Altogether these results suggest that TNC promotes survival but not proliferation. Several questions arise from these observationssuch as how does TNC protect CTC in LVI from apoptosis? Which cells express TNC in the LVI and in parenchymal metastasis? Which cellular source of TNC is involved in this process? Does TNC act as a physical barrier in the LVI shielding CTCs from the effects of shear stress and immune cell attack? Or does TNC trigger signaling that allows tumor cells to adapt to the unfriendly microenvironment in the blood stream and in the target organ? These questions will be discussed here.

5.1.4. Fibroblasts at the rim of LVI are candidate TNC providers

The current literature provides evidence that both stromal and tumor cells can express TNC in different stages of tumor progression. For example, by injecting 4T1 mammary tumor cells into TNC WT and TNC KO mice, O'Connell and colleagues found that TNC produced by S100A4+ (FSP1+) stromal cells are important for metastatic colonization (O'Connell et al., 2011). In a xenograft mouse model, TNC deficiency in human breast cancer cells significantly impaired metastatic initiation and outgrowth in the lung. Moreover, the author also observed that if the metastasis surface reached a certain size, αSMA expressing myofibroblasts become an important source of TNC (Oskarsson et al., 2011). These results point at distinct functions of stromal and cancer cell derived TNC in the metastatic process (Oskarsson T, 2011 Nat Med).

However, no information was available whether TNC is expressed in LVI and if so what the cellular source and the impact on metastasis would be. Therefore, we used the NT193 orthotopic grafting model, also displaying LVI, that upon injection of cells into the mammary gland of an immune competent host spontaneously develops lung metastasis

(Sun, Velazquez-Quesada et al., in preparation). We downregulated TNC by shRNA technology in NT193 cells and grafted shC and shTNC cells into a WT and TNC KO host, respectively. We observed the highest metastatic index in WT mice that were grafted with shC tumor cells. Moreover, irrespective of the TNC levels in the cells, there was a tendency towards more metastasis in the WT in comparison to TNC KO host. We also found that LVI were in general bigger in a WT than in a TNC KO host. Assessing survival and proliferation by tissue staining revealed that cells in LVI proliferated, yet independent of TNC which is similar to the MMTV-NeuNT model. In contrast, we saw the lowest apoptosis index when shC cells were grafted into a WT host in comparison to a TNC KO host. Altogether, these results demonstrate that stromal TNC is important for enhancing survival of cells in LVI.

Upon grafting of shC cells the corresponding LVI expressed TNC when cells were grafted into a WT host, yet not when grafted in a TNC KO host. Also in LVI derived from shTNC cells (generating tumors with largely reduced TNC levels) we found TNC expressed in LVI when cells were grafted in a WT yet not in a TNC KO host. We also found both in the grafting and transgenic model that TNC is located at the rim of the LVI yet not in the center. These results reveal that stromal cells are the major source of TNC. To further address the stromal source of TNC we co-stained LVI with an antibody against TNC and markers for fibroblasts such as FSP1, αSMA and vimentin, (Kalluri et al., 2006). In contrast to vimentin and αSMA, we observed signal overlap of the TNC and FSP1 staining suggesting that FSP1 expressing cells likely express TNC which previously was described in another model by O'Connell (O`Connell et al., 2011). We also observed an intact layer of elongated vimentin positive cells around LVI suggesting that vimentin expressing cells presumably fibroblasts likely play important role in establishing the microenvironment in the LVI. In addition, Duda and colleagues (Duda et al., 2010) found that viability of circulating metastatic cancer cells is higher if they are incorporated in heterotypic tumor–stroma cell fragments. They also found that partial depletion of the carcinoma-associated fibroblasts, which spontaneously spread to the lung tissue along with metastatic cancer cells, significantly decreased the number of metastases and extends survival after primary tumor resection. Together with our previous findings, we conclude that TNC presumably secreted by FSP1+ cells plays an important role in survival of tumor cells in LVI. However, the origin, activation and destination of the fibroblasts/myeloid cells in the LVI remains

largely unknown. Therefore, a potential impact of TNC on myeloid cells in LVI needs to be further investigated.

5.1.5. TNC promote survival in CTC potentially involving EMT

We wanted to find an explanation for how TNC promotes survival of tumor cells in the LVI. Can TNC act as a physical barrier in the LVI? Given that TNC is expressed at the periphery of the LVI, we considered that TNC may form a protective niche (together with other ECM molecules) which is reminiscent of what we had described for TNC tracks observed in several tumors (Spenlé et al., 2015). We determined a potential co-expression of TNC with lamnin (LM), an essential component of the basement membrane which can have barrier function (Yurchenco et al., 2011; Ritié et al., 2012), and for fibronectin (FN), an ECM molecule that is frequently coexpressed together with TNC in matrix tracks (Chiquet-Ehrismann et al., 1988; Spenlé et al., 2015). We observed that indeed both ECM molecules were expressed at the periphery of the LVI yet in discrete layers that were distinct of the TNC layer. CTC form a “tumor nest” that is enveloped by a capsule composed of TNC layer facing the CTC, followed by a layer of FN/LM. Since TNC is located at the inner layer of the capsule, we may consider that the ECM layer acts as a physical shield to protect cells within the LVI from the sheer stress generated in the circulation.

These observations brought us to search another explanation for the survival promoting role of TNC in LVI. Given that ECM components have been demonstrated to bind growth factors they can act as a reservoir for growth factors and presenters of growth factors (Lu et al., 2012). We considered that TNC may act as survival signalling presenter that modulates adaptation to the unfriendly microenvironment in the blood stream. In a recent study of human breast cancer, mesenchymal markers indicative of EMT were expressed within the cancer cells comprising CTC clusters. This mesenchymal transformation may provide survival signals that attenuate the apoptotic outcome of CTCs (Yu et al., 2013). Also, emerging evidence suggests that EMT related transcripts, cytokines, genes and epigenetic changes are involved in increasing the treatment resistance in breast cancer (Daveet al., 2012; Mallini et al., 2014; Huang et al., 2015; Zheng et al., 2015; Fischer et al., 2015; Robson et al., 2006). Because of this link between EMT and apoptosis in tumor

progression, we hypothesized that TNC may promote survival of CTC inside LVI through EMT.

By quantifying expression of vimentin (a classical mesenchymal marker of EMT) we noticed that more vimentin expressing cells were present in the tumor nests of WT LVI than in TNC KO LVI while the E-cadherin expression was similar between genotypes. This result suggests that TNC may promote partial EMT in LVI. Whether increased expression of mesenchymal markers (vimentin positive) by TNC results from enhanced proliferation of cells with such a phenotype or whether more cells undergo EMT needs to be addressed in the future.

In order to further confirm the role of TNC in inducing EMT, we treated NT193 cells with TNC in monolayer or spheroid cultures and observed a morphological change reminiscent of EMT. Moreover, IF staining revealed loss of E-cadherin and gain of vimentin expression. We also confirmed a change in expression of several EMT markers by qRTPCR and western blot. In particular, we saw increased mRNA levels for snail, slug, ZEB1, vimentin, PAI-1, MMP9 and TNC yet not for twist nor FN. On the contrary mRNA levels for E-cadherin were reduced. As determined by western blot, protein levels of vimentin and E-cadherin were increased and reduced, respectively upon treatment with TNC. Other studies provided evidence for a role of TNC in EMT as e.g. a close correlation between TNC expression and gastrulation and formation of the neural crest, endocardial cushion, and secondary palate indicated a potential role of TNC in EMT (Jones et al., 2000). In breast cancer patients, TNC was found to be co-expressed with vimentin (Dandachi et al., 2001). In colorectal cancer patients, TNC was also found to be positively correlated with a EMT signature (Takahashi et al., 2013). Furthermore, Chiquet-Ehrismann R and colleagues observed detachment and disruption of cell-cell contacts in MCF-7 cells upon treatment with conditioned medium containing TNC as well as after seeding cells on a TNC substratum (Chiquet-Ehrismann et al., 1989; Martin et al., 2003). It has also been shown that TNC can induce an EMT-like morphology in MCF-7 cells by binding to αvβ6 and αvβ1 integrins (Katoh et al., 2013). Both *in vitro* results and *in vivo* observations suggest a role of TNC in promoting EMT or an EMT-like phenotype.

Finally, we determined whether NT193 cells having undergone EMT by TNC are more resistant to an apoptotic stimulus. Indeed, pre-treatment of NT193 cells with TNC for 24 hours promoted cell survival against staurosporine-induced apoptosis as measured by the

caspase-3/7 activity assay. All together, these observations support our hypothesis that TNC promotes survival through inducing EMT in tumor cells. However, the in vitro experiments were done under static conditions. Whether tumor cells better tolerate shear stress under flowing conditions, or resist other apoptotic stimuli upon TNC-induced partial EMT needs to be investigated in the future.

5.1.6. TNC-induced EMT in NT193 cells involves TGF-β signaling

Transforming growth factor-β (TGF-β) is a potent pleiotropic molecule that is implicated in diverse biological processes. Interestingly, TGF-β is involved in the tight control of both apoptosis and EMT, which are key to TGF-β's role in physiological and pathological events (Song et al., 2007). In human breast cancer, a strong TGF-β signatures was found in mesenchymal CTC clusters (Yu et al., 2013). It has been intensively reported that increased expression and activation of TGF-β1 signaling promotes an epithelial plasticity response that may progress to EMT and increase cancer cell invasion and dissemination (Thiery et al., 2009; Kalluri et al., 2009; Katsuno et al., 2013). Dominant-negative forms of TβRII or TβRI, and pharmacological inhibition of the kinase activity of TβRI, blocked TGF-β-induced EMT in many cell types (Portella et al., 1998; Valcourt et al., 2005; Lamouille et al., 2007; Lamouille et al., 2014) whereas a constitutively activated TβRI protein initiates EMT (Piek et al., 1999). All this evidence suggests that TGF-β family signalling has a predominant role in EMT.

Recently, Alcaraz and colleagues found that the fibrinogen-like (FBG) domain of the matrix glycoprotein tenascin-X (TNX) that has some homolgy to TNC can activate latent TGF-β into an active molecule by changing the conformation of the small latent TGF-β complex. This signaling subsequently induced EMT in mammary epithelial cells (Alcaraz et al., 2013). It has also been reported that TNC deficiency can attenuate TGF-β-mediated fibrosis in the lung indicating a potential role of TNC in latent TGF-β activation (Carey et al., 2010). In addition, the fifth fibronectin type III-like (TNCfnIII5) domain of TNC has been shown to have a high binding affinity for various growth factors including TGF-β1(De Laporte et al., 2013). All these observations indicate that TNC may act as a resevoir and/or presenter of TGF-β and regulate multiple physiological and pathological processes *in vivo* though this activity.

Given a role of TGF-β and TNC in EMT, and the TGF- β binding ability of TNC, we asked whether TNC may promote tumor cell EMT through TGF-β signaling. Therefore, we treated cells with TGF-β (control) or TNC and the TGF-β signaling inhibitor GW. We found that GW reverted the mesenchymal to an epithelial phenotype as demonstrated by high expression of E-cadherin and low expression of vimentin. This occurred not only in cells treated with TGF-β but also upon exposure to TNC. We also determined downstream signaling upon TNC treatment by western blot and observed that both canonical and non-canonical TGF-β signaling is induced by TNC as levels of p-Smad2/3, p-Akt and p-Erk1/2 were increased in comparison to control conditions which was similar to treatment with TGF-β. Again, TNC signaling was TGF-β signaling dependent as it was blocked by GW. These observations suggest that TNC-induced EMT in NT193 involves TGF-β signaling. However, the underlying molecular mechanism such as latent TGF-β complex binding of TNC, and binding of TNC to other potential co-receptors and integrins as well as the releasing and anchoring signals need to be investigated in the future.

5.1.7. Role of TNC in mediating platelet-induced EMT in LVI

Another important question is the source of the TGF-β in the LVI. Platelets have an important role in the microenvironment of the bloodstream, where they aggregate around cancer cells almost immediately after they enter the bloodstream (Labelle et al., 2011). Platelets can release a series of active molecules like TGF-β and ATP which aid the tumor cell extravasation and metastatic seeding (Schumacher et al., 2013; Labelle et al., 2011). In addition, Labelle and colleagues proposed that mesenchymal transformation of epithelial cells in the blood is mediated by TGF-β released from platelets (Labelle et al., 2011) Hence, platelet could be one important source of TGF-β in TNC-induced EMT in our model. By tissue staining, we found platelets as integral component of LVI surrounded by LM, suggesting a potential early role in the formation of LVI. We also noticed an overlap of CD41 and TNC expression at the rim of the LVI. By quantification of CD41 we found less platelets in LVI of TNC KO mice than in WT mice suggesting that TNC can promote the abundance of the platelet in LVI. In support with this in vivo observation, Schaff and colleagues (Schaff et al., 2011) found that platelets can efficiently adhere to TNC and get activated under both static and flow conditions through a complex including integrin α2β1 and glycoprotein Ib-IX complex respectively. Together with the vicinity of the CD41 and

TNC staining we hypothesize that TGF-β released from platelet potentially bind TNC thus impacting on the properties of CTC within LVI such as EMT. In addition, we investigated EMT in NT193 cells upon addition of platelets. We saw that indeed platelets induced EMT as documented by western blot: E-cadherin levels dropped and vimentin expression increased. Similar to TNC, also platelet-induced EMT was blocked with GW suggesting that platelet-induced EMT is TGF-β signaling dependent in NT193. A platelet depletion experiment could tell what roles platelets play in vivo, yet this difficult to accomplish due to complications with bleeding in the absence of platelets.

5.1.8. TNC induced EMT promotes tumor cell migration and may facilitate the extravasation of the LVI

Besides the anti-apoptotic function of TNC-induced EMT, the increased cell migration is another functional impact of TNC-induced EMT. We used a wound closure assay and saw increased migration of cells with TNC (over control conditions), which was similar to TGF-β treatment. Again, this effect was blocked with GW indicating that TNC-induced EMT enables NT193 cell motility through TGF-β signaling.

As TNC increases tumor cell motility through TGF-β *in vitro*, it is possible that tumor cell migration and extravasation are potentially induced by a similar mechanism. LVI were described as precursors of parenchymal metastasis in the MMTV-Neu model (Siegel et al., 2003). When we compared the ratio of LVI to parenchymal metastasis between genotypes of MMTV-NeuNT mice we found more parenchymal metastasis in lungs of WT mice. A similar observation was also found in the NT193 grafting model. These results suggest that TNC may play a role in the progression of LVI into parenchymal metastasis, presumably by promoting extravasation. Recent evidence has shown that EMT contributes to the progression of solid tumors by permitting detachment of cells from their primary site and by inducing a migratory phenotype, allowing cells to invade the local tissue and entering into the lymphatic system or blood circulation (Heerboth et al., 2015; Yilmaz et al., 2009). Despite a large number of publications supporting a role for EMT in tumor metastasis, the link between EMT and tumor cell exravasation is still lacking.

Drake and colleagues found that TEM4-18 cell with an EMT-like morphology and near complete loss of E-cadherin enhanced transendothelial migration (TEM) and colonized the target organ at a higher rate in SCID mice compared the PC-13 cells that did not undergo TEM in vitro. Knockdown of Zeb1 in TEM4-18 cells partially restored the epithelial

morphology and reduced TEM (Drake et al., 2009). However, other studies suggest that epithelial tumor cells are also capbale to metastasis. Tsuji and colleagues (Tsuji et al., 2009) compared the invasive and metastatic properties of normal epithelial and hamster cheek pouch carcinoma cells having undergone EMT. When injecting these 2 tumor cells subcutanously, both cells developed tumors, however, only the EMT cells locally invaded the tissue and were detected in the blood, consistent with previous findings that EMT promotes invasion. Surprisingly, when injecting these two cells intravenously, only the epithelial cells gave rise to metastasis but not cells with an EMT phenotype. When the two cell lines were differently labeled, mixed and injected subcutaneously, lung metastasis only consisted of epithelial cells. Although the EMT cells can be detected in the blood, none of them were detected in the lung metastasis. In agreement with this finding, Antoine and colleagues (Karnoub et al., 2007) reported that epithelial-like MDA.MB.231 cells are enhanced in metastasis from a subcutaneous site when mixed with mesenchymal stem cells, however, the resulting metastatic tumors did not contain detectable mesenchymal cells. These results suggest that only epithelial cells have a propensity for colonization and proliferation but not the EMT phenotype cells, while the EMT phenotype may facilitate the epithelial cells undergoing transendothelial migration. In our model, by tissue staining for CK8/18 (combined with TNC) or E-cadherin (together with vimentin), respectively, we investigated CTC leaving LVI and invading the parenchymal lung tissue. These cells mostly expressed CK8/18 and E-cadherin with the vimentin positive cell at the invasive front which is reminiscent of collective tumor cell migration and invasion (Cheung et al., 2015). It is possible that the mesenchymal phenotype cells at the invasive front in our LVI could lead the epithelial tumor cells migrating towards the lung parenchymal by breaking the endothelial cell junction and basement membrane underlying the endothelium, and hence facilitate the collective migration of the follower epithelial cells. From this immunofluoresent staining together with the enhanced proportion of parenchymal metastasis by TNC we may suspect that TNC can induce EMT in LVI and in turn promote extravasation of the LVI.

5.1.9. TNC may promote extravasation though the endothelial cell layer

The interplay between CTCs and endothelial cells plays also an important role during extravasation. When we characterized LVI by IF on sequential lung tissue sections,

staining for CD31 revealed two layers of endothelial cells, one derived from the blood vessel and one directly surrounding the LVI, seen in both genetic and grafting models. Consistent with our findings, the same phenotype was observed in another genetically engineered mouse model (Oshima et al., 2004; Sugino et al., 2002). In human primary tumors, a percentage of tumor LVI was also found to be enveloped by endothelial cells, and these structures were particularly prevalent in renal cell carcinomas, hepatocellular carcinomas and follicular thyroid carcinomas (Sugino et al., 2004). However, there has not been many report describing the meaning of this phenomenon in the metastatic process. Moreover, little attention has been paid to the existence of tumor LVI-associated endothelia in human cancers other than follicular carcinoma of the thyroid.

Sinusoid structures surrounding tumor nests is an important feature of the vasculature in the primary tumors carrying endothelium-coated tumor LVI. In fact, renal cell carcinomas, hepatocellular carcinomas and follicular thyroid carcinomas are known as hypervascular tumors which have the appearance of sinusoidal capillaries (Sabo et al., 2001; Wong et al., 1999; Macchiarini et al., 1992). In these cancers, the tumor nests and surrounding sinusoidal vessels presumably form the LVI “unit”, which is subsequently budded and disseminates. Therefore, this budding process is not cancer cell invasion dependent but depends on the remodeling of the tissue structure through tumor-stroma interactions. The CTC clusters with an endothelial layer covering may be mechanically arrested in an arteriole and then intravascularly proliferates. During the extravasation, instead of disrupting the vascular walls, the CTC clusters may bud to the parenchyma of the target organ by fusing of the covering endothelial layer and the endothelial cells lining the vessel wall (Sugino et al., 2002; Al-Mehdi et al., 2000).

Another hypothesis of the formation of this endothelial cell covering LVI is proposed by LAPIST and colleagus (Lapis et al., 1988). During extravasation, in the arterioles, the individual tumor CTC became encompassed by endothelial cells after tumor cell attachment. Then the tumor cell in the LVI can outgrow from the intact endothelial lining of the arteriole. Owing to the proliferation of the tumor cells, tumor colonies encompassed by endothelial cells expand within the lumen. When these intravascular growing LVI completely fill the lumen, the tumor cells extravasate from the vessel after the endothelial layer is mechanically disrupted (Lapis et al., 1988).

When we quantified the abundance of LVI with a monolayer of endothelial cells we found that LVI lacking an endothelial layer were more frequent in TNC KO mice than in WT mice, suggesting that TNC positively impacts on the endothelialization process. The endothelial cell layer of the LVI may be involved in extravasation through a mechanism proposed by Sugino et al (2002) and involves andothelial layer fusion resutling in ingestion of the LVI. Indeed, we obtained visual evidence for such a scenario. It has been shown that TNC has multiple roles in tumor vascular remodeling such as promoting endothelial cell proliferation, adhesion, migration, invasion and tube formation (Delaney et al., 2006; Castellon et al., 2002; Ishiwata et al., 2005; Martina et al., 2010). TNC exerts a Janus activity on endothelial cells that may explain that TNC promotes the abundance of new vessels in a tumor that are poorly functional (Saupe et al., 2013). While inducing apoptosis through direct interaction with TNC, endothelial cells also receive an angiogenesis promoting signal from TNC through paracrine signaling inducing a pro-angiogenic secretome comprising pro-angiogenic Ephrin-B2 in glioblastoma cells and other not yet identified factors in carcinoma associated fibroblasts (Rupp et al., 2016). What molecules TNC induces in the LVI that promote endothelial layering remains to be determined. Nevertheless, the correlation of enhanced apoptosis in the TNC KO LVI, reduced endothelial layering and reduced extravasation suggests an important role of the endothelial layer in extravasation. Thus an endothelial cell targeting drug such as bevacizumab could be envisaged as treatment to counteract LVI extravasation.

5.1.10. Plasticity of tumor cells in parenchymal metastasis

We investigated expression of mesenchymal (vimentin, αSMA) and epithelial cell markers (ErbB2, CK8/18) in parenchymal metastatic tissue and observed that cells had a mixed phenotype expressing both epithelial and mesenchymal markers. This observation suggests that a MET process may not occurr in parenchymal lung metastasis, or the other way around, that a second round of EMT happens after the lung metastasis has reached a certain size.

We also analyzed proliferation and apoptosis in lung parenchymal tissue and observed Ki67 positive cells in all parenchymal metastases indicating expansion of the tumor nests in the lung rather than dormancy. It is well reported that MET is required for colonization

and metastasis as an EMT phenotype is associated with growth arrest (Brabletz et al., 2012; Tsai et al., 2012; Ocaña et al., 2012).

The plasticity of tumor cells in metastasis remains largely unclear. The transient and reversible EMT/MET/EMT process in the physiological settings needs to be better studied. Here, we observed the existence and role of epithelial-mesenchymal plasticity in metastasis of MMTV-NeuNT derived mammary tumors without artificially modifying EMT regulators which is consistent with another recent paper that also showed this plasticity in a non-EMT manipulated breast tumor mouse model (MMTV-PyMT) (Beerling et al., 2016). In addition, it is important to better understand the switch of MET to EMT in the metastatic site for future therapeutic strategies against metastasis. Targeting MET, thereby maintaining dormancy of the metastatic tumor cell might be counterproductive because it would facilitate tumor cells to disseminate.

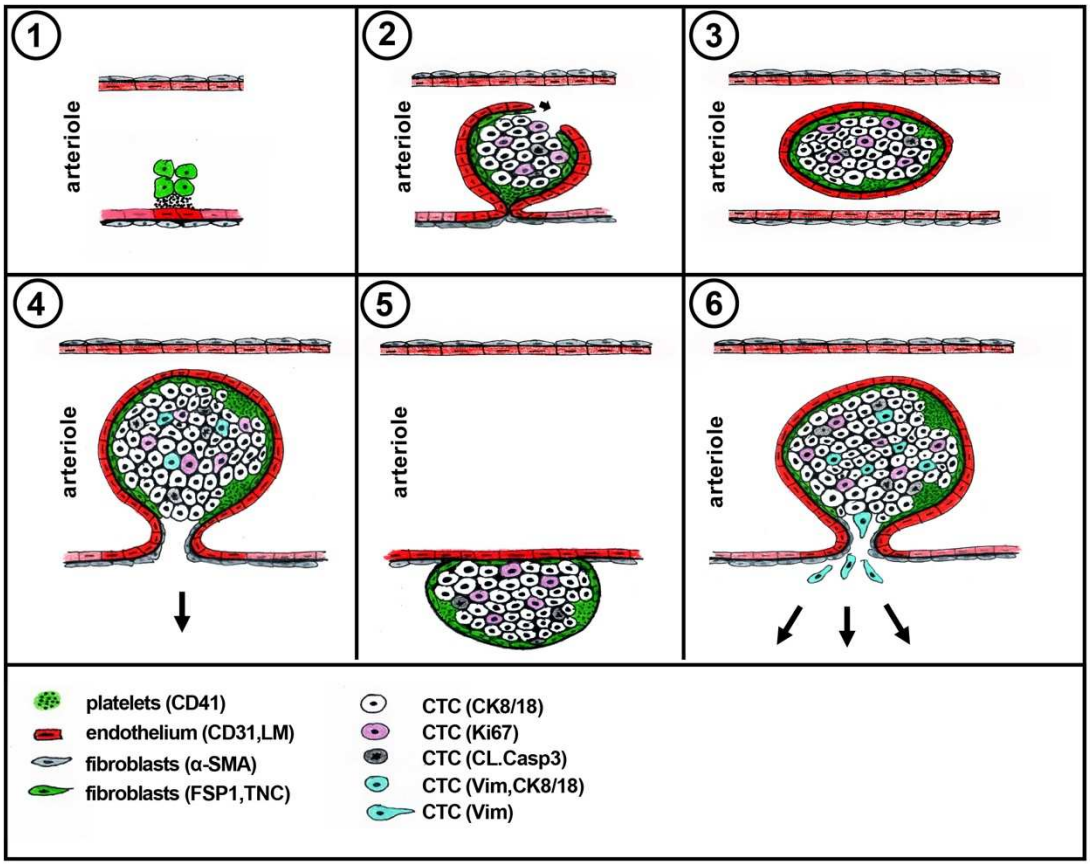

Figure 6. Summary cartoon of TNC enhancing parenchymal lung mestastasis by two mechanisms with LVI as critical precursors

A comprehensive view of the presented results in relation to knowledge from the literature is presented in **Fig. 6.** Upon release of CTC from a primary tumor CTC may be trapped by platelets at the lung arteriole wall. At this step CTC may express TNC which is consistent with results from another model where TNC expression in CTC was shown to be essential for breast cancer lung metastasis (Oskarsson et al., 2013). We have shown that TNC promotes abundance of platelets suggesting a potential role in platelet attachment which may involve a mechanism described in thrombus formation (Schaff et al., 2011) (**panel 1**). Attachment may induce remodeling of the lung vessel endothelium triggering endothelialization, thus leading to a monolayer of endothelial cells around CTC, generating a LVI. In addition to endothelial cells also FSP1+ myeloid cells, found underneath the LVI basement membrane (thick black lining), are involved in endothelialization. FSP1+ cells are already present in the arteriole wall where they do not express TNC which is in contrast to the LVI, where FSP1+ cells are positive for TNC (green) (**panel 2**). Two possibilities are consistent with our observations: (i) LVI may be released into the circulation (**panel 3**, as we have seen "floating" LVI) and then attach to another arteriole or (ii) LVI remain attached to the arteriole wall and continue to expand (**panel 4**). Walls of arterioles that are occupied by LVI are positive for activated macrophages (F4/80) indicating activation of the endothelium which may contribute to fusion of endothelial cells from the LVI and the arteriole which is in agreement with the model proposed by Sugino et al., (2002). An LVI may be ingested and grow into parenchymal metastasis (**panel 5**). During expansion of an already attached LVI some CTC proliferate indicating stem like properties, other CTC die via apoptosis. Cell survival is promoted by TNC. In addition to E-cadherin, some CTC express vimentin indicating partial EMT of CTC which is enhanced by TNC (**panel 6**). TNC-driven EMT may involve TGF-β as TNC triggers EMT in cultured NT193 cells in a TGF-β dependent manner. Some cells at the invading front are exclusively vimentin+, altogether reminiscent of single cell and collective cell invasion during the extravasation step (**panel 6**). Altogether these events result in parenchymal metastasis which is increased by TNC and may involve TGF-β signaling in MMTV-Neu tumors (Siegel et al., 2003).

5.1.11. Perspectives:

Although my work has revealed multiple roles of TNC in breast cancer lung metastasis in the MMTV-NeuNT model in particular that TNC enhances metastasis by enhancing survival and EMT in CTC, and increasing abundance of platelets and endothelial cells in LVI many questions concerning the mechanism of TNC - promoted lung metastasis remain to be studied in the future.

1. Only small CTC clusters less than dozens of tumor cells can be monitored or captured by current CTC capturing technologies. A subset of the CTC clusters for instance a CTC cluster larger than 100 cells which may have an even worse metastatic potential then the small CTC clusters need to be monitored intravitally by different kinds of imaging instead of the static pathohistological analysis. A non-invasive molecular imaging based on TNC could be one candidate to monitor the LVI behavior during metastasis since TNC is always observed at the rim of LVI in TNC WT mice.

2. The role of TNC during intravasation is still unknown, whether it is tumor invasiveness dependent and/or independent needs to be further investigated. We have observed that TNC does not only increase the endothelial layering of CTCs clusters but also found that TNC increases tumor cell migration and invasion. We hypothesize that endothelial layer fusion and LVI ingestion as well as EMT contribute to TNC-promoted extravasation. These possibilities need to be experimentally addressed in NT193 metastasis by e.g. targeting endothelial cells (bevacizumab) and EMT (TGF-β receptor inhibitor).

3. We have found that stromal TNC presumably fibroblasts derived TNC plays the major role in promoting LVI survival. However, our evidence of co-staining of the fibroblasts marker and TNC is correlative. A functional relevance has to be addressed in a fibroblast ablation inducible mouse model using *S100A4-tk* mice (Iwano et al., 2001; O'Connell et al., 2013). Absence of TNC expression around LVI is expected in a S100A4/FSP1 depleted mouse. In addition, origin, activation and destiny of fibroblasts in the LVI could be investigated in such a mouse model.

4. We have observed that a partial EMT exists in the TNC expressing LVI. Whether the expression of mesenchymal markers (vimentin postive) by these adherent cells (E-cadherin) results from proliferation of a single cell that has undergone EMT into a cluster

of cells or, alternatively, from the continuous mesenchymal transformation of preexisting CTC clusters within the bloodstream need to be determined.

5. We have observed that TNC can promote survival of tumor cells through inducing EMT. However, the in vitro apoptosis assay was done under static condition. Whether TNC-induced EMT-tumor cells can tolerate better shear stress, or other apoptotic stimuli (such as hypoxia, chemotherapy or immune cell induced cytotoxicity) needs to be addressed in the future.

6. We have observed that TGF-β signaling is involved in TNC-induced EMT, however, the molecular mechanism is unknown. A potential binding of the latent TGF-β complex to TNC, as well as co-receptors and integrins and signals involved in releasing of anchored TGF-β need to be further investigated. By treating NT193 cells with different purified domains of TNC such FNIII repeats or FBG domain, we may gain insight whether these domains are pottentially involved in binding TGF-β. A loss of function approach for TGF-β R1 and co-receptor LRP6 (Ren et al., 2013), TNC binding integrins such as α9β1 (Yokosaki et al., 1994), α11β1 (Alcaraz et al., 2014) or α5β1/syndencan-4 (Kanno et al., 1994) may provide more detailed mechanistic insight into the roles of TNC in TGF-β signaling.

7. We have observed that, similar to TNC, platelet-induced EMT was also blocked with GW suggesting that platelet-induced EMT is TGF-β signaling dependent in NT193 cells. A platelet depletion experiment may be done in both MMTV-NeuNT TNC WT and KO host to further evaluate the role of TNC in mediating platelet-induced EMT in the LVI in vivo. Yet, a continuous depletion of platelets may induce bleeding. A drawback of the NT193 model in this context is its long latency of 11-14 weeks. Thus it is questionable whether low enough levels of platelets can be reached to see an effect on NT193 LVI and metastasis.

8. Finally, we have observed that in the parenchymal metastasis, cells had a mixed phenotype expressing both epithelial and mesenchymal markers suggesting the plasticity of the tumor cells in metastasis sites. The transient and reversible EMT/MET/EMT process in the physiological in vivo settings, non-experimentally induced EMT during metastasis needs to be better studied. A better intravital imaging, a better lineage tracing system such as *MMTV-Neu/ Rosa26-RFP-GFP/Fsp1-cre* mouse model **(Fischer et al., 2015)** could be applied to answer this complex question.

Second manuscript

5.2. Tenascin-C promotes tumor cell migration through integrin α9β1 inhibiting YAP

In single cell migration, EMT is a rigid process following extensive alterations in gene transcription (Jahn et al., 2012). In contrast, mesenchymal-to-amoeboid transition (MAT) involves rapid changes in the migratory mode that arises as a reply to the local properties of the TME and is reversible and transiently regulated (Paňková et al., 2010). The actin polymerisation state determines whether cells migrate in a mesenchymal (actin stress fiber dependent) or amoeboid (actin polymerisation independent or stress fiber inhibited) mode (Bergert et al., 2012). Since TNC as substratum impairs actin stress fiber formation (Huang et al., 2001; Saupe et al., 2013), we asked whether an anti-adhesive FN/TNC substratum promotes amoeboid-like cell migration.

We have described a novel tumor cell autonomous signaling mechanism where TNC inhibits actin stress fibers and YAP through integrin α9β1 downregulating genes that in their repressed state promote tumor cell migration. In support of this mechanism, we found that tenascin-C reduces expression of YAP target genes in a glioblastoma model. The provided link may have clinical relevance as a signature comprising CTGF, Cyr61 and Cdc42EP3 at low levels, resembling the tumor cell autonomous effect of tenascin-C in vitro, correlates with worst survival of osteosarcoma and glioblastoma cancer patients. Our results suggest that tumor cell-expressed tenascin-C stimulating migration may be important for tumor malignancy. We provided a comprehensive view on a signaling pathway initiated by TNC, employing integrin α9β1 impacting on the actin cytoskeleton, repressing YAP and inhibiting gene expression, thus promoting cell migration. This information has diagnostic and therapeutic potential.

5.2.1 TNC downregulates gene expression through inhibition of actin stress fibers which in turn abolishes MKL1 and YAP activities in tumor cells

As it was incompletely understood how TNC regulates cell adhesion and migration, we have revisited the effect of TNC on cell adhesion in context of FN. TNC competes syndecan-4 binding to FN, thus blocking integrin α9β1 mediated cell adhesion and actin

stress fiber formation (Huang et al., 2001) which results in a pro-tumorigenic gene expression profile (Ruiz et al., 2004). We had previously shown that through this mechanism the tumor suppressor like molecule TPM1 is downregulated. Restoring syndecan-4 function either through a peptide mimicking the syndecan-4 binding site in FN or by overexpressing syndecan-4 (yet not syndecans 1 or 2), TPM1 expression was turned on and induced cell spreading on the FN/TNC substratum. This was blocked upon knockdown of TPM1 ((Ruiz C et al., 2004, Lange et al., 2008). Now, I have provided a novel link of TNC to TPM1 repression showing that TNC impairs MKL1. MKL1 itself is repressed by TNC at mRNA and protein level. This mechanism is also relevant for TNC to downregulate other genes (Cdc42EP3, DKK1 and TNC). As SRF luciferase reporter activity was not reduced we consider that TNC may impair other MKL1 activities such as the SAP-associated MKL1 function (Gurbuz et al., 2014), a possibility that needs to addressed in future studies.

In addition to MKL1 we have identified YAP to be impaired by TNC through its inhibition of actin stress fibers. We identified CTGF, Cyr61, Ccd42EP3 as target genes of TNC that are regulated in a YAP dependent manner. We found TNC itself as a novel YAP regulated gene. Whether regulation of TNC and the other target genes is direct and occurs through the TEAD binding sites in the respective promoters needs to be seen in the future. Altogether, we identified three groups of genes that TNC represses through its impact on MKL1 (TPM1), YAP (CTGF, Cyr61) or MKL1 and YAP (Cdc42EP3, TNC and DKK1).

5.2.2 TNC promotes amoeboid like migration in tumor cells through integrin α9β1 impairing YAP

Our results suggest that TNC specific transwell migration has properties of amoeboid migration (Paňková K et al., 2010) as cells are rounded, have no actin stress fibers and do not form focal adhesions nor have active FAK and paxillin (Huang et al., 2001; Ruiz et al., 2004; Lange et al., 2008; Orend et al., 2003; Lange et al., 2007). Amoeboid migration depends on ROCK (Paňková et al., 2010) which we have shown here (by chemical inhibition) to be required for TNC specific 3D migration. We have identified integrin α9β1 as novel upstream regulator of TNC-induced 3D migration. Although integrin α9β1 is known as receptor for TNC (Yokosaki et al., 1998), little was known how binding to TNC affects cell behaviour. Here, we have demonstrated for the first time that integrin α9β1 is

promoting 3D migration by TNC and link this effect to blocking actin stress fibers and inhibiting YAP.

We have shown that a direct interaction of tumor cells with TNC could be a key event in cancer malignancy by promoting motility through integrin α9β1 signaling. Future studies need to address whether this signaling leads to TNC repression in tumor cells. TNC is copiously expressed by stromal cells including fibroblasts and immune cells (O'Connell et al., 2011; Goh et al., 2010) and thus may generate a TNC rich TME that could promote tumor malignancy by additional non-tumor cell autonomous events such as angiogenesis (Saupe et al., 2013; Rupp et al., 2016).

In tumor tissue TNC is often coexpressed together with FN and other ECM molecules forming matrix tracks that serve as niches for tumor and stromal cells (Spenlé et al., 2015). These ECM rich niches may have different stiffness properties than adjacent areas devoid of matrix as they offer many integrin binding opportunities for cells. In support, in GBM it was recently shown that high TNC is correlated with tissue stiffness (Miroshnikova et al., 2016). Here, we have demonstrated that TNC regulates its own expression by a negative feedback loop. TNC may also reduce cellular tension due to its anti-adhesive properties. Altogether, this may contribute to balancing stiffening and cellular tension in cancer cells thus potentially contributing to their survival (**Fig.7**).

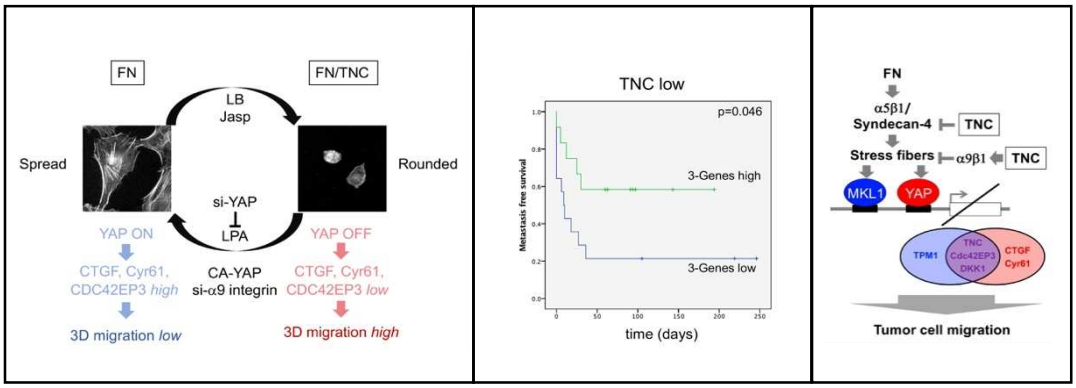

Figure 7. Kaplan Meier survival analysis in osteosarcoma patients and summary of TNC effects on actin polymerisation, gene expression and tumor cell migration

5.2.3 TNC can induce different modes of tumor cell migration

We have shown that soluble TNC can promote mesenchymal migration in NT193 cells through induction of EMT which occurred in a TGF-β dependent manner. This observation is in agreement with an EMT-like phenotype induced by TNC in other epithelial cell lines such as NMuMG cells (Alcaraz et al., 2014) and MCF-7 cells (Nagaharu et al., 2011; Martin et al., 2003). When we plated cells with a mesenchymal phenotype such as KRIB osteosarcoma or T98G glioblastoma cells on an anti-adhesive FN/TNC substratum they changed their appearance into an amoeboid like migratory phenotype. Both approaches are tumor relevant as soluble TNC may mimick fragmented TNC due to proteolytoc cleavage that can occur in the TME whereas TNC presented as substratum may represent matrix incorporated TNC. Our results showed that both soluble TNC as well as substratum-presented TNC can promote different modes of tumor cell migration by distinct mechanisms. Future studies have to address whether NT193 cells that have undergone EMT by TNC will further undergo MAT. This could be highly relevant as amoeboid cells are even more difficult to target than mesenchymal tumor cells.

Amoeboid and mesenchymal types of invasiveness are two modes of migration that are mutually interchangeable. It has been reported that tumor cells are able to use Rho/ROCK-dependent and Rho/ROCK-independent modes of invasiveness with respect to the spatial organization of surrounding collagen fibers. In mesenchymally migrating MDA-MB-231 breast carcinoma cells, Rho/ ROCK independent migration can only be used when the collagen fibers are pre-aligned perpendicularly to the tumor-ECM boundary, whereas Rho/ROCK mediated contractility is used in this cell type for the active, protease-independent reorganization of filaments in cases where the filaments are not pre-aligned (Provenzano et al., 2008). We may speculate that acting as a growth factor reservoir, TNC may induce EMT and promote survival and extravasation of the tumor cells. However, locally singularized mesenchymal tumor cells may respond to TNC by undergoing rapid changes towards an amoeboid-like migratory phenotype (**Fig. 8**). Where, when and how TNC containing ECM can trigger the switch of the mesenchymal to amoeboid migration needs to be further investigated.

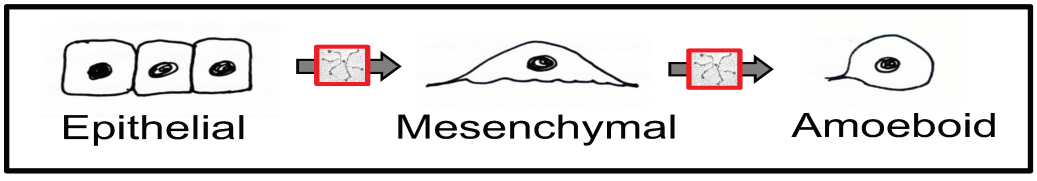

Figure 8. Summary cartoon of TNC effects on tumor cell migration

5.2.4 Perspectives

We have shown that by modulating tumor cell adhesion TNC regulates gene expression. We described a novel tumor cell autonomous signaling pathway where TNC inhibits actin stress fibers and YAP through integrin α9β1, which is required for TNC to promote amoeboid-like tumor cell migration. However, there are still many questions concerning the roles of TNC in amoeboid-like migration that need to be studied in the future.

1. We have shown that TNC specific transwell migration has properties of amoeboid migration as cells are rounded, have no actin stress fibers, do not form focal adhesions nor have active FAK and paxillin and ROCK dependent 3D transwell migration. Whether this mode of migration is protease independent which is another hallmark of amoeboid migration has to be addressed. Therefore, cells should be plated on fibrillar collagen with defined pore size and cell migration to the other side of the Boyden chamber should ne measured as described (Sahai et al., 2007; Gadea et al., 2008; Belletti et al., 2008). If TNC promotes amoeboid migration, cells will be able to pass through a collagen gel with a pore size that is at least the size of a nucleus (6 µm) and does not require MMPs which can be addressed with specific inhibitors. Furthermore, a potential impact of a TNC substratum on migration of melanoma cells such as A375M2 (representing a well-known cancer type that migrates in an amoeboid manner) should be determined.

2. We have shown that integrin α9β1 knockdown induced actin stress fibers and abolished TNC specific 3D transwell migration indicating that integrin α9β1 could be an upstream molecular regulator of TNC-induced cell rounding and amoeboid-like migration. It will be interesting to know whether these in vitro results have any relevance for tumor cell behaviour *in vivo*. Therefore, behaviour of cells with an integrin α9β1 knockdown or inhibition of YAP (by Verteporfin (Johnson et al., 2014)) upon grafting into an immune compromised mouse should be determined. If our identified signaling pathway is relevant *in vivo* it is expected that knockdown of integrin α9β1 results in inhibition of YAP and will reduce expression of the YAP target CTGF. By using KRIB cells that are (poorly) metastatic or another e.g. highly metastatic melanoma cell line it can be addressed whether integrin α9β1 knockdown has an impact on metastasis. Similarly, inhibition of

YAP by Verteporfin may lead to reduced CTGF expression and less (amoeboid-like) motile cells and potentially reduced metastasis.

6. Summary

In conclusion, I had used several in vitro and in vivo models to study the roles of TNC in tumor migration and metastasis.

We used the stochastic MMTV-NeuNT and a novel associated syngeneic orthotopic breast cancer model engineered to present abundant and no/low TNC. We observed that TNC promotes tumor onset and increases lung metastasis. We further revealed an important role of stromal TNC in metastasis and that TNC is a prominent component of parenchymal metastasis and lymphovascular invasions (LVI). We document that LVI are nests of proliferating circulating tumor cells, that are enveloped by TNC, fibronectin, laminin, a layer of endothelial cells and fibroblasts, and contain platelets. Moreover, TNC increases platelet abundance and endothelial cell layering of LVI. TNC also elevates tumor cell survival in LVI, partial epithelial-to-mesenchymal transition (EMT) and breaching into the lung parenchyma. In cultured cells through TGF-β signaling, TNC induces EMT and survival. We conclude that the presence and function of TNC in LVI may offer novel opportunities for cancer targeting.

We also showed that by modulating tumor cell adhesion TNC regulates gene expression. We describe a novel tumor cell autonomous signaling pathway where TNC inhibits actin stress fibers and YAP through integrin α9β1, which is required for TNC to promote tumor cell amoeboid-like migration. In support, expression of YAP target genes is elevated in a glioblastoma model upon knockdown of TNC. The provided link could have clinical relevance as a signature comprising CTGF, Cyr61 and Cdc42EP3 at lower levels, resembling the TNC effect in vitro, correlates with worst survival of osteosarcoma and glioblastoma cancer patients. Our results suggest that TNC matters as soon as it is expressed where stimulation of tumor cell migration may be important for TNC promoting tumor malignancy. To our knowledge this is the first report that provides a full view on a signaling pathway initiated by tenascin-C, employing integrin α9β1, its impact on the actin cytoskeleton, YAP and gene expression thus promoting amoeboid-like migration. This information could be of diagnostic and therapeutic value.

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Résumé en français suivi des mots-clés en français

Les métastases sont la principale cause de décès chez les patients atteints d'un cancer. Lors du développement métastatique, les cellules tumorales disséminées (CTD) doivent franchir certaines étapes clés avant de coloniser des organes distants de la tumeur primaire. Notre hypothèse est que la TNC pourrait jouer différents rôles dans la migration des cellules cancéreuses et par conséquent dans le développement métastatique. Considérant l'actine comme un réservoir de facteurs de croissance, la TNC pourrait induire la TEM ainsi que la survie et l'extravasation des cellules tumorales. Cependant, des cellules cancéreuses individualisées localement pourraient répondre à la TNC en initiant des changements rapides menant à un phénotype migratoire de type amiboïde. L'objectif de cette thèse a été d'étudier comment la TNC stimule le développement métastatique dans le cancer du sein au niveau cellulaire et moléculaire en utilisant des modèles tumoraux et cellulaires.

Mots clés :
Ténascine-C ; Cancer ; Cellules tumorales disséminées ; Lymphovasculaire invasion ; EMT ; Amiboïde migration ; YAP ; TGF- β ; Prognostic

Résumé en anglais suivi des mots-clés en anglais

A high TNC expression correlates with lung metastagenicity and was shown to promote experimental lung metastasis, but the underlying mechanisms are poorly understood. The results of my thesis have provided insight into the roles of TNC in metastasis suggesting that TNC contributes to extravasation by impacting on survival, endothelialization, EMT and migration. Moreover, I have identified TGF-β signaling and integrin α9β1 as important pathway and molecule, respectively to be employed by TNC. Whether both molecule/pathway play a similar role in the investigated models of breast cancer, osteosarcoma and glioblastoma remains to be seen.

Keywords :

Tenascin-C ; Cancer ; Circulating tumor cell ; lymphovascular invasion ; EMT ; Amoeboid migration ; YAP ; TGF- β ; Prognosis